University of Basilicata

International PhD Programme
"Applied Biology and Environmental Safeguard"

"*DEVELOPMENT AND OPTIMIZATION OF SYSTEM SUITABLE FOR THE PRODUCTION OF BIOACTIVE MOLECULES STARTING FROM UNICELLULAR PLANTS AND/OR FROM MICROORGANISMS*"

Scientific Disciplinary Sector
BIO/13 – Applied Biology

PhD Coordinator
Prof. Patrizia FALABELLA

PhD Student
Dr. Ilaria PAPPALARDO

Tutor
Prof. Vittoria INFANTINO

XXXV Cycle

INDEX

ABSTRACT……………………………………………………………………………………**4**

LIST OF FIGURES……………………………………………………………………………6

LIST OF TABLES………………………………………………………………………………8

1. **INTRODUCTION**…………………………………………………………………………**9**

1.1 BIOACTIVE MOLECULES…………………………………………………………10

1.1.1 Main classes of bioactive molecules……………………………………12

1.2 MICROALGAE……………………………………………………………………………21

1.2.1 Classification…………………………………………………………………25

1.2.2 Microalgae biomass……………………………………………………………29

1.2.3 Factors affecting biomass production…………………………………32

1.2.4 Biochemical composition of microalgae………………………………33

1.3 MICROALGAL BIOACTIVE MOLECULES: BIOTECHNOLOGICAL POTENTIAL USES……………………………………………………………………………35

1.3.1 Pharmaceutical applications……………………………………………36

1.3.2 Nutraceuticals/Food applications………………………………………37

1.3.3 Cosmeceutical applications………………………………………………38

1.3.4 Feed………………………………………………………………………………38

1.3.5 Proteomics………………………………………………………………………39

1.4 RESEARCH OBJECTIVES……………………………………………………………40

2. **MATERIALS AND METHODS**……………………………………………………**41**

2.1 MICROALGAL STRAINS………………………………………………………………42

2.1.1 *Haematococcus pluvialis*…………………………………………………42

2.1.2 Cell growth………………………………………………………………………44

2.1.3 Optical density…………………………………………………………………46

2.1.4 Cell counting……………………………………………………………………46

2.1.5 Wet biomass……………………………………………………………………47

2.1.6 Analysis of phylogenetic distances……………………………………47

2.1.7 Evaluation of enzymatic profile and chemical composition of microalgal strains………………………………………………………………47

2.2 EXTRACTS PREPARATION…………………………………………………………47

2.2.1 Extracts preparation…………………………………………………………47

2.2.2 Protein determination………………………………………………………48

2.3 *IN VITRO* TESTS…………………………………………………………………………49

2.3.1 Cell culture………………………………………………………………………49

2.3.2 Cell viability analysis.....52
2.3.3 3T3-L1 preadipocyte cell differentiation.....53
2.3.4 Cell viability analysis in differentiated 3T3-L1 preadipocytes.....54
2.3.5 ROS Assay.....54
2.3.6 NO• Assay.....55
2.3.7 IL-6 and TNFα detection.....55
2.3.8 Quantification of lipid content by Oil Red O staining method.....56
2.3.9 Quantitative analysis of intracellular lipid accumulation.....56
2.3.10 *In vitro* lipolysis.....56
2.3.11 Mitochondrial mass analysis.....56
2.3.12 Mitochondrial membrane potential analysis.....57
2.3.13 Mitochondrial superoxide analysis.....57
2.3.14 Western blot.....58
2.3.15 IL-6 and TNFα detection.....59
2.3.16 Statistical analysis.....59
3. RESULTS.....60
3.1 CHARACTERIZATION OF MICROALGAL STRAINS.....61
3.1.1 Microalgal strains growth.....61
3.1.2 Analysis of phylogenetic distances.....62
3.1.3 Microalgal enzymatic profile evaluation.....63
3.1.4 Microalgal biochemical composition.....63
3.2 EXTRACTS PREPARATION.....65
3.3 *IN VITRO* TEST.....67
3.3.1 Effect of microalgal extracts on primary human fibroblasts viability.....67
3.3.2 Effect of microalgal extracts on differentiated U937 cell viability.....69
3.3.3 ANTI-INFLAMMATORY EFFECT OF MICROALGAL EXTRACTS.....70
3.3.3.1 Microalgal extracts lower ROS levels.....71
3.3.3.2 Microalgal extracts affect NO• production.....71
3.3.3.3 Microalgal extracts reduce the secretion of the pro-inflammatory cytokines IL-6 and TNFα.....72
3.3.4 EFFECT OF MICROALGAL EXTRACTS ON ADIPOCYTE FUNCTION.....73
3.3.4.1 Effect of microalgal extracts on mouse 3T3-L1

preadipocyte viability……..……………………………………...73
3.3.4.2 Differentiation of mouse 3T3-L1 preadipocytes…………75
3.3.4.3 Effect of microalgal extracts on mouse 3T3-L1 adipocyte viability………………………..…………………………………..76
3.3.4.4 Effect of microalgal extracts on lipid content in mouse 3T3-L1 adipocytes…………………………………………………77
3.3.4.5 Effect of microalgal extracts on *in vitro* lipolysis…………………………………………………………………79
3.3.4.6 Effect of microalgal extracts on mitochondrial function………………………………………………………………80
3.3.4.7 Treatment with microalgal extracts regulates the expression of markers of adipogenesis………………………82
3.3.4.8 Microalgal extracts reduce levels of pro-inflammatory cytokines IL-6 and TNFα in adipocytes………………………84
4. DISCUSSION……………………………………………………………………………85
5. CONCLUSIONS……………………………………………………………………….90
6. REFERENCES…………………………………………………………………………92

ABSTRACT

In recent years, the interest in algae has increased significantly thanks to their health benefits, such as dietary supplementation but also potential alternative to classic drugs. Microalgae are new model organisms for a wide range of biotechnology applications, including the production of biodiesel and the bioremediation of waste water but also the natural food and nutraceutical food supplements sector thanks to the development of products with benefits for human health and nutrition. Rich in proteins, carbohydrates, mineral salts, vitamins and fatty acids, the cultivated microalgae are harvested and dried to be transformed into powder and then used in the preparation of food supplements and easy-to-eat products such as bread, pasta, biscuits and drinks. In particular, they are rich in micronutrients such as β-carotene (which is transformed into vitamin A in the body), astaxanthin (carotenoid with antioxidant action), vitamin B12, and omega 3 and omega 6 polyunsaturated fatty acids with interesting biological activities, including powerful antioxidant and anti-inflammatory properties presenting themselves as valuable supplements in the prevention of various pathologies and in the management and treatment of physiological imbalances. Microalgae are also considered a potential source of bioactive compounds with anti-obesity activity. Algal compounds showing potential anti-obesity activities include fucoxanthin, fluorotannine, fucoidan and alginates. Studies are currently focusing on how to use algae to manage obesity and related diseases. Inhibition of lipases, especially pancreatic lipase is one of the main therapeutic targets of anti-obesity drugs. The current market-approved anti-obesity drug, orlistat, acts through this mechanism, namely irreversibly blocking pancreatic lipase. Microalgae are also a promising source of industrial enzymes, thanks to their many positive and beneficial properties. To date, several microalgal enzymes have been studied with different industrial applications (for example, biofuel production, health care and bioremediation) and the modification of the enzymatic sequences involved in the production of lipids and carotenoids has produced promising results. In many cases, however, entire biosynthetic pathways/systems leading to the synthesis of potentially important bioactive compounds still need to be fully characterized.

We investigated the effect of the microalgal extracts of *Haematococcus Pluvialis* and two of its mutants on inflammation and adipocyte function using models of inflammation and obesity *in vitro*. In particular, treatment with FBR1 and FBR2 mutants inhibited both the levels of some inflammatory markers such as reactive oxygen species, nitric oxide, interleukin 6, tumor necrosis factor-alpha and lipid droplet accumulation. It also reduced the expression of the transcription factor PPARγ (peroxisome proliferator-activated receptor γ) and the metabolic protein ACLY (ATP citrate lyase). Therefore, the results suggest that the two extracts may have potential therapeutic implications for the treatment of inflammation and obesity.

LIST OF FIGURES

Figure 1.1: Growing interest in bioactive molecules and its industrial applications……………………………………………………………………………11

Figure 1.2: Bioactive molecule extracted using conventional methods…………………………………………………………………………………12

Figure 1.3: Structures of typical carotenes and xanthophylls…………………..13

Figure 1.4: Structures of typical phenolic compounds…………………………...15

Figure 1.5: Lock-and-key model that explains the selectivity of enzymes…………………………………………………………………………………………17

Figure 1.6: Growing interest in microalgae and its industrial applications…………………………………………………………………………………23

Figure 1.7: Number of publications by category of microalgae applications over time……………………………………………………………………………………...24

Figure 1.8: Spectrum of phenotypes and sizes of algal species……………......26

Figure 1.9: Summary of algal phylogeny……………………………………………..27

Figure 1.10: Cyanobacteria Structure………………………………………………..28

Figure 1.11: The production of microalgae biomass……………………………...30

Figure 1.12: Systems of microalgae cultivation………………………………….....31

Figure 2. 1: Life cycle stages of *H. pluvialis*………………………………………………42

Figure 2.2: Life cycle of *H. pluvialis*………………………………………………………43

Figure 2.3: A trichome of the cyanobacterium *Arthrospira platensis*………………………………………………………………………………………44

Figure 2.4: Extracts preparation………………………………………………………48

Figure 2.5: Human primary fibroblasts………………………………………………50

Figure 2.6: U937 cell line……………………………………………………………...50

Figure 2.7: Mouse 3T3-L1 cell line………………………………………………….....51

Figure 2.8: Flow diagram showing preparation and use of CellTiter-Glo® Reagent…………………………………………………………………………………………52

Figure 2.9: Scheme of 3T3-L1 preadipocyte cell differentiation………………...53

Figure 2.10: DCFH-DA reaction in ROS Assay……………………………………....54

Figure 2.11: DAF-FM diacetate reaction in NO• Assay…………………………......55

Figure 2.12: Overview of Western blotting procedure…………………………..59
Figure 3.1: Growth curves for HP, FBR1, FBR2 and AP…………………………..61
Figure 3.2: Dendrogram showing greater genotypical similarities between FBR1 and HP than FBR2 (UPGMA method)……………………………………………62
Figure 3.3: Genetic distances between species…………………………………...63
Figure 3.4: Microalgal extracts and AP in Petri dishes……………………………………………………………………………………………..65
Figure 3.5: Petri dishes in the stove…………………………………………………...66
Figure 3.6: Microalgal and AP extracts……………………………………………….67
Figure 3.7: Fibroblasts cells viability……………………………………………….68
Figure 3.8: Differentiated U937 cell viability…………………………………….…70
Figure 3.9: Microalgal and AP extracts lowered ROS level…………………......71
Figure 3.10: Microalgal and AP extracts lowered NO• levels. …………………...72
Figure 3.11: Microalgal and AP extracts lowered IL-6 and TNFα levels …….73
Figure 3.12: Effect of microalgal and AP extracts on mouse 3T3-L1 preadipocyte viability……………………………………………………………………….…74
Figure 3.13: Differentiation of 3T3-L1 preadipocytes into adipocytes………75
Figure 3.14: Formation of lipid droplets in adipocytes…………………………...76
Figure 3.15: Effect of microalgal and AP extracts on mouse 3T3-L1 adipocyte viability…………………………………………………………………………………………77
Figure 3.16: Microscopic observation of the preadipocytes differentiation into adipocytes, monitored with Oil Red O. ……………………………………….....78
Figure 3.17: Effect of microalgal and AP extracts on lipid content, monitored through Oil Red O staining…………………………………………………………………79
Figure 3.18: Effect of microalgal and AP extracts on *in vitro* lipolysis…………………………………………………………………………………………80
Figure 3.19: Effect of microalgal and AP extracts on mitochondrial parameters……………………………………………………………………………………...81
Figure 3.20: Effect of microalgal and AP extracts on transcription factor PPARγ gene expression……………………………………………………………………...82
Figure 3.21: Effect of microalgal and AP extracts on metabolic protein ACLY gene expression…………………………………………………………………………...83

Figure 3.22: Effect of microalgal and AP extracts on IL-6 and TNFα levels. ………………………………………………………………………………………………………....84

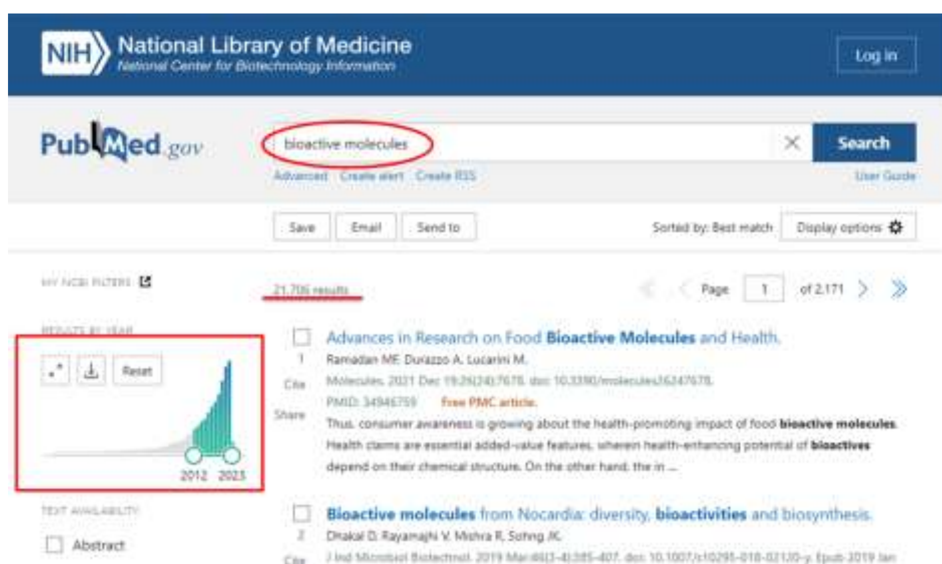
LIST OF TABLES

Table 1.1. Enzyme classification: main classes of enzymes in EC system…...19
Table 1.2. A selection of enzymes used in industrial processes……………….....20
Table 2.1. Modified Bold 3N Medium composition………………………………....45
Table 3.1: Enzymatic profile of microalgal and AP strains……………………….....63
Table 3.2: Biochemical composition of microalgal strains and AP……………....64
Table 3.3: Composition of fatty acids in microalgal strains………………………...65
Table 3.4: Wet and dry biomass weight of microlgal and AP extracts………...66
Table 3.5: Protein dosage of microalgal and AP extracts………………………….67

1. INTRODUCTION

1.1 BIOACTIVE MOLECULES

The term "bioactive molecule" refers to any substance of very variable chemical composition widely distributed in nature that activates its functionality after interacting with a cell, tissue or living organism [1]. This interaction involves a number of effects, the main of which is the modification of metabolism [2]. Bioactive molecules exist in different forms such as polysaccharides, vitamins, carotenoids, enzymes, peptides, antioxidants, phenolic compounds, phytosterols and new lipids, bacteriocles, immunomodulatory compounds and amino acids [3]. Bioactive molecules have become increasingly important for the exhibition of different biological activities including anti-inflammatory, antioxidants, anticancer, antifungal, antimicrobial, cardioprotective, neuroprotective, antidiabetic and many others [4]. Thanks to these beneficial properties, the production of bioactive molecules is in great demand in several pharmaceutical and nutraceutical industries. To give a recent view of the interest aroused in the context of international research within this topic, a search throughout the PubMed database was carried out using the string "*bioactive molecules*", the search returned 26.160 documents covering the time interval from 1972 to 2022. In the last ten years (from 2012-2023), research has returned 21.706 documents. The string "*bioactive molecules AND industrial application*" gave 868 documents related to the last 22 years (***Figure 1.1***).



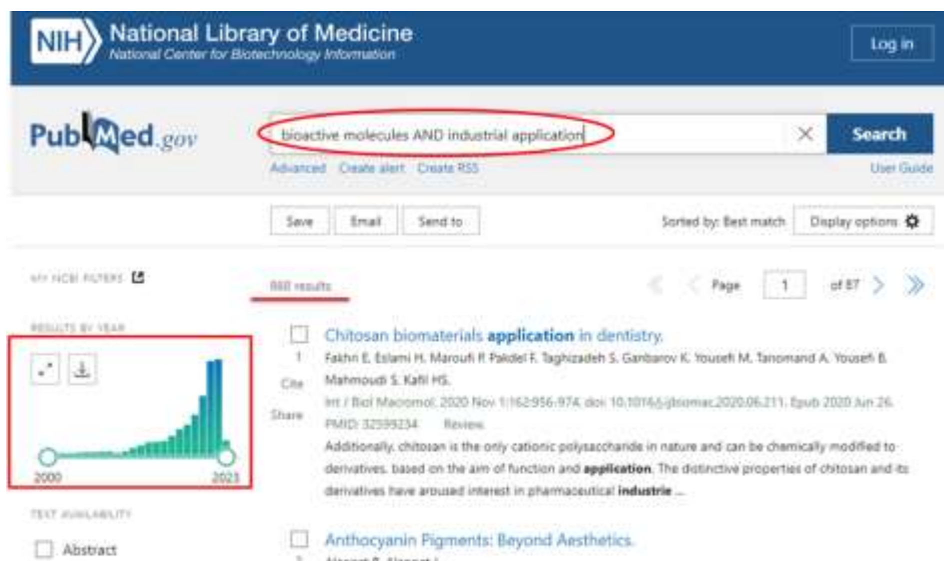


Figure 1.1: Growing interest in bioactive molecules and its industrial applications. The image shows the results of the research on PubMed by inserting different keywords such as "*bioactive molecules*" and "*bioactive molecules AND industrial applications*" [https://pubmed.ncbi.nlm.nih.gov/, 3 February 2023].

Over the years many approaches and techniques have been developed and used for the extraction, purification and processing of different bioactive molecules. These techniques involve the use of chemical and mechanical methods that exploit extreme temperatures and aggressive chemicals to purify and process bioactive molecules from their initial sources (***Figure 1.2***). However, researchers have found some shortcomings in the use of these conventional techniques, such as, for example, low investment benefits or even negative aspects such as product degradation, inefficient bioactivity and low product yield [5]. To overcome these obstacles, scientists have worked for over 50 years to develop techniques that are not only innovative in their methodology but are highly environmentally friendly, have shorter operating times and offer a high quality product and better performance [6].

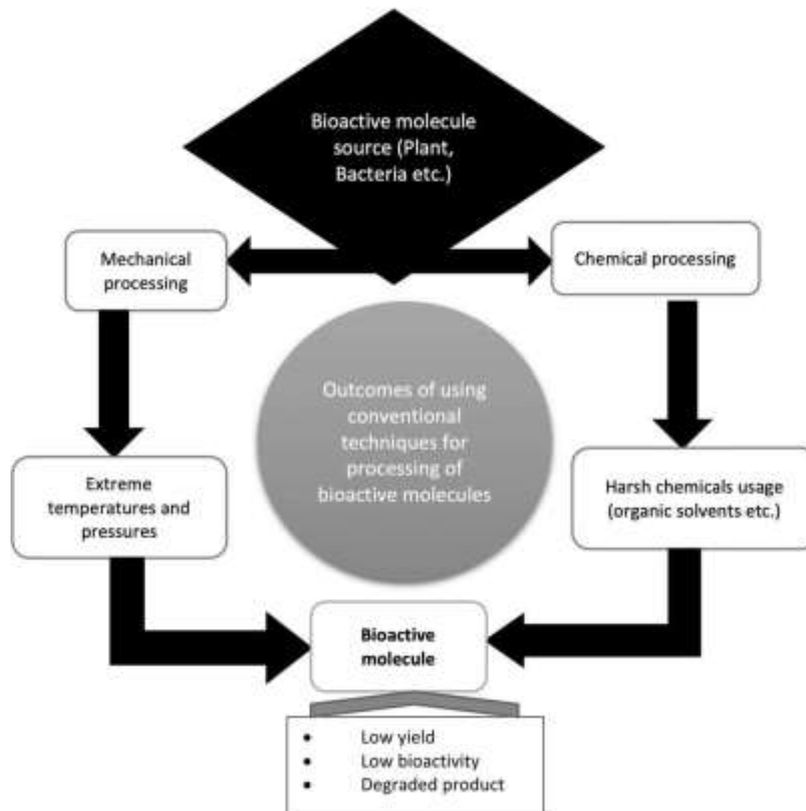


Figure 1.2: Bioactive molecule extracted using conventional methods [5].

1.1.1 Main classes of bioactive molecules

In the following section will be presented a brief overview of the major classes of bioactive molecules.

- ***Carotenoids***

Carotenoids are a class of long chain organic pigments of lipid nature characterized by a bright color (yellow, orange, red and purple). They are widely spread in nature (up to 2018 about 850 had been reported) and are present in photosynthetic bacteria, in some species of archaea and fungi, algae, plants and animals. Most carotenoids consist of eight isoprenenic units with a skeleton of 40 carbon atoms. Their structures generally have a polythene chain with nine double

conjugated bonds and a terminal group at both ends of the polythene chain [7]. Carotenoids are classified into carotenes and xanthophylls. The former, such as *α*-carotene, *β*-carotene, *β*, *ψ*-carotene (*γ*-carotene) and lycopene, are hydrocarbons. The latter, such as astaxanthin, fucoxanthin, lutein, zeaxanthin, *β*-cryptoxanthin and peridinin, contain oxygen atoms in the structure such as hydroxy, carbonyl, aldehyde, carboxyl, epoxide and furanoxide groups. Some xanthophylls are present as esters of fatty acids, glycosides, sulfates and protein complexes. The structures of the xanthophylls show a marked diversity. In ***Figure 1.3*** are shown the structures of the typical carotenes and xanthophylls. Most carotenoids have a skeleton of 40 carbon atoms although some have a skeleton of 45 or 50 carbon atoms and are called superior carotenoids. Carotenoids composed of carbon skeletons with less than 40 carbon atoms are called apocarotenoids [8].

It is believed that the beneficial effects of carotenoids are closely related to their role as antioxidants. *β*-carotene can show additional benefits due to its ability to be converted into vitamin A. In addition, lutein and zeaxanthin can be protective in eye diseases because they absorb the harmful blue light that enters the eye [9].

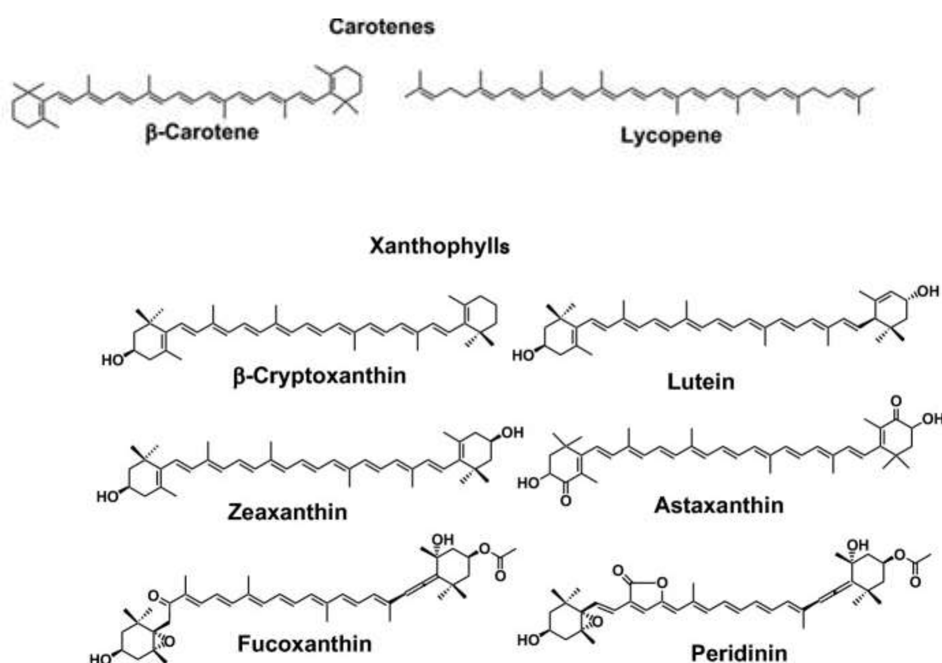


Figure 1.3: Structures of typical carotenes and xanthophylls.

- ***Vitamins***

Vitamins represent a very heterogeneous set of small organic molecules that cannot be synthesized by vertebrates but are necessary to perform specific biological functions for the normal growth and maintenance of a human's health [10]. Discovered in 1911 by the Polish physician Kazimierz Funk, who extracted for the first time from bran a substance capable of treating beri beri, vitamins are essential nutrients to health whose regular intake, through food, can have important effects in the prevention of numerous diseases, including various forms of anemia, nervous system disorders, up to certain types of cancer [10]. The term "vitamin" comes from the union of two terms, vital and amine, but since later we realized that not all are amines, the letter "e" has been removed. Vitamins can be divided into two large groups in according to their solubility in water or fat which further affects their phamacokinetic properties. Water solubles are not accumulative by the body and therefore must be taken daily with food. They include all B vitamins, folic acid, vitamin H, PP and C. The fat solubles are absorbed together with dietary fats and accumulated in the liver. They include vitamins A, D, E and K. The deficiency is then manifested by a failure to take for a long time [11], [12].

- ***Phenolic compounds***

Phenolic compounds are a class of natural bioactive molecules found primarily in plant tissues, divided into phenolic acids and polyphenols (***Figure 1.4***). These compounds are found combined with mono and polysaccharides, bound to one or more phenolic groups, or may present as derivatives, such as esters or methyl esters [13]. Polyphenols include flavonoids, tannins, lignans and coumarins, compounds found naturally in fruits, vegetables, cereals, roots and leaves among other plant products. Phenolic compounds showed interesting bioactivities, such as antioxidant, antimicrobial, anti-inflammatory and antiproliferative activities, among others, which aroused great interest in their use by several industries [14].

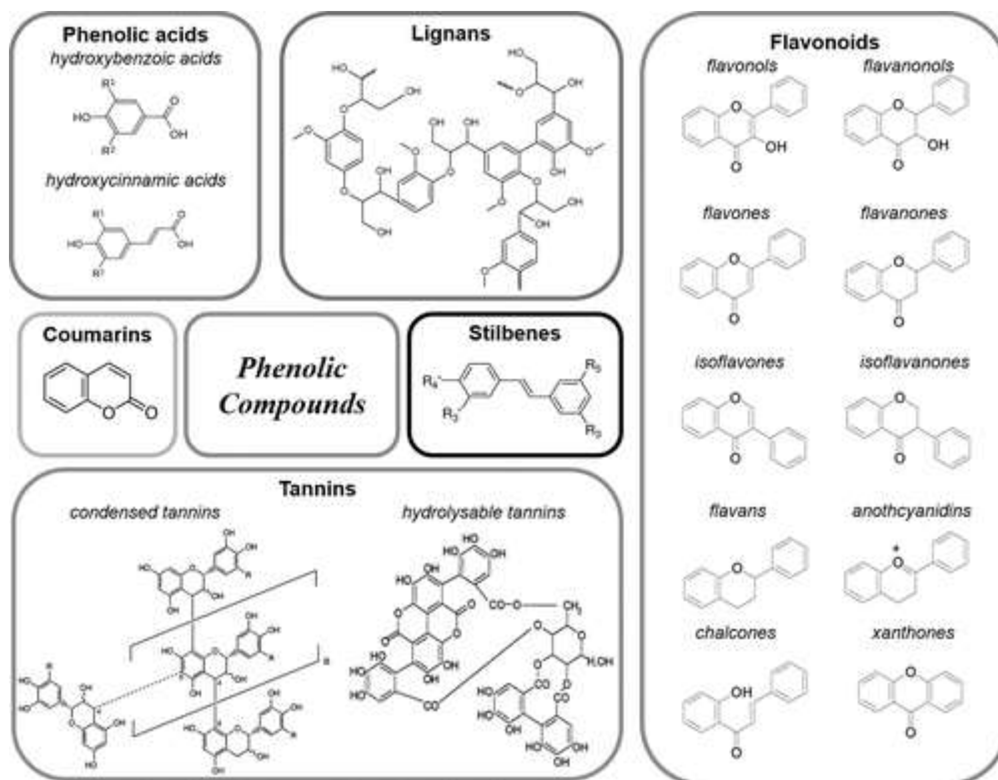


Figure 1.4: Structures of typical phenolic compounds.

- ***Lipids***

Lipids are hydrophobic molecules insoluble in water but soluble in apolar solvents. From a purely biological point of view, lipids are essential molecules that function as the main structural components of living cells and play important roles in various cellular functions [15]. For decades it was believed that lipids were molecules that stored energy and constituted cell membranes. But, thanks to the advent of advanced analytical techniques and biological assays, more and more lipid classes and functions are coming to light. It is now known that lipids are involved in numerous cell signaling pathways, including those that regulate cell proliferation, survival, apoptosis, and cellular metabolism [16]. Thanks to their excellent biocompatibility, lipids are also used as efficient and safe vectors for drug administration in various formulations. Sources such as The Lipid Library (http://lipidlibrary.aocs.org) and Cyberlipids (http://www.cyberlipid.org) divide lipids into "simple" and "complex" groups, where the former are those that produce at most two types of distinct entities following hydrolysis (e.g.

acylglycerol: fatty acids and glycerol) and the latter (e.g. glycerophospholipids: fatty acids, glycerol, and headgroup) produce three or more hydrolysis products. In contrast, LipidBank (www.lipidbank.jp) [17] in Japan defines a third major group called "derived" lipids (alcohols and fatty acids derived from the hydrolysis of simple lipids). In 2005, the International Lipid Classification and Nomenclature Committee on the initiative of the LIPID MAPS Consortium developed and established a comprehensive lipid classification system based on well-defined chemical and biochemical principles and using a framework designed to be extensible, flexible, scalable and compatible with modern computer technology [18]. Dysregulated lipid levels have been reported in various diseases, such as cancer, obesity, diabetes and cardiovascular diseases, autoimmune and neurodegenerative [19]. This, combined with their important roles in various biological conditions, suggests that lipids could be potential therapeutic targets in the treatment of diseases.

- ***Polysaccharides***

Polysaccharides are complex carbohydrate polymers consisting of more than two monosaccharides covalently bound by glycosidic bonds in a condensation reaction. Being relatively large macromolecules, polysaccharides are often insoluble in water. Starch, cellulose and glycogen are some examples of polysaccharides. Polysaccharides show a number of pharmacological activities such as anticancer, antiviral, antibacterial, immune activation activity, and hypoglycemic. In addition, polysaccharides have been reported to exhibit various biological activities, such as anti-inflammatory, antioxidant, anticomplementation, anticoagulant and growth enhancement of probiotic bacteria [20].

- ***Enzymes***

Enzymes are the large biomolecules defined as catalysts of biological processes [21]. They are proteins that have the power to accelerate the chemical reactions typical of vital processes from 100 million to 10 billion times more and they do it like all other catalysts: they only allow thermodynamically possible reactions, at

the end of the process they are unaltered and never alter the balance of reactions. Thanks to the development of recombinant technology and protein engineering, enzymes have evolved as an important molecule that has been widely used in various industrial and therapeutic purposes. For example, they play important roles in the production of sweeteners and in the modification of antibiotics, or are used in washing powders and various cleaning products. They also play a key role in analytical devices and tests that have clinical, forensic and environmental applications. The term “enzyme” was first used in 1877 by Wilhelm Friedrich Kühne, professor of physiology at the University of Heidelberg, when he described the ability of yeast to produce alcohol from sugars, and derives from the Greek words *εν* (meaning "inside") e *ζυμον* (meaning 'yeast') [22]. The first enzyme, diastase, was discovered by the French chemist Anselme Payen in 1833 [23]. From the 1980s onwards, many scientists began to apply genetic engineering techniques in order to improve the production of enzymes and also to alter the properties of enzymes by engineering proteins. Nobel laureate Emil Fischer in 1894 suggested that the specificity of the enzymes was due to the fact that both the enzyme and the substrate possess specific complementary geometric shapes that exactly fit each other [24]. Although this model, termed "the lock and key model" (***Figure 1.5***), explains the specificity of the enzyme, it fails to explain the stabilization of the transition state achieved by the enzymes.

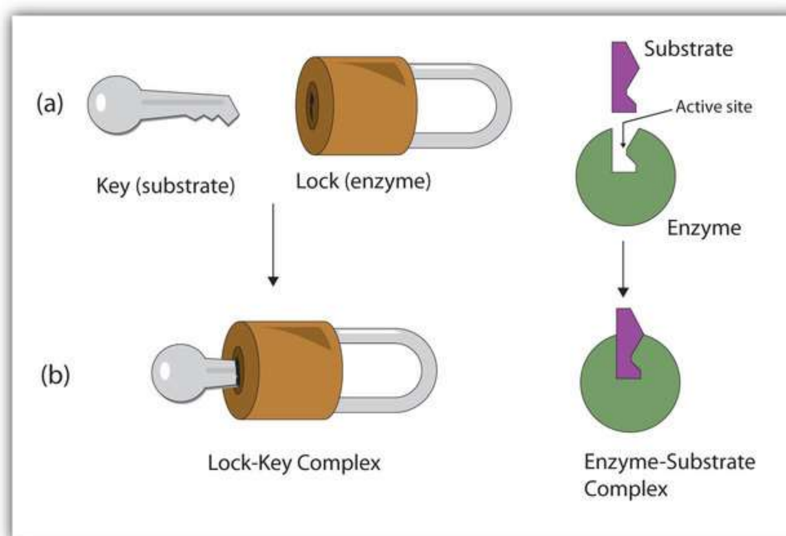


Figure 1.5*: *Lock-and-key model that explains the selectivity of enzymes [25].

Most enzymes are much larger than the substrates they act on and only a small part of the enzyme (about 2-4 amino acids) is directly involved in catalysis [26]. The region of the enzyme where the reaction occurs is called the active site. Proteins take on a tertiary structure, so the enzyme will take on a tertiary structure and the reaction will take place in a specific area of this structure. The molecule that binds to the active site and on which the enzyme acts is the substrate. The enzyme then establishes interactions with the substrate which binds to the active site and thus forms an enzyme-substrate complex. Once the catalytic reaction has taken place, the products leave the surface of the enzyme free to react with another substrate molecule.

Enzymes generally have common names (often called "trivial names") related to the reaction they catalyze, with the suffix -*ase* (e.g. dehydrogenase, oxidase, carboxylase), although individual proteolytic enzymes generally have the suffix -*in* (e.g. chymotrypsin, trypsin, papain). Often the name also indicates the substrate on which the enzyme acts (e.g. glucose oxidase, alcohol dehydrogenase, pyruvate decarboxylase). However, some trivial names (e.g. invertase, diastase, catalase) provide little information about the substrate, product or reaction involved. In general, enzymes are classified according to the reactions they catalyze. In fact, a unique number identifies every single enzyme, each number is made up of four distinct sections separated by a dot. The numbering responds to a precise classification scheme that has been in use since 1961 and was introduced by the Commission on Enzymes (EC) of the International Union of Biochemistry (IUB) [27]. The first part of the EC number refers to the reaction that the enzyme catalyzes (***Table 1.1***). The remaining digits have different meanings depending on the nature of the reaction identified by the first digit. Enzymes are divided into 6 classes. The 6 classes are divided into subclasses and sub-subclasses for specify in more detail the type of reaction and the nature chemistry of the species involved. Each enzyme is identified by a unique number (E.C., Enzyme Commission number) consisting of four distinct sections, separated by a point:

EC A.B.C.D.

A: membership class (type of reaction)

B: sub-class (substrate type)

C: sub-sub-class (type of acceptor, or group removed, possible coenzyme)

D: serial number of the enzyme (the position of the enzyme in the sub-class)

Table 1.2 gives examples of a few industrially important classes of enzymes.

First EC digit	Enzyme class	Reaction type
1.	Oxidoreductases	Oxidation/reduction
2.	Transferases	Atom/group transfer (excluding other classes)
3.	Hydrolases	Hydrolysis
4.	Lyases	Group removal (excluding 3)
5.	Isomerases	Isomerization
6.	Ligases	Joining of molecules linked to the breakage of a pyrophosphate bond

Table 1.1: Enzyme classification: main classes of enzymes in EC system.

First EC digit	Enzyme class	Industrial enzymes
1.	Oxidoreductases	Catalases Glucose oxidases Laccases
2.	Transferases	Fructosyltransferases Glucosyltransferases
3.	Hydrolases	Amylases Cellulases Lipases Mannanases

		Pectinases Phytases Proteases Pullulanases Xylanases
4.	Lyases	Pectate lyases Alpha-acetolactate decarboxylases
5.	Isomerases	Glucose isomerases Epimerases Mutases Lyases Topoisomerases
6.	Ligases	Argininosuccinate Glutathione synthase

Table 1.2: A selection of enzymes used in industrial processes.

Enzymes are omnipresent, we find them in animals, plants and microorganisms, as they catalyze and coordinate the complex reactions of cellular metabolism.

Until the 1970s, enzymes of animal and vegetable origin were mainly used in the industrial field. In the food industry these enzymes were preferred as they are considered free from the toxicity and contamination problems associated with enzymes of microbial origin. But thanks to the increase in demand and the development of fermentation technology, the competitive cost of microbial enzymes has been recognized and they have become more widely used. Compared to enzymes of plant and animal origin, microbial enzymes have a number of advantages, such as economic, technical and ethical. The use of microorganisms for the production of enzymes is favored by the possibility of producing a huge quantity in a short time and in a small production plant. Furthermore, the microbial product is clearly preferable as it is free from the ethical issues surrounding the use of animals. Their ease of extraction is also an advantage [28]. Most of the microbial enzymes used in biotechnological processes

are extracellular, with a considerable simplification of the extraction and purification processes. Intracellular microbial enzymes are also often easier to obtain than their animal or plant equivalents, as they generally require fewer extraction and purification steps. The extraction of animal and plant enzymes involves a step of transporting the source to the extraction plant, while when microorganisms are used, the same plant can generally be used for production and extraction. Note that commercially important animal and plant enzymes are often found within only one organ or tissue, so the remaining material is essentially a waste product, which needs to be disposed of. Not to mention that enzymes of plant and animal origin show a wide variation in yield and may only be available at certain times of the year, while none of these problems are associated with microbial enzymes [28].

From a technical point of view, microbial enzymes show properties that make them more suitable for commercial exploitation than their equivalents of animal and vegetable origin. For example, when the process must operate at elevated temperatures the high temperature stability of the enzymes of thermophilic microorganisms is often useful. The genetic manipulation of microorganisms for the production of new or altered enzymes is technically much simpler, less expensive and no more subject to ethical concerns than that of animals and plants [28].

1.2 MICROALGAE

Algae are a group of photosynthetic autotrophs that normally live in freshwater and marine environments but can also colonize other spaces including deserts, volcanic waters, highly acidic and frozen soils [29].

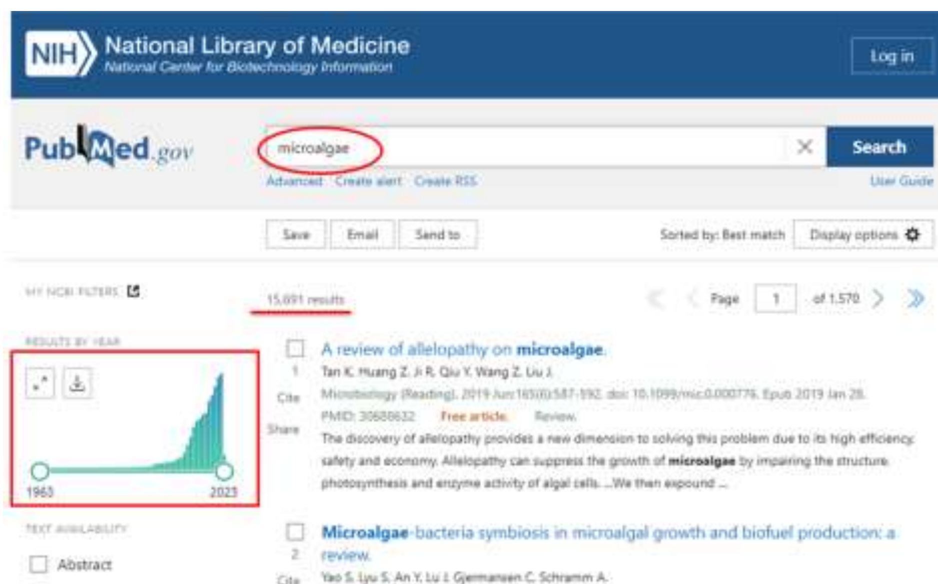
In addition to being the basic component of food chains in global ecosystems, they are known for producing atmospheric oxygen through photosynthesis, a process that transforms water and carbon dioxide into carbohydrates using solar energy.

The algae group is heterogeneous and includes macroalgae, commonly known as marine algae, which are macroscopic and multicellular organisms, and

microalgae, which are microscopic organisms commonly found in marine and fresh waters [30]. They can adapt to a wide range of temperatures, salinities and pH values, different light intensities and conditions. They can grow in artificial basins or deserts, alone or in symbiosis with other organisms.

The use of microalgae dates back to the dawn of time. Although they were used as food for indigenous peoples more than a thousand years ago, the production of microalgal biomass really began to develop in the middle of the last century, starting with Germany, when post-war societies were concerned about strong population growth and its implications for food safety [31].

There is a growing interest in seaweed and their derivatives ranging from physico-mediating, food supplements, pharmaceuticals and dyes, as demonstrated by the ***Figures 1.6*** in which are shown the results related to the search on PubMed by entering several keywords.



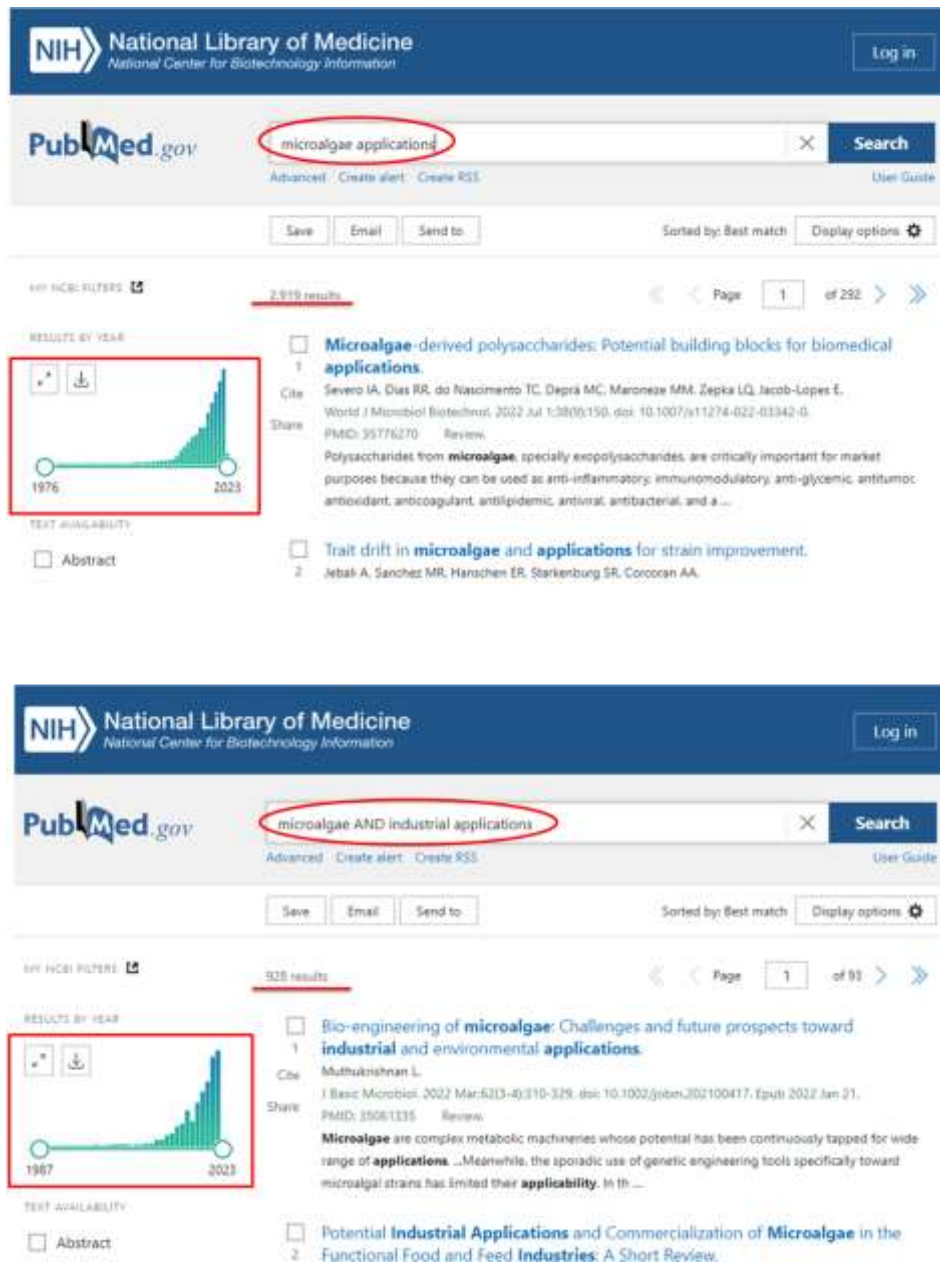


Figure 1.6: ***Growing interest in microalgae and its industrial applications***. The image shows the results of the research on PubMed by inserting different keywords such as "*microalgae*", "*microalgae applications*" and "*microalgae AND industrial applications*" [https://pubmed.ncbi.nlm.nih.gov,/, 3 February 2023].

Today, algae are considered an emerging panacea for society for numerous reasons. First of all, as photosynthetic organisms, they can derive their energy from light and carbon from inorganic sources, and compared to higher plants,

microalgae show better annual photon-to-biomass conversion efficiencies (about 3% versus <1% for higher plants) and no inherent sensitivity to seasonality [4-5]. This aspect, combined with the great diversity of the strains, allows to produce precious molecules such as proteins, lipids, carbohydrates, pigments with high yields. In addition, they can be grown on wastewater or non-agricultural land without pesticides, thus without compromising the production of food or other crop products. Furthermore, they are able to recycle atmospheric carbon dioxide while minimizing the associated environmental impacts. For these reasons, microalgae biotechnology is of growing interest. Today it can be divided into four main research fields which are wastewater treatment, CO_2 sequestration, biofuel production and high value added molecule production. Taking a look at the scientific literature, it is clear that the focus of microalgae applications is increasingly shifting towards the production of high added value molecules rather than environmental applications (***Figure 1.7***).

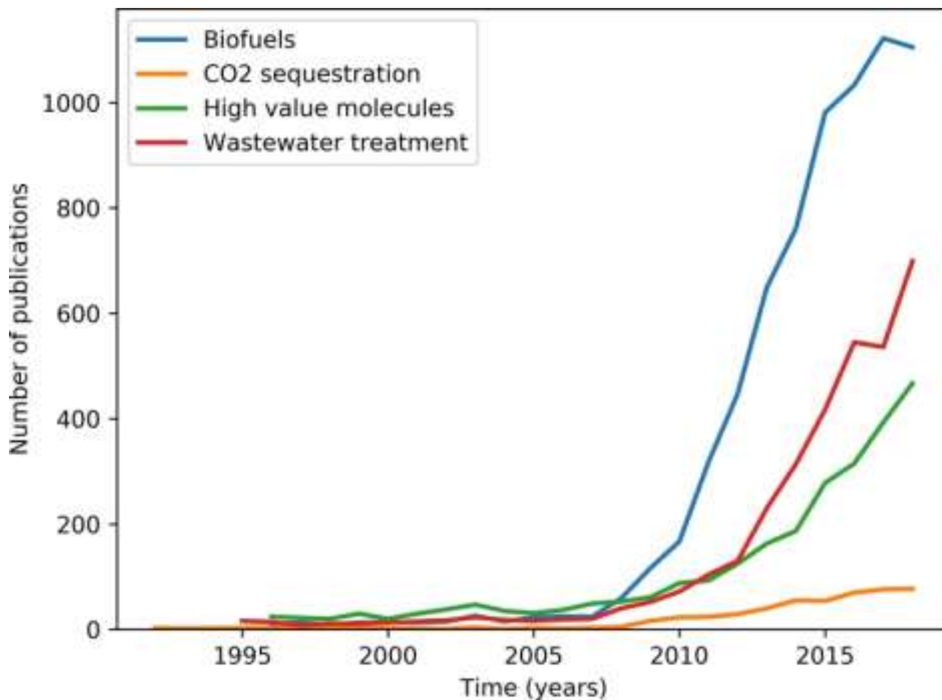


Figure 1.7: Number of publications by category of microalgae applications over time [29].

At present, about 40.000 species of microalgae have been described and identified, but probably this one number largely underestimates the real ones.

1.2.1 Classification

The term "microalgae" refers to both microscopic algae and oxygenated photosynthetic bacteria. Therefore the first distinction is between eukaryotes and prokaryotes, a distinction linked to the presence or absence of intracellular membrane-enclosed organelles. These structures would have been acquired by eukaryotes from evolution through endosymbiosis. Hence, eukaryotes are larger, more complex and can be unicellular or multicellular while prokaryotes are simple and small unicellular organisms.

Microalgal species show great heterogeneity although they have some common morphological and metabolic characteristics. Cell size and morphology change according to species (***Figure 1.8***). The cell diameter it can reach up to 50 µm. The morphology can also vary in the same species depending on of the reproductive cycle or environmental conditions and can present themselves in coccoid, filamentous, flagellate form, amoeboid and palmelloid [76-78].

Microalgae can also have different organizations, including single cells or colonies.

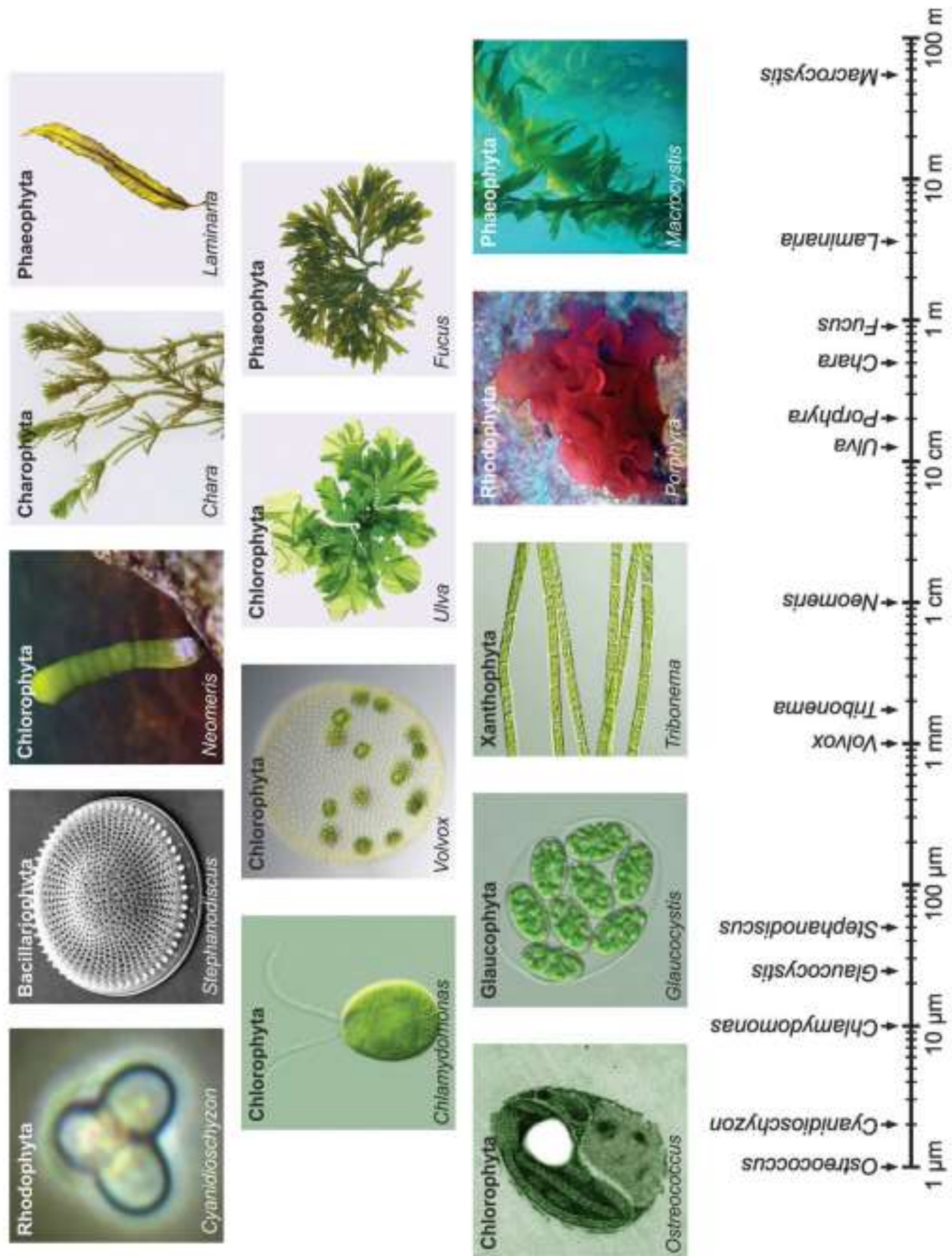


Figure 1.8: Spectrum of phenotypes and sizes of algal species [35].

The phylogeny of algae is characterized by a series of serial endosymbioses (***Figure 1.9***). The first that originated eukaryotic algae involved the incorporation of a cyanobacterium by a eukaryote to produce the chloroplasts of a clade comprising glaucocytophytes, red and green algae. Further secondary and tertiary endosymbioses generated other algal groups [37].

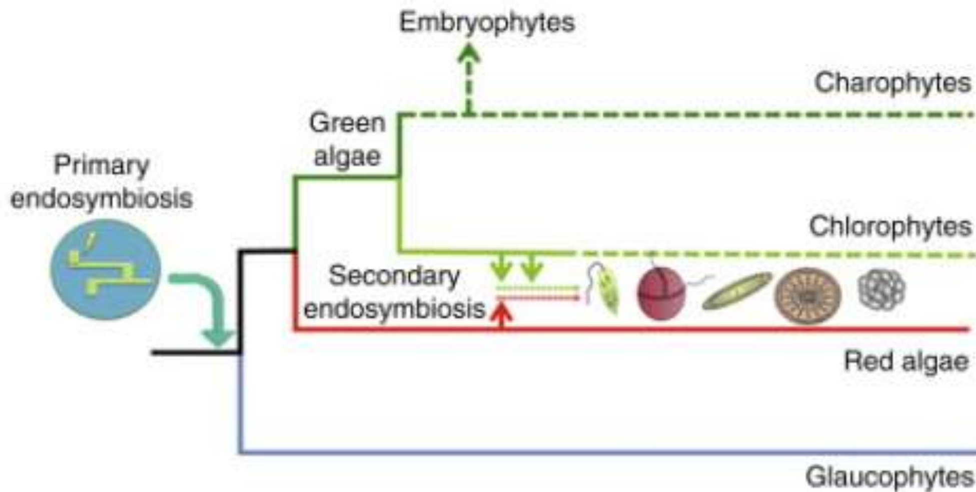


Figure 1.9*: *Summary of algal phylogeny [37].

Generally, microalgae have been classified on the basis of their photosynthetic pigments. However, other criteria should be taken into account, including cytological and morphological characters, the constituents of the cell wall and the chemical nature of the storage products. A further classification could be done using specific short gene sequences [38]. The classification system has changed many times over the years and there is still no consensus among taxonomists around the world on the use of one classification over another. One of the latest classification models includes two main domains, Prokaryota and Eukaryota. The prokaryotic domain includes only *Cyanophyta* and *Prochlorophyta*. The eukaryotic domain, the most abundant, includes nine phyla: *Glaucophyta*, *Rhodophyta* (red algae), *Heterokontophyta*, *Haptophyta*, *Cryptophyta*, *Dinophyta*, *Euglenophyta*, *Chlorachniophyta* and *Chlorophyta* (green algae) [39].

- *Cyanobacteria*

Called, before 1960, blue-green algae, they have been classified together with green algae, red algae and brown algae as photosynthetic microbes. They owe their characteristic coloring to the presence of photosynthetic pigments, such as chlorophyll α, phycocyanins and phycobiliproteins. They exist in the environment as unicellular, filamentous or colonial forms surrounded by a mucilaginous sheath [40]. Their structure is very varied although they have common elements, including the cell wall consisting of peptidoglycan on the outside, periplasmic space and cell membrane inside (***Figure 1.10***). Sometimes on their surface they have piles, of different lengths, which allow motility or the possibility of establishing connections with neighboring cells. Cyanobacteria often have an extracellular polymeric sheath (EPS), consisting of mucilage and, sometimes, of polysaccharides, which has the role of protecting cells from drying and allowing them to survival even in the most hostile conditions [41]. The genetic material (non-circular DNA) is contained in the central protoplasm associated with proteins. While the peripheral protoplasm is composed of thylakoids and phycobilisomes. Cyanobacteria are able to fix nitrogen playing an important role in the cycles of various elements at the level of aquatic ecosystems.

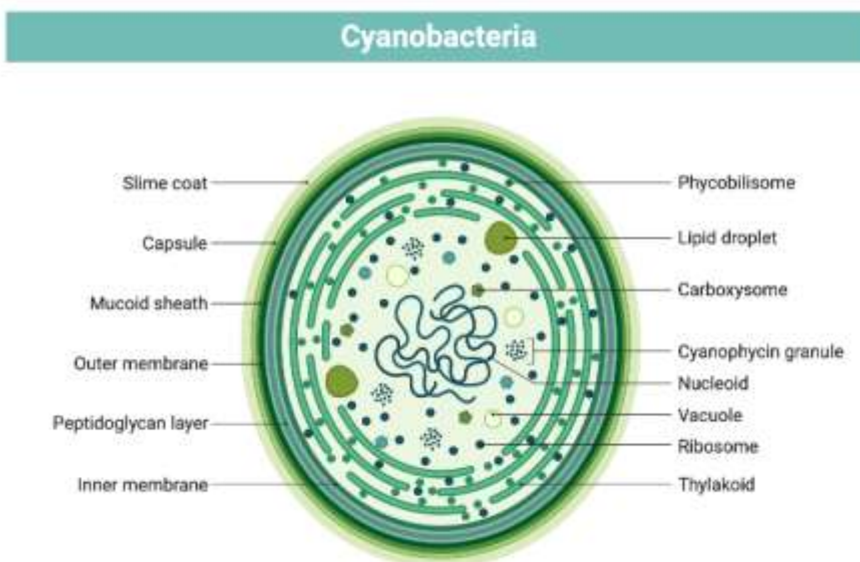


Figure 1.10*: *Cyanobacteria Structure.

- *Archaeplastida*

Glaucocytophytes are a small group of single-celled freshwater eukaryotic algae [37]. The plastids of these algae have similarities with cyanobacteria in the peptidoglycan layer between the membranes of the inner and outer plastid envelope and with pigment-containing phycobilisomes. They produce oxygen through photosynthesis, contain free circular DNA and can be both non-flagellated and biflagellated [11-12].

Rhodophytes are a large group of eukaryotic algae, some of which are unicellular, others are multicellular and many reach a high level of complexity. As the name suggests, they are often red due to the presence of high levels of phycoerythrin although there are also red algae, particularly freshwater ones, blue-green in color, and some almost black [37]. Rhodophytes have different morphological forms and are not flagellated.

Chlorophyta, also known as green algae, are the algae closest to higher plants; in fact they have chlorophyll A and B, they accumulate starch and some have cellulose walls. Some chlorophytes are unicellular, others are multicellular and have significant complexity [37]. They are divided into: *Chlorophyceae, Ulvophyceae and Charophyceae.*

1.2.2 Microalgae biomass

The process of producing microalgae biomass includes three phases: cultivation, harvesting and dehydration as shown in ***Figure 1.11***.

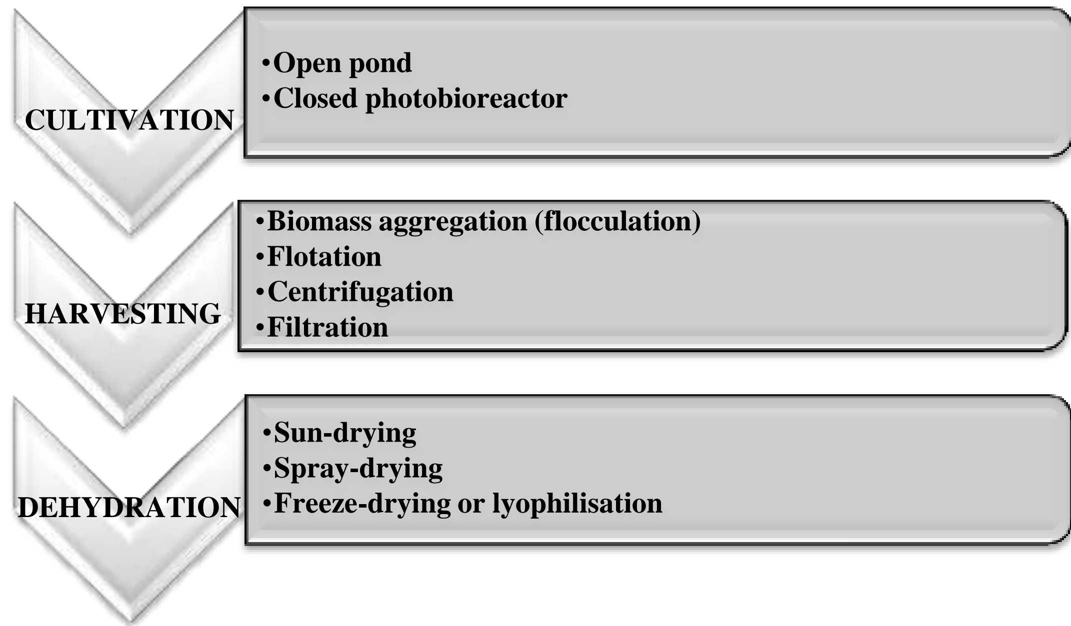


Figure 1.11: The production of microalgae biomass.

Three methods of conditions for the microalgae cultivation are distinguished: photoautotrophs, heterotrophs and mixotrophics. The first is the most commonly used one. Microalgae use light as an energy source and as a carbon source inorganic carbon (e.g. carbon dioxide) to generate chemical energy. In heterotrophic cultivation, microalgae can grow under photoautotrophic conditions, but also use organic carbon in the absence of light. Mixotrophic cultivation involves the introduction of alternative carbon sources, with a reduction in ambient light and the supply of CO_2 [44]. Two systems have been developed for the cultivation of microalgae: open pond and closed photobiorector (PBR) technologies (***Figure 1.12 A-B***). The former is classified into two systems: natural waters (ponds, lakes and lagoons) and artificial ponds (circular and canalizable). Open pond is cheaper for large-scale algal biomass production than PBR. The latter, however, provides an excellent and controlled closed culture system for cultivation, preventing danger or contamination from mold, bacteria, protozoa and competition from other microalgae. There are three types of PBR classified into tubular (TPBR), vertical column (VCPBR) and flat (FP-PBR) [86-87].

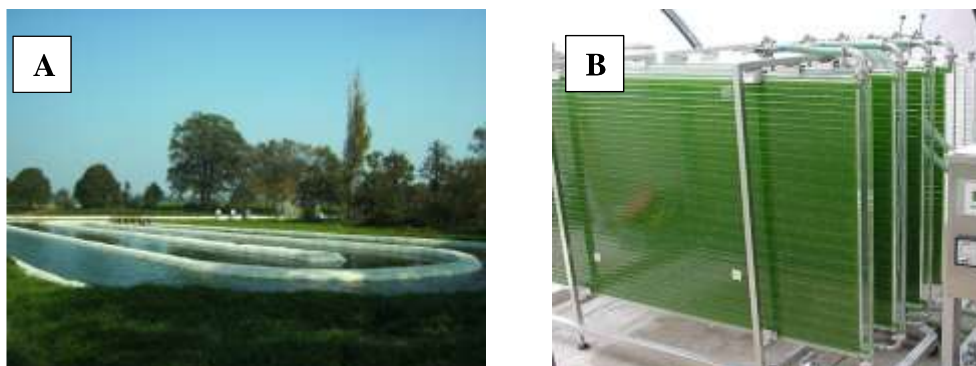


Figure 1.12: Systems of microalgae cultivation. (**A**) open pond cultivation. Picture: JanB46, Wikipedia, CC BY-SA 3.0. (**B**) flat panel bioreactor. Picture: IGV Biotech, Wikipedia, CC BY-SA 3.0.

Microalgal biomass can be harvested using different techniques, often used in combinations to increase its effectiveness. The choice of collection method depends on several factors such as density, size and desired end products. Through flocculation, microalgae cells are aggregated together to form a larger particle known as floc, by adding flocculant agents such as multivalent cations and cationic polymers to the medium that helped neutralize the surface charge of cells. Flotation is a technique that involves the use of small bubbles that stick to the cells of microalgae to promote the floating of the same on the surface of the culture soil for easy harvesting. This technique is convenient due to low operating costs with easy operation procedure and high biomass collection. Centrifugation recovers microalgal biomass from the culture medium based on the density of each component and particle size using centrifugal force. This technique is quick, easy and efficient, but the cost can increase due to the high energy input and maintenance required. In addition, internal cell damage may occur, resulting in a loss of delicate nutrients. Finally, filtration allows the algae biomass to be isolated from the liquid culture medium through the use of a semi-permeable porous membrane. However, this method incurs fouling and clogging and therefore requires frequent filter or membrane changes increasing processing costs [86-87].

In order to avoid deterioration or to prolong the shelf life, the biomass is dried or dehydrated immediately. The cheapest method available is sun drying which is based exclusively on solar energy. Spray drying can be used, through which a dry powder is produced from a fine spray of suspension droplets in continuous contact

with hot air in a large vessel. It's a very fast and very efficient drying method, though quite expensive. Freeze-drying is widely used on a laboratory scale and allows the dehydration of frozen products by means of a sublimation mechanism. For large-scale biomass production can be expensive [47].

1.2.3 Factors affecting biomass production

Important indicators of the effectiveness of the microalgae strain are the level of biomass accumulation and the productivity of biologically active substances. These parameters are influenced by different conditions, such as the composition of the culture medium, temperature, pH, growth phase, collection method and illumination.

Temperature is an important factor to control as it has a strong impact on biochemical processes, suffice it to note that the decrease in the optimum temperature reduces the assimilation of carbon, while an increase reduces the photosynthetic activity of microalgae. Most algae in nature grow at temperatures between 15 and 30º C, while most *in vitro* algae grow at temperatures between 20 and 25º C [44].

pH is an important parameter in the cultivation of microalgae, as it determines the solubility of minerals and carbon dioxide in the medium, as well as the direct effect on the microalgae themselves. Generally the optimal pH varies from 6 to 8, although some species may adapt in the larger range. Attention should also be paid to the salinity of the solution for optimal growth.

An ideal culture medium should contain inorganic elements such as nitrogen (N) and phosphorus (P), which may vary depending on the crop species. After carbon, which represents about 50% of the fraction of elements in the biomass of microalgae, nitrogen occupies second place, with a concentration of 1% to 14% in the dry mass. The reduction in nitrogen concentration leads to the predominant synthesis of lipids and polysaccharides. The concentration of phosphorus in the dry biomass of microalgae can vary from 0.05% to 3.3%. For adequate growth of microalgae, the soil must contain other nutrients: trace elements. The main trace elements are Mg, S, Ca, Na, Cl, Fe, Zn, Cu, Mo, Mn, B and Co, with an emphasis

on Mg, S and Fe. Nutrient limitation directly impacts biomass growth, photosynthesis processes in microalgae and the synthesis of biologically active substances.

Optimal conditions for the growth of microalgae depend on the light intensity, wavelength and photoperiod to which cells are exposed during cultivation. Photosynthetically active radiation, useful for microalgae, is in the range of 400 to 700 nm from light radiation, which corresponds to 50% of solar radiation and with an intensity of 800 to 1000 W/m^2 [48]. On a laboratory scale, LED lights with an intensity of about 200-400 µm photon/m^2/s are used. In an attempt to adapt the growth cycles to natural conditions, light-dark cycles are often reproduced. Most microalgae achieve optimal growth levels after 16 hours of light/8 hours of darkness.

Effective stirring of the culture medium is important as it keeps the cells in suspension, eliminates thermal separation, distributes nutrients and increases the efficiency of gas exchange. Shaking can reduce the degree of auto-darkening and the likelihood of photoinhibition by evenly distributing light between all microalgae cells [49].

1.2.4 Biochemical composition of microalgae

At the biochemical level microalgae produce a number of molecules including carbohydrates, proteins, lipids and nucleic acids, as well as essential vitamins and minerals, the content of which varies according to the specific strain and in response to biotic and abiotic factors, such as light intensity, photoperiod, temperature, nutrients and growth phase.

- *Carbohydrates*

Carbohydrates together with mono, oligo and polysaccharides, have both structural and metabolic functions. Carbohydrates can be found bound to proteins or lipids, while polysaccharides represent the main structural characteristics of the cell wall and are produced in a variety of forms depending on the group of microalgae. Cyanophytes accumulate glycogen, Chlorophyta synthesizes starch in the form of amylopectin and amylose, while Rhodophyta produces a carbohydrate

polymer known as floridean starch [50]. The use of microalgal carbohydrates in the food sector is limited, while polysaccharides are becoming increasingly popular in the cosmetics industry [51].

- *Proteins*

Proteins represent an integral component of the microalgal membrane and light collection complex, including numerous catalytic enzymes involved in photosynthesis. The total protein content in biomass depends on the type of microalgae and varies from 30% to 70% of the dry weight [52]. It has been reported that some microalgae contain proteins soluble in their cytoplasm. The amino acid profile of the microalgae has no lower biological value than the animal. Due to the presence of non-protein components that could alter the color and taste of the product, their application in food is limited [53].

- *Lipids*

The fatty acid profile of microalgae is characterized by a mixture of saturated and unsaturated fatty acids C16 and C18 as well as longer carbon chains, including many omega fatty acids. The lipid content in microalgae varies from 20% to 50% of the dry weight and such lipids can be used for energy accumulation, as energy substrates, as structural components of the cell membrane and for metabolic processes [54]. The type of microalgae and the conditions of growth determine the number of lipids and the presence or position of double bonds in the carbon chain. In the presence of optimal conditions, the conversion of fatty acids into glycerol-based membrane lipids can occur, while in adverse conditions it can increase the synthesis of neutral lipids, such as triacylglycerols. Most microalgae contain polyunsaturated fatty acids (PUFA) such as arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid. The main saturated fatty acid is palmitic acid [55].

- *Pigments and vitamins*

Microalgae contain different pigments such as carotenoids, phycobylins and chlorophylls. Compared to some plants, microalgae have a higher carotenoid and chlorophyll content [56]. Depending on the type of microalgae, carotenoids can be stored in the chloroplast stroma or cytosol and play an important role in photosynthesis. They can be used as antioxidants. Phycobylins, highly water-

soluble pigments found in the stroma of chloroplasts *Cyanobacteria*, *Rhodophyta*, *Glucophyta* and some cryptocurrencies, are widely used in the food industry as dyes and in molecular biology as fluorescent markers [56]. Chlorophylls, fat-soluble green pigments involved in the photosynthesis process, represent 0.5% to 1.0% of the dry weight microalgal. In microalgae we find a good content of vitamins A, B_1, B_2, B_6, B_{12}, C, E, biotin, folic acid, pantothenic acid, etc [44].

1.3 MICROALGAL BIOACTIVE MOLECULES: BIOTECHNOLOGICAL POTENTIAL USES

Microalgae and cyanobacteria are valuable sources of important bioactive and biologically relevant molecules, mainly due to their enormous biodiversity and the consequent variability in their biochemical composition. In addition, the nature of the chemicals produced can be manipulated by changing the means and conditions of cultivation. The bioactive compounds produced by microalgae have a variety of health benefits such as antioxidants, anti-inflammatory, antitumor or antiobesity effects. Thanks to the extraordinarily rich variety of species and molecules you can be sure that new effects and/or new molecules have yet to be discovered. The interest in microalgae has significantly increased in relation to the upsurge in demand for sustainable biomass for the production of important bioactive components with various potential applications, especially therapeutic. The use of microalgae in many fields has grown in various sectors ranging from the exploitation of renewable energy to the utilization of high-value products. However, the applications of microalgae in industries are concentrated in a few specific species that have a high economic value. The most sought-after genera in the global market are spirulina and *Chlorella* in the form of dried biomass due to various health benefits [47]. Among the myriad of bioactive compounds exploited for commercial purposes are fatty acids, carotenoids, vitamins, enzymes and polysaccharides. According to the latest analysis, the global microalgae market is expected to reach 3318 million dollars by 2022, driven mainly by demand from both pharmaceutical and nutraceutical industries [57] due to the escalation of chronic diseases and its interest in natural alternatives. It has been reported that countless microalgae-derived compounds are beneficial for skin health and are

currently gaining attention in many aspects of cosmeceuticals. A significant increase in the demand for algae in the global market is also due to the progress of new areas of application of microalgae in aquaculture and biofuel production.

Below will be a review of the major industrial applications of bioactive microalgal compounds.

1.3.1 Pharmaceutical applications

Although only a limited number of microalgae with pharmaceutical applications are currently available, many studies have documented the health benefits of their bioactive compounds for the prevention and improvement of diseases such as diabetes, obesity, cardiovascular disease, cancer, inflammation, Alzheimer's disease, depression and bacterial, fungal and viral infections [58]–[60]. Factors limiting the marketing of such products include low extraction yield and high production cost, without neglecting a potential risk of serious side effects, allergic reactions and accumulation of heavy metals and toxins in some species of microalgae [61]. Bioactive derivatives of microalgae with anticancer properties have been extensively studied in recent years [62]. Among bioactive compounds with anticancer properties, microalgal pigments such as astaxanthin, β-carotene, lutein, violaxanthin and fucoxanthin have been very promising. Another promising anticancer agent is phycocyanin, a protein pigment of the phycobiliprotein group isolated from S*pirulina platensis* and *Limnothrix sp.*. C-phycocyanin showed inhibitory activity in liver cancer cell lines (HepG2) [63], human leukemia cells (K562) [64] and against lung cancer cell lines (A549 and NSCLC) [65]. In another study, *Limnothrix phycocyanin sp.* has improved the anticancer properties of the anticancer drug Topetecan against the cell line of prostate cancer (LNCap) [66]. While phycocyanin isolated from *Limnothrix sp.* NS01 with two α and β subunits (17 and 20 kDa) showed antiproliferative activity in human breast cancer cell lines (MCF-7) [67]. Several compounds derived from microalgae have been studied for its cardioprotective effect. Carotenoids have been shown to possess antioxidant properties, which are important for preventing cell damage caused by free radicals associated with chronic cardiovascular disease

and stroke [68]. *β*-carotene extracted from *D. Salina* has been shown to have protective effects against atherosclerosis in both mice and humans. Another group of bioactive with cardioprotective properties are polyunsaturated fatty acids (PUFA), in particular omega-3 fatty acids such as DHA, EPA and α-linoleic acid (ALA) which have been shown to reduce blood cholesterol and improve hypertension. Of these fatty acids, DHA is the only commercially available PUFA.

1.3.2 Nutraceuticals/Food applications

Several microalgae have been classified as "Generally Recognised as Safe" (GRAS) and approved by the U.S. Food and Drug Administration (FDA). For this reason, algae are widely used in industries especially for food and nutraceutical applications. Among the bioactive compounds, most used in this field are proteins, vitamins, minerals, natural pigments and fatty acids. *Chlorella* and spirulina species are the most sought after in the global microalgae market because of their high protein content (50-70% dry weight protein) and the wide spectrum of other nutrients, namely minerals, vitamins, lipids, carbohydrates, pigments and other trace elements [69]. In the food sector, the two above-mentioned species or their proteins have been incorporated into various types of foods, such as milk products, bread, biscuits, instant noodles and pasta to produce functional foods enriched with proteins [70], [71]. Microalgae contain a distinctive lipid profile, particularly fatty acids with potential commercial value. The ability to produce and accumulate high amounts of PUFA, makes microalgae even more valuable from a nutraceutical point of view. Omega-3 fatty acids are known as a good source of highly recognized and recommended dietary supplements for their health benefits, especially in disease prevention [72] and human nutrition. Seaweed oil is often used in liquid or capsule form and is generally consumed by vegetarians and populations with low-fish diets, enriched or fortified in different foods such as dairy products, nutritional bars, bakery products and so on to further increase the nutritional value of the product. Natural microalgal pigments are used as biocolorants or food additives in various products as an alternative to synthetic sources that have various health implications.

1.3.3 Cosmeceutical applications

Microalgae extracts are widely applied in the cosmetic industry, especially for skin and hair care products especially in contexts such as antioxidant protection and photoaging, areas in which there is still much to be explored in terms of products intended for the treatment of the skin appendages, modulation of fatty adipochines and skin microbiota [73]. The most widely used species of microalgae include *Arthrospira sp., C. vulgaris, D. salina, S. platensis, Chondrus Crispus, Mastocarpus stellatus, Ascophyllum nodosum, Alaria esculenta* and *N. oculate* [74]. For example, *β*-carotene is used in the prevention of free radicals triggering premature aging, lutein has protective action against UV radiation and can be obtained from different microalgae (*Scenedesmus salina, Chlorella, C. vulgaris, Scenedesmus obliquss, D. salina and Mougeotia sp*.); lycopene instead has anti-aging properties [75]; fucoxanthin, mainly extracted from *Phaeodactylum tricornotum*, *Odontella aurita* and *Isochrysis aff. Galbanum* shows antioxidant activity and prevents oxidative stress. The pigments of microalgae are used as excellent pigmenting agents or even in tanning pills as they give various colors ranging from green, yellow, brown and red constituting a valid alternative to synthetic dyes. In Asian women, for example, the whitening action of the skin is exploited through the inhibition of the enzyme tyrosinase, which plays a vital role in the biosynthesis of melanin. The pigments of zeaxanthin and astaxanthin of different species of microalgae such as *N. oculate* or *H. pluvalis* are indicated for their anti-tyrosinase ability [47].

1.3.4 Feed

Microalgal biomass is widely used in feed formulations for different animals such as cattle, fish, goats, lambs, poultry, pigs and rabbits, thanks to the high protein and carbohydrate content. It has been shown that the use of feed based on microalgae as well as improving the immune response and fertility affects the physiology of the animal. The addition of 5-15% algal biomass to animal feed can be safely used as a partial replacement of conventional proteins. The quality of microalgal proteins has a functionality comparable or superior (such as foam,

emulsifier, solubilizer, surfactant or gelling agent) to that of other commercial protein sources [76]. For example, *Chlorella* biomass has been shown to be easily digested by up to 5% in the form of paste [77]. As for fish, however, microalgae can provide an important source of essential fatty acids such as DHA and EPA that are not synthesized by fish [78]. Microalgae, such as *C. vulgaris* and *A. platensis*, have amino acid profiles very similar to that of soya, which is currently the main source of protein used in feed. In addition, microalgal biomass is a potential source of minerals just think of *C. vulgaris*, *A. platensis*, *Micractinium reisseri*, *Nannochloris bacillaris* and *Tetracysti ssp*. that show a very high iron content compared to soy [79].

1.3.5 Proteomics

Genetic engineering has been used to manipulate microalgae allowing the production of new molecules useful for biotechnology applications [80]. In particular, the proteomics of microalgae can provide information on how genes of interest are expressed as proteins and this will give an impetus to finding proteins with biotechnological applications such as anti-inflammatory proteins [81].

For example, *C. reinhardtii* has been extensively studied for the production of therapeutic proteins and in it have been expressed more than 100 proteins [82]. The advantages of using microalgae are primarily in terms of cost and energy, but also the lower chance of contamination and greater simplification of downstream processing. Note that the malaria detection protein that is used in ELISA tests has been successfully expressed and produced in the chloroplast of *C. reinhardtii* [83]. It has been described that the phycobiliproteins of cyanobacteria and red algae have antioxidant, hepatoprotective, anti-inflammatory, immunomodulatory and anticancer effects [59], [84]. Peptides isolated from *Chlorella pyrenoidosa* and *Chlorella vulgaris* showed antihypertensive and antitumor activity [85].

A protein isolated from *Chlorella sorokiniana* has high value bioactive peptides with nutraceutical and pharmaceutical application, using proteomics techniques [86].

1.4 RESEARCH OBJECTIVES

In recent decades, macroalgae and microalgae have shown a renewed commercial interest in the production of natural metabolites for nutraceutical, pharmaceutical, energy and food purposes. Modern lifestyle, characterized by high fat and sugar intake, as well as reduced physical activity, contributes to the development of metabolic and inflammatory diseases, such as obesity, diabetes, hypertension, cancer and other chronic pathologies. Nutrition can play an important role in preventing these disorders, as desirable is finding functional ingredients. In this context, microalgae represent an excellent source of functional ingredients, thanks to the high content of bioactive molecules with valuable health benefits.

The aim of this research project was to investigate biological activity and potential applications of microalgae in order to develop tools, products and procedures meeting the growing demand for bioactive molecules from microalgae. In particular, it aimed mainly to identify and evaluate microalgae species producing bioactive molecules that can be exploited in different industrial contexts. Focus was paid to the genomic-functional characterization of the selected genotypes. We have evaluated the effects of microalgae on some inflammatory and adipogenic markers for a potential application in the nutraceutical and/or pharmacological field.

2. MATERIALS AND METHODS

2.1 MICROALGAL STRAINS

In this work we used different microalgal strains all belonging to the collection of Bioinnova Srls. Specifically a strain belongs to *Haematococcus Pluvialis* (HP, wild type), and the remaining two are selected genotypes of HP derived from a specific process of mutagenesis induced by high selective pressure that led the two species to change randomly, responding to environmental stresses in a different way (FBR1 and FRB2).

2.1.1 Haematococcus pluvialis

Haematococcus pluvialis is a green unicellular freshwater biflagellate microalga that belongs to the class *Chlorophyceae*, order *Volvocales*, and family *Haematococcaseae* distributed in many habitats around the world (***Figure 2.1***) [87]. *H. pluvialis* is an important source of bioactive compounds such as proteins, carotenoids and fatty acids. In particular, it is known to be the largest natural producer of astaxanthin, a secondary carotenoid that gives the vibrant pink color to the tissues of fish and crustaceans that feed on it (and the flamingos that, at the top of the food chain, feed in turn on crustaceans) [129-130]. *H. pluvialis* is characterized by a complex but at the same time fascinating life cycle. In ***Figure 2.1*** are reported the common morphotypes observed: green mobile macroozoids, palmelloids and aplanospores [131-132].

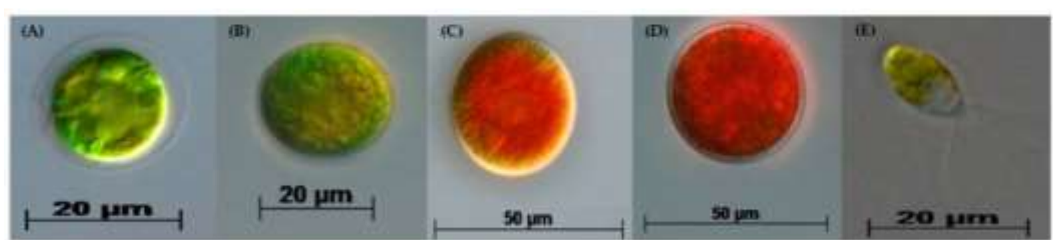


Figure 2.1*: *Life cycle stages of H. pluvialis. (**A**) green motile macrozooid, (**B**) early stage palmelloid, (**C**) late-stage palmelloid, (**D**) aplanospore (haematocyst) and (**E**) green motile microzooid [91].

In favorable growth conditions it is found mainly in the vegetative state in the form of macrozoids (spheroidal, green and biflagellated cells), which are characterized by high motility [133-134]. In this state, macrozoids can divide by mitosis to generate 32 daughter cells (microzoids) within the cell wall of the

mother cell (***Figure 2.2***). In the case, however, of growth-limiting conditions with nutrient deprivation and/or exposure to intense light or anything that can lead to oxidative stress, macrozoids begin to lose flagella, stop dividing and undergo the so-called process of "engraving".

The cell wall begins to thicken (palmella stage), until the formation of an ultra-resistant coating consisting of a trilaminar sheath and a secondary wall resistant to acetolysis that allows them to survive extreme chemical-physical conditions. Then, palmella enters the haematocyst stage (or haplanospora) and begins to synthesize and accumulate within lipid bodies high amounts of carotenoids to protect themselves from radiation damage and oxidative stress [87].

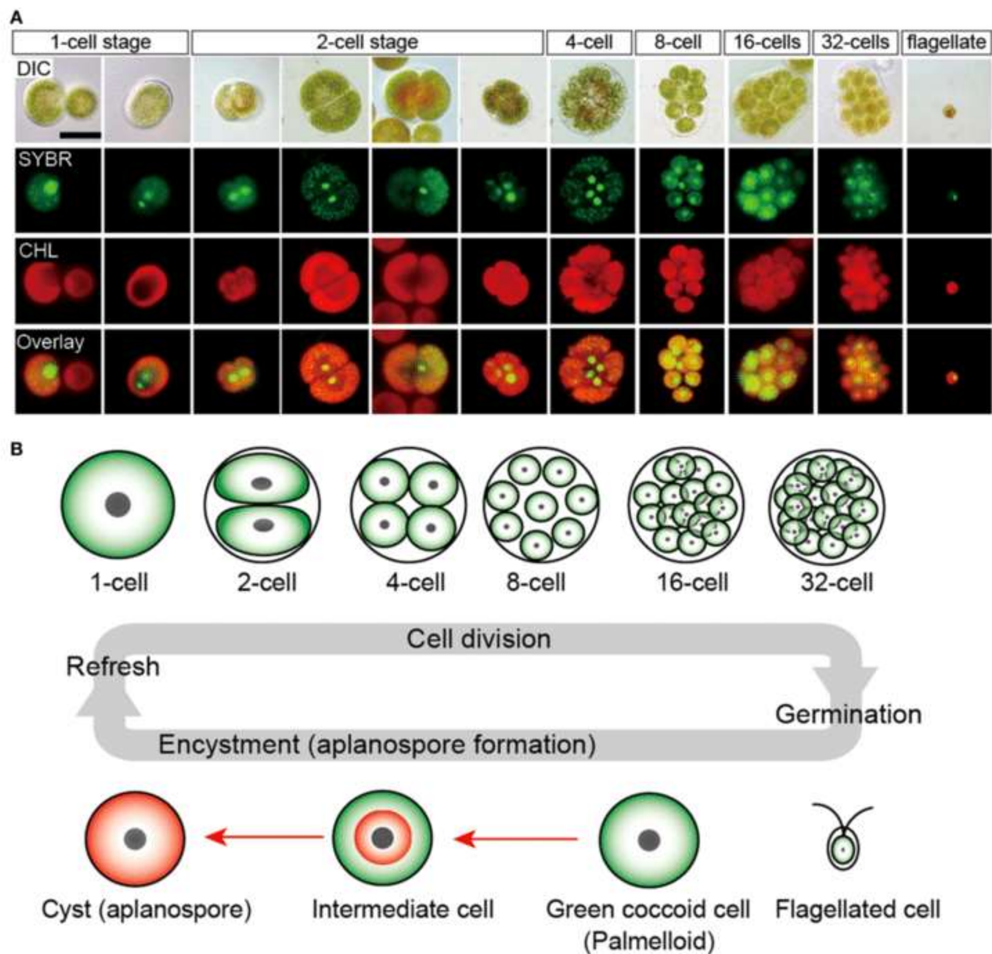


Figure 2.2: Life cycle of H. pluvialis. (**A**) Fluorescence microscopy images, showing the 1-to 32-cell stages, and the flagellated stage. (**B**) Life cycle of *H. pluvialis* [87].

A strain belonging to *Arthrospira platensis* (AP) was used as reference in some experiments.

Arthrospira platensis is a cyanobacteria (formerly blue algae) alkali and filamentous multicellular belonging to the family of *Phormidiaceae* or *Microcoleaceae* and the genus *Arthrospira*, commonly called "spirulina". *Arthrospira spp*. are distributed naturally in alkaline lakes and ponds around the world. The mature trichomes of *Arthrospira* are a few millimetres long and consist of cylindrical cells of 3-12 μm in diameter (***Figure 2.3***). The trichome is usually arranged like a narrow helix, but under different growing conditions this can turn into a free spiral and even straight filaments. They reproduce by fragmentation of the mature trichome into shorter filaments by breaking up specialized intercalary cells called necridians. These smaller fragments then increase again in length by division of individual cells in the filament followed by elongation of cells [36].

Figure 2.3: A trichome of the cyanobacterium Arthrospira platensis [36].

Arthrospira platensis has a wide variety of applications, from food use to pharmacology largely attributable to its content of phycobiliprotein, mainly C-phycocyanin.

2.1.2 Cell growth

Cell cultures were performed in different media to assess which was the optimal one for microalgae growth. The media and glass containers used were autoclaved (VAPORMATIC 770, ASAL S.r.l.), for 20 minutes, at 1 atm and 120° C.

For the *H.pluvialis* strains, the Modified Bold 3N Medium (UTEX, University of

Texas, Austin), whose chemical composition is shown in the ***Table 2.1*** has been preferentially used. The sterile, ready-to-use soil is stored at 4º C and has a pH of 6.2, ensuring ideal growth conditions.

Components	**Amount**	**Stock Solution Concentration**	**Final concentration**
$NaNO_3$	30 mL/L	10 g/400mL dH_2O	8.82 mM
$CaCl_2$•$2H_2O$	10 mL/L	1 g/400mL dH_2O	0.17 mM
$MgSO_4$•$7H_2O$	10 mL/L	3 g/400mL dH_2O	0.3 mM
K_2HPO_4	10 mL/L	3 g/400mL dH_2O	0.43 mM
KH_2PO_4	10 mL/L	7 g/400mL dH_2O	1.29 mM
NaCl	10 mL/L	1 g/400mL dH_2O	0.43 mM
P-IV Metal Solution	6 mL/L		
Soilwater: GR+Medium	40 mL/L		
Vitamin B12	1 mL/L		
Biotin Vitamin Solution	1 mL/L		
Thiamine Vitamin Solution	1 mL/L		

Table 2.1: Modified Bold 3N Medium composition.

The strain of *A.platensis* instead grew in a medium consisting of 2 g/L of fertilizer based on (KNO_3), potassium phosphate (K_3PO_4) and iron sulphate (FeO_4S), 16 g/L of sodium bicarbonate ($NaHCO_3$) and 1 g/L of sodium chloride (NaCl).
Cells were grown under a light intensity of 120 mmol photons $m^{-2}s^{-1}$ on a 16 h: 8 h light/dark cycle at 25° C. Cultures were not supplied with extra source of CO_2 and were shaken by mechanical agitator (g24 environmental incubator shaker, American Laboratory Trading) at 70 rpm as reported in [94]. Algal growth was

assessed by measuring optical density at 750 nm (SPECTROstar® Nano, BMG Labtech) and cell counts by light microscopy (Zeiss Axioplan) using the Burker chamber (BLAUBRAND).

2.1.3 Optical density

Optical density (OD) or absorbance (A) is a parameter used to quantify cell density in the culture medium. Generally, for unicellular microorganisms, the absorbance is proportional to the number of cells and the optical density is calculated as the logarithm of the ratio of the detected strength for the white signal (I), or only the growth medium, and the sample of cells (I) in the growth medium (A = log Io/I).

Measurements were made using a spectrophotometer (SPECTROstar® Nano, BMG Labtech). The samples were diluted if necessary to avoid absorbance values of more than one unit, in which case the linearity relationship between optical density and cell number is lost. The wavelength used for spectrophotometric measurement was 750 nm, following the example of the studies conducted by Griffiths *et al*. [95]. This spectral region does not fall within the absorption peaks of the main microalgal pigments and allows the estimation of cell density.

2.1.4 Cell counting

The cell count was performed using a Burker camera (BLAUBRAND). A small portion of the sample (10 µL) was spread by capillarity covering the entire area of the counting grid, consisting of nine squares delimited by three parallel lines, each of which contains sixteen smaller bounded, in turn, by two parallel lines. By convention the cells present in the four squares at the corners of the chamber are counted. The count was carried out with an optical microscope (ZEISS Axioplan) at a 10X magnification. The number of cells per millilitre of suspension was obtained by multiplying the average of the cells counted in the four squares considered, by the chamber volumetric conversion factor, 10^4, and the dilution factor used.

2.1.5 Wet biomass

At the end of the growth cycle (plateau phase) wet biomass was also quantitatively evaluated. In detail, 5 mL of samples were taken after one week of growth for analysis. The samples, collected in 15 mL falcon, were centrifuged (Thermo Fisher Scientific MR23i centrifuge) at room temperature and 4.500 g for 3 minutes to separate the pellet from the liquid phase. The pellets were weighed and stored at -20º C for subsequent analysis.

2.1.6 Analysis of phylogenetic distances

For phylogenetic reconstruction the UPGMA method (group of unweighted pairs with arithmetic averages) was used. The analysis was conducted by an external laboratory. In short, the three species HP, FBR1 and FBR2 were genetically differentiated by 50-loci differential analysis using eight random primers (a RAPD PCR was performed).

2.1.7 Evaluation of enzymatic profile and chemical composition of microalgal strains

The qualitative and quantitative enzymatic profile assessment and the chemical composition analysis of the microalgal strains under study were carried out by external laboratories. Colorimetric and spectroscopic methods were used respectively. The Soxhlet method was used for the analysis of the total fat content. The content and type of fatty acids were obtained with the gas chromatograph (GC Analysis).

2.2 EXTRACTS PREPARATION

We then processed the samples to be analysed. The lysates were dosed to evaluate their protein content.

2.2.1 Extracts preparation

For the preparation of microalgal extracts several steps have been followed. First, the wet biomass was dried in the oven for 1 h at 60º C (***Figure 2.4 A***). The dry

biomass was recovered and placed in a mortar, the RIPA lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, Sodium deoxycholate 0,5 %, sodium dodecylsulfate 0,1 %, 1 mM EDTA, NP-40 1%) was added and the mechanical lysis with a pestle was carried out (***Figure 2.4 B***). The samples were transferred, after syringing (***Figure 2.4 C***), to new eppendorf and were left in incubation in rotation 20 min at 4° C (***Figure 2.4 D***). A first centrifugation at 14.000 rpm for 10' was followed. The supernatants were sonicated (3 cycles of 1 min) (***Figure 2.4 E***) and centrifuged again (14.000 rpm for 10 min). They were then subjected to 4 cycles of freezing/defrosting (5 min to 65° C and 5 min to 100° C) and centrifuged (14.000 rpm for 10 min). The extracts obtained have been stored at -20º C until they are used.

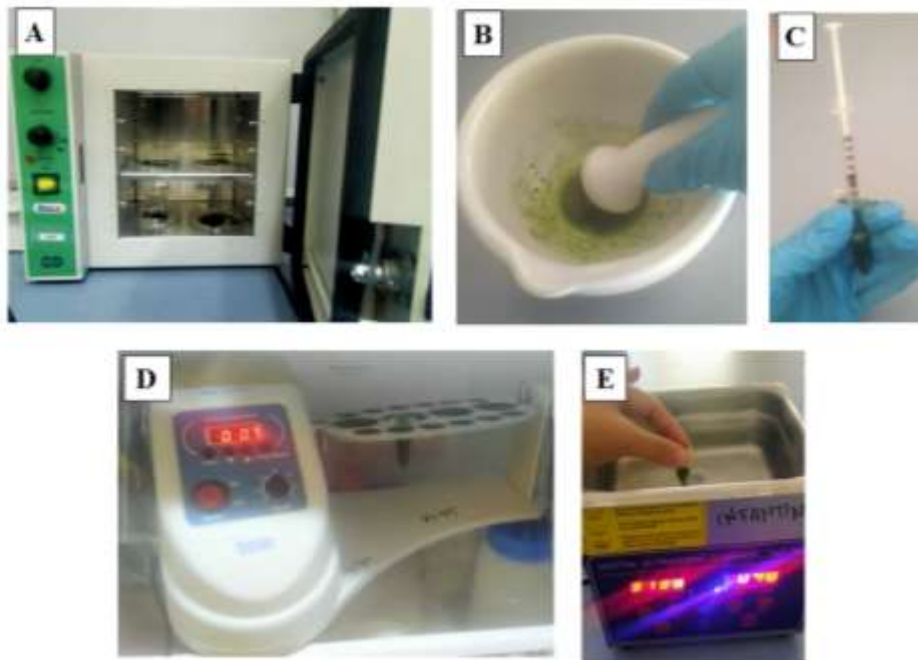


Figure 2.4: Extracts preparation.

2.2.2 Protein determination

The protein content was determined by Bradford’s method, a fast and fairly sensitive method based on the displacement of the maximum absorbance of the dye Coomassie Brilliant Blue G-250 from 465 to 595 nm after binding to proteins

denatured in solution [96]. The protein concentration was determined using a standard curve constructed with known concentrations of bovine serum albumin (BSA, 0.25 - 0.5 - 1 - 2 µg/mL).

2.3 *IN VITRO* TESTS

We then move on to the second phase, namely *in vitro* experiments to assess the effect of extracts on the anti-inflammation and adipocyte function.

2.3.1 Cell cultures

Fibroblasts are cells typical of connective tissue, capable of producing the components of the extracellular matrix (***Figure 2.5***). They present a vacuolized cytoplasm surrounding an elliptical nucleus and stained with one or two nucleoli. Fibroblasts produce collagen, glycosaminoglycans, elastic and reticular fibers, and glycoproteins found in extracellular matter.

During growth they divide and synthesize the basic substances. Tissue damage stimulates fibroblasts and induces mitosis. Such cells can give rise to others such as bone cells, adipocytes and muscle cells, all of mesenchymal and mesodermal origin. They are also found in the scar tissue, possess a modest migratory capacity and can intervene in scarring. TGFβ stimulates fibroblasts to produce extracellular matrix substances (such as collagen) and decreases the production of enzymes that degrade ECM such as metalloproteases.

Fibroblasts purchased from Galliera Genetic Bank and grow in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, San Jose, CA, USA) supplemented with Fetal Bovine Serum (FBS) at 15% (v/v), L-Glutamine 2 mM, penicillin 100 U/mL and streptomycin 100 µg/mL at a temperature of 37° C in the presence of 5% CO_2 and in a water-saturated atmosphere.

Figure 2.5: Human primary fibroblasts.

U937 is a human hematopoietic cell line stabilized from a 37-year-old male histiocytic lymphoma and is used as a monocyte/macrophage differentiation model (***Figure 2.6***) [97]. U937 cells mature and differentiate in response to a number of stimuli, adopting the morphology and characteristics of mature macrophages. They are myeloid line cells and secrete a large number of cytokines and chemokines both constitutively such as IL-1 and GM-CSF and in response to soluble stimuli. TNFα and recombinant GM-CSF independently promote the production of IL-10 [97].

U937 (Interlab Cell Line Collection (ICLC) HTL94002) grow in suspension in soil the RPMI-1640 supplemented with FBS at 10% (v/v), L-Glutamine 2 mM, penicillin 100 U/mL and streptomycin 100 µg/mL at a temperature of 37° C in the presence of 5% CO_2 and in a water-saturated atmosphere. Pro-monocytic U937 cells were differentiated to macrophages by 10 ng/mL phorbol 12-myristate 13-acetate (PMA).

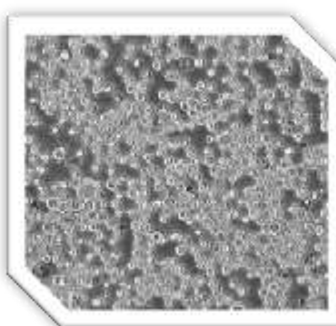


Figure 2.6: U937 cell line.

3T3-L1 is a cell line of mouse embryonic fibroblasts, originally derived from Swiss mouse embryonic tissue by Dr. Howard Green of Harvard Medical School [98]. The designation '3T3' refers to the abbreviation "3-day transfer, inoculum $3x10^5$ cells". The primary mouse embryonic fibroblast cells were transferred (the "T") every 3 days (the first "3"), and inoculated at the rigid density of $3x10^5$ cells per 20 cm^2 flat (the second "3") continuously. Spontaneously immortalised cells with a stable growth rate were established after 20-30 generations in culture and then referred to as "3T3" cells. L1 is a continuous sub-unit of 3T3 (Swiss albino) developed through clonal isolation (***Figure 2.7***). 3T3-L1 murine adipocytes have been key in metabolic disease research for over 30 years and are used for understanding the underlying cellular mechanisms associated with diabetes, obesity and related disorders. Murine 3T3-L1 preadipocytes cell were purchased from ATCC and were maintained in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, San Jose, CA, USA), supplemented with FBS at 10% (v/v), L-Glutamine 2 mM, penicillin 100 U/mL and streptomycin 100 µg/mL at 37° C, in the presence of 5% CO_2 and in an atmosphere saturated with water. 3T3-L1 cell splitting was performed by using trypsin-EDTA solution: cells were washed once with phosphate buffered saline (PBS), incubated with trypsin-EDTA solution for 5 minutes at 37°C to detach adherent cells from flasks and complete medium was added to inactivate trypsin.

Figure 2.7*: Mouse 3T3-L1 cell line.*

2.3.2 Cell viability analysis

The effect of HP, FBR1, FBR2 and AP (20, 50 and 100 µg/mL) on cell proliferation was evaluated by CellTiter-Glo® 2.0 Cell Viability Assay (G9242 Promega, Madison, WI, USA) according to the manufacture's protocol. Briefly, fibroblasts, U937/PMA and 3T3-L1 preadipocytes were seeded in 96-well plates with a cell density of $1,5x10^4$, $8x10^3$, $1x10^4$ per well respectively and incubated overnight. The day after cells were trated with microalgal extracts. 24, 48 and 72 hours later, CellTiter-Glo® Luminescence stain was added and the luminescence signal was read by the plate reader (GloMax, Promega) [99]. (***Figure 2.8***). Cells viability was also determined using a Millipore Scepter™ handheld automated cell counter (Merck Millipore, Darmstadt, Germany) 24, 48 and 72 h after incubation, according to the manufacturer's instructions [100].

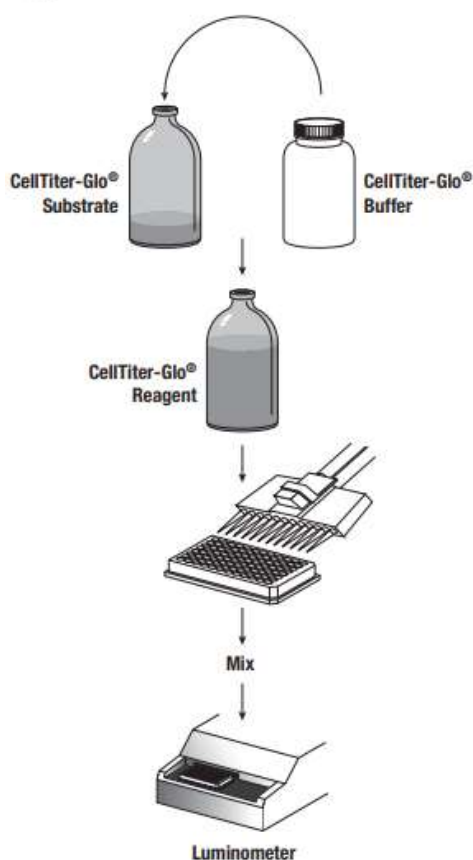


Figure 2.8: Flow diagram showing preparation and use of CellTiter-Glo® Reagent.

2.3.3 3T3-L1 preadipocyte cell differentiation

For adipocyte differentiation, 3T3-L1 preadipocytes were allowed to grow in DMEM until they reached a 100% confluence, changing the medium every 2-3 days. Two days after the cells were confluent, the medium was replaced with Medium induction MDI [Day 0] (DMEM/ high glucose content 10% FBS, 1 μM dexamethasone, 0.5 mM IBMX and 10 μg/mL insulin).

On day 3 the MDI induction medium was removed from the cells and replaced with insulin medium (DMEM/ high glucose containing 10% FBS and 10 μg/mL insulin). From day 8 onwards it was added fresh DMEM to cells, with refreshment every two days. Between days 8 and 12 completely differentiated cells should be obtained. The differentiation was monitored by Red Oil O staining and its quantification on days 4 and 8. ***Figure 2.9*** represents the differentiation scheme.

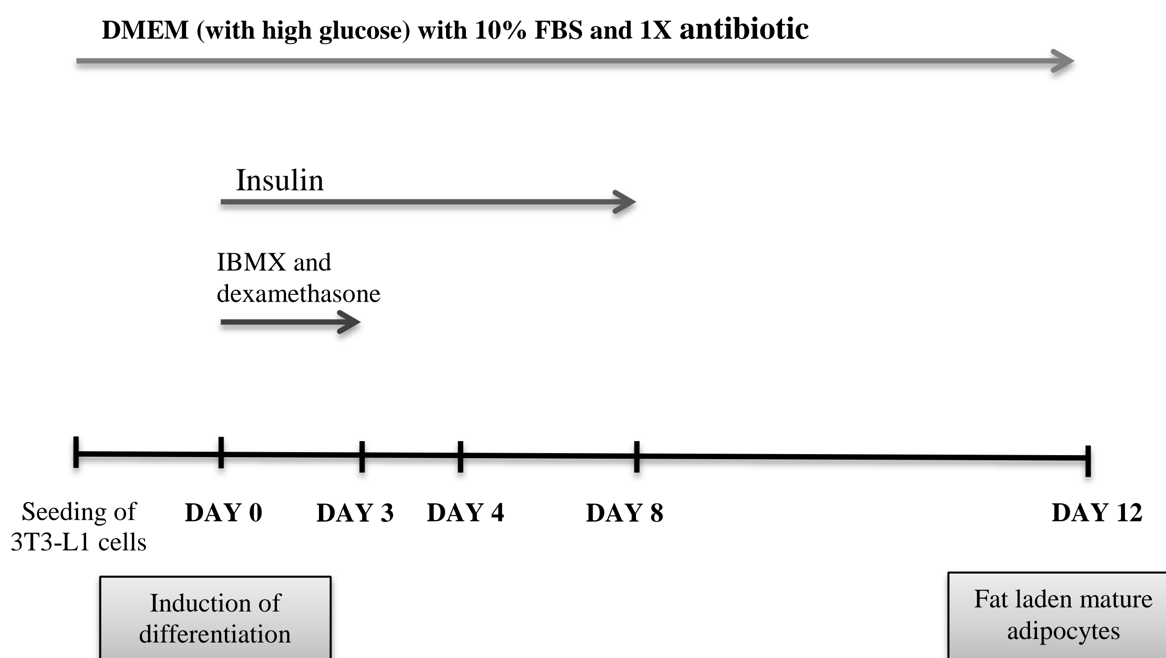


Figure 2.9: Scheme of 3T3-L1 preadipocyte cell differentiation.

2.3.4 Cell viability analysis in differentiated 3T3-L1 preadipocytes

3T3-L1 preadipocytes were sown in 96-well plate with a cell density of $1x10^4$ per well and differentiated as described in 2.3.3. On day 8, differentiated adipocytes were treated with extracts of HP, AP, FBR1 and FBR2 at concentrations of 20, 50 and 100 µg/mL for 24/48 and 72 h. Cell viability was evaluated as described in 2.3.2.

2.3.5 ROS Assay

To assess ROS levels, differentiated U937 were activated from 100 ng/mL lipopolysaccharide from *Salmonella enterica Serotype typhimurium* (LPS, Sigma Aldrich, St. Louis, MO, USA) in the presence or absence of microalgal extracts and AP at 20 µg/mL concentration. After 24 hours, the cells were collected, washed with PBS, and incubated in the dark at 37° C for 30 minutes with 10 µM of 6-Carboxy-2',7'-Dichlorodihydrofluorescein Diacetate (H_2DCFDA, Thermo Fisher Scientific) to determine ROS concentration. Cell permeant H_2DCFDA is a chemically reduced form of fluorescein. Upon cleavage of the acetate groups by intracellular esterases and oxidation, the nonfluorescent H_2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) (***Figure 2.10***).

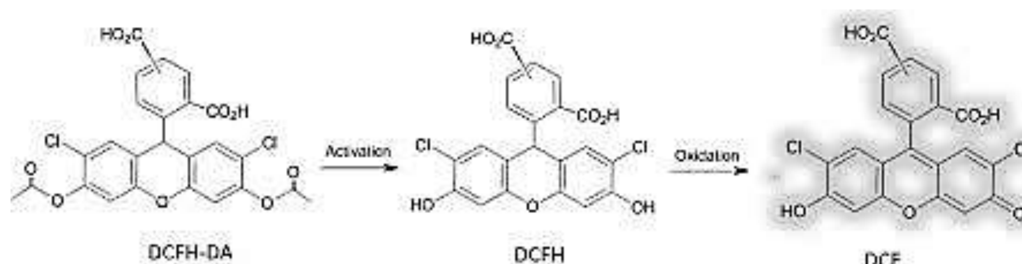


Figure 2.10: DCFH-DA reaction in ROS Assay. 2'-7'dichlorofluorescin diacetate (DCFH-DA), a cell permeant reagent, is deacetylated by cellular esterases to a non-fluorescent compound, DCFH, which is then oxidized by ROS into the fluorescent compound DCF.

2.3.6 NO• Assay

To assess NO• levels, differentiated U937 were activated by LPS in the presence or not of microalgal extracts and AP at 20 µg/mL concentration. After 24 hours, the cells were collected, washed with PBS and incubated in the dark at 37 ºC for 30 minutes to measure concentration of NO•, using 4-Amino-5-Methylamino-2',7'-Difluoropluorescein diacetate (DAF-FM diacetate, Thermo Fisher Scientific). DAF-FM is not fluorescent until it reacts with NO• and forms a fluorescent benzotriazole (***Figure 2.11***).

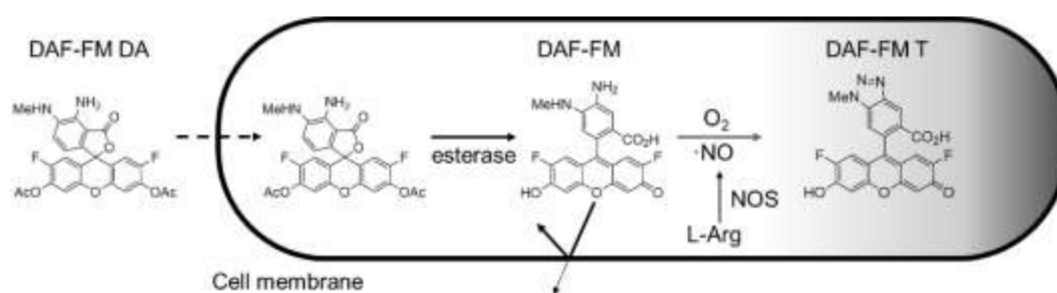


Figure 2.11: DAF-FM diacetate reaction in NO• Assay. Diaminofluorescein-FM diacetate (DAF-FM DA) is non-fluorescent and cell permeable reagent that in cells is deacetylated by esterases in cell impermeable DAFFM, which is reacts with NO• to generate the fluorescent compound DAF-FM T.

2.3.7 IL-6 and TNFα detection

Differentiated U937 were seeded in 24-well plates at a density of 5×10^5 cells/well, treated with microalgal extracts and AP 20 µg/mL for 1 hour and then activated with 1 µg/mL of LPS. After 24 hours, cell-free supernatants were collected and assayed for the concentration of IL-6 and TNFα by Mouse IL-6 ELISA (Cat. n° 32670069U1, ImmunoTools, Friesoythe, Germany) and Mouse TNFα ELISA (Cat.n° 32673019U1, ImmunoTools) using specific matched-pair antibodies and recombinant cytokines as standards following the manufacturer's recommendations.

2.3.8 Quantification of lipid content by Oil Red O staining method

To study the accumulation of intracellular lipids, Oil Red O staining (ORO staining) was performed. In short, differentiated 3T3-L1 adipocytes were treated with microalgal extracts and AP at a concentration of 20 µg/mL. After 24 h adipocytes were washed twice with PBS and fixed with 4% paraformaldehyde for 1 h at room temperature. It was followed by a washing phase with PBS and one with 60% isopropanol. Then the cells were stained with filtered 0.5% Oil Red O solution (Sigma-Aldrich) (60% isopropanol 40% water) for 1 h, washed three times with distilled water and observed by using Evos Floid Cell Imaging Station.

2.3.9 Quantitative analysis of intracellular lipid accumulation

After colouring the lipid droplets with ORO solution, adipocytes were solubilised by incubating the plate with 100% isopropanol for 15 minutes. Then lipid absorption/accumulation at 492 nm was measured by a microplate reader.

2.3.10 *In vitro* lipolysis

Lipolysis assay was performed following the manufacturer's instructions of lipolysis colorimetric assay kit (Lipolysis (3T3-L1) Colorimetric Assay Kit, MAK211, Sigma-Aldrich). In short, 3T3-L1 adipocytes are cultivated and differentiated in a 96-well plate until the oil drops are visible with a microscope. On day 8 the cells are treated with extracts for 24 hours. We wash the cells twice with Lipolysis Wash Buffer and add the Lipolysis Assay Buffer. The next step is to stimulate lipolysis by incubating samples in which isoproterenol has been added. After 3 h of incubation 20-50 µL of the medium are transferred in a new 96-well plate, the Lipolysis Assay Buffer is added up to a volume of 50 µL. Add Reaction Mix to each well and incubate for 30 minutes in the dark. Then measure the absorbance at 570 nm.

2.3.11 Mitochondrial mass analysis

To determine the changes in mitochondrial mass of adipocytes was used a green-fluorescent probe (MitoTracker® Green FM, MTG, Thermo Fisher Scientific)

which appears to localize to mitochondria regardless of mitochondrial membrane potential, as described in [101]. After treatment with microalgal extracts and AP for 24 h, the medium was removed and added prewarmed (37° C) staining solution containing 25 nM MitoTracker® probe. After 30 min of incubation at 37° C in the dark, cells were washed with PBS and analyzed by Evos Floid Cell Imaging Station (magnification 20X). MTG has excitation and emission peaks at 490 and 516 nm, respectively. Images are representative of three independent experiments. For a quantitative analysis, the fluorescence was also measured (Ex/Em 490/516 nm) by using a GloMax plate reader.

2.3.12 Mitochondrial membrane potential analysis

To examine changes in mitochondrial membrane potential of adipocytes was used a red-fluorescent dye MitoTracker™ Red CMXRos (MTR, Thermo Fisher Scientific), that stains mitochondria in live cells and its accumulation is dependent upon membrane potential, as described in [102]. In brief, adipocytes were seeded and treated with microalgal extracts and AP. After 24 h of incubation, the medium was removed and cells were staining with a prewarmed (37° C) 25 nM MitoTracker™ probe for 30 min at 37° C in the dark. Then, cells were washed with PBS and analyzed by Evos Floid Cell Imaging Station (magnification 20X). MTR has excitation and emission peaks at 579 and 599 nm, respectively. Image analysis was performed with ImageJ to quantify the intensity of mitochondrial red. Images are representative of three independent experiments. For a quantitative analysis of the mitochondrial membrane potential, the fluorescence was also measured (Ex/Em 579/599 nm) by using a GloMax plate reader.

2.3.13 Mitochondrial superoxide analysis

To detect mitochondrial ROS, especially superoxide, was used MitoSOX™ Red mitochondrial superoxide indicator (MS, Thermo Fisher Scientific), a novel fluorogenic dye specifically targeted to mitochondria in live cells, following the manufacturer's instructions. In detail, adipocytes were seeded and treated as previously described. Cells were staining with 5 µM MitoSOX™ reagent solution

for 45 min at 37° C in the dark. After incubation, adipocytes were washed with PBS and analyzed by Evos Floid Cell Imaging Station (magnification 20X). MS has excitation and emission peaks at 510 and 580 nm, respectively [103]. Image analysis was performed with ImageJ to quantify the intensity of mitochondrial red. A quantitative analysis of the mitochondrial superoxide levels was obtained by using a GloMax plate reader at 510/580 nm. Images are representative of three independent experiments.

2.3.14 Western blot

3T3-L1 adipocytes were lysed in RIPA buffer with protease inhibitor cocktail and quantified using the BCA Protein Assay Kit. Thirty micrograms of total protein per well were separated by SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis on gel) and then electroblotted on nitrocellulose membranes. The membranes were stained with Ponceau solution to verify the correct transfer, washed with distilled water to remove the staining. The membranes were blocked for 1 hour in a tris-buffered saline solution (TBST) containing 5% non-fat dry milk and 0.5% Tween 20, and then immunostained at 4º C overnight with primary antibodies directed against PPARγ (7273, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ATP citrate lyase (ab157098, Abcam) or anti-*β*-actin antibodies (ab8227, Abcam). The next day, the membranes were washed three times for 5 minutes in TBST and, after 1 hour of incubation with HRP Goat antiRabbit IgG secondary antibody (Santa Cruz Biotechnology), the immunoreactions were detected using the substrate of horseradish peroxidase ECL WesternBright (Advansta, Menlo Park, CA, USA) at the Chemidoc survey, XRS system equipped with Image Lab software for image acquisition and densitometric analysis (Bio-Rad Laboratories, Hercules, CA, USA) (***Figure 2.12***). The level of a specific protein from each sample has been normalized with respect to the respective *β*-actin signal. The protein expression level in the control sample was considered to be 1. Each result from treated the samples were expressed as a proportion of the control sample.

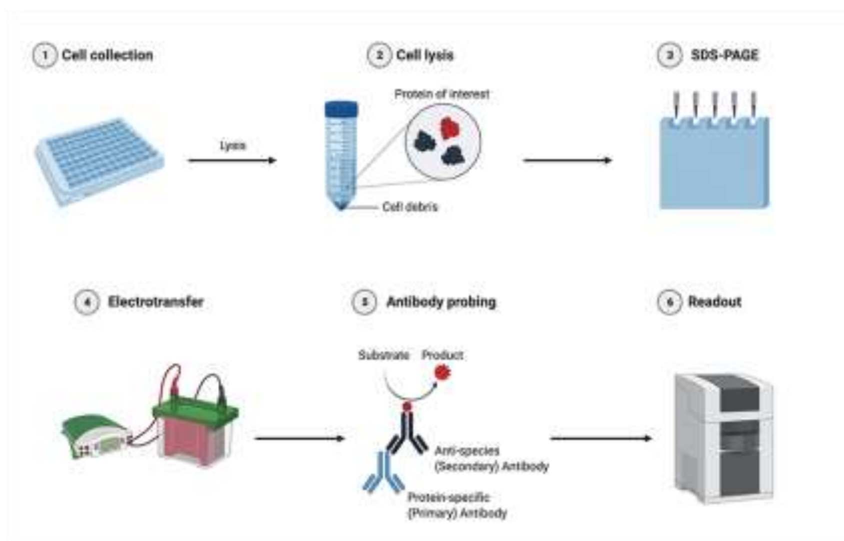


Figure 2.12*: *Overview of Western blotting procedure. Cells are treated, collected and lysed before to be subjected to separation according to their molecular weight in SDS-PAGE. Following electro transfer, the immunoreaction involved primary specific antibody and secondary antibody conjugated to HRP, allow to recognize the protein of interest. Images are acquired at ChemiDoc.

2.3.15 IL-6 and TNFα detection

Adipocytes were seeded in 24-well plates at a density of 5×10^5 cells/well, treated with microalgal extracts and AP 20 μg/mL for 24 h. After incubation, cell-free supernatants were collected and assayed for the concentration of IL-6 and TNFα by Mouse IL-6 ELISA (Cat. n° 32670069U1, ImmunoTools) and Mouse TNFα ELISA (Cat.n° 32673019U1, ImmunoTools) using specific matched-pair antibodies and recombinant cytokines as standards following the manufacturer's recommendations.

2.3.16 Statistical analysis

Data are presented as mean values ± standard deviation (SD) of, at least, three independent experiments run in triplicate. Comparisons of more than two groups were evaluated using *one-way ANOVA* followed by Dunnett’s multiple comparison tests. The statistical methods used for each experiment are detailed in the figure legends. The asterisks in the figures denote statistical significance (*$p<$ 0.05; **$p<$ 0.01; and ***$p<$ 0.001).

3. RESULTS

3.1 CHARACTERIZATION OF MICROALGAL STRAINS

The first step of the PhD project was to identify the optimal growth conditions for *Haematococcus Pluvialis* and the two mutants FBR1 and FBR2 and characterize them from the biochemical point of view.

3.1.1 Microalgal strains growth

To identify the best microalgal growth conditions we tested several conditions, including the material and shape of the culture containers or the different composition of the culture media. However, once the optimal conditions (reported in paragraph 2.1.3), *H.pluvialis* followed the trends reported in the bibliography. Growth was monitored by measuring optical density. The three strains of green microalgae showed a growth trend reaching the stationary phase within 4-6 days, in line with the values reported in the literature. Growth of *A.platensis* was also examined.

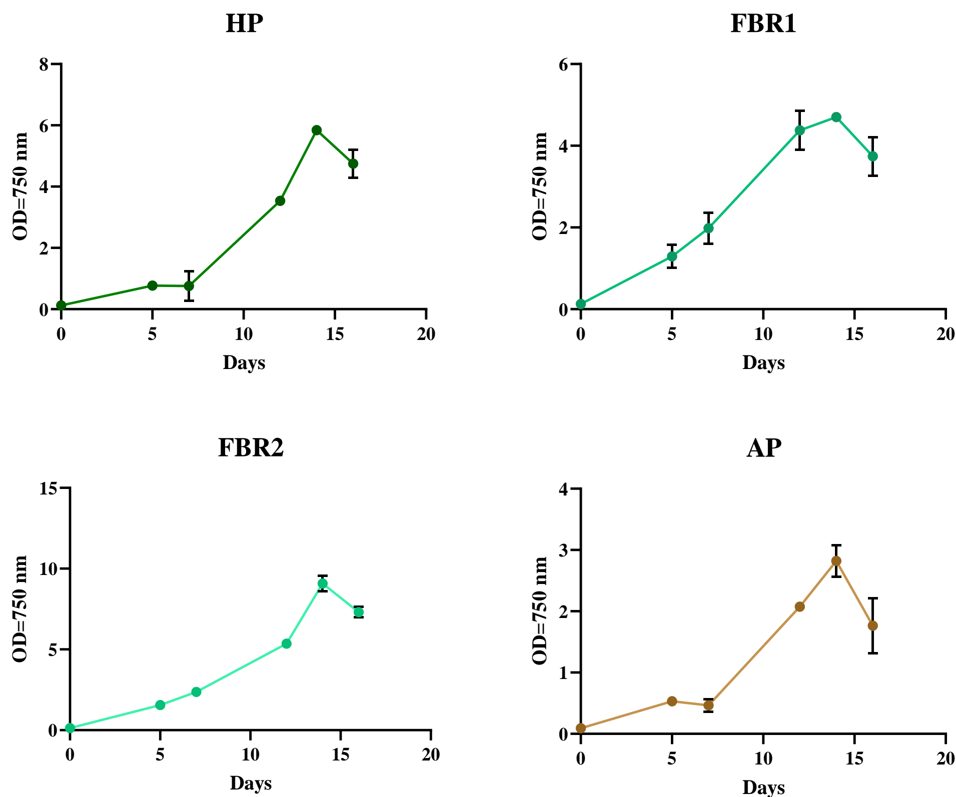


Figure 3.1: Growth curves for HP, FBR1, FBR2 and AP.

In ***Figure 3.1***, we note that the growth of all three strains of microalgae and cyanobacteria followed a sigmoidal pattern, and the two mutants reached the plateau stage earlier than the wild type strain. During the exponential phase, 1 mL of the crops were withdrawn and reimnoculated in a fresh medium to keep the crops healthy.

3.1.2 Analysis of phylogenetic distances

For the reconstruction of the phylogenetic distances between HP and the two mutants FBR1 and FBR2, the UPGMA method has been used, according to which it is assumed that the evolution speed of the sequences is constant along all the branches of the tree. It uses an iterative clustering algorithm that proceeds by associating sequences or clusters of sequences more similar to each other.

Figure 3.2: Dendrogram showing greater genotypical similarities between FBR1 and HP than FBR2 (UPGMA method).

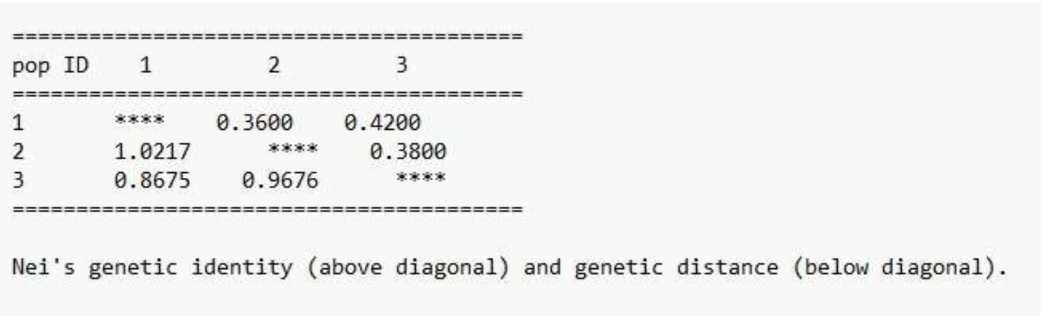
From the dendrogram in ***Figure 3.2*** it is clear that FBR1 and HP are genetically closer than FBR2, detached in a second cluster.

In particular, ***Figure 3.3*** shows the genetic distances between species:

HP and FBR1 = 0.8675

HP and FBR2 = 0.9676

FBR1 and FBR2 = 1.0217



```
========================================
pop ID    1         2         3
========================================
1       ****    0.3600    0.4200
2       1.0217      ****    0.3800
3       0.8675    0.9676      ****
========================================

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).
```

Figure 3.3: Genetic distances between species.

3.1.3 Microalgal enzymatic profile evaluation

The analysis of the enzymatic profile showed the presence of proteases, lipases and amylases with differences in the various strains, as shown in ***Table 3.1***:

Description	Proteases	Lipases	Amylases
HP		X	
FBR1	X	X	
FBR2	X	X	X
AP		X	

Table 3.1: Enzymatic profile of microalgal strains and AP.

The quantitative analysis, however, did not provide us with significant differences in the microalgal strains tested (data not shown).

3.1.4 Microalgal biochemical composition

The biochemical composition of the microalgal strains studied showed the following data (***Tables 3.2, 3.3***). Among the microalgal strains analyzed, FBR2 showed the highest content of total phenols and fats, as well as the highest antioxidant power. The evaluation of fatty acid composition showed the presence of saturated and polyunsaturated Ω3, Ω6 and Ω9 fatty acids. Interestingly, the best ratio Ω3:Ω6 was observed in FBR2.

	Total fat		ABTS		Total phenolic		FRAP	
	%	SD	mg TE/g lyophilisate	SD	mg AGE/g lyophilisate	SD	mg TE/ g lyophilisate	SD
HP	16,15	1,87	35,71	1,46	17,36	0,553	46,46	1,84
FBR1	16,88	1,25	24,49	0,981	10,15	0,125	39,06	2,12
FBR2	38,4	3,07	74,00	2,25	24,61	0,469	119,67	3,13
AP	12,62	1,14	97,28	0,3	19,61	0,3964	261,12	6,95

Table 3.2: Biochemical composition of microalgal strains and AP.

	FBR1	FBR2	HP
C 4			Nd
C 6			nd
C 8	0,107	0,089284	0,109617
C 10	0,804117	0,312493	nd
C 12	1,072156	1,752193	1,571178
C 14	4,020585	5,345863	4,750073
C 14:1	0,589686	0,881677	0,840398
C 15	1,125764	1,328095	1,315405
C 15:1	0,214431	0,189728	0,372698
C 16	24,33794	26,3387	22,4715
C 16:1	0,643294	1,060244	0,73078
C 17	1,018548	1,026763	2,521193
C 17:1	0,268039	0,379456	1,059632
C 18	19,78128	17,37684	16,88103
C 18:1 T 9	0,268039	0,147318	0,621163
C 18:1 C 9	22,30085	23,11332	22,65419
C 18:2 C 9	11,36485	8,850249	10,92517
C 20	0,911333	0,658467	0,438468
C 18:3 C6	1,286587	1,104886	1,205788
C 20:1	0,48247	0,502221	0,621163
C 18:3 C9	2,19792	2,198612	2,703888
C 21	0,305564	0,200888	0,36539
C 20:2	0,375255	0,24553	0,584624
C 22	0,160823	0,167407	0,255773
C 20:3 C8 C11 C14	0,321647	0,145086	0,423853

C 22:1	1,393803	1,774514	1,680795
C 20:3 C11 + C 23	0,225153	0,256691	0,511546
C 20:4 N6	1,720811	1,763353	1,096171
C 22:2	0,214431	0,100444	0,182695
C 24	0,091133	0,01116	0,073078
C 20:5	1,125764	1,484342	1,4981
C 24:1	0,466388	0,256691	0,475007
C 22:6	0,804117	0,937479	1,059632
Total	100	100	100

Table 3.3: Composition of fatty acids in microalgal strains (% of total fatty acids, nd= not detected).

3.2 EXTRACTS PREPARATION

For the preparation of microalgal extracts we started from wet biomass. At the end of the growth cycle, 5 mL of samples were taken, collected in 15 mL falcon and centrifuged (Thermo Fisher Scientific MR23i centrifuge) at room temperature and 4.500 g for 3 minutes to separate the pellet from the liquid phase. We then tiled the samples and weighed (***Figure 3.4, Table 3.4***).

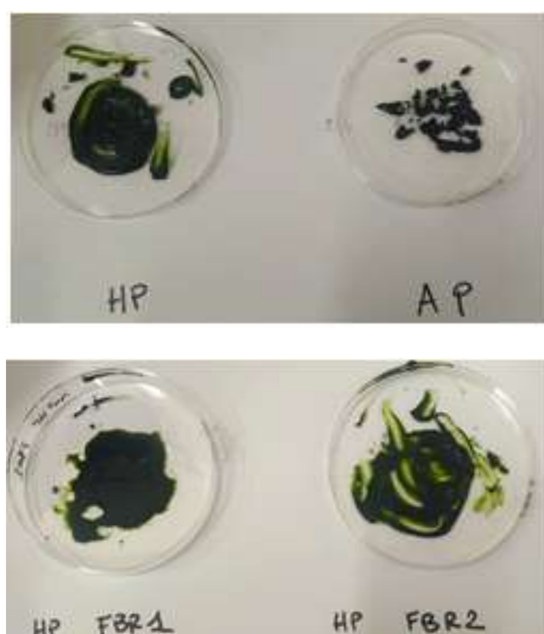


Figure 3.4*: *Microalgal and AP extracts in Petri dishes.

	Wet biomass weight (mg)	Dry biomass weight (mg)
H. pluvialis	630	132
FBR1	720	146
FBR2	780	145
A.platensis	620	110

Table 3.4: Wet and dry biomass weight of microlgal extracts and AP.

The Petri dishes were placed in the stove and the samples were dried for 1 h at 60°C to obtain dry biomass (***Figure 3.5***).

Figure 3.5: Petri dishes in the stove.

Once dried, we collected the samples and filled them with RIPA buffer in a mortar. The lysed samples were transferred to new eppendorf, after syringing and incubated at 4° C for 20 minutes. After a first centrifugation to remove the pellet, the samples were sonified, followed by 4 freezing/thawing cycles. After the last centrifugation the samples were dosed to evaluate the protein content by the Bradford method (***Figure 3.6***, ***Table 3.5***). Because the samples had marked stains, we checked whether they could interfere with the Bradford dosage in any way, but

fortunately, we got virtually zero absorbance values.

Figure 3.6: Microalgal and AP extracts.

SAMPLE	**PROTEIN CONCENTRATION (µg/µL)**
H.pluvialis	1
FBR1	1,8
FBR2	1,3
A.platensis	1,3

Table 3.5: Protein dosage of microalgal extracts and AP.

3.3 *IN VITRO* TEST

Once we obtained the aqueous microalgal extracts we tested *in vitro* their effect on inflammation and adipocyte function.

3.3.1 Effect of microalgal extracts on primary human fibroblasts viability

Primary human fibroblasts were treated for 24, 48 and 72 hours with increasing concentrations of HP, FBR1, FBR2 and AP (20, 50 and 100 µg/mL). As shown in ***Figure 3.7*** HP at the highest concentration reduces cell viability by 35% at all

times (***Figure 3.7 A***). A slight antiproliferative activity was observed at the lowest tested concentration of 20 µg/mL, where reductions in cell numbers relative to untreated cells (Control) were 8%, 11% and about 18% respectively at 24, 48 and 72 h. FBR1 shows toxicity above 50% only at the highest concentration 100 ug/mL at 72 h, while at the lowest concentration it reduces cell viability by 12%, 19% and 22% respectively at 24, 48 and 72 h (***p <0.001, **p <0.01, Dunnett's multiple comparison test) (***Figure 3.7 B***). FBR2, on the other hand, shows no fibroblasts toxicity except at the greater concentration at 48 and 72 h (22 and 23% compared to the Control, *p <0.05, Dunnett's multiple comparison test) (***Figure 3.7 C***). Finally, AP induces a slight reduction in the number of cells at the highest concentration (15%, 22% and 28% respectively at 24, 48 and 72 h) (**p <0.01, Dunnett's multiple comparison test) (***Figure 3.7 D***).

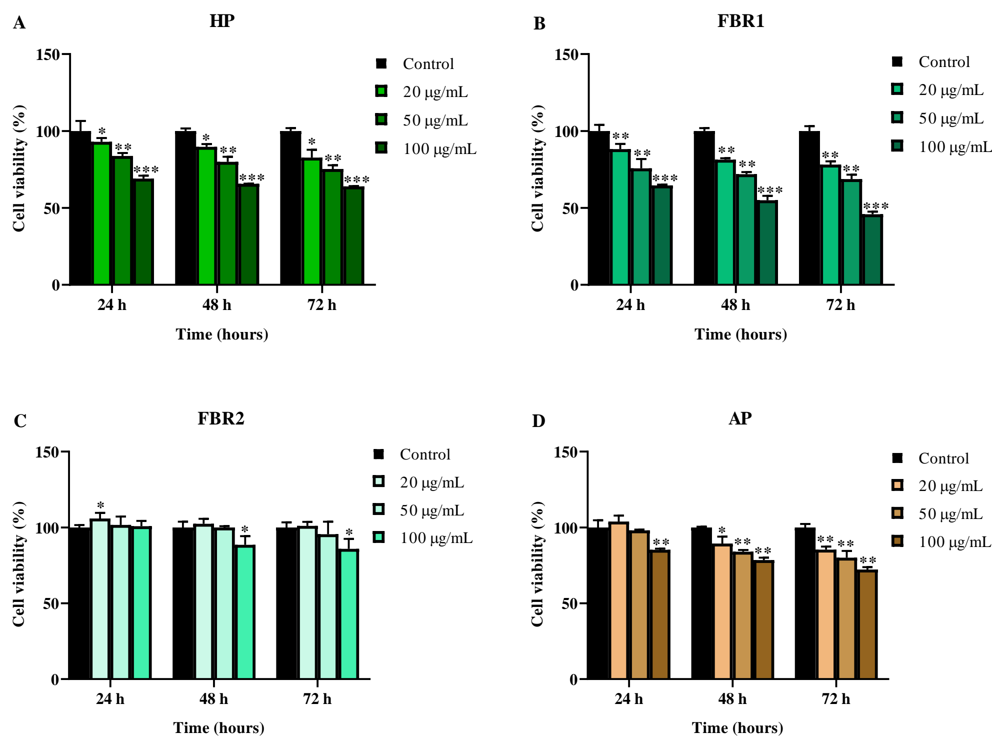


Figure 3.7: Fibroblasts cells viability. Primary human fibroblasts were treated with increasing concentrations of HP, FBR1, FBR2 and AP, ranging from 20 to 100 µg/mL, and cell viability was assessed by CellTiter-Glo® 2.0 Cell Viability Assay after 24, 48 and 72 hours exposure. The mean values ± SD of three independent experiments with four replicates in each are shown. Where indicated differences were significant according to *one-way ANOVA* followed by Dunnett's

multiple comparison test (*** p<0.001, ** p<0.01, * p<0.05).

3.3.2 Effect of microalgal extracts on differentiated U937 cell viability

PMA-differentiated U937 cells were treated for 24, 48 and 72 hours with increasing concentrations of HP, FBR1, FBR2 and AP (20, 50 and 100 μg/mL). As shown in ***Figure 3.8*** HP at the highest concentration reduces cell viability by about 50% at all times (***Figure 3.8 A***). A slight antiproliferative activity was observed at the lowest tested concentration of 20 μg/mL, where cell number reductions compared to untreated cells (Control) were respectively 17%, 21% and about 28% at 24, 48 and 72 h. FBR1 shows toxicity around 50% only at the highest concentration 100 ug/mL, while at the lowest concentration it reduces cell viability by 15%, 19% and 25% respectively at 24, 48 and 72 h (***p <0.001, **p <0.01, Dunnett's multiple comparison test) (***Figure 3.8 B***). FBR2, on the other hand, does not show the toxicity of fibroblasts except at the higher concentration at 48 and 72 h (8 and 12% compared to the control, *p <0.05, Dunnett's multiple comparison test) (***Figure 3.8 C***). Finally, AP induces a reduction in the number of cells at the highest concentration (25%, 30% and 35% respectively at 24, 48 and 72 h) (***p <0.001,**p <0.01, Dunnett's multiple comparison test) (***Figure 3.8 D***).

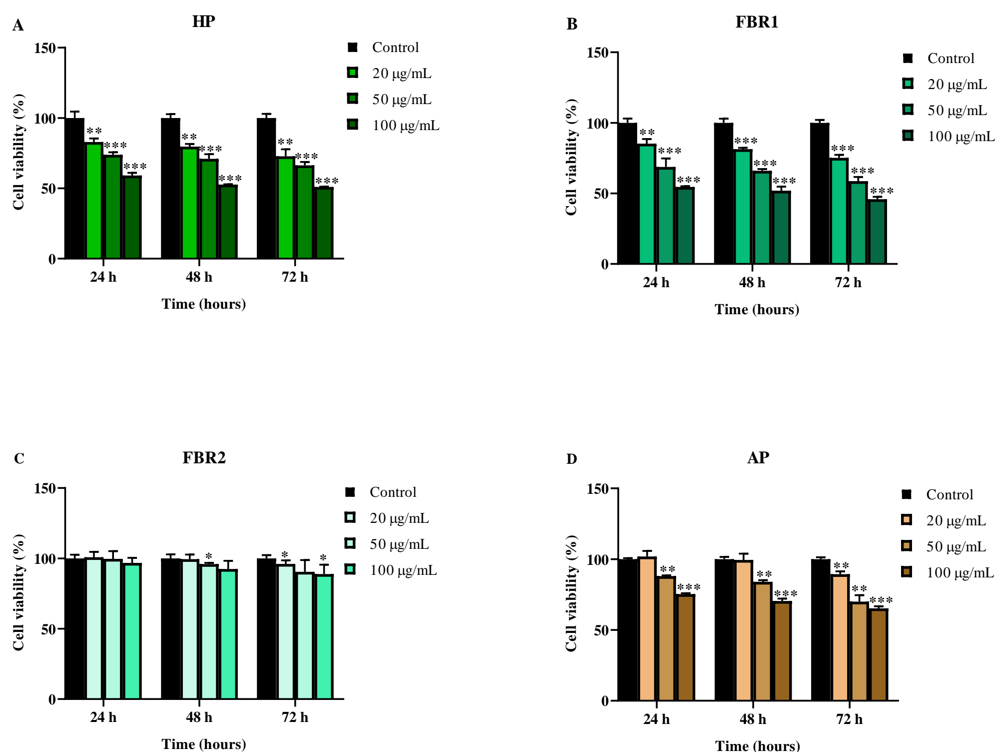


Figure 3.8: Differentiated U937 cell viability. PMA-differentiated U937 cells were treated with increasing concentrations of HP, FBR1, FBR2 and AP, ranging from 20 to 100 µg/mL, and cell viability was assessed by CellTiter-Glo® 2.0 Cell Viability Assay after 24, 48 and 72 hours exposure. . The mean values ± SD of three independent experiments with four replicates in each are shown. Where indicated differences were significant according to *one-way ANOVA* followed by Dunnett's multiple comparison test (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).

In light of these results, we decided to run all the subsequent experiments to assess the effect of microalgal extracts on inflammation using the lowest and non-toxic concentration, 20 µg/mL.

3.3.3 ANTI-INFLAMMATORY EFFECT OF MICROALGAL EXTRACTS

To evaluate the anti-inflammatory activity of microalgal extracts we decided to measure the levels of some mediators of inflammation such as ROS, NO•, IL-6 and TNFα.

3.3.3.1 Microalgal extracts lower ROS levels

When macrophages were activated with LPS, we found a sharp increase in ROS levels compared to untreated cells (***Figure 3.9***, CTRL *vs*. LPS $^{###}p < 0.001$). Our extracts at the concentration of 20 µg/mL significantly reduced ROS production. In more detail, HP decreased ROS levels by 18%, FBR1 by 26% and AP (used as reference) by 20% compared to cells treated with LPS alone. The FBR2 extract showed the best effect, reducing ROS levels by 28%.

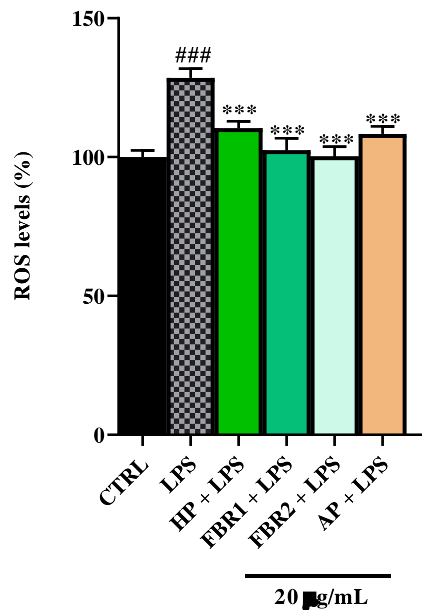


Figure 3.9: Microalgal and AP extracts lowered ROS levels. Macrophages were treated with LPS in the presence or absence of HP, FBR1, FBR2 and AP. Following 24 hours, ROS levels were evaluated and expressed as percentage of unstimulated cells (CTRL, set at 100 %). The mean values ± SD of three independent experiments with four replicates in each are shown. Where indicated differences were significant according to *one-way ANOVA* followed by Dunnett's multiple comparison test (p<0.001).

3.3.3.2 Microalgal extracts affect NO• production

Nitric oxide is another metabolite involved in the inflammatory process. In LPS-triggered human macrophages cell line NO• levels increase compared to untreated control cells (***Figure 3.10***, CTRL *vs*. LPS $^{###}p < 0.001$). Microalgal extracts at a concentration of 20 ug/mL significantly reduced nitric oxide levels. In particular, HP reduced NO• levels by 17%, FBR1 by 20% and AP (used as reference) by

13% compared to cells treated only with LPS. Again FBR2 showed the greatest effect, reducing NO• levels by 21%.

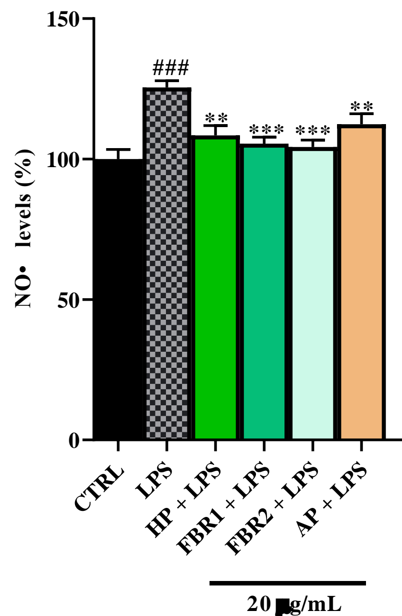


Figure 3.10: Microalgal and AP extracts lowered NO• levels. Macrophages were treated with LPS in the presence or absence of HP, FBR1, FBR2 and AP. Following 24 hours, NO• levels were evaluated and expressed as percentage of unstimulated cells (CTRL, set at 100 %). The mean values ± SD of three independent experiments with four replicates in each are shown. Where indicated differences were significant according to *one-way ANOVA* followed by Dunnett's multiple comparison test (p<0.01, p<0.001).

3.3.3.3 Microalgal extracts reduce the secretion of the pro-inflammatory cytokines IL-6 and TNFα

When stimulated with lipopolysaccharides, macrophages secrete several mediators including IL-6 and TNFα, which have a primary role for the onset of inflammation [104]. Therefore, to probe the effect of microalgal extracts on IL-6 and TNFα secretion, PMA-differentiated U937 cells were stimulated with LPS in the presence or absence of each extract at a concentration of 20 µg/mL (***Figure 3.11 A-B***). Twenty-four hours later, IL-6 and TNFα levels were measured in cell culture media. LPS induced a significant release of IL-6 and TNFα (***Figure 3.11 A-B***; LPS *vs*. CTRL, $^{###}$p <0,001). The extracts were all active in lowering IL-6 and TNFα levels compared to cells activated only with LPS. Interestingly, FBR2

extract was again the most effective, showing a 20% and 15% reduction in IL-6 and TNFα levels respectively, compared to LPS-only cells.

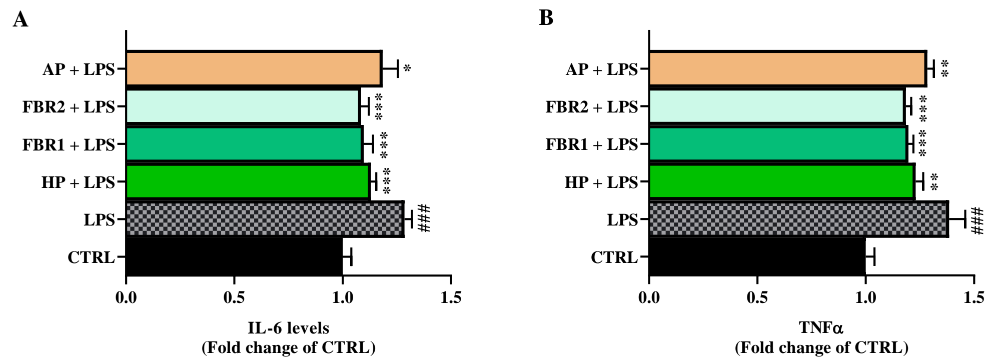


Figure 3.11: Microalgal and AP extracts lowered IL-6 and TNFα levels. Macrophages were treated with LPS in the presence or absence of HP, FBR1, FBR2 and AP. Following 24 hours, IL-6 (**A**) and TNFα (**B**) levels were evaluated and expressed as percentage of unstimulated cells (CTRL, set at 100 %). The mean values ± SD of three independent experiments with four replicates in each are shown. Where indicated differences were significant according to *one-way ANOVA* followed by Dunnett's multiple comparison test ($p<0.05$, $p<0.001$).

3.3.4 EFFECT OF MICROALGAL EXTRACTS ON ADIPOCYTE FUNCTION

To evaluate the effect of microalgal extracts on the adipocyte function we decided to monitor lipid accumulation, the expression of any adipogenic markers such as PPARγ and ACLY, as well as pro-inflammatory cytokine levels IL-6 and TNFα.

3.3.4.1 Effect of microalgal extracts on mouse 3T3-L1 preadipocyte viability

Mouse 3T3-L1 preadipocytes were treated for 24, 48 and 72 hours with increasing concentrations of HP, FBR1, FBR2 and AP (20, 50 and 100 µg/mL). As shown in ***Figure 3.12,*** HP - at a maximum of tested concentration - reduces cell viability by more than 50% at all times (***Figure 3.12 A***). At the lowest tested concentration of 20 µg/mL the number of cells is reduced compared to untreated (Control) cells by 27%, 40% and about 68% respectively at 24, 48 and 72 h. FBR1 shows maximum

antiproliferative effect at 100 µg/mL concentration at 72 h, while the number of cells at the lowest concentration reduces cell viability by 22%, 19% and 25% respectively at 24, 48 and 72 h (***p <0.001, Dunnett's multiple comparison test) (***Figure 3.12 B***). FBR2, on the other hand, does not show antiproliferative activity in preadipocytes except at the concentration of 100 µg/mL at 48 h (about 21% compared to the control, **p <0.01, Dunnett's multiple comparison test) (***Figure 3.12 C***). Finally, AP does not affect cell viability only at the lowest concentration (***Figure 3.12 D***).

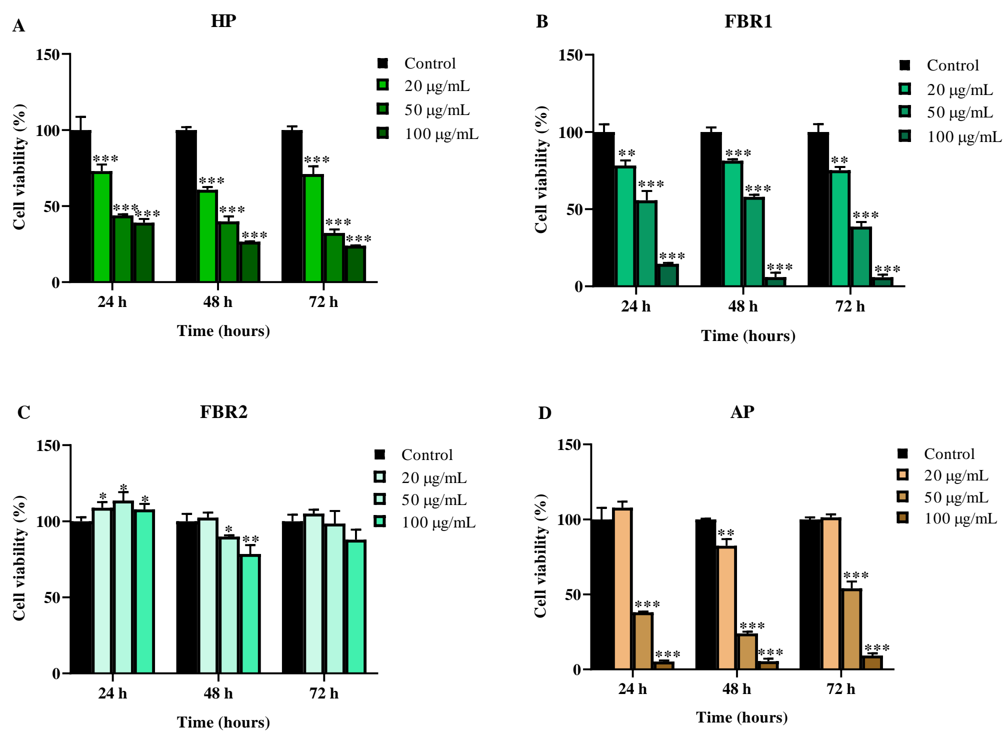


Figure 3.12: Effect of microalgal extracts and AP on mouse 3T3-L1 preadipocyte viability. Mouse 3T3-L1 preadipocytes were treated with increasing concentrations of HP, AP, FBR1 and FBR2 ranging from 20 to 100 µg/mL, and cell viability was assessed by CellTiter-Glo® 2.0 Cell Viability Assay after 24, 48 and 72 hours exposure. The mean values ± SD of three independent experiments with four replicates in each are shown. Where indicated differences were significant according to *one-way ANOVA* followed by Dunnett's multiple comparison test (***p<0,001, ** p<0,01, * p<0,05).

3.3.4.2 Differentiation of mouse 3T3-L1 preadipocytes

To perform all the experiments we first differentiated the preadipocytes. In detail, we sowed and grew the preadipocytes. Two days after reaching the confluence we replaced the medium with MDI. On the third day the induction medium was replaced with an insulin-containing medium. From the eighth day onwards, the insulin-containing medium was removed and DMEM was added. Complete differentiation of preadipocytes into adipocytes is achieved between the eighth and the 12th day. We monitored the stages of differentiation with ORO staining on days 0, 4 and 8 and with the relative quantification of lipid accumulation as shown in ***Figure 3.13 A-B***. Note a different cell morphology since the 4 days of differentiation. In fact the differentiated cells have become round and large, surrounded by lipid droplets, which stained red by the Red Oil O.

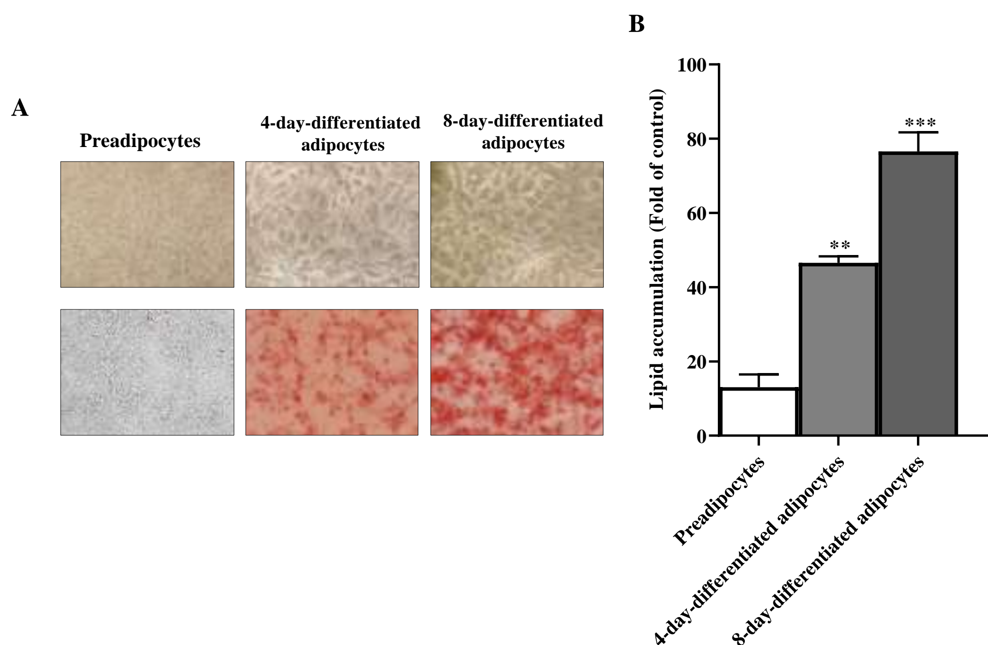


Figure 3.13: Differentiation of 3T3-L1 preadipocytes into adipocytes, monitored with Oil Red O staining and relative quantification of lipid accumulation on days 0, 4 and 8. The mean values ± SD of three independent experiments with four replicates in each are shown. Differences were significant (***p<0,001, **p<0,01).

With a greater magnification it is possible to observe the formed lipid droplets (***Figure 3.14***).

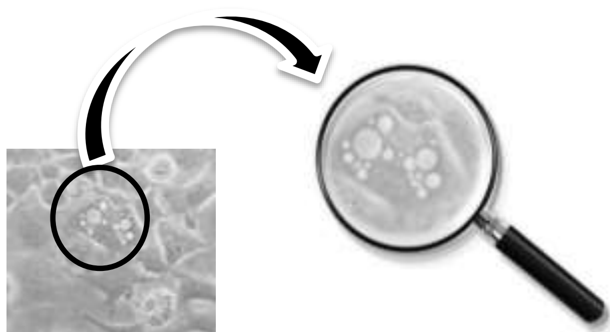


Figure 3.14: Formation of lipid droplets in adipocytes.

3.3.4.3 Effect of microalgal extracts on mouse 3T3-L1 adipocyte viability

3T3-L1 cells were cultured in adipocyte differentiation media for 8 days. On the 12th day we treated adipocytes for 24, 48 and 72 hours with increasing concentrations of HP, FBR1, FBR2 and AP (20, 50 and 100 µg/mL). As shown in ***Figure 3.15*** HP is not toxic only at the lowest concentration at all times (***Figure 3.15 A***). FBR1 shows maximum toxicity at a concentration of 100 µg/mL, resulting in a reduction in the number of cells by 86%, 89% and 95% at 24, 48 and 72 h respectively (***p <0.001, Dunnett's multiple comparison test) (***Figure 3.15 B***). FBR2, on the other hand, shows no significant antiproliferative effect on adipocytes (***Figure 3.15 C***). Finally, AP does not affect cell viability at the lowest concentration (***Figure 3.15 D***).

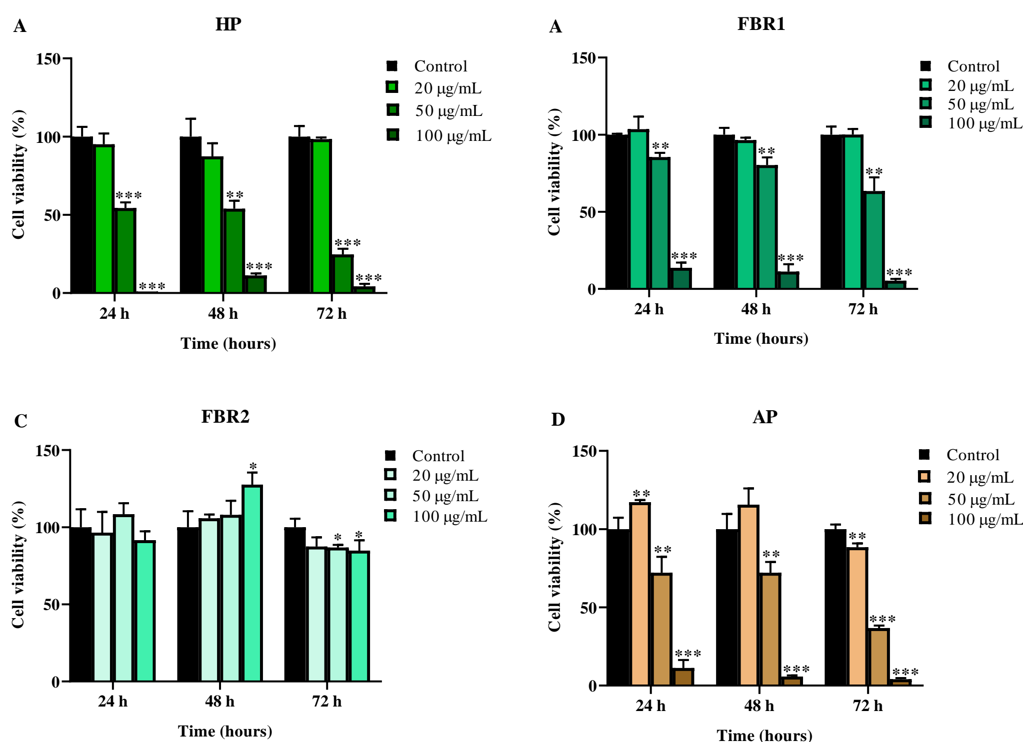


Figure 3.15: Effect of microalgal and AP extracts on mouse 3T3-L1 adipocyte viability. Mouse 3T3-L1 adipocytes were treated with increasing concentrations of HP, AP, FBR1 and FBR2 ranging from 20 to 100 µg/mL, and cell viability was assessed by CellTiter-Glo® 2.0 Cell Viability Assay after 24, 48 and 72 hours exposure. The mean values ± SD of three independent experiments with four replicates in each are shown. Where indicated differences were significant according to *one-way ANOVA* followed by Dunnett's multiple comparison test (***p<0,001, ** p<0,01, * p<0,05).

In light of these results, we decided to run all the subsequent experiments to assess the effect of microalgal extracts on adipocyte function and metabolism using the lowest and non-toxic concentration, 20 µg/mL.

3.3.4.4. Effect of microalgal extracts on lipid content in mouse 3T3-L1 adipocytes

The dye Oil Red O can be strongly combined with triglycerides. To study intracellular lipid accumulation, Oil Red O staining was performed. Briefly, the differentiated adipocytes 3T3-L1 were treated with 20 µg/mL of microalgal

extracts for 24 h. After staining, the fatty droplets were displayed as a "ring" and the images were obtained by fluorescent microscopy. The dye Oil Red O was eluted using isopropanol and the absorbance was determined by Microplate Reader. Based on microscopic observation, the differentiation of preadipocytes into adipocytes induced significant intracellular lipid accumulation, as shown in ***Figure 3.16 A-B***.

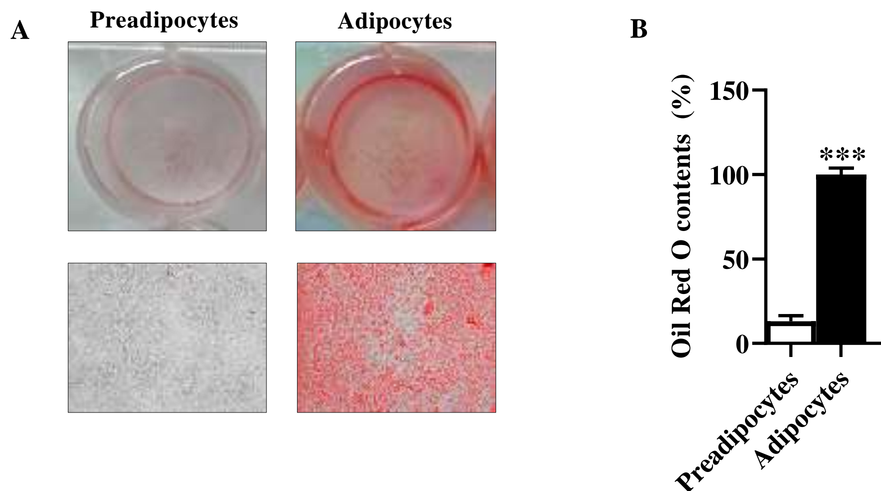


Figure 3.16: Microscopic observation of the preadipocytes differentiation into adipocytes*, *monitored with Oil Red O. (**A**) Staining and relative quantification of lipid accumulation (**B**). The images are representative of three independent experiments. The mean values ± SD of three independent experiments with four replicates in each are shown. Differences were significant (***$p<0,001$).

Treatment with HP and AP contributed to the accumulation of intracellular lipid droplets (***Figure 3.17 A***). The relative quantitative result of staining with Red Oil O showed that lipid accumulation in 3T3-L1 cells was significantly increased after treatment with HP and AP compared to untreated cells (***Figure 3.17 B***). In particular, *H.Pluvialis* extract increased lipid accumulation by 14% and *A.platensis* extract by 28%. Only FBR2 extract improved lipid accumulation by about 10% compared to untreated adipocytes (***Figure 3.17 A-B***).

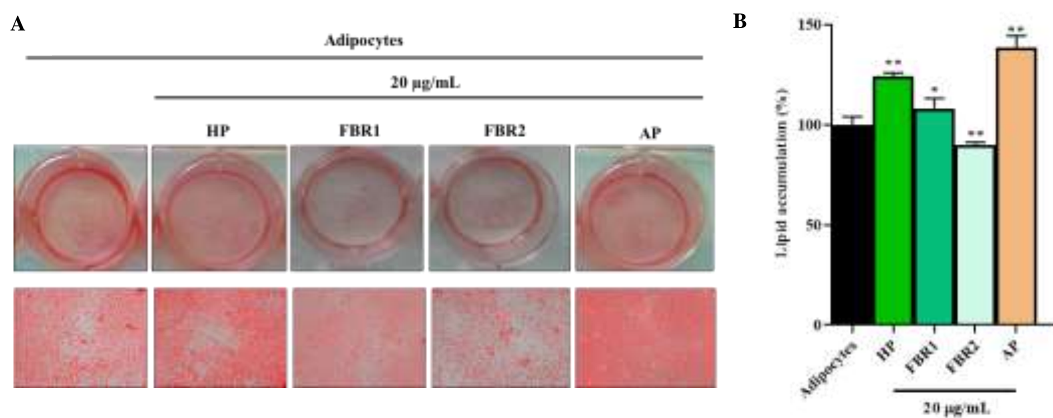


Figure 3.17: Effect of microalgal and AP extracts on lipid content, monitored through Oil Red O staining. (**A**) Staining and relative quantification of lipid accumulation (**B**). The images are representative of three independent experiments. The mean values ± SD of three independent experiments with four replicates in each are shown. Differences were significant (*p<0,05, **p<0,01).

3.3.4.5 Effect of microalgal extracts on *in vitro* lipolysis

How do you notice from the chart in ***Figure 3.18*** the extracts that showed *in vitro* lipolytic action are FBR2 and FBR1, increasing the release of glycerol significantly compared to untreated adipocytes. HP and AP, by contrast, have reduced the release of glycerol.

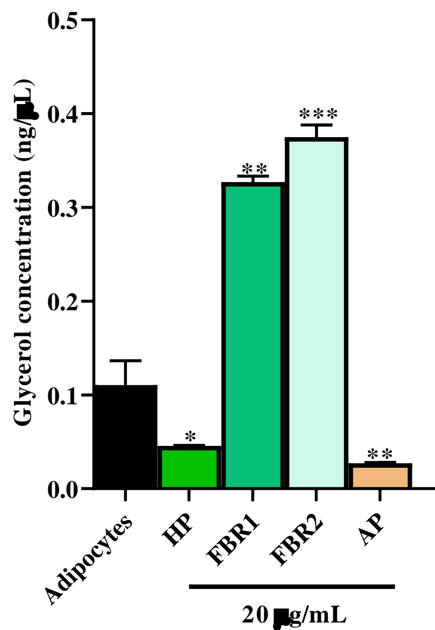


Figure 3.18: Effects of microalgal and AP extracts on in vitro lipolysis. The mean values ± SD of three independent experiments with four replicates in each are shown. Differences were significant (***p<0,001, **p<0,01, *p<0,05).

3.3.4.6 Effect of microalgal extracts on mitochondrial function

Then, we stained adipocytes with different fluorogenic dyes evaluating the mitochondrial mass, membrane potential and oxidative stress. When the adipocytes were stained with MitoTracker Green, we did not observe any decrease in its intensity following treatment with microalgal extracts. FBR2 showed an increase in mitochondrial mass over untreated cells about 11% (***Figure 3.19 A***).

When membrane potential was analysed using MitoTracker Red CMXRos no significant differences were observed with respect to untreated cells (***Figure 3.19 B***). On the other hand, when we evaluated the effect of microalgal extracts on mitochondrial oxidative stress and superoxide radical production, we observed a significant reduction in the production of mitochondrial superoxide anion following both FBR1 and FBR2 addition (**p<0.01, *p<0.05, Dunnett's multiple comparison test) (***Figure 3.19 C***).

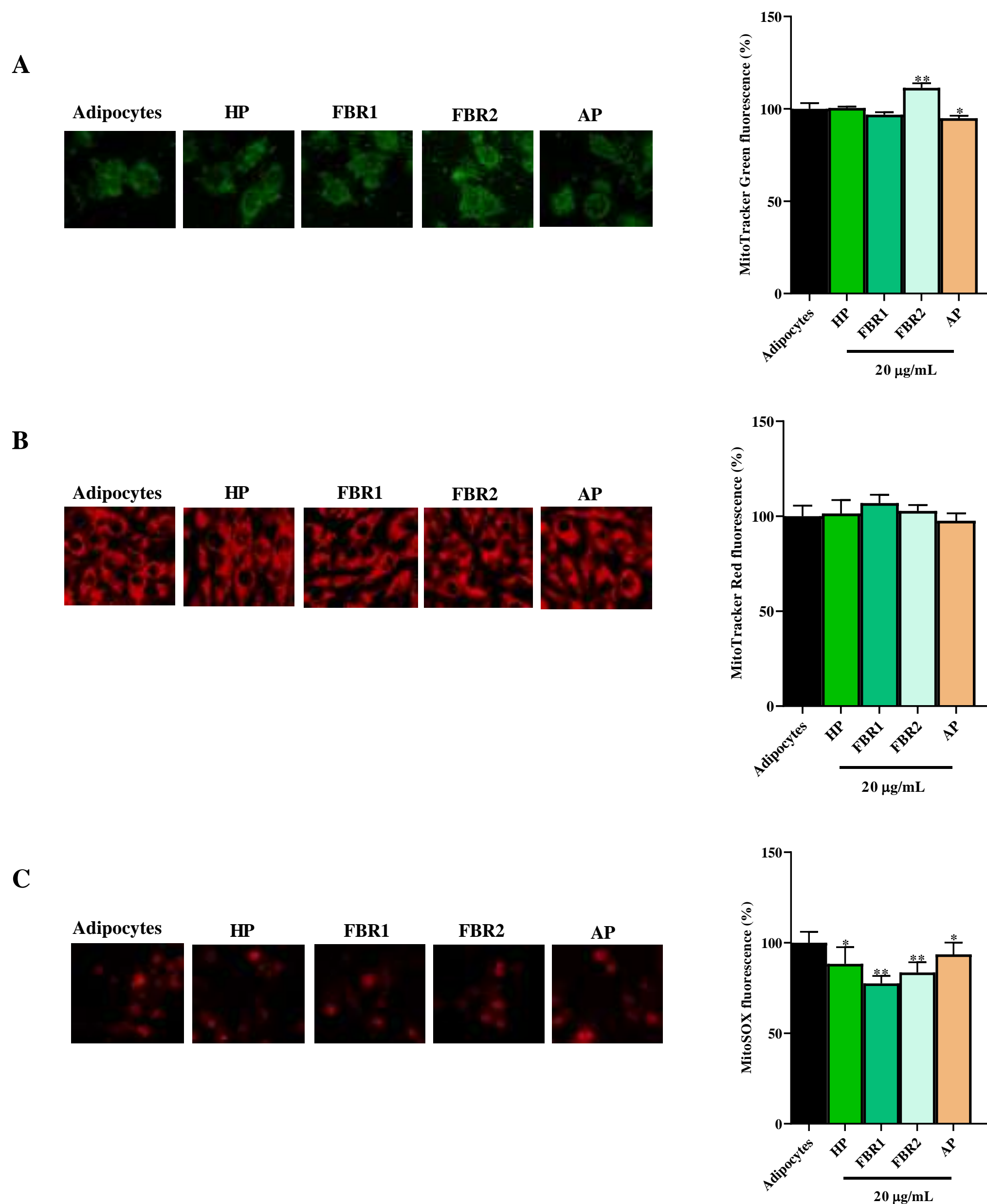


Figure 3.19: Effect of microalgal and AP extracts on mitochondrial parameters. The adipocytes treated with microalgal extracts were stained with MitoTracker Green FM (**A**), MitoTrackerσ Red CMXRos (**B**) and MitoSOX red mitochondrial superoxide indicator (**C**) and displayed under the fluorescence microscope (magnification 20X). Images and related bar graphs in (**A-C**) are representative of three independent experiments with similar results. The mean values ± SD of three independent experiments with four replicates in each are shown. Where indicated differences were significant according to *one-way ANOVA* followed by Dunnett's multiple comparison test (** p<0,01, * p<0,05).

3.3.4.7 Treatment with microalgal extracts regulates the expression of adipogenic markers

We then investigated the microalgal extracts effect on the expression of adipogenic markers in 3T3-L1 adipocytes.

As shown in ***Figure 3.20***, the differentiation of preadipocytes into adipocytes induced an increase in the expression of adipogenic protein PPARγ. Treatment with microalgal extracts, in particular FBR1 and FBR2, has been associated with a lower expression of PPARγ.

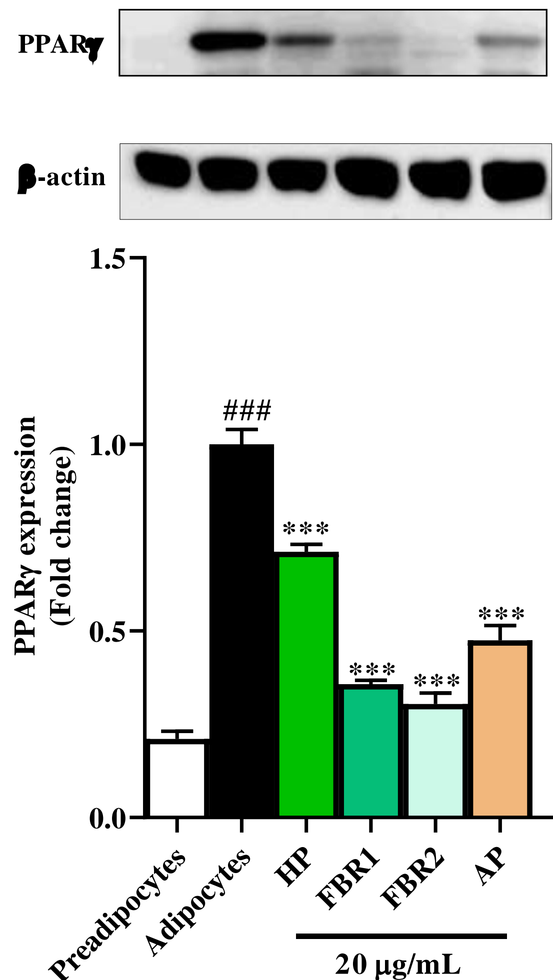


Figure 3.20: Effect of microalgal and AP extracts on adipogenic protein PPARγ gene expression. Western blots of adipogenic protein PPARγ expression after microalgal extracts treatments. The specific bands were quantified and are presented as graphs and normalized against

β-actin. Bar chart reports the mean values ± SD of three independent experiments, each in triplicate.Protein expression levels in the control sample were taken as 1. According to *one-way ANOVA*, differences were significant ($p<0.05$). where the indicated differences between samples and their controls are significant (***$p<0.001$, Dunnett's multiple comparison test, # *vs*. preadipocytes, * *vs*. adipocytes).

As shown in ***Figure 3.21***, the differentiation of preadipocytes into adipocytes induced an increase in the expression of the adipogenic protein ACLY. Treatment with HP and FBR1, was associated with a higher expression of ACLY, while treatment with FBR2 and AP with a lower expression.

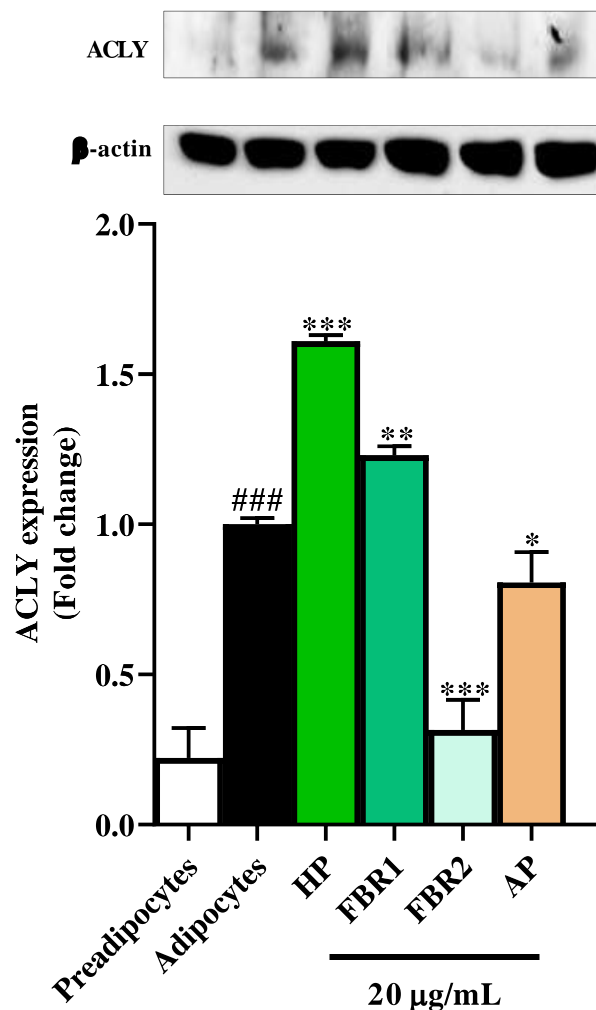


Figure 3.21: Effect of microalgal and AP extracts on adipogenic protein ACLY gene expression. Western blots of adipogenic protein ACLY expression after microalgal extracts treatments. The specific bands were quantified and are presented as graphs and normalized against β-actin. Bar

chart reports the mean values ± SD of three independent experiments, each in triplicate.Protein expression levels in the control sample were taken as 1. According to *one-way ANOVA*, differences were significant (p<0.05). where the indicated differences between samples and their controls are significant (***p<0.001, **p < 0.01, *p<0.05, Dunnett's multiple comparison test, # *vs*. preadipocytes, * *vs.* adipocytes).

3.3.4.8 Microalgal extracts reduce levels of pro-inflammatory cytokines IL-6 and TNFα in adipocytes

In ***Figure 3.22 A*** and ***B***, there is a significant reduction in the levels of both pro-inflammatory cytokines IL-6 and TNFα following treatment of adipocytes with microalgal extracts. FBR2 is, in both cases, the most active extract.

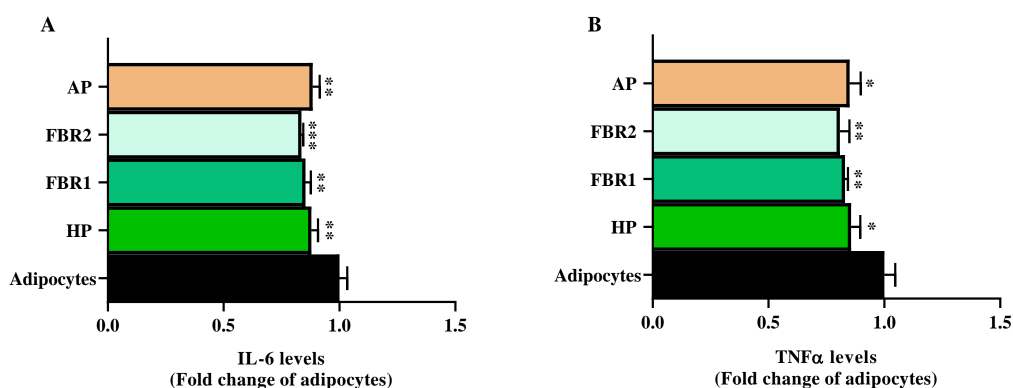


Figure 3.22: Effect of microalgal and AP extracts on IL-6 and TNFα levels. Adipocytes were treated with HP, FBR1, FBR2 and AP. Following 24 hours, IL-6 (**A**) and TNFα (**B**) levels were evaluated and expressed as percentage of adipocytes (set at 1). Mean values ± SD of three replicate independent experiments with five replicates in each are shown. According to *one-way ANOVA*, differences were significant (p<0.05). Dunnett's multiple comparison test was run as post-hoc test to compare treatment groups with control group; where indicate, differences were statistically significant (*** p<0.001, ** p<0.01, * p<0.05).

4. DISCUSSION

Nowadays microalgae are gaining more and more popularity thanks to their high nutrient content. Microalgae, as a source of bioactive molecules, are used in the pharmaceutical and cosmetic industry [105], [106]. The research continuously confirms the beneficial effects of the components of microalgae and their biological properties. Among these components, polysaccharides such as starch, cellulose and β-1,3 glucan are used in the pharmaceutical and cosmetic industries, as well as in food supplements [105]. The same applies to lipids such as hydrocarbons and polyunsaturated fatty acids. Many of the bioactive compounds displaying strong antibacterial, antifungal and antiviral properties have found application in the production of vaccines, antibiotics, agrochemicals and cosmetics [107]. Enzymes, amino acids and polypeptides are used in the food and pharmaceutical industry, while pigments, including carotenoids or chlorophylls, are widely used in food technology, chemical and pharmaceutical industry. In light of these data, we decided to focus on characterizing enzymes and compound from microalgae to meet the growing demand for industrial enzymes. During the first year we selected a strain of *Haematococcus pluvialis* and two of its mutants, FBR1 and FBR2. We have therefore studied and identified the best conditions for growth. *Haematococcus pluvialis* has a very varied and complete biochemical composition. It is characterized by a high protein content of 29% - 45% per dry weight (dw). In the green phase, the total lipid content varies from 20% to 25%, with about 10% of lipids composed mainly of polyunsaturated fatty acids (PUFAs) deposited in chloroplasts [89]. It should also not be forgotten that *H.pluvialis* is the best source of one of the most powerful natural antioxidants, which the astaxanthin [87]. Among C*yanobacteria,* spirulina (*Arthrosphira platensis)* also shows an interesting nutritional composition, including macronutrients such as carbohydrates, lipids, proteins, vitamins and minerals, essential for basic human nutrition. Moreover, in addition to macro and micronutrients, spirulina possesses other compounds with biological activities, including unsaturated fatty acids, amino acids, carotenoids and phenolic compounds, responsible for different biological activities [108].

Qualitative and quantitative analyses of the enzyme profile, carried out by an

external laboratory, revealed no significant differences between the different strains used. Subsequently, we decided to focus on metaboloma, that is, the study and profiling of metabolites in a cell. In this regard, the analyses have shown that the mutant FBR2 displays the highest content of total fats and phenols compared to the other strains (***Table 3.2***). The results of the analysis of fatty acid composition showed the presence of PUFA with the best ratio of omega-3 and omega-6 in FBR2 (***Table 3.3***). After evaluating the biochemical composition of the strains in the study and evaluating the possible applications of the metabolites, we decided to test their application in the nutraceutical field. In particular we decided to evaluate the anti-inflammatory and anti-adipogenic activity *in vitro*.

Obesity is a complex multifactorial disorder and a potential risk factor for the development of diabetes mellitus, cardiovascular disease, hypertension, dyslipidemia, chronic inflammation and certain types of cancer. The prolonged imbalance in energy homeostasis is the basis of the onset of overweight and obesity. An increase in body weight is closely related to an increase in the mass of white adipose tissue [109], [110]. Adipose tissue, today, is recognized as a vital endocrine organ because it not only stores excess triglycerides, but also produces a variety of adipochins (leptin, adiponectin, resistin, etc.) and activates cytokines that affect the regulation of energy homeostasis, insulin resistance, inflammation, immunity and metabolic disorders [111]–[113]. Adipogenesis is the process during which fat accumulation occurs in the visceral, subcutaneous, peritoneal and abdominal regions leading to obesity [114]. Adipose tissue contains several types of cells, including endothelial cells, blood cells, fibroblasts, preadipocytes, macrophages, other immune cells and mature adipocytes [115]. When there is an increase in the number of adipocytes due to excessive accumulation of triglycerides, become the main cell type and thus form adipose tissue [116]. The cellular process of adipogenesis involves the expression of key genes at the transcriptional level such as PPAR and the induction of lipogenic genes such as SREBP, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) [117]. There are also other signaling pathways such as AKT, AMPK and MAPK that can regulate energy homeostasis and adiposity [118]–[120]. Molecules or drugs that regulate the expression of key genes/proteins involved in adipogenesis and

lipogenesis pathways can be used as targets for the treatment of obesity disorders [121]. An important role could also be played by ATP citrate lyase, an enzyme between lipid and carbohydrate metabolism. Several infectious conditions also increase pro-inflammatory cytokines such as IL-6 and TNFα which in turn activate other cytokines and cause inflammation [122]. Obesity also induces low-grade chronic inflammation resulting in the secretion and altered expression of inflammatory cytokines, adipochins, eicosanoids, etc. which alter the process of energy homeostasis and cause obesity-related comorbidities [123]. Therefore, therapies capable of modulating the inflammatory state of adipose tissue and inhibiting adipogenesis are highly considered for the treatment of obesity. Since natural product-based therapies are considered safe with no or minimal side effects compared to synthetic drugs, there is increasing interest among researchers and the public in this regard.

Experiments to assess the anti-inflammatory properties of microalgal extracts were performed on human differentiated U937 macrophage cells activated with LPS. The extracts reduced the secretion of the pro-inflammatory cytokines IL-6 and TNFα and together with the production of ROS and NO• (***Figures 3.9, 3.10, 3.11A-B***). Especially the mutant FBR2 has revealed the best anti-inflammatory ability. These data confirm the anti-inflammatory benefits of microalgae already reported in the literature. As for *H.pluvialis*, anti-inflammatory activity is generally associated with the presence of astaxanthin [124], [125]. Numberless studies have reported that bioactive compounds can improve obesity by mechanisms including downregulation of adipogenesis and upregulation of thermogenesis [126], [127]. In this study, we demonstrated that FBR1 and FBR2 reduce the accumulation of lipid droplets by inhibiting expression of PPARγ adipogenic marker during 3T3-L1 differentiation (***Figures 3.20***). In addition, the expression of ATP citrate lyase, an acyltransferase important for the biosynthesis of fatty acids [130], is also reduced after treatment with FBR2 (***Figure 3.21***).

Lipolysis is a critical function of white adipose tissue [131]. Lipolysis is regulated by nutritional factors together with hormonal and biochemical signals [132]. Disregulation of lipolysis is associated with obesity, diabetes, and metabolic syndromes. To evaluate the effect of extracts on lipolysis we used a colorimetric

kit containing a synthetic catecholamine (isoproterenol) that activates *β*-adrenergic receptors. This activates adenylate cyclase, converting ATP to cAMP. cAMP then activates the hydrolysis of triglycerides by hormone-sensitive lipase. Lipolysis is determined by measuring a colorimetric product with absorbance at 570 nm proportional to the amount of glycerol present. Lipolysis was promoted by both FBR1 and FBR2 strains (***Figure 3.18***).

At the mitochondrial level the extracts did not induce dysfunction as revealed by specific fluorescent stainings (***Figure 3.19***). In addition, pro-inflammatory cytokine levels IL-6 and TNFα were reduced after treatment with extracts (***Figure 3.22 A-B***).

Briefly, in this doctoral thesis, the potential contribution of microalgal extracts in modulating the inflammatory and adipogenic response has been investigated. In particular, FBR2 extract has been shown to be the most active in counteracting the inflammatory and adipogenic response.

5.CONCLUSIONS

The microalgal extracts studied showed promising anti-inflammatory and anti-adipogenic properties. Especially FBR2 extract reduced secretion of IL-6 and TNFα together with ROS and NO• levels. It also suppressed lipid accumulation by reducing the expression of key adipogenic proteins, such as PPARγ and ACLY in 3T3-L1 adipocytes. Moreover, FBR2 reduced secretion of pro-inflammatory cytokines IL-6 and TNFα in addition to induce more lipolysis. These effects are most likely due to the increased presence of fatty acids with the best ratio of omega 3 and 6 polyunsaturated fatty acids, which have a number of biological properties such as anti-inflammatory. Due to the anti-inflammatory effect, supplementation with FBR2 could be a promising approach for the treatment of overweight and obesity, metabolic disorders characterized by an increasing prevalence in the juvenile population.

REFERENCES

[1] Guaadaoui, Abdelkarim, and et al., “What is a bioactive compound? A combined definition for a preliminary consensus,” *Int. J. Nutr. Food Sci.*, vol. 3.3, pp. 174–179, 2014.

[2] S. González, “Dietary Bioactive Compounds and Human Health and Disease,” *Nutrients*, vol. 12, no. 2, p. 348, Jan. 2020, doi: 10.3390/nu12020348.

[3] M. Indira, T. C. Venkateswarulu, K. Abraham Peele, M. Nazneen Bobby, and S. Krupanidhi, “Bioactive molecules of probiotic bacteria and their mechanism of action: a review,” *3 Biotech*, vol. 9, no. 8, p. 306, Aug. 2019, doi: 10.1007/s13205-019-1841-2.

[4] E. Ivanišová, M. Kačániová, T. A. Savitskaya, and D. D. Grinshpan, “Medicinal Herbs: Important Source of Bioactive Compounds for Food Industry,” in *Herbs and Spices - New Processing Technologies*, IntechOpen, 2021. doi: 10.5772/intechopen.98819.

[5] R. Shakoor, N. Hussain, S. Younas, and M. Bilal, “Novel strategies for extraction, purification, processing, and stability improvement of bioactive molecules,” *J. Basic Microbiol.*, Oct. 2022, doi: 10.1002/jobm.202200401.

[6] A. K. Jha and N. Sit, “Extraction of bioactive compounds from plant materials using combination of various novel methods: A review,” *Trends Food Sci. Technol.*, vol. 119, pp. 579–591, Jan. 2022, doi: 10.1016/j.tifs.2021.11.019.

[7] G. Britton, S. Liaaen-Jensen, and H. (Eds. . Pfander, “Carotenoids: handbook.,” *Springer Sci. Bus. Media*, 2004.

[8] T. Maoka, “Recent progress in structural studies of carotenoids in animals and plants,” *Arch. Biochem. Biophys.*, vol. 483, no. 2, pp. 191–195, Mar. 2009, doi: 10.1016/j.abb.2008.10.019.

[9] N. I. Krinsky and E. J. Johnson, “Carotenoid actions and their relation to health and disease,” *Mol. Aspects Med.*, vol. 26, no. 6, pp. 459–516, Dec.

2005, doi: 10.1016/j.mam.2005.10.001.

[10] R. D. Semba, “The Discovery of the Vitamins,” *Int. J. Vitam. Nutr. Res.*, vol. 82, no. 5, pp. 310–315, Oct. 2012, doi: 10.1024/0300-9831/a000124.

[11] J. Chawla and D. Kvarnberg, “Hydrosoluble vitamins,” 2014, pp. 891–914. doi: 10.1016/B978-0-7020-4087-0.00059-0.

[12] R. A. Youness, A. Dawoud, O. ElTahtawy, and M. A. Farag, “Fat-soluble vitamins: updated review of their role and orchestration in human nutrition throughout life cycle with sex differences,” *Nutr. Metab. (Lond).*, vol. 19, no. 1, p. 60, Sep. 2022, doi: 10.1186/s12986-022-00696-y.

[13] I. O. Minatel, C. V. Borges, M. I. Ferreira, H. A. G. Gomez, C.-Y. O. Chen, and G. P. P. Lima, “Phenolic Compounds: Functional Properties, Impact of Processing and Bioavailability,” in *Phenolic Compounds - Biological Activity*, InTech, 2017. doi: 10.5772/66368.

[14] B. R. Albuquerque, S. A. Heleno, M. B. P. P. Oliveira, L. Barros, and I. C. F. R. Ferreira, “Phenolic compounds: current industrial applications, limitations and future challenges,” *Food Funct.*, vol. 12, no. 1, pp. 14–29, 2021, doi: 10.1039/D0FO02324H.

[15] E. Fahy, D. Cotter, M. Sud, and S. Subramaniam, “Lipid classification, structures and tools,” *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids*, vol. 1811, no. 11, pp. 637–647, Nov. 2011, doi: 10.1016/j.bbalip.2011.06.009.

[16] B. Alabdulkarim, Z. A. N. Bakeet, and S. Arzoo, “Role of some functional lipids in preventing diseases and promoting health,” *J. King Saud Univ. - Sci.*, vol. 24, no. 4, pp. 319–329, Oct. 2012, doi: 10.1016/j.jksus.2012.03.001.

[17] Watanabe Kiyohiro, Etsuko Yasugi, and Mieko Oshima, “‘How to search the glycolipid data in “LIPIDBANK for Web”, the newly developed lipid database in Japan.,’” *Trends Glycosci. Glycotechnol.*, vol. 12.65, pp. 175-184., 2000.

[18] E. Fahy *et al.*, “Update of the LIPID MAPS comprehensive classification system for lipids,” *J. Lipid Res.*, vol. 50, pp. S9–S14, Apr. 2009, doi: 10.1194/jlr.R800095-JLR200.

[19] A. Leuti, D. Fazio, M. Fava, A. Piccoli, S. Oddi, and M. Maccarrone, "Bioactive lipids, inflammation and chronic diseases," *Adv. Drug Deliv. Rev.*, vol. 159, pp. 133–169, 2020, doi: 10.1016/j.addr.2020.06.028.

[20] A. K. Duttaroy, "Polysaccharides and their bioactivity and biomedical applications," in *Evidence-Based Nutrition and Clinical Evidence of Bioactive Foods in Human Health and Disease*, Elsevier, 2021, pp. 139–154. doi: 10.1016/B978-0-12-822405-2.00010-4.

[21] A. Smith, "Oxford dictionary of biochemistry and molecular biology: Revised Edition. ," *Oxford Univ. Press*, 2000.

[22] W. Kühne, "Über das Verhalten verschiedener organisirter und sog. ungeformter Fermente. Verhandlungen des naturhistorisch-medicinischen Vereins zu Heidelberg. ," *New Ser 1*, pp. 190-193., 1877.

[23] A. . and J. F. P. Payen, "Annales de Chimie et de Physique," vol. 53, 1833.

[24] Fischer E., " Einfluss der configuration auf die wirkung der enzima.," *Berichte der Dtsch. Chem. Gesellschaft* , pp. 2985–2993, 1894.

[25] Liubov Poshyvailo, "Modelling and simulations of enzyme-catalyzed reactions," *DOI 10.13140/RG.2.2.30420.63369*, Jun. 2015.

[26] "The Catalytic Site Atlast presso The European Bioinformatics Institute," 2007.

[27] "Webb EC. Enzyme nomenclature 1992: reccomemdations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology on the nomenclature and classification of enzymes.," *Acad. Press*, 1992.

[28] P. K. Robinson, "Enzymes: principles and biotechnological applications.," *Essays Biochem.*, vol. 59, pp. 1–41, 2015, doi: 10.1042/bse0590001.

[29] W. Levasseur, P. Perré, and V. Pozzobon, "A review of high value-added molecules production by microalgae in light of the classification.," *Biotechnol. Adv.*, vol. 41, p. 107545, doi: 10.1016/j.biotechadv.2020.107545.

[30] J. Sen Tan *et al.*, "A review on microalgae cultivation and harvesting, and

their biomass extraction processing using ionic liquids.," *Bioengineered*, vol. 11, no. 1, pp. 116–129, 2020, doi: 10.1080/21655979.2020.1711626.

[31] J. J. Milledge, "Commercial application of microalgae other than as biofuels: a brief review," *Rev. Environ. Sci. Bio/Technology*, vol. 10, no. 1, pp. 31–41, Mar. 2011, doi: 10.1007/s11157-010-9214-7.

[32] G. Perin, A. Bellan, A. Bernardi, F. Bezzo, and T. Morosinotto, "The potential of quantitative models to improve microalgae photosynthetic efficiency," *Physiol. Plant.*, vol. 166, no. 1, pp. 380–391, May 2019, doi: 10.1111/ppl.12915.

[33] R. E. Blankenship *et al.*, "Comparing Photosynthetic and Photovoltaic Efficiencies and Recognizing the Potential for Improvement," *Science (80-.).*, vol. 332, no. 6031, pp. 805–809, May 2011, doi: 10.1126/science.1200165.

[34] A. Richmond and Q. Hu, "Handbook of microalgal culture: applied phycology and biotechnology," no. ISBN 978-1-118-56716-6., 2013.

[35] A. Hallmann, "Algae Biotechnology – Green Cell-Factories on the Rise," *Curr. Biotechnol.*, vol. 4, no. 4, pp. 389–415, Jan. 2016, doi: 10.2174/2211550105666151107001338.

[36] M. A. Borowitzka, "Biology of Microalgae. In Microalgae in Health and Disease Prevention ," *Acad. Press*, no. ISBN 9780128114056., pp. 23–72, 2018.

[37] J. A. Raven and M. Giordano, "Algae.," *Curr. Biol.*, vol. 24, no. 13, pp. R590-5, Jul. 2014, doi: 10.1016/j.cub.2014.05.039.

[38] S. Groendahl, M. Kahlert, and P. Fink, "The best of both worlds: A combined approach for analyzing microalgal diversity via metabarcoding and morphology-based methods," *PLoS One*, vol. 12, no. 2, p. e0172808, Feb. 2017, doi: 10.1371/journal.pone.0172808.

[39] I. Hamed, "The Evolution and Versatility of Microalgal Biotechnology: A Review. ," *Compr. Rev. Food Sci. Food Saf*, vol. 15, no. doi:10.1111/1541-4337.12227/FORMAT/PDF., pp. 1104–1123, 2016.

[40] H. Qamar, K. Hussain, A. Soni, A. Khan, T. Hussain, and B. Chénais, "Cyanobacteria as Natural Therapeutics and Pharmaceutical Potential: Role in Antitumor Activity and as Nanovectors.," *Molecules*, vol. 26, no. 1, Jan. 2021, doi: 10.3390/molecules26010247.

[41] D. Kumar, P. Kastanek, and S. P. Adhikary, "Exopolysaccharides from Cyanobacteria and Microalgae and Their Commercial Application," *Curr. Sci.*, vol. 115, no. 2, p. 234, Jul. 2018, doi: 10.18520/cs/v115/i2/234-241.

[42] M. Iino and H. Hashimoto, "INTERMEDIATE FEATURES OF CYANELLE DIVISION OF CYANOPHORA PARADOXA (GLAUCOCYSTOPHYTA) BETWEEN CYANOBACTERIAL AND PLASTID DIVISION1," *J. Phycol.*, vol. 39, no. 3, pp. 561–569, Jun. 2003, doi: 10.1046/j.1529-8817.2003.02132.x.

[43] J. P. Sexton and M. W. Lomas, "Microalgal Systematics," in *Microalgae in Health and Disease Prevention*, Elsevier, 2018, pp. 73–107. doi: 10.1016/B978-0-12-811405-6.00004-9.

[44] V. Dolganyuk *et al.*, "Microalgae: A Promising Source of Valuable Bioproducts," *Biomolecules*, vol. 10, no. 8, p. 1153, Aug. 2020, doi: 10.3390/biom10081153.

[45] J. Sen Tan *et al.*, "A review on microalgae cultivation and harvesting, and their biomass extraction processing using ionic liquids," *Bioengineered*, vol. 11, no. 1, pp. 116–129, Jan. 2020, doi: 10.1080/21655979.2020.1711626.

[46] J. Jerney and K. Spilling, "Large Scale Cultivation of Microalgae: Open and Closed Systems," 2018, pp. 1–8. doi: 10.1007/7651_2018_130.

[47] V. Balasubramaniam, R. D.-N. Gunasegavan, S. Mustar, J. C. Lee, and M. F. Mohd Noh, "Isolation of Industrial Important Bioactive Compounds from Microalgae," *Molecules*, vol. 26, no. 4, p. 943, Feb. 2021, doi: 10.3390/molecules26040943.

[48] X. Zhang, X. Tang, B. Zhou, S. Hu, and Y. Wang, "Effect of enhanced UV-B radiation on photosynthetic characteristics of marine microalgae Dunaliella salina (Chlorophyta, Chlorophyceae)," *J. Exp. Mar. Bio. Ecol.*,

vol. 469, pp. 27–35, Aug. 2015, doi: 10.1016/j.jembe.2015.04.002.

[49] L. Zhu, "Microalgal culture strategies for biofuel production: a review," *Biofuels, Bioprod. Biorefining*, vol. 9, no. 6, pp. 801–814, Nov. 2015, doi: 10.1002/bbb.1576.

[50] M. V. Busi, J. Barchiesi, M. Martín, and D. F. Gomez-Casati, "Starch metabolism in green algae," *Starch - Stärke*, vol. 66, no. 1–2, pp. 28–40, Jan. 2014, doi: 10.1002/star.201200211.

[51] M. Raposo, R. De Morais, and A. Bernardo de Morais, "Bioactivity and Applications of Sulphated Polysaccharides from Marine Microalgae," *Mar. Drugs*, vol. 11, no. 1, pp. 233–252, Jan. 2013, doi: 10.3390/md11010233.

[52] C. E. C. C. Ejike, S. A. Collins, N. Balasuriya, A. K. Swanson, B. Mason, and C. C. Udenigwe, "Prospects of microalgae proteins in producing peptide-based functional foods for promoting cardiovascular health," *Trends Food Sci. Technol.*, vol. 59, pp. 30–36, Jan. 2017, doi: 10.1016/j.tifs.2016.10.026.

[53] E. W. Becker, "Micro-algae as a source of protein," *Biotechnol. Adv.*, vol. 25, no. 2, pp. 207–210, Mar. 2007, doi: 10.1016/j.biotechadv.2006.11.002.

[54] S. Bellou, M. N. Baeshen, A. M. Elazzazy, D. Aggeli, F. Sayegh, and G. Aggelis, "Microalgal lipids biochemistry and biotechnological perspectives," *Biotechnol. Adv.*, vol. 32, no. 8, pp. 1476–1493, Dec. 2014, doi: 10.1016/j.biotechadv.2014.10.003.

[55] A. Villarruel-López, F. Ascencio, and K. Nuño, "Microalgae, a Potential Natural Functional Food Source – a Review," *Polish J. Food Nutr. Sci.*, vol. 67, no. 4, pp. 251–263, Dec. 2017, doi: 10.1515/pjfns-2017-0017.

[56] M. Koller, A. Muhr, and G. Braunegg, "Microalgae as versatile cellular factories for valued products," *Algal Res.*, vol. 6, pp. 52–63, Oct. 2014, doi: 10.1016/j.algal.2014.09.002.

[57] "Meticulous Research Algae Products Market by Value (Medium Value, High Value, And Low Value), Products (Hydrocolloids, Carotenoids, Omega-3 PUFA, Spirulina, Chlorella), Application (Food and Feed, Nutraceutical, Cosmetics, Chemicals)—Forecast. [(accessed on November

2022)]; Available online: https://www.meticulousresearch.com/product/algae-products-market-forecast-2022”.

[58] I. Deniz, M. O. Ozen, and O. Yesil-Celiktas, “Supercritical fluid extraction of phycocyanin and investigation of cytotoxicity on human lung cancer cells,” *J. Supercrit. Fluids*, vol. 108, pp. 13–18, Feb. 2016, doi: 10.1016/j.supflu.2015.10.015.

[59] E. Talero, S. García-Mauriño, J. Ávila-Román, A. Rodríguez-Luna, A. Alcaide, and V. Motilva, “Bioactive Compounds Isolated from Microalgae in Chronic Inflammation and Cancer,” *Mar. Drugs*, vol. 13, no. 10, pp. 6152–6209, Sep. 2015, doi: 10.3390/md13106152.

[60] J. Rumin, E. Nicolau, R. Gonçalves de Oliveira Junior, C. Fuentes-Grünewald, and L. Picot, “Analysis of Scientific Research Driving Microalgae Market Opportunities in Europe,” *Mar. Drugs*, vol. 18, no. 5, p. 264, May 2020, doi: 10.3390/md18050264.

[61] P. J. Harvey and A. Ben-Amotz, “Towards a sustainable Dunaliella salina microalgal biorefinery for 9-cis β-carotene production,” *Algal Res.*, vol. 50, p. 102002, Sep. 2020, doi: 10.1016/j.algal.2020.102002.

[62] K. Martínez Andrade, C. Lauritano, G. Romano, and A. Ianora, “Marine Microalgae with Anti-Cancer Properties,” *Mar. Drugs*, vol. 16, no. 5, p. 165, May 2018, doi: 10.3390/md16050165.

[63] G. Prabakaran, P. Sampathkumar, M. Kavisri, and M. Moovendhan, “Extraction and characterization of phycocyanin from Spirulina platensis and evaluation of its anticancer, antidiabetic and antiinflammatory effect,” *Int. J. Biol. Macromol.*, vol. 153, pp. 256–263, Jun. 2020, doi: 10.1016/j.ijbiomac.2020.03.009.

[64] Y. Liu, L. Xu, N. Cheng, L. Lin, and C. Zhang, “Inhibitory effect of phycocyanin from Spirulina platensis on the growth of human leukemia K562 cells.,” *J. Appl. Phycol.*, vol. 12, no. 2, pp. 125–130, 2000, doi: 10.1023/A:1008132210772.

[65] S. Hao *et al.*, “The In Vitro Anti-Tumor Activity of Phycocyanin against

Non-Small Cell Lung Cancer Cells," *Mar. Drugs*, vol. 16, no. 6, p. 178, May 2018, doi: 10.3390/md16060178.

[66] M. Gantar, S. Dhandayuthapani, and A. Rathinavelu, "Phycocyanin Induces Apoptosis and Enhances the Effect of Topotecan on Prostate Cell Line LNCaP," *J. Med. Food*, vol. 15, no. 12, pp. 1091–1095, Dec. 2012, doi: 10.1089/jmf.2012.0123.

[67] M. Safaei *et al.*, "Development of a novel method for the purification of C-phycocyanin pigment from a local cyanobacterial strain Limnothrix sp. NS01 and evaluation of its anticancer properties," *Sci. Rep.*, vol. 9, no. 1, p. 9474, Dec. 2019, doi: 10.1038/s41598-019-45905-6.

[68] M. Raposo, A. de Morais, and R. de Morais, "Carotenoids from Marine Microalgae: A Valuable Natural Source for the Prevention of Chronic Diseases," *Mar. Drugs*, vol. 13, no. 8, pp. 5128–5155, Aug. 2015, doi: 10.3390/md13085128.

[69] B. Capelli and G. R. Cysewski, "Potential health benefits of spirulina microalgae*," *Nutrafoods*, vol. 9, no. 2, pp. 19–26, Apr. 2010, doi: 10.1007/BF03223332.

[70] A. Niccolai *et al.*, "Development of new microalgae-based sourdough 'crostini': functional effects of Arthrospira platensis (spirulina) addition," *Sci. Rep.*, vol. 9, no. 1, p. 19433, Dec. 2019, doi: 10.1038/s41598-019-55840-1.

[71] S. Grahl, M. Strack, R. Weinrich, and D. Mörlein, "Consumer-Oriented Product Development: The Conceptualization of Novel Food Products Based on Spirulina (*Arthrospira platensis*) and Resulting Consumer Expectations," *J. Food Qual.*, vol. 2018, pp. 1–11, Jul. 2018, doi: 10.1155/2018/1919482.

[72] A. Tomdio, M. Ritchie, and A. C. Miller, "Omega-3 Fatty Acids and Cardiovascular Disease Prevention.," *Am. Fam. Physician*, vol. 100, no. 4, pp. 209–210, Aug. 2019.

[73] M. A. Alam, Jing-Liang Xu, and eds. Zhongming Wang, "Microalgae biotechnology for food, health and high value products.," *Springer*, 2020.

[74] H. M. . Iqbal, “b b High-value compounds from microalgae with industrial exploitability ndash A review b b,” *Front. Biosci.*, vol. 9, no. 3, p. 490, 2017, doi: 10.2741/s490.

[75] E. Jacob-Lopes, Maria Isabel Queiroz, and eds. Leila Queiroz Zepka, “Pigments from microalgae handbook. ,” *Springer*, 2020.

[76] I. GILL and R. VALIVETY, “Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications,” *Trends Biotechnol.*, vol. 15, no. 10, pp. 401–409, Oct. 1997, doi: 10.1016/S0167-7799(97)01076-7.

[77] H. R. Bokesch *et al.*, “A Potent Novel Anti-HIV Protein from the Cultured Cyanobacterium *Scytonema varium*,” *Biochemistry*, vol. 42, no. 9, pp. 2578–2584, Mar. 2003, doi: 10.1021/bi0205698.

[78] F. M. Pratiwy and Dian Yuni Pratiwi, “‘The potentiality of microalgae as a source of DHA and EPA for aquaculture feed: A review.’ ,” *Int. J. Fish. Aquat. Stud.* , vol. 8.4, pp. 39–41, 2020.

[79] M. L. Amorim, J. Soares, J. S. dos R. Coimbra, M. de O. Leite, L. F. T. Albino, and M. A. Martins, “Microalgae proteins: production, separation, isolation, quantification, and application in food and feed,” *Crit. Rev. Food Sci. Nutr.*, vol. 61, no. 12, pp. 1976–2002, Jul. 2021, doi: 10.1080/10408398.2020.1768046.

[80] M. Vaudel *et al.*, “Exploring the potential of public proteomics data,” *Proteomics*, vol. 16, no. 2, pp. 214–225, Jan. 2016, doi: 10.1002/pmic.201500295.

[81] F. J. Fernández-Acero *et al.*, “Valorisation of the microalgae Nannochloropsis gaditana biomass by proteomic approach in the context of circular economy,” *J. Proteomics*, vol. 193, pp. 239–242, Feb. 2019, doi: 10.1016/j.jprot.2018.10.015.

[82] Y. Gong, H. Hu, Y. Gao, X. Xu, and H. Gao, “Microalgae as platforms for production of recombinant proteins and valuable compounds: progress and prospects,” *J. Ind. Microbiol. Biotechnol.*, vol. 38, no. 12, pp. 1879–1890, Dec. 2011, doi: 10.1007/s10295-011-1032-6.

[83] S. Shamriz and H. Ofoghi, “Expression of Recombinant PfCelTOS Antigen

in the Chloroplast of Chlamydomonas reinhardtii and its Potential Use in Detection of Malaria," *Mol. Biotechnol.*, vol. 61, no. 2, pp. 102–110, Feb. 2019, doi: 10.1007/s12033-018-0140-1.

[84] L.-H. Zheng *et al.*, "Antitumor Peptides from Marine Organisms," *Mar. Drugs*, vol. 9, no. 10, pp. 1840–1859, Oct. 2011, doi: 10.3390/md9101840.

[85] X. Wang and X. Zhang, "Separation, antitumor activities, and encapsulation of polypeptide from *Chlorella pyrenoidosa*," *Biotechnol. Prog.*, vol. 29, no. 3, pp. 681–687, May 2013, doi: 10.1002/btpr.1725.

[86] L. A. Tejano, J. P. Peralta, E. E. S. Yap, F. C. A. Panjaitan, and Y.-W. Chang, "Prediction of Bioactive Peptides from Chlorella sorokiniana Proteins Using Proteomic Techniques in Combination with Bioinformatics Analyses," *Int. J. Mol. Sci.*, vol. 20, no. 7, p. 1786, Apr. 2019, doi: 10.3390/ijms20071786.

[87] M. M. R. Shah, Y. Liang, J. J. Cheng, and M. Daroch, "Astaxanthin-Producing Green Microalga Haematococcus pluvialis: From Single Cell to High Value Commercial Products," *Front. Plant Sci.*, vol. 7, Apr. 2016, doi: 10.3389/fpls.2016.00531.

[88] R. G. Fassett and J. S. Coombes, "Astaxanthin: A Potential Therapeutic Agent in Cardiovascular Disease," *Mar. Drugs*, vol. 9, no. 3, pp. 447–465, Mar. 2011, doi: 10.3390/md9030447.

[89] M. Mularczyk, I. Michalak, and K. Marycz, "Astaxanthin and other Nutrients from Haematococcus pluvialis-Multifunctional Applications.," *Mar. Drugs*, vol. 18, no. 9, Sep. 2020, doi: 10.3390/md18090459.

[90] M. Wayama, S. Ota, H. Matsuura, N. Nango, A. Hirata, and S. Kawano, "Three-Dimensional Ultrastructural Study of Oil and Astaxanthin Accumulation during Encystment in the Green Alga Haematococcus pluvialis," *PLoS One*, vol. 8, no. 1, p. e53618, Jan. 2013, doi: 10.1371/journal.pone.0053618.

[91] T. Butler, G. McDougall, R. Campbell, M. Stanley, and J. Day, "Media Screening for Obtaining Haematococcus pluvialis Red Motile Macrozooids Rich in Astaxanthin and Fatty Acids," *Biology (Basel).*, vol. 7, no. 1, p. 2,

Dec. 2017, doi: 10.3390/biology7010002.

[92] S. Boussiba and A. Vonshak, “Astaxanthin Accumulation in the Green Alga Haematococcus pluvialis1,” *Plant Cell Physiol.*, vol. 32, no. 7, pp. 1077–1082, Oct. 1991, doi: 10.1093/oxfordjournals.pcp.a078171.

[93] M. Harker, A. J. Tsavalos, and A. J. Young, “Factors responsible for astaxanthin formation in the Chlorophyte Haematococcus pluvialis,” *Bioresour. Technol.*, vol. 55, no. 3, pp. 207–214, Mar. 1996, doi: 10.1016/0960-8524(95)00002-X.

[94] R. P. Radice *et al.*, “An innovative protocol to select the best growth phase for astaxanthin biosynthesis in H. pluvialis.,” *Biotechnol. Reports*, vol. 31, p. e00655, Sep. 2021, doi: 10.1016/j.btre.2021.e00655.

[95] M. J. Griffiths, C. Garcin, R. P. van Hille, and S. T. L. Harrison, “Interference by pigment in the estimation of microalgal biomass concentration by optical density,” *J. Microbiol. Methods*, vol. 85, no. 2, pp. 119–123, May 2011, doi: 10.1016/j.mimet.2011.02.005.

[96] C. L. Kielkopf, W. Bauer, and I. L. Urbatsch, “Bradford Assay for Determining Protein Concentration,” *Cold Spring Harb. Protoc.*, vol. 2020, no. 4, p. pdb.prot102269, Apr. 2020, doi: 10.1101/pdb.prot102269.

[97] C. Sundström and K. Nilsson, “Establishment and characterization of a human histiocytic lymphoma cell line (U-937),” *Int. J. Cancer*, vol. 17, no. 5, pp. 565–577, May 1976, doi: 10.1002/ijc.2910170504.

[98] George J. Todaro and Howard Green, “Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines,” *J Cell Biol* , vol. 17(2), pp. 299–313, May 1963.

[99] S. Kamiloglu, G. Sari, T. Ozdal, and E. Capanoglu, “Guidelines for cell viability assays,” *Food Front.*, vol. 1, no. 3, pp. 332–349, Sep. 2020, doi: 10.1002/fft2.44.

[100] K. Ongena, C. Das, J. L. Smith, S. Gil, and G. Johnston, “Determining Cell Number During Cell Culture using the Scepter Cell Counter,” *J. Vis. Exp.*, no. 45, Nov. 2010, doi: 10.3791/2204.

[101] M. Marsico *et al.*, “Mitochondria-Mediated Apoptosis of HCC Cells Triggered by Knockdown of Glutamate Dehydrogenase 1: Perspective for Its Inhibition through Quercetin and Permethylated Anigopreissin A,” *Biomedicines*, vol. 9, no. 11, p. 1664, Nov. 2021, doi: 10.3390/biomedicines9111664.

[102] P. Convertini, F. Tramutola, V. Iacobazzi, P. Lupattelli, L. Chiummiento, and V. Infantino, “Permethylated Anigopreissin A inhibits human hepatoma cell proliferation by mitochondria-induced apoptosis,” *Chem. Biol. Interact.*, vol. 237, pp. 1–8, Jul. 2015, doi: 10.1016/j.cbi.2015.05.005.

[103] A. Wojtala, M. Bonora, D. Malinska, P. Pinton, J. Duszynski, and M. R. Wieckowski, “Methods to monitor ROS production by fluorescence microscopy and fluorometry.,” *Methods Enzymol.*, vol. 542, pp. 243–62, 2014, doi: 10.1016/B978-0-12-416618-9.00013-3.

[104] P. Singh, S. Kaur, A. Sharma, G. Kaur, and R. Bhatti, “TNF-α and IL-6 inhibitors: Conjugates of N-substituted indole and aminophenylmorpholin-3-one as anti-inflammatory agents,” *Eur. J. Med. Chem.*, vol. 140, pp. 92–103, Nov. 2017, doi: 10.1016/j.ejmech.2017.09.003.

[105] A. Molino *et al.*, “Microalgae Characterization for Consolidated and New Application in Human Food, Animal Feed and Nutraceuticals,” *Int. J. Environ. Res. Public Health*, vol. 15, no. 11, p. 2436, Nov. 2018, doi: 10.3390/ijerph15112436.

[106] M. B. Ariede, T. M. Candido, A. L. M. Jacome, M. V. R. Velasco, J. C. M. de Carvalho, and A. R. Baby, “Cosmetic attributes of algae - A review,” *Algal Res.*, vol. 25, pp. 483–487, Jul. 2017, doi: 10.1016/j.algal.2017.05.019.

[107] E. A. Specht and S. P. Mayfield, “Algae-based oral recombinant vaccines,” *Front. Microbiol.*, vol. 5, 2014, doi: 10.3389/fmicb.2014.00060.

[108] D. G. Bortolini *et al.*, “Functional properties of bioactive compounds from Spirulina spp.: Current status and future trends,” *Food Chem. Mol. Sci.*, vol. 5, p. 100134, Dec. 2022, doi: 10.1016/j.fochms.2022.100134.

[109] P. S. MacLean, J. A. Higgins, E. D. Giles, V. D. Sherk, and M. R.

Jackman, “The role for adipose tissue in weight regain after weight loss,” *Obes. Rev.*, vol. 16, no. S1, pp. 45–54, Feb. 2015, doi: 10.1111/obr.12255.

[110] I. Romieu *et al.*, “Energy balance and obesity: what are the main drivers?,” *Cancer Causes Control*, vol. 28, no. 3, pp. 247–258, Mar. 2017, doi: 10.1007/s10552-017-0869-z.

[111] M. Coelho, T. Oliveira, and R. Fernandes, “State of the art paper Biochemistry of adipose tissue: an endocrine organ,” *Arch. Med. Sci.*, vol. 2, pp. 191–200, 2013, doi: 10.5114/aoms.2013.33181.

[112] C. R. Balistreri, C. Caruso, and G. Candore, “The Role of Adipose Tissue and Adipokines in Obesity-Related Inflammatory Diseases,” *Mediators Inflamm.*, vol. 2010, pp. 1–19, 2010, doi: 10.1155/2010/802078.

[113] J. Shi, J. Fan, Q. Su, and Z. Yang, “Cytokines and Abnormal Glucose and Lipid Metabolism,” *Front. Endocrinol. (Lausanne).*, vol. 10, Oct. 2019, doi: 10.3389/fendo.2019.00703.

[114] K. R. Silva and L. S. Baptista, “Adipose-derived stromal/stem cells from different adipose depots in obesity development,” *World J. Stem Cells*, vol. 11, no. 3, pp. 147–166, Mar. 2019, doi: 10.4252/wjsc.v11.i3.147.

[115] M. S. Burhans, D. K. Hagman, J. N. Kuzma, K. A. Schmidt, and M. Kratz, “Contribution of Adipose Tissue Inflammation to the Development of Type 2 Diabetes Mellitus,” in *Comprehensive Physiology*, Wiley, 2018, pp. 1–58. doi: 10.1002/cphy.c170040.

[116] A. Armani *et al.*, “Cellular models for understanding adipogenesis, adipose dysfunction, and obesity,” *J. Cell. Biochem.*, vol. 110, no. 3, pp. 564–572, Apr. 2010, doi: 10.1002/jcb.22598.

[117] M. Park, J. Han, and H.-J. Lee, “Anti-Adipogenic Effect of Neferine in 3T3-L1 Cells and Primary White Adipocytes,” *Nutrients*, vol. 12, no. 6, p. 1858, Jun. 2020, doi: 10.3390/nu12061858.

[118] J. Boucher, A. Kleinridders, and C. R. Kahn, “Insulin Receptor Signaling in Normal and Insulin-Resistant States,” *Cold Spring Harb. Perspect. Biol.*, vol. 6, no. 1, pp. a009191–a009191, Jan. 2014, doi: 10.1101/cshperspect.a009191.

[119] M. López, "Hypothalamic AMPK and energy balance," *Eur. J. Clin. Invest.*, vol. 48, no. 9, p. e12996, Sep. 2018, doi: 10.1111/eci.12996.

[120] Z. Song, Y. Wang, F. Zhang, F. Yao, and C. Sun, "Calcium Signaling Pathways: Key Pathways in the Regulation of Obesity," *Int. J. Mol. Sci.*, vol. 20, no. 11, p. 2768, Jun. 2019, doi: 10.3390/ijms20112768.

[121] M. Balaji, M. S. Ganjayi, G. E. N. Hanuma Kumar, B. N. Parim, R. Mopuri, and S. Dasari, "A review on possible therapeutic targets to contain obesity: The role of phytochemicals," *Obes. Res. Clin. Pract.*, vol. 10, no. 4, pp. 363–380, Jul. 2016, doi: 10.1016/j.orcp.2015.12.004.

[122] S. Kany, J. T. Vollrath, and B. Relja, "Cytokines in Inflammatory Disease," *Int. J. Mol. Sci.*, vol. 20, no. 23, p. 6008, Nov. 2019, doi: 10.3390/ijms20236008.

[123] A. Ritter, N.-N. Kreis, F. Louwen, and J. Yuan, "Obesity and COVID-19: Molecular Mechanisms Linking Both Pandemics," *Int. J. Mol. Sci.*, vol. 21, no. 16, p. 5793, Aug. 2020, doi: 10.3390/ijms21165793.

[124] Z. Kohandel, T. Farkhondeh, M. Aschner, A. M. Pourbagher-Shahri, and S. Samarghandian, "Anti-inflammatory action of astaxanthin and its use in the treatment of various diseases," *Biomed. Pharmacother.*, vol. 145, p. 112179, Jan. 2022, doi: 10.1016/j.biopha.2021.112179.

[125] M. X. Chang and F. Xiong, "Astaxanthin and its Effects in Inflammatory Responses and Inflammation-Associated Diseases: Recent Advances and Future Directions," *Molecules*, vol. 25, no. 22, p. 5342, Nov. 2020, doi: 10.3390/molecules25225342.

[126] A. M. Cypess and C. R. Kahn, "Brown fat as a therapy for obesity and diabetes," *Curr. Opin. Endocrinol. Diabetes Obes.*, vol. 17, no. 2, pp. 143–149, Apr. 2010, doi: 10.1097/MED.0b013e328337a81f.

[127] J. Choi, K.-J. Kim, E.-J. Koh, and B.-Y. Lee, "Gelidium elegans Regulates the AMPK-PRDM16-UCP-1 Pathway and Has a Synergistic Effect with Orlistat on Obesity-Associated Features in Mice Fed a High-Fat Diet," *Nutrients*, vol. 9, no. 4, p. 342, Mar. 2017, doi: 10.3390/nu9040342.

[128] S. Giri *et al.*, "AICAR inhibits adipocyte differentiation in 3T3L1 and

restores metabolic alterations in diet-induced obesity mice model," *Nutr. Metab. (Lond).*, vol. 3, no. 1, p. 31, Dec. 2006, doi: 10.1186/1743-7075-3-31.

[129] S. A. Habinowski and L. A. Witters, "The Effects of AICAR on Adipocyte Differentiation of 3T3-L1 Cells," *Biochem. Biophys. Res. Commun.*, vol. 286, no. 5, pp. 852–856, Sep. 2001, doi: 10.1006/bbrc.2001.5484.

[130] X. Feng, L. Zhang, S. Xu, and A. Shen, "ATP-citrate lyase (ACLY) in lipid metabolism and atherosclerosis: An updated review," *Prog. Lipid Res.*, vol. 77, p. 101006, Jan. 2020, doi: 10.1016/j.plipres.2019.101006.

[131] M. Ahmadian, Y. Wang, and H. S. Sul, "Lipolysis in adipocytes.," *Int. J. Biochem. Cell Biol.*, vol. 42, no. 5, pp. 555–9, May 2010, doi: 10.1016/j.biocel.2009.12.009.

[132] R. E. Duncan, M. Ahmadian, K. Jaworski, E. Sarkadi-Nagy, and H. S. Sul, "Regulation of lipolysis in adipocytes.," *Annu. Rev. Nutr.*, vol. 27, pp. 79–101, 2007, doi: 10.1146/annurev.nutr.27.061406.093734.