UNIVERSITÀ DEGLI STUDI DI NAPOLI "FEDERICO II"

FACOLTÀ DI AGRARIA

DOTTORATO DI RICERCA IN

"SCIENZE E TECNOLOGIE DELLE PRODUZIONI AGRO-ALIMENTARI"

XXVII CICLO

DEVELOPMENT OF NEW ACTIVE INGREDIENTS FROM MICROALGAE BIOMASS FERMENTATION FOR HUMAN HEALTHCARE APPLICATIONS

Tutor:

Chiar.mo Prof. Vincenzo Fogliano

Co-Tutor:

Dott. Fabio Apone

PhD Student:

Adriana De Lucia

Year 2014/2015

SUMMARY

PREFACE

Since ancient times microalgae have represented a source of bioactive molecules that can be harnessed for commercial uses. The first use of microalgae by humans dates back 2000 years, when the Chinese used the cyanobacteria *Nostoc* as a food source during a famine (Spolaore et al., 2006[1]). Nowadays, there is a huge interest on natural products obtained from marine organisms that can promote the state of health and well-being for humans and other animals.

During my PhD course, in collaboration with the company Arterra Bioscience - Naples, I explored the potential industrial applications of some microalgae species, cultivated at the Agriculture Department of the University of Naples (Portici, Italy). In this study the attention was mainly focused on the characterization of two microalgae species: *Arthrospira maxima* and *Galdieria sulphuraria*. The microalga *A. maxima*, commercially known as Spirulina, is rich in protein and other essential nutrients, such as phenolic acids, tocopherols and β-carotene, which are known for their antioxidant properties. *G. sulphuraria* is a thermophile unicellular microalga, that lives in extreme conditions of temperature and acidity.

The study on Spirulina is described in chapter 1, while the characterization of *G. sulphuraria* ended up with a patent deposit, as reported in chapter 2.

CHAPTER 1

Abstract

Microalgae are microscopic photosynthetic organisms that produce a wide range of metabolites, such as proteins, fatty acids, carbohydrates, carotenoids and vitamins, useful for different specific industrial applications, including health care, food, cosmetics and energy production. The cyanobacteria Spirulina (*Arthrospira maxima*) is one of the most promising source of important metabolites, particularly proteins, lipids and phytochemicals, thus it was the focus of the research aimed at developing new active ingredients for health care applications.
In order to obtain valuable products from Spirulina, a total lysate, obtained from the microalga, was subjected to three types of fermentation processes: one based on the use of indigenous bacteria (auto-fermentation), those developed spontaneously in the lysate; the second based on the use of the mix Yovis® (Sigma-Tau), added after a sterilization procedure of the lysate; and the third performed by employing enzymes (carbohydrase and Alcalase), thus without using any microorganism.
The three extracts obtained from the fermentation processes were investigated for their antioxidant activity in protecting human cells against free radicals, then, more specifically, for inducing hydration and reduce dehydration in cultured skin cells. The results of the study underlined that the Spirulina extracts, either those derived from microbial treatments or enzymatic digestion, were significantly more efficient than the untreated samples in protecting cell membrane from oxidative stress. Moreover, those extracts obtained by enzymatic treatment showed the best hydrating and moisturizing capacity.
In conclusion, the products derived from Spirulina fermentation have potential applications in both the cosmetic field, as hydrating and moisturizing agents for skin care, and nutraceutics, as food supplements that increase the natural defense response of the cells and guarantee a more efficient balance of nutrients in the body.

INTRODUCTION

1. INTRODUCTION

1.1 Microalgae

Microalgae or microphytes are microscopic photosynthetic organisms, typically found in both freshwater and marine environments. Microalgae are classified as Protists. They are unicellular or multicellular organisms without specialized tissues; this simple cellular organization distinguishes the Protists from other eukaryotes, such as fungi, animals and plants. They exist individually or in chains and groups, and their sizes can range from one to few hundreds micrometers, depending on the species (Fig.1). Like plants, microalgae contain chlorophyll and are capable to convert light to chemical energy via photosynthesis. They are essential for life on earth because they produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photo-autotrophically. In the past, natural waters (lakes, lagoons, ponds) or artificial ponds were used to grow algae, more recently closed photobioreactors have been employed. Today, a range of microalgae species is produced in hatcheries and is used in a variety of ways for commercial purposes. Studies have estimated main factors in the success of a microalgae hatchery system as the dimensions of the container/bioreactor where microalgae are cultured, exposure to light/irradiation and concentration of cells within the reactor (Xu et al., 2009[2]).

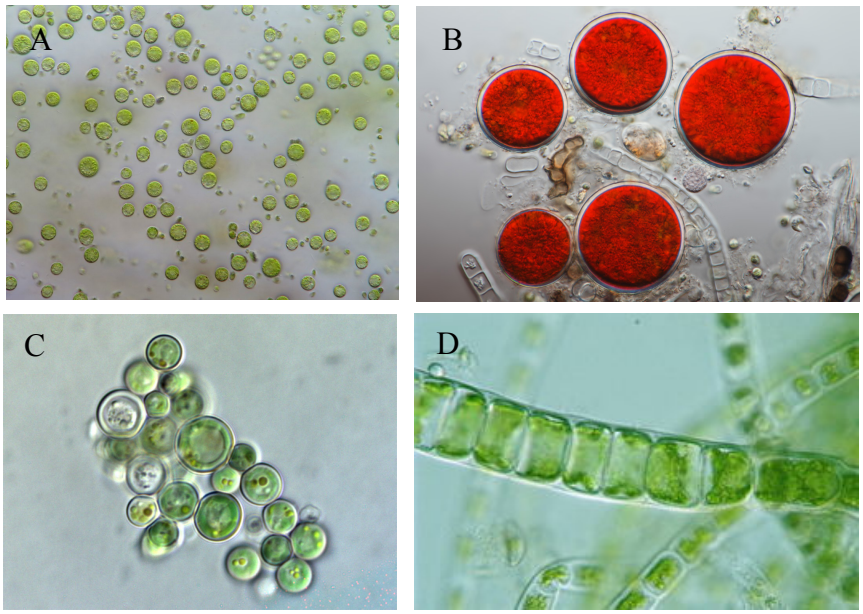


Figure 1. Different examples of microalgae: *Chlorella vulgaris* (A); *Haematococcus pluvialis* (B); *Galdieria sulphuraria* (C); *Spirulina platensis* (D).

Microalgae contain numerous bioactive compounds that can be exploited for commercial use. Most part of microalgae species can be used to produce a wide range of metabolites such as proteins, fatty acids, carbohydrates, carotenoids or vitamins for health, food, cosmetics and for energy production (Priyadarshani and Biswajit, 2012[3]). The chemical composition of microalgae is not a constant factor but on the opposite varies over a wide range, both depending on the species and on the cultivation conditions. Moreover, it is possible to induce microalgae to

synthesize desired compounds by changing environmental factors, like temperature, illumination, pH, CO_2 supply, salt and nutrients, especially those compounds that are essential for human and animal diet (Danesi et al., 2004[4]; Colla et al., 2007[5]; Ogbonda et al., 2007[6]). For example, the large variety of fatty acids, such as the omega-3, produced by several species of microalgae represent the basic foodstuff for numerous aquaculture species, especially filtering bivalves and fish, which are unable to synthesize them by their own.

Besides the use of microalgae as food for cultured aquatic species, they have been very important for human diet as well, thus microalgae production has become central in many commercial applications concerning food and health care, with the consequent need of increasing production techniques and productivity. Compared to other natural sources of bioactive ingredients, microalgae have many advantages such as their huge biodiversity, the possibility to grow in arid land and with limited fresh water consumption and the flexibility of their metabolism, which could be adapted to produce specific molecules. All these factors led to very sustainable production making microalgae eligible as one of the most promising foods for the future, particularly as source of proteins, lipids and phytochemicals (Buono et al., 2014[7]). **Table 1** reports a comparison of the general compositions of human food sources with that of different algae.

Commodity	Protein	Carbo-hydrate	Lipid
Bakers' yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
Anabaena cylindrica	43–56	25–30	4–7
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51–58	12–17	14–22
Dunaliella salina	57	32	6
Porphyridium cruentum	28–39	40–57	9–14
Scenedesmus obliquus	50–56	10–17	12–14
Spirulina maxima	60–71	13–16	6–7
Synechococcus sp.	63	15	11

Table 1. General composition of different human food sources and algae (% of dry matter) (Becker, 2007[8]).

1.1.1 Microalgae in nutraceutics and cosmetics

The oldest documented use of microalgae as food was 2000 years ago, when the Chinese used the cyanobacteria *Nostoc* as a food source during a famine (Spolaore et al., 2006[1]). Another type of microalgae, the cyanobacteria *Arthrospira maxima* was a common food source among populations in Chad and Aztecs in Mexico as far back as the 16th century (Whitton, B., and M. Potts. 2000. The ecology of Cyanobacteria: their diversity in time and space[9]). Since the past, cultured microalgae were used as direct feed for humans and land-based farm animals, and as feed for such as fish and crustaceans. Commercial use of microalgae as sources of specific chemicals began with *D. salina* for the production of β-carotene in the 1970s (Borowitzka and Borowitzka, 1989[10]) followed by the use of *Haematococcus pluvialis* as a source of astaxanthin (Lorenz and Cysewski, 2000[11]) and *Crypthecodinium cohnii* for long-chain polyunsaturated fatty acid (PUFA) and docosahexaenoic acid (DHA) (Kyle et al., 1998[12]). On the other hand, interest in applied algal culture continued with studies on the use of algae as photosynthetic gas exchangers for space travel and for generating renewable energy sources (Pulz and Scheibenbogen 1998[13]; Borowitzka 1999[14]). Therefore, today there is a huge interest among both consumers and industries on products, obtained from marine

and fresh water organisms, that can promote health and well-being (Sloan, 1999[15]). Microalgae are a potentially great source of natural compounds, such as carotenoids, phycobilins, fatty acids, polysaccharides, vitamins that could be used as biologically active compounds (Gouveia et al., 2005[16]). When foods or part of them have these properties, they can be generically named as functional foods. The concept of functional food was developed at the beginning of the 80s in Japan, as a way to reduce the high health costs derived from a population with high life expectations (Arai, 1996[17]). In Europe, in the second half of the 90s, a working group coordinated by the European Section of the International Life Science Institute (ILSI), and supported by the European Commission, was created to promote inside the IV Framework Program the action FUFOSE (Functional Food Science in Europe) to stimulate the scientific study on functional foods. From this project a definition for functional food was generated. Namely, a food can be considered "functional" if, besides its nutritious effects, it has a demonstrated benefit for one or more functions of the human organism, improving the state of health or well-being or reducing the risk of disease (Diplock et al., 1999[18]; Kalra, 2003[19]). In this definition it is necessary to emphasize three important and new aspects: a) the functional effect is different that the nutritious; b) the functional effect must be demonstrated satisfactorily; c) the benefit can consist in an improvement of a physiological function or in a reduction of risk of developing a pathological process. Besides, functional foods must have a series of additional characteristics as, for instance, the need of effectiveness in their beneficial action at the normal consumed doses. The beneficial action exercised by functional foods is due to a component or a series of ingredients that either are not present in the analogous conventional food or are present at lower concentrations. These ingredients are called functional ingredients.

Nowadays, over 470 nutraceutical and functional food products are commercially available (Eskin and Tamir, 2005[20]) (Table 2). The current estimated global market size for nutraceutical products is 30 to 60 billion dollars, primarily in the United States, Japan, and Europe, with a potential short-term growth market demand of over 197 billion dollars (Benkouider, 2005[21]). Different types of microalgae are well known to be used as food supplements and nutraceuticals, such as *Nostoc* (Deng et al., 2008[22]), *Botryococcus* (Dayananda et al., 2010[23]), *Chlamydomonas*, *Scenedesmus* (Ceron-Garcia et al., 2010[24]) and *Porphyridium* (Dvir et al., 2009[25]), especially for their capability of producing necessary vitamins including: A (Retinol), B1 (Thiamine), B2 (Riboflavin), B3 (Niacin), B6 (Pyridoxine), B9 (Folic acid), B12 (Cobalamin), C (L-Ascorbic acid), D, E (Tocopherol), and H (Biotin).

Besides the food market, the use of microalgae has been established in cosmetic market as well. The use of algal proteins or derivatives is important in conferring moisture retention on hair and skin, since they show a strong affinity with hair or skin to improve their nourishments. In particular, the extracts form *Arthrospira* and *Chlorella* are examples of microalgae already characterized in the skin care market (Stolz and Obermayer, 2005[26]): Protulines (Exsymol, Monaco) is a product from *Arthrospira* with the function of repairing the signs of early skin aging, while, Dermochlorella (France) is an extract from *Chlorella vulgaris*, involved in collagen synthesis in the skin. Two new products were launched by Pentapharm (Basel, Switzerland): an extract from *Nannochloropsis oculata* with skin-tightening properties and an ingredient from *Dunaliella salina*, which showed the ability to stimulate cell proliferation and turnover and to positively influence the energy metabolism of skin (Stolz and Obermayer, 2005[26]). More recently, a new characterization for cosmetic activities regarded an extract derived from the microalga *Botryococcus braunii*, suggesting that a *B. braunii* water soluble extract could improve skin barrier function and prevent premature skin ageing (Buono et al., 2012[27]). Other examples of microalgae species used in cosmetic products are *Ascophyllum nodosum*, *Alaria esculenta and Chondrus crispus*. *Ascophyllum nodosum* shows anti-aging, anti-wrinkles and anti-puff properties (CAS #84775-78-0). Kalpariane represents a product from *Alaria esculenta* extract with the anti-aging anti-collagenase and anti-elastase activities, thanks to its high concentrations of fatty acids (Verdy et al., 2011[28]). Moreover, Carrageenan extract, from alga *Chondrus crispus*, is an excellent moisturizing agent (CAS #244023-79-8).

Company	Species/group	Applications area	Reference
Green fuel Technologies Corporation (USA), Valcent Products Inc., (USA)	*Botryococcus braunii* / Chlorophyta	Biofuel	Trivedi, 2001; Pulz and Gross, 2004; Gomez and Gonzalez, 2004; Acien Fernandez et al. 2005; Spolaore et al. 2006; Ladygina et al. 2006; Raja et al. 2007c
Nikken Sohonsha Corporation (Japan), Ocean Nutrition (Canada)	*Chlorella vulgaris*/ Chlorophyta	Health food supplement, feed surrogates, aquaculture, Immune system, anti-flu	
Cyanotech (USA), Earthrise Nutritionals (USA), Panmol/ Madaus (Austria), Parry Nutraceuticals (India)	*Spirulina platensis* / Cyanophyta	Nutritional supplement to inhibit replication and infectivity of viruses including HIV, CMV, HSV and influenza A, immunological diagnostics, aquaculture feed/pigments, food colouring also helps immune system	
Nikken Sohonsha Corporation (Japan), Nature Beta Technologies (Israel), Cognis, Henkel-Cognis nutrition and Health (Australia), Cyanotech (USA), Tianjin Lantai Biotechnology (China), Dutch State Mines (The Netherlands), Inner Mongolia Biological Eng. (China), Parry Nutraceuticals (India), Proalgen Biotech (India), ABL Biotechnologies (India)	*Dunaliella salina*/ Chlorophyta	Health food supplement, feed, β-carotene capsules	
Mera Pharmaceuticals Inc., (USA), Cyanotec (USA), Bioreal (USA), Alga Tech (Israel), Dutch State Mines (The Netherlands), Parry Nutraceuticals (India)	*Haematococcus pluvialis* / Chlorophyta	Astaxanthin, health food, aquaculture, pharmaceuticals, feed additives and Treating carpal tunnel syndrome	
Phycotransgenics (USA), Algal biotechnology	*Chlamydomonas reinhardtii*/ Chlorophyta	Transgenic microalgae for animal health/feed, bioremediation, environmental monitoring and biopesticides, developing recombinant protein technology	
InnovalG, France	*Odontella aurita* / Bacillariophyta	Pharmaceuticals, cosmetics, baby food and Anti-inflammatory	
	Porphyridium cruentum / Rhodophyta	Pharmaceuticals, cosmetics, infant formulas, soil conditioning	
Innovative Aquaculture Products Ltd., (Canada)	*Isochrysis galbana* / Chlorophyta	Animal nutrition, aquaculture, mass oil production	
	Phaeodactylum tricornutum / Bacillariophyta	Nutritional supplements, aquaculture, fuel production	

Table 2: Examples of microalgae with their application areas (Raja et al., 2008[29]).

1.1.2 Biotechnological processes applied to microalgae

In order to obtain functional foods, food products were initially enriched with vitamins and/or minerals, such as vitamin C, vitamin E, folic acid, zinc, iron, and calcium (Sloan, 2000[30]). Later, the approach changed by the addition of several micronutrients such as omega-3 fatty acids, linoleic acids, phytosterols, soluble fibres (inulin and fructo-oligosaccharides, called prebiotics), trying to promote consumers health or to prevent different diseases (Shahidi, 2004[31]; van Kleef et al., 2005[32], Sloan, 2002[33]). Also, foods can be changed to contain viable microorganisms that can benefit human health; these products are called probiotics and are able to improve the activity in the intestinal tract and the immune system, among other functions. Lactic acid bacteria including *Lactobacillus acidophilus*, *L. johnsonii*, *L. reuteri*, *L. casei shirota*, etc., represents the microorganisms usually added to the food (Sanders, 2000[34]). Novel bio-processing technologies are developing for isolation of some bioactive substances with bioactive properties. Some of them involve certain bio-transformation processes mediated by enzymes and microorganisms (Kim and Mendis, 2006[35]). The process by which microorganisms and their enzymes bring about these desirable changes in food materials is known as fermentation. Technically, fermentation is a metabolic process in which an organism converts a carbohydrate, such as starch or a sugar, into an alcohol or an acid. The bacteria use food as a substrate for their propagation. Natural fermentation precedes human history; the earliest evidence of a fermentation process started with the production of yogurt, bread and wine (Hutkins, 2008[36]). Since the earliest evidences, fermentation, applied to foods, was mainly processed to enhance properties such as the taste, aroma, shelf-life, texture and nutritional value of foods. Nowadays, fermentation and enzymatic digestion represent the most utilized biotechnological approach for the development of new bioactive ingredients (Shahidi, 2009[37]). There are several industrial applications of fermented products. Fermentation has been reported to lead to antioxidant peptides in soybean (Wang et al., 2014[38]), polyphenols like theaflavins and tearubigins in black tea (Halder et al., 1998[39]), isoflavanoids in beer (Lapcik et al., 1998[40]) and phenolic compounds in beans (Chung et al., 2002[41]), flavonoids, flavonols, and several phenolic acids contained in red cabbage sprouts (Hunaefi et al., 2013[42]). Fermented soybean products such as natto, tempeh, and douche also contain antioxidative peptides due to the action of fungal proteases (Wang et al., 2008[43]). During the fermentation of soybean by fungi, isoflavone glycosides are hydrolysed to release free isoflavone aglycone by fungal β-glucosidase (Chiou and Cheng, 2001[44]) and therefore render health benefits to fermented soybean products. Black tea fermentation has been reported to lead to the formation of theaflavins and thearubigins, which are polyphenols that account for the various characteristics of black tea. The volatile aroma in the black tea is also associated with fermentation, which leads to the formation of volatile aroma aglycones (Halder et al., 1998[39]). It is widely known that tea possess antioxidant, anticancer, anti-inflammatory and antimicrobial properties; all these activities may be associated with fermentation (Shahidi, 2009[37]). In fermented marine food sauces such as, blue mussel sauce and oyster sauce, enzymatic hydrolysis has already been done by microorganisms, and bioactive peptides can be purified without further hydrolysis. In addition, marine processing by-products contain bioactive peptides with valuable functional properties (Kim and Mendis, 2006[35]).

Besides the use of fermented products, the addition of a specific enzyme to some plant derivatives has been recognized in the food industry, and the main objective is to improve the utilization of nutrients from raw materials, leading to value-added processed products. Over the last decade, the use of plant protein hydrolysates in human nutrition has broadly expanded. They are often used in different nutritional formulations, such as supplementation of drinks to enhance their nutritional and functional properties, or special medical diets. Proteins are essential components because they are a source of amino acids useful for growth and maintenance and provide functional properties to foods. Due to the increasing costs and limited supplies of animal proteins, and since vegetable protein is the most abundant source of protein on Earth, a number of vegetable proteins have been investigated for possible incorporation into formulated foods (Hrckova et al., 2002[45]). In general, food proteins are hydrolysed for many reasons ranging from the improvement of nutritional and functional properties, texture characteristics to the removal of odour, flavour, and toxic or components with low nutritional value. Since the obtained peptides have a smaller molecular size than proteins, thus their functional properties are different: increased solubility over pH range, decreased viscosity, and significant changes in foaming, gelling and emulsifying properties. Most of the basic research on food protein hydrolysis is based on soy protein (Cui et al., 2013[46]). Despite the long history of

fermentation technology, fermented food items produced from algae have yet to be developed. Many studies were conducted on methane fermentation of seaweeds during the 1970s and 1980 (Aquaculture Associates, 1981[47]), but few researches were conducted on fermentation of algae for foods production. Nowadays, there is great interest on the potential use of microalgae to produce biofuels through biotechnological technologies (Goh and Lee, 2010[48]**)**. A recent study on *Chlorella* cell wall degradation enhances its using in biofuels production (Gerken et al., 2012[49]). Under nitrogen starvation, *Scenedesmus obliquus* is able to accumulate carbohydrates to be used for bioethanol production (Ho et al., 2012[50]). One of the few examples of cosmetic application of a fermented algae is represented by peptides or protein hydrolysates derived from *Porphyra* spp. and wakame seaweeds under appropriate conditions (Hagino and Saito, 2010[51]).

1.2 *Arthrospira maxima* - Spirulina

Arthrospira maxima, usually named Spirulina, is the best known and most cultivated genus of Cyanobacteria because of its unique nutritional properties. Cyanobacteria are Gram-negative photosynthetic prokaryotes with a long evolutionary history. Spirulina is a ubiquitous blue-green microalgae, where cells are joined from end to end to form twisted filaments (Fig. 2). They are generally found in tropical and subtropical regions in warm water bodies with high alkalinity. The blue-green colour of these microalgae is due to a mix of photosynthetic pigments like chlorophyll, carotenoids and phycocyanin, the latter one is responsible for the blue color of the organism.

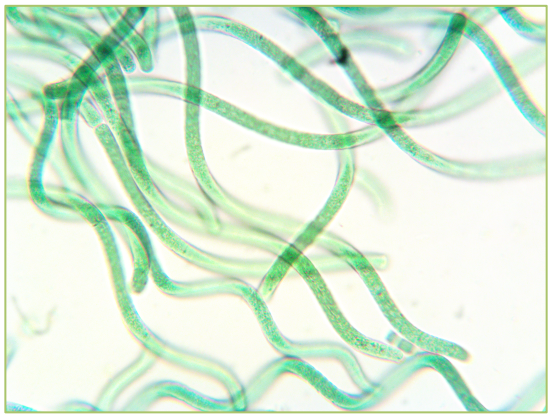


Figure 2 . Spirulina maxima cultivated in a microalgae pilot plant at Agriculture Department of University of Naples (Portici, Italy).

The blue-green microalgae Spirulina, was an important source of nutrients in the traditional diet of natives of Africa and Mexico. It has been found to be a rich natural source of proteins, carotenoids, Ω-3 and Ω-6 polyunsaturated fatty acids, provitamins, and other nutrients such as vitamin A, vitamin E, and selenium (Wu et al., 2005[52]). Two main species are known: *A. plantensis* and *A. maxima*. *Arthrospira* whole biomass and extracts are reported to show various biological properties due to their bioactive compounds. Proximate analysis of Spirulina was started in the 1970s and it indicated that this microalgae was an excellent source of proteins and was also rich in other nutrients (Becker, 2007[8]). Table 3, indicates the different proximate analysis results from different institutions and producers. The entire genome of *Arthrospira maxima,* with a genome size of 5.4 Mb, has been sequenced and is available through the Departments of Energy's Joint Genome Institute in raw form.

Component	FOI, France	SAC, Thailand	IPGSR, Malaysia	BAU, Bangladesh
Crude protein	65	55–70	61	60
Soluble carbohydrate	19		14	
Crude lipid	4	5–7	6	7
Crude fiber	3	5–7		
Ash	3	3–6	9	11
Moisture		4–6	6	9
Nitrogen free extract (NFE)		15–20	4	17

FOI = French Oil Institute; SAC = Siam Algae Co. Ltd; IPGSR = Institute of Post-graduate Studies and Research laboratory, University of Malaya; BAU = Bangladesh Agricultural University

Table 3: Proximate analysis of Spirulina. Source: FAO 2008.

1.2.1 History

Arthrospira maxima has a history of being consumed along the alkaline lakes of Chad and Niger and also by the Aztecs living in the valley of Mexico. The Aztecs referred to it as Tecuitalat meaning stone excrement, while in Chad it was called Dihe and was sold in the local markets as dried cakes.

The first commercial large-scale production of Spirulina was in the early 1970s in Texcoco, Mexico. Nowadays, it is commercialized in the market of most countries around the world. The largest commercial producers of Spirulina are located in the United States, Thailand, India, Taiwan, China, Pakistan, Burma (a.k.a. Myanmar), Greece and Chile.

1.2.2 Spirulina culturing and industrial applications

Spirulina grows quickly and produces 20 times more protein by surface unit than soy beans (Henrikson, 1994[53]). When comparing the growth of Spirulina and the agricultural crop cycles, the difference in the time of production is noticeable. In agriculture, the harvest is obtained after several months of cultivation, while Spirulina is continually produced (Switzer, 1980[54]).

The cultivation and the production of Spirulina can be in closed or in open systems; closed systems include laboratory photo bioreactors while the open system includes ponds (Fig. 3).

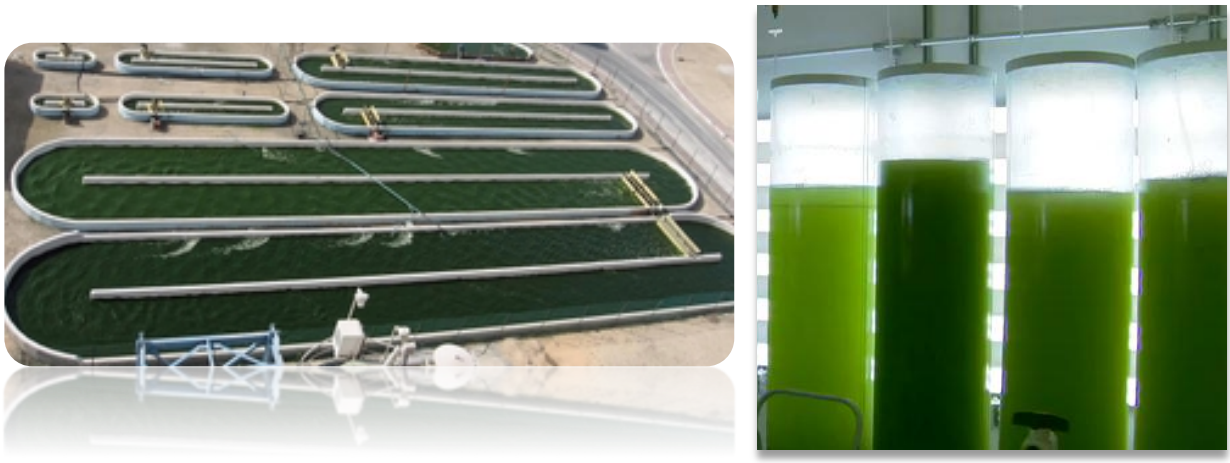


Figure 3: Different techniques of production of Spirulina: in open pond (on the left) and in bioreactors (on the right).

Zarrouk medium (Zarrouk, 1996[55]) is the most commonly used medium for Spirulina and also preferred for industrial production. The main factors to consider during the cultivation of Spirulina are: luminosity, temperature, inoculation size, stirring speed, dissolved solids, pH, water quality, macro and micro nutrients presence (Sanchez et al., 2003[56]). In particular, Spirulina, such as other microalgae, are organisms that live in complex habitats submitted to extreme conditions (changes of salinity, temperature, nutrients, UV-vis irradiation). Therefore, they must adapt rapidly to the new environmental conditions to survive, producing a great variety of secondary metabolites, biologically active, which cannot be found in other organisms. Moreover, it is possible to induce microalgae to synthesize desired compounds by changing environmental factors, like temperature, illumination, pH, CO_2 supply, salt and nutrients. It means we could use them as natural reactors, leading to an enrichment of some bioactives (Plaza et al., 2009[57]).

Spirulina has found wide applications in diverse areas such as agriculture, food, pharmaceuticals, perfumeries, medicine and science. Since the past, it is used as fertilizer of depleted soils because of its natural nitrogen content (Littler et al., 2006[58]). The biomass of blue-green algae is also used as feed for farm animals and birds (Spolaore et al., 2006[1]). *Arthrospira*, like other microalgae, has been considered recently as a promising biomass feedstock with great potential for biodiesel production, especially because of its easy culture system, it can achieve high cell densities and can produce fatty acids efficiently (EL Shimi et al., 2013[59]). Biologically produced fuel is gaining popularity as fossil fuels are becoming more expensive and scarce. Therefore, its role in bioremediation and wastewater treatment is an emerging area of interest (Fig. 4).
Moreover, Spirulina has a long history of safe usage as food and food supplement because of its nutritional value (Belay, 2002[60]). It is classified as GRAS (Generally Recognized as Safe) by FDA (Food and Drug Administration), which ensures its use as food without risk to health.

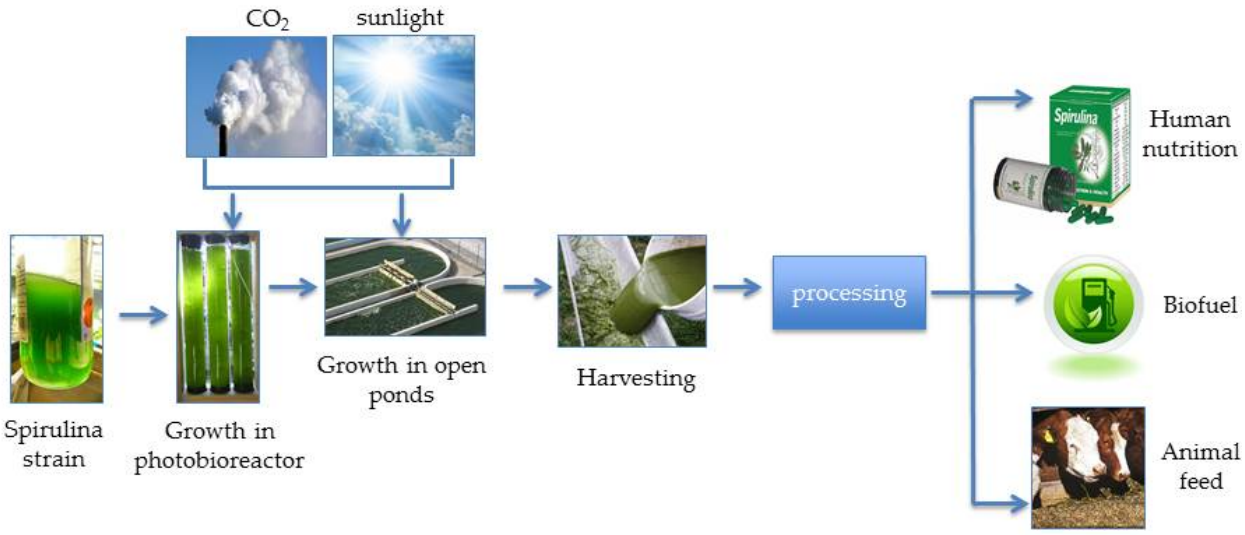


Figure 4. Examples of industrial applications of Spirulina.

Nowadays, a lot of attention is focused on the use of nutraceuticals, which includes food and supplement products that claim to provide health benefits beyond their basic nutritional value (Kim and Wijesekara, 2010[61]). Faithful to this model, the cosmetic industry during the past 10-15 years has observed a gradual shift in the use of ingredients towards more biologically derived products or products of fermentation processes. Today, consumers are more aware of nutritional products that contribute to both skin health and disease prevention. Cosmetics companies are making huge investments in biotechnology to create advanced products able to promote welfare and quality of life, with an extra touch of nutrition.

However, there is growing demand to isolate new function proteins or bioactive peptides from microalgae. Therefore, much of the work on bioactivity of microalgae especially on Spirulina has been on the entire biomass (Harnedy and FitzGerald, 2011[62]). Some algae species have induced biological activities, which are associated with proteins, protein hydrolysates or peptides that can affect their standing beyond their nutritional values. Their bioactivities make them potential candidates for multifunctional applications such as functional food, nutraceuticals, pharmaceuticals and cosmetic ingredients.

It has been claimed that Spirulina and its extracts have various health promoting effects: such as preventing cancers, the alleviation of hyperlipidemis, suppression of hypertension, stimulate the immunological system, growth promotion of intestinal *Lactobacillus*, reduce the nephrotoxicity of pharmaceuticals and toxic metals and provide protection against the harmful effect of radiation (Belay et al., 1993[63]), suppression of elevated serum glucose level. These properties have been attributed to different compounds such as phenolics, phycobiliproteins, carotenoids, organic acids, sulphated polysaccharide spirulan and polyunsaturated fatty acids (Vonshak, 1997[64]; Khan et al., 2005[65]). For these reasons, *A. platensis* is widely used in commercial cultivation (Dejsungkranont et al., 2012[66]).

Besides, it has also been reported to have antimicrobial activities against some pathogenic bacteria (Mendiola et al., 2007[67]). Antioxidative phycocyanin and phycocyanobilin are isolated from *Arthrospira platensis*. Phycocyanin, one of the major constituents of *A. platensis* responsible for its biological activity, has significant antioxidant and anti-inflammatory properties (Bhat and Madyastha, 2000[68]). Phycocyanobilin, an open chain tetrapyrrole chromophore covalently attached to the apoproteina constituent of phycocyanin in *A. platensis*, plays an important role in some of the biological properties exhibited by phycocyanin. Thus, the characterization of phycocyanobilin and its separation from *A. platensis* are worthwhile for increasing its attractive biological and commercial possibilities.

Concerning the cosmetic application, the high protein content of Spirulina represents a requested source of essential amino acids that constitute collagen and, moreover, can contribute to the maintenance of the hydrated and moisturized state of the skin (Microlife[69]).

Spirulina is also one of the few microorganisms that contain γ-linolenic acid, which is a rare fatty acid that is believed to prevent the frequency and severity of acne outbreaks and keep skin fresh and healthy looking.
Cosmetics companies use biotechnology both to discover and manufacture cosmetic active ingredients, which are then evaluated for efficacy on skin cell cultures and on human skin *in vivo*. Among the range of activities highly requested by the cosmetic market, particular attention is focused on products having high antioxidant activity, in term of protection of the skin against free radicals, and high hydration capacity, which is the ability to retain and uniformly distribute the moisture throughout the different skin layers.

AIM OF THE THESIS

2. AIM OF THE THESIS

Microalgae are one of the most promising sources of active ingredients for industrial applications, such as functional food and skin care products. They contain a wide range of secondary metabolites that can be used as biologically active compounds.

In the present study we chose Spirulina (*Arthrospira maxima*) as source of active molecules to develop products with potential applications in human health care. Since ancient times, Spirulina has been used as nutraceutical product thanks to its high content of protein. Based on this assumption, Spirulina was subjected to biotechnological processes, such as auto-fermentation, lactic acid fermentation and enzymatic digestion. The main goal of my PhD thesis was characterizing biologically and comparing the different products derived from these processes to develop new active ingredients with specific properties for food and/or cosmetic applications.

Arterra Bioscience srl, an Italian research-based biotech company, was chosen as partner of my PhD program, since it has strong expertise in developing new active ingredients with industrial applications, ranging from cosmetics to agriculture.

Spirulina biomass was cultured in open ponds at Agriculture Department of University of Naples, then fermented at Microbiology Department of University of Naples (Portici) in collaboration with the group of Prof. Ercolini. Finally, Spirulina derived extracts were analysed for the different biological activities, concerning antioxidant, hydration and moisturizing activities, at laboratories of Arterra Bioscience srl.

MATERIALS AND METHODS

3. MATERIALS AND METHOD

3.1 Cultivation of Spirulina

Arthrospira maxima (Spirulina), strain SAG 84.79 (University of Göttingen, Germany), was cultivated in a microalgae pilot plant at Agriculture Department of University of Naples (Portici, Italy) in July of 2012. Spirulina was cultured in open pond (deep 25 cm, total volume 2500 L/pond) using Zarrouk medium (Zarrouk, 1966[55]) and the parameters at harvesting were: pH 10.2 ± 0.2, temperature 27.5 ± 2.2°C, mean daily irradiation of 25.3 MJ/m^2/die, OD_{560} 1.0±0.1, conductivity 24.2 ±1.4 mS/cm. $NaHCO_3$ and NaCl were food grade; KNO_3 and K_2HPO_4 were technical grade; K_2SO_4, $CaCl_2$, EDTA, $MgSO_4$, $FeSO_4$ and A5 solution were of analytical grade supplied by Sigma Aldrich (Milan, Italy). The biomass was harvested using a pre-filter of 380 µm and a nylon filter of 47 µm and washed three times with sterile water to reduce salts content. After washing Spirulina biomass was pressed to remove the highest possible amount of interstitial water, packed in plastic bags and finally frozen at -20 °C. Dry weight (DW) of samples was obtained gravimetrically at 105 °C overnight. Nutritional analyses made with standard methods on Spirulina dried in vented oven at 52 °C for 5 h are reported in table 4. The tools in contact with the microalgae were properly sterilized in order to minimize the possible contamination throughout the process. Microbiological analyses were performed on Spirulina in tanks (St) after filtration (Sf) and after pressing (Sp).

Nutrition values of *A. maxima* oven-dried **Values in g/100 g of products**	
Protein	56.8
Total Fat	6.6
Saturated Fat	2.7
Total carbohydrate	16.9
Dietary fiber	7.3
Moisture	9.24
C-phycocyanin	6.3
Total carotenoids	0.84
β-carotene	0.32
chlorophyll	0.87
Calcium	0.46
Iron	0.12
Potassium	1.1
Vitamin B12 (mg/100g)	2.6
Calories (cal/100g)	354.2

Table 4. Nutritional analysis of *A.maxima* dried in vented oven at 52 °C for 5 h.

3.2 Sample preparation

Fresh biomass containing 30g of dry Spirulina per litre of distilled water was prepared. Further, this initial preparation was used to obtain various samples, as reported in the flowchart of figure 5. The samples were denoted as reported in table 6.

3.2.1 Crude extract

Fresh biomass has been thawed in the refrigerator at 4 °C. The biomass was then diluted with MilliQ water to speed up the thawing and breaking. For an efficient extraction, some glass-beads were put inside the biomass sample. The extracts were centrifuged at 4000 rpm for 15 minutes to separate the pellet from the supernatant. For the purpose of this thesis, Spirulina extract refers to the supernatant sample that was lyophilized directly without being subjected to other processes.

3.2.2 Auto-fermented sample

The initial biomass (30g/L) was set to undergo natural fermentation without addition of inoculum. Biomass contains some native bacteria that provoke fermentation in culture conditions. The microflora associated with Spirulina crops is generally scarce and non-pathogenic. In fact, the high alkalinity of the crop environment (pH 10-11) is an excellent barrier against contamination, e.g. by bacteria, yeast, fungi or algae. The fermentation was allowed to take place at 37 °C and samples were taken at time 0 and 24 hours. These samples were denoted as SEA (Spirulina extract auto-fermented).

3.2.3 Fermentation mediated by Yovis®

The initial biomass was divided into 2 batches and subjected to autoclaving at 121 ºC for 15 minutes. One of the samples was subjected to analysis after autoclaving without further treatment (no inoculation) and was denoted as SEA. The other batch was fermented using a commercial probiotic mix Yovis®. 1g of Yovis® was dissolved in 100ml of solution and then added (800ml) to the autoclaved Spirulina biomass. The biomass was then incubated at 37 °C and samples were taken at time 0 and 48 hours. This sample was denoted as SEA-Y.

3.2.3.1 Commercial Probiotic mix (Yovis®)

Yovis®, a commercial probiotic mix from Sigma-Tau (Italy), was used for the fermentation of samples. 1g of the mix contains the following concentration of freeze dried bacteria:

- *S. salivarius* subsp. *Thermophilus*. minimum 2.04 x 10^{11} cfu
- *Bifidobacteria (B. breve, B. infantis, B. longum*) minimum 9.3 x 10^{10} cfu
- *L. acidophilus* minimum 2 x 10^{9} cfu
- *L. plantarum* minimum 2.20 x 10^{8} cfu
- *L. casei* minimum 2.20 x 10^{8} cfu
- *L. delbrueckii* subsp. *bulgaricus* minimum 3 x 10^{8} cfu
- *S.faecium* minimum 3 x 10^{7} cfu

3.2.4 Enzymatic digestion

Spirulina suspension was prepared by adding 25 ml of distilled water to 1 g of Spirulina. The suspension was adjusted to pH 6 using 1 N NaOH and stirred in the presence of magnetic beads for 4 hours at 10-15 °C to provoke microalgae breaking. For carbohydrates treatments, Spirulina suspension was mixed with 50 mM citrate buffer (pH 5.0) in 125-ml Erlenmeyer flasks at a loading rate of 2 % w/v. Hydrolysis was carried out in a water bath at 50 °C for 2 hours. All treatments were conducted in triplicate. Aliquots (500 µl) were taken to measure the amount of sugar content.

Then, 10 ml of 1 M potassium phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.5) and sodium azide (0.1%, w/v) was added to the mixture and stirred using a magnetic stirrer. The pH of the mixture was adjusted to 7.5 with 1 N NaOH. The flask was then placed in a water bath shaker operating at 140 rpm. The enzymatic hydrolysis was started by adding 420 mg (1259 U/L) of Immobilized Alcalase (Novozyme, Novo Nordisk, Bagsvaerd, Denmark) and carried out for 2 h. During hydrolysis, 100 µl samples were taken at different time points and the increase of the formed

primary ammines was measured through OPA test.

3.2.4.1 OPA assay

In order to understand if enzymatic digestion was overcome, an OPA (o-Phthaldialdehyde) assay was performed. A solution of 10 mg of o-Phthaldialdehyde, 10 μl of β-mercapthoethanol, 200 μl EtOH, 20 ml Na_2CO_3 50 mM pH 10.5 was prepared. A series of dilutions ranging from 0 μg/ml to 2 μg/ml of Glycine (Gly) were made using deionized water. Samples and standards were placed in cuvettes with 1 ml of OPA reagent solution and incubated for 2 minutes, thoroughly shaking at room temperature. The OPA solution reacted with the primary amino groups and generated a product with absorbance at 340 nm. The quantity of the product was determined using a spectrophotometer.

3.2.5 Centrifugation

After several processes, all the samples were centrifuged at 4000 rpm for 15 minutes to separate the pellet from the supernatant. The later was used in subsequent experiments. The supernatant was used based on the hypothesis that it contains molecules such as small peptides, reported in literature to possess high bioactivity.

3.3 Microbiology analysis

The objective of this experiment was to determine the presence and activity of microorganisms involved in the fermentation process of Spirulina. The samples used for the microbiological analysis were directly taken at time 0 and at time points of 24 and 48 hours after the fermentation. The samples were not subjected to centrifugation and various dilutions were prepared using ringer solution and standard plate count method.

3.4 Oxygen radical absorbance capacity (ORAC assay)

In order to calculate the antioxidant power of the extracts, we performed an *in vitro* Oxygen Radical Absorbance Capacity (ORAC) assay. 25 μl of compound dilution in phosphate buffer was aliquoted into 96 well plate and 150 μl of fluorescein solution, 8.5 nM in phosphate buffer, was added to each sample. After incubation at 37 °C for 15 min, 25 μl of AAPH solution (153 mM in phosphate buffer) was pipetted into each well and the progress of the reaction was monitored at 535 nm, using a fluorescence multiwell reader (EnVision, PerkinElmer). The fluorescence was measured every minute for 60 min. Antioxidant power of the mixture was calculated according to the method described by Huang et al., 2002[70]. The net area under the curve (AUC) of the samples and standards, represented by different dilutions of 6-hydroxy-2,5,7,8-tetramethylchroman2-carboxylic acid (Trolox) was calculated. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Net AUC was obtained by subtracting the AUC of the blank from that of the sample or the standard. ORAC values of the samples were expressed as μmol of Trolox equivalents (TE) per g of lyophilized extract.

3.5 SDS-PAGE analysis

Electrophoresis of all extracts fractions were carried out in the Mini-PROTEAN II (BIO-RAD, USA) using 15% polyacrylamide gel with SDS (SDS- PAGE) (Laemmli, 1970[71]). The concentration of the stacking gel was 5%. The marker (BIORAD, Precision Plus Protein M.W. 10kDa-250kDa) was used to identify the approximate size of the bands. The samples were prepared by adding 30 μl of loading buffer (SDS reducing buffer: 125 mM Tris-HCl, pH 6.8; 4% of SDS, 10% β-mercaptoethanol, 20% glycerol, 0.2% (w/v) of bromophenol blue) to 10 μg of protein of each extract and heating for 5 min at 95 °C. Further, 30 μl of each sample and 10 μl of marker were applied onto gel into different wells. Following electrophoresis (100 V for 60 min in Tris-glycine pH 8.4) the gel was stained in 0.25% (w/v) Coomassie blue R-250 (Sigma) in methanol:acetic acid:water solution (50:10:40) for 1 h and destained with methanol:acetic acid:water solution (50:10:40) to get rid of the background.

3.6 BIOACTIVITY ANALYSIS

3.6.1 Cell viability assay

In order to estimate the cytotoxicity of the different preparations, we performed cell viability assay (MTT) on murine immortalized fibroblasts (NIH_3T_3, murine embryonic fibroblasts, ECACC, Salisbury, UK) and on immortalized keratinocytes (HaCaT), according to the method described by Mosmann et al. (1983[72]). The MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) enters the cells and passes into the mitochondria, where it is reduced to an insoluble, colored, dark purple formazan product. Only viable cells with active metabolism convert MTT into the formazan product. Treatments were performed with different concentrations of Spirulina extracts (from 0.5 to 0.01 g/L). The keratinocytes were seeded at density of 1.8 x 10^5 and fibroblasts at density of 1.5 x 10^5 in the 96 well plates. After 8 hours, the ingredients were added to the cells for 48 hours. The plates were incubated at 37 °C and 5% CO_2. After 48 hours of treatments, the media was carefully removed and the cells rinsed with PBS (Phosphate buffered saline). The cells were incubated with 100 µl/well of sterile MTT solution I, containing: 10 mM Hepes, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM glucose and 0.5 mg/ml of colorimetric substrate (MTT) in PBS buffer at pH 7.4. The plates were incubated for 3 hours a 37 °C in dark. The cells were then solubilized with 100 µl/well of MTT solution II, containing: 10% TritonX100, 0.1 N HCl in isopropanol, and the plate incubated overnight at room temperature. The developed colour was then quantified at 595 nm by a multiwell spectrophotometer Victor3 (PerkinElmer). The absorbance values were normalized by setting the control group (untreated cells) to 100%. Subsequently, the viability of the treated cells was expressed as a percentage of untreated cells (Fig. 8).
All materials were obtained from Sigma-Aldrich.

3.6.2 Membrane ROS (Lipid peroxidation)

1.5×10^4 NIH_3T_3 cells, maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS under 5% CO_2 at 37 °C, were seeded in 96 well plates and grown for 20 h to let them attach to the bottom of the well. The cells were then incubated with different concentrations of Spirulina extracts or Tocopherol (Vitamin E) 100 µM, used as positive control, for 2.5 h. At the end, the cells were washed in PBS and the cell auto-fluorescence value (background) measured at the plate reader, using 490 nm as excitation and 535 nm as emission wavelength. The cells were then incubated with the dye C11-BODIPY (Invitrogen) at 37 °C for 30 min. After an additional wash in PBS, the cells were treated with H_2O_2 150 µM and incubated for 30 min. The fluorescence of the samples was thus measured using the instrument EnVision (PerkinElmer), and from each measure the cell auto-fluorescence value was subtracted. The values obtained from stressed cells (with the pre-treatment with the compounds) were expressed as percentage to the stressed control (without compounds treatment), set as 100%.

3.6.3 DNA Repair/Protection (COMET assay)

The comet assay (single-cell gel electrophoresis) is a simple and sensitive technique for the detection of DNA damage at the level of the individual cell. The term "comet" refers to the pattern of DNA migration through the electrophoresis gel, which often resembles a comet, where the length of the comet tail (damaged DNA) relative to the head (nuclear region), reflects the number of DNA breaks.
Fibroblasts NIH_3T_3, maintained in DMEM with 10% FCS under 5% CO_2 at 37 °C, were plated at density of 1.5 x 10^5 per well in 6 well plates, for 6 hours. After 18 h of incubation with test compounds, cells were treated with H_2O_2 175 µM for 2.5 h and detached from the plate using 1 ml/well of cell dissociation non-enzymatic solution. Cells were transferred to Eppi-tubes, spun at 1500 rpm and washed once with PBS. The cell pellet was resuspended in 20 µl of PBS and 80 µl of Low Melting point Agarose (LPMA) was added to each tube, and the solution immediately dropped onto a Normal Melting Agarose (NMA) pre-coated slide. Coverslip slides were placed on the top, without squeezing the cells. Then, coverslips were gently slid off without scraping the agarose layer containing the cells. Thus, the slides were placed in cold Lysis solution for at least 2 h at 4 °C and then left 10 min in an electrophoresis

tank, filled with an alcaline buffer for 10 min to allow DNA unwinding and the expression of alkali-labile damage, and then power supply was turned on to 24 volts. Slides were electrophoresed for 20 min and at the end placed in cold Neutralization buffer for at least 10 min. After drying the slides, cells were stained with a 10 μg/ml solution of ethidium bromide, covered with a coverslip and scored at a fluorescence microscope.

3.6.4 Hydration and moisturizing activity

3.6.4.1 Semi-quantitative Reverse Transcriptase/Polymerase Chain Reaction (RT/PCR)

1.5 x 10^5 HaCaT cells, maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS under 5% CO_2 at 37 °C, were seeded in 6 well plates and grown for 6 h to let them attach to the bottom of the well. The cells were then incubated for 6 hours with the test compounds, and then collected for total RNA extraction. Total RNA was extracted with the GenElute Mammalian Total RNA Purification Kit (SIGMA-ALDRICH, Milan, Italy) according to the manufacture's instructions and treated with DNAse I at 37 °C for 30 min to eliminate any contaminating genomic DNA. The first strand cDNA was synthesized from 1-2 μg using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). RT-PCR was performed using gene specific primers and the QuantumRNA™ 18S internal standard (Ambion) according to manufacturer's instructions. The QuantumRNA™ kit contained primers to amplify 18S rRNA along with competimers that reduced the amplified 18S rRNA product within the range to allow it to be used as endogenous standard.
The PCR products obtained were loaded on 1.5% agarose gel, and the amplification bands were visualized and quantified with the Geliance 200 Imaging system (Perkin Elmer). The amplification band corresponding to the gene analyzed was normalized to the amplification band corresponding to the 18S. The values obtained were finally converted into percentage values by considering the measure of the untreated controls as 100%.

Gene Specific Primers used:

AQP3-Fw: 5' GATCAAGCTGCCCATCTA 3'

AQP3-Rv: 5' TGGGCCAGCTTCACATTCT 3'

HAS3-Fw: 5' AGCCTATGTGACGGGCTA 3'

HAS3-Rv: 5' TCCCCACTTCTGCATGATG 3'

FLG-Fw: 5' AGAGCTGAAGGAACTTCTGG 3'

FLG-Rv: 5' GTGTCATAGGCTTCATCC 3'

SMIT-Fw: 5' ATGAGAGCTGTACTGGACAC 3'

SMIT-Rv: 5' GTCATGCCAATGAGCAGGAT 3'

3.6.4.2 Permeability assay

The water permeability assay was set up using a well established method as described by Zelenina et al., 2002[73], 2003[74], 2004[75].
1.8 x 10^4 HaCaT cells, maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS under 5% CO_2 at 37 °C, were seeded in 96 well plates and grown for 6 h to let them attach to the bottom of the well. The cells were then incubated with different Spirulina extracts or Retinoic acid 1 μM, used as positive control, for 18 h.
The cells were loaded with 10 μM calcein-AM (calcein-acetoxymethyl ester, Santa-Cruz Biotechnology) for 1 h at 37 °C in Hanks' balanced salt solution (HBSS). Upon addition of calcein/acetoxymethyl ester, the dye enters the cells and becomes fluorescent on de-esterification. The poly-anionic nature of calcein renders its membrane impermeable, and thus it is retained within the cell under normal physiological conditions. After washing with PBS, the cells were alternatively stressed with sorbitol 500 mM and deionized H_2O with cobalt chloride 1 mM, which quenches the fluorescence outside the cell at physiological pH. The intensity of the remaining fluorescence in the

adherent cells was measured at 480 nm excitation and 535 nm emission by using a fluorescence multi-well plate reader (EnVision, Perkin Elmer). Fluorescence images were recorded after the stress and the relative fluorescence was quantified as percentage, considering the measurement of the stressed control as 100%.

3.7 Statistical analysis

The obtained results are average of three independent experiments each one conducted in triplicate. Data are presented as mean±SD for the different experiments while for the determination of differences, the student t-test GraphPad was used. Significant difference was set at $p < 0.05$.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

4.1 Samples preparation

Arthrospira maxima was grown in open pond, showing a daily productivity of 7.4 ± 3.5 g $m^{-2}d^{-1}$ (Langellotti et al., submitted[76]). Frozen Spirulina was aseptically added to ultrapure water (30 g/L) and was whisked with Mixer (Continental Equipment Super mixer and tilting, Asal 715) for 15 min. In order to obtain various samples, this initial preparation was divided into 3 batches.

1) One was pre-autoclaved (121 °C, 15 min) in order to decontaminate the biomass from indigenous bacteria. This sample was denoted as SEA (Spirulina Extract Autoclaved). An aliquot of autoclaved Spirulina was further incubated at 37 °C for 48 h with a probiotic mix Yovis®. The code of this preparation was SEA-Y (Spirulina Extract Autoclaved and Fermented with Yovis®), and in the bioassays its activity was compared with that of its control SEA. 2) A second batch of fresh biomass was set to undergo natural fermentation without addition of inoculum. Biomass contains some native bacteria that provoke fermentation in culture conditions. The fermentation took place at 37 °C for 24 hours. This sample was denoted as SEA-F (Spirulina Extract Auto-Fermented) and it was compared to its control Spirulinal Extract (SE), the untreated sample. 3) A third batch of fresh biomass was digested by carbohydrases and proteases, and it was denoted as SED (Spirulina Extract Digested). Also for this sample, the control was always Spirulina Extract (SE).

After treatments, all the samples were centrifuged at 4000 g for 15 min. Supernatants (crude aqueous extracts) were collected and lyophilized (Modulyo, 2000). The experiments are summarized in the flowchart of figure 5. The code of each sample in the experimental plan is reported in table 6.

All samples were filtered to 0.45 μm in order to obtain extracts without bacterial contamination necessary for further bioassays. The concentration of the samples was calculated considering the weight of the lyophilized supernatant.

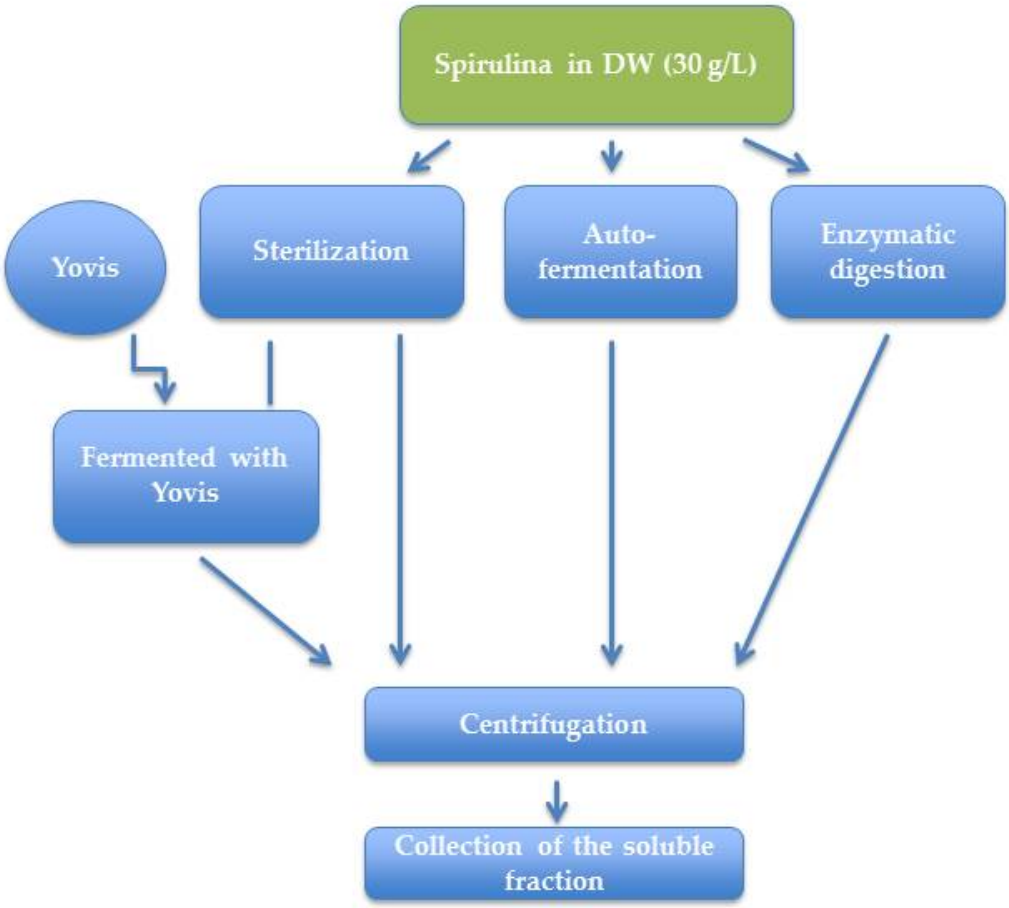


Figure 5: Flowchart of the experimental plan.

CODE	Sample Description
SE	Spirulina extract
SEA-F	Spirulina auto-fermented extract
SED	Spirulina enzymatically digested extract
SEA	Spirulina extract after autoclaving (control of SEA-Y)
Yovis®	Commercial probiotic starter culture
SEA-Y	Spirulina extract after autoclaving and fermented with Yovis®

Table 6: Samples description and codes.

4.2 Molecular mass of Spirulina extracts

In order to analyse the level of protein degradation of the obtained different fermented products, we performed a SDS-page by running the samples reported in table 6, in a 15% polyacrylamide gel and staining the protein bands by Coomassie blue. The band corresponding to the C-phycocyanin (17 kD), visible in the total extract in lane 2, was still visible in the lane 3 of the sample subjected to auto-fermentation, indicating that this type of process did not produce any degradation of the protein (Figure 7). On the opposite, the enzymatic digestion (lane 4), the autoclaving process (lane 5) and the autoclaving plus fermentation (lane 6) produced protein degradation, thus no clear protein band corresponding to the C-phycocyanin was detectable. This indicated that the C-phycocyanin, which represented almost 15% of the whole weight of the microalga, had been digested into smaller peptides by the sterilization process itself, by the bacterial proteases during the fermentation or by enzyme the Alcalase.

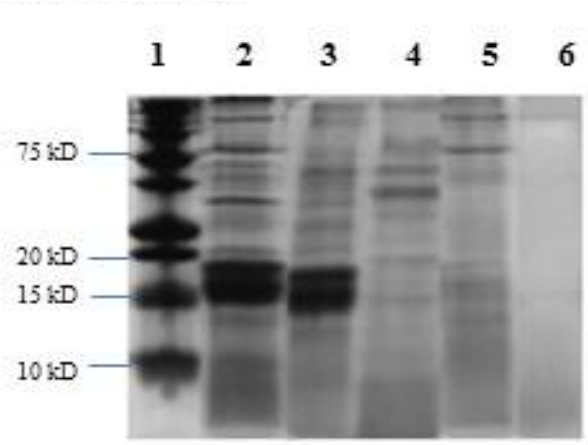


Figure 7. SDS-PAGE analysis of Spirulina extracts. Lane 1: Marker; lane 2: Spirulina total extract; lane 3: Spirulina after autofermenting process; lane 4: Spirulina derived by enzymatic digestion; lane 5: Spirulina after autoclaving process; lane 6: Autoclaved Spirulina after fermentation by Yovis® (10 μg of total proteins of each lane).

4.3 Spirulina citotoxicity

The Spirulina extracts, prepared as described in Materials and Methods and showed in table 6, were first tested in the MTT assay on NIH_3T_3 fibroblasts and HaCaT keratinocytes, to define the right concentrations of use in the following experiments and to determine the maximum non-cytotoxic concentration. The results showed that all the concentrations lower than 0.1 g/L did not affect the viability of the cells (Figure 8), and for all of them the DL50 was calculated as 1 g/L. Therefore, the concentrations of 0.1 g/L and 0.02 g/L were used for all the following cellular bioassays.

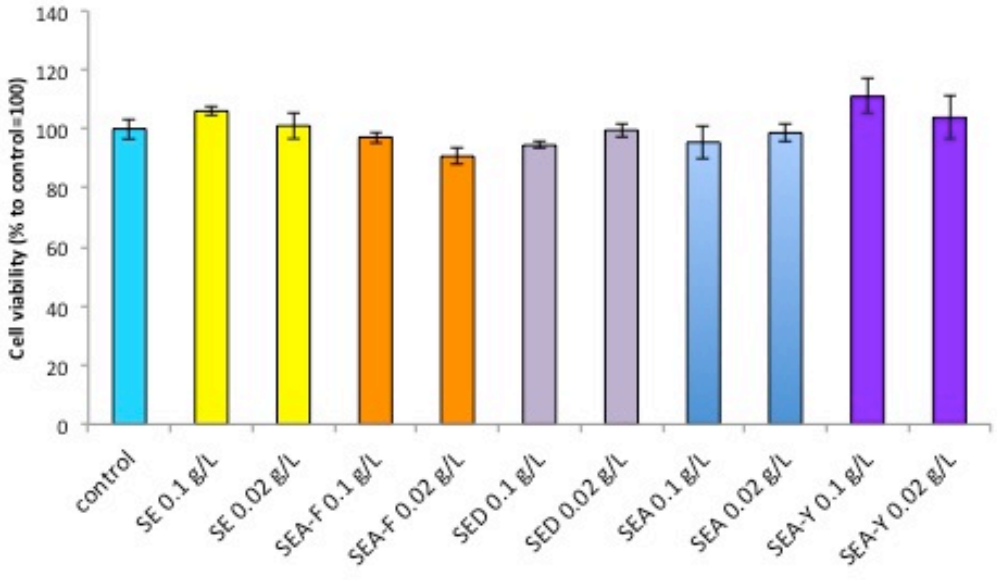


Figure 8: MTT assay. Fibroblasts were treated for 48 h with different concentrations of different Spirulina extracts. After treatments, the percentage of vital cells was measured for each sample using the MTT dye. The values are means of three independent measures obtained from one representative experiment.

4.4 Antioxidant effect of Spirulina derivatives

According to the literature, Spirulina, such as other microalgae, is considered as a rich source of proteins with antioxidant properties (Herrero et al., 2005[77]; Chidambara Murthy et al., 2005[78]; Tannin-Spitz et al., 2005[79]). To evaluate the capacity of Spirulina extracts to neutralize the dangerous free radicals in the cells, we conducted a series of experiments to measure the total antioxidant power of the mixture both *in vitro* and in cultured skin cells. Spirulina is well-known to have antioxidant properties, which are attributed to molecules such as phycocyanin, β-carotene, tocopherol, γ-linoleic acid and phenolic compounds (Chopra and Bishnoi, 2008[80]).

Wu et al. (2005[52]) reported that Spirulina extract has a higher antioxidant activity than the microalga *Chlorella*, because of its higher content of phenolic compounds. Thus, in our experiments we compared the fermented extracts of Spirulina with the extracts of total biomass in order to demonstrate the best antioxidant efficacy.

As *in vitro* assay we performed an ORAC (Oxygen Radical Absorbance Capacity), where the different dilutions of the samples were mixed with fluorescein and the reaction started by the addition of the oxidant AAPH. The value of reducing capacity was of 360 μmol TE/g for SE and 346 μmol TE/g for SEA. After the auto-fermentation of SE or fermentation by Yovis® of SEA, the ORAC values changed from 360 and 346 into 332 or 340 μmol TE/g, respectively. Thus, both the autoclaving process and the fermentation by Yovis® had no significant effect on the antioxidant power. Differently, the ORAC value of SED changed from 360 μmol TE/g into 380 μmol TE/g, indicating that the digested samples had a higher antioxidant capacity compared to the non-digested ones. This meant that probably during the digestion process the main components, proteins, vitamins and antioxidants molecules, were transformed or hydrolysed into simpler compounds with higher antioxidant power. Therefore, these ORAC values, ranging from 332 to 380 μmol TE/g, were higher than those reported for other antioxidant rich algal extracts (Li et al., 2001[81]; Plaza et al., 2010[82]).

Although the ORAC assay measures the reducing potential of the test materials, however it cannot provide any information on the antioxidant capacity in living cells. On the other hand, measuring the amount of Reactive Oxygen Species (ROS) present in the cell membranes by the ROS assay gives important clues about the real protective effect of the test materials under stress conditions.

ROS are also a physiological products formed during aerobic life as a result of the metabolism of oxygen, thus, they can also damage the cellular membrane or intracellular molecules (especially DNA) if not efficiently removed by the antioxidant defence mechanism of the cell (Cooke et al., 2010[83]).

To evaluate the capacity of Spirulina extracts to scavenge the ROS produced on the cell membrane after oxidative stress, we treated mammalian immortalized keratinocytes (HaCaT) with two different concentrations of the extracts. The results indicated that the amount of ROS produced in the cells after treatment with 450 μM H_2O_2 was significantly reduced by Spirulina extracts. The extracts SEA-F and SEA-Y at both the concentrations and SEA at 0.1 g/L inhibited ROS formation, which was comparable to the effect produced by Tocopherol, a compound known for its strong antioxidant power on the cells. SE showed a modest inhibitory effect on ROS production (17%) when used at 0.02 g/L. The results of the ROS assay indicated that the extracts subjected to the autoclaving process had a higher protecting capacity against ROS compared to the untreated ones, probably because the heating resulted in a change of the nature and the size of the compounds originally present in the extract SE. Moreover, fermentation by Yovis® restored the antioxidant capacity of SEA by 40% at 0.02 g/L.

The auto-fermenting process enhanced the capacity of Spirulina to protect the cell membranes against ROS production. At both concentrations of 0.1 g/L and 0.02 g/L, the extracts reduced the amount of ROS, induced by H_2O_2 treatment, by about 33% ($p<0.05$). Finally, when the cells were pre-treated with SED, at both the concentrations, the keratinocytes were protected and the ROS production was decreased by about 25%. These results suggested that also the enzymatic digestion improved the antioxidant capacity of the Spirulina extracts, even if in a smaller extend than the auto-fermentation and Yovis® fermentation processes.

The attenuation of the H_2O_2 induced stress obtained after treatment with Spirulina extracts could be explained both by a direct interaction with the stressing agent (the compound mixture may interact with H_2O_2 neutralizing its oxidizing action on the cellular components) and by an activation of the defence response mechanism to stress by triggering specific signaling pathways in the cells.

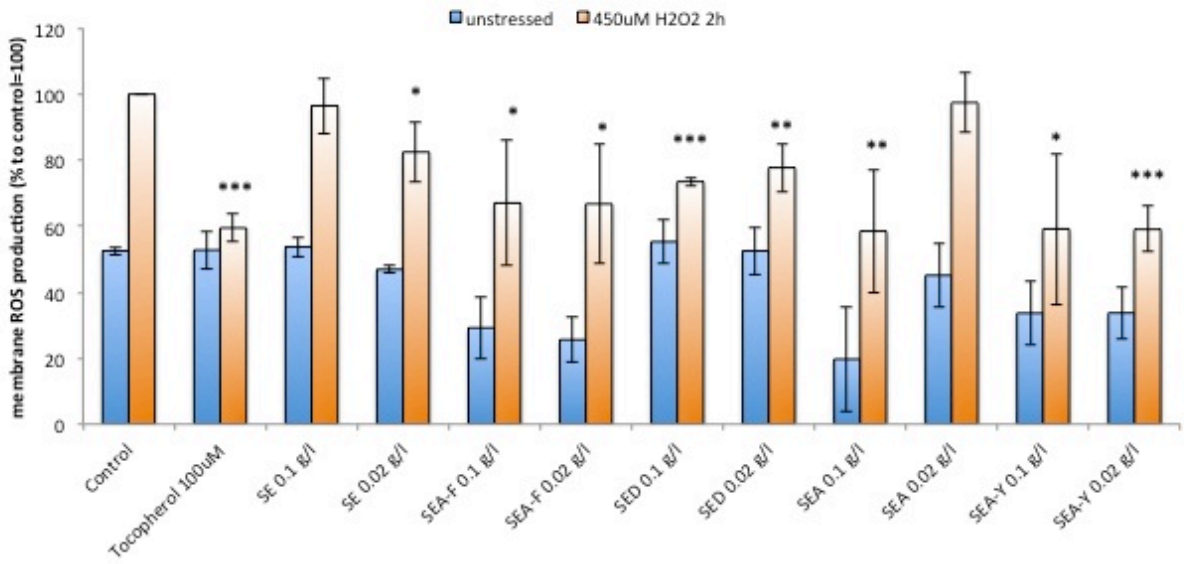
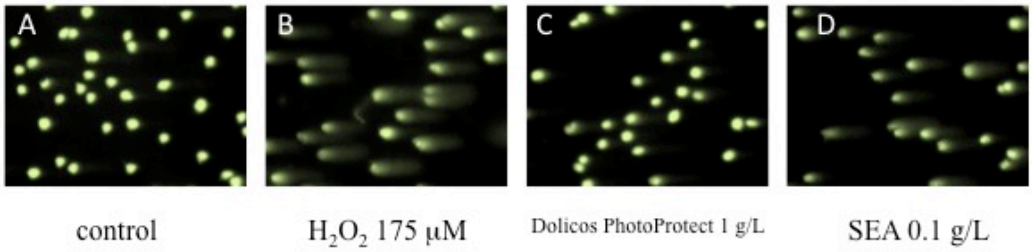


Figure 9. Lipid peroxidation in HaCaT cells after incubation for 2.5 hours with the Spirulina extracts and Tocopherol. Data are presented as percentage to the stressed control without treatment, arbitrarily set as 100%.

In order to confirm the ability of Spirulina extracts to protect the cells against oxidative stress, it was measured the

amount of nuclear DNA damage after hydrogen peroxide treatment by performing a Comet assay. The cells were first treated with different Spirulina extracts for 18 hours and then stressed with H_2O_2 175 μM. This assay allowed to evaluate the amount of DNA damage by measuring the length of a fluorescent nuclear "comet tail", which was proportionally related to the entity of the DNA damage produced in the cell nuclei.
The comet tail length was significantly reduced ($p<0.05$) from 30 to 40% in the cells pre-treated with SEA-F and SEA underlying that the DNA protective capacity of the extracts was preserved during the sterilization process. On the other hand, the enzymatically digested Spirulina (SED) did not show any protective effect on DNA from H_2O_2 induced damage. As positive control of the assay, an extract derived from *Dolichos biflorus* cell cultures, already known for its effect to shield H_2O_2 induced DNA damages, was used (Bimonte et al., 2013[84]).



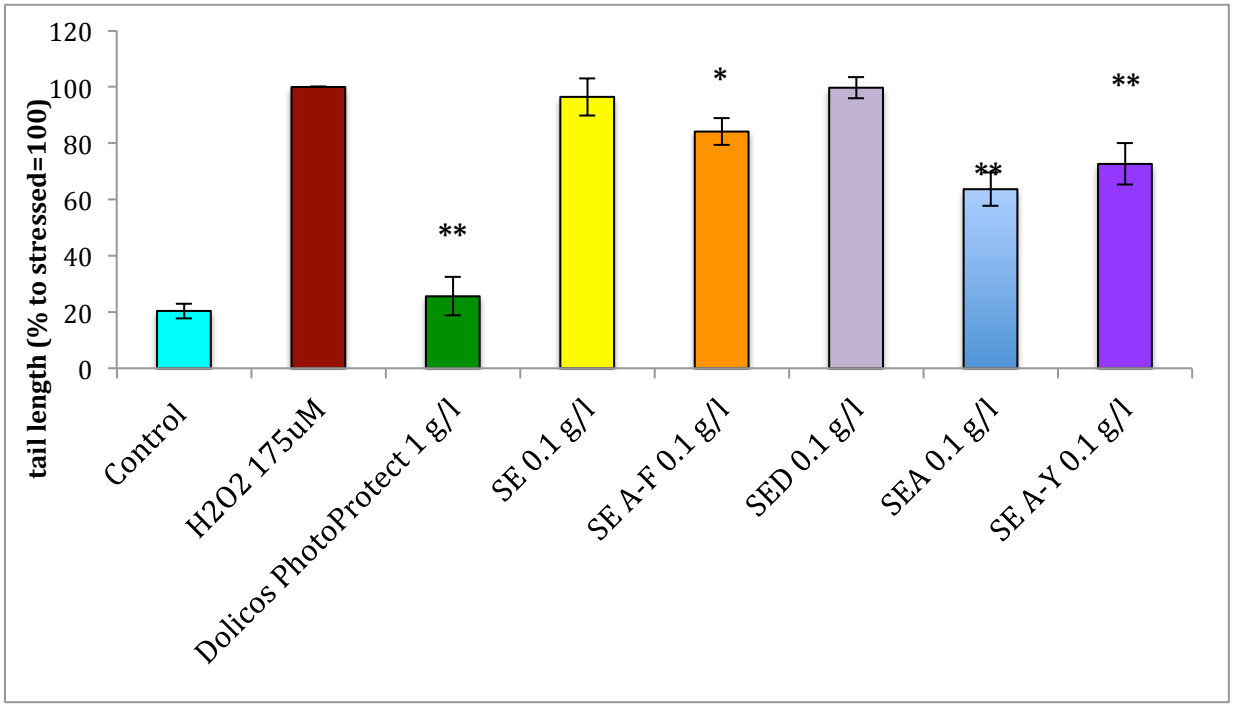


Figure 10. Comet assay. The pictures show the damages to cell nuclear DNA produced by treatment with different extracts of Spirulina compared to an untreated control. Cells in the panel D were previously incubated with SEA for 18 h. Each value was calculated in microns as average of 100 different measures, and then expressed as percentage to the untreated control, arbitrarily set as 100%.

4.5 Hydration and moisturizing capacity

Besides the oxidative stress protection, there is much interest in studying the capacity of compounds and extracts to protect human cells from de-hydration and osmotic stress, which may alter significantly the cell functionality and homeostasis, in particularly in the skin.

Skin is the outer tissue covering of the body, thus it represents an effective barrier between the organism and the environment, which is indispensable for the prevention of the invasion of microorganisms, chemical compounds and allergens, and the maintenance of moisture levels of the skin. Skin is composed of three layers: the epidermis, dermis, and hypodermis. The epidermis is a stratified squamous, keratinized epithelium composed of the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC) (Watt, 1989[85]) (Fig. 11).

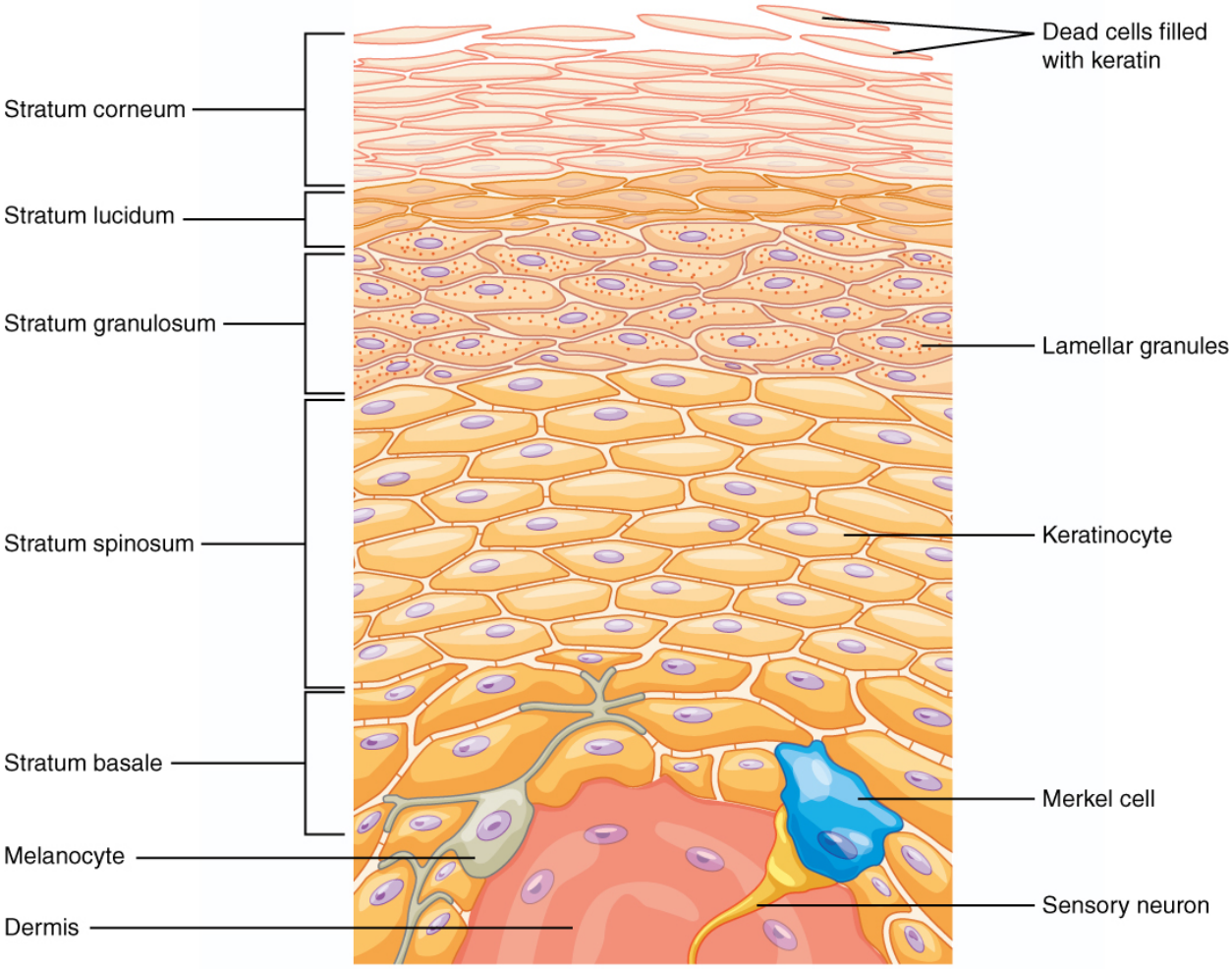
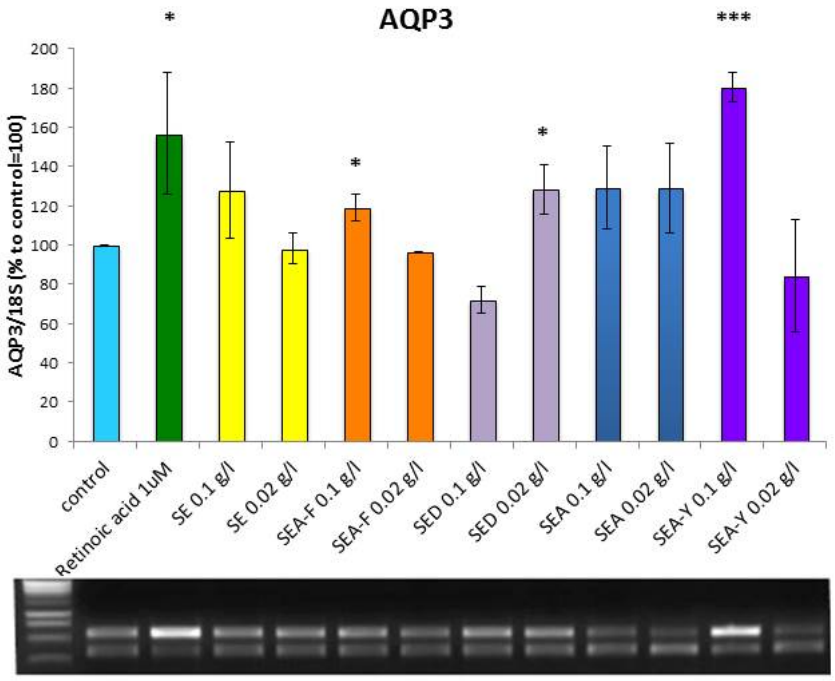


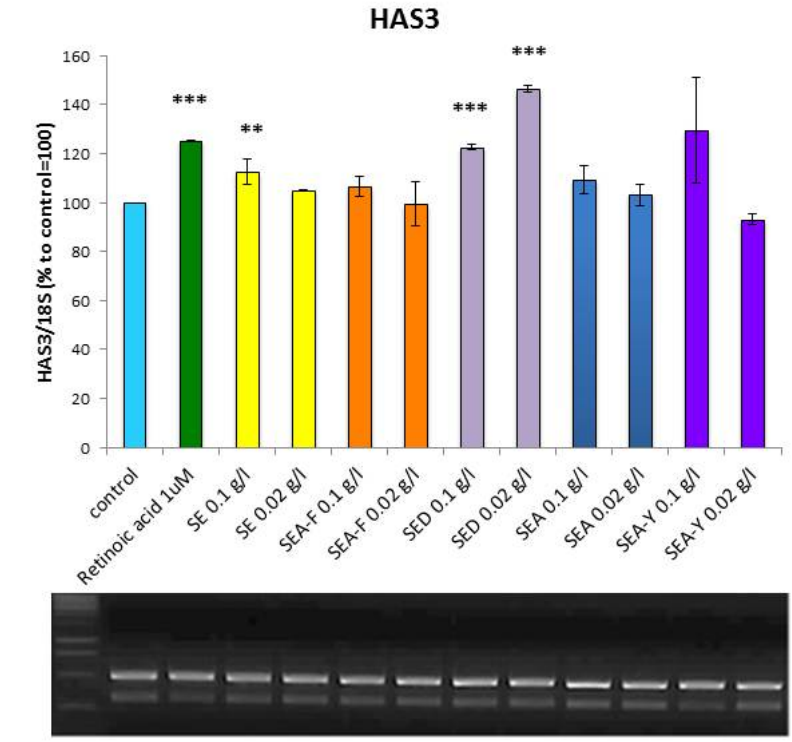
Figure 11. The most superficial layer of the skin is the epidermis. It is divided in five layers: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. Source: http://cnx.org/contents/IntegumentarySystem.

A decrease in SC water content is found in a number of common skin diseases, such as AD, eczema, psoriasis, senile xerosis, and hereditary ichthyosis (Hara et al., 2002[86]). Dehydration is also one of the major characteristics of aged skin.

To measure the capacity of the Spirulina extracts to protect cells from dehydration, we analyzed the gene expression of proteins specifically involved in the maintenance of water balance in human skin cells, such as Aquaporin-3 (AQP3), Hyaluronan Synthase-3 (HAS3), and Filaggrin (FLG) (Fig. 12). AQP3 is an integral membrane channel in epidermal keratinocytes and mediates water and glycerol movement into and out of the skin. HAS3 is the enzyme that polymerizes low molecular weight hyaluronic acid (HA) in keratinocytes (Ijuin et al., 2001[87]). HA plays a predominant role in the epidermis and in the dermis, enabling these tissues to maintain their hydration level thanks to its high water retention ability. HA holds the water together and keeps body smooth, moist and lubricated. The level of hyaluronic acid decreases with age and this is intimately linked with dehydration, loss of elasticity and the appearance of wrinkles (Ghersetich et al., 1994[88]). This leads to an increase in the hyaluronic acid content of the dermis and the epidermis, improving the structure of the extracellular matrix, and helping to ensure skin flexibility and firmness as well as moisture.

FLG is a filament-associated protein that binds to keratin fibres in epidermal cells and is important for the skin's waterproof properties.
SE and SEA-F increased gene expression of AQP3 by 28% and 19% at 0.1g/L, respectively, while SED increased AQP3 gene expression by 28% only at the concentration 0.02 g/L. Moreover, SED induced HAS3 gene expression by 22% and 46% at 0.1 g/L and 0.02 g/L, respectively.
Also SEA induced gene expression of AQP3 by 29% at both the concentrations. The effect produced by the lactic acid fermented samples was even higher: they induced AQP3 and HAS3 gene expression by 80% and 29% at 0.1 g/L, respectively, showing a higher hydrating capacity compared to the autoclaved Spirulina (SEA) used at the same dose. Retinoic acid, a vitamin A derivative, known for its capacity to promote cell hydration, was used as positive control in the experiments (Cao et al., 2008[89]; Bellemère et al., 2008[90]; Sayo et al., 2013[91]).
Water is also retained in the cells of the epidermis thanks to the NMF (natural moisturizing factor), which consists of molecules present in the corneocyte cells of the stratum corneum. Thanks to these substances, the skin is able to fix water in the epidermis and to maintain the hydration level necessary for its proper functioning. The main part of the NMF is composed by small peptides or amino acids derived from the degradation of filaggrin. FLG is expressed as a profilaggrin of >400kDa in humans, which is a major component of keratohyalin granules in the stratum granulosum of the epidermis (Dale et al., 1985[92]; Presland et al., 2005[93]). When keratinocytes move upward from the stratum basale to the stratum corneum, they dramatically change their shapes becoming dead cells without intracellular organelles and containing keratin bundles and lipids to constitute the stratum corneum (Candi et al., 2005[94]). At the stratum granulosum-to-stratum corneum transition, each filaggrin filament is processed by certain proteases to generate the filaggrin monomer. The resulting filaggrin monomers are joined to keratin cytoskeleton to form microfibrils (Dale et al., 1978[95]). Keratin-bound filaggrin is degraded into amino acids, which constitute a part of the natural moisturizing factor in the upper stratum corneum (Tarcsa et al., 1996[96]; Mechin et al., 2005[97]; Nachat et al., 2005[98]). In this respect, a number of compounds that naturally occur in many medicinal herbs and plants, such as oleanolic acid, ursolic acid, and pentacyclic triterpenoids, have been shown to improve skin barrier function by increasing FLG expression (Lim et al., 2007[99]). On the basis of the obtained results regarding the AQP3 and HAS3 expression, we investigated the capacity of Spirulina extracts to induce another important gene involved in cell hydration, filaggrin. More specifically, FLG has a key role in keeping the skin moisturized, guaranteeing the proper corneocyte cohesion and avoiding excessive water loss through the upper epidermal layers. Moreover, FLG is an important source of NMF upon degradation, further contributing to the maintenance of the necessary water retention capacity of the skin and its osmotic proprieties (Tarcsa et al, 1996[96]; Mechin et al, 2005[97]; Nachat et al, 2005[98]).
In accordance with the results described above, both SEA and SEA-Y induced FLG expression by 75% and by 59% respectively, at the concentration of 0.1 g/L, an effect even higher that the positive control CellPulse, an hydro-soluble extract derived from *Coffea bengalensis* cell cultures (Bimonte et al., 2011[100]).
The higher activity of the samples derived from auto-fermenting process, digestion by enzymes or fermentation by Yovis® compared to the control Spirulina extract or Spirulina autoclaved, could be explained by the effect of the autoclaving, followed by fermentation process, on the proteins contained in the Spirulina lysate, which produce small size peptides and molecules capable of passing through the plasma membrane more easily and thus responsible for the activation of signal cascade mechanisms ending to AQP3, HAS3 and FLG expression.


AQP3
AQP3/18S (% to control=100)
*
*
*
***
200
180
160
140
120
100
80
60
40
20
0
control
Retinoic acid 1uM
SE 0.1 g/l
SE 0.02 g/l
SEA-F 0.1 g/l
SEA-F 0.02 g/l
SED 0.1 g/l
SED 0.02 g/l
SEA 0.1 g/l
SEA 0.02 g/l
SEA-Y 0.1 g/l
SEA-Y 0.02 g/l


HAS3
HAS3/18S (% to control=100)
***
**
***
***
160
140
120
100
80
60
40
20
0
control
Retinoic acid 1uM
SE 0.1 g/l
SE 0.02 g/l
SEA-F 0.1 g/l
SEA-F 0.02 g/l
SED 0.1 g/l
SED 0.02 g/l
SEA 0.1 g/l
SEA 0.02 g/l
SEA-Y 0.1 g/l
SEA-Y 0.02 g/l

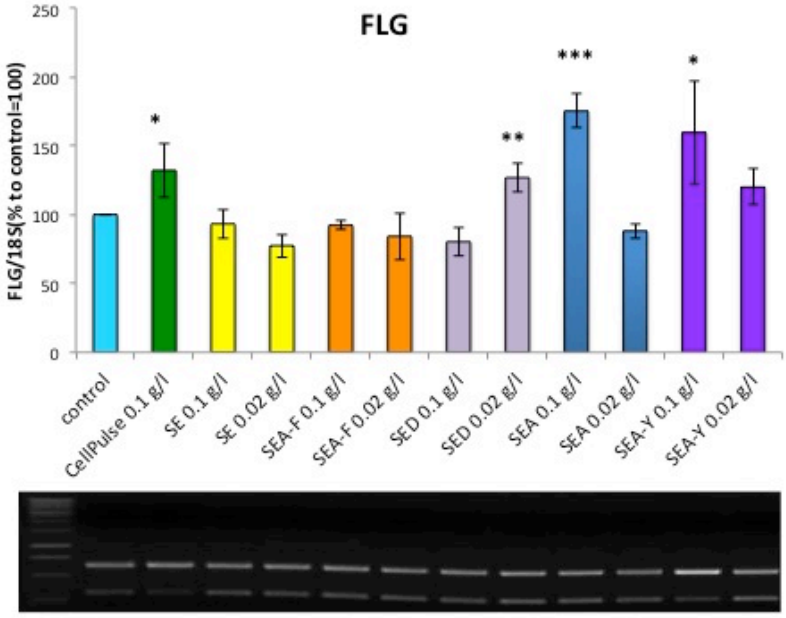


Figure 12. RT-PCR analysis of gene expression in HaCaT cells treated with two different concentrations of Spirulina extracts. Cell samples were treated with the extracts indicated for 6 h. The pictures show the amplification bands of the genes AQP3, HAS3 and FLG. The amplified bands were quantified using Geliance 200 Imaging software and the values obtained normalized to the internal standard 18S. All the values were reported in the graphs above the pictures and expressed as percentage to the control, set as 100%.

4.6 Protection from osmotic stress

Another activity which is particularly requested for ingredients specifically developed for skin care applications is the capacity to protect cells against osmotic stress, due to either hyper or hypotonic pressure.

Regulation of cell and total body water content is an essential homeostatic function. The skin of animals and humans is challenged by exposure to an environment that varies in humidity from 0 to 100%. Less than 100% relative humidity can produce significant dehydration or osmotic stress on the epidermis, which then must respond and protect the organism from water loss. A decrease in skin water content is found in a number of common skin diseases, such as AD, eczema, psoriasis, senile xerosis, and hereditary ichthyosis (Hara et al., 2002[86]). The major function of the epidermis is to provide protection from this loss of water and dehydration (Nolte et al., 2011[101]).

Cells adapt to the hypertonicity by a variety of biophysical (cell shrinkage and a subsequent regulatory volume increase), biochemical (opening/closing of ion channels; increased transport or synthesis of compatible osmolytes; enzyme activation) and genetic (induction of selected gene expression) mechanisms (Burg and Garcia-Perez, 1992[102]; Burg, 1995[103]).

In order to keep a well-hydrated and functional skin, it is necessary that all the small compounds, responsible for osmotic balance and moisturization, are correctly distributed throughout the upper skin layers. In particular, water is retained in the cells of the epidermis thanks to the NMF, which consists of molecules present in the corneocyte cells of the stratum corneum. The main part of the NMF is composed by small peptides or amino acids derived from the degradation of FLG, as already mentioned. Functionally related to FLG, SMIT is a gene involved in the protection from osmotic stress. SMIT-1 is the sodium-dependent myo-inositol cotransporter-1, which utilizes the electrochemical gradient of Na^+ across the plasma membrane to import myo-inositol (MI), with two Na^+ coupled to each molecule of MI (Hager et al., 1995[104]). Myo-inositol is a crucial constituent of living cells. It serves as an organic osmolyte, and its level increases when cells are under a hypertonic environment (Garcia and Burg, 1991[105], Handler and Kwon, 1996[106]). MI is also the precursor of phosphatidylinositol (PI), a signal transduction molecule.

Phosphorylation of MI generates a number of different phosphatidylinositol phosphates (PIPs) derivatives with important physiological functions, such as cell survival, growth, vesicular trafficking, and glucose homeostasis (Katso et al., 2001[107]).

To evaluate the ability of Spirulina derived extracts to protect the keratinocytes from osmotic stress, the cells were treated for 6 h with different Spirulina extracts. SEA-Y induced SMIT expression by 59% ($p<0.01$) and 31% ($p<0.05$) at 0.1 g/L and 0.02 g/L, respectively, while SED increased SMIT expression by 21% ($p<0.01$) at 0.1 g/L (Fig. 13). In agreement with the previous results, both the autoclaving process and lactic bacteria fermentation produced the most evident effect on the capacity of the cells to overcome osmotic stress conditions, most likely due to the formation of osmolites able to protect better the cell membrane from stressing agents that altered the correct water homeostasis in the cells.

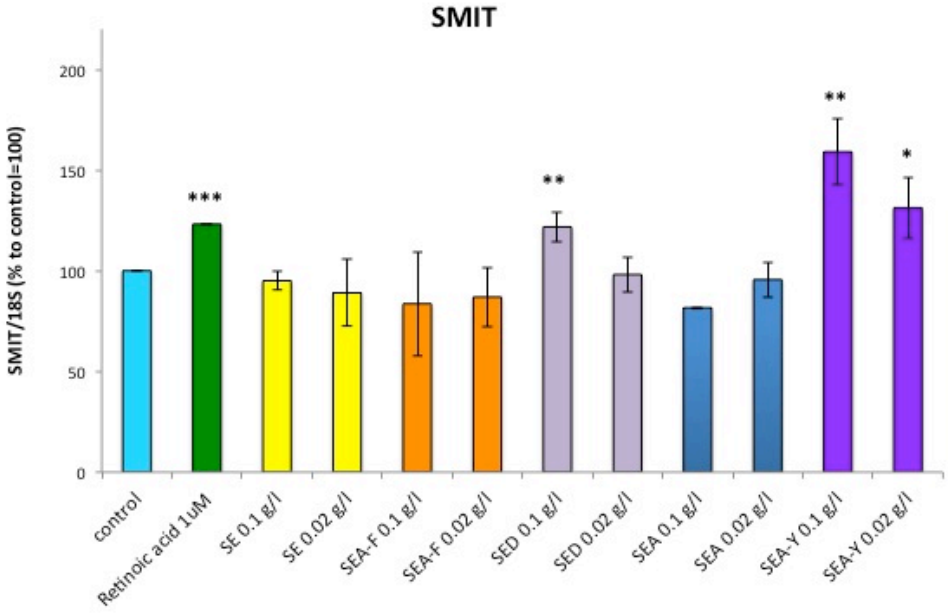


Figure 13. RT-PCR analysis of SMIT expression in HaCaT cells treated with two different Spirulina extracts. The amplified bands were quantified using Geliance 200 Imaging software and the values obtained normalized to the internal standard 18S. All the values were reported as percentage to the control, set as 100%.

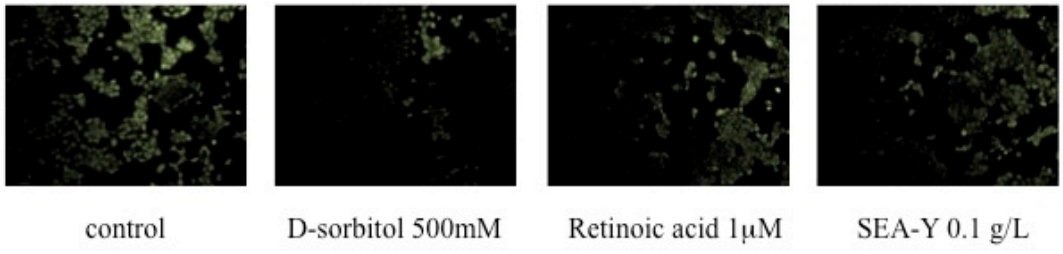
4.7 Permeability assay

The capacity of extracts or compounds to protect the cells against osmotic stress can be also evaluated by measuring how cultured cells respond to hypo or hyper-osmotic environments by swelling or shrinking, respectively. In order to confirm the gene expression results, we set up a permeability assay, based on protocols described by Zelenina et al., 2002[73], 2003[74], 2004[75], adapted to our cell system.

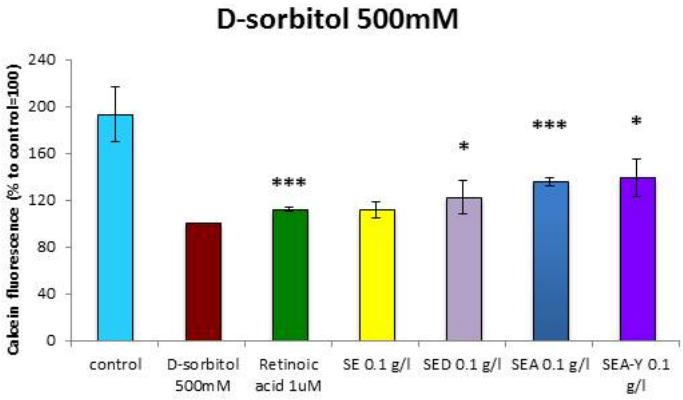
The keratinocytes HaCaT were pretreated with or without the different Spirulina extracts and then exposed to hyper-osmotic stress (sorbitol 500 mM) or hypo-osmotic stress (deionized water). The application of the stress produced a significant alteration of membrane permeability, measured by changes in the fluorescence emission. The calcein, contained in the cells, was released after the membrane damage, resulting in the decrease of the fluorescent signal. The Spirulina extracts inhibited osmotic stress damages in the cells by slowing down either the swelling or the shrinking process, caused by sorbitol or distilled water, respectively. In figure 14, it is shown how the hyperosmotic shock applied to the cells could be partially recovered by the treatment with SED, SEA and SEA-Y, similarly to retinoic acid. For the hypo-osmotic stress, caused by distilled water, the recovery was only detectable for the samples SED and SEA.

Consistent with the gene expression analysis, the results of this assay supported the role of the SED, SEA and SEA-Y extracts as protectants against cell dehydration and osmotic stress associated phenomena.

A



B



C

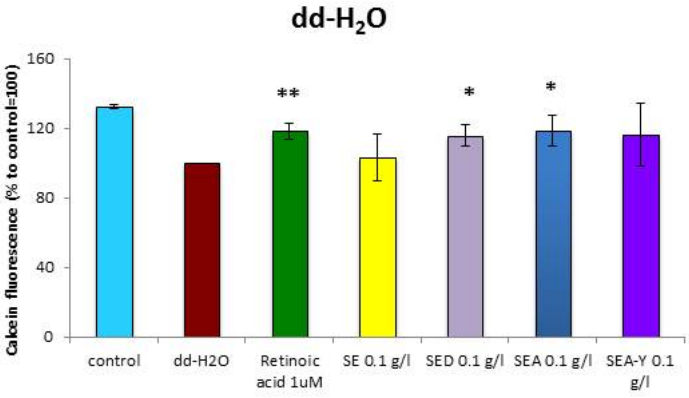


Figure 14. HaCat cells were incubated with different Spirulina extracts or Retinoic acid 1 μM, used as positive control, for 18 h. Cell water permeability was analyzed by the method described above and shown in (A). The relative fluorescence intensity was reported as percentage to the control, set as 100%. In the graph B was reported the water permeability in presence of hyper osmotic stress (D-sorbitol 500mM). The graph C reported the water permeability after hypo-osmotic stress (deionized water).

CONCLUSIONS

5. CONCLUSIONS

Microalgae have been exploited for millennia as sources of several bioactive ingredients. Currently, they have several applications from human and animal nutrition, pharmaceutics to cosmetics, both as extracts and purified molecules (e.g., fatty acids, pigments, stable isotope biochemical). In table 5 is reported a list of the main microalga species cultivated in different areas of the world and their main application field. However, microalgae are still not a well-studied group from a biotechnological point of view (Spolaore et al., 2006[1]).

Alga	Annual production	Producer country	Application and product
Arthrospira	3000 t dry weight	China, India, USA, Myanmar, Japan	Human and animal nutrition, cosmetics, phycobiliproteins
Chlorella	2000 t dry weight	Taiwan, Germany, Japan	Human nutrition, aquaculture, cosmetics
Dunaliella salina	1200 t dry weight	Australia, Israel, USA, China	Human nutrition, cosmetics, β-carotene
Aphanizomenon flos-aquae	500 t dry weight	USA	Human nutrition
Haematococcus pluvialis	300 t dry weight	USA, India, Israel	Aquaculture, astaxanthin
Crypthecodinium cohnii	240 t DHA oil	USA	DHA oil
Shizochytrium	10 t DHA oil	USA	DHA oil

Table 5. Present state of microalgal production (Pulz and Gross, 2004[108]).

In the research study that I conducted during my PhD course, the microalga *A. maxima* was selected due to its ability to grow more easily both in the laboratory and in large-scale facilities, and for its features of being a potential interesting source of proteins and therapeutic compounds. In order to make the extracts obtained from Spirulina even more valuable in terms of industrial applicative output, different types of fermentation processes were conducted and the derived products analyzed for their efficacy in cellular based tests. The assumption of this research was based on several studies that demonstrated the advantages of having small peptides and processed macromolecules with higher biological activity than their bigger counterparts, higher specificity (minimal unspecific binding), and often lower toxicity due to minimal accumulation in tissues (Marx, 2005[109]; Ayoub and Scheidegger, 2006[110]).

Spirulina lysate showed to be a perfect candidate for this type of study, since this microalga was extremely rich in functional macromolecules (proteins, polysaccharides and essential fatty acids) that could be easily processed using fermentation (Estrada et al., 2001[111]).

The C-phycocyanin, the most represented protein in Spirulina hydro-lysate, was a good molecular marker to follow the different steps of the fermentation process, each associated with a different degree of macromolecule degradation, as confirmed by the SDS-PAGE analysis conducted in the unfermented samples as well in the fermented ones. Three types of fermentation processes were compared in this study, one based on the use of indigenous bacteria (auto-fermentation), those developed spontaneously in the lysate; the second based on the use of the mix Yovis® (Sigma-Tau), added after a sterilization procedure of the lysate; and the third performed by employing enzymes (carbohydrase and Alcalase), thus without using any microorganism.

The results of the study underlined that the Spirulina lysates treated either by biological or enzymatic fermentation were significantly more efficient than the untreated samples in protecting cell membrane from lipid peroxidation, even though their total antioxidant power, measured by ORAC assay, was slightly lower in the sample fermented by Yovis®. This can be explained with the consideration that during the fermentation process many redox reactions occur and many phenolic compounds and macromolecules may partially loose their capacity to transfer electrons. This phenomenon does not necessarily means that these compounds loose their capacity to protect the cells against ROS formation, which is totally dependent on the ability of these molecules to interact with the cell constituents and to penetrate the cells through the membrane. Based on this assumption, smaller compounds, that result from the fermentation, would be much more permeable to membranes and more suitable to fullfil a biological protective

action in the cell. The effect of protection on nuclear DNA was also detectable in some of the treatments with the fermented samples, indicating that fermented products (auto-fermented and fermented by Yovis®) were able to penetrate cell membrane and prevent DNA damage by oxidative stress.
Gene expression analysis, analogously to the previous studies, revealed that in cultured keratinocytes the fermented samples were able to induce the genes involved in hydration AQP3, FLG and HAS3, suggesting also a role as signaling complexes, capable to trigger signal transduction pathways, possibly through the activation of receptors either outside or inside the cells. Surprisingly, the samples that were the most active in inducing the expression of hydration genes were those treated enzymatically. SED showed the strongest effect at the dose of 0.02 g/L for all tested genes. The induction of these genes, involved in the hydration process indicates an increase of the capacity of the cells to retain water, and guarantee a correct water balance homeostasis within the tissues. This is a very important characteristic claimed by many cosmetic products, as water dehydration is one of the main problem related to aged skin. Moreover, in the SMIT gene expression analysis, related to the osmotic stress protection, and in the membrane permeability determination, the enzymatically digested sample, SED, at concentration of 0.1 g/L, showed again the highest protection activity, confirming the potential role of this type of product in enhancing both a correct water distribution and osmolite balance in the upper skin layers.
There are other examples in literature of the development of potential industrial products derived from biotechnological processing. A fermentation of the alga *Gracilaria tikvahiae* is an example of a cosmetic active ingredient involved in stimulating proteasome activity in skin cells (Chavan et al., 2013[112]). Indonesian tempeh and Oriental soy sauce are well known examples of indigenous fermented foods that have been industrialized and marketed globally (Hachmeister and Fung 1993[113]; Sugiyama 1984[114]). Another example of cosmetic application of a fermented algae is represented by peptides or protein hydrolysates derived from *Porphyra* spp. and wakame seaweeds under appropriate conditions (Hagino and Masanobu, 2003[51]).
In conclusion, the products derived from Spirulina fermentation can find application in both the cosmetic field, as hydrating and moisturizing agents for skin care, and in nutraceuticals, as food supplements able to increase the natural defense response of the cells and to guarantee a more efficient balance of nutrients in the body.
In particular, Arterra Bioscience srl, the collaborating company of my PhD course, has already shown considerable interest to include one or more products, deriving either from Spirulina microbial fermentation or enzymatic digestion, in its portfolio of active ingredients, and explore the chance to propose them in the cosmetic mar

ACKNOWLEDGEMENTS

We acknowledge Caisial from University of Naples Federico II, for preparation and submission of the Spirulina extracts. This work is supported by POR Campania FSE 2007–2013, Project CARINA.

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CHAPTER 2

MI2014A 000186 Composizioni cosmetiche comprendenti estratti derivati dalla microalga *Galdieria sulphuraria*, particolarmente indicate per ridurre gli effetti dannosi causati dall'acne.

M. Bimonte, A. De Lucia, A. Tito, A. Carola, F. Apone, G. Colucci, V. Fogliano, S. Buono, Martello A., Langellotti L., Pollio A., Pinto G.

La presente invenzione si riferisce all'uso in campo cosmetico di estratti cellulari derivati dalla microalga appartenente al genere *Galdieria*, preferibilmente alla specie G. *sulphuraria* e alle relative composizioni dermocosmetiche comprendenti detti estratti aventi effetti seboregolatori, antibatterici e di stimolazione del sistema di difesa della pelle, utili per alleviare gli effetti dannosi causati dall'acne sulla pelle. L'invenzione concerne ulteriormente un metodo per ottenere i suddetti estratti.

Come è noto, la pelle svolge funzioni vitali nel nostro organismo, quali la regolazione della temperatura corporea, la percezione sensoriale, l'azione protettiva e di difesa contro patogeni e radiazioni UV, che possono venir compromesse da numerose patologie.

Una delle funzioni più importanti della pelle è proprio quella protettiva, che è assicurata dalla presenza del sebo, una miscela complessa di lipidi, trigliceridi, cere ed esteri del colesterolo, steroli, squalene, paraffina ed acidi grassi liberi (insaturi e saturi). Il sebo è fondamentale per la formazione del

film idrolipidico che ricopre la pelle ed il cuoio capelluto, in quanto protegge la superficie cutanea dalle aggressioni chimiche (detergenti, solventi, inchiostri, ecc.) e batteriche, lubrifica ed impermeabilizza la superficie, permettendo alla pelle di mantenere un giusto livello di idratazione. Il sebo è prodotto da piccoli organi specializzati, le ghiandole sebacee, che sono localizzate in tutta la superficie della pelle, ad eccezione dei palmi delle mani e delle piante dei piedi. Le ghiandole sebacee non hanno una distribuzione omogenea nella pelle, ma si fanno molto più abbondanti e produttive in aree cutanee particolari, come quelle del viso e del cuoio capelluto, dove la funzione protettiva è ancora più importante. Le ghiandole sebacee sono generalmente associate ad un follicolo pilifero ed insieme ad esso formano l'unità pilo-sebacea. Una corretta regolazione dell'attività della ghiandola sebacea e di conseguenza della produzione di sebo, è quindi estremamente importante per mantenere una pelle sana e meno soggetta a patologie.

L'attività della ghiandola sebacea è influenzata da numerosi fattori, tra questi i più importanti sono l'età dell'individuo ed il livello di un ormone androgeno, il testosterone. Infatti la massiccia attivazione delle ghiandole sebacee si ha soprattutto in età puberale, quando è più intensa la produzione del testosterone. La concentrazione del testosterone, ormone tipico dell'uomo ma presente in minori quantità anche nella donna, influenza quindi in modo determinante la produzione di sebo, in quanto proprio a

livello della ghiandola sebacea esso viene convertito dall'enzima 5-α reduttasi in diidrotestorone, un derivato che attiva la biosintesi del sebo. Un'elevata concentrazione di testosterone, e quindi di diidrotestorone, porta ad una eccessiva produzione di sebo, definita seborrea, che conferisce alla pelle un aspetto lucido ed untuoso ed è spesso il fattore scatenante di una patologia più grave: l'acne. Proprio per questo motivo è fondamentale per l'organismo garantire sempre una giusta produzione di sebo per avere una pelle sana e funzionale.

L'acne è un processo infiammatorio caratterizzato da lesioni del follicolo pilosebaceo. Essa insorge in soggetti in età giovanile, di regola nel periodo puberale, in quelle regioni del corpo più ricche di ghiandole sebacee, come viso e torace. Il processo infiammatorio dell'acne si origina da un eccessivo accumulo di sebo nella ghiandola sebacea dei follicoli piliferi, il sebo prodotto rappresenta il terreno di coltura per i batteri, quali *Propionibacterium acnes* e *Staphylococcus epidermidis*, che abitualmente colonizzano il follicolo pilifero. In condizioni normali, quando il sebo fluisce liberamente fuori dai pori, le popolazioni batteriche si mantengono a livelli bassi, ma un'eccessiva produzione di sebo ed un suo eccessivo accumulo nella ghiandola sebacea porta ad un proliferare incontrollato di batteri.

I batteri proliferano e producono enzimi proteolitici in grado di ledere la parete del follicolo pilifero e riversare il contenuto della ghiandola sebacea nel derma sottostante, stimolando lo scatenarsi

di una reazione infiammatoria (Pawin et al., 2004).

L'organismo umano ha sviluppato nel corso dell'evoluzione dei meccanismi di difesa contro l'attacco di microrganismi: tali meccanismi comprendono una prima risposta immunitaria aspecifica (o immunità innata) ed una seconda risposta specifica (o immunità acquisita). L'immunità innata è la prima linea di difesa per evitare l'ingresso ed il proliferare di agenti patogeni nell'organismo e per sconfiggerne l'eventuale azione patogena.
L'immunità acquisita è invece la seconda linea nella difesa da agenti patogeni che agisce nel momento in cui l'immunità innata non riesce a distruggere l'agente.

Le β-defensine, piccoli peptidi cationici, sono tra i maggiori componenti dell'immunità innata; esse presentano una spiccata attività antimicrobica, antivirale ed antimicotica. La loro particolare natura cationica gli permette di distruggere la membrana batterica, carica negativamente, legandosi ad essa, e di lasciare intatte le membrane delle cellule umane, che hanno carica neutrale. Inoltre le β-defensine stimolano la risposta specifica richiamando le cellule del sistema immunitario, quali macrofagi e neutrofili, e promuovono il processo di rimarginazione delle ferite. Nei pazienti affetti da acne, l'espressione della β-defensine 2 (hBD-2) risulta aumentata nelle ghiandole sebacee (Chronnell et al., 2001), dove verosimilmente esse esplicano un ruolo importante nel proteggere dall'invasione microbica.

L'acne, essendo caratterizzata dalla formazione di

comedoni, papule, pustole e talvolta di noduli e cicatrici, è molto spesso associata a disagi di tipo estetico e di carattere sociale, e questo spiega l'elevata attenzione che la cosmetica e la dermatologia hanno dedicato alla cura di questa patologia.

Ad oggi, sono utilizzati diversi farmaci per il trattamento dell'acne, anche se ognuno di questi sembra agire su di uno solo degli eventi che portano allo scatenarsi dell'acne; inoltre alcuni di questi farmaci presentano delle controindicazioni.

Tra questi l'isotretinoina, un derivato della vitamina A che inibisce la produzione di sebo. Tuttavia questo composto presenta numerose controindicazioni ed un elevato rischio teratogeno, pertanto è fortemente sconsigliato il suo uso nelle donne in gravidanza o addirittura in quelle che non hanno ancora avuto figli.

Anche gli antibiotici, quali tetraciclina, eritromicina e la nadifloxacina, sono utilizzati per la cura dell'acne in quanto inibiscono la crescita dei batteri nella ghiandola sebacea, ma il principale limite nell'impiego di questi è l'insorgenza della resistenza agli antibiotici da parte dei batteri stessi.

Sulla base di quanto sopra esposto appare evidente l'esigenza di disporre di nuovi composti in grado di contrastare l'insorgenza dell'acne, che siano al tempo stesso efficaci e sicuri, e che siano capaci di agire su più di uno degli eventi che portano allo scatenarsi della patologia.

E' noto nell'arte che le microalghe sono dotate di spiccate proprietà farmacologiche e terapeutiche grazie

alle numerose molecole attive in esse contenute: vitamine, aminoacidi, fitocolloidi, polisaccaridi, carotenoidi, ecc.

Negli ultimi anni l'utilizzo degli estratti ottenuti da microalghe è aumentato molto in prodotti cosmetici, con applicazioni che vanno dalla cura del viso, a quella del corpo e capelli.

In particolare, la microalga *Galdieria sulphuraria* rappresenta una specie di particolare interesse per la sua straordinaria capacità di crescere in condizioni estreme, come ad esempio ambienti estremamente acidi e temperature elevate (Ott and Seckbach, 1994), e per la sua versatilità nella crescita, che rende detta alga capace di adattarsi ai cambiamenti ambientali (Oesterhelt et al. 2007). A tale proposito, la sua presenza all'interno del cratere del vulcano Solfatara, e in particolare nel sito di Pisciarelli, Agnano, Napoli, è stata documentata da diversi anni (Merola et al.1981): le temperature superiori ai 60°C e l'acidità (pH compresi tra 0 e 4) rendono questo ambiente idoneo alla crescita di pochissimi organismi, tra cui questa microalga rossa. La sua versatilità metabolica, dovuta alla capacità di produrre un vasto repertorio di enzimi metabolici, in parte ottenuti mediante un processo di trasferimento genico orizzontale (HGT) da batteri ed archea che convivono nello stesso ambiente (Schönknecht et al., 2013), rende questa microalga capace di adattarsi ai cambiamenti ambientali e di crescere sia alla luce, in autotrofia, sia al buio, in eterotrofia, in presenza di un'ampia gamma di substrati organici (Gross et al., 1998).

Tuttavia non esistono studi relativi all'utilizzo dell'estratto di questa specie di microalga come principio attivo, sfruttabile a livello cosmetico in formulazioni di diverso genere.

Gli autori della presente invenzione hanno trovato che estratti cellulari derivati dalla microalga appartenente al genere *Galdieria*, preferibilmente alla specie *G. sulphuraria*, testati su cellule della pelle in coltura, si sono dimostrati in grado di: i) diminuire (inibire) l'attività dell'enzima 5α-reduttasi; ii) inibire la crescita dei batteri; iii) indurre l'espressione delle β-defensine; iv) accelerare il processo di rimarginazione delle ferite.

Le cellule di *G. sulphuraria* utilizzate nella presente invenzione sono state inizialmente raccolte nei pressi della solfatara di Pozzuoli da parte del gruppo del Dipartimento di Biologia, Orto Botanico, via Foria 223, 80139 – NAPOLI.

Gli estratti cellulari derivati dalla microalga appartenente al genere *Galdieria*, preferibilmente alla specie *G. sulphuraria*, mostrano avere, quindi, delle spiccate proprietà per alleviare gli effetti dannosi della eccessiva produzione di sebo (iperseborrea) e dell'acne, grazie alle proprietà di seboregolazione, antibatteriche e di stimolazione del sistema di difesa della pelle. Costituisce pertanto un primo aspetto della presente invenzione un metodo per ottenere un estratto da cellule in coltura liquida della microalga *Galdieria*, preferibilmente della specie *G. sulphuraria*, comprendente le seguenti fasi a)-g):

a) allestimento delle colture algali monospecifiche di

Galdieria, preferibilmente della specie *G. sulphuraria,* e crescita delle cellule in coltura liquida in un appropriato terreno di coltura;

b) separazione delle cellule dal mezzo di crescita;

c) omogeneizzazione delle cellule;

d) centrifugazione dell'omogenato per isolare l'estratto idrosolubile, rappresentato dal sopranatante.

La microalga *Galdieria*, preferibilmente della specie *G. sulphuraria*, può essere fatta crescere in autotrofia, eterotrofia o mixotrofia, in diversi tipi di terreni di coltura acidi: è sufficiente che i terreni contengano fonti organiche o inorganiche di azoto, fosforo, carbonio e zolfo, e abbiano un pH variabile da 0 a 5. L'intervallo della temperatura di crescita ottimale è compreso tra 20 °C e 55 °C, essendo una microalga che si adatta bene a diverse temperature.

Secondo una forma di realizzazione preferita, la microalga *Galdieria*, preferibilmente della specie *G. sulphuraria*, è fatta crescere in eterotrofia ad una temperatura da 26 a 37 ^{0}C.

Un terreno di coltura particolarmente preferito della fase a) comprende: 1,32 g/L di $(NH_4)_2SO_4$, 0,6 g/L di K_2HPO_4, 0,1 g/L di NaCl, 0,3 g/L di $MgSO_4$, 0,00996 g/L di $FeSO_4$, 0,02 g/L di $CaCl_2$, 30g/L glucosio, 0,0199 g/L di $MnCl_2$, 0,211 g/L di H_3BO_3, 0,01 g/L $CuSO_4$, 0,005g/L Na_2MoO_4, 0,005 g/L di $CoCl_2$, 0,014 g/L di $ZnCl_2$ (Allen, 1968) e dove detto terreno di coltura ha pH 1,5, preferibilmente ottenuto con H_2SO_4.

Secondo una forma preferita di realizzazione di tale metodo di estrazione, l'omogeneizzazione della

fase c) avviene in un mortaio con PBS 1X (tampone fosfato salino). Alternativamente, possono essere utilizzati tamponi salini differenti (come citrati, carbonati, bicarbonati, acetati, tricina, HEPES) e metodi automatizzati, come frullatori, lame d'acciaio collegati a motori o omogeneizzatori da laboratorio.

Preferibilmente, l'allestimento delle colture cellulari di *Galdieria*, preferibilmente *Galdieria sulphuraria*, e crescita delle cellule in coltura liquida nella fase a) sono in eterotrofia: la crescita delle cellule può essere condotta rispettando un rapporto inoculo/medium di 1:4 (per esempio inoculando 200 ml di sospensione di cellule in 1L di mezzo). La coltura viene poi areata per mezzo di una pompa ad aria e/o di un agitatore a pale rotanti, che oltre a garantire il sufficiente apporto di ossigeno (O_2) per la crescita, permettono anche di mantenere omogeneo il mezzo di crescita ed evitare fenomeni di addensamento della coltura sul fondo. La crescita delle cellule viene condotta ad una temperatura variabile tra 26°C e 37°C, al buio. Dopo circa 3 settimane si raggiunge la corretta densità (6 g/L) e le cellule, dopo il controllo al microscopio, possono essere raccolte per il processamento.

Secondo una forma preferita di realizzazione del metodo secondo l'invenzione, prima della separazione dal mezzo di crescita, le cellule possono ulteriormente essere sottoposte ad almeno un trattamento selezionato tra: trattamento di tipo fisico, scelto tra trattamento al calore, al freddo o irradiazione UV; e/o trattamento di tipo chimico, con almeno un composto scelto tra

proteine, lipidi, zuccheri, sali, piccole molecole organiche, o loro combinazioni. Questi trattamenti hanno lo scopo di stimolare nelle cellule la produzione di metaboliti secondari di interesse e aumentare così l'efficacia cosmetica degli estratti derivati dalla microalga.

La separazione delle cellule dal mezzo di crescita della fase b), può essere effettuata mediante centrifugazione in continuo da 1000 a 5000 g, preferibilmente tra 3000 e 4000 g e ancora più preferibilmente a 3300 g, o sedimentazione e successiva filtrazione mediante membrane con porosità non superiore ai 10 micron. Le cellule vengono poi lavate in una soluzione fisiologica o PBS e asciugate per rimuovere l'acqua presente. In alternativa il pellet (sedimento di centrifugazione) delle cellule può essere liofilizzato in modo da rimuovere completamente tutta l'acqua presente.

Nella fase c) il pellet di cellule viene omogeneizzato in presenza di PBS 1X (NaCl 136 mM, KCl 2,7 mM, NaH2PO4 12 mM, KH2PO4 1,76 mM, pH 7,4), preferibilmente in rapporto di 2:1 volume/peso. In questo passaggio, la sabbia di silice può essere utilizzata per facilitare la rottura delle cellule. L'omogeneizzazione può essere effettuata in un contenitore idoneo, quale un mortaio di ceramica e con un pestello anch'esso di ceramica, precedentemente raffreddati. Per volumi superiori a 200 g si possono utilizzare recipienti più grandi, anche metallici, dove le cellule possono essere omogeneizzate con lame metalliche, utilizzando omogeneizzatori da laboratorio o industriali, frullatori o presse.

Nella fase d), si ha l'ottenimento dell'estratto idrosolubile: una volta ottenuto un lisato cellulare omogeneo tramite l'omogeneizzazione dello stadio c), il campione di cellule viene centrifugato, ad esempio con un valore di rpm compreso tra 4.000 e 8.000 rpm per circa 15 minuti a 4 °C, per precipitare le componenti insolubili.

Il sopranatante ottenuto dalla centrifugazione viene prelevato: esso costituisce l'estratto idrosolubile dell'invenzione.

L'estratto ottenuto nella fase d) può essere portato a secco (è sottoposto ad essiccazione) in un liofilizzatore o in una camera di essiccazione o in uno spray-dryer per eliminare l'acqua.

In una forma preferita dell'invenzione l'estratto secco ottenuto con il metodo dell'invenzione è diluito in un solvente organico, preferibilmente ad una concentrazione variabile tra lo 0,1 e 10% p/v.

L'estratto secco viene preferibilmente diluito in una miscela di glicerolo e acqua, preferibilmente in un rapporto preferito di 50:1, preferibilmente ad una concentrazione variabile tra lo 0,1 e 10% p/v. Alternativamente al glicerolo, l'estratto secco ottenuto con il metodo dell'invenzione è diluito ad una concentrazione variabile tra lo 0,1 e 10% p/v in un altro solvente organico compatibile con le formulazioni cosmetiche, come un solvente stearato, palmitato e/o butilen-glicole.

Queste soluzioni contenenti l'estratto liofilizzato/essiccato rappresentano la materia prima che può essere utilizzata, opportunamente diluita,

nelle formulazione dei prodotti cosmetici finiti.

Inoltre, le suddette cellule di microalga di *Galdieria*, preferibilmente *Galdieria sulphuraria*, possono essere precedentemente indotte da specifici trattamenti e condizioni di crescita per produrre maggiori quantità di composti attivi (es. ad azione seboregolatoria o in generale benefica sulle cellule della pelle), prima di essere sottoposte al procedimento di estrazione.

Costituisce quindi un secondo aspetto della presente invenzione l'estratto idrosolubile ottenibile mediante il processo a)-d).

In particolare, l'estratto ottenibile secondo il metodo dell'invenzione può essere utilizzato per il trattamento e/o la prevenzione cosmetica della iperseborrea, dell'acne e/o della pelle tendente all'acne.

Inoltre, costituisce un terzo aspetto della presente invenzione una composizione comprendente l'estratto idrosolubile ottenuto mediante il processo a)-d) come sopra definito.

In particolare, detta composizione può essere utilizzata per il trattamento e/o la prevenzione cosmetica della seborrea, dell'acne e/o della pelle tendente all'acne.

Detto estratto idrosolubile ottenibile mediante il processo a)-d) può essere compreso in un veicolo cosmeticamente accettabile, eventualmente insieme ad eccipienti e/o adiuvanti cosmeticamente accettabili.

Veicoli preferiti che possono essere impiegati sono liposomi, preferibilmente liposomi multilamellari,

ciclodestrine e/o silicati.

Secondo un aspetto preferito, le composizioni dell'invenzione che comprendono un estratto ottenibile con il metodo dell'invenzione sono in una qualsiasi forma galenica per l'applicazione cutanea, preferibilmente in forma di crema, gel, lozione, soluzione, dispersione, emulsione O/A, emulsione A/O, latte, shampoo, sapone, stick, matita, spray, olio.

Secondo un aspetto maggiormente preferito, le composizioni dell'invenzione possono essere in forma di creme, gel, lozioni cosmetiche per l'applicazione cutanea.

Costituisce un quarto aspetto della presente invenzione l'uso cosmetico dell'estratto come sopra definito.

In particolare, detto estratto e/o la composizione cosmetica che lo comprende, può essere utilizzato per ridurre (inibire) l'attività dell'enzima 5α-reduttasi e/o stimolare le difese dell'organismo contro agenti patogeni e/o combattere l'insorgenza dell'acne.

Costituisce un ulteriore aspetto dell'invenzione un estratto ottenibile secondo il metodo dell'invenzione o una composizione che lo comprende, per l'uso nell'inibizione dell'enzima 5α-reduttasi a livello cutaneo e/o nella stimolazione delle difese dell'organismo a livello cutaneo contro agenti patogeni.

Costituisce un ulteriore aspetto dell'invenzione un estratto ottenibile secondo il metodo dell'invenzione o una composizione che lo comprende, per l'uso nella prevenzione e/o nel trattamento della

caduta dei capelli.

Costituisce un ulteriore aspetto dell'invenzione un metodo di trattamento cosmetico per alleviare gli effetti dannosi dell'acne, comprendente l'applicazione sulla cute in necessità di trattamento di una quantità cosmeticamente efficace di una composizione cosmetica come sopra definita, preferibilmente contenente concentrazioni da 0.1 a 2% di ingrediente attivo, costituito dall'estratto della microalga diluito in solvente organico.

La presente invenzione verrà ora descritta a titolo illustrativo, ma non limitativo, secondo sue forme preferite di realizzazione con particolare riferimento alle figure dei disegni allegati, in cui:

- **la figura 1** riporta il grafico del saggio di citotossicità (MTT) che mostra come l'estratto idrosolubile di *Galdieria sulphuraria* ottenuto con il metodo dell'invenzione non abbia effetto tossico sui cheratinociti a concentrazioni uguali o inferiori a 0,01 % p/v (1 mg/ml). In ordinata è riportato il numero di cellule vitali espresso in valore percentuale rispetto al controllo, uguale a 100%.

- **la figura 2** mostra l'analisi dell'espressione genica della 5-α reduttasi (a due diverse concentrazioni di estratto secco dell'invenzione : 0,002% e 0,01% p/v) dopo 6 ore dal trattamento con l' estratto di *G. sulphuraria* rispetto ad un campione di controllo non trattato; i valori riportati nel grafico sono espressi come percentuali rispetto al campione del controllo non trattato (stabilito arbitrariamente come 100%).

- **la figura 3** mostra l'effetto dell'estratto di *G. sulphuraria* sulla crescita dei batteri *Staphyilococcus epidermidis, Staphylococcus aureus* e *Propionibacterium acnes*. Sull'asse delle y sono riportati i valori di crescita dei batteri espressi come percentuali rispetto al campione di controllo non trattato (stabilito arbitrariamente come 100%).

- **la figura 4** mostra l'effetto dell'estratto di *G. sulphuraria* (a due diverse concentrazioni: 0,002% e 0,01% p/v) sulla espressione della β-defensina 2, dopo 6 ore di trattamento; i valori riportati nel grafico sono espressi come percentuali rispetto al campione di controllo stabilito arbitrariamente come 100%.

- **la figura 5** mostra l'effetto dell'estratto di *G. sulphuraria* sulla rimarginazione di una ferita simulata in cellule in coltura. Sull'asse delle Y è riportata la riduzione delle dimensioni della ferita in cellule trattate con l'estratto della microalga e confrontata con quella in cellule di controllo non trattate, stabilita arbitrariamente come 100%.

A titolo esemplificativo, ma non limitativo della presente invenzione, si riportano di seguito alcuni esempi relativi alla preparazione dell'estratto idro-solubile ottenuto da cellule di *G. sulphuraria*, ed alla loro attività biologica in campo cosmetico.

ESEMPIO 1

Preparazione dell'estratto idrosolubile ottenuto da cellule di *G. sulphuraria* e saggi biologici per valutare le sue proprietà in campo cosmetico.

MATERIALI E METODI

Estrazione della miscela idrosolubile dalle cellule di *Galdieria sulphuraria*:

100 g di cellule di *G. sulphuraria* sono state risospese in due volumi di PBS 1X (NaCl 136 mM, KCl 2,7 mM, NaH_2PO_4 12 mM, KH_2PO4 1,76 mM, pH 7,4) ed omogeneizzate in un frullatore meccanico. L'omogeneizzato è stato centrifugato a 4000 rpm per 15 min a 4°C e il sopranatante prelevato e congelato a –80°C: esso costituisce l'estratto idrosolubile. L'estratto è stato poi liofilizzato in modo da ottenere una polvere, che è stata risospesa in acqua alla concentrazione variabile tra 10 e 1% p/v per eseguire i saggi biologici.

L'estratto è stato testato su cellule HaCaT (linea di cheratinociti umani) per stabilirne le dosi di utilizzo e la tossicità. L'estratto è stato poi utilizzato in saggi cellulari specifici per valutare la sua attività cosmetica come seboriduttore, antibatterico, per la sua capacità di innalzare la risposta immunitaria di difesa della pelle e di accelerare la rimarginazione di una ferita.

Saggio di citotossicità

Questo saggio si basa sull'uso del MTT [3-(4,5-dimetiltiazolil)-2,5-difeniltetrazoliobromuro]descritto da Mosmann nel 1983 per la prima volta. E' basato sulla capacità dell'enzima deidrogenasi mitocondriale delle cellule vitali di idrolizzare l'anello tetrazolico del MTT (di colore giallo chiaro) e formare cristalli di formazano (di colore blu scuro). Tali cristalli sono impermeabili alle membrane cellulari e si accumulano nel citoplasma delle cellule metabolicamente attive. Il

numero di cellule vive e sane è così direttamente proporzionale al livello di formazan prodotto.
Cellule HaCaT, in numero iniziale di 1 x 10^4 per pozzetto, sono state fatte crescere in piastre da 96 pozzetti in mezzo di coltura DMEM (Dulbecco's Modified Eagle Medium) (Lonza), supplementato con il 10% di Fetal Bovine Serum, per circa 8 ore. Dopo il trattamento con concentrazioni crescenti dell'estratto per circa 48 ore, le cellule sono state lavate in PBS ed incubate con 100 ul/pozzetto di "tampone di reazione" contenente: 10 mM di Hepes, 1,3 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM di glucosio e 0,5 mg/ml di substrato colorimetrico MTT in tampone PBS a pH 7,4. Dopo 3 ore di incubazione a 37°C, 5% CO2, ad ogni pozzetto sono stati aggiunti 100 µl di soluzione solubilizzante contenente 10% di Triton X100, 0,1 N di HCl in isopropanolo assoluto. Dopo 2 ore, la reazione colorimetrica è stata misurata a 595 nm con il lettore di piastre Victor3.

Analisi dell'espressione dei geni 5-α reduttasi e della beta defensine 2 in cellule trattate con l'estratto:

Per l'analisi dell'espressione del gene 5-α reduttasi (SRD5A1) sono stati utilizzati fibroblasti primari neonatali, mentre per il gene per le la beta defensine 2 (hBD2) sono stati utilizzati cheratinociti umani immortalizzati (HaCat).

Fibroblasti primari neonatali (HDFn) o Cheratinociti immortalizzati (HaCaT) in numero iniziale di 1,2 x 10^5 per pozzetto, sono stati cresciuti in piastre da 6 pozzetti in mezzo di coltura DMEM (Lonza),

supplementato con il 10% di Fetal Bovine Serum per 16 ore.

Le cellule sono state poi incubate con due diverse concentrazioni dell'estratto della microalga per 6 ore (come di seguito descritto). Al termine dell'incubazione, le cellule sono state raccolte e l'RNA estratto utilizzando un kit acquistato dalla Sigma. I campioni di RNA sono stati sottoposti ad un trattamento con DNasi I (Ambion) per eliminare il DNA genomico contaminante. 2 µl di ciascun campione sono stati caricati su gel di agarosio 1% in presenza di "loading dye" (0,25% Blu di bromofenolo, 0.25% xilene cianolo FF, 40% saccarosio in acqua) e quantizzati utilizzando come riferimento uno specifico marker per RNA (Riboruler RNA ladder, Fermentas). Per la quantificazione è stato usato il software Gene tools (Perkin Elmer). 300 ng di RNA totale è stato retrotrascritto usando l'enzima Trascrittasi Inversa (Fermentas).

Le reazioni di RT-PCR semiquantitativa sono state condotte utilizzando come standard interni la coppia di primer universali 18S primer/competimer (Ambion) in rapporto 3:7 per SRD5A1 e 2:8 per hBD2. I prodotti di PCR sono stati separati su gel d'agarosio 1,5%, visualizzati utilizzando lo strumento Geliance (Perkin Elmer) ed analizzati densitometricamente utilizzando il software Genetools. I valori riportati nei grafici delle figure 2 e 4 rappresentano i rapporti tra l'intensità della banda relativa al gene in analisi e quella della banda relativa allo standard di riferimento 18S, in modo da avere un valore correlato

alla reale espressione di quel gene e non dipendente dalla quantità di RNA o di reagenti di PCR presenti in quel campione.

La sequenza dei primers specifici per l'amplificazione è la seguente:

SRD5A1-Fw: ATGTTCCTCGTCCACTACGG

SRD5A1-Rv: GGAGGTACCACTCATGATGC

hBD2-Fw: ATGAGGGTCTTGTATCTCCT

hBD2-Rv: TCATGGCTTTTTGCAGCATT

Saggio di crescita batterica

Per la crescita batterica di *S.aureus* e *S.epidermidis*, che crescono in condizioni di aerobiosi, è stato seguito il seguente protocollo:

una colonia di batteri è stata fatta crescere per 18 ore a 37°C in agitazione nel mezzo di coltura triptone soia (Sigma-Aldrich). La coltura è stata successivamente diluita 1:50 in mezzo di coltura fresco e 100 µl della diluizione sono stati distribuiti in piastra da 96 pozzetti in presenza di differenti concentrazioni (da 0,01% a 2,5% p/v di estratto secco dell'estratto della microalga. La piastra è stata incubata a 37°C in agitazione per 18 ore. La crescita batterica è stata misurata a 595 nm, usando un lettore di piastre Victor 3 (PerkinElmer).

Per la crescita di *P.acnes* che cresce in anaerobiosi, quindi in assenza di ossigeno, è stato seguito il seguente protocollo. Una colonia di *P.acnes* è stata cresciuta in Reinforced Clostridium Medium (Oxiod) a 37 °C fino a raggiungere OD600=1.0 in condizioni di anaerobiosi usando il sistema Anaerogen AN 0025A (Oxiod). Successivamente 100 ul della coltura

sono stati distribuiti in piastra da 96 pozzetti in presenza di differenti concentrazioni (da 0,01% a 2,5% p/v di estratto secco dell'estratto della microalga. La piastra è stata incubata a 37° per 18 ore in condizioni di anaerobiosi usando il sistema Anaerogen Wzip Compact (Oxiod). La crescita dei batteri è stata misurata a 595nm, usando il lettore di piastra Victor 3 (PerkinElmer).

Saggio sulla rimarginazione della ferita.

Cellule HDFn, in numero iniziale di 1,5 x 10^5 per pozzetto, sono state fatte crescere in piastre da 6 pozzetti in mezzo di coltura DMEM (Lonza), supplementato con il 10% di Fetal Bovine Serum (FBS) per 12 ore, affinchè raggiungano la confluenza e si distribuiscano uniformemente su tutta la superficie del pozzetto.

Con la punta di una pipetta si genera una ferita interrompendo la continuità dello strato cellulare.

Le cellule vengono sottoposte ad un lavaggio con un tampone salino, PBS, per rimuovere quelle staccate in seguito al taglio. Dopo il trattamento con 0,01 e 0,002% dell'estratto secco della microalga per 7 ore in un mezzo di coltura DMEM (Lonza), supplementato con 0,5% di FBS e 10 μg/ml di mytomycin C: quest'ultimo componente viene aggiunto alle cellule per bloccarne la proliferazione e valutarne solo la migrazione. L'area delimitata dai bordi delle cellule, che rappresenta la ferita, viene misurata al tempo 0 ed a 7 ore dal trattamento con il software Image J (Wayne Rasband, National Instituite of Heath, USA), e comparata con quella dei campioni di cellule non trattate. La

migrazione delle cellule porta ad una riduzione dell'area delimitata, riduzione che viene stabilita arbitrariamente pari al 100% nelle cellule di controllo non trattate.

RISULTATI

Studio della citotossicità degli estratti di microalga *Galdieria sulphuraria*.

Allo scopo di determinare le concentrazioni di estratto da utilizzare per tutti i successivi saggi, sono stati condotti esperimenti di citotossicità. Sono state saggiate tre concentrazioni dell'estratto secco, comprese tra 0,0025 e 0,04% (da 25 a 400 μg/ml) su cheratinociti HaCaT. Come mostrato nella Figura 1, l'estratto risulta non avere alcun effetto tossico sulle cellule a partire da concentrazioni inferiori o uguali allo 0,01%.

Studio dell'effetto dell'estratto di *Galdieria sulphuraria* sull'espressione del gene 5 α-reduttasi I, enzima chiave nella produzione del sebo.

Allo scopo di valutare la capacità dell'estratto di influenzare la produzione di sebo, sono stati condotti esperimenti di RT-PCR per valutare i livelli di espressione dell'enzima responsabile della sua produzione nella ghiandola sebacea, la 5α-reduttasi I. Per l'analisi dell'espressione, le cellule sono state trattate con due diverse concentrazioni dell'estratto secco della microalga (0,002%, 0,01%) per 6 ore. Dopo il trattamento, le cellule sono state raccolte, l'RNA estratto e l'espressione genica analizzata mediante RT-

PCR. Come si evince dalla Figura 2, il trattamento con l'estratto idrosolubile ha ridotto significativamente l' espressione del gene 5α–reduttasi ad entrambe le concentrazioni utilizzate: la concentrazione più alta (0,01%) produce una riduzione dell'espressione del gene pari a circa il 45%. Questo risultato suggerisce che il trattamento con l'estratto, riducendo espressione del gene 5α–reduttasi, possa ridurre la produzione di sebo sulla pelle.

Studio della capacità dell'estratto di *Galdieria sulphuraria* di inibire la crescita batterica.

L'aumento della produzione di sebo porta ad una crescita incontrollata di batteri che normalmente colonizzano la ghiandola sebacea. Allo scopo quindi di valutare la capacità dell'estratto di inibire la crescita batterica, sono stati condotti esperimenti su *Staphylococcus epidermidis* e *Propionibacterium acnes*, batteri presenti nella ghiandola sebacea, e su *Staphylococcus aureus*, batterio patogeno che causa foruncoli.

I batteri sono stati fatti crescere in presenza di concentrazioni da 0,01 a 2,5% dell'estratto secco della microalga per 16 ore e la loro crescita, quantificata mediante lettura spettofotometrica a 595 nm, è stata confrontata con quella dei batteri cresciuti in assenza di composti.

Come si evince dalla figura 3, l'estratto è capace di inibire totalmente la crescita di *S. epidermidis* alla concentrazione di 2,5%, e di ridurre la crescita di *S.aureus* e *P.acnes* del 35% alla stessa

concentrazione.

Studio della capacità dell'estratto di *Galdieria sulphuraria* di stimolare i meccanismi di difesa della pelle.

Allo scopo di valutare la capacità dell'estratto di stimolare l'espressione di geni coinvolti nella risposta immunitaria innata della pelle, sono stati condotti esperimenti di RT-PCR per valutare i livelli di espressione della beta-defensina 2, uno dei peptidi antimicrobici prodotto dai cheratinoci.
Per l'analisi dell'espressione, le cellule HaCaT sono state trattate con due diverse concentrazioni dell'estratto secco della microalga (0,002%, 0,01%) per 6 ore. Dopo il trattamento, le cellule sono state raccolte, l'RNA estratto e l'espressione genica analizzata mediante RT-PCR.

Come si evince dalla Figura 4, il trattamento con l'estratto idrosolubile stimola significativamente l' espressione del gene della beta defensina-2 ad entrambe le concentrazioni utilizzate: la concentrazione più bassa (0,002%) produce un effetto più evidente sull'espressione del gene, inducendo un aumento dell'espressione pari a circa il 160%. Questo risultato suggerisce che il trattamento con l'estratto, stimolando l'espressione del gene hβD-2, innalza i meccanismi di difesa dell'organismo, combattendo la crescita dei batteri e richiamando le cellule del sistema immunitario.

Studio della capacità dell'estratto di *Galdieria sulphuraria* di rimarginare una ferita.

Sulla base della capacità dell'estratto di aumentare l'espressione della beta-defensina 2, è stata valutata anche la capacità dell'estratto di stimolare il processo di rimarginazione di una ferita. A questo scopo sono stati effettuati esperimenti di "wound healing" (rimarginazione delle ferite) su fibroblasti primari in piastra. Le cellule "ferite", come descritto nella sezione materiali e metodi, sono state trattate per 7 ore con 0,01 e 0,002% di estratto secco della microalga *G. sulphuraria* e la dimensione della ferita è stata valutata mediante misurazione dell'area delimitata dalla ferita stessa. Come mostrato in figura 5, l'estratto di *G. sulphuraria* è capace di ridurre la grandezza della ferita di circa il 20% rispetto alla dimensione della ferita delle cellule di controllo non trattate.

Questo risultato suggerisce che l'estratto è in grado di stimolare il processo di rimarginazione di una ferita, proprietà importante nella patologia dell'acne, che causa spesso significative lesioni dovute alla formazione di pustole e foruncoli.

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RIVENDICAZIONI

1. Metodo per ottenere un estratto idrosolubile da cellule in coltura liquida della microalga *Galdieria*, preferibilmente della specie *G. sulphuraria*, comprendente le seguenti fasi a)-g):

a) allestimento delle colture algali monospecifiche di *Galdieria* e crescita delle cellule in coltura liquida in un appropriato terreno di coltura;

b) separazione delle cellule dal mezzo di crescita;

c) omogeneizzazione delle cellule;

d) centrifugazione dell'omogenato per isolare l'estratto idrosolubile, rappresentato dal sopranatante.

2. Metodo secondo la rivendicazione 1, in cui la microalga *Galdieria*, preferibilmente della specie *G. sulphuraria*, è fatta crescere nella fase a) in eterotrofia ad una temperatura compresa tra 26 e 37° C.

3. Metodo secondo una qualsiasi delle rivendicazioni precedenti, in cui il terreno di coltura della fase a) comprende 1,32 g/L di $(NH_4)_2SO_4$, 0,6 g/L di K_2HPO_4, 0,1 g/L di NaCl, 0,3 g/L di $MgSO_4$, 0,00996 g/L di $FeSO_4$, 0,02 g/L di $CaCl_2$, 30 g/L glucosio, 0,0199 g/L di $MnCl_2$, 0,211 g/L di H_3BO_3, 0,01 g/L $CuSO_4$, 0,005g/L Na_2MoO_4, 0,005 g/L di $CoCl_2$, 0,014 g/L di $ZnCl_2$ e dove detto terreno di coltura ha pH 1,5.

4. Metodo secondo una qualsiasi delle rivendicazioni precedenti, in cui detta fase c) avviene in un mortaio con PBS 1X.

5. Metodo secondo una qualsiasi delle rivendicazioni precedenti, in cui l'estratto idrosolubile ottenuto dalla fase d) è sottoposto ad

essiccazione.

6. Metodo secondo una qualsiasi delle rivendicazioni precedenti, in cui le cellule sono ulteriormente sottoposte ad almeno un trattamento scelto tra un trattamento di tipo fisico, come riscaldamento, raffreddamento o irradiazione UV; e/o un trattamento di tipo chimico con almeno un composto scelto tra: proteine, lipidi, zuccheri, sali, piccole molecole organiche o loro combinazioni.

7. Estratto idrosolubile ottenibile con il metodo secondo una qualsiasi delle precedenti rivendicazioni.

8. Estratto idrosolubile secondo la rivendicazione 7, compreso in un veicolo cosmeticamente accettabile, eventualmente insieme ad eccipienti e/o adiuvanti cosmeticamente accettabili.

9. Estratto idrosolubile secondo la rivendicazione 8, in cui il veicolo cosmeticamente accettabile è un liposoma, preferibilmente un liposoma multi lamellare, una ciclodestrina e/o un silicato.

10. Composizione cosmetica comprendente un estratto secondo una qualsiasi delle rivendicazioni 7-9.

11. Composizione secondo la rivendicazione 10 in una qualsiasi forma galenica per l'applicazione cutanea, preferibilmente in forma di crema, gel, lozione, soluzione, dispersione, emulsione O/A, emulsione A/O, latte, shampoo, sapone, stick, matita, spray, olio.

12. Uso dell'estratto secondo una qualsiasi delle rivendicazioni 7-9 o della composizione secondo una qualsiasi delle rivendicazione 10-11 in campo

cosmetico.

13. Uso cosmetico secondo la rivendicazione 12 per il trattamento dell'acne, della iperseborrea e/o della pelle tendente all'acne.

14. Estratto secondo una qualsiasi delle rivendicazioni 7-9 o composizione definita secondo una qualsiasi delle rivendicazione 10-11, per l'uso nella prevenzione e/o il trattamento dell'acne, della iperseborrea e/o della pelle tendente all'acne.

15. Estratto secondo una qualsiasi delle rivendicazioni 7-9 o composizione secondo una qualsiasi delle rivendicazione 10-11, per l'uso nell'inibizione dell'enzima 5α-reduttasi a livello cutaneo e/o nella stimolazione delle difese dell'organismo a livello cutaneo contro agenti patogeni.

16. Estratto secondo una qualsiasi delle rivendicazioni 7-9 o composizione secondo una qualsiasi delle rivendicazione 10-11, per l'uso nella prevenzione e/o nel trattamento della caduta dei capelli.

17. Metodo di trattamento cosmetico per alleviare i sintomi dell'acne, comprendente l'applicazione sulla cute da trattare di una quantità cosmeticamente efficace (da 0.0001 a 0.01% p/v) di un estratto secondo una qualsiasi delle rivendicazioni 7-9 o composizione secondo una qualsiasi delle rivendicazione 10-11.

Composizioni cosmetiche comprendenti estratti derivati dalla microalga *Galdieria sulphuraria*, particolarmente indicate per ridurre gli effetti dannosi causati dall'acne.

RIASSUNTO

La presente invenzione si riferisce all'uso in campo cosmetico di estratti cellulari derivati dalla microalga rossa appartenente al genere *Galdieria*, preferibilmente alla specie G. *sulphuraria* e alle relative composizioni dermocosmetiche comprendenti detti estratti aventi effetti seboregolatori, antibatterici e di stimolazione del sistema di difesa della pelle, utili per alleviare gli effetti dannosi causati dall'acne sulla pelle. L'invenzione concerne ulteriormente un metodo per ottenere i suddetti estratti.

Figura 1

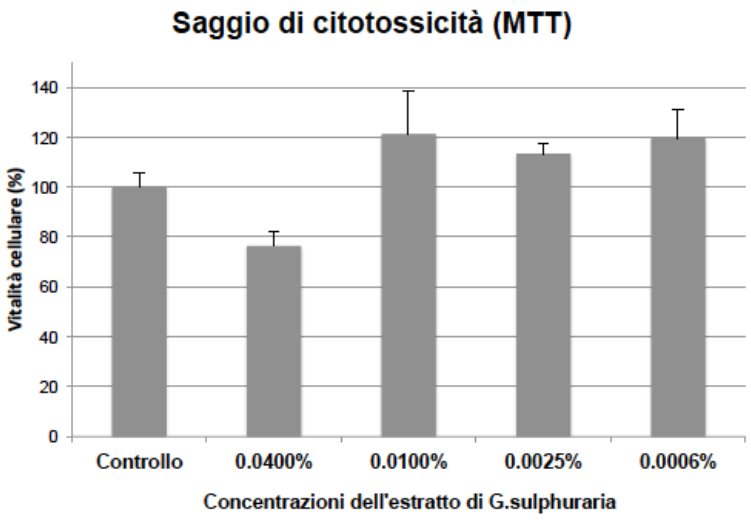


Figura 2

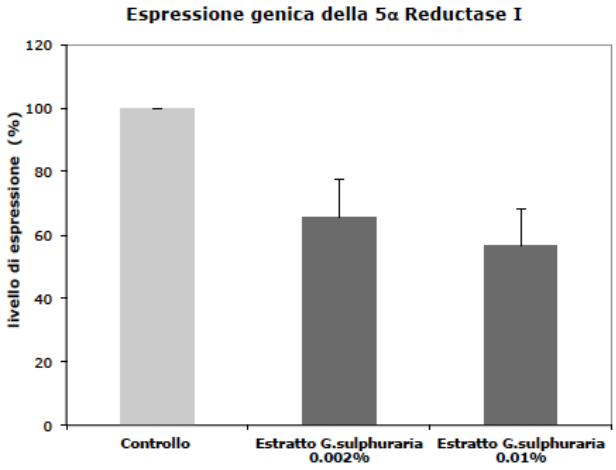


Figura 3

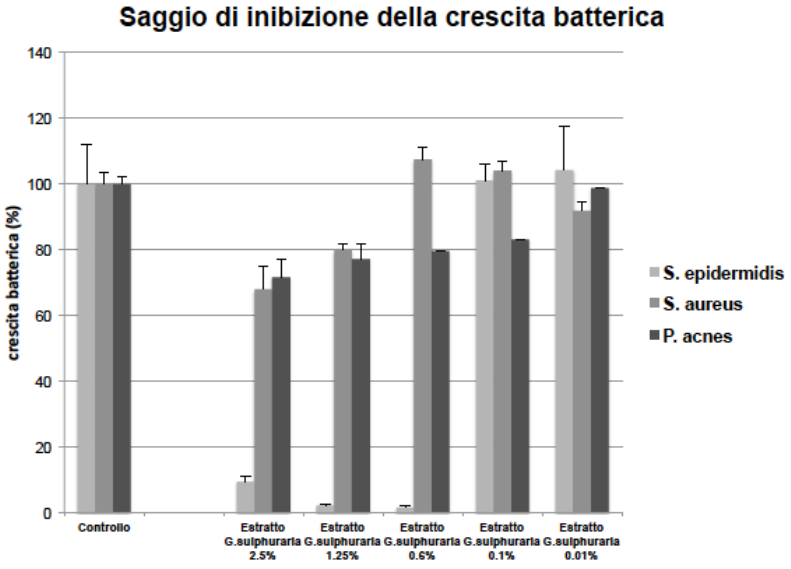


Figura 4

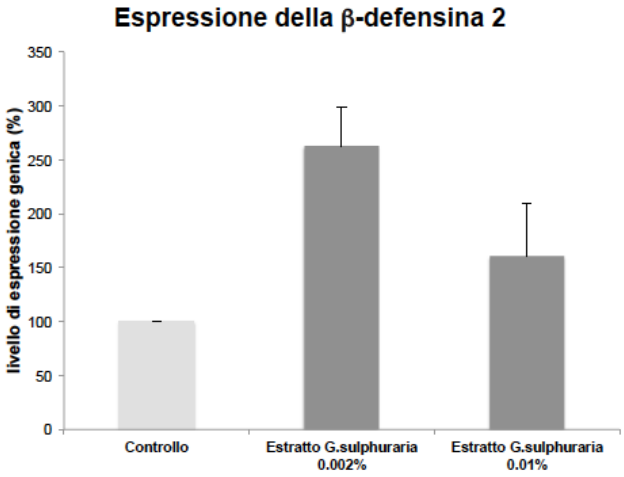
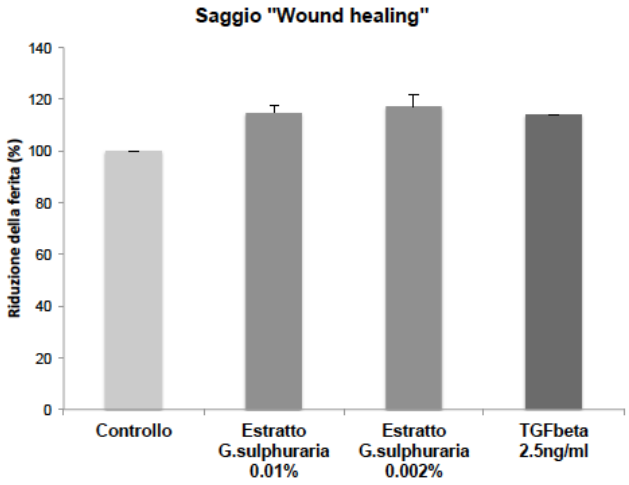


Figura 5


Saggio "Wound healing"
Riduzione della ferita (%)
140
120
100
80
60
40
20
0
Controllo
Estratto G.sulphuraria 0.01%
Estratto G.sulphuraria 0.002%
TGFbeta 2.5ng/ml

List of publications

M. Bimonte, A. Carola, **A. De Lucia**, A. Tito, S. Buono, A.L. Langellotti, V. Fogliano, G. Colucci, F. Apone. 2015. The water- soluble extract of *Galdieria sulphuraria* has beneficial activities on oily and acne prone skin (Submitted).

A. Tito, M. Bimonte, A. Carola, **A. De Lucia**, A. Barbulova, A. Tortora, G. Colucci, F. Apone. 2015. An oil-soluble extract of *Rubus Idaeus* cells enhances hydration and water homeostasis in skin cells (Submitted to *International Journal of Cosmetic Science*).

A. De Lucia, A.L. Langellotti, A. La Storia, S. Buono, G. Graziani, D. Ercolini, G. Colucci, F. Apone, V. Fogliano. 2015. Bioactive ingredients from fermented Arthrospira maxima biomass (Submitted to *Journal of Agricultural and Food Chemistry*).

A.L. Langellotti, S. Buono, **A. De Lucia**, L. Castaldo, G. Graziani, P. Vitaglione, F. Apone, G. Colucci, V. Fogliano. 2015. Bioactives from thermal processed Arthrospira maxima (Spirulina) biomass (Submitted to *Food and function*).