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Eco-Integrated Production and Formulation of *Arthrospira platensis* Dietary Supplements

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Abstract

The increasing demand for sustainable and high-nutritional food sources has directed considerable attention toward microalgae, particularly *Arthrospira platensis* (Spirulina), due to its notable protein content and bioactive compounds. However, the high production costs associated with Spirulina cultivation remain a major limitation to its industrial expansion. This study proposes an integrated and eco-innovative strategy aimed at developing a Spirulina-based dietary supplement by optimizing cultivation efficiency, enhancing extraction yields of bioactive compounds, and improving bioavailability. Spirulina was cultivated in vertical tubular photobioreactors utilizing mine water supplemented with Zarrouk medium, achieving biomass yields and pigment concentrations comparable to conventional systems while significantly reducing dependence on freshwater resources. Notably, cultivation in mine water resulted in a higher protein content (52.64 ± 2.51 g·100 g^{-1} DW) and improved protein-to-carbohydrate and protein-to-lipid ratios. To maximize the recovery of functional compounds, a green extraction protocol combining phosphate-buffered saline (PBS) maceration with ultrasound-assisted extraction (UAE) was developed. This method led to significant increases in the yields of total fatty acids (+60.48%), polyphenols (+65.99%), carotenoids (+30.84%), and overall antioxidant activity (+42.95%), although a partial reduction in protein and carbohydrate recovery was observed. To further enhance bioavailability and stability, Spirulina extracts were encapsulated into liposomes (Lps), bilosomes (Bls), and gelatin-enriched bilosomes (G-Bls). These nanocarriers exhibited favourable physicochemical characteristics, including small particle sizes (101.8–129.7 nm), high encapsulation efficiencies (>80%), and stable negative surface charges. Among the formulations, G-Bls demonstrated superior performance, offering gastric resistance, controlled intestinal release, high biocompatibility, and effective mitigation of H_2O_2-induced oxidative stress in Caco-2 cells. Overall, this work presents a sustainable and scalable platform for the valorisation of Spirulina biomass, reinforcing circular economy principles and supporting its application in dietary supplements designed to promote sports performance, manage oxidative stress, and improve metabolic health.

1. Introduction

Spirulina (*Cyanophyceae; Arthrospira spp*.) is a filamentous cyanobacterium naturally adapted to high-temperature, alkaline environments where microbial competition is limited, making it an ideal model for ecological and biotechnological studies [1]. Among the 23 currently accepted species within the *Arthrospira* genus, *A. platensis* and *A. maxima* are the most widely cultivated and commercialized [2]. Spirulina is globally recognized as a "superfood" due to its exceptional nutritional profile. It contains 50–70% protein by dry weight, including all essential amino acids in proportions comparable to those of animal-derived proteins, which makes it particularly appealing to vegetarian and vegan consumers. Additionally, it provides 3-9% lipids, mainly polyunsaturated fatty acids, 15–25% carbohydrates in the form of polysaccharides, and up to 25% of phycocyanin, a potent antioxidant pigment. Its composition is further enriched by B-complex vitamins, vitamin E, β-carotene, minerals, and bioactive compounds such as polyphenols and sterols, all of which contribute to its health-promoting properties. However, despite its high nutritional value, Spirulina's rigid peptidoglycan cell wall and tightly bound protein–pigment complexes limit digestibility and reduce the bioavailability of its nutrients [3-5]. To address these challenges, various mechanical and physical pretreatments have been employed to disrupt the cell wall and improve the solubility and extractability of intracellular compounds [6]. In this context, ultrasound-assisted extraction (UAE) has emerged as a promising, sustainable and efficient alternative to traditional extraction methods to recover valuable biomolecules. By exploiting acoustic cavitation, UAE enhances mass transfer, enabling the yield of bioactive compounds with high efficiency, reduced solvent and energy use, and shorter processing times. Nevertheless, its large-scale industrial application remains limited due to high equipment costs, challenges in scalability, and the complexity of process control [7].

To meet the growing global demand for spirulina-based products, cultivation systems need to be optimised. Currently, open raceway ponds, which leverage natural sunlight to reduce energy requirements and enable large-scale biomass production, remain the industry standard. In contrast closed photobioreactor (PBR) systems offer precise control over critical growth conditions such as light, temperature, and nutrient supply. Commercial implementation of both approaches is constrained by high capital and operating expenses, stringent technical demands, biological contamination, regulatory requirements, and sustainability concerns [8]. To reduce production costs, recent proposals have advocated using wastewater and agro-industrial byproducts as alternative cultivation media, integrating renewable energy sources,

and repurposing decommissioned sites, cultivation facilities. This approach aligns with circular-bioeconomy principles by promoting resource and space efficiency and reducing environmental impact, while meeting growing demand across diverse sectors including food, nutraceuticals, pharmaceuticals, cosmetics, and agricultural bio-inputs [9]. Among different applications, dietary supplements represent the largest and most dynamic market segment, projected to reach approximately USD 897.61 million by 2027, with a compound annual growth rate (CAGR) of 10.5%. The popularity of Spirulina-based supplements is driven by evolving consumer trends such as increased health awareness, the shift toward plant-based diets, and demand for natural, minimally processed, and clean-label products, trends that position Spirulina as a key ingredient in the wellness market [10].

Despite the considerable promise of Spirulina as a source of health-promoting nutrients, the development of Spirulina-based supplements remains at an early stage. There are substantial challenges in the design of dosage forms, the physicochemical characterisation and the stability of the supplements, especially regarding the enhancement of bioavailability for its key active constituents. In recent times, liposomal delivery systems and their modifications have gained prominence as advanced platforms in the formulation of nutraceuticals and dietary supplements. Liposomes are spherical vesicles composed of one or more phospholipid bilayers. These bilayers are structurally analogous to those found in biological membranes. These nanoscale carriers have the capacity to encapsulate a wide array of bioactive agents, thereby safeguarding them from gastrointestinal degradation while promoting targeted cellular uptake. The utilisation of these nanoparticles has been demonstrated to enhance bioavailability, allow for the control of release kinetics, and improve overall therapeutic efficacy [11]. Concurrently, the regulatory landscape for dietary supplements is evolving, with increasing emphasis on evidence-based safety and efficacy standards. Regulatory bodies are moving toward more structured frameworks, highlighting the importance of standardized production methods, rigorous quality control, and scientifically validated product claims [12].

2. Scope of the thesis

The overarching objective of this Ph.D. project was to design and optimize an advanced Spirulina-based dietary supplement applying a multifaceted approach that integrated innovative cultivation methods, sustainable extraction techniques, and advanced formulation strategies.

The primary focus of this study was to investigate the feasibility of cultivating *Arthrospira platensis* using mine water in vertical photobioreactors under batch conditions. The cultivation system was developed and implemented in collaboration with LiveGreen, an agro-technology start-up based in Oristano (Sardinia, Italy). Mine water was sourced from 500 meters underground from the warm water basin of the historic Monte Sinni coal mine in Carbonia-Iglesias, southwestern Sardinia. Three trials were conducted using conventional Zarrouk medium as a control, mine water alone, and mine water enriched with Zarrouk medium. However, mine water alone was unable to support growth. Following a 12-day cultivation period, the mine-water batch exhibited comparable biomass yield and pigment levels to the control, while demonstrating a significantly elevated protein content. These results confirm the viability of mine water as a sustainable alternative to distilled water for Spirulina cultivation, substantially reducing primary water sources consumption and operational costs while supporting circular bioeconomy principles.

The second objective was to develop a green and effective extraction method capable of recovering a wide spectrum of nutritionally relevant bioactive compounds from Spirulina. This protocol involved an initial maceration in phosphate-buffered saline (PBS, pH 7.4) followed by ultrasound-assisted extraction (UAE). The resulting extract exhibited high recovery in bioactive compounds, including phenols, pigments (chlorophyll a, carotenoids, and phycobiliproteins), and essential fatty acids. The process has shown satisfactory results in extracting total proteins and carbohydrates, consistent with existing literature. In comparison with the raw biomass, the resultant extract demonstrated a complete nutritional profile and a significant enhancement in antioxidant capacity. This finding demonstrates the efficacy of the proposed eco-innovative extraction strategy in maximising bioactive metabolite recovery while concurrently minimising solvent consumption and energy output.

In the culminating phase of this project, Spirulina extract was encapsulated in three phospholipid nanovesicle formulations: liposomes (Lps), bilosomes (Bls) and gelatin-enriched bilosomes (G-Bls). Concurrently, in collaboration with the Pharmaceutical Technology Unit of Department of Life and Environmental Sciences (DiSVA), the oral

supplement prototypes were characterised and tested in vitro using Caco-2 cell models. The systems exhibited uniform nanoscale size, low polydispersity, strong negative charge, high entrapment efficiency and long-term (90-day) stability. They demonstrated protection under gastric conditions with controlled intestinal release and, particularly for G-Bls, superior biocompatibility, mucoadhesive-mediated uptake and antioxidant efficacy against H_2O_2-induced stress. These properties underscore their potential for advanced clinical nutrition and performance supplementation. This project delivers a framework that generates significant economic, environmental, and social value by aligning bioprocessing with circular-bioeconomy principles. Optimising cultivation and extraction workflows reduced freshwater and energy inputs in microalgal bioproduct systems, lowering production costs and consumption while catalysing local enterprise development and job creation.

Implementing eco-innovative extraction technologies minimised environmental impact by curtailing solvent use and energy demand, exemplifying sustainable process intensification. Concurrently, advanced delivery systems demonstrated enhanced bioactive bioavailability and therapeutic efficacy, highlighting benefits for public health initiatives. These outcomes establish a robust foundation for next-generation advances in the nutraceutical and dietary-supplement sectors and underscore a clear pathway toward a resilient, circular bioeconomy model.

3. State of art

Spirulina, an ancient filamentous cyanobacterium dating back 2.5–3.5 billion years to the Proterozoic era, played a foundational role in the oxygenation of Earth's atmosphere and the evolution of photosynthetic eukaryotes via primary endosymbiosis [13,14].

Historical documentation reveals its traditional use as a dietary component, most notably by the Aztecs, who harvested blue-green algae from Lake Texcoco to produce *tecuitlatl*, a protein-rich cake-like food referenced by Bernal Díaz del Castillo in 1521 [15]. Mid-20th-century ethnobotanical investigations by Pierre Dangeard and Jean Leonard later confirmed the organism's identity and traditional use, laying the groundwork for its scientific characterization as Spirulina [16]. Since the 1970s, growing awareness of global malnutrition and resource scarcity has driven extensive research into Spirulina as a sustainable food source, leading to the development of large-scale production systems and institutional endorsements, such as its designation as the "best food for the future" by the United Nations World Food Conference in 1974 and its recognition as a high-quality food by the WHO and FAO in subsequent decades [17–19].

In the past two decades, *Spirulina* has attracted widespread interest due to its exceptional nutritional profile, comprising between 50-70% protein by dry weight, all essential amino acids, polyunsaturated fatty acids, complex polysaccharides, phycobiliproteins, and diverse micronutrients, including B vitamins, β-carotene, and minerals. This distinctive formulation has led to its application in various fields, including human nutrition and functional foods, as well as in the production of sustainable feed additives for livestock, poultry, aquaculture, and pet food industries. Spirulina supplantation has been shown to enhance growth performance, immune modulation, and overall product quality [4].

Furthermore, the compound's antioxidant, anti-inflammatory, immunomodulatory, antiviral, and antineoplastic properties have contributed to an increased scientific focus on its potential utilisation in the fields of preventive and therapeutic medicine [20,21]. Advances in parallel have facilitated its integration into cosmetic formulations for skin protection and age control [22,23], as well as into agriculture, where it is employed as a biofertilizer and bio stimulant to enhance crop yield and resilience while reducing reliance on synthetic agrochemicals [24,25]. Notably, recent studies have highlighted Spirulina's potential as a suitable food source for long-duration space missions, given its high nutrient density, photosynthetic efficiency, and adaptability to extreme conditions [26].

Despite this broad spectrum of applications, key challenges persist in optimizing its cultivation under resource-efficient conditions, enhancing the extraction of bioactive compounds using green technologies, and developing advanced delivery systems to improve nutrient stability and bioavailability.

These emerging needs underscore the necessity for integrated, multidisciplinary approaches, to unlock the full biotechnological potential of Spirulina within the framework of a sustainable, circular bioeconomy.

4. Spirulina in the global market: growth, trends and opportunities

The global microalgae market is experiencing robust growth, valued at approximately USD 11.86 billion in 2023 and expected to reach around USD 25.72 billion by 2033 at a CAGR of 8.05% [26]. This expansion is driven by rising demand for bio-based products and sustainable, nutrient-dense food sources amid increasing global population pressures, alongside significant investments in research and development to advance production technologies [27].

Within this dynamic market, Spirulina stands as the most widely cultivated microalga and dominates the sector, holding 50.4% of the market share in 2024 [28]. Spirulina market valued at approximately USD 0.16 billion in 2024 and projected to reach USD 0.23 billion by 2032, fuelled by a compound annual growth rate (CAGR) of 4.4%.

The Spirulina market is commonly segmented both by product form and applications. Among products form, powdered Spirulina remained the dominant form, accounting for approximately 34% of global sales. This is primarily due to the ease with which it can be dosed, its incorporation into functional foods, and its long shelf life. Tablets and capsules constituted the second-largest segment, a position favoured for their precise dosing and high consumer convenience in the context of dietary supplementation. Liquid formulations, including ready-to-drink beverages and tinctures, are gaining popularity in the health and wellness sector due to their rapid absorption and portability. Flakes and granules are used in specialty foods for their texture and visual appeal, while phycocyanin extracts are the fastest-growing segment, driven by demand for clean-label colorants and expected to outpace bulk forms in growth over the next five years [29].

Among applications, nutraceutical and dietary supplement sector currently account for approximately 70% of global spirulina production, driven by its high protein content, potent antioxidant properties, and broad applicability in functional foods. It is projected that this market segment will grow from USD 300 million in 2023 to approximately USD 537.25 million by 2033, reflecting a compound annual growth rate (CAGR) of 6%. Beyond human nutrition, additional opportunities continue to emerge in the fields of animal feed, cosmetics, and, more recently, sustainable applications such as bioplastics and biofertilizers [30].

North America currently leads the global Spirulina market of regional share and generating the highest revenue among all regions. This leadership reflects strong consumer health and wellness awareness, robust demand for dietary supplements, a demand which is being supported by government policies.

Within the European market, Germany, France, and the United Kingdom represent key growth hubs for Spirulina-based products. This expansion is supported by the rise in plant-based diets and increased consumer awareness around health and sustainability. The range of applications extends to the domains of dietary supplements, cosmetics, and sustainable animal feed. Furthermore, favourable regulatory frameworks and growing investment in algae farming technologies are accelerating market development across the region.

In Asia, countries such as India, China, and Japan are experiencing rapid market expansion for Spirulina-based products. This growth is driven by government-led initiatives promoting sustainable food production and anti-malnutrition strategies, alongside rising disposable incomes. These factors collectively support increasing demand for affordable, high-quality Spirulina across nutritional, pharmaceutical, and agricultural sectors [29].

The Spirulina industry is moderately consolidated, with a small number of large, vertically integrated enterprises that dominate production and downstream processing. Cyanotech Corporation, a company with its headquarters in Hawaii, markets bulk Spirulina Pacifica® powder and high purity phycocyanin. These products are intended for use in the dietary supplement and food colourant sectors on a global scale. Earthrise Nutritionals, a DIC Corporation subsidiary, runs the world's largest outdoor Spirulina farm in California. It also pioneered commercial-scale cultivation and natural phycocyanin colorants under the LINABLUE® trademark. Parry Nutraceuticals (part of India's EID Parry) produces cost-effective organic spirulina powder and tablets at its Onnaiyur facility. It has recently secured the first EU import licence for such products [30,31]

The future growth of the Spirulina industry is contingent upon sustained research and development initiatives aimed at enhancing downstream processing methodologies, harmonising regulatory compliance, and optimising scalability.

5. The multifunctional role of Spirulina-based supplements in metabolic health, immune support, and physical performance

Spirulina-based supplements are increasingly recognised for their broad-spectrum health benefits, including immune enhancement, antioxidant protection, and support in managing metabolic and neurodegenerative disorders [32]. The biological effects are attributed to its dense nutritional profile, including complete proteins, B vitamins, essential fatty acids, minerals such as iron and magnesium, and antioxidant compounds such as phycocyanin and β-carotene [33]. This comprehensive biochemical profile positions Spirulina as a versatile and high-value functional ingredient in the nutraceutical sector.

In sports nutrition, Spirulina is valued for its high protein content, particularly its branched-chain amino acids (leucine, isoleucine and valine, BCAAs) which support muscle protein synthesis, energy metabolism, and post-exercise recovery. Antioxidants in Spirulina also counteract exercise-induced oxidative stress, enhancing performance and reducing fatigue [34]. Furthermore, Spirulina's immunomodulatory properties, which enhance macrophage activity, natural killer (NK) cell proliferation, and T cell activation, as well as exert anti-inflammatory effects through redox-sensitive pathways, make it beneficial for individuals under physical stress or with compromised immune function [35–37]. In metabolic health, Spirulina has shown promise in improving lipid profiles, reducing LDL and triglycerides, and increasing HDL, particularly in obese individuals [38-40]. It may also contribute to drug therapy and physical activity, glycaemic control in type 2 diabetes by improving insulin sensitivity and regulating glucose metabolism [41]. Additionally, moderate reductions in blood pressure in hypertensive patients suggest a vase-protective role through bioactive peptides with vasodilatory and antioxidative effects [42,43].

Neuroprotective benefits of Spirulina have been increasingly reported, including the inhibition of α-synuclein aggregation and amyloid-β toxicity, which are key pathological features in Alzheimer's and Parkinson's disease models. Additionally, Spirulina demonstrates protective effects against iron-induced neurotoxicity, a hallmark of neurodegenerative disorders, by modulating oxidative stress and stabilizing cellular structures, thereby potentially mitigating neuronal damage and slowing disease progression [44].

Despite these promising findings, the current evidence is limited by sizes, durations, and inconsistencies in formulation, dosage, and bioavailability assessments. The influence of gut microbiota and interindividual variability in nutrient absorption is also not well understood.

To validate the clinical potential of Spirulina-based supplements, larger, long-term trials are needed to address these gaps and ensure safety and efficacy across diverse populations.

6. Toxicology, clinical outcomes, and quality control in Spirulina-based supplements

A growing body of toxicological and clinical evidence supports the safety of Spirulina-based supplements. Preclinical studies in animal models have shown no adverse effects, even at high oral doses of up to 10 $g \cdot kg^{-1}$, as corroborated by clinical chemistry and histopathological assessments [45–48]. Complementary findings from randomized controlled and open-label clinical trials indicate that daily Spirulina supplementation, at doses ranging from 1 to 8 $g \cdot day^{-1}$ for durations of 8 to 24 weeks, is well tolerated across various populations, including both healthy individuals and patients with specific clinical conditions [49–51].

Considering these data, the U.S. Dietary Supplement Information Executive Committee (DSI-EC) classifies Spirulina as a Class A substance, indicating no significant health risks under standard consumption conditions [52].

Nonetheless, safety concerns persist, primarily regarding contamination during cultivation. Although Spirulina itself is non-toxic, it can co-occur with toxin-producing cyanobacteria in open-pond or poorly regulated systems. This may result in contamination with hepatotoxic microcystin, which have been identified in some commercial supplements at microgram-per-gram levels [53–55]. The World Health Organization (WHO) provides provisional limits for microcystin exposure in drinking water (1 $\mu g \cdot L^{-1}$) yet harmonized international standards for dietary supplements are still lacking [56].

Further risks include contamination with heavy metals (e.g., arsenic, cadmium, lead, mercury), particularly in products cultivated near industrial or polluted areas. Adulteration with pharmacologically active substances or low-quality raw materials also undermines the safety and nutritional value of commercial products [57].

To ensure quality and consumer protection, advanced analytical techniques—such as Fourier-transform infrared (FTIR) spectroscopy, ultra-high-performance liquid chromatography–mass spectrometry (UHPLC-MS), and stable isotope analysis—are increasingly applied to authenticate identity, purity, and traceability [58,59].

While Spirulina is generally well tolerated, mild adverse effects including gastrointestinal discomfort, allergic reactions, and insomnia have been reported [60]. Rare but serious cases of rhabdomyolysis and hepatotoxicity have also been documented; however, causality remains unclear due to limited data and potential confounders [61,62]. The recommended daily intake for healthy adult ranges from 3 to 10 $g \cdot day^{-1}$, with an upper threshold of 30 $g \cdot day^{-1}$ advised for individuals with elevated metabolic demands, such as professional athletes [63].

7. Regulatory and strategic frameworks governing Spirulina in the European union: from food law to bioeconomic transition

From a regulatory standpoint, Spirulina is subject to various legal frameworks in the European Union, depending on its intended use. As a dietary supplement, it is classified as a foodstuff under Regulation (EC) No 178/2002, which establishes foundational principles on food safety, traceability, and consumer protection [64]. Its production and distribution must comply with hygiene requirements under Regulation (EC) No 852/2004 [65]; while labelling and consumer information are governed by Regulation (EU) No 1169/2011 [66].

Despite Spirulina's established nutritional and potential therapeutic profile, Regulation (EC) No 1924/2006 does not authorise specific health claims. Evaluations by the European Food Safety Authority (EFSA) have found claims such as "tone and vitality" and "glycaemic health" to be scientifically unsubstantiated [67,68]. However, products meeting compositional criteria may use generic nutrition claims such as "source of protein" or vitamin-related claims permitted under the same framework.

Though exempt from novel food authorisation under Regulation (EU) 2015/2283 due to pre-1997 consumption [69], Spirulina extract is not listed as an approved food additive under Regulation (EC) No 1333/2008 [70]. Consequently, its use as a colouring agent or additive in conventional foods is currently prohibited unless a novel food application is submitted and authorised. Moreover, Spirulina marketed as an organic product must comply with Regulation (EU) 2018/848, which outlines production standards specific to aquaculture and algae [71].

In the United States, Spirulina is regulated as a dietary ingredient under the Dietary Supplement Health and Education Act and is self-affirmed as Generally Recognized as Safe (GRAS) for uses including natural blue colouring (21 CFR 73.530) [72,73]. Comparable regulatory systems exist in China and Japan, while Codex Alimentarius advocates for harmonised international standards focusing on traceability, toxicological assessment, and sustainability [75].

The European Union has recognised Spirulina's strategic role within the broader bioeconomy. Initiatives such as the 2012 Innovating for Sustainable Growth: A Bioeconomy for Europe identified microalgae, including Spirulina, as key bioresources for circular economy development [75]. This vision was expanded in the 2013 Blue Growth Strategy, promoting sustainable algal cultivation within marine sectors [76], and further supported by the 2014 Innovation in the Blue Economy Communication, which targeted regulatory and financial obstacles to microalgae scale-up [77].

Building on these initiatives, the 2018 EU Protein Plan explicitly identified Spirulina as a priority microbial protein to reduce reliance on imported soy and animal-based protein sources by 2030 [78]. This goal aligns with the European Green Deal and its Farm to Fork Strategy, which advocates for climate-neutral, low-footprint protein sources, such as algae, to strengthen food system resilience by 2050 [79,80].

To implement these policy frameworks, the Horizon Europe programme (2021–2027) has launched funding calls for Spirulina cultivation system optimisation and downstream processing [81]. Simultaneously, the Common Agricultural Policy (CAP) Strategic Plans and the EU4Algae Stakeholder Platform promote spirulina production through eco-schemes, knowledge exchange, and community engagement in coastal and rural areas [82,83]. Additionally, the Blue Invest Readiness Assistance programme has mobilised private capital into Spirulina ventures, supporting their transition to scalable, circular economy models [84]. Together, these policies create a favourable ecosystem for public and private investment in Spirulina, positioning it as a cornerstone in Europe's shift toward sustainable protein sources, climate resilience, and food security.

8. Common Materials and Methods

8.1. Chemicals

The chemical materials presented in this section encompass those employed across all three of the subsequent chapters.

Methanol (MeOH), hydrochloric acid (HCl), sodium hydroxide (NaOH), and chloroform ($CHCl_3$) were ultra-residue solvents of analytical grade, sulfuric acid (96%, 0.5 N), sodium hydroxide (32%, 0.5 N and 1 N), phenol, KCl, $CaCl_2$, Na_2CO_3, $CuSO_4$, Na and K tartrate, Na_2SO_4 anhydrous, Na_2HPO_4, NaH_2PO_4, D-glucose, α-amylase, methyl red indicator, 2, 2 diphenyl-1-picrylhydrazyl were reagent grade, and the internal standard (IS) tritridecanoin (TAF C13:0) were reagent grade solvents and chemical, the salts used for the Zarrouk medium (Table 1) were of analytical grade, purchased from Sigma Aldrich (Merck KGaA, Darmstadt, Germany). 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) was analytic grade, purchased from BDH, UK (Copens Scientific, Selangor, Malaysia). The PBS solution was prepared in the laboratory by weighing 1.09 g of Na_2HPO_4, 0.32 g of NaH_2PO_4, and 9 g of NaCl, dissolving in MilliQ water till a 1 L final volume was achieved, and adjusting the pH to 7.4 with HCl (0.1 M), reaching a final concentration of Na_2HPO_4 (8 mM), NaH_2PO_4 (3 mM), and NaCl (154 mM). Thiamine hydrochloride (B1) > 98.0%, nicotinamide (B3) > 99.0%, pyridoxine hydrochloride (B6) > 98.0% were purchased from TCI Europe N.V. (Tokyo Chemical Industry, Zwijndrecht, Belgium), riboflavin (B2) 99.43% was purchased from TargetMol Chemicals Inc. (Boston, Massachusetts), vitamin B12 ≥ 98% and L-ascorbic acid 99% were purchased from Indagoochem (Barcelona, Spain), folic acid (B9) 97.0 - 102.0% was purchased from Glentham, Life Sciences Ltd (Corsham, United Kingdom). Lipoid S75 (soybean phospholipids with 70% phosphatidylcholine, S75) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Sodium deoxycholate (SDC), gelatine and cholesterol were purchased from Merck (Milan, Italy). Cell medium, fetal bovine serum, penicillin, streptomycin, fungizone, and all the other reagents for cell studies were purchased from Thermo-Fisher Scientific Inc. (Waltham, MA, US). MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) was purchased from Thermo Fisher Scientific Inc (Waltham, MA, USA). Double-deionized water, with a conductivity of less than 18.2 MΩ, was produced using a Milli-Q system (Millipore, Bedford, MA, USA). The simulated gastric solution at pH 1.20 (SGF) was prepared by dissolving 1.75 g of NaCl in 94 mL of Milli-Q water and adding 6 mL of HCl 1 M. The simulated intestinal solution at pH 7.00 (SIF) was prepared dissolving

7.26 g of Na_2HPO_4, 3.56 g of NaH_2PO_4, and 17.54 g of NaCl in 1 L of Milli-Q water. Double-deionized water with a conductivity of less than 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

8.2. Methods

The methodologies described in this section encompass those employed across the next three chapters, as they are shared among them. Chapter-specific methods will be presented in their corresponding sections.

8.2.1. Ashes

The samples (0.5 g) were carbonized at 525 °C in a porcelain crucible for 8 h. Ashes were expressed as g·100 g^{-1} DW.

8.2.2. Total Proteins

Protein content was evaluated following the Kjeldahl method [85]. Samples (500 mg) were put in a Kjeldahl flask with 0.5 g of Na_2SO_4, 10 mg of $CuSO_4$, and 20 mL of H_2SO_4 96%. The samples were digested in a Speed-Digester K-436 BÜCHI (Labortechnik GmbH, Essen, Germany) at 400 °C until the solution became colourless. The Kjeldahl flasks were then removed, cooled, and introduced into a distillation system VAPODEST 300 (C. Gerhardt GmbH & Co. KG, Königswinter, Ger-many). One hundred mL of MilliQ water and 80 mL of NaOH (32%, w/v) were automatically added to the Kjeldahl flask. The distillate was recovered in a 250 mL receiving flask, with 10 mL of H_2SO_4 (0.5 N) and methyl red indicator (10 drops). Distillation lasted 4 min, and the obtained solution was titrated with NaOH (0.5 N) till it changed from red to light yellow. The total protein content was expressed as g·100 g^{-1} DW:

$$\% \text{ protein} = ((a - b)\, c \cdot 100 \cdot K)/g$$

a: mL of H_2SO_4 0.5 N added to the collection flask (10 mL)
b: mL of titrant used (NaOH 0.5 N)
c: conversion factor mL of H_2SO_4 0.5 N in g of nitrogen (0.007)
K: general nitrogen–protein conversion factor (6.25)
g: grams of sample

8.2.3. Total Carbohydrates

Carbohydrate analysis was conducted according to Dubois et al. [86]. Samples (15 mg) was put in a glass tube with 5 mL of HCl (1 M), sonicated in a water bath (15 min.), and extracted in a boiling water bath (1 h at 100 °C). 100 µL of the extract were collected in a glass tube with 1 mL of phenol solution (5% in deionized water, w/v) and 5 mL of H_2SO_4 (95%). The mixture was mixed, left at room temperature for 30 min in the dark, and measured at 488 nm using a Cary 50 UV–Vis spectrometer (Varian Inc., Palo Alto, CA, USA).
Quantification was assessed by a 5-point calibration curve of D-glucose (20 - 100 $mg \cdot L^{-1}$) with r2 ≥ 0.997. This analysis was carried out in triplicate, and total carbohydrates were expressed as D-glucose equivalents $g \cdot 100\ g^{-1}$ DW.

8.2.4. Total Lipids

Total lipids quantification was done according to Chen et al. and Pasquini et al. [87, 88]. After suspending the samples in PBS, saponification with a NaOH 1 N/MeOH solution (75/25), and centrifugation, the supernatant was transferred to a falcon tube and mixed with $CHCl_3$/MeOH (2/1) and a KCl solution 0.88% (w/v). 1 mL of the organic phase was transferred to a 2 mL HPLC vial and evaporated under nitrogen, and the remaining fat residue was weighed. The analysis was done in triplicate, and total lipids were expressed as $g \cdot 100\ g^{-1}$ DW.

8.2.5. Neutral detergent fiber (NDF)

A FIWE Advance Automatic Fiber Analyzer, VELP Scientifica (Usmate Velate (MB), Italy) was used to assess the Neutral detergent fiber (NDF) according to Van Soest et al. [89]. Samples (500 mg) with 0.5 g of Na_2SO_3 were placed in a P2 glass crucible and subjected to digestion (1.5 h) using a neutral surfactant mixture (6.81 g $Na_2B_4O_7$ x $10H_2O$ + 18.61 g EDTA + 30 g $C_{12}H_{25}NaO_4S$ + 10 mL triethylene glycol + 4.56 g Na_2HPO_4 in 1000 mL of double-deionized water) and α-amylase. The samples were dried in an oven for 4 h (100 °C) and mineralized for 5 h at 525 °C. The analysis was carried out in triplicate, and NDF% concentration was expressed as $g \cdot 100\ g^{-1}$ DW:

$$\% \text{ NDF} = (W1 - W0) * 100 / \text{sample (g)}$$

W1 = Crucible weight (g) + sample weight after drying
W0 = Crucible weight (g) + sample weight after mineralization
Sample (g) = grams of sample weighted

8.2.6. Chlorophyll a (Chl a) and carotenoids (Carot)

The concentration of Chl a and total carotenoids was determined according to Singh et al. [90]. Samples (20 mg) were placed in a 15 mL falcon tube with MeOH (4 mL); after 2 min of agitation in a vortex, the falcon was left in a thermostatic bath at 70 °C for 3 min. The tubes were cooled at ambient temperature and centrifuged at 3154× g, 10 °C for 5 min (Centrifuge 5810 R, Eppendorf AG 22,331 Hamburg) to precipitate cell debris. The bright green supernatant was analysed at 470, 666, and 750 nm in a UV–Vis spectrometer (Cary 50, Varian Inc., Palo Alto, CA, USA). Chl a and total carotenoid concentration was calculated according to Chamizo et al. [91].

$$\text{Chl a mg}\cdot\text{L}^{-1} = 12.9447\ (A665 - A750)$$

$$\text{Carot mg}\cdot\text{L}^{-1} = (4.08 \times (A470 - A750) - 0.0117 \times \text{Chl a mg} \cdot \text{L}^{-1})$$

$$\text{Chl a mg}\cdot\text{g}^{-1} = (\text{Chl a mg}\cdot\text{L}^{-1}\ \text{Vol Solv(L)/g Sample}) \times \text{DF}$$

$$\text{Carot mg}\cdot\text{g}^{-1} = (\text{Carot mg}\cdot\text{L}^{-1}\ \text{Vol Solv(L)/g Sample}) \times \text{DF}$$

Analyses were carried out in triplicate, and the amount of Chl a and total carotenoids were expressed as mg·100 mg^{-1} DW.

8.2.7. Phycocyanin (Pc), allophycocyanin (Apc), and phycoerythrin (Pe)

The samples (10 mg) were dissolved in $CaCl_2$ (4 mL, 1%, w/v), according to Dianursanti et al. [92]. The procedure comprises 3 freezing cycles (1 h at − 80 °C each) followed by thawing with warm water. The concurrent effect of the thermal shock and $CaCl_2$ accelerated cell lysis, allowing the extraction of the water-soluble cellular components. Finally, the tubes were centrifuged at 3154× g, 10 °C (Centrifuge 5810 R, Eppendorf AG 22,331 Hamburg) for 10 min to precipitate unwanted cellular components. The bright blue supernatant was analysed by UV–Vis spectrometer at 565, 620, 652, and 750 nm (Cary 50, Varian Inc.). Pc, Apc, and Pe concentrations were calculated according to Zavřel et al. [93].

$$\text{Pc mg}\cdot\text{L}^{-1} = (((\text{ABS620} - \text{ABS750}) - 0.474 \times (\text{ABS652} - \text{ABS750}))/5.34$$

$$\text{Pc\%} = (\text{Pc mg}\cdot\text{L}^{-1} = \text{x Vol Solv(L) mg S}^{-1}) \times 100 \times \text{DF}$$

$$\text{Apc mg}\cdot\text{L}^{-1} = = ((\text{ABS652} - \text{ABS750}) - 0.208 \times (\text{ABS620} - \text{ABS750}))/5.09$$

$$\text{Apc\%} = (\text{Apc mg}\cdot\text{L}^{-1} = \text{x Vol Solv(L) mg S}^{-1}) \times 100 \times \text{DF}$$

$$\text{Pe mg}\cdot\text{L}^{-1} = ((\text{ABS565} - \text{ABS750}) - 2.41 \times (\text{Pc mg mL}^{-1}) - 0.849 \times (\text{Apc mg}\cdot\text{L}^{-1}))/9.62$$

$$\text{Pe\%} = (\text{Pe mg}\cdot\text{L}^{-1}\text{x Vol Solv(L) mg S}^{-1}) \times 100 \times \text{DF}$$

**S= sample; DF= dilution factor*.

Analyses were carried out in triplicate, and the final concentration of Pc, Apc, and Pe were expressed as mg·100 mg^{-1} DW.

8.2.8. Total polyphenols

Total polyphenols were assessed using the Folin–Ciocalteu reagent method, according to Manconi et al. [94]. Ten mg of the samples were placed in a 15 mL falcon tube with 10 mL of methanol. The tubes were shaken for 1 min and were subjected to an ultrasound-assisted extraction (UAE) at 100% power (400 W, 30 kHz; 1 s ON and 1 s OFF, 3 min.). Finally, the samples were centrifuged at 3154× g at 10 °C for 15 min (Centrifuge 5810 R, Eppendorf AG 22,331 Hamburg). 100 µL of the extract and 100 µL of the Folin-Ciocalteu reagent were put into a 15 mL falcon tube and vortexed (1 min). The mixture, and 400 µL of a sodium carbonate solution (20% w/v), was agitated and sheltered (60 min, 18°C in the dark). The total amount of polyphenols was assessed at 765 nm using a UV–Vis spectrometer (Cary 50, Varian Inc., Palo Alto, CA, USA) by comparing the absorbance with a 7-point calibration curve of Gallic

acid (2.5 - 200 µg ·mL^{-1}; r2 ≥ 0.997). Analyses were carried out in triplicate, and total polyphenols were expressed as mg · g^{-1} gallic acid eq DW.

8.2.9. Fatty acid extraction and GC-MS analysis

Fatty acids (FA) were extracted according to Breuer et al. [95]. Briefly, 50 mg of the samples were weighed in a 15 mL Falcon (F1) with a $CHCl_3$/MeOH solution (4 mL, 4:5, v/v) containing tritridecanoin acid (IS at 50 mg·L^{-1}). After mixing, 2.5 mL of NaCl solution (1 M in MilliQ water and 50 mM in 2-amino-2-hydroxymethyl-propane-1,3-diol-Tris) was added, and the pH was adjusted to 7.0 using an HCl solution. The sample was vortexed in the presence of glass beads (5 min), sonicated in ice (10 min.), and centrifuged at 3154× g at 15°C (5 min). The $CHCl_3$ layer was transferred to a 12 mL glass tube, whereas the residual sample was re-extracted with 1 mL of $CHCl_3$ and finally assembled in the glass tube. The $CHCl_3$ phases were brought to dryness under a nitrogen flow. The lipidic residue was subjected to transesterification of fatty acids by adding 3 mL of a methanol solution at 5% (v/v) sulfuric acid. The solution was placed on a thermostatic plate at 70 °C for 3 h, with a 30 s vortex every 30 min. After cooling, 3 mL of MilliQ water and 3 mL of hexane were added, and the tube was vortexed for 1 min and centrifuged at 3154× g at 15°C for 5 min. The hexane phase was transferred to a 2 mL amber vial and analysed with an Agilent 8860 Gas Chromatograph (GC) (Agilent Technologies, Santa Clara, CA, USA) coupled with a 5977C GC/MSD Detector. The column was an HP-5ms Ultra Inert (Agilent GC Column, 30 m x 0.25 mm, and 0.25µm thickness). The injector temperature was 250 °C. He was the carrier gas at 1.5 mL·min^{-1}; the injection volume was 1 µL, with a split ratio 1:2.

The oven temperature gradient was T = 0 50 °C (4 min), T = 26 160 °C (5 °C·min^{-1}), T = 76 210 °C (1 ° C·min^{-1}), and T = 93.5 280 °C (4 ° C·min^{-1}). The transfer line temperature was 280 °C. Ions were generated at 70 eV with positive EI and recorded at 1.5 scans·s^{-1} over 50 to 550 m·z^{-1}. The electro multiplier was set at 1162 V. The analysis was conducted in triplicate.

The content of each fatty acid (FA mg·g^{-1} DW) was determined using the following formula:

FA mg·g^{-1} = (IS mg · sample^{-1})· {(Area of individual FAME)/ [(Area of C13:0 FAME(IS)*Rel.Resp.Factor individual FAME)]}/ (biomass (g))

The concentration of the IS mg · sample^{-1} was calculated with the following formula:

IS mg·sample^{-1}= 0.004 L·[IS]·(3·MW FA C13:0)/ (MW TAF C13:0)

[IS]= 50 mg·L^{-1}; MW FA C13:0: 214. 34; MW TAF C13:0: 684,08.

The relative response factor of individual FAME was calculated with the following formula:

Rel. Resp. Factor individual FAME =
(conc. C13:0 FAME in calibration sol./conc. individual FAME in calibration sol.) * (Area of individual FAME in calibration sol./Area of C13:0 FAME in calibration sol.)

8.2.10. DPPH method

2, 2 diphenyl-1-picrylhydrazyl (DPPH) test was carried out as described by Manca et al. [96]. Total polyphenol of the samples was mixed with a DPPH methanolic solution (25 μM) at a ratio of 1:50. Samples were incubated for 30 min in the dark at room temperature. Analyses were performed at 517 nm in a UV–Vis spectrophotometer (Cary 50, Varian Inc.). Quantification was assessed by a 5-point calibration curve of Trolox (0.025 – 1 mM, r2 value ≥ 0.997).

The test was carried out in triplicate, and the antioxidant activity was expressed as mg·g^{-1} Trolox eq DW, or as percentage with the following formula:

Antioxidant activity %= (ABS DPPH - ABS sample)/ ABS DPPH·100

8.2.11. Water-soluble vitamins

The water-soluble vitamin extraction and analysis were carried out according to Rakuša et al., with minor changes [97]. The samples (20 $mg \cdot mL^{-1}$ in 0.5% glacial Acetic acid) were homogenized by vortex for 1 min, sonicated in an ice bath for 10 min, kept in the dark (10 min at 5 °C), and centrifuged at 3154× g (10 min at 15 °C). The solution was filtered with a 0.45 µm nylon filter just before injection.

An ultra-high performance liquid chromatograph UHPLC Infinity II (Agilent Technologies, Santa Clara, CA, USA), equipped with a diode array detector (DAD), on reversed-phase Infinity Lab Poroshell 120 EC-C18, 3.0 x 150 mm, 2.7 □m, 1000 bar, Agilent settled at 20 °C. The mobile phase included A: 25 mM $NaH_2PO_4 \cdot H_2O$, and B: methanol, with the following gradient T = 0 min-97% A, T = 9 min-85% A, T = 10 min-80% A, T = 20 min-75% A, T = 30 min-45% A, post-run 10 min. The flow rate was 0.3 $mL \cdot min^{-1}$, and the injection volume was 10 µL. UV-vis detection was made at 246 nm (B1), 290 nm (B2), 260 nm (B3), 210 nm (B6), 268 nm (B9), 362 nm (B12), and 244 nm (C). Vitamins were identified by comparing retention time and UV-vis spectra. Stock standard solutions of the single vitamins were prepared in dark volumetric flasks with concentrations around 1000 $mg \cdot L^{-1}$ in 0.1 M EDTA (chelating agent, stabilizer) and stored at 5 °C. Vitamin B9 was prepared in NaOH 1 mM due to its low water solubility. A 5-point calibration curve (0.25 - 5 $mg \cdot L^{-1}$, $r^2 \geq 0.997$) was prepared by diluting the intermediate working standards solution at 100 $mg \cdot L^{-1}$ in 0.5% (v/v) glacial acetic acid before use. Glacial acetic acid was used to improve the solubility of vitamins, facilitate their separation from other components, and prevent the degradation of sensitive vitamins (B1 and B2).

9. Use of Enriched Mine Water to Grow the Cyanobacterium *Arthrospira platensis* in Photobioreactors.

This chapter is based on the article:

Milia, M.; Andreotti, V.; Giglioli, A.; Pasquini, V.; Addis, P.; Angioni, A. Use of Enriched Mine Water to Grow the Cyanobacterium *Arthrospira platensis* in Photobioreactors. *Foods* **2025**, *14*, 1665. https://doi.org/10.3390/foods14101665

9.1. Abstract

The demand for sustainable and high-nutritional food sources is forcing the industrial sector to seek alternatives to animal proteins. Microalgae and macroalgae showed remarkable contents of protein and bioactive compounds, offering a promising solution for the food industry. However, the high production cost represents the main concern related to microalgae sector. Thus, innovation strategies that can reduce production costs, preserve the environment and improve the nutritional characteristics of microalgae are requested. The exploitation of sewage water from dismissed mines could lead to energy savings in production, thereby creating new industrial opportunities for microalgae cultivation. *Arthrospira platensis* (Spirulina) can be grown in open ponds and photobioreactors; the composition of the growth medium and the light radiation could affect its biochemical composition. This work investigated the influence of mine water with the addition of Zarrouk growth medium on the biochemical composition of the final dried Spirulina. The trials were performed in vertical tubular photobioreactors (PBRs) exposed to specific light radiance. Samples were compared with standard growing condition using distilled water with the addition of Zarrouk medium. Spirulina strains showed a good tolerance to medium/high concentrations of Cl^-, SO_4^{2-} and nitrogen in mine water. The experiment lasted 12-days, showing significant differences in protein, lipids, and carbohydrates between trials. Spirulina grown in mine water enriched with Zarrouk' salts (MWZ) showed higher protein levels 52.64 ± 2.51 g·100 g^{-1} DW. On the other hand, Spirulina grown in classic Zarrouk medium with distilled water (DWZ) had higher lipids and carbohydrates levels, accounting for 9.22 ± 1.01 and 31.72 ± 1.57 g·100 g^{-1} DW. At the end of the experiment both trials showed similar growth and pigment concentration. This study presents a validated, resource-efficient approach for cultivating Spirulina in vertical tubular photobioreactors using mine water supplemented with Zarrouk medium. The system achieves biomass yields comparable to conventional cultivation methods, while significantly

reducing dependence on freshwater. By converting sustainable and constant water supply into inputs for biomass generation, the method reinforces circular economy principles and supports the achievement of the sustainable development goals.

9.1. Introduction

Macro and microalgae can contribute to meeting the next generation's food demand and environmental sustainability [98]. Despite the increasing market size, microalgal production is still limited in comparison to the global algal output. This disparity can largely be ascribed to the high production costs and the relatively modest yields that are observed when scaling up from laboratory to industrial cultivation. Microalgae have a remarkable nutritional composition with high-value proteins, lipids, carbohydrates, active enzymes, pigments, sterols, and vitamins [99]. Microalgae and cyanobacteria are currently employed in several applications, including food, pharmaceuticals, agriculture, wastewater treatment, aquaculture, cosmetics and biofuels. [100-104]. Moreover, the World Health Organization has labelled microalgae as a superfood, a rich source of biologically active compounds that could be used as functional ingredients [105]. Nowadays, the cyanobacterium *Arthrospira platensis* Gomont, 1892 (Spirulina), due to its characteristics and growth conditions, is the most commercialized species in the bio-resource market, with a 2025 global market of 522.6 million USD and forecast to reach 884.6 million USD by 2032 [106].

Spirulina macronutrients range from 50-70% protein, 3-9% lipid, and 15-25% carbohydrate; moreover, it contains chlorophyll a (Chl a), carotenoids, phycobiliproteins and essential fatty acids [3].

Spirulina naturally thrives in alkaline waters of lakes and confined ponds, typically in warm areas [107]. Domestication can utilize diverse cultivation methodologies, including photobioreactors or open ponds, operating in batch or semi-continuous modes. Open ponds in outdoor cultivation conditions showed higher duplication times and lower biomass productivity. However, the high variability of these systems (such as temperature and light intensities) can deeply influence the growth performance of microalgae and biochemical composition [3]. In semi-continuous production, the microalga is harvested at the late-logarithmic phase, resulting in a higher content of macronutrients. Following harvesting, the cultivation system is restored to support new growth [108].

Spirulina does not compete with traditional agricultural production; however, the price of the Zarrouk growth medium can lead to high production costs in large-scale growing farms, decreasing the market demand. Therefore, numerous studies have evaluated substituting the Zarrouk medium with less expensive substrates capable of producing Spirulina biomass of comparable quality, such as fish farming, piggery, brewery, winery, industrial, and dairy wastewater [109-115].

Mining sites offer extensive areas and water resources that can be converted into aquaculture facilities for microalgae cultivation [116]. The conversion of decommissioned mining areas into microalgae cultivation sites represents a significant opportunity to achieve environmental, social and economic benefits [117].

The historic coal mine of Monte Sinni (Carbonia-Iglesias, southwestern Sardinia) includes more than 30 km of tunnels and sits above a warm water basin. Daily, about 3000 m^3 of water is pumped from -500 m underground to avoid flooding the galleries. The water temperature is constant at about 42 °C, reaching 38 °C at the first surface storage tank. Nowadays, its primary destination is the sustenance of a nearby river used to irrigate the cultivated fields in the area [118].

The present study investigates the feasibility of growing *A. platensis* with the mine effluent implemented with conventional Zarrouk medium in vertical tubular photobioreactors (PBRs). The biomass production, photosynthetic pigments amount, biochemical composition, total lipids, proteins, carbohydrates, neutral detergent fiber (NDF) and ash were evaluated to assess the performance of the cyanobacterium.

Enriched mine water for Spirulina cultivation represents a sustainable innovation aligned with the European Blue Economy and Green Deal principles, with economic, environmental, and social benefits. contributing to sustainability in financial, ecological, and social terms.

9.2. Methods

The methods applied are reported in Chapters 8.2.1 to 8.2.7.

9.2.1. Microalgae culture and photobioreactors

Livegreen SRL (Arborea, Sardinia, Italy) provided the microalgal strains of *A. platensis*. Pre-culture inoculums cultivated in Zarrouk medium [16] were permanently kept in Erlenmeyer flasks in Pyrex glass with a total capacity of 5 L at 30 °C, irradiated with 80 µmol photons · $m^{-2} \cdot s^{-1}$, and mixed with continuous aeration.

The experiments were carried out using four vertical PBRs in polymethyl methacrylate with a capacity of 25 L.

PBRs were placed in a vertical steel structure in a monitored room. They consisted of an upper cap with three accessways for the filling system, ventilation, and heating, and a lower cap with a tap for recovery and cleaning. The filling medium was stored in a 150 L tank connected to the PBRs by a pump system managed with a control panel.

The PBRs were illuminated by three types of LED light lamps (24 hours a day) anchored to the support structure and oriented to allow maximum light exposure. The following light spectra characterized LED lamps: 1) white 380 - 760 nm, maximum 437 nm, and 630 nm; 2) red 625 - 675 nm, maximum 650 nm; and 3) blue 400 - 475 nm, maximum 450 nm. All lamps operated simultaneously with the same light intensity of 80 µmol · m^{-2} · s^{-1}.

Immersed heating probes maintained the temperature at 33 ± 2 °C (Aquarium Heater, 3613010, 60-100 L, 230 V, 75 W, Eheim, Thermocontrol).

Three experiments were carried out simultaneously with different mediums: a) deionized water (DWZ), b) mine water (MWZ), both enriched with Zarrouk medium (Table 1), and c) mine water (MW) (Table 2). On average, the medium's pH was 11.13 ± 2.14 (RSD%).

Table 1. Chemical composition of the Zarrouk medium.

Ingredients	$g \cdot L^{-1}$
$NaHCO_3$	16.80
K_2HPO_4	0.50
$NaNO_3$	2.50
K_2SO_4	1.00
NaCl	1.00
$MgSO_4 \cdot 7H_2O$	0.20
EDTA-$Na_2 \cdot 2H_2O$	0.08
$CaCl_2 \cdot 2H_2O$	0.04
$FeSO_4 \cdot 2H_2O$	0.01
H_3BO_3	2.860
$MnCl_2 \cdot 4H_2O$	1.810
$ZnSO_4 \cdot 7H_2O$	0.222
$Na_2MoO_4 \cdot H_2O$	0.007
$CuSO_4 \cdot 5H_2O$	0.079

Table 2. Chemical composition of the mine water.

Physicochemical Properties	Values (conc ± RSD%)
COD ($mg \cdot O_2^{-1}$)	5.80 ± 0.10
NO_3^- ($mg \cdot L^{-1}$)	2.07 ± 0.03
NO_2^- ($mg \cdot L^{-1}$)	0.02 ± 0.01
NH_4^- ($mg \cdot L^{-1}$)	3.34 ± 0.49
SO_4^{2-} ($mg \cdot L^{-1}$)	547.2 ± 1.31
Hardness °F	19.4 ± 0.5
Cl^- ($mg \cdot L^{-1}$)	606.6 ± 1.53
PO_4^{3-} ($mg \cdot L^{-1}$)	4.72 ± 0.07
Fe ($mg \cdot L^{-1}$)	<0.001
Ba ($mg \cdot L^{-1}$)	0.33 ± 0.01
B ($mg \cdot L^{-1}$)	0.31 ± 0.03
Al ($mg \cdot L^{-1}$)	0.014 ± 0.003
As ($mg \cdot L^{-1}$)	0.035 ± 0.005
Zn ($mg \cdot L^{-1}$)	0.004 ± 0.001
F^- ($mg \cdot L^{-1}$)	5.85 ± 0.02
pH	8.3

9.2.2. Experimental design

Four PBRs were used for each treatment in batch condition, filled with 20 L of medium. Before transferring Spirulina into the PBRs, an aliquot from the Erlenmeyer flasks was collected to determine the dry weight (DW). Therefore, Spirulina was placed in each PBR to reach an initial concentration of 200 $mg \cdot L^{-1}$ DW. Control sampling was performed daily, collecting 100 mL of culture from each PBR, dividing the sample into three replicate flasks, and maintaining it under agitation before physical analysis. Before sampling, each PBR was refilled with distilled water to compensate for evaporation.

Trials lasted 12 days when the late logarithmic phase was achieved. The pH was measured daily with a bench-top pH meter (Hanna Instruments, Rome, Italy).

At the end of the experiment, the entire culture was collected, filtered at 0.25 mm, washed two times with deionized water, and lyophilized. Freeze-dried samples were used for biochemical analysis after being finely ground in a mortar. Each study was carried out in triplicate.

9.2.3. Statistical analysis

Differences in biomass production (DW), phycocyanin (Pc, $mg \cdot L^{-1}$), allophycocyanin (Apc, $mg \cdot L^{-1}$), chlorophyll a (Chl a, $mg \cdot L^{-1}$), and total carotenoids (Carot, $mg \cdot L^{-1}$) were assessed with permutational analyses of variance (PERMANOVA) tests using Trials (2 fixed levels) and Days (12 fixed levels) as orthogonal sources of variance. Differences in protein, carbohydrate, lipid, ash and NDF were assessed with permutational analyses of variance (PERMANOVA) tests using Trials (2 fixed levels). PERMANOVA was conducted on Euclidean distance-based resemblance matrices of normalized data, using 999 random permutations of the appropriate units in univariate and multivariate contexts. When significant differences were observed, pairwise tests were also carried out to ascertain patterns of differences among treatments and/or sampling times.

All statistical analyses were performed using the routines included in the PRIMER 7+ software [119]. Multivariate differences were assessed in all photosynthetic pigments (Pc, Apc, Chl a, Carot) between trials, with a biplot after a canonical analysis of the principal coordinates (CAP), which allows identifying an axis through the multivariate cloud of points that is best at separating a priori groups [120].

9.3. Results

After 24 h from the inoculum, Spirulina in MW separates from the culture medium, forming a gelatinous mass represented by dead cells, and moves to the medium surface. Therefore, it was no longer considered throughout the study. This outcome can be attributed to the oxidative stress induced by heavy metals, the excessively low levels of nutrients, and the suboptimal pH in the mine water. During the 12 days of the experiments, the growth (expressed as DW) showed distinct patterns in DWZ and MWZ. In DWZ, Spirulina had an almost constant value in the first 5 days and then increased steadily until the 12th day. On the other hand, MWZ showed a wave-like trend up to day 5, followed by a consistent upward pattern till day 12. On day 12, both Trials showed the highest biomass at comparable amounts (Figure 1). Statistical analysis showed substantially equivalent biomass DW in the two Trials, except on days 1, 3, and 9, which highlighted higher growth in DWZ (Table 3).

The different growth patterns on other days did not affect the overall development of the culture, leading to similar values at the late-logarithmic phase in both trials.

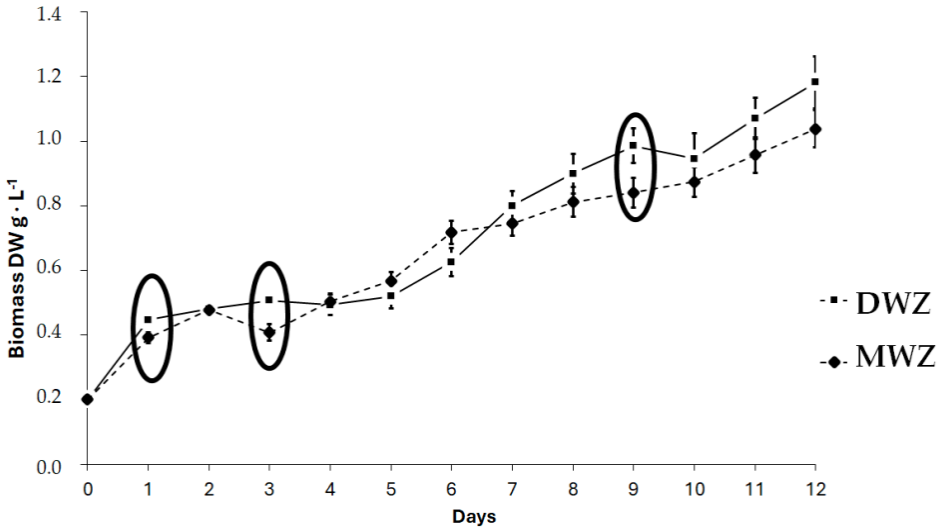


Figure 1. Biomass DW evolution (g·L^{-1}) during Spirulina growth in the two trials. DWZ used bi-distilled water with Zarrouk medium, and MWZ used mine waters with Zarrouk medium. Error bars indicate the standard deviation.

The points inside the circles showed statistical differences.

Table 3. Results of univariate PERMANOVA testing for the effects of different culture media (DWZ, MWZ) and the days of the experiment (12 days) on DW ($g \cdot L^{-1}$) and photosynthetic pigments (Pc, Apc, Chl a, Carot) ($mg \cdot L^{-1}$).

		Dry Weight		Chl *a*		Carot		Pc		Apc	
	Days	t	P (MC)	t	P (MC)	t	P (MC)	t	P (MC)	t	P (MC)
DWZ vs. MWZ	1	2.460	*	1.854	ns	4.638	***	6.278	***	1.651	ns
	2	0.124	ns	2.571	*	4.341	***	6.354	***	4.729	***
	3	2.986	**	1.410	ns	1.964	*	2.644	*	4.770	***
	4	0.239	ns	2.561	*	3.014	*	1.143	ns	3.988	***
	5	1.027	ns	2.351	*	2.819	**	2.947	**	5.553	***
	6	1.619	ns	0.646	ns	1.238	ns	0.946	ns	3.134	**
	7	0.919	ns	2.311	*	2.663	*	2.294	*	6.723	***
	8	1.140	ns	0.401	ns	0.042	ns	2.757	*	2.054	ns
	9	2.038	*	1.617	ns	1.941	ns	1.074	ns	0.656	ns
	10	0.786	ns	0.482	ns	0.150	ns	0.538	ns	1.831	ns
	11	1.347	ns	1.062	ns	0.317	ns	0.763	ns	0.709	ns
	12	1.462	ns	0.887	ns	1.776	ns	0.508	ns	0.993	ns

** $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = non statistically significant*

The percentage composition of photosynthetic pigments in the biomass obtained in the experiment ($mg \cdot 100\ mg^{-1}$ DW) showed significant differences between Trials. The pairwise test showed that all pigments varied among trials only during the first 8 days of the experiment. The values in DWZ were 1.2 times higher for carotenoids, Chl a, and Pc and 1.3 times higher for Apc. From days 9 to 12, no differences were observed among trials (Table 3).

DWZ showed a constant increase in Chl a and carotenoids with an r^2 of 0.994 and 0.988, respectively. MWZ showed an irregular increase with an r2 of 0.971 and 0.973, respectively. Pc and Apc had an opposite trend with r^2 of 0.833, and 0.919 and 0.944 and 0.857 in DWZ and MWZ, respectively (Figures 2 and 3, Table 3). Metal cations bind to phycobiliproteins, quenching their light absorbance, thus resulting in lower apparent concentration. The absorbance spectra for Apc and Pc shows little shift in λmax but some decrease in total absorbance, suggesting that only moderate structural perturbations occur upon metal binding. The mechanism of heavy metal binding to phycobiliproteins is not fully understood, limiting their widespread quantitative applications. However, the different substrate medium did not affect the final amount of the photosynthetic pigments in the Spirulina biomass.

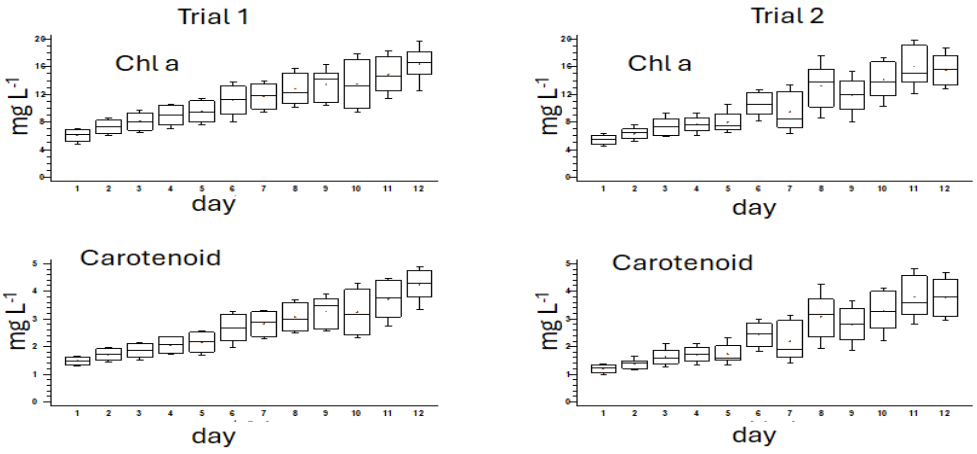


Figure 2. Box-and-whisker plot of the variation of Chl a and carotenoids ($mg \cdot L^{-1}$) in the two trials along the 12 days of the experiment. Error bars indicate the standard deviation.

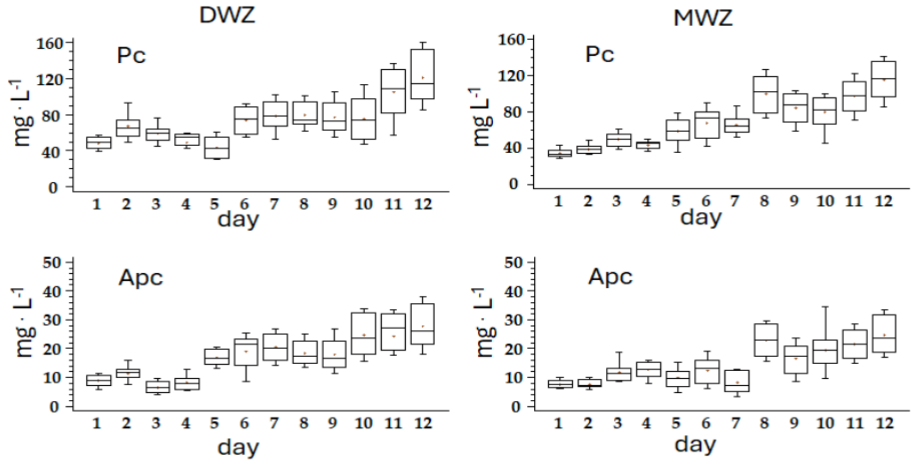


Figure 3. Box-and-whisker plot of the variation of PC and APC ($mg \cdot L^{-1}$) in the two trials along the 12 days of the experiment. Error bars indicate the standard deviation.

Spirulina at the end of the experiment showed differences among trials in proteins, carbohydrates, and lipids. The content of lipids and carbohydrates was higher in DWZ, whereas the protein content was higher in MWZ On the other hand, the content of NDF and ashes was not significantly different among trials (Tables 5 and 6).

Table 5. Biochemical composition (g·100 g^{-1} ± RSD%) of Spirulina in DWZ and MWZ.

	Lipids	Carbohydrates	Proteins	NDF	Ashes
DWZ	9.22 ± 1.01	31.72 ± 1.57	46.03 ± 2.14	2.69 ± 0.37	9.00 ± 0.32
MWZ	6.25 ± 0.88	25.66 ± 2.12	52.64 ± 2.51	2.34 ± 0.11	8.18 ± 1.41

Table 6. Results of univariate PERMANOVA testing the effects of different culture media (DWZ and MWZ) on the lipids, carbohydrates, proteins, NDF, and ashes (g·100 g^{-1}).

	Source	DF	MS	Pseudo-F	P (MC)
Lipids	Trial (Tr)	1	5.36	19.6	**
	Res	6	0.27		
Carbohydrates	Trial (Tr)	1	5.45	21.2	**
	Res	6	0.26		
Proteins	Trial (Tr)	1	5.09	16.02	**
	Res	6	0.32		
NDF	Trial (Tr)	1	2.48	3.29	ns
	Res	6	0.75		
Ashes	Trial (Tr)	1	1.24	1.3	ns
	Res	6	0.95		

DF = degree of freedom; MS = mean squares; Pseudo-F = F; P(MC) = probability level after Monte Carlo simulations.
** = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ns = $p > 0.05$.*

9.4. Discussion

Spirulina biomass production and its bioproducts are estimated to exhibit significant growth over the next 5-10 years. However, spirulina cultivation still faces many limitations.

The primary limits are represented by the excessive cost of the growth medium and apparatus, and the extraction technology applied. Zarrouk medium is one of the earliest and most widely used for Spirulina cultivation [121]. Since its development, many authors have investigated more economical alternative nutrient sources [122, 123]. Ragaza et al. reviewed the alternative mediums, highlighting the dependence of the microalgae on light, availability of appropriate nutrients, and temperature, concluding that the cost reduction of spirulina culture media, as well as the adoption of low-cost production techniques, is a prerequisite for its successful utilization in aquafeeds [122]. AlFadhly et al. identified critical bottlenecks of Spirulina cultivation, emphasizing temperature, light intensity, and the physicochemical properties of the culture medium as key factors influencing growth performance [123]. Circular economy strategies have encouraged the use of microalgae in Multi-Trophic Aquaculture systems, in the remediation programs of agricultural wastewaters, and in the bioremediation of anthropogenic activity-related water contamination [102,103, 124-128]. However, wastewater has two main problems, turbidity and susceptibility to contamination. Turbidity decreases light penetration, influencing microalgae growth and bioactive compounds synthesis, therefore dilution of the wastewater is required [129]. The growth of spirulina is inhibited by biocontamination of the medium. Yuan et al. reported a study on the use of recycled medium, highlighting the necessity to subject it to decontaminating treatments to avoid inhibition on the growth of microalgae [130]. To mitigate the development of bacteria and fungi, it is essential to maintain high salinity levels and high alkalinity conditions [131]. Moreover, Spirulina can remove chemical contaminants such as heavy metals from the environment, concentrating them and becoming potentially toxic for food and feed [132]. Pereira et al. studied the mixotrophic cultivation of Spirulina, replacing part of the standard medium with cheese whey. The authors found increased phenolic compounds with 10% whey and increased growth rates [133]. These results were confirmed by Athanasiadou et al., who reported the need for whey pretreatment by filtration at 1 μm to decrease turbidity and avoid pathogen contamination [134].

Papadopulos et al. investigated the use of brewery wastewater to cultivate Spirulina. The microalga showed good yield and biochemical composition. However, many heterotrophic bacteria were detected, limiting the biomass use in food and feed products [135].

Spennati et al. reported data from the use of winery wastewater; the yield of protein, carbohydrates and polyphenols was lower than conventional growing conditions, even if the total biomass was similar [136].

The presence of toxic compounds (e.g. copper) and the unbalanced concentration of inorganic N or P could interfere with the micro-algal-bacterial consortium's physiology, growth and metabolism [137]. Therefore, the transition from laboratory to industrial development remains unfinished. This study focused on exploring unused mine water as a growth medium for spirulina in controlled systems. Before modifying the existing growth medium, the goal was to achieve positive results for both the biomass's application in food and feed and the cultivation's environmental and social impact.

Dismissed mine sites represent a high social and environmental cost. The redevelopment of these sites into an environmentally sustainable and productive farm represents one of the strategies of the European Commission.

The Monte Sinni mine can afford high quantities of water at an optimal temperature for Spirulina cultivation, avoiding heating systems and substantially reducing the cultivation cost. Temperature, light intensity, and pH were kept constant in the three trials. The trials were conducted in vertical PBRs using laboratory equipment; the temperature was kept constant at 33 °C similar to the mining site conditions. This was the mean temperature of the water rising from the underground and kept moving in PBRs on the surface after the first surface storage tank. The chemical composition of the mine water showed that nitrogen, phosphate, iron, and micronutrient amounts were insufficient to allow strain growth. This fact was highlighted by MW, which was carried out with the mine water without implementing the Zarrouk medium, lasting for 24 h.

Elevated chloride (Cl^-) concentrations in the mine water substrate could negatively impact Spirulina growth, decreasing pigments and dry weight in the exponential growth phase of MWZ. Grettenberger et al. reviewed the limiting factors to Spirulina growth. These authors highlighted the accumulation of ROS at elevated levels of Cl-, negatively affecting photosynthesis [138]. However, in contrast to the higher plants, the cyanobacteria exhibited a high capacity to adapt to different environmental conditions and a significantly higher salt stress resistance. This adaptability has been supported in many studies conducted on natural populations of Spirulina [139-143] in a laboratory environment [144- 148] and outdoor mass cultures [142,148] exposed to different growth conditions.

At the late-logarithmic phase (plateau), MWZ showed no significant disparities in pigment concentration compared to DWZ, suggesting that the culture had adequately adapted to the

mine water composition. Moreover, the results were consistent with literature data reporting values of macronutrients ranging from 50-70% protein, 3-9% lipid, and 15-25% carbohydrate [3,149,150].

Pelizer et al. reported the biochemical composition of Spirulina grown at different inoculum levels. At the same inoculum concentration (200 $mg \cdot L^{-1}$), they reported a protein content of 55.2% DW, which matched the 52.6% DW observed in MWZ, but was higher than the 46.03% DW recorded in DWZ. In contrast, lipid and carbohydrate concentrations showed inverse trends compared to the present study, with carbohydrates being more abundant than lipids in both Trials. These differences in final lipid and carbohydrate concentrations can be attributed to variations in the culture system and experiment duration [151].

The increase in the protein-to-carbohydrate ratio from 1.45 to 2.05, and protein-to-lipid from 5.00 to 8.48 highlighted in MWZ, suggested that the mine water medium improved protein synthesis at the expense of carbohydrate and lipid accumulation. This behaviour agrees with literature data reporting that important levels of SO_4^{-2} increase Spirulina's growth performance and biochemical composition. According to Magwell et al, sulphate assimilation in microalgae involves activation by ATP sulphurylase, followed by reduction to sulphide, which is incorporated into cysteine, a precursor for methionine and other sulphur-containing biomolecules. Increased availability of sulphate stimulates key enzymes and promotes increased production of sulphur-containing amino acids. Supplementation with sulphate salts such as $MgSO_4$ and K_2SO_4 has been associated with increased biomass yield, protein content and cysteine and methionine levels, without affecting pigment or lipid profiles [152].

During the starvation phase and stress conditions, characterised by nitrogen limitation, an increase in lipids and carbohydrates was observed. In such conditions, excess carbon produced through photosynthesis or glycolysis is redirected towards the synthesis of carbon-rich compounds. The primary consequence of carbohydrate accumulation is the enhanced fixation of carbon into storage polysaccharides, such as glycogen or starch, which serve as an energy reserve. Concurrently, the process of lipid metabolism is promoted through multiple biochemical pathways, including the gamma-aminobutyric acid (GABA) pathway, glycolysis, and the tricarboxylic acid (TCA) cycle. This results in the conversion of surplus carbon into fatty acids and subsequent lipid storage [153, 154]. Literature data reporting Spirulina cultivation with wastewater from dairy, brewery and winery industries highlighted the low nitrogen concentration of the different medium leading to a decrease in the protein content and an increase in lipid and carbohydrates. [123,126, 127].

This suggested that nitrogen levels at the end of the present study were lower in DWZ than in MWZ.

9.5. Conclusions

The mine represents an inexhaustible water source at a constant temperature, ideal for growing Spirulina with significant energy savings and reductions in production costs. The mineral profile of mine water, which is characterized by a high concentration of SO_4^{2-}, can potentially enhance Spirulina's protein content and overall nutritional profile. The pigment concentration analysis showed similar values at the end of the experiment, indicating that the mine water could be successfully used for Spirulina cultivation. Spirulina strains confirmed the high adaptability to different environmental conditions during growth, allowing comparable final DW yields with the standard growing conditions represented by distilled water with the addition of the Zarrouk medium.

However, when dealing with water from mining sites, it is imperative to conduct rigorous and continuous water quality monitoring to manage risks from potential contaminants, such as trace metals or excess ions, to ensure that these factors do not compromise the safety and nutritional integrity of the biomass.

Moreover, integrating these techniques with renewable energy production systems can further save production costs and marketing opportunities.

The Spirulina biomass cultivated in mine water could have various applications. Its protein-rich content makes it suitable for nutritional applications, including use in animal feed and aquaculture, as well as a phagostimulant compound, while its bioactive compounds, such as phycocyanin and antioxidants, could be valuable for pharmaceutical, nutraceutical, and cosmetic applications. From an economic perspective, this strategy offers a competitive advantage by reducing dependency on conventional water sources and supporting the large-scale production of Spirulina-based products. From an environmental perspective, utilizing a readily available resource mitigates water wastage and promotes resource efficiency in accordance with European Blue Economy and Green Deal principles. From a social perspective, this approach can potentially drive job creation and stimulate local economies in mining regions.

10. Eco-innovative approach for the extraction of *Arthrospira platensis* to enhance antioxidants and nutritional value.

This chapter is based on the article:

Milia, M.; Pasquini, V.; Addis, P.; Angioni, A. Eco-Friendly Extraction to Enhance Antioxidants and Nutritional Value in *Arthrospira platensis*. *Foods* **2025**, *14*, 1510. https://doi.org/10.3390/foods14091510

10.1 Abstract

The cyanobacterium *Arthrospira platensis* (Spirulina) has a global production of approximately 18,000 tons annually. Spirulina has notable nutritional benefits and is a key component of dietary supplements. However, efficiently extracting its bioactive compounds poses challenges. This study aims to develop an eco-innovative method to enhance Spirulina's antioxidant and nutritional values. The extraction protocol included a maceration step in phosphate-buffered saline (PBS, pH 7.4) at 5 °C for 48 h, followed by ultrasound-assisted extraction (UAE) at 400 W, 30 kHz, with a frequency of 30 cycles per min (consisting of 1 s on/off cycles, for a total of six extraction cycles). The proposed methodology allows the quantitative recovery of high-value compounds from Spirulina raw material (control) with increased the yields of total lipids (+20.29%), total fatty acids (+60.48%), allophycocyanin (Apc, +41.41%), phycoerythrin (Pe, +81.42%), carotenoids (+30.84%), and polyphenols (+65.99%), leading to a boost in antioxidant activity (+42.95%). Conversely, the recoveries of proteins (-16.65%), carbohydrates (-18.84%), and phycocyanin (Pc, -0.77%) were incomplete. This study suggests a green extraction approach using PBS coupled with UAE, with promising energy and cost savings, for potential applications in the dietary supplement sector.

10.1. Introduction

Spirulina (*Cyanophyceae; Arthrospira spp*.) represents the most widely cultivated cyanobacteria, with over 18,000 tons of biomass produced annually, dominating the current microalgae market. The global Spirulina market size reached US$ 577.0 million in 2023. The International Market Analysis Research and Consulting Group (IMARC) expects the market to reach US$ 1,287.8 million by 2032, exhibiting a growth rate (CAGR) of 9.33% during the period 2024-2032 [155,156]. Spirulina is a fast-growing, adaptable, and protein-rich superfood [157] containing around 50-70% protein by dry weight [3, 158-160]. In addition, it has bioactive components with high therapeutic potential, such as polyphenols, carotenoids, phycobiliproteins (PBPs), carbohydrates, vitamins, sterols, polyunsaturated fatty acids (PUFAs), and minerals [161,162]. Accordingly, most of the world's Spirulina production is intended for dietary supplements, especially in sports nutritional products, to maintain, restore, or enhance energy levels and immune function, reducing oxidative stress and improving general well-being [163-165]. Dietary supplements containing Spirulina raw material or extracts are marketed as pills, tablets, capsules, liquids, and powders [166]. Spirulina-based products are characterized by a protective effect against muscle damage related to overtraining; they reduce blood lactate dehydrogenase, serum glucose, triglyceride, and cholesterol levels [167].

Spirulina amino acid composition fulfils FAO nutrition requisites [168]; however, the protein digestibility of microalgae used as single-cell protein represents a key limiting factor in humans and animals, decreasing the availability of essential amino acids [157, 169-171]. Devi et al. evaluated the amino acid digestibility of Spirulina in humans, implementing a dual isotope method. The authors found 85.2% means amino acid digestibility with values ranging from 77.5% (lysine) to 95.3% (phenylalanine) [172]. Relatively low algae amino acid digestibility has been related to the cell walls, macronutrient composition, enzyme specificity, anti-nutritional factors, fiber, and absorptive capacities within the gastrointestinal tract [4, 173-176].

Thus, the hydrolysis of covalent bonds in the cell wall matrix is recommended to facilitate proteins and other biochemical compounds extraction for their use in food and supplements applications, enhancing protein digestibility and solubility [177-181], extracting phenolics [182,183], Chl a [184, 30], PBPs [185-187], lipids and carotenoids [188-190]. Target compound recoveries are highly dependent on the extraction conditions applied. Conventional extraction depends on mechanical and chemical processes, with high production costs, long

extraction times, and elevated temperatures. Prolonged time and high temperature can lead to the degradation of bioactive compounds, reducing the extracts' overall bioactivity [191-194]. These approaches often result in the co-extraction of unwanted materials, loss of selectivity, and residual sol-vents hazardous to human health and the environment [195,196]. These limitations require efficient and environmentally friendly extraction technologies to save time and energy, using green solvents in line with ecologically sustainable strategies [197,198]. In recent years, microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) have shown promising capacity in terms of both extraction efficiency and eco-sustainability [7,199-202]. The cavitation movement imparted to the solution by UAE leads to the breaking of hydrophobic and hydrogen bonds, creating a series of microscopic channels with a sponge effect in the tissue, improving the solubility of proteins and allowing the compounds of interest to be released more efficiently [179-181] maintaining integrity and reducing the interference of impurities [200,203]. UAE has shown high efficiency, reduced solvent consumption, moderate extraction times and costs, and ease of management. Furthermore, it allows faster and more scalable production from the laboratory to the industry [204].

UAE technique, coupled with maceration as pre-treatment, showed increased ex-traction of bioactive compounds with high antioxidant activity [205,206].

However, some disadvantages should be considered when scaling up this technology to an industrial level. UAE can have low uniformity when big samples are processed since ultrasound waves decrease in intensity with distance from the emitter; moreover, pasty matrices reduce the penetration of the waves, which requires technical adjustment for proper action. The system scale-up needs higher energy consumption and problems in controlling the equipment parameters; therefore, to effectively have a broader impact, coupling with clean energy, such as wind power conversion, solar energy conversion and hydraulic conversion, is mandatory.

The extraction yield was influenced by the target compounds' polarity, chemical stability, solvent, temperature, and time [205,207]. Among the "green solvents", PBS is economical, non-toxic, and poses no risk of harmful residues or environmental contamination. PBS, maintaining a stable pH in the extraction environment, could prevent protein denaturation and lipid peroxidation and allow obtaining a high yield of polyphenols, phycobiliprotein, and photosynthetic pigments [167, 205,208].

In this work, the macronutrient and bioactive compound composition was investigated to evaluate the feasibility and efficacy of the UAE technique coupled with low-temperature PBS

maceration in extracting biomolecules from *Artrospira platensis* to obtain an extract with potential applications in the dietary supplement sector.

10.2. Methods

The methods applied are reported in Chapters 8.2.1 to 8.2.11.

10.2.1. Raw material and processing

Spirulina grown in raceway ponds were concentrated and dried in the factory following the commercial protocol (LiveGreen, Società Agricola SRL, Arborea, Oristano, Sardinia, Italy). The dried Spirulina was powdered with a porcelain mortar, and an exact amount was mixed with 300 mL of PBS (pH 7.4) with a ratio of 1:14 (w/v), according to Benedetti et al. [209, 55] and Tavanandi et al. [185, 56]. The suspension was mixed with a magnetic stirrer for 1 h, macerated for 48 h in the dark at 5 °C, and subjected to UAE using an ultra-sonic GM extractor 1 (GM Solution, Cagliari, Italy). The UAE operational condi-tions were 30 cycles/min at 100% power (400 W, 30kHz; 1 s ON and 1 s OFF) for 6 ex-tractions. Every 30 cycles, the solution was allowed to stand for 1 min. The entire process was carried out in an ice bath to avoid degradation of the target thermosensitive compounds. After centrifugation, the supernatant was collected, freeze-dried (LIO5P DIGITAL Cinquepascal srl, Trezzano sul Naviglio, Milan, Italy), and weighed. The biochemical analysis was carried out on the dried spirulina raw material (control) and the freeze-dried biomass from UAE extraction (extract).

10.2.2. Statistical analysis

The experiments were done in triplicate, and data were reported as the mean ± RSD%. One-way analysis of variance (ANOVA) was performed using the complete set of data acquired as a source of variation. Before performing the ANOVA test, variance homogeneity was confirmed with Cochran's test. The statistical analyses were performed using STATGRAPHICS PLUS 5.1 professional edition (Statistical Graphics Corp., Rockville, MD, USA).

10.3. Results

The biochemical analysis of the control and extract samples revealed significant differences in macronutrient composition (Table 1). After the extraction process, the total macronutrient content was as follows: total carbohydrates 12.63 ± 6.06, protein 52.23 ± 0.50, lipids 6.21 ± 5.61, fiber 0.35 ± 8.70, and ash 18.55 ± 0.64 g·100 g^{-1} (mean ± RSD% DW). During extraction, carbohydrates and protein were only partially recovered, at 81.16% and 83.35%, respectively. Conversely, lipids showed an increase of 20.29%, whereas the neutral detergent fiber (NDF) decreased by nearly 90% (Table 7). The ash fractions were significantly enriched, increasing by 120%.

Table 7. Biochemical composition (mean g·100 g^{-1} DW) and yield% of control and extract.

Macronutrient	Control	Extract	Yield% Control vs. Extract
	g·100 g^{-1} DW		
Carbohydrate	15.57 ^{ay}	12.63 ^{b}	−18.84
Lipid	5.16 ^{bx}	6.21 ^{a}	+20.29
Protein	62.66 ^{az}	52.23 ^{b}	−16.65
Fiber	3.38 ^{az}	0.35 ^{b}	−89.52
Ash	8.41 ^{bz}	18.55 ^{a}	+120.66

Means differences (a, b) between columns were tested using one-way ANOVA with Cochran's Correction for multiple comparisons. x=p < 0.05, y=p < 0.01, z=p < 0.001.

Total carotenoid and Chl a showed a higher value in the extracts of almost 30% (Table 2). Apc (4.58 ± 5.16 (mg·100 g^{-1} ± RSD% DW) and Pe (1.20 ± 4.33 (mg·100 g^{-1} ± RSD% DW) were higher in the extract, at +41% and +81% ($p < 0.001$), respectively. Meanwhile, the Pc concentration showed no significant differences ($p > 0.05$) between the control and the extract (Table 8).

Table 8. Photosynthetic pigments (mg·100 g^{-1}), phycobiliproteins, total polyphenols (mg·g^{-1} gallic ac. eq.), and antioxidant activity (mg·g^{-1} Trolox eq.) obtained in control and extract analysis

Bioactive Compounds	Control	Extract	Yield% Control vs. Extract
Chl a	0.49 ^{by}	0.64 ^{a}	+32.33
Total carotenoids	0.09 ^{by}	0.12 ^{a}	+30.84
Pc	10.46 ^{a}	10.38 ^{a}	−0.77
Apc	3.24 ^{bz}	4.58 ^{a}	+41.41
Pe	0.66 ^{bz}	1.20 ^{a}	+81.42
Total polyphenols	5.94 ^{bz}	9.86 ^{a}	+65.99
Vitamin B3	3.49 ^{az}	1.54 ^{b}	−55.87
DPPH assay	14.76 ^{by}	21.10 ^{a}	+42.95

Means differences (a, b) between columns were tested using one-way ANOVA with Cochran's Correction for multiple comparisons. x=p < 0.05, y=p < 0.01, z=p < 0.001

Total polyphenols concentration was 5.94 ± 2.05 and 9.86 ± 5.64 (mg·g^{-1} ± RSD% gallic ac. eq.) ($p < 0.001$), in control and extract, respectively. In addition, DPPH assay showed a higher value in the extract compared to the control, 21.10 ± 4.58 vs 14.76 ± 6.40 (mg·g^{-1} ± RSD% Trolox eq) (Table 8).

The vitamin B group (B1, B3, B2, B6, B9, and B12) and vitamin C were investigated in control and extracts; however, only B3 was detected with values almost half in the extract (1.54 ± 3.10 mg·100 g^{-1}± RSD%) compared to control (3.49 ± 5.04 (mg·100 g^{-1} ± RSD%) (Table 8).

Six compounds comprised 95% of the total fatty acids (FA), exhibiting an increase of nearly 60% in the extract. The extraction procedure did not alter the FA profile; palmitic acid and linoleic acid were the most abundant, averaging 48.35% and 33.40%, respectively. Oleic acid and g-linolenic acid accounted for 5.78% and 7.93%, followed by palmitoleic acid at 2.76%. Stearic acid accounts for only 1.78% of the total FA.

When comparing the fatty acids in raw material and the extract, palmitoleic acid, g-linolenic acid, and linoleic acid exhibited the most significant differences. Their concentrations varied from 0.53 ± 10.89% to 1.01 ± 7.12%, from 1.62 ± 9.36% to 2.77 ± 7.11%, and from 6.90 ± 6.63% to 11.51 ± 8.29% ($p < 0.01$) (mg·g^{-1} ± RSD% DW) in raw material and the extract, respectively (see Table 3). In contrast, palmitic, oleic, and stearic acids ranged from 10.41 ±

10.75% to 16.05 ± 8.90%, from 1.22 ± 10.56% to 1.94 ± 6.94 ($p < 0.05$), and from 0.40 ± 16.84% to 0.56 ± 17.47 (mg·g^{-1} ± RSD% DW) in raw material and the extract, respectively. Statistical analyses revealed significant differences in the concentrations (mg·g^{-1}) between the extract and raw material, with p values ranging from < 0.05 to < 0.01, except for stearic acid, which showed no significant difference ($p > 0.05$).

The total SFA, PUFA, and MUFA were statistically higher in the extracts. The PUFA/SFA rate showed a statistical difference between groups, while MUFA/SFA and PUFA/MUFA rates did not change (Table 9).

Table 9. Average total fatty acid (FAME) composition content (mg·g^{-1} DW) and yield% of extract vs raw material.

FAME	N° C	Control	Extract	Yield% Control vs. Extract
Palmitoleic acid	C 16:1	0.54 ^{by}	1.00 ^{a}	+88.62
Palmitic acid	C 16:0	10.40 ^{bx}	16.05 ^{a}	+54.18
γ-linolenic acid (GLA)	C 18:3 (n–6)	1.63 ^{by}	2.77 ^{a}	+71.15
cis-linoleic acid (LA)	C 18:2 (n–6)	6.90 ^{by}	11.5 ^{a}	+66.71
cis-Oleic acid	C 18:1 (n–9)	1.23 ^{bx}	1.94 ^{a}	+58.85
Stearic acid	C 18:0	0.40 ^{a}	0.56 ^{a}	+40.40
Total FAME (mg·g^{-1})		21.18 ^{by}	33.98 ^{a}	+60.48
Total PUFA (mg·g^{-1})		1.75 ^{by}	2.95 ^{a}	+67.55
Total MUFA (mg·g^{-1})		8.52 ^{bz}	14.28 ^{a}	+67.91
Total SFA (mg·g^{-1})		10.8 ^{by}	16.6 ^{a}	+53.67
PUFA/SFA		0.79 ^{bx}	0.86 ^{a}	
MUFA/SFA		0.16 ^{a}	0.18 ^{a}	
PUFA/MUFA		4.86 ^{a}	4.85 ^{a}	

Means differences (a, b) between columns were tested using one-way ANOVA with Cochran's Correction for multiple comparisons. x=p < 0.05, y=p < 0.01, z=p < 0.001.

10.4. Discussion

This paper reports on the results of using PBS coupled with UAE to evaluate the feasibility of producing an extract rich in bioactive compounds. Previous research and projects on Spirulina have used common laboratory solvents such as methanol, chloroform, and acetone to extract bioactive compounds, with standard extraction methods such as liquid–liquid and Soxhlet extraction, heat treatment, and maceration. The current trend is to replace solvents with green technologies to fulfil the EU directive on environmentally sustainable production. Therefore, the extraction conditions were selected based on the available literature supporting green solvents as a substitute for standard extraction procedures. Preliminary trials investigated specific solvent extraction rates before setting out the experiment (unpublished data).

The combined extraction method of PBS and UAE was chosen due to the ability of PBS buffer to stabilize proteins and maintain their structural integrity while effectively extracting phycobilin [210, 211]. In addition, it facilitates the extraction of water-soluble carbohydrates, particularly those with a low molecular weight or polar sugars; however, a pH greater than 6 could limit yields [212].

PBS also solubilizes polar lipids, such as glycerophospholipids (GPLs), and contributes to disrupting protein–lipid complexes. This allows for cell wall destabilization, enhancing membranes and improving the extraction of Chl a and carotenoids [213].

UAE was chosen for its capacity to perform cell disruption in less time and at a lower temperature than other methods, releasing bioactive compounds more efficiently. The combination of PBS and UAE results in a more efficient extraction system. PBS enhances the efficiency of UAE by stabilizing the compound structure (maintaining a stable pH) and increasing acoustic cavitation due to its salt content. This combined effect intensifies cell disruption and improves mass transfer, promoting the release of intracellular compounds from Spirulina [214].

The recent literature confirms the adaptability of Spirulina to various environmental conditions, including temperature, light intensity, and nutrient availability [188-190].

The observed biochemical composition of *Arthrospira platensis* (Table 1) aligns with data from the literature, which indicate that its protein content ranges from 50% to 70% dry weight (DW), carbohydrates comprise from 15% to 25% DW, and lipids account for 3% to 9% DW [215,216].

Following the extraction process, the total carbohydrate recovery was 81.16%, lower than the recovery reported by Liu et al. [217]. They utilized an ultrasound-assisted extraction (UAE)

pre-treatment at 500 W for 10 min, followed by hot water extraction at 80 °C, achieving a maximum recovery rate of 84.09%. In contrast, the current method employed milder heat conditions and shorter UAE times to minimize the risk of the loss and degradation of bioactive compounds, thereby preserving the nutritional quality of the extract, as reported by Shen et al., who reported that excessive ultrasound power, together with a high frequency and long extraction time, can damage active ingredients [218].

In addition, the findings of this study demonstrated higher recovery rates than those reported by Lupatini et al. [219], who achieved carbohydrate and protein recoveries ranging from 41.52% to 75.56% using more rigorous extraction conditions, including 35 min of sonication at 37 kHz (100% power) followed by 50 min of agitation under alkaline conditions (pH 9) at 30 °C.

The incomplete carbohydrate recovery stated in this study can be attributed to several factors. The cell wall is made of insoluble carbohydrates, such as glucan and peptidoglycan polymers, which form a multilayered structure stabilized by amino acid residues, which cause difficulties in extraction [220]. Additionally, uronic acids and extracellular polymeric substances form gels or aggregates that are more resistant to solubilization than intracellular polysaccharides. Finally, the solubility of glycogen is influenced by factors such as the type of solvent used, extraction temperature, pH, and extraction time [221].

Purdi et al. reported a high protein recovery of 76.83% when using UAE. The recovery shown in this paper (83.35%) confirm UAE's effectiveness in extracting proteins from the matrix [174]. Studies on pecan and lupin proteins have indicated that higher recoveries can be attributed to the extraction process's ability to effectively break hydrophobic and hydrogen bonds, thus releasing water-soluble proteins [179-181].

The unusually high ash values can be attributed to the salt added during extraction with the phosphate-buffered saline (PBS) solution.

The lipid content in the control was 5.16 g·100 g^{-1} (DW), which aligns with the literature reporting values between 4% and 9% [169, 222- 2241]. The complete breakdown of the cell wall resulted in a significantly higher lipid concentration in the extract, with an increase of 20.29% [188-190]. This phenomenon is attributed to UAE cavitation, which alters the structure and morphology of plant cell tissue, disrupting or deforming cell walls, making them more porous while breaking down the neutral detergent fiber (NDF) microstructure (including hemicellulose, cellulose, and lignin) [217]. This modification facilitates a higher extraction efficiency and leads to an overall decreased recovery of NDF at 10.48% [218].

Previous works have compared green extraction to standard solvent extractions of the lipid fraction. The yield obtained does not differ consistently among extraction methods; the highest lipid content was obtained with the Bligh and Dyer method (11.6%), followed by high-pressure ethanol (11.4%) [223]. The literature has identified palmitic acid (30–45%), linoleic acid (15–20%), and γ-linolenic acid (GLA) (20–30%) as the primary fatty acids characterizing the lipid profile of Spirulina [219, 221- 224]. The data reported in this paper confirm the trend of these constituents, although the absolute amounts cannot be directly compared due to differences in quantification methods.

The increased lipid content in the extracts could account for the higher levels of fatty acids observed, which showed an increase of nearly 60%, consistent with the reported literature [223]. The extraction process has been shown to selectively impact the polyunsaturated fatty acid (PUFA) to saturated fatty acid (SFA) ratio, resulting in a more favourable fatty acid composition [225].

Cyanobacteria synthesize chlorophyll a (Chl a) similarly to higher plants [226]. The Chl a content is influenced by various environmental parameters, including light intensity and nutrient availability, with nitrogen playing a critical role [227]. The reported concentrations of Chl a range from 0.5 to 1.1 mg·100 mg^{-1} DW [122,228], representing approximately 9–12% of the lipid fraction [184].

In the present study, the Chl a concentration in the control samples was measured at 0.49 mg·100 mg^{-1} DW, aligning with Orthesin et al.'s findings [229]. They extracted Chl a using a 90% methanol-based microwave-assisted extraction (MAE) protocol (40 W irradiation for 5 min), resulting in a maximum yield of 0.50 mg·100 mg^{-1} DW. However, the extracts we obtained from UAE showed higher values, reaching 0.64 mg·100 mg^{-1} DW (Table 2). In contrast, Park et al. reported lower values for Spirulina (0.26 and 0.47 mg·100 mg^{-1} DW) using acetone extraction with three consecutive 10 min sonication cycles [230]. These findings are consistent with Orthesin's findings, indicating a lower extraction efficiency for acetone than methanol.

Choi et al. reported nearly three times higher concentrations for *Arthrospira maxima* extracted with a 70% ethanol solution at 80 °C for 24 h (1.38 mg·100 mg^{-1} DW) and with the same solution at 40 °C for 4 h using UAE at 40 kHz (1.80 mg·100 mg^{-1} DW) [231]. The UAE method in their study resulted in a 31% increase in the Chl a concentration, comparable to the 30.61% increase observed in this experiment.

The phycobiliprotein extraction values in this study are consistent with the existing literature, which states that phosphate-buffered saline (PBS) helps to preserve the native structure and

stability of phycobiliproteins. UAE has proven effective in extracting these compounds without causing degradation [185-187, 232]. Chia et al. reported recoveries of −4.9% for phycocyanin (Pc) and −38% for allophycocyanin (Apc) from Spirulina using UAE (30% amplitude, 5 s on-5 s off, 10 min sonication) combined with liquid biphasic flotation (LBF) (100 cc·min−1 air flow rate, 7 min flotation time) [233]. Similarly, Obeid et al. reported a 5% recovery for Pc using UAE (12 kHz and 200 W) and membrane filtration (10 kDa-cutoff membranes) [234]. The results of this study, however, showed higher recoveries of −0.77% for Pc and +41.41% for Apc. Rodrigues et al. used UAE at 25 kHz and 25 °C for 30 min with ionic liquids at pH 6.5, reporting an extraction order of Apc > Pc > Pe; this study observed a different order of Pe > Apc > Pc, likely due to variations in extraction conditions [186].

Da Silva et al. reported a total polyphenol concentration of 2.07 ± 0.01 $mg \cdot g^{-1}$ of gallic acid equivalents (DW) from methanol extracts obtained through UAE technology [235]. In contrast, El-Baky et al. found values ranging from 4.51 to 16.96 $mg \cdot g^{-1}$ gallic acid eq. (DW) when Spirulina was grown in varying salinities and extracted by sonication with ethanol at 4 °C [236]. The extraction method used in this study allowed for the recovery of significant amounts of total polyphenols, which increased by nearly 70% in the extracts.

The DPPH assay showed an antioxidant activity of 21.10 ± 4.58 $mg \cdot g^{-1}$ Trolox eq. DW, consistent with Seghiri et al., who reported an antioxidant activity of 23 $mg \cdot g^{-1}$ Trolox eq. DW in Spirulina methanolic extracts [237]. An enhanced antioxidant activity is usually attributed to a synergistic effect among the components of the antioxidant pool [238-240]. In this study, polyphenols increased by almost 66%, phycobiliproteins by 12%, carotenoids by 31%, and Chl a by 32%. The antioxidant properties of Chl a alone are still in the preliminary stages of investigation. In contrast, the antioxidant effects of the other compound families are well known and have been reported in the literature. Polyphenols have a scavenging effect on free radicals and enhance antioxidant enzyme activity [237]. Phycobiliproteins have anti-inflammatory and antioxidant capacities by neutralizing hydroxyl, alkoxyl, and peroxyl radicals, stabilizing cell membranes [241]. Furthermore, carotenoids protect lipid membranes by quenching singlet oxygen, preventing lipid peroxidation, and donating electrons to neutralize radicals [242]. Thus, the increased antioxidant activity observed in this study may be linked to higher concentrations and the synergistic action of polyphenols, phycobiliproteins, and carotenoids in the extracts.

Data on *Arthrospira spp.* in the literature indicates significant levels of group B vitamins, especially vitamin B12 analogues and substitutes, and important amounts of the fat-soluble pro-vitamins A, K, and E [161,165]. However, in this study, only vitamin B3 was detected.

Factors such as strain, cultivation system, environmental conditions, and processing techniques can influence the preservation and concentration of vitamins in Spirulina [230]. The lower levels in the extracts may be attributed to the UAE process, which can create localized heat and free radicals that negatively impact the concentrations of heat-sensitive compounds, including vitamins [243].

10.5. Conclusions

The combined use of ultrasonic extraction (UAE) and maceration in phosphate-buffered saline (PBS) has proven effective in extracting bioactive compounds, including phenols, pigments (chlorophyll a, carotenoids, and phycobiliproteins), and essential fatty acids. These compounds significantly enhance the antioxidant properties of the extract. The process has shown satisfactory results in extracting total proteins and carbohydrates, consistent with existing literature; however, different extraction conditions should be explored to achieve higher yields of proteins and carbohydrates.

The cellulosic cell wall accounts for about 10% of the dry matter of algae, which impacts amino acids and other bioactive compounds accessibility, making them indigestible for humans and other non-ruminants. The resulting extracts have a low fiber level, allowing a higher nutrient bioavailability. Given this composition, the extracts have potential as a food supplement for athletes and bodybuilders, where the high protein content could aid in muscle recovery, especially in low-carbohydrate or ketogenic diets. The potential benefits of the extract include support for maintaining muscle mass and improving glycaemic management in individuals with insulin resistance or type 2 diabetes, due to its favourable ratio of polyunsaturated fatty acids (PUFAs) to saturated fatty acids (SFAs). Natural antioxidants are considered valuable therapeutic agents for reducing illnesses related to oxidative stress. The antioxidant effects of the extract could positively influence inflammation and play a preventive role in protecting against the generation of free radicals.

The methodology employed is still far from achieving conclusive laboratory-scale results and requires improvements to enhance recovery rates and to scale up for industrial applications. This could involve using green energy sources, which would lower processing costs and increase the product's value.

11. Physicochemical characteristics of phospholipid vesicles for Spirulina-based dietary supplement delivery

This chapter is based on the article submitted for publication:
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11.1. Abstract

Spirulina (*Arthrospira platensis*) is a cyanobacteria widely used as dietary supplement, in sports nutrition and in metabolic diseases such as diabetes, obesity and metabolic syndrome. Spirulina cell structure limits digestibility and reduces the availability of bioactive compounds. The extraction processes coupled with encapsulation can enhance the bioavailability of nutritional and antioxidant compounds, protecting them from degradation, preserving their functional activity, and supporting controlled release. The physicochemical properties of liposomes (Lps), bilosomes (Bls), and gelatin-enriched bilosomes (G-Bls) with incorporated Spirulina extracts were investigated. The delivery systems exhibited small particles size (101.8 ± 0.5 to 129.7 ± 1.2 nm), homogeneous distribution (polydispersity index (PDI) 0.17 ± 6.67 to 0.33 ± 9.06), negative surface charges (-31.9 ± 5.2 to 31.1 ± 6.4 mV) and high entrapment efficiency (> 80%). G-Bls demonstrated effective retention of the extract, with a low release rate at pH 1.2 (41.8% ± 6.1) and controlled release at pH 7.0 (52.5% ± 3.0). Biocompatibility studies on Caco-2 cells showed that G-Bls maintained high cell viability at 200 μg · mL^{-1} (87.89% ± 10.35), and significantly mitigated H_2O_2 induced oxidative stress at 20 and 200 μg · mL^{-1}, increasing cell viability by 23.47% and 19.28%. G-Bls are a promising delivery system for enhancing the stability, bioavailability, and protective effects of Spirulina extract, supporting their potential application in dietary supplements aimed at promoting sports performance and recovery, mitigating exercise-induced oxidative stress, and managing metabolic disorders.

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11.2. Introduction

Arthrospira platensis (Spirulina) is a filamentous, spiral-shaped prokaryotic cyanobacterium that thrives in extreme environments, withstanding high temperatures, light irradiation, and strong alkaline conditions. Spirulina is recognized for its high nutritional value characterized by a high protein content (50–70% DW), carbohydrates (15–25% DW), and important bioactive compounds including fatty acids, carotenoids, phycocyanin, and polyphenols [3,156,20]. The oral bioavailability of active compounds represents the bottleneck for Spirulina consumption. Moreover, Spirulina bioactive compounds can be subjected to degradation from enzymatic activity within the gastrointestinal tract, and their limited intestinal permeability. Therefore, the development of appropriate dietary supplements able to protect Spirulina bioactive compounds and boost their bioavailability represents an important challenge [244-248]. Dietary supplements are regulated in the European Union by the General Food Law Regulation (EC) No 178/2002 and are defined as concentrated sources of nutrients and bioactive compounds, such as vitamins, minerals, amino acids, essential fatty acids and plant extracts [64]. Dietary supplements can prevent physiological and nutritional deficiencies and are classified according to type and function. The most important factors that affect nutrient intake are ensuring proper nutrition, reducing the risk of age-related disorders, and protecting body tissues. [249]. Primary consumers include active adults particularly woman (25–65 years), from medium high level school education, peoples affected by metabolic conditions, and young individuals with healthy lifestyle conduct [250,251]. Among the various drug delivery systems liposomes are among those must studied. Liposomes are small, spherical vesicles with one or more phospholipid bilayers that mimic cell membranes, are composed of cholesterol and biocompatible phospholipids, with a diameter between 0.5 and 5 micrometres. Liposomes have hydrophobic and lipophilic characteristics and are extensively used for their ability to encapsulate proteins, carbohydrates, lipids, vitamins and minerals, facilitating targeted delivery, sustained release, and protection against chemical degradation, thereby enhancing metabolic efficacy [252,253]. There are many different types of liposomes, and many preparatory methods, which mostly include lipid solubilization by organic solvents, forming a thin lipid film by evaporation, hydration and physicochemical characterization of liposome properties [254]. Conventional liposomes have demonstrated instability within the gastrointestinal tract and diminished efficiency in retaining active agents, which limits their therapeutic application. [255]. To address these limitations, advanced lipid vesicles such as bile-salt enriched liposomes (Bls) have been developed. These vesicles

showed enhanced flexibility and structural integrity, and consequently achieved a higher entrapment efficiency, particularly for compounds that are poorly water-soluble [256]. The strong structure of these vesicles minimizes premature leakage under harsh acidic and enzymatic gastrointestinal conditions and improves permeation across the intestinal mucosa, enhancing oral bioavailability [257,258]. In recent years, increasing attention has been given to the development of gelatin-based phospholipid vesicles. Gelatin is a natural biopolymer derived from collagen hydrolysis, is highly valued for its biocompatibility, biodegradability, low immunogenicity, and is classified as "Generally Recognized as Safe" (GRAS) by the U.S. FDA. Its main structure comprises a three-dimensional shape formed for the 50% by three amino acids (glycine, proline, hydroxyproline) and lower amounts of alanine, arginine, aspartic and glutamic acid. Among various biopolymers, gelatin stands out for its ability to maintain particle size distribution and entrapment efficiency. Gelatin-based delivery systems enhance oral bioavailability and facilitate the uptake of active pharmaceutical ingredients by protecting them during the gastrointestinal transit [259,260].

However, aggregation, sedimentation in aqueous dispersions, and potential oxidation and lipid degradation persist, requesting further stabilization strategies [261]. Freeze-drying techniques utilizing sugars (e.g., mannitol, trehalose, or sucrose) or biopolymers (such as chitosan or alginate) as cryoprotectants have demonstrated significant efficacy in maintaining particle size distribution reducing aggregation and degradation and entrapment efficiency, thereby enhancing the long-term stability of the formulations. [262]. In this study different delivery systems consisting of liposomes (Lps), bilosomes (Bls), and gelatin-enriched bilosomes (G-Bls) incorporating Spirulina extract (Chapter 10) were developed to enhance stability, oral bioavailability, and controlled release of the bioactive compounds. The phospholipid vesicles were added of mannitol to increase the shelf life, the antioxidant capacity and the cytoprotective effects against oxidative stress induced by hydrogen peroxide in Caco-2 cells, was studied.

The phospholipid vesicles were evaluated in terms of mean diameter (MD), polydispersity index (PDI), surface charge (ζ potential), entrapment efficiency (EE%), shelf life, release profile, biocompatibility, and cytoprotective effects against hydrogen peroxide-induced oxidative stress in Caco-2 cells.

11.3. Methods

11.3.1. Phospholipid vesicles preparation

Phospholipid vesicles were prepared mixing Lipoid S75 (120 mg·mL^{-1}) and cholesterol (20 mg·mL^{-1}) in a glass vial (Lps), adding 20 mg·mL^{-1} of SDC for Bls and further 80 mg·mL^{-1} of gelatin for G-Bls.

The lipid mixtures were hydrated with 2 mL of PBS for 2 hours to facilitate phospholipid swelling, followed by sonication (4 s on / 2 s off, 15 cycles, 14 µm probe amplitude) using a high-intensity ultrasonic disintegrator (Soniprep 150, MSE Crowley, London, UK) to produce small and homogeneous vesicles. Spirulina extract (20 mg·mL^{-1}) was subsequently incorporated into the preformed vesicles and subjected to a second sonication step (4 s on/2 s off, 7 cycles, 10 µm probe amplitude), and a 5 min rest interval between cycles (Table 10).

Table 10. Composition of Lps, Bls, and G-Bls vesicles.

Vesicles	Extract	S75	Cholesterol	SDC	Gelatine	PBS
			mg·mL^{-1}			mL
Lps	20	120	20	-	-	2
Bls	20	120	20	20	-	2
G-Bls	20	120	20	20	80	2

11.3.2. Phospholipid vesicles physical characterization

Phospholipid vesicles mean diameters (MD, nm) and polydispersity index (PDI) were determined by photon correlation spectroscopy (Zetasizer ultra; Malvern Instruments, Worcestershire, UK). The surface charge (ζ potential, mV) was measured according to the mixed-mode measurement-phase analysis (M3-PALS). To ensure optical clarity and to prevent particle attenuation of the laser beam, each sample was diluted with water (1:100) before analysis. To remove the unloaded drug from dispersions, vesicles (1 mL) were dialyzed for 2 hours in 1 L of distilled water at 25 °C (Spectra/Por® dialysis membranes,12–14 kDa MW cut-off, 3 nm pore size; Spectrum Laboratories Inc., DG Breda, The Netherlands). The entrapment efficiency was calculated on the supernatant obtained after particle disruption in methanol (1:50, v/v), by comparing the antioxidant activity before and after the purification process using the DPPH (2,2-diphenyl-1-picrylhydrazyl) (Chapter 8.2.10) colorimetric test according to Paseban et al. [263]. Entrapment efficiency was further validated by quantifying Chl a total carotenoid (Chapter 8.2.6) and total polyphenols (Chapter 8.2.8).

11.3.3. Long term stability

Lps, Bls and G-Bls vesicles were prepared using mannitol as a cryoprotectant at 120 and 220 mg · mL^{-1} (maximum solubility in water at 25 °C). The phospholipid vesicles were freeze-dried in an Operon freeze-dryer (FDB-8603, Operon Advantech, Korea) at 0.4 mbar for 48 h, and stored at room temperature (25 °C) in hermetically sealed dark glass vials for 90 days. The powder was rehydrated to its original volume (2 mL), and the mean vesicle diameter, polydispersity index, and surface charge were measured.

11.3.4. pH sensitivity of phospholipid vesicles

To assess the pH-dependent stability of the vesicles, Lps, Bls, and G-Bls dispersions were incubated at 1% in acidic medium simulating pH gastric conditions (pH 1.2) for 2 hours and in the neutral medium simulating pH intestinal conditions (pH 7.0) for 6 hours at 37 °C. The incubation was performed in an orbital shaker-incubator (BS-1 ES20, BIOSAN, Rome, Italy) under constant stirring (170 rpm). After incubation, the MD and PDI were measured to assess vesicle stability under different pH conditions.

11.3.5. In vitro release of spirulina extract

Spirulina extract (2 mL) in aqueous dispersion or loaded in Lps, Bls, G-Bls were placed in polycarbonate dialysis tubes (Spectra/Por® membranes: 12–14 kDa MW cut-off, with pores of 3 nm; Spectrum Laboratories Inc., USA) and left under constant stirring (300 rpm) in a falcon filled with 25 mL of the acidic medium simulating pH gastric fluid (pH 1.20) at 37 °C for 2 h, and with 25 mL of a the neutral medium simulating pH intestinal fluid (pH 7.00) for 6 h. At selected times (0, 15, 45, 90, and 120 min in the gastric medium and 0, 1.5, 3, 4.5, and 6 hours in the intestinal medium), a 20 µL aliquot was withdrawn for analysis. The released extract was quantified using the DPPH colorimetric method (Chapter 8.2.10) and expressed as a percentage of antioxidant activity over time. To maintain sink conditions, an equal volume of fresh medium was added after each sampling [264]. All experiments were performed in triplicate.

11.3.6. Cell Culture and biocompatibility of phospholipid vesicles

The MTT colorimetric test was utilized to evaluate the biocompatibility of the dispersion and phospholipid vesicles [265]. The human colon adenocarcinoma (Caco-2, ATCC HTB-37, USA) cells were cultured as monolayers in 75 cm2 flasks at 37 °C with 100% humidity and 5% carbon dioxide. The growth media used was Dulbecco's Modified Eagle Medium (DMEM) containing glucose and L-glutamine. The media was supplemented with 10% fetal bovine serum, 1% penicillin (10,000 units·mL-1) and streptomycin (10,000 units·mL-1). Cells were cultured for 10 days to reach a fully differentiated state. Subsequently, cell division was halted for an additional 20 days. This step helps maintain the differentiated state and prevents the cells from proliferating, which could alter their mature characteristics. Following these steps, the cells were treated with trypsin and then re-plated into 96-well plates at a density of 1×10^4 cells per well and allowed to attach and stabilize for 24 h [264]. The cells were then exposed to spirulina extract dispersion, Lip, Bls, and G-Bls at concentrations of 2, 20, and 200 $\mu g \cdot mL^{-1}$ for 48 h.

After the treatment period, the cell culture medium was removed and replaced with a solution of MTT (0.5 $mg \cdot mL^{-1}$ in PBS, pH 7.40). The MTT reagent is metabolized by viable cells to form insoluble formazan crystals. After three hours the formazan crystals were dissolved in 100 µL of DMSO. The optical density of the resulting solution was measured at 570 nm using a microplate reader (Multiskan EX, Thermo Fisher Scientific, Inc., Waltham, MA, USA). Each sample was tested in triplicate, and untreated cells served as a positive control.

11.3.7. Protective effect of phospholipid vesicles against damage induced in cells by hydrogen peroxide (H_2O_2)

The ability to protect adenocarcinoma cells (Caco-2, ATCC HTB-37, USA) from oxidative stress by hydrogen peroxide (H_2O_2) was determined according to Castangia et al. [264]. Caco-2 cells were seeded in 96-well plates at a density of 1 × 10^4 cells per well and allowed to adhere for 24 hours. The cells were added with spirulina extract in dispersion or incorporated in Lps, Bls and G-Bls at 20 and 200 μg mL^{-1} of extract and exposed to oxidative stress by the addition of H_2O_2 at 1e-3 %. Caco-2 cells treated with H_2O_2, and untreated cells (representing 100% viability) were used as control samples. After 4 hours of incubation the cells were washed with PBS and their viability was assessed using the MTT assay (Chapter 11.3.6.).

11.3.8. Statistical analysis

All experiments were conducted in triplicate, and differences among the extracts were evaluated using ANOVA followed by Tukey's post-hoc test to identify significant pairwise differences or Dunnett's multiple comparisons ($p < 0.05$). Statistical analysis was performed with GraphPad Prims 8.3.0(538).

11.4. Results

11.4.1. Preparation and characterization of phospholipid vesicles

Pre-formulation studies were carried out with different concentrations of the extract (5, 10, 20, 30, and 40 mg·mL^{-1}), different amounts of Lipoid S75 (60 and 120 mg·mL^{-1}), SDC (10 and 20 mg·mL^{-1}), gelatin (40, 80, and 120 mg·mL^{-1}), and cryoprotectants (mannitol, sucrose, trehalose, lactose, and mannose). The optimal composition was selected based on vesicle stability, homogeneity, and entrapment performance and was used in the present study (data not reported).

The analysis showed no statistically significant differences ($p > 0.05$) in MD and PDI between Lps and Bls indicating a comparable size distribution. In contrast, G-Bls exhibited a significantly larger MD (129.7 ± 1.2 nm, $p < 0.0001$) and a higher PDI value (0.33 ± 9.06, $p < 0.01$), due to the physicochemical properties of gelatin. All phospholipid vesicles had comparable negative surface charge (ζ) ($p > 0.05$) ranging from -31.9 (Lps) to -31.1 (Bls) (Table 11).

Table 11. Characterization of vesicles by means of mean diameter (MD), polydispersity index (PDI), zeta potential (ZP) and entrapment efficiency (EE). Mean values ± standard deviations from at least 6 replicates are reported

Vesicle	MD (nm)	PDI	ζ Potential (mV)	EE (%) DPPH	EE (%) Tot. polyphen.	EE (%) Tot. carot.	EE (%) Chl a
Lps	101.8 ± 0.5 a*	0.19 a	-31.9 ± 5.2 a	94.7 ± 6.0 a	94.4 ± 11.2 a	99.2 ± 11.5 a	84.2 ±8.3 a
Bls	103.2 ± 1.5 a	0.17 a	-31.1 ± 6.4 a	89.5 ± 5.3 a	94.3 ± 10.3 a	89.9 ± 4.6 a	85.2 ± 11.0 a
G-Bls	129.7 ± 1.2 b$^{(e)}$	0.33 b$^{(c)}$	-31.3 ± 4.2 a	95.4 ± 3.8 a	82.1 ± 2.7 a	91.1 ± 1.8 a	86.6 ± 6.0 a

** Different letters in the column indicate statistical differences. Letter in brackets identified p value (b: $p < 0.05$; c: $p < 0.01$; d: $p < 0.001$; e: $p < 0.0001$).*

The antioxidant activity using the DPPH colorimetric assay, revealed no significant differences in quenching efficiency between the phospholipid vesicles ($p > 0.05$), with values ranging from 89.5 ± 5.3% for Bls to 95.4 ± 3.8% for G-Bls (mean ± RSD%).

EE% was confirmed by quantifying total polyphenols, chlorophyll a (Chl a), and total carotenoids. No statistically significant differences were observed among the tested formulations ($p > 0.05$), with EE% values ranging from 82.1 ± 2.7% for G-Bls (Tot polphenols) to 99.2 ± 11.5% for Lps (Tot carot), indicating a consistently high encapsulation performance across different bioactive components

Following a 15-day storage period at 5 °C, all phospholipid vesicles exhibited significant physicochemical changes indicative of instability, in comparison with freshly prepared. MD increased markedly ($p < 0.0001$), reaching 512.3 ± 11.1 nm for Lps, 443.8 ± 10.5 nm for Bls, and 466.1 ± 13.9 nm for G-Bls, suggestive of aggregation. PDI demonstrated a significant increase for Lps and Bls ($p < 0.0001$), and a more modest rise for G-Bls ($p < 0.01$) further reflecting colloidal destabilization. All formulations showed a significant increase in negative ζ-potential ($p < 0.01$). Therefore, vesicle preparations were integrated with mannitol at 120 and 220 mg · mL^{-1}. MD, PDI and ζ potential were assessed immediately after freeze-drying for 72 h and rehydration in water (1:100 v/v) (Figure 4).

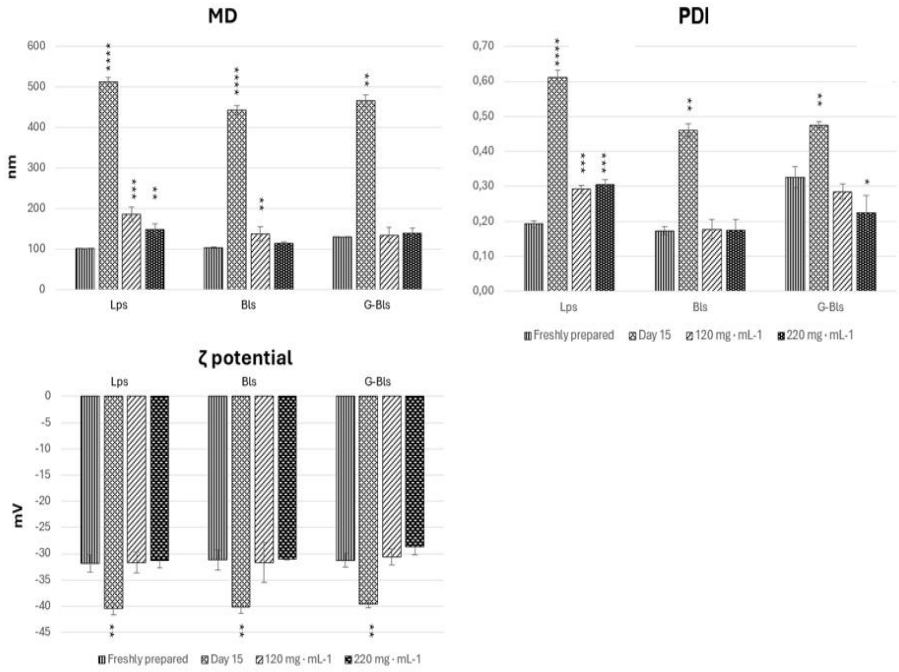


Figure 4. Physicochemical properties of the freshly prepared vesicles, after 15 days of storage at 5 °C and with mannitol addition followed by freeze-drying and rehydration. Data reported as mean values ± standard deviations (n = 6).

** p < 0.05, ** p < 0.01, *** p <0.001, **** p < 0.0001 express the statistical difference between extract free dispersion (control) and other groups, based on ANOVA Tuckey's comparison.*

MD of Lps increased from 101.8 ± 0.5 (mean nm ± RSD%) in the fresh phospholipid vesicles to 186.1 ± 9.5 ($p < 0.001$) with 120 mg·mL^{-1}of mannitol and t to 147.8 ± 9.8 nm ($p < 0.01$) at the 220 mg·mL^{-1} ($p < 0.05$, within the two concentrations). This trend suggests that mannitol concentrations have different effect depending to the concentration, promoting particle enlargement at lower concentration, possibly due to osmotic effects, and size reduction at higher amounts, potentially due to enhanced stabilization or prevention of aggregation. Bls exhibited a similar trend with increase from 103.2 ± 1.5 nm to 137.1 ± 17.6 nm at 120 mg·mL^{-1} mannitol ($p < 0.05$), and 115.0 ± 2.7 nm at 220 mg·mL^{-1} ($p > 0.05$). Between mannitol concentration there was no statistical difference ($p > 0.05$). G-Bls showed a modest size increase from 129.7 ± 1.5 nm to 134.7 ± 19.2 nm and 139.8 ± 12.7 nm without significant differences between the three experiments ($p > 0.05$). PI increased in Lps from 0.19 ± 0.01 (mean ± RSD%) to 0.29 ± 0.01 and 0.31 ± 0.01 ($p < 0.0001$) with crescent mannitol addition, suggesting a broader size distribution, while no differences were highlighted between

mannitol trials. In contrast, Bls maintained a consistent PI around 0.17-0.18 ($p > 0.05$), while G-Bls demonstrated a decrease trend from 0.33 ± 0.03 to 0.28 ±0.02 ($p > 0.05$) and 0.22 ± 0.05 ($p < 0.05$), indicating improved uniformity increasing mannitol content. The zeta potential remained stable across all phospholipid vesicles and within the two mannitol concentrations ($p > 0.05$). Freeze-dried solid samples were kept in the dark at 25 °C for 90 days to assess long-term stability. Afterwards, vesicles were rehydrated with water to the original volume (2 mL), equilibrated at room temperature for 30 minutes and measured to evaluate physicochemical characteristics.

The MD, PDI, and ζ potential of all vesicles remained stable over time, showing no significant differences ($p > 0.05$) compared to the aqueous dispersions at once after the freeze-drying process. Overall, G-Bls demonstrated optimal storability with negligible particle size variation, stable PDI, and minimal zeta potential changes, whereas Lps appeared the least stable due to their substantial size increase, Bls vesicles displayed intermediate stability (Table 12).

Table 12 Characterization of freeze-dried vesicles with mannitol added at 120 and 220 $mg \cdot mL^{-1}$ by means of mean diameter (MD), polydispersity index (PDI), ζ potential. Mean values ± standard deviations from at least 6 replicates are reported.

Phospholipid vesicles	Mannitol ($mg \cdot mL^{-1}$)	Days	MD (nm)	PDI	ζ potential (mV)
Lps	120	0	186.2 a*	0.29 a	-31.7 a
		90	199.8 a(a)	0.29 a(ab)	-29.3 a(ab)
	220	0	147.8 a	0.31 a	-31.3 a
		90	154.7 a(b)	0.32 a(a)	-31.3 a(a)
Bls	120	0	137.1 a	0.18 a	-31.7 a
		90	137.1 a(c)	0.18 a(c)	-31.7 a(a)
	220	0	115.0 a	0.17 a	-31.3 a
		90	121.0 a	0.22 a(c)	-28.7 a(b)
G-Bls	120	0	134.7 a	0.29 a	-30.7 a
		90	139.5 a(c)	0.31 a(a)	-31.3 a(a)
	220	0	131.8 a	0.22 a	-28.7 a
		90	131.6 a(c)	0.22 a(bc)	-30.3 a(ab)

**Different letters indicate statistical differences within vesicles among days of storage (T=0, T=90); letters in brackets indicate statistical differences among vesicles with equal mannitol addition at 90 d ($p > 0.05$).*

11.4.2. pH sensitivity of phospholipid vesicles

The stability of the phospholipid vesicles under simulated gastrointestinal conditions were evaluated in terms of MD and PDI (Table 4). Lps maintained structural stability across pH variations, allowing for controlled release without major disruptions showing a significant increase in size from 101.78 ± 0.48% (freshly prepared) to 141.0 ± 1.91% nm (mean ± RSD%) after 2 hours at pH 1.2, and 163.4 ± 16.5 nm after 6 hours at pH 7.0 ($p < 0.001$), and no statistical difference between the pH 1.2 and 7.0 ($p > 0.05$). Similarly, the PDI also increased from 0.19 ± 0.01 (freshly prepared) to 0.32 ± 4.04 at pH 1.2 and 0.39 ± 3.32 at pH 7.0 (both $p < 0.001$). Bls exhibited a significant increase in size from 103.2 ± 1.5 nm (freshly prepared) to 146.9 ± 3.2 nm at pH 1.2 ($p < 0.001$), and a decrease to 115.3 ± 1.16 nm at pH 7.0 ($p < 0.05$ relative to freshly prepared, $p < 0.001$ when comparing pH 1.2 vs. 7.0). The PDI increased from 0.17 ± 6.67 to 0.30 ± 16.50 at pH 1.2 ($p < 0.001$), and to 0.47 ± 2.67 at pH 7.0 ($p < 0.05$ compared to pH 1.2). The larger increase in size and heterogeneity at acidic pH suggests that Bls may undergo partial destabilization in gastric conditions. G-Bls showed the most substantial increase in size, from 129.73 ± 1.18 nm (freshly prepared) to 239.7 ± 2.9 nm at pH 1.2 ($p < 0.0001$), and 221.2 ± 1.3 nm at pH 7.0 ($p < 0.0001$, $p > 0.05$ compared to pH 1.2). The PDI decreased from 0.33 ± 9.06 to 0.22 ± 6.37 at pH 1.2 ($p < 0.01$), and to 0.20 ± 10.19 at pH 7.0 ($p < 0.001$, $p > 0.05$ compared to pH 1.2), suggesting that gelatin promoted significant swelling and size increase, but ensured a more controlled structure at physiological pH.

Table 4. Physicochemical properties of vesicles measured after exposure for 2 h at pH 1.2 and 6 h at pH 7.0.

	pH	MD (nm)	PDI
Lps	Freshly prepared	101.8 ± 0.5 c	0.19 b
	1.2 2h	141.0 ± 1.9 b**	0.32 a*
	7.0 6h	163.4 ± 1.6 ab**	0.39 a*
Bls	Freshly prepared	103.2 ± 1.6 c	0.17 c
	1.2 2h	142.9 ± 3.2 b****	0.30 b*
	7.0 6h	115.3 ± 1.2 a**	0.47 a***
G-Bls	Freshly prepared	129.7 ± 1.2 b	0.33 a
	1.2 2h	239.7 ± 2.9 a****	0.22 b**
	7.0 6h	221.2 ± 1.3 a****	0.20 b***

** Different letters indicate statistical differences within vesicles among trials (pH 1.2, and pH 7.0) and freshly prepared (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).*

11.4.3. *In vitro* release of Spirulina extract

At pH 1.2, the release followed the series Lps>Bls>G-Bls and was lower than the free dispersion (after 15 min ~24% vs ~40%) (Figure 5). After 2 hours, the extract dispersion released 54.2% ± 8.3, while vesicles showed similar release profiles ranged from 41.8% ± 6.2 in G-Bls to 45.5% ± 8.7 (mean ± SD%) in Lps ($p > 0.05$). This behaviour underscores the protective role of vesicles in limiting early drug exposure to the harsh gastric environment. At pH 7.0 the releasing trend remained the same, with Lps less efficient in preserving the extract. After 1.5 h, almost 50% was released from the vesicles, reaching 66.9 ± 15.6 after 6 h in Lps. The free dispersion reached 73.3% ± 2.9 at 6 hours, whereas Bls (58.9% ± 10.9, $p < 0.001$) and G-Bls showed the lower release, accounting for 52.5% ± 3.0 ($p < 0.0001$ compared to control and $p < 0.001$ respect with Lps) (mean ± SD%), suggesting that gelatin reinforced the vesicle structure, delaying diffusion. These trends are in line with MD and PDI studies. G-Bls were larger but more homogeneous and exhibited greater stability at low pH, but swelled at neutral pH, forming a denser matrix that restricted rapid payload diffusion, potentially extending the therapeutic window and reducing dosing frequency.

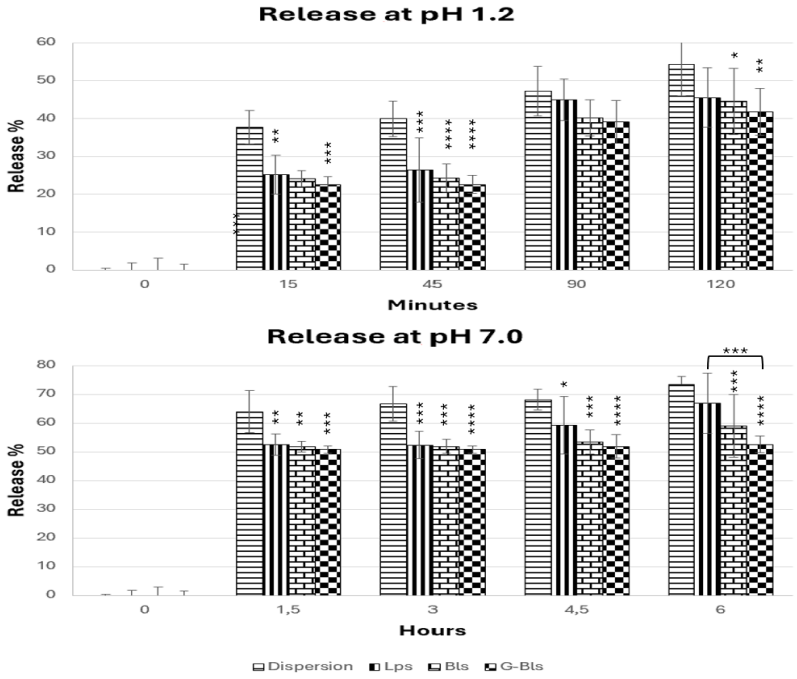


Figure 5. Release of the Spirulina extract from the dispersion and the vesicles, at pH 1.20 for 2 h and at pH 7.00 for 6 h.

** $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ express the statistical difference between extract free dispersion (control) and other groups, based on ANOVA Tuckey's comparison.*

11.4.4. Biocompatibility of Spirulina extract dispersion and phospholipid vesicles in CACO-2 cells

In vitro biocompatibility was assessed using human colon adenocarcinoma (Caco-2) cells treated with spirulina extract in dispersion and incorporated into G-Bls, Bls, Lps at final concentrations of 2, 20, and 200 $\mu g \cdot mL^{-1}$. Cell viability was determined using the MTT colorimetric test (Figure 6). The untreated control maintained a baseline viability of 100%. Treatment with the spirulina extract dispersion showed high biocompatibility at all tested concentrations, with viability ranging from 101.4% ± 7.4 (mean % ± RSD%) to 114.8% ± 19.8 ($p > 0.05$ compared to control and within concentrations). Lps exhibited an inverse trend with lower viability at 2 $\mu g \cdot mL^{-1}$ and 20 $\mu g \cdot mL^{-1}$, around 75%, and an increased viability at 200 $\mu g \cdot mL^{-1}$, accounting for 100.5% ± 12.5, suggesting cellular adaptation. Bls and G-Bls showed a similar trend with high biocompatibility at 2 $\mu g \cdot mL^{-1}$ (~125%), decreasing at higher concentrations of the extracts till ~ 87%.

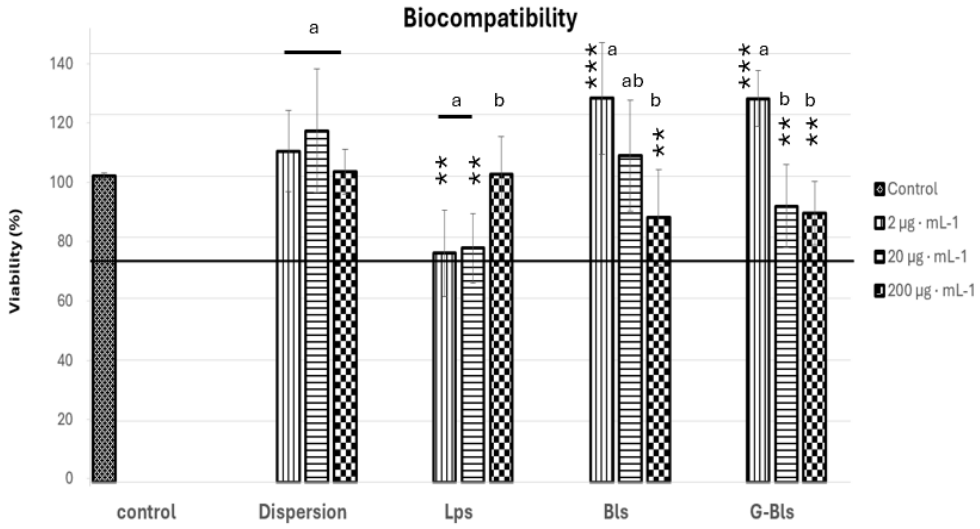


Figure 6. Viability of Caco-2 cells after 48 h of incubation with Spirulina extract dispersion or vesicles at three concentrations (2, 20, and 200 $\mu g \cdot mL^{-1}$). Data reported as mean values ± standard deviations (n = 6).

** $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ express the statistical difference between groups, based on ANOVA Tuckey's comparison. Letters indicated statistical differences within each group, based on ANOVA with Tukey's post hoc test.*

11.4.5. Protective effect of dispersion and phospholipid vesicles in CACO-2 cells against H_2O_2 oxidative stress induced.

The stressed control group exhibited a significant decrease in cell viability to 57.3% ± 11.4 compared to untreated cells ($p < 0.0001$), confirming the cytotoxic impact of oxidative stress. Treatment with Spirulina extract dispersion at 20 $\mu g \cdot mL^{-1}$ did not significantly improve viability, although a slight increase was observed (64.6% ± 11.5; $p > 0.05$), while at 200 $\mu g \cdot mL^{-1}$ viability significantly increased by 25.0% (71.7% ± 10.1; $p < 0.01$), indicating a dose-dependent protective response. Lps showed a slight improved viability by 14.5% (65.6% ± 9.5) at 20 $\mu g \cdot mL^{-1}$ with respect to stressed cells ($p > 0.05$) and by 25.4% (72.4% ± 9.1) at 200 $\mu g \cdot mL^{-1}$ ($p < 0.01$), suggesting enhanced delivery capacity at higher concentrations. Bls induced statistically significant improvements at both concentrations, with viability increasing by 19.52% (68.5% ± 5.5) at 20 $\mu g \cdot mL^{-1}$ ($p < 0.05$) and by 24.5% (71.4% ± 12.4) at 200 $\mu g \cdot mL^{-1}$ ($p < 0.01$) reflecting efficient bioactive protection even at the lower dose.

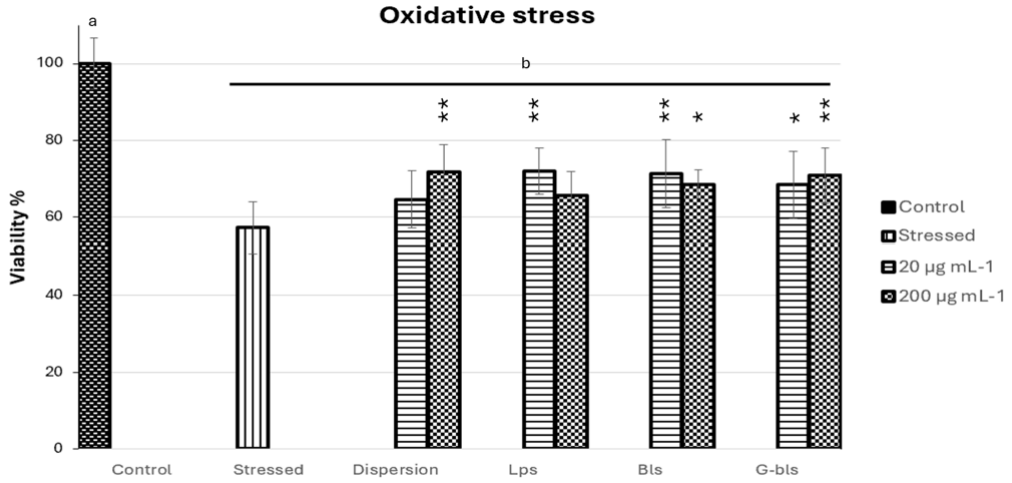


Figure 7. Viability of Caco-2 cells stressed with H_2O_2 and treated with Spirulina extract dispersion or vesicles at 20 and 200 $\mu g \cdot mL^{-1}$. Data are reported as mean percentages relative to untreated healthy cells (n = 8).

** Indicate statistical differences between H2O2 damaged cells (stressed) and cells treated with Dispersion, Lps, Bls, and G-Bls (** = p < 0.01, ***= p < 0.001, **** = p < 0.0001), based on ANOVA with Tukey's post hoc test (p < 0.05). Letters indicate statistical differences within untreated cells (control) and each group.*

11.5. Discussion

Phospholipid vesicles for oral delivery have a long history starting in the late 1970s, with the entrapment of drugs for pharmacological applications. The vesicles with particle sizes below 1000 nm, have demonstrated the ability to sustain, target, and traverse the intestinal biological barrier, thereby preserving the integrity of the bioactive agent [266]. Conventional liposomes have large particle size which hardly permeate the intestinal epithelia. This type of liposomes exploits the ability to release the active components into the gastrointestinal lumen and subsequent permeate the intestinal membrane. However, this approach is useful only on small molecules, when dealing with biomacromolecules it is mandatory to have liposomes able to protect from the intestinal environmental characteristics and to carry them through the gastrointestinal membrane. Among various oral delivery liposomes, Lps, Bls and G-Bls, represent promising delivery strategies for improving oral drug retention and controlled release in small intestine, intestine lymphatic, and colon. The physicochemical proprieties of phospholipid vesicles component is a key factor in determining their size, stability and aggregation tendencies, as well as their interaction with biological system [267,268]. Phospholipid vesicles loaded with spirulina extract demonstrated favourable size (101.8 to 129.7 nm), PDI (0.17-0.33), colloidal stability (-31.15 to -31.86 mV) and effective entrapment (over 82%). These results align with literature standards, where Lps vesicles typically measure 50–150 nm, Bls up to 200 nm [269,270], PDI ≤ 0.3 indicates uniform size distribution [271], and ζ potential above ±30 mV suggests good stability against aggregation [272]. Machado et al. observed larger lecithin-based phenolic Spirulina extract Lps (269–292 nm), higher PI (0.38–0.52), and milder zeta potentials (~–11 mV) [273]. Zewail et al. Reported Bls Spirulina-loaded Bls used as antiaging drug delivery system, with higher particle sizes (226.5–251.3 nm), PDI values (0.31–0.50), and similar zeta potentials ranging from –28.4 to –34.5 mV [274]. Slight variation in physic-chemical parameters was attributable to different composition. Entrapment efficiency (EE%) of the drug is a challenging analytical task. Unlike the study of EE% of a single active ingredient, the total amount of drug encapsulated is a complicated issue when dealing with natural extracts such as plant or micro and macroalgae extracts. HPLC analysis is a powerful technique to qualify and quantify active ingredients in complex matrices. However, the considerable number of compounds in the Spirulina extract and the different chemical classes limit the performance of HPLC. Therefore, the entrapment efficiency (EE%) was assessed using the DPPH assay, which provides an indirect functional estimate of the bioactive compound content retained within the vesicles. Antioxidant activity

remains high across all vesicles ranging between 89–95% ($p > 0.05$), suggesting that the entrapment process effectively preserved and protected loaded spirulina extract, confirming the proficiency of each system. To further substantiate these findings, the EE% was confirmed by quantifying total polyphenols, chlorophyll a (Chl a), and total carotenoids. Multiple studies have consistently demonstrated the high entrapment efficiency of Spirulina bioactive compounds in various nanocarrier systems. Machado et al. [273] and Gibis et al. [275] reported over 80% EE for polyphenols in liposomes, highlighting their strong interaction with the liposomal bilayer. Similar values were seen for chlorophyll a in protein-based particles (83.6–96.3%) by Agarry et al. [276]. Carotenoids also showed excellent incorporation, with Moraes et al. [277] reporting up to 100% EE for β-carotene, and Zewail et al. [274] confirming high EE% (85.2–89.5%) in Bls. Phospholipid vesicles that initially became unstable in aqueous dispersion after 15 days, were stabilized by freeze-drying with two mannitol concentrations and storing in solid form for 90 days. Using 220 $mg \cdot mL^{-1}$ of mannitol, resulted in a pronounced enhancement of stability, ensuring spatial isolation of particles, maintaining the desired physicochemical characteristics over extended storage [278] This effect was more evident in Bls and G-Bls, where sodium deoxycholate significantly enhanced freeze-drying stability by reducing interfacial tension and forming a protective lyophilic matrix, [279] while gelatin prevented ice-crystal damage, preserving particle size [280,281]. Gastrointestinal pH changes affected the structural stability of Lps and Bls, undergoing a partial destabilization with respect to freshly prepared vesicles. In this study, simulated gastro and intestinal fluids were used as a simplified in vitro model to investigate the preliminary behaviour of phospholipid vesicles in an aqueous environment mimicking the pH conditions of the gastrointestinal tract. Phospholipid vesicles for oral delivery face pH and ionic variations during gastrointestinal transit, which significantly affect their physicochemical and release properties. At pH 1.2, all formulations (Lps, Bls, G-Bls) showed increased size and reduced stability, with G-Bls exhibiting pronounced size enlargement but improved uniformity. Despite structural differences, similar release profiles (~42-46%) were observed, due to protonation-induced charge neutralization [282]. At pH 7.0, G-Bls were larger but more homogeneous and stable than Lps, which exhibited higher release (~67% vs. 53%), due to gelatin's network-forming properties, which support sustained drug release, potentially extending the therapeutic window and reducing dosing frequency [283,284]. Spirulina extract exhibited high biocompatibility and the absence of cytotoxic effects on Caco-2 cells at concentrations of 2, 20, and 200 $\mu g \cdot mL^{-1}$,confirming its safety. Lps showed a moderate reduction in cell viability at lower concentrations (2 and 20 $\mu g \cdot mL^{-1}$), with viability rates of

~75%, these results were > 70% limit conventionally considered as the threshold of cytotoxic potential in vitro, according to EN ISO 10993-5:2009 [285]. Notably, a concentration-dependent recovery toward 100% viability was observed at higher Lps doses. Bls formulations yielded enhanced cell viability (115–126%) at lower concentrations, showing a moderate decline to 82% at elevated doses. Finally, G-Bls showed similar behavior to Bls, with a reduction in cell viability at higher concentrations (89%). G-Bls showed enhanced safety profiles with respect to Lps and Bls at higher concentrations, which can be correlated to mucoadhesive properties that influence the uptake of nanoparticles by intestinal cells [286,287]. The spirulina extract mitigated H_2O_2-induced oxidative stress, thereby increasing the viability of Caco-2 cells in a dose-dependent manner. Specifically, the extract increased viability by 12.7% ($p > 0.05$ vs stressed cells) at a dose of 20 μg-mL-¹ and 25.0 % ($p < 0.01$) at 200 $\mu g \cdot mL^{-1}$. These observations corroborate the findings of Abdel-Moneim et al., who proved a similar dose-dependent radical-scavenging efficacy of spirulina extract [288]. Overall, both free and incorporated Spirulina extract treatments conferred significant cytoprotection against oxidative damage. These findings demonstrate that phospholipid vesicles preserve the antioxidant efficacy of bioactive compounds. This property makes them ideal for use as delivery systems in the development of dietary supplements, particularly those designed for sporting individuals, and in interventions targeting metabolic disorders associated with oxidative stress.

11.6. Conclusions

Phospholipid vesicle systems incorporating spirulina extract have been proved to exhibit optimal physicochemical attributes, characterised by uniform particle size, low polydispersity index, strong negative zeta potential and high encapsulation efficiency. These characteristics have been shown to remain constant over a 90-day storage period, thereby confirming the long-term stability of the systems. All formulations afforded protection of the active ingredient under gastric acidity and enabled pro release at intestinal pH, confirming their suitability for oral delivery. Notably, G-Bls exhibited superior biocompatibility on Caco-2 monolayers, an effect attributed to gelatin-mediated mucoadhesive interactions that enhance cellular uptake while mitigating the cytotoxicity typically associated with lipid carriers. Antioxidant evaluations revealed that vesicular preparations supported radical-scavenging efficacy comparable to the free extract, showing effective release and preservation of bioactivity. The data presented herein collectively underscores the significance of G-Bls as a delivery platform, which exhibits considerable promise in the development of advanced spirulina-based dietary supplements. These supplements are designed to address the specific requirements of athletes and to provide therapeutic benefits in the context of metabolic disorders associated with oxidative stress. It is recommended that future investigations include in vivo studies with the aim of elucidating bioavailability, pharmacokinetics and therapeutic outcomes. Such studies should also assess interactions with the gut microbiota, optimise dosing regimens and address long-term safety. Furthermore, it is essential to address commercial translation challenges, specifically process scalability, real-world stability and regulatory compliance.

12. Project conclusion, key limitations, and future research directions

This project established a sustainable, circular-bioeconomy framework for valorising Spirulina biomass via integrated cultivation, extraction and encapsulation, culminating in the development of an advanced Spirulina-based dietary supplement tailored for athletes and individuals with metabolic disorders.

Cultivation in sulphate rich mine water proved technically and economically viable, yielding biomass with enhanced protein and nutritional quality, comparable pigment and dry weight metrics to control, and markedly reduced energy costs and primary water inputs in line with European Blue Economy and Green Deal objectives.

An optimized extraction protocol combining phosphate buffered saline maceration with ultrasonic assisted extraction efficiently recovered phenolics, pigments, total fats with favourable PUFA/SFA ratio, yielding low fibre extracts with superior antioxidant activity ideal for rapid nutrient delivery in sports nutrition and glycaemic control. Although protein and carbohydrate recovery were satisfactory, further process refinement and scale-up are needed.

Encapsulation of Spirulina extract within gelatin-enriched liposomes, bilosomes and G-Bls produced nanometric vesicles with narrow size distributions, strong negative ζ-potentials and high entrapment efficiencies, while preserving antioxidant activity. Among these, G-Bls emerged as the best performing oral delivery vehicle. They demonstrated excellent gastric stability and a controlled, prolonged release at intestinal pH. In vitro assays on Caco-2 cells confirmed their superior biocompatibility and antioxidant efficacy, attributable to gelatin-mediated mucoadhesive interactions. Moreover, G-Bls retained their physicochemical properties for up to 90 days post-lyophilization. Incorporating G-Bls into dietary supplements provides an innovative way to promote health and well-being by utilising nanotechnology to enhance bioavailability, absorption and effectiveness of Spirulina bioactive ingredients, minimizes required dosages.

The findings of this study propose a viable model that enhances natural resources while concomitantly reducing costs, energy consumption and environmental impact. The project demonstrates how sustainability and innovation can act in synergy, transforming nutritional constraints into benefits for health, economic development, and competitiveness. Nevertheless, key limitations must be addressed. The variability in mine effluent composition and potential contaminants demands rigorous monitoring and standardized cultivation protocols.

Moreover, UAE technique require refinement to achieve higher efficiency and scalability, with future improvements potentially leveraging green energy technologies to enhance economic and environmental sustainability. Future work should focus on energy-integrated bioreactor design, detailed scale-up studies for both extraction and formulations, Additionally, long-term in vivo assessments of bioavailability, pharmacokinetics, gut microbiota interactions, and safety will be essential to accelerate regulatory approval, commercial adoption, and clinical translation of this cost-competitive, sustainable platform.

13. References

1. Belay, A. Biology and Industrial Production of Arthrospira (Spirulina). In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, 2nd ed.; Richmond, A., Hu, Q., Eds.; Elsevier: Amsterdam, The Netherlands, 2013; pp. 339–358. https://doi.org/10.1016/B978-0-12-397981-8.00014-9
2. Sinetova, M. A.; Kupriyanova, E. V.; Los, D. A. Spirulina/Arthrospira/Limnospira—Three names of the single organism. *Foods* **2024**, *13*(17), 2762. https://doi.org/10.3390/foods13172762
3. Milia, M.; Corrias, F.; Addis, P.; Chini Zitelli, G.; Cicchi, B.; Torzillo, G.; Andreotti, V.; Angioni, A. Influence of Different Light Sources on the Biochemical Composition of Arthrospira spp. Grown in Model Systems. *Foods*, **2022**, *11*, 399. https://doi.org/10.3390/foods11030399
4. Spínola, M. P.; Mendes, A. R.; Prates, J. A. Chemical Composition, Bioactivities, and Applications of Spirulina (Limnospira platensis) in Food, Feed, and Medicine. *Foods* **2024**, *13*, 3656. https://doi.org/10.3390/foods13223656
5. Athiyappan, K.D.; Routray, W.; Paramasivan, B. Phycocyanin from *Spirulina*: A Comprehensive Review on Cultivation, Extraction, Purification, and Its Application in Food and Allied Industries. *Food Hum.* **2024**, *2*, 100235. https://doi.org/10.1016/j.foohum.2024.100235
6. Spínola, M.P.; Costa, M.M.; Prates, J.A.M. Studies on the impact of selected pretreatments on protein solubility of Arthrospira platensis microalga. *Agriculture* **2023**, *13*, 221. https://doi.org/10.3390/agriculture13010221
7. Shen, L.; Pang, S.; Zhong, M.; Sun, Y.; Qayum, A.; Liu, Y.; Rashid, A.; Xu, B.; Liang, Q.; Ma, H.; Ren, X. A comprehensive review of ultrasonic assisted extraction (UAE) for bioactive components: Principles, advantages, equipment, and combined technologies. *Ultrason. Sonochem*. **2023**, *101*, 106646. https://doi.org/10.1016/j.ultsonch.2023.106646
8. Thevarajah, B.; Nishshanka, G.K.S.H.; Premaratne, M.; Nimarshana, P.H.V.; Nagarajan, D.; Chang, J.S.; Ariyadasa, T.U. Large-scale production of Spirulina-based proteins and c-phycocyanin: A biorefinery approach. *Biochem. Eng. J*., **2022**, *185*, 108541. https://doi.org/10.1016/j.bej.2022.108541
9. Razzak, S. A.; Bahar, K.; Islam, K. O.; Haniffa, A. K.; Faruque, M. O.; Hossain, S. Z.; Hossain, M. M. Microalgae cultivation in photobioreactors: Sustainable solutions for a greener future. *Green Chem. Eng.* **2024**, *5*(4), 418–439. https://doi.org/10.1016/j.gce.2023.10.004
10. Allied Market Research. *Spirulina Market Size, Share, Competitive Landscape and Trend Analysis Report, by Type, Application and Formulation: Global Opportunity Analysis and Industry Forecast, 2020–2027*. Available online: https://www.alliedmarketresearch.com/spirulina-market (accessed on 10 March 2025).

11. Rudzińska, M.; Grygier, A.; Knight, G.; Kmiecik, D. Liposomes as Carriers of Bioactive Compounds in Human Nutrition. *Foods* **2024**, *13*(12), 1814. https://doi.org/10.3390/foods13121814
12. Dwyer, J. T.; Coates, P. M.; Smith, M. J. Dietary supplements: Regulatory challenges and research resources. *Nutrients* **2018**, *10*(1), 41. https://doi.org/10.3390/nu10010041
13. Frei, R.; Gaucher, C.; Poulton, S. W.; Canfield, D. E. Fluctuations in Precambrian atmospheric oxygenation recorded by chromium isotopes. *Nature* **2009**, *461*(7261), 250-253. https://doi.org/10.1038/nature08266
14. Sánchez-Baracaldo, P.; Cardona, T. On the origin of oxygenic photosynthesis and Cyanobacteria. *New Phytol.* **2020**, *225*(4), 1440-1446. https://doi.org/10.1111/nph.16249
15. Sanchez, M.; Bernal-Castillo, J.; Rozo, C.; Rodríguez, I. *Spirulina (Arthrospira): An Edible Microorganism: A Review. Univ. Sci.* **2003**, *8*(1), 7-24.
16. Dangeard, P. Sur une algue bleue alimentaire pour l'homme: *Arthrospira platensis* (Nordst.) Gomont. *Actes Soc. Linn. Bordeaux Extr. PV* **1940**, *91*, 39-41.
17. Zarrouk, C. Contribution à l'étude d'une cyanophycée influencée de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de *Spirulina maxima* (Setch. et Gardner) Geitler. Ph.D. Thesis, University of Paris, Paris, France, 1966.
18. World Health Organization. The work of WHO: 1992–1993, biennial report of the Director-General to the World Health Assembly and to the United Nations. https://iris.who.int/handle/10665/39102
19. Ahsan, M.; Habib, B.; Parvin, M.; Huntington, T. C.; Hasan, M. R. A review on culture, production and use of spirulina as food for humans and feeds for domestic animals. FAO Fisheries and Aquaculture Circular (FAO), (1034). https://openknowledge.fao.org/handle/20.500.14283/I0424E
20. Shah, M. A. R.; Zhu, F.; Cui, Y.; Hu, X.; Chen, H.; Kayani, S. I.; Huo, S. (2024). Mechanistic insights into the nutritional and therapeutic potential of Spirulina (Arthrospira) spp.: Challenges and opportunities. *Trends Food Sci. Technol.* **2024,** *104648*. https://doi.org/10.1016/j.tifs.2024.104648
21. Ragusa, I.; Nardone, G. N.; Zanatta, S.; Bertin, W.; Amadio, E. Spirulina for skin care: A bright blue future. *Cosmetics*, **2021***,8*(1), 7. https://doi.org/10.3390/cosmetics8010007
22. Im, S. T.; Heo, S. Y.; Kang, N.; Heo, S. J.; Lee, S. H. The potential of Spirulina maxima extract-treated conditioned medium from adipose-derived mesenchymal stem cells for hair growth promoting effect on human dermal papilla cells. *Regenerative Therapy*, **2025**, *29*, 184-191. https://doi.org/10.1016/j.reth.2025.03.013
23. Gonçalves, J.; Freitas, J.; Fernandes, I.; Silva, P. Microalgae as biofertilizers: A sustainable way to improve soil fertility and plant growth. *Sustainability* **2023**, *15*, 12413. https://doi.org/10.3390/su151612413

24. Sarsekeyeva, F.K.; Sadvakasova, A.K.; Sandybayeva, S.K.; Kossalbayev, B.D.; Huang, Z.; Zayadan, B.K.; Allakhverdiev, S.I. Microalgae- and cyanobacteria-derived phytostimulants for mitigation of salt stress and improved agriculture. *Algal Res*. **2024**, *103686*. https://doi.org/10.1016/j.algal.2024.103686
25. Fisher, K.E.; Saban, K.E.; Broddrick, J.T.; Settles, A.M. Space Algae-2 Ground and Lunar Analog Studies in Preparation for Long-Duration Propagation of Cyanobacteria in Spaceflight. In *Proceedings of the 53rd International Conference on Environmental Systems (ICES)*, Louisville, Kentucky, USA, July 21–25, 2024; International Conference on Environmental Systems: Louisville, KY, USA, 2024; Abstract ICES-2024-184.
26. Fortune Business Insights. *Microalgae Market Size, Share & Industry Analysis, by Species (Spirulina, Chlorella, Nannochloropsis, Haematococcus, Isochrysis, Chlamydomonas, and Others), by Application (Food and Feed), and Regional Forecast, 2025–2032* [Report ID: FBI110314]. Available online: https://www.fortunebusinessinsights.com/microalgae-market-110314 (accessed on 10 March 2025)
27. Show, P. L. Global market and economic analysis of microalgae technology: Status and perspectives. *Bioresource Technology*, **2022**, *357*, 127329. https://doi.org/10.1016/j.biortech.2022.127329
28. Maddiboyina, B.; Vanamamalai, H. K.; Roy, H.; Ramaiah, G.; Kavisri, M.; Moovendhan, M. Food and drug industry applications of microalgae Spirulina platensis: A review. J. Basic *Microbiol*. **2023**, *63*, 573-583. https://doi.org/10.1002/jobm.202200704.
29. Business Research Insights. Spirulina market size, share, growth, and industry analysis, by type (Spirulina powder, Spirulina tablet, Spirulina extracts), by application (health products, feed) and regional forecast to 2033 (Report No. 118051). Available online: https://www.businessresearchinsights.com/market-reports/spirulina-market-118051 (accessed on 10 March 2025).
30. The Brainy Insights. Spirulina dietary supplements market size by form (powder, tablets/capsules, liquid and granules), end user (food and beverages, nutraceuticals, feed, and pharmaceuticals), distribution channel (offline and online), regions, global industry analysis, share, growth, trends, and forecast 2024 to 2033 (Report ID: TBI-14554). Available online: https://www.thebrainyinsights.com/report/spirulina-dietary-supplements-market-14554 (accessed on 29 October 2024).
31. Virtue Market Research. Spirulina market research report – segmentation by product type (Arthrospira platensis, Arthrospira maxima); by application (dietary supplements, food and beverages, pharmaceuticals, cosmetics and personal care, animal feed); by distribution channel (direct sales/B2B, retail stores, online retail/e-commerce); and region; size, share, growth analysis | forecast (2024–2030). Available online: https://virtuemarketresearch.com/report/spirulina-market (accessed on 29 October 2024).

32. Sharma, R.; Rana, A. ALGALELIXIRS: Unraveling the Multifaceted Impact of Spirulina in Human Health. *Food Biosci.* **2024**, *105365*. https://doi.org/10.1016/j.fbio.2024.105365.
33. Soni, R. A.; Sudhakar, K.; Rana, R. S. *Spirulina*–From Growth to Nutritional Product: A Review. *Trends Food Sci. Technol.* **2017**, *69*, 157–171. https://doi.org/10.1016/j.tifs.2017.09.010
34. Guo, Y.; Wu, L.; Zhu, Z.; et al. Investigation of Peptides Containing Branched-Chain Amino Acids from *Arthrospira platensis* Through a Peptidomics Workflow. *Food Bioprocess Technol.* **2024**, *17*, 516–527. https://doi.org/10.1007/s11947-023-03147-5
35. Calella, P.; Cerullo, G.; Di Dio, M.; Liguori, F.; Di Onofrio, V.; Galle, F.; Liguori, G. Antioxidant, Anti-Inflammatory and Immunomodulatory Effects of Spirulina in Exercise and Sport: A Systematic Review. *Front. Nutr.* **2022**, *9*, 1048258. https://doi.org/10.3389/fnut.2022.1048258
36. Kalafati, M.; Jamurtas, A. Z.; Nikolaidis, M. G.; Paschalis, V.; Theodorou, A. A.; Sakellariou, G. K.; Kouretas, D. Ergogenic and Antioxidant Effects of Spirulina Supplementation in Humans. *Med. Sci. Sports Exerc.* **2010**, *42*(1), 142–151. https://doi.org/10.1249/MSS.0b013e3181ac7a45
37. Naeini, F.; Zarezadeh, M.; Mohiti, S.; Tutunchi, H.; Ebrahimi Mamaghani, M.; Ostadrahimi, A. Spirulina Supplementation as an Adjuvant Therapy in Enhancement of Antioxidant Capacity: A Systematic Review and Meta-Analysis of Controlled Clinical Trials. *Int. J. Clin. Pract.* **2021**, *75*, e14618. https://doi.org/10.1111/ijcp.14618.
38. Prete, V.; Abate, A. C.; Di Pietro, P.; De Lucia, M.; Vecchione, C.; Carrizzo, A. Beneficial Effects of Spirulina Supplementation in the Management of Cardiovascular Diseases. *Nutrients* **2024**, *16*(5), 642. https://doi.org/10.3390/nu16050642
39. Hernández-Lepe, M. A.; Wall-Medrano, A.; Juárez-Oropeza, M. A.; Hernández-Torres, R. P. Spirulina and Its Hypolipidemic and Antioxidant Effects. *J. Med. Food* **2020**, *23*(7), 746–757. https://doi.org/10.1089/10966200152744463
40. Shah, M. A. R.; Zhu, F.; Cui, Y.; Hu, X.; Chen, H.; Kayani, S. I.; Huo, S. Mechanistic Insights into the Nutritional and Therapeutic Potential of *Spirulina* (Arthrospira) spp.: Challenges and Opportunities. *Trends Food Sci. Technol.* **2024**, *104648*. https://doi.org/10.1016/j.tifs.2024.104648
41. Ray, K. K.; Seshasai, S. R.; Wijesuriya, S.; Sivakumaran, R.; Nethercott, S.; Preiss, D.; Erqou, S.; Sattar, N. Effect of Intensive Control of Glucose on Cardiovascular Outcomes and Death in Patients with Diabetes Mellitus: A Meta-Analysis of Randomised Controlled Trials. *Lancet* **2009**, *373*, 1765–1772. https://doi.org/10.1016/S0140-6736(09)60697-8
42. Carrizzo, A.; Conte, G.M.; Sommella, E.; Damato, A.; Ambrosio, M.; Sala, M.; Scala, M.C.; Aquino, R.P.; De Lucia, M.; Madonna, M.; et al. Novel potent decameric peptide of Spirulina platensis reduces blood pressure levels through a PI3K/AKT/eNOS-dependent mechanism. *Hypertension* **2019**, *73*, 449–457. https://doi.org/10.1161/HYPERTENSIONAHA.118.11801.

43. Majewski, M.; Klett-Mingo, M.; Verdasco-Martin, C.M.; Otero, C.; Ferrer, M. Spirulina extract improves age-induced vascular dysfunction. *Pharm. Biol*. **2022**, *60*, 627–637. https://doi.org/10.1080/13880209.2022.2047209
44. Trotta, T.; Porro, C.; Cianciulli, A.; Panaro, M. A. Beneficial Effects of Spirulina Consumption on Brain Health. *Nutrients* **2022**, *14*(3), 676. https://doi.org/10.3390/nu14030676
45. Hutadilok-Towatana, N.; Reanmongkol, W.; Satitit, S.; Panichayupakaranant, P.; Ritthisunthorn, P. A Subchronic Toxicity Study of *Spirulina platensis*. *Food Sci. Technol. Res.* **2008**, *14*(4), 351–351. https://doi.org/10.3136/fstr.14.351
46. Hutadilok-Towatana, N.; Reanmongkol, W.; Panichayupakaranant, P. Evaluation of the Toxicity of Arthrospira (Spirulina) platensis Extract. J. Appl. Phycol. 2010, 22, 599–605. https://doi.org/10.1007/s10811-009-9499-5
47. Gutiérrez-Salmeán, G.; Fabila-Castillo, L.; Chamorro-Cevallos, G. Nutritional and Toxicological Aspects of *Spirulina* (*Arthrospira*). *Nutr. Hosp.* **2015**, *32*(1), 34–40. https://doi.org/10.3305/nh.2015.32.1.9001
48. Karkos, P. D.; Leong, S. C.; Karkos, C. D.; Sivaji, N.; Assimakopoulos, D. A. Spirulina in Clinical Practice: Evidence-Based Human Applications. *Evid. Based Complement. Alternat. Med.* **2011**, *2011*, 531053. https://doi.org/10.1093/ecam/nen058
49. Karkos, P.D.; Leong, S.C.; Karkos, C.D.; Sivaji, N.; Assimakopoulos, D.A. Spirulina in clinical practice: Evidence-based human applications. *Evid. Based Complement. Alternat. Med*., **2011**, *531053*. https://doi.org/10.1093/ecam/nen058
50. Lympaki, F.; Giannoglou, M.; Magriplis, E.; Bothou, D.L.; Andreou, V.; Dimitriadis, G.D.; Markou, G.; Zampelas, A.; Theodorou, G.; Katsaros, G.; Papakonstantinou, E. Short-term effects of Spirulina consumption on glycemic responses and blood pressure in healthy young adults: Results from two randomized clinical trials. *Metabolites*, 2022, **12**(12), 1180. https://doi.org/10.3390/metabo12121180
51. Machowiec, P.; Ręka, G.; Maksymowicz, M.; Piecewicz-Szczęsna, H.; Smoleń, A. Effect of Spirulina supplementation on systolic and diastolic blood pressure: Systematic review and meta-analysis of randomized controlled trials. *Nutrients*, 2021, **13**(9), 3054. https://doi.org/10.3390/nu13093054
52. Marles, R. J.; Barrett, M. L.; Barnes, J.; Chavez, M. L.; Gardiner, P.; Ko, R.; ... & Griffiths, J. United States Pharmacopeia Safety Evaluation of Spirulina. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*(7), 593–604. https://doi.org/10.1080/10408391003721719
53. Roy-Lachapelle, A.; Solliec, M.; Bouchard, M. F.; Sauvé, S. Detection of Cyanotoxins in Algae Dietary Supplements. *Toxins (Basel)* **2017**, *9*(3), 76. https://doi.org/10.3390/toxins9030076
54. Papadimitriou, T.; Kormas, K.; Vardaka, E. Cyanotoxin Contamination in Commercial Spirulina Food Supplements. *J. Consum. Prot. Food Saf.* **2021**, *16*, 227–235. https://doi.org/10.1007/s00003-021-01324-2

55. Marsan, D. W.; Conrad, S. M.; Stutts, W. L.; Parker, C. H.; Deeds, J. R. Evaluation of Microcystin Contamination in Blue-Green Algal Dietary Supplements Using a Protein Phosphatase Inhibition-Based Test Kit. *Heliyon* **2018**, *4*(3), e00573. https://doi.org/10.1016/j.heliyon.2018.e00573
56. World Health Organization. *Chemical fact sheets: Microcystins (cyanobacterial toxins).* Available online: https://www.who.int/publications/m/item/chemical-fact-sheets--microcystins-%28cyanobacterial-toxins%29 (accessed on 10 October 2024).
57. Al-Dhabi, N. A. Heavy Metal Analysis in Commercial Spirulina Products for Human Consumption. *Saudi J. Biol. Sci.* **2013**, *20*(4), 383–388. https://doi.org/10.1016/j.sjbs.2013.04.006
58. Kejžar, J.; Jagodic Hudobivnik, M.; Nečemer, M.; Ogrinc, N.; Masten Rutar, J.; Poklar Ulrih, N. Characterization of Algae Dietary Supplements Using Antioxidative Potential, Elemental Composition, and Stable Isotopes Approach. *Front. Nutr.* **2021**, *7*, 618503. https://doi.org/10.3389/fnut.2020.618503
59. Gallardo-Velázquez, T.; Osorio-Revilla, G.; Zuñiga-de Loa, M.; Rivera-Espinoza, Y. Application of FTIR-HATR Spectroscopy and Multivariate Analysis to the Quantification of Adulterants in Mexican Honeys. *Food Res. Int.* **2009**, *42*(3), 313–318. https://doi.org/10.1016/j.foodres.2008.11.003
60. Peluso, I.; Magrone, T.; Valencia, D. V.; Chen, C. O.; Palmery, M. Antioxidant, Anti-inflammatory, and Microbial-modulating Activities of Nutraceuticals and Functional Foods. *Oxid. Med. Cell. Longev.* **2017**, *2017*, 7658617. https://doi.org/10.1155/2017/7658617
61. Mazokopakis, E. E.; Karefilakis, C. M.; Tsartsalis, A. N.; Milkas, A. N.; Ganotakis, E. S. Acute Rhabdomyolysis Caused by Spirulina (Arthrospira Platensis). *Phytomedicine* **2008**, *15*(6–7), 525–527. https://doi.org/10.1016/j.phymed.2007.10.002
62. Iwasa, M.; Yamamoto, M.; Tanaka, Y.; Kaito, M.; Adachi, Y. Spirulina-associated Hepatotoxicity. Am. J. *Gastroenterol.* **2002**, *97*(12), 3212–3213. https://doi.org/10.1111/j.1572-0241.2002.06099.x
63. ElFar, O.A.; Billa, N.; Lim, H.R.; Chew, K.W.; Cheah, W.Y.; Munawaroh, H.S.H.; Show, P.L. Advances in delivery methods of *Arthrospira platensis* (spirulina) for enhanced therapeutic outcomes. *Bioeng.* **2022**, *13*(1), 10245–10261. https://doi.org/10.1080/21655979.2022.2100863
64. European Parliament; Council of the European Union. *Regulation (EC) No 178/2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety*. Off. J. Eur. Union 2002, L 31, 1–24. Available online: https://eur-lex.europa.eu/eli/reg/2002/178/oj (accessed on 10 March 2025).
65. European Parliament; Council of the European Union**.** Regulation (EC) No 852/2004 on the hygiene of foodstuffs. *Off. J. Eur. Union* **2004**, *L 139*, 1–54. Available online: https://eur-lex.europa.eu/eli/reg/2004/852/oj (accessed on 10 March 2025).

66. European Parliament; Council of the European Union**.** Regulation (EU) No 1169/2011 on the provision of food information to consumers. *Off. J. Eur. Union* **2011**, *L 304*, 18–63. Available online: https://eur-lex.europa.eu/eli/reg/2011/1169/oj (accessed on 10 March 2025).
67. European Commission. Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods; Article 13(1). Official Journal of the European Union L 404, 30 December 2006; pp. 9–25. Available online: https://eur-lex.europa.eu/eli/reg/2006/1924/oj/eng (accessed on 10 March 2025)
68. European Commission. EU register of health claims. In *Nutrition and Health Claims*; Labelling and Nutrition; Food Safety, Directorate-General for Health and Food Safety, European Commission: Brussels, Belgium, 2025. Available online: https://food.ec.europa.eu/food-safety/labelling-and-nutrition/nutrition-and-health-claims/eu-register-health-claims_en (accessed on 10 March 2025)
69. European Parliament; Council of the European Union**.** Regulation (EU) 2015/2283 on novel foods, amending Regulation (EU) No 1169/2011 and repealing Regulations (EC) No 258/97 and (EC) No 1852/2001. *Off. J. Eur. Union* **2015**, *L 327*, 1–22. Available online: https://eur-lex.europa.eu/eli/reg/2015/2283/oj (accessed on 10 March 2025).
70. European Parliament and Council. Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on Food Additives. *Official Journal of the European Union*, L 354, 31 December 2008, pp. 16–33. Publications Office of the European Union: Luxembourg, 2008. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32008R1333 (accessed on 10 March 2025).
71. European Parliament; Council of the European Union. Regulation (EU) 2018/848 on organic production and labelling of organic products, and repealing Council Regulation (EC) No 834/2007. *Off. J. Eur. Union* **2018**, *L 150*, 1–92. Available online: https://eur-lex.europa.eu/eli/reg/2018/848/oj (accessed on 10 March 2025).
72. Dietary Supplement Health and Education Act of 1994. *Public Law 103-417*, 103rd Congress, 1994. Available online: https://www.congress.gov/bill/103rd-congress/senate-bill/784/text (accessed on 10 March 2025).
73. U.S. Food and Drug Administration**.** 21 CFR § 73.530 – Spirulina extract. *Electronic Code of Federal Regulations*. Available online: https://www.law.cornell.edu/cfr/text/21/73.530 (accessed on 10 March 2025).
74. WHO. Codex Alimentarius: Food Standards, Guidelines, and Codes of Practice. In *Codex Alimentarius 2023: Ensuring Food Safety and Quality Standards*, Proceedings of the World Health Organization Conference, Geneva, Switzerland, March 2023; FAO, Ed.; World Health Organization: Geneva, Switzerland, 2023. Available online: https://www.fao.org/fao-who-codexalimentarius/en/

75. European Commission. Innovating for Sustainable Growth: A Bioeconomy for Europe. In *Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions*, Proceedings of the EU Strategy for a Sustainable Bioeconomy, Brussels, Belgium, February 2012; Publications Office of the European Union: Luxembourg, 2012; COM(2012) 60 final. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52012DC0060 (accessed on 10 March 2025).
76. European Commissio**n.** Blue Growth: Opportunities for Marine and Maritime Sustainable Growth. In *Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions*, Proceedings of the EU Maritime Policy Framework, Brussels, Belgium, September 2012; Publications Office of the European Union: Luxembourg, 2012; COM(2012) 494 final. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52012DC0494 (accessed on 10 March 2025).
77. European Commission. Innovation in the Blue Economy: Realising the Potential of Our Seas and Oceans for Jobs and Growth. In Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions, Proceedings of the EU Maritime and Innovation Policy Framework, Brussels, Belgium, May 2014; Publications Office of the European Union: Luxembourg, 2014; COM(2014) 254 final. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52014DC0254 (accessed on 10 March 2025).
78. European Commission. Report on the Development of Plant Proteins in the European Union. In Communication from the Commission to the European Parliament and the Council, Proceedings of the EU Strategy on Sustainable Protein Sources, Brussels, Belgium, November 2018; Publications Office of the European Union: Luxembourg, 2018; COM(2018) 757 final. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52018DC0757 (accessed on 10 March 2025).
79. European Commission. The European Green Deal. In *Communication from the Commission to the European Parliament, the European Council, the Council, the European Economic and Social Committee and the Committee of the Regions*, Proceedings of the EU Strategy for Sustainable Growth and Climate Neutrality, Brussels, Belgium, December 2019; Publications Office of the European Union: Luxembourg, 2019; COM(2019) 640 final. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52019DC0640 (accessed on 10 March 2025).
80. European Commission. A Farm to Fork Strategy for a Fair, Healthy and Environmentally-Friendly Food System. In *Communication from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions*, Proceedings of the European Green Deal Policy Framework, Brussels, Belgium, May 2020; Publications Office

of the European Union: Luxembourg, 2020; COM(2020) 381 final. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:52020DC0381 (accessed on 10 March 2025).

81. European Parliament; Council of the European Union. Regulation (EU) 2021/695 Establishing Horizon Europe – the Framework Programme for Research and Innovation, Laying Down its Rules for Participation and Dissemination. In *Official Journal of the European Union*, Proceedings of the European Framework for Research and Innovation, Brussels, Belgium, April 2021; Publications Office of the European Union: Luxembourg, 2021; L 170, 1–68. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32021R0695 (accessed on 10 March 2025).
82. European Parliament; Council of the European Union**.** Regulation (EU) 2021/2115 Establishing Rules on Support for Strategic Plans to Be Drawn Up by Member States under the Common Agricultural Policy (CAP Strategic Plans). In *Official Journal of the European Union*, Proceedings of the EU Agricultural and Rural Development Framework, Brussels, Belgium, December 2021; Publications Office of the European Union: Luxembourg, 2021; L 435, 1–186. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32021R2115 (accessed on 10 March 2025).
83. European Commission. EU4Algae: European Algae Stakeholder Platform. In European Algae Initiative for Sustainable Growth and Innovation, Proceedings of the Launch of the EU4Algae Stakeholder Platform, Brussels, Belgium, 2022; Publications Office of the European Union: Luxembourg, 2022. Available online: https://eu4algae.eu (accessed on 10 March 2025).
84. European Commission**.** BlueInvest Readiness Assistance Programme: Supporting Investment in Sustainable Blue Economy Startups and SMEs. In *Blue Economy Investment and Innovation Framework*, Proceedings of the European Commission Blue Economy Initiatives, Brussels, Belgium, 2020; Publications Office of the European Union: Luxembourg, 2020. Available online: https://blue-invest.eu (accessed on 10 March 2025).
85. Kirk, P.L. Kjeldahl method for total nitrogen. Anal. Chem., 1950, 22(2), 354-358. https://doi.org/10.1021/ac60038a038.
86. DuBois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.T.; Smith, F. Colorimetric method for determination of sugars and related substances. Anal. Chem., 1956, 28(3), 350-356. https://doi.org/10.1038/168167a0
87. Chen, Y.; Vaidyanathan, S. A simple, reproducible and sensitive spectrophotometric method to estimate microalgal lipids. *Anal. Chim. Acta* **2012**, *724*, 67-72. https://doi.org/10.1016/j.aca.2012.02.049
88. Pasquini, V.; Biancacci, C.; Milia, M.; Moccia, D.; Solari, P.; Angioni, A.; Addis, P. "Effects of different culture media on growth, composition, quality and palatability of the green algae Ulva sp. cultivated in cylindrical photobioreactors", *Algal Res*. **2024**, *84*, 103749. https://doi.org/10.1016/j.algal.2024.103749

89. Van Soest, P.V.; Robertson, J.B.; Lewis, B.A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci*., **1991**, *74*(10), 3583-3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.
90. Singh, M.; Singh, M.P. Chlorophyll content and its relation to productivity of crops and weeds in a tropical agroecosystem at Gujar Lake Margin, India. *Ecoprint* **2016**, *23*, 39–44. https://doi.org/10.3126/eco.v23i0.20650.
91. Chamizo, S.; Adessi, A.; Torzillo, G.; De Philippis, R. Exopolysaccharide features influence growth success in biocrust-forming cyanobacteria, moving from liquid culture to sand microcosms. *Front. Microbiol.* **2020**, *11*, 2719. https://doi.org/10.3389/fmicb.2020.568224.
92. Dianursanti, D.; Hafidzah, M.A. The enhancement of phycocyanin yield and purity from Spirulina platensis using freeze-thawing method on various solvents. In AIP Conference Proceedings (Vol. 2344, No. 1), 5th International Symposium of Biomedical Engineering, ISBE 2020 - Depok, Virtual, Indonesia, 28/07/2020-29/07/2020. https://doi.org/10.1063/5.0047581.
93. Zavřel, T.; Chmelík, D.; Sinetova, M.A.; Červený, J. Spectrophotometric determination of phycobiliprotein content in cyanobacterium Synechocystis. *J. Vis. Exp.,* **2018**, *139,* e58076. https://doi.org/10.3791/58076.
94. Manconi, M.; Marongiu, F.; Castangia, I.; Manca, M.L.; Caddeo, C.; Tuberoso, C.I.G.; D'Hallewin, G.; Bacchetta, G.; Fadda, A.M. Polymer-associated liposomes for the oral delivery of grape pomace extract. *Colloids Surf. B Biointerfaces*, **2016,** *146,* 910-917. https://doi.org/10.1016/j.colsurfb.2016.07.043.
95. Breuer, G.; Evers, W.A.; de Vree, J.H.; Kleinegris, D.M.; Martens, D.E.; Wijffels, R.H.; Lamers, P.P. Analysis of fatty acid content and composition in microalgae. *J. Vis. Exp.* **2013**, (*80*). https://doi.org/10.3791/50628.
96. Manca, M.L.; Castangia, I.; Zaru, M.; Nácher, A.; Valenti, D.; Fernàndez-Busquets, X.; Fadda, A.M.; Manconi, M. Development of curcumin loaded sodium hyaluronate immobilized vesicles (hyalurosomes) and their potential on skin inflammation and wound restoring. *Biomaterials*, **2015**, *71*, 100-109. https://doi.org/10.1016/j.biomaterials.2015.08.034.
97. Rakuša, Ž.T.; Grobin, A.; Roškar, R. A comprehensive approach for the simultaneous analysis of all main water-soluble vitamins in multivitamin preparations by a stability-indicating HPLC-DAD method. *Food Chem.* **2021**, *337*, 127768. https://doi.org/10.1016/j.foodchem.2020.127768.
98. Thakura, A.; Sharmaa, D.; Sainib, R.; Suhagc, R.; Thakur, D. Cultivating blue food proteins: Innovating next-generation ingredients from macro and microalgae *Biocatal. Agric. Biotechnol*. **2024**, *60*, 103278. https://doi.org/10.1016/j.bcab.2024.103278
99. Pulz, O.; Gross, W. Valuable products from biotechnology of microalgae. *Appl. Microbiol. Biotechnol*. **2004**, *65*, 635–648. https://doi.org/10.1007/s00253-004-1647-x

100. Su, M.; Bastiaens, L.; Verspreet, J.; Hayes, M. Applications of microalgae in foods, pharma and feeds and their use as fertilizers and biostimulants: Legislation and Regulatory aspects for consideration. *Foods* **2023**, *12,* 3878. https://doi.org/10.3390/foods12203878
101. Camacho, F.; Macedo, A.; Malcata, F. Potential industrial applications and commercialization of Microalgae in the functional food and feed industries: A Short Review. *Mar. Drugs* **2019**, *17*, 312. https://doi.org/10.3390/md17060312
102. Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A. Commercial applications of microalgae. *J. Biosci. Bioeng.* **2006,** *101*(6), 201-211. https://doi.org/10.1263/jbb.101.87
103. Andreotti, V.; Chindris, A.; Brundu, G.; Vallainc, D.; Francavilla, M.; García, J. Bioremediation of aquaculture wastewater from Mugil cephalus (Linnaeus, 1758) with different microalgae species. Chem. *Ecol.*, **2017**, *33*(8), 750–761. https://doi.org/10.1080/02757540.2017.1378351
104. Andreotti, V.; Solimeno, A.; Rossi, S.; Ficara, E.; Marazzi, F.; Mezzanotte, V.; García, J. Bioremediation of aquaculture wastewater with the microalgae Tetraselmis suecica: Semi-continuous experiments, simulation and photo-respirometric tests. *Sci. Total Environ*. **2020,** *738*, 139859. https://doi.org/10.1016/j.scitotenv.2020.139859
105. Chacón-Lee, T.L.; González-Mariño, G.E. Microalgae for "Healthy" Foods—Possibilities and Challenges. *Compr. Rev. Food Sci. Food Saf*. **2010**, *9*, 655-675. https://doi.org/10.1111/j.1541-4337.2010.00132.x
106. Market Research reports. Spirulina Market Analysis & Forecast: 2025-2032. https://www.coherentmarketinsights.com/market-insight/spirulina-market-972#:~:text=Spirulina%20Market%20Size%20and%20Trends,7.8%25%20from%202025%20to%202032. (accessed on 03 April 2025)
107. Sili, C.; Torzillo, G.; Vonshak, A. Arthrospira (Spirulina). In Ecology of Cyanobacteria II: Their Diversity in Space and Time; Whitton, B.A., Ed.; Springer Science+Business Media B.V.: Berlin/Heidelberg, Germany, 2012.
108. Sarker, N.K.; Kaparaju, P. A critical review on the status and progress of microalgae cultivation in outdoor photobioreactors conducted over 35 Years. *Energies*, **2023**, *16*, 3105. https://doi.org/10.3390/en16073105
109. Vieira Costa, J.A.; Bastos Freitas, B.C.; Martins Rosa, G.; Moraes, L.; Greque Morais, M.; Mitchell, B.G. Operational and economic aspects of Spirulina-based biorefinery. *Bioresour. Technol*. **2019**, *292*, 121946. https://doi.org/10.1016/j.biortech.2019.121946
110. Nogueira, S.; Souza Junior, J.; Damasceno Maia, H.; Sousa Saboya, J.P.; Lobo Farias, W.R. Use of Spirulina platensis in treatment of fish farming wastewater. *Rev. Ciênc. Agron*. **2018**, *49*(4), 599-606. https://doi.org/10.5935/1806-6690.20180068
111. Depraetere, O.; Foubert, I.; Muylaert, K. Decolorisation of piggery wastewater to stimulate the production of Arthrospira platensis. *Bioresour. Technol*., **2013**, *148*, 366–372. https://doi.org/10.1016/j.biortech.2013.08.165

112. Papadopoulos, K.P.; Economou, C.N.; Markou, G.; Nicodemou, A.; Koutinas, M.; Tekerlekopoulou, A.G.; Vayenas, D.V. Cultivation of Arthrospira platensis in brewery wastewater. *Water*, **2022**, *14*, 1547. https://doi.org/10.3390/w14101547

113. Spennati, E.; Casazza, A.A.; Dehghani, F.; Perego, P.; Converti, A.; Padula, M.P.; Valtchev, P. Winery waste valorisation as microalgae culture medium: A step forward for food circular economy. *Sep. Purif. Technol.*, **2022**, *293*, 121088. https://doi.org/10.1016/j.seppur.2022.121088

114. Ljubic, A.; Safafar, H.; Holdt, S.L.; Jacobsen, C. Biomass composition of Arthrospira platensis during cultivation on industrial process water and harvesting. *Appl. Phycol*. **2018**, *30*(1), 943–954. https://doi.org/10.1007/s10811-017-1332-y

115. Athanasiadou, V.; Klontza, E.E.; Dimitriou-Christidis, P.; Fountoulakis, M.; Lekkas, D.F. Evaluation of Arthrospira (Spirulina) platensis growth on cheese whey in the context of circular economy. Sustain. *Chem. Pharm*. **2023**, *34*, 101173. https://doi.org/10.1016/j.scp.2023.101173

116. Nicknig, M.A.; Azevedo, A.C.D.; de Oliveira, H.A.; Schneider, I.A.H. Cultivation of microalgae (Scenedesmus sp.) using coal mining wastewater and separation via coagulation/flocculation and dissolved air flotation (DAF). *Minerals,* **2024**, *14*, 426. https://doi.org/10.3390/min14040426

117. Levett, A.; Gagen, E.J.; Levett, I.; Erskine, P.D. Integrating microalgae production into mine closure plans. *J. Environ. Manag.*, **2023**, *337*, 117736. https://doi.org/10.1016/j.jenvman.2023.117736

118. Bigi, S.; Tartarello, M.C.; Ruggiero, L.; Graziani, S.; Beaubien, S.E.; Lombardi, S. On-going and future research at the Sulcis site in Sardinia, Italy –characterization and experimentation at a possible future CCS pilot. *Energy Procedia*, **2017**, *114*, 2742 – 2747. http://creativecommons.org/licenses/by-nc-nd/4.0/).

119. Anderson, M.J.; Gorley, R.N.; Clarke, K.R. PERMANOVA+ for primer: Guide to software and statistical methods. PRIMER-E, Auckland, New Zealand, 2008.

120. Anderson, M.J.; Willis, T.J. Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. *Ecology*, **2003**, *84*, 511–525. https://doi.org/10.1890/0012-9658(2003)084 [0511: CAOPCA]2.0.CO;2

121. Vonshak, A. Laboratory techniques for the culturing of microalgae. In Handbook for microalgal mass culture (Richmond, A. (ed.)), CRC Press, Boca Raton, FL, 1986, 117-45.

122. Ragaza, J.A.; Hossain, M.S.; Meiler, K.A.; Velasquez, S.F.; Kumar, V. A review on Spirulina: alternative media for cultivation and nutritive value as an aquafeed. *Rev. Aquac*. **2020**, *12*(4), 2371–2395. https://doi.org/10.1111/raq.12439

123. AlFadhly, N.K.; Alhelfi, N.; Altemimi, A.B.; Verma, D.K.; Cacciola, F. Tendencies affecting the growth and cultivation of genus Spirulina: An investigative review on current trends. *Plants*, **2022**, *11*(22), 3063. https://doi.org/10.3390/plants11223063

124. Ansari, F.A.; Singh, P.; Guldhe, A.; Bux, F. Microalgal cultivation using aquaculture wastewater: Integrated biomass generation and nutrient remediation. *Algal Res*., **2017**, *21*, 169–177. http://dx.doi.org/10.1016/j.algal.2016.11.015

125. Lowrey, J.; Brooks, M.S.; McGinn, P.J. Heterotrophic and mixotrophic cultivation of microalgae for biodiesel production in agricultural wastewaters and associated challenges—a critical review. *J. Appl. Phycol.*, **2014**, *27*, 1485-1498. http://dx.doi.org/10.1007/s10811-014-0459-3

126. Nasir, N.M.; Bakar, N.S.; Lananan, F.; Abdul Hamid, S.H.; Lam, S.S.; Jusoh, A. Treatment of African catfish, Clarias gariepinus wastewater utilizing phytoremediation of microalgae, Chlorella sp. with Aspergillus niger bio-harvesting. *Bioresour. Technol*., **2015**, *190*, 492-498. http://dx.doi.org/10.1016/j.biortech.2015.03.023

127. Lananan, F.; Abdul Hamid, S.H.; Din, W.N.S.; Ali, N.A.; Khatoon, H.; Jusoh, A.; Endut, A. Symbiotic bioremediation of aquaculture wastewater in reducing ammonia and phosphorus utilizing effective microorganism (EM-1) and microalgae (Chlorella sp.). *Int. Biodeterior. Biodegrad*., **2014**, *95*, 127-134. http://dx.doi.org/10.1016/j.ibiod.2014.06.013

128. Wang, Y.; Ho, S.; Cheng, C.; Guo, W.; Nagarajan, D.; Ren, N.; Lee, D.; Chang, J. Perspectives on the feasibility of using microalgae for industrial wastewater treatment. *Bioresour Technol*., **2016**, *222*, 485–497. http://dx.doi.org/10.1016/j.biortech.2016.09.106

129. Yusni, E.; Hartono, E.S. Growth of Spirulina sp. microalgae in the media of fish waste treatment from Cemara market, Medan, North Sumatera. *Earth Environ. Sci*., **2021**, *782*, 042002. http://dx.doi.org/10.1088/1755-1315/782/4/042002

130. Yuan, D.; Yaoa, M.; Wanga, L.; Lia, Y.; Gonga, Y.; Hu, Q. Effect of recycling the culture medium on biodiversity and population dynamics of bio-contaminants in Spirulina platensis mass culture systems. *Algal Res.*, **2019**, *44,* 101718. https://doi.org/10.1016/j.algal.2019.101718

131. Mufidatun, A.; Koerniawan, M.D.; Siregar, U.J.; Suwanti, L.T.; Budiman, A.; Suyono, E.A. The Effect of pH on Contamination Reduction and Metabolite Contents in Mass Cultures of Spirulina (Arthrospira platensis Gomont). Int. *J. Adv. Sci. Eng. Inf. Technol*., **2023**, *13*(1), 84-90. https://doi.org/10.18517/ijaseit.13.1.16582

132. Al-Dhabi, N.A. Heavy metal analysis in commercial Spirulina products for human consumption. Saudi *J. Biol. Sci*., **2013**, *20*(4), 383–388. https://doi.org/10.1016/j.sjbs.2013.04.006.

133. Pereira, M.I.B.; Aguiar, E.M.; Chagas, B.M.E.; Borba, L.H.F.; Sassi, R.; Medeiros, G.F.; Silva, E.P.E.; Rangel, A.H.N. Mixotrophic cultivation of Spirulina platensis in dairy wastewater: Effects on the production of biomass, biochemical composition and antioxidant capacity. *PLoS ONE*, **2019**, *14*(10): e0224294. https://doi. org/10.1371/journal.pone.0224294

134. Athanasiadou, V.; Klontza, E.E.; Dimitriou-Christidis, P.; Fountoulakis, M.; Lekkas, D.F. Evaluation of Arthrospira (Spirulina) platensis growth on cheese whey in the context of circular economy. *Sustain. Chem. Pharm.*, **2023**, *34*, 101173. https://doi.org/10.1016/j.scp.2023.101173.

135. Papadopoulos, K.P.; Economou, C.N.; Markou, G.; Nicodemou, A.; Koutinas, M.; Tekerlekopoulou, A.G.; Vayenas, D.V. Cultivation of Arthrospira platensis in Brewery Wastewater. *Water*, **2022**, *14*, 1547. https://doi.org/10.3390/w14101547

136. Spennati, E.; Casazza, A.A.; Dehghani, F.; Perego, P.; Converti, A.; Valtchev, P. Winery waste valorisation as microalgae culture medium: A step forward for food circular economy. *Sep. Purif. Technol*., **2022**, *293* 121088. https://doi.org/10.1016/j.seppur.2022.121088

137. Mohsenpour, S.F.; Hennige, S.; Willoughby, N.; Adeloye, A.; Gutierrez, T. Integrating micro-algae into wastewater treatment: A review. *Sci. Total Environ*., **2021**, *752* 142168. https://doi.org/10.1016/j.scitotenv.2020.142168

138. Grettenberger, C.L.; Abou-Shanab, R.; Hamilton, T.L. Limiting factors in the operation of photosystems I and II in cyanobacteria. *Microb. Biotechnol*., **2024**, *17*(8), e14519. https://doi.org/10.1111/1751-7915.14519

139. Komárek, J.; Lund, J.G. What is Spirulina platensis in fact? In Archiv für Hydrobiologie. Supplementband. Untersuchungen des Elbe-Aestuars, 1st ed.; 1990; Vol. 85, pp. 1–13.

140. Vonshak, A.; Tomaselli, L. Arthrospira (Spirulina): Systematics and Ecophysiology. In The Ecology of Cyanobacteria, 2nd ed.; Whitton, B.A.; Potts, M., Eds.; Springer: Dordrecht, The Netherlands, 2000; pp. 505–522. https://doi.org/10.1007/0-306-46855-7_18

141. Woldie, A.A.; Chowdhary, A.K.; Sekine, M.; Kishi, M.; Zegeye, M.B.; Kurosawa, N.; Toda, T. Growth characteristics and molecular identification of indigenous Limnospira strains from Ethiopian soda lakes as a protein source. *Biocatal. Agric. Biotechnol*., **2024**, *60*, 103336. https://doi.org/10.1016/j.bcab.2024.103336

142. Etisa, D.; Mebrat, Y. Recent changes in the phytoplankton community of Soda Lake Chitu, Ethiopia, in response to some environmental factors. *Fish. Aquat. Sci*., **2024**, *27*(1), 23–34. https://doi.org/10.47853/FAS.2024.e4

143. Verma, K.; Mohanty, P. Changes of the photosynthetic apparatus in Spirulina cyanobacterium by sodium stress. *Z. Naturforsch. C*, **2000**, *55*(1–2), 16–22. https://doi.org/10.1515/znc-2000-1-205

144. Gong, H.; Tang, Y.; Wang, J.; Wen, X.; Zhang, L.; Lu, C. Characterization of photosystem II in salt-stressed cyanobacterial Spirulina platensis cells. *Biochim. Biophys. Acta Bioenerg*., **2008**, *1777*(6), 488–495. https://doi.org/10.1016/j.bbabio.2008.03.018

145. Saif, A.H.A. Indoor and Outdoor Culture of Spirulina (Arthrospira platensis) Grown in Different Salinity in Sultanate of Oman. Master's Thesis, University Putra Malaysia, Selangor, Malaysia, 2017

146. Ho, S.H.; Liao, J.F.; Chen, C.Y.; Chang, J.S. Combining light strategies with recycled medium to enhance the economic feasibility of phycocyanin production with Spirulina platensis. *Bioresour. Technol*., **2018**, *247*, 669–675. https://doi.org/10.1016/j.biortech.2017.09.165

147. Beshi, M.; Assaye, H.; Tilahun, G.; Kishi, M.; Tanaka, K.; Addisu, S.; Toda, T. Optimization of Growth Conditions for Arthrospira fusiformis in Laboratory and Outdoor Cultivation Settings. *SSRN*, **2024**, *4917755*. https://ssrn.com/abstract=4917755

148. Jeeji, B.; Cv, S. On coiling and uncoiling of trichomes in the genus Spirulina. *Algol Stud.*, **1980**, *26*, 32–47.

149. Torzillo, G.; Vonshak, A. Environmental Stress Physiology with Reference to Mass Cultures. In Handbook of microalgal culture: applied phycology and biotechnology, 1st ed.; Richmond, A., Hu, Q., Eds.; Wiley: Chichester, UK, 2013; pp. 154–196. https://doi.org/10.1002/9781118567166.ch6.

150. Habib, M.A.B.; Parvin, M.; Huntington, T.C.; Hasan, M.R. A Review on Culture, Production and Use of Spirulina as Food for Humans and Feeds for Domestic Animals and Fish. FAO Fisheries and Aquaculture Circular. No. 1034 2008, FAO, Rome. Available from: http://www.fao.org/3/i0424e/i0424e00.pdf.

151. Pelizer, L.H.; Danesi, E.D.G.; de O Rangel, C.; Sassano, C.E.; Carvalho, J.C.M.; Sato, S.; Moraes, I.O. Influence of inoculum age and concentration in Spirulina platensis cultivation. *J. Food Engin.*, **2003**, *56*(4), 371–375. https://doi.org/10.1016/S0260-8774(02)00209-1

152. Magwell, P.F.R.; Minyaka, E.; Fotsop, O.W.; Leng, M.S.; Lehman, L.G. Influence of sulphate nutrition on growth performance and antioxidant enzyme activities of Spirulina platensis. *J. Agric. Sci*. **2021**, *13*(10), 115. https://doi.org/10.5539/jas.v13n10p115

153. Udayan, A.; Pandey, A.K.; Sirohi, R.; Sreekumar, N.; Sang, B.I.; Sim, S.J.; Pandey, A. Production of microalgae with high lipid content and their potential as sources of nutraceuticals. *Phytochem. Rev.*, **2023**, *22*(4), 833–860. https://doi.org/10.1007/s11101-021-09784-y

154. Tarazona Delgado, R.; Guarieiro, M.D.S.; Antunes, P.W.; Cassini, S.T.; Terreros, H.M.; Fernandes, V.D.O. Effect of nitrogen limitation on growth, biochemical composition, and cell ultrastructure of the microalga Picocystis salinarum. *J. Appl. Phycol*., **2021**, *33*, 2083–2092. https://doi.org/10.1007/s10811-021-02462-8

155. Spirulina Market Report by Species (Arthrospira Platensis, Arthrospira Maxima), Form (Powder, Tablet and Capsule, Liquid, Granule and Gelling Agent), Distribution Channel (Business to Business, Business to Consumer), Application (Nutraceuticals, Food and Beverages, Cosmetics, Animal Feed, and Others), and Region 2024-2032. Report ID: SR112025A3535. www.imarcgroup.com/Spirulina-market, (accessed on 11 March 2025)

156. Lafarga, T.; Sánchez-Zurano, A.; Villaró, S.; Morillas-España, A.; Acién, G. Industrial production of Spirulina as a protein source for bioactive peptide generation. *Trends Food Sci Technol*, **2021**, *116*, 176-185. https://doi.org/10.1016/j.tifs.2021.07.018.

157. Rehman Shah, M.A.; Zhu, F.; Cui, Y.; Hu, X.; Chen, H.; Kayani, S.I.; Huo, S. Mechanistic insights into the nutritional and therapeutic potential of Spirulina (Arthrospira) spp.: Challenges and

opportunities. *Trends Food Sci Technol*, **2024,** *151*, 104648. https://doi.org/10.1016/j.tifs.2024.104648

158. Saraswathi, K.; Kavitha, C.N. Spirulina: Pharmacological Activities and Health Benefits. *J Young Pharma*, **2023**, *15*(3), 441-447. https://doi.org/10.5530/jyp.2023.15.59.

159. Seyidoglu, N.; Inan, S.; Aydin, C. A prominent superfood: Spirulina platensis. In Superfood and functional food - The development of superfoods and their roles as medicine. Shiomi, N., Waisundara, V., Eds. IntechOpen, 2017, 12, 1-27. http://doi.org/10.5772/66118.

160. Gutierrez-Salmeán, G.; Fabila-Castillo, L.; Chamorro-Cevallos, G. Nutritional and toxicological aspects of Spirulina (Arthrospira). *Nutr. Hosp*., **2015**, *32(*1), 34-40. http://doi.org/10.3305/nh.2015.32.1.9001.

161. Wu, J.Y.; Tso, R.; Teo, H.S.; Haldar, S. The utility of algae as sources of high-value nutritional ingredients, particularly for alternative/complementary proteins to improve human health. *Front. Nutr.* **2023**, *10*, 1277343. http://doi.org/10.3389/fnut.2023.1277343.

162. Sorrenti, V.; Castagna, D.A.; Fortinguerra, S.; Buriani, A.; Scapagnini, G.; Willcox, D.C. Spirulina microalgae and brain Health: A scoping review of experimental and clinical evidence. *Mar. Drugs* **2021**, *19*, 293 https://doi.org/10.3390/md19060293.

163. Goncalves Bortolini, D.; Maciel, G.M.; de Andrade Arruda Fernandes, I.; Pedro, A.C., Vieira Rubio, F.T.; Guiherme Branco, I.; Haminiuk, C.W.I. Functional properties of bioactive compounds from Spirulina spp.: Current status and future trends. *Food Chem: Mol Sci*, **2022**, *5*, 100134. https://doi.org/10.1016/j.fochms.2022.100134

164. Frenandes de Carvalho, L.; Botelho Moreira, J.; Souza Oliveira, M.; Vieira Costa, J.A. Novel food supplements formulated with Spirulina to meet athletes' needs. *Braz. Arch. Biol. Technol*., **2018**, *61*, e17160656. https://doi.org/10.1590/1678-4324-2017160656

165. Bastos de Freitas, B.C.; Duarte Santos, T.; Botelho Moreira, J.; Zanfonato, K.; Greque de Morais, M.; Vieira Costa, J.A. Novel foods: A meal replacement shake and a high-calorie food supplemented with Spirulina biomass. *Int. Food Res. J*., **2019**, *26*(1), 59-65.

166. Lafarga, T.; Fernández-Sevilla, J.M.; González-López, C.; Acién-Fernández, F.G. Spirulina for the food and functional food industries. *Food Res. Int***.**, **2020,** *137*, 109356. https://doi.org/10.1016/j.foodres.2020.109356.

167. Nikolova, K.; Petkova, N.; Mihaylova, D.; Gentscheva, G.; Gavrailov, G.; Pehlivanov, I.; Andonova, V. Extraction of Phycocyanin and Chlorophyll from Spirulina by "Green Methods". *Separations*, **2024**, *11*(2), 57. https://doi.org/10.3390/separations11020057.

168. Lupatini, A.L.; Colla, L.M.; Canan, C.; Colla, E. Potential application of microalga Spirulina platensis as a protein source. *J. Sci. Food Agric*., **2017**, *97*(3), 724-732. https://doi.org/10.1002/jsfa.7987.

169. Becker, E.W. Micro-algae as a source of protein. *Biotechnol. Adv*., **2007**, *25*(2), 207-210. . https://doi.org/10.1016/j.biotechadv.2006.11.002.

170. Wild, K.J.; Steingaß, H.; Rodehutscord, M. Variability of in vitro ruminal fermentation and nutritional value of cell-disrupted and nondisrupted microalgae for ruminants. *Glob. Change Biol. Bioenergy*, **2019**, *11*(1), 345-359. https://doi.org/10.1111/gcbb.12539.

171. Tessier, R.; Calvez, J.; Khodorova, N.; Gaudichon, C. Protein and amino acid digestibility of 15 N Spirulina in rats. *Eur. J. Nutr*., **2021**, *60*, 2263-2269. https://doi.org/10.1007/s00394-020-02368-0.

172. Devi, S.; Varkey, A.; Sheshshayee, M.S.; Preston, T.; Kurpad, A.V. Measurement of protein digestibility in humans by a dual-tracer method. *Am. J. Clin. Nutr.*, **2018**, *107*(6), 984-991. https://doi.org/10.1093/ajcn/nqy062.

173. Janczyk, P.; Franke, H.; Souffrant, W.B. Nutritional value of Chlorella vulgaris: effects of ultrasonication and electroporation on digestibility in rats. *Anim. Feed Sci. Technol*., **2007**, *132*(1-2), 163-169. https://doi.org/10.1016/j.anifeedsci.2006.03.007.

174. Purdi, T.S.; Setiowati, A.D.; Ningrum, A. Ultrasound-assisted extraction of Spirulina platensis protein: physicochemical characteristics and techno-functional properties. *J. Food Meas. Charact*., **2023**, *17*(5), 5474-5486. https://doi.org/10.1007/s11694-023-02051-y.

175. Schwenzfeier, A.; Lech, F.; Wierenga, P.A.; Eppink, M.H.; Gruppen, H. Foam properties of algae soluble protein isolate: Effect of pH and ionic strength. *Food Hydrocoll*., **2013**, *33*(1), 111-117. https://doi.org/10.1016/j.foodhyd.2013.03.002.

176. Boisen, S.; Eggum, B.O. Critical evaluation of in vitro methods for estimating digestibility in simple-stomach animals. *Nutr. Res. Rev*., **1991**, *4*(1), 141-162. https://doi.org/10.1079/NRR19910012.

177. Bleakley, S.; Hayes, M. Algal proteins: extraction, application, and challenges concerning production. *Foods*, **2017**, *6*(5), 33. https://doi.org/10.3390/foods6050033

178. Dai, L.; Hinrichs, J.; Weiss, J. Emulsifying properties of acid-hydrolyzed insoluble protein fraction from Chlorella protothecoides: Formation and storage stability of emulsions. *Food Hydrocol*l., **2020**, *108*, 105954. https://doi.org/10.1016/j.foodhyd.2020.105954

179. Jain, S.; Anal, A.K. Optimization of extraction of functional protein hydrolysates from chicken egg shell membrane (ESM) by ultrasonic assisted extraction (UAE) and enzymatic hydrolysis. *LWT-Food Sci. Technol*., **2016**, *69*, 295-302. https://doi.org/10.1016/j.lwt.2016.01.057.

180. Wang, Q.; Wang, Y.; Huang, M.; Hayat, K.; Kurtz, N.C.; Wu, X.; Zheng, F. Ultrasound-assisted alkaline proteinase extraction enhances the yield of pecan protein and modifies its functional properties. Ultrason. Sonochem., **2021**, 80, 105789. https://doi.org/10.1016/j.ultsonch.2021.105789.

181. Lo, B.; Kasapis, S.; Farahnaky, A. Effect of low frequency ultrasound on the functional characteristics of isolated lupin protein. *Food Hydrocoll*., **2022**, *124*, 107345. https://doi.org/10.1016/j.foodhyd.2021.107345.

182. Arranz, S.; Silván, J.M.; Saura-Calixto, F. Nonextractable polyphenols, usually ignored, are the major part of dietary polyphenols: A study on the Spanish diet. *Mol. Nutr. Food Res*., **2010**, *54*(11), 1646-1658. https://doi.org/10.1002/mnfr.200900580.

183. Shahidi, F.; Yeo, J. Insoluble-bound phenolics in food. *Molecules*, **2016**, *21*(9), 1216. https://doi.org/10.3390/molecules21091216

184. Minchev, I., Petkova, N.; Milkova-Tomova, I. Ultrasound-assisted extraction of chlorophylls and phycocyanin from Spirulina platensis. *Biointerface Res. Appl. Chem*., **2020**, *11*(2), 9296-9304. https://doi.org/10.33263/BRIAC112.92969304.

185. Tavanandi, H.A.; Mittal, R.; Chandrasekhar, J.; Raghavarao, K.S.M.S. Simple and efficient method for extraction of C-Phycocyanin from dry biomass of Arthospira platensis. *Algal Res*., **2018**, *31*, 239-251. https://doi.org/10.1016/j.algal.2018.02.008.

186. Rodrigues, R.D.P.; de Castro, F.C.; de Santiago-Aguiar, R.S.; Rocha, M.V.P. Ultrasound-assisted extraction of phycobiliproteins from Spirulina (Arthrospira) platensis using protic ionic liquids as solvent. *Algal Res*., **2018**, *31*, 454-462. https://doi.org/10.1016/j.algal.2018.02.021.

187. Setyoningrum, T.M.; Nur, M.A. Optimization of C-phycocyanin production from S. platensis cultivated on mixotrophic condition by using response surface methodology. *Biocatal. Agric. Biotechnol*., **2015**, *4*(4), 603-607. http://dx.doi.org/10.1016/j.bcab.2015.09.008.

188. Castejón, N.; Señoráns, F.J. Simultaneous extraction and fractionation of omega-3 acylglycerols and glycolipids from wet microalgal biomass of Nannochloropsis gaditana using pressurized liquids. *Algal Res*., **2019**, *37*, 74-82. https://doi.org/10.1016/j.algal.2018.11.003.

189. Tang, D.Y.Y.; Khoo, K.S.; Chew, K.W., Tao, Y.; Ho, S.H., Show, P.L. Potential utilization of bioproducts from microalgae for the quality enhancement of natural products. *Bioresour. Technol*., **2020**, *304,* 122997. https://doi.org/10.1016/j.biortech.2020.122997.

190. Blanco-Llamero, C.; Señoráns, F.J. Biobased solvents for pressurized liquid extraction of Nannochloropsis gaditana Omega-3 lipids. *Mar. Drugs*, **2021**, *19*(2), 107. https://doi.org/10.3390/md19020107.

191. Jha, A.K.; Sit, N. Extraction of bioactive compounds from plant materials using combination of various novel methods: A review. *Trends Food Sci Technol*, **2022**, *119*, 579-591. https://doi.org/10.1016/j.tifs.2021.11.019.

192. Pohndorf, R.S.; Camara, Á.S.; Larrosa, A.P.; Pinheiro, C.P.; Strieder, M.M.; Pinto, L.A. Production of lipids from microalgae Spirulina sp.: Influence of drying, cell disruption and extraction methods. *Bio. Bioener*. **2016**, *93,* 25-32. https://doi.org/10.1016/j.biombioe.2016.06.020.

193. Getachew, A.T.; Jacobsen, C.; Holdt, S.L. Emerging technologies for the extraction of marine phenolics: Opportunities and challenges. *Mar. Drugs*, **2020**, *18*(8), 389. https://doi.org/10.3390/md18080389.

194. Jaeschke, D.P.; Teixeira, I.R.; Marczak, L.D.F.; Mercali, G.D. (2021). Phycocyanin from Spirulina: A review of extraction methods and stability. *Food Res. Int*., **2021**, *143*, 110314. https://doi.org/10.1016/j.foodres.2021.110314.

195. Kaur, N.; Khunger, A.; Wallen, S.L.; Kaushik, A.; Chaudhary, G.R.; Varma, R.S. Advanced green analytical chemistry for environmental pesticide detection. *Curr. Opin. Green Sustain*., **2021**, *30*, 100488. https://doi.org/10.1016/j.cogsc.2021.100488.

196. Bognár, S.; Putnik, P.; Šojić Merkulov, D. Sustainable green nanotechnologies for innovative purifications of water: Synthesis of the nanoparticles from renewable sources. *Nanomaterials*, **2022**, *12*(2), 263. https://doi.org/10.3390/nano12020263

197. Marjanović, B.; Benković, M.; Jurina, T.; Sokač Cvetnić, T.; Valinger, D.; Gajdoš Kljusurić, J.; Jurinjak Tušek, A. Bioactive Compounds from Spirulina spp.—Nutritional Value, Extraction, and Application in *Food Industry. Separations*, **2024**, *11*(9), 257. https://doi.org/10.3390/separations11090257.

198. Alotaiby, S.; Zhao, X.; Boesch, C.; Sergeeva, N.N. Sustainable approach towards isolation of photosynthetic pigments from Spirulina and the assessment of their prooxidant and antioxidant properties. *Food Chem*., **2024**, *436*, 137653. https://doi.org/10.1016/j.foodchem.2023.137653.

199. Awad, A.M.; Kumar, P.; Ismail-Fitry, M.R.; Jusoh, S.; Ab Aziz, M.F.; Sazili, A.Q. Green extraction of bioactive compounds from plant biomass and their application in meat as natural antioxidant. *Antioxidants*, **2021**, *10*(9),1465. https://doi.org/10.3390/antiox10091465.

200. Janicka, P.; Płotka-Wasylka, J.; Jatkowska, N.; Chabowska, A.; Fares, M.Y.; Andruch, V.; Kaykhaii, M.; Gębicki, J. Trends in the new generation of green solvents in extraction processes. *Curr. Opin. Green Sustain*., **2022**, *37*, 100670. https://doi.org/10.1016/j.cogsc.2022.100670.

201. García-Oms, S.; Sánchez-Bonet, D.; Belda-Antolí, M.; Padrón-Sanz, C.; Lloris-Carsi, J.M.; Cejalvo-Lapeña, D. Optimisation of a green ultrasound-assisted extraction (UAE) methodology for obtaining maximum antioxidant activity from red algae and determination of the co-extracted compounds. J. *Appl. Phycol*., **2024**, *36*(3), 1433-1444. https://doi.org/10.1007/s10811-023-03157-y.

202. Al Fuhaid, L.; Wellman, G.B.; Kharbatia, N.; Farinha, A.S.; Vrouwenvelder, J.S.; Verpoorte, R.; Choi, Y.H.; Witkamp, G.J.; Fortunato, L. Green extraction of pigment from astaxanthin-producing algae using natural deep eutectic solvents. *Algal Res*., **2024**, *82*, 103668. https://doi.org/10.1016/j.algal.2024.103668.

203. Ruiz-Domínguez, M.C.; Medina, E.; Salinas, F.; Bugueño, W.; Fuentes, J.L.; Vílchez, C.; Garbayo, I.; Cerezal-Mezquita, P. Methodological optimization of supercritical fluid extraction of valuable bioactive compounds from the acidophilic microalga Coccomyxa onubensis. *Antioxidants*, 2022, *11*(7), 1248. https://doi.org/10.3390/antiox11071248.

204. Martins, R.; Mouro, C.; Pontes, R.; Nunes, J.; Gouveia, I. Ultrasound-assisted extraction of bioactive pigments from Spirulina platensis in natural deep eutectic solvents. *Bioresour. Bioprocess*., 2023, *10*(1), 88. https://doi.org/10.1186/s40643-023-00692-x

205. Baghizadeh K.B.; Goli, M.; Shahi, S. The survey of bioactive compounds extraction from Spirulina platensis algae by ultrasound-assisted ethanolic maceration. *J. Food Sci. Technol. (Iran),* 2023, *20*(135), 45-57. https://doi.org/10.22034/FSCT.19.135.45.

206. Elferjane, M.R.; Milutinović, V.; Jovanović Krivokuća, M.; Taherzadeh, M.J.; Pietrzak, W.; Marinković, A.; Jovanović, A.A. Vaccinium myrtillus L. leaf waste as a source of biologically potent compounds: optimization of polyphenol extractions, chemical profile, and biological properties of the extracts. *Pharmaceutics*, **2024**, *16*(6), 740. https://doi.org/10.3390/pharmaceutics16060740.

207. Pogorzelska-Nowicka, E.; Hanula, M.; Pogorzelski, G. Extraction of polyphenols and essential oils from herbs with green extraction methods – An insightful review. *Food Chem*., **2024**, *460*(1), 140456. https://doi.org/10.1016/j.foodchem.2024.140456.

208. Parshina, E.Y.; Liu, W.; Yusipovich, A.I.; Gvozdev, D.A.; He, Y.; Pirutin, S.K.; Klimanova, E.A.; Maksimov, E.G.; Maksimov, G.V. Spectral and conformational characteristics of phycocyanin associated with changes of medium pH. *Photosynth. Res*., **2024**, *161*, 93-103. https://doi.org/10.1007/s11120-023-01068-0.

209. Benedetti, S.; Rinalducci, S.; Benvenuti, F.; Francogli, S.; Pagliarani, S.; Giorgi, L.; Canestrari, F. Purification and characterization of phycocyanin from the blue-green alga Aphanizomenon flos-aquae. *J. Chromatogr. B* **2006**, *833*(1), 12–18. https://doi.org/10.1016/j.jchromb.2005.10.010.

210. Kovaleski, G.; Kholany, M.; Dias, L.M.S.; Correia, S.F.H.; Ferreira, R.A.S.; Coutinho, J.A.P.; Ventura, S.P.M. Extraction and purification of phycobiliproteins from algae and their applications. *Front. Chem.*, **2022**, *10*, 1065355. https://doi.org/10.3389/fchem.2022.1065355

211. Pavani, P.; Kumar, K.; Rani, A.; Venkatesu, P.; Lee, M.J. The influence of sodium phosphate buffer on the stability of various proteins: Insights into protein-buffer interactions. *J. Mol. Liq*., **2021**, *331*, 115753. https://doi.org/10.1016/j.molliq.2021.115753

212. Charoensiddhi, S.; Lorbeer, A.J.; Lahnstein, J.; Bulone, V.; Franco, C.M.; Zhang, W. Enzyme-assisted extraction of carbohydrates from the brown alga Ecklonia radiata: Effect of enzyme type, pH and buffer on sugar yield and molecular weight profiles. *Process Biochem*., **2016**, *51*(10), 1503–1510. https://doi.org/10.1016/j.procbio.2016.07.014

213. Saini, R.K.; Prasad, P.; Shang, X.; Keum, Y.S. Advances in lipid extraction methods—a review. Int. J. *Mol. Sci*., **2021**, *22*(24), 13643. https://doi.org/10.3390/ijms222413643

214. Salazar-González, C.; Mendoza Ramos, C.; Martínez-Correa, H.A.; Lobatón García, H.F. Extraction and concentration of spirulina water-soluble metabolites by ultrafiltration. Plants, **2024**, 13(19), 2770. https://doi.org/10.3390/plants13192770

215. Saharan, V.; Jood, S. Nutritional composition of Spirulina platensis powder and its acceptability in food products. *Int. J. Adv. Res*., **2017**, *5*(6), 2295-2300. https://doi.org/10.21474/IJAR01/4671.
216. Anvar, A.A.; Nowruzi, B. Bioactive Properties of Spirulina: A Review. *Microb. Bioact*., **2021**, *4*(1), 134-142. https://doi.org/10.25163/microbbioacts.412117B0719110521.
217. Liu, Q.; Yao, C.; Sun, Y.; Chen, W.; Tan, H.; Cao, X.; Xue, S.; Yin, H. Production and structural characterization of a new type of polysaccharide from nitrogen-limited Arthrospira platensis cultivated in outdoor industrial-scale open raceway ponds. *Biotechnol Biofuels*, **2019**, *12*, 131. https://doi.org/10.1186/s13068-019-1470-3
218. Shen, L.; Pang, S.; Zhong, M.; Sun, Y.; Qayum, A.; Liu, Y.; Rashid, A.; Xu, B.; Liang, Q.; Ma, H.; Ren, X. A comprehensive review of ultrasonic assisted extraction (UAE) for bioactive components: Principles, advantages, equipment, and combined technologies. *Ultrason. Sonochem*., **2023**, *101*, 106646. https://doi.org/10.1016/j.ultsonch.2023.106646.
219. Lupatini, A.L.; de Oliveira Bispo, L.; Colla, L.M.; Costa, J.A.V.; Canan, C.; Colla, E. Protein and carbohydrate extraction from S. platensis biomass by ultrasound and mechanical agitation. *Food Res. Int*., **2017**, *99*, 1028–1035. https://doi.org/10.1016/j.foodres.2016.11.036.
220. Wan, D.; Wu, Q.; Kuča, K. Spirulina. - In Nutraceuticals - Efficacy, Safety and Toxicity, 1st ed.; Gupta, R.C., Ed.; Academic Press/Elsevier, USA, 2016; Chapter 42, pp. 569-583. https://doi.org/10.1016/B978-0-12-802147-7.00042-5.
221. Bai, L.; Zhu, P.; Wang, W.; Wang, M. The influence of extraction pH on the chemical compositions, macromolecular characteristics, and rheological properties of polysaccharide: The case of okra polysaccharide. *Food Hydrocoll*., **2020**, *102,* 105586. https://doi.org/10.1016/j.foodhyd.2019.105586.
222. Senila, L.; Kovacs, E.; Roman, C. Chemical Characterization, Lipid Profile, and Volatile Compounds in Chlorella sp. and Spirulina platensis: A Promising Feedstock for Various Applications. *Molecules*, **2025**, *30*, 1499. https://doi.org/10.3390/molecules30071499
223. Neag, E.; Stupar, Z.; Varaticeanu, C.; Senila, M.; Roman, C. Optimization of Lipid Extraction from Spirulina spp. by Ultrasound Application and Mechanical Stirring Using the Taguchi Method of Experimental Design. *Molecules*, **2022**, *27*, 6794. https://doi.org/10.3390/molecules27206794
224. Ramadan, M.F.; Asker, M.M.S.; Ibrahim, Z.K. Functional Bioactive Compounds and Biological Activities of Spirulina platensis Lipids. Czech *J. Food Sci*., **2008**, *26*(3), 211–222. https://doi.org/10.17221/2567-CJFS
225. Chaiklahana, R.; Chirasuwana, N.; Lohab, V.; Bunnag, B. Lipid and fatty acids extraction from the cyanobacterium Spirulina. *Sci. Asia*, **2008**, *34*, 299-305. https://10.2306/scienceasia1513-1874.2008.34.299
226. Muhling, M.; Belay, A.; Whitton, B.A. Variation in fatty acid composition of Arthrospira (Spirulina) strains. J. *Appl. Phychol*. **2005**, *17*, 137–146. https://doi.org/10.1007/s10811-005-7213-9.

227. Soni, R.A.; Sudhakar, K.; Rana, R.S. Spirulina–From growth to nutritional product: A review. *Trends Food Sci. Technol*. **2017**, *69,* 157–171. https://doi.org/10.1016/j.tifs.2017.09.010

228. Orthesin, N.; Hidayat, I.T.; Wahyuni, W.T.; Syafitri, U.D.; Herbani, Y.; Sari, Y.W. Optimization of Chlorophyll Extraction from dried Spirulina platensis using low power microwave assisted extraction method. IOP Conf. Ser. Earth Environ. Sci., 2024, 1359, 012021. https://doi.org/10.1088/1755-1315/1359/1/012021

229. Minchev, I.; Petkova, N.; Milkova-Tomova, I. Ultrasound-assisted extraction of chlorophylls and phycocyanin from Spirulina platensis. *Biointerface Res. Appl. Chem*., **2020**, *11*, 9296–9304. https://doi.org/10.33263/BRIAC112.92969304

230. Park, W.S.; Kim, H.J.; Li, M.; Lim, D.H.; Kim, J.; Kwak, S.S.; Chang, C.M.; Ferruzzi, M.G.; Ahn, M.J. Two classes of pigments, carotenoids and C-phycocyanin, in Spirulina powder and their antioxidant activities. *Molecules*, **2018**, *23*(8), 2065. https://doi.org/10.3390/molecules23082065

231. Choi, W.Y.; Lee, H.Y. Enhancement of Chlorophyll a production from marine Spirulina maxima by an optimized ultrasonic extraction process. *Appl. Sci*., **2018**, *8*(1), 26. https://doi.org/10.3390/app8010026

232. Sadewo, B.R.; Dewayanto, N.; Rochmadi, R.; Juniawan, A.S.; Budiman, A. Optimization study of phycocyanin ultrasound-assisted extraction process from Spirulina (Arthrospira platensis) using different solvent. *Egypt. J. Chem*., **2024**, *67*(7), 589–608. https://doi.org/10.1155/2024/589608

233. Chia, S.R.; Chew, K.W.; Leong, H.Y.; Manickam, S.; Show, P.L.; Nguyen, T.H.P. Sonoprocessing-assisted solvent extraction for the recovery of pigment-protein complex from Spirulina platensis. *Chem. Eng. J.* **2020**, *398*, 125613. https://doi.org/10.1016/j.cej.2020.125613

234. Obeid, S.; Rida, H.; Peydecastaing, J.; Takache, H.; Ismail, A.; Pontalier, P.Y. Coupling ultrasound and membrane filtration for the fractionation of Spirulina platensis sp. and the recovery of phycocyanin and pigment-free proteins. *Biotechnol. Lett*. **2025**, *47*(1), 8. https://doi.org/10.1007/s10529-024-03541-9.

235. da Silva, M.F.; Casazza, A.A.; Ferrari, P.F.; Aliakbarian, B.; Converti, A.; Pedros Bezerra, R.; Figueiredo Porto, A.L.; Perego, P. Recovery of phenolic compounds of food concern from Arthrospira platensis by green extraction techniques. *Algal Res*., **2017**, *25,* 391-401. https://doi.org/10.1016/j.algal.2017.05.027.

236. El-Baky, H.H.A., El Baz, F.K., El-Baroty, G.S. Production of phenolic compounds from Spirulina maxima microalgae and its protective effects. *Afric. J. Biotechnol*., **2009**, *8*(24), 7059-7067.

237. Seghiri, R.; Kharbach, M.; Essamri, A. Functional composition, nutritional properties, and biological activities of Moroccan Spirulina microalga. *J. Food Qual*., **2019**, art ID 3707219, *1*-11. https://doi.org/10.1155/2019/3707219.

238. Stajčić, S.; Ćetković, G.; Tumbas Šaponjac, V.; Travičić, V.; Ilić, P.; Brunet, S.; Tomić, A. Bioactive compounds and the antioxidant activity of selected vegetable microgreens: A correlation study. *Processes*, **2024**, *12*(8), 1743. https://doi.org/10.3390/pr12081743.

239. Yamauchi, M.; Kitamura, Y.; Nagano, H.; Kawatsu, J.; Gotoh, H. DPPH measurements and structure—activity relationship studies on the antioxidant capacity of phenols. *Antioxidants*, **2024**, *13*(3), 309. https://doi.org/10.3390/antiox13030309.

240. Saroyo, H.; Arifah, I.A.N. Antioxidant activity using DPPH & FRAP method and their correlation with the levels of phenolic and flavonoid compounds from Nemba plants (Azadirachta Indica A. Juss). *J. Nutrac. Herb. Med*., **2021**, *3*(2), 10-20. https://doi.org/ 10.23917/jnhm.v3i2.15658.

241. Pinero Estrada, J.E.; Bermejo Bescós, P.; Villar del Fresno, A.M. Antioxidant activity of different fractions of Spirulina platensis protein extract. *Farmaco*, **2021**, *56*, 5-7, 497-500. https://doi.org/10.1016/S0014-827X(01)01084-9

242. Fiedor, J.; Burda, K. Potential role of carotenoids as antioxidants in human health and disease. *Nutrients*, **2014**, *6*(2), 466–488. https://doi.org/10.3390/nu6020466.

243. Picot-Allain, C.; Mahomoodally, M.F.; Ak, G.; Zengin, G. Conventional versus green extraction techniques — a comparative perspective. *Curr. Opin. Food Sci*., **2021**, *40*, 144-156. https://doi.org/10.1016/j.cofs.2021.02.009

244. McClements, D. J.; Li, F.; Xiao, H. The nutraceutical bioavailability classification scheme: classifying nutraceuticals according to factors limiting their oral bioavailability. *Annu. Rev. Food Sci. Technol*. **2015**, *6*(1), 299–327. https://doi.org/10.1146/annurev-food-032814-014043.

245. Aquino, R. P.; Auriemma, G.; Conte, G. M.; Esposito, T.; Sommella, E.; Campiglia, P.; Sansone, F. Development of chitosan/mannitol microparticles as a delivery system for the oral administration of a Spirulina bioactive peptide extract. *Molecules* **2020**, *25*(9), 2086. https://doi.org/10.3390/molecules25092086.

246. Adjali, A.; Clarot, I.; Chen, Z.; Marchioni, E.; Boudier, A. Physicochemical degradation of phycocyanin and means to improve its stability: a short review. *J. Pharm. Anal*. **2022**, *12*(3), 406–414. https://doi.org/10.1016/j.jpha.2021.12.005

247. Liu, L.; McClements, D. J.; Liu, X.; Liu, F. Overcoming biopotency barriers: advanced oral delivery strategies for enhancing the efficacy of bioactive food ingredients. *Adv. Sci.* **2024**, *11*(44), 2401172. https://doi.org/10.1002/advs.202401172.

248. Zhang, F.; Li, Z.; Duan, Y.; Abbas, A.; Mundaca-Uribe, R.; Yin, L.; Wang, J. Gastrointestinal tract drug delivery using algae motors embedded in a degradable capsule. *Sci. Robot.* **2022**, *7*(70), eabo4160. https://doi.org/10.1126/scirobotics.abo4160.

249. Coppens, P. The importance of food supplements for public health and well-being. In Hidden Hunger and the Transformation of Food Systems: How to Combat the Double Burden of Malnutrition? Biesalski, H.K.; Koletzko, B., Eds.; World Review of Nutrition and Dietetics, Vol. 121; S. Karger AG: Basel, Switzerland, 2020; pp. 66–72. https://doi.org/10.1159/000507524

250. 2020 CRN Consumer Survey on Dietary Supplements. Available online at https://www.crnusa.org/resources/2020-crn-consumer-survey-dietary-supplements. (accessed on 27 March 2025)

251. Djaoudene, O.; Romano, A.; Bradai, Y. D.; Zebiri, F.; Ouchene, A.; Yousfi, Y.; Madani, K. A global overview of dietary supplements: Regulation, market trends, usage during the COVID-19 pandemic, and health effects. *Nutrients* **2023**, *15*(15), 3320. https://doi.org/10.3390/nu15153320.
252. Barani, M.; Sangiovanni, E.; Angarano, M.; Rajizadeh, M.A.; Mehrabani, M.; Piazza, S.; Gangadharappa, H.V.; Pardakhty, A.; Mehrbani, M.; Dell'Agli, M., Nematollahi, M.H. Phytosomes as Innovative Delivery Systems for Phytochemicals: A Comprehensive Review of Literature. *Intern. J. Nanomed.*, **2021**, *16*, 6983-7022. https://doi.org/10.2147/IJN.S318416
253. Šturm, L.; Poklar Ulrih, N. Basic Methods for Preparation of Liposomes and Studying Their Interactions with Different Compounds, with the Emphasis on Polyphenols. *Int J Mol Sci.*, **2021**, *22*(12), 6547. https://doi.org/10.3390/ijms22126547.
254. He, H.; Lu, Y.; Qi, J.; Zhu, Q.; Chen, Z.; Wu, W. Adapting liposomes for oral drug delivery. *Acta Pharm Sin B*., **2019,** *9*(1), 36-48. https://doi.org/10.1016/j.apsb.2018.06.005.
255. Mondal, D.; Mandal, R. P.; De, S. Addressing the superior drug delivery performance of bilosomes─ A microscopy and fluorescence study. *ACS Appl. Bio Mater.* **2022**, *5* (8), 3896-3911. https://doi.org/10.1021/acsabm.2c00435
256. Nayak, D.; Rathnanand, M.; Tippavajhala, V. K. Unlocking the potential of bilosomes and modified bilosomes: A comprehensive journey into advanced drug delivery trends. *AAPS PharmSciTech* **2023**, *24*, 238. https://doi.org/10.1208/s12249-023-02696-4.
257. Zhang, B.; Xue, A.; Zhang, C.; Yu, J.; Chen, W.; Sun, D. Bile salt liposomes for enhanced lymphatic transport and oral bioavailability of paclitaxel. *Die Pharmazie* **2016**, *71* (6), 320-326. https://doi.org/10.1691/ph.2016.5184
258. Jain, S.; Harde, H.; Indulkar, A.; Agrawal, A. K. Improved stability and immunological potential of tetanus toxoid containing surface engineered bilosomes following oral administration. *Nanomedicine* **2014**, *10*(2), 431-440. https://doi.org/10.1016/j.nano.2013.08.012.
259. Milano, F.; Masi, A.; Madaghiele, M.; Sannino, A.; Salvatore, L.; Gallo, N. Current trends in gelatin-based drug delivery systems. *Pharmaceutics* **2023**, *15*(5), 1499. https://doi.org/10.3390/pharmaceutics15051499
260. Parashar, P.; Kumar, P.; Gautam, A.K.; Singh, N.; Bera, H.; Sarkar, S.; Saraf, S.A.; Saha, S. Gelatin-based nanomaterials in drug delivery and biomedical applications. In *Biopolymer-Based Nanomaterials in Drug Delivery and Biomedical* Applications; Grumezescu, A.M., Ed.; Academic Press: Cambridge, MA, USA, 2021; pp. 407–426. https://doi.org/10.1016/B978-0-12-820874-8.00020-8
261. van den Hoven, J. M.; Metselaar, J. M.; Storm, G.; Beijnen, J. H.; Nuijen, B. Cyclodextrin as membrane protectant in spray-drying and freeze-drying of PEGylated liposomes. *Int. J. Pharm.* **2012**, *438*(1-2), 209-216. https://doi.org/10.1016/j.ijpharm.2012.08.046.

262. Guan, P.; Lu, Y.; Qi, J.; Wu, W. Readily restoring freeze-dried probilosomes as potential nanocarriers for enhancing oral delivery of cyclosporine A. Colloids Surf. B Biointerfaces **2016**, *144*, 143-151. https://doi.org/10.1016/j.colsurfb.2016.04.006

263. Paseban, K.; Noroozi, S.; Gharehcheloo, R.; Haddadian, A.; Robattorki, F. F.; Dibah, H.; Gharaghie, T. P. Preparation and optimization of niosome encapsulated meropenem for significant antibacterial and anti-biofilm activity against methicillin-resistant Staphylococcus aureus isolates. *Heliyon* **2024**, *10*(16), e35651. https://doi.org/10.1016/j.heliyon.2024.e35651.

264. Castangia, I.; Corrias, F.; Jiménez, F. J. L.; Aroffu, M.; Fulgheri, F.; Perra, M.; Manca, M. L. Formulation and testing of cutting-edge food supplements tailored for glucose and oxidation controlling, converting artichoke by-products in inulin-rich antioxidant phytocomplex loaded into zein liposomes. *Food Biosci*. **2024**, *62*, 105482. https://doi.org/10.1016/j.fbio.2024.105482.

265. Manca, M. L., Zaru, M., Manconi, M., Lai, F., Valenti, D., Sinico, C., & Fadda, A. M. Glycerosomes: A new tool for effective dermal and transdermal drug delivery. *Int. journal of pharma.***2013**, *455*(1-2), 66-74. https://doi.org/10.1016/j.ijpharm.2013.07.060

266. Ensign LM, Cone R, Hanes J, Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers, *Adv. Drug Deliv. Rev.* **2012,** *64*, 557–570, https://doi.org/10.1016/J.ADDR.2011.12.009.

267. Singh, S.; Dash, A.K. Physical properties, their determination, and importance in pharmaceutics. In *Pharmaceutics, Basic Principles and Application to Pharmacy Practice*; Singh, S.; Dash, A.K. Eds. Academic Press: Cambridge, MA, USA, 2024; pp. 67–113. https://doi.org/10.1016/B978-0-323-99796-6.00002-3

268. Dwivedi, C.; Verma, S. Review on preparation and characterization of liposomes with application. *Int. J. Sci. Innov. Res.* **2013**, *2(2)*, 486–508.

269. Nsairat, H.; Khater, D.; Sayed, U.; Odeh, F.; Al Bawab, A.; Alshaer, W. Liposomes: Structure, composition, types, and clinical applications. *Heliyon* **2022**, *8*(5), e09394. https://doi.org/10.1016/j.heliyon.2022.e09394

270. Ansari, I.; Shakeel, K. A review on bilosomes: Advanced drug delivery system. *Int. J. Pharm. Sci. Med.* **2023**, *8*(7), 48–57. https://doi.org/10.47760/ijpsm.2023.v08i07.005

271. Danaei, M.; Dehghankhold, M.; Ataei, S.; Hasanzadeh Davarani, F.; Javanmard, R.; Dokhani, A.; Mozafari, M.R. Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. *Pharmaceutics* **2018**, *10*(2), 57. https://doi.org/10.3390/pharmaceutics10020057

272. Lowry, G.V.; Hill, R.J.; Harper, S.; Rawle, A.F.; Hendren, C.O.; Klaessig, F.; Rumble, J. Guidance to improve the scientific value of zeta-potential measurements in nanoEHS. *Environ. Sci. Nano* **2016**, *3*(5), 953–965. https://doi.org/10.1039/C6EN00136J

273. Machado, A. R.; Pinheiro, A. C.; Vicente, A. A.; Souza-Soares, L. A.; Cerqueira, M. A. Liposomes loaded with phenolic extracts of Spirulina LEB-18: Physicochemical characterization and

behaviour under simulated gastrointestinal conditions. *Food Res. Int.* **2019**, *120*, 656-667. https://doi.org/10.1016/j.foodres.2018.11.023.

274. Zewail, M.; Gaafar, P.M.; Youssef, N.A.H.A.; Ali, M.E.; Ragab, M.F.; Kamal, M.F.; Abbas, H. Novel *Spirulina platensis* bilosomes for combating UVB-induced skin damage. *Pharmaceuticals* **2022**, *16*(1), 36. https://doi.org/10w.3390/ph16010036

275. Gibis, M.; Vogt, E.; Weiss, J. Encapsulation of polyphenolic grape seed extract in polymer-coated liposomes. *Food Funct.* **2012**, *3*(3), 246–254. https://doi.org/10.1039/c1fo10181a

276. Agarry, I.E.; Wang, Z.; Cai, T.; Wu, Z.; Kan, J.; Chen, K. Utilization of different carrier agents for chlorophyll encapsulation: Characterization and kinetic stability study. *Food Res. Int.* **2022**, *160*, 111650. https://doi.org/10.1016/j.foodres.2022.111650

277. Moraes, M.; Carvalho, J. M. P.; Silva, C. R.; Cho, S.; Sola, M. R.; Pinho, S. C. Liposomes encapsulating beta-carotene produced by the proliposomes method: Characterization and shelf life of powders and phospholipid vesicles. *Int. J. Food Sci. Technol.* **2013**, *48*(2), 274–282. https://doi.org/10.1111/j.1365-2621.2012.03184.x.

278. Allison, M.; Molina, T.J.; Anchordoquy, T.J. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. *Biochim. Biophys. Acta Biomembr.* **2000**, *1468*(1–2), 127–138. https://doi.org/10.1016/s0005-2736(00)00251-0

279. Geh, K. J.; Hubert, M.; Winter, G.). Progress in formulation development and sterilisation of freeze-dried oligodeoxynucleotide-loaded gelatine nanoparticles. *Eur. Pharm. Biopharm.* **2018**, *129*, 10-20. https://doi.org/10.1016/j.ejpb.2018.05.016

280. Zillies, J. C.; Zwiorek, K.; Hoffmann, F.; Vollmar, A.; Anchordoquy, T. J.; Winter, G.; Coester, C. (2008). Formulation development of freeze-dried oligonucleotide-loaded gelatin nanoparticles. *Eur. Pharm. Biopharm.* **2008**, *70*(2), 514-521. https://doi.org/10.1016/j.ejpb.2008.04.026

281. Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. *Adv Drug Deliv Rev*. **2013**, **65**(1):36-48. https://doi.org/10.1016/j.addr.2012.09.037.

282. Muhaimin, M.; Chaerunisaa, A.Y.; Bodmeier, R. Impact of dispersion time interval and particle size on release profiles of propranolol HCl and carbamazepines from microparticle blends system. *Sci Rep.* **2022**, *12*(1), 10360. https://doi.org/10.1038/s41598-022-14678-w

283. Vijayakumar, V.; Subramanian, K. Diisocyanate mediated polyether modified gelatin drug carrier for controlled release. *Saudi Pharm. J.* **2014**, *22*, 43–51. https://doi.org/10.1016/j.jsps.2013.01.005

284. Battogtokh, G.; Joo, Y.; Abuzar, S. M.; Park, H.; Hwang, S. J. Gelatin coating for the improvement of stability and cell uptake of hydrophobic drug-containing liposomes. *Molecules,* **2022**, *27*, 1041. https://doi.org/10.3390/molecules27031041

285. ISO 10993-5:2009. Biological evaluation of medical devices—part 5: tests for in vitro cytotoxicity. International Organization for Standardization (2009).

286. Abdel-moneum, R.; Abdel-Rashid, R. S. Bile salt stabilized nanovesicles as a promising drug delivery technology: A general overview and future perspectives. *J. Drug Deliv. Sci. Technol.* **2023**, *79*, 104057.https://doi.org/10.1016/j.jddst.2022.104057

287. Kumar, R.; Islam, T.; Nurunnabi, M. Mucoadhesive Carriers for Oral Drug Delivery. *Journal of Controlled Release* **2022**, 351, 504–559. https://doi.org/10.1016/j.jconrel.2022.09.024.

288. Abdel-Moneim, A. M. E.; El-Saadony, M. T.; Shehata, A. M.; Saad, A. M.; Aldhumri, S. A.; Ouda, S. M.; Mesalam, N. M. Antioxidant and antimicrobial activities of Spirulina platensis extracts and biogenic selenium nanoparticles against selected pathogenic bacteria and fungi. *Saudi J. Biol. Sci.* **2022**, *29*(2), 1197–1209. https://doi.org/10.1016/j.sjbs.2021.09.046.

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