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Maximizing Spirulina's value in a biorefinery approach

CAROLINA ASCENSO DOS SANTOS
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Maximizing *Spirulina*'s value in a biorefinery approach

Carolina Ascenso dos Santos

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Orientador: Eng. Nídia de Sá Caetano

Júri:

Presidente:

- MARIA MANUELA DIAS MACHADO

Vogais:

- SIMONE BARREIRA MORAIS
- NIDIA DE SÁ CAETANO

Porto, outubro 2024

Para ti, avô.

Abstract

With the pressing global environmental challenges there is an increasing need for sustainable and circular bioresource management. This thesis explores the multifaceted potential of *Spirulina* in various sectors, leading to the idealisation of a biorefinery.

The study begins by assessing *Spirulina*'s properties, culture conditions and applications that boost its potential for a sustainable biorefinery development, emphasizing its versatility in producing high-value compounds and its contributions to several industrial sectors. Experimentally, *Spirulina* was cultivated and high-value compounds, such as lipids, phycocyanin and antioxidants, were obtained with the use of eco-friendly extraction methods. Additionally, *Spirulina* was incorporated as a functional ingredient in butter. Sensorial analyses were conducted revealing positive consumer acceptance and the potential for *Spirulina* to enhance the nutritional profile of everyday foods. Then, *Spirulina* was tested as an organic soil corrective. Through comparative tests, it was shown that *Spirulina* improved germination, by 150%, and plant growth, underscoring its potential in promoting sustainable agricultural practices. Finally, aggregating the results and inputs from the literature, the concept of a *Spirulina*-based biorefinery was explored, identifying viable process routes and multiple processes integration, such as the reuse of waste streams. The results indicate that with strategic process optimization, *Spirulina*-based biorefineries could contribute significantly to reducing environmental negative impact while offering economically viable solutions, supporting a circular bioeconomy.

Key-words: Biostimulant; Food enrichment; Green chemistry; Phycocyanin; Sustainability

Resumo

À luz dos desafios globais presentes, como o esgotamento dos recursos naturais, a acumulação de resíduos e as alterações climáticas, existe uma necessidade crescente de uma gestão sustentável e circular dos recursos biológicos. Esta tese pretende explorar o potencial multifacetado da *Spirulina*, focando-se nas suas aplicações em vários setores, incluindo a alimentação e a agricultura, conduzindo à idealização de uma biorrefinaria e, em última análise, à mitigação dos referidos desafios.

O estudo começa por avaliar as propriedades, condições de cultura e aplicações da *Spirulina* que demonstram o seu potencial para o desenvolvimento sustentável da biorrefinaria, enfatizando a sua versatilidade na produção de compostos de elevado valor e as suas contribuições para diversos setores industriais.

No trabalho experimental, a *Spirulina* foi cultivada em diversas condições controladas, nomeadamente efluentes de setores alimentares (soro de queijo). Foi possível obter compostos de alto valor, como lipídios, ficocianina e antioxidantes, com o uso de métodos com base na química verde de extração e processamento. Além disso, a *Spirulina* foi incorporada como ingrediente funcional na manteiga. Foram realizadas análises sensoriais que revelaram a aceitação positiva do consumidor e, consequentemente, o potencial da *Spirulina* para melhorar o perfil nutricional dos alimentos do dia a dia. Além das aplicações alimentares, *a Spirulina* foi testada como corretivo orgânico do solo. Através de testes comparativos de germinação, demonstrou-se que a utilização de resíduos de *Spirulina* obtidos durante o seu processamento resultou na melhor germinação (1,5 vezes mais) e crescimento das plantas. Esta secção sublinha o seu potencial na promoção de práticas agrícolas sustentáveis, reduzindo a dependência de fertilizantes sintéticos e melhorando a saúde do solo. Finalmente, agregando resultados obtidos com os resultados de outros estudos presentes na literatura, foi explorado o conceito de uma *biorrefinaria* baseada na *Spirulina*, identificando esquemas processuais viáveis e integração de múltiplos processos, como a recuperação do meio de cultura e a reutilização de fluxos de resíduos, demonstrando a viabilidade da *Spirulina* no apoio à construção de uma bioeconomia circular. Os resultados indicam que, com a otimização estratégica de processos, as biorrefinarias baseadas na *Spirulina* poderiam contribuir significativamente para reduzir os impactos ambiental negativos, oferecendo soluções economicamente viáveis.

Palavras-chave: Bioestimulante; Enriquecimento alimentar; Ficocianina; Química verde; Sustentabilidade;

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List of Abbreviations, Acronyms and Symbols

%Ashes	Ashes content in the biomass, dry basis
%CH	Carbohydrate content in the biomass, dry basis
%Lip	Lipid content in the biomass, dry basis
%N	Total nitrogen content
%Prot	Protein content in the lyophilized biomass, dry basis
abs	Absorvance,
A&O	Aiba and Ogawa culture medium
AOAC	AOAC (Association of Official Agricultural Chemists) International
B&D	Bligh and Dyer method
$B\&D_G$	Greener Bligh and Dyer method (with ethanol, ethyl acetate and water)
CB100	Culture residual medium Biostimulant
CB25	Culture residual medium Biostimulant with 25% concentration
CB50	Culture residual medium Biostimulant with 50% concentration
COD	Chemical Oxygen Demand
C-PC	Phycocyanin content in the biomass
$C\text{-}PC_E$	Phycocyanin concentration in the extract
E	Total energy in 100 g of the lyophilised biomass
FB	Phycocyanin extraction residue Biostimulant
LCA	Life Cycle Assessment
LEDs	Light-emitting diodes
m	Mass of the lyophilised biomass
P_E	Phycocyanin extract purity
PUFA	Polyunsaturated fatty acids
TEA	Techno-Economic Analysis

1. Introduction

1.1. Framework and relevance of the study

The global population, according to the United Nations, is projected to increase to a staggering 9.6 billion by 2050 and will inevitably result in an increased demand for food, by at least 60% (Mendes, et al., 2022; Probst, et al., 2015). Simultaneously, there has been a global increase in demand for foods of animal origin in developing countries, towards higher intakes of protein. This, adding up with the fact that these countries are where the most population growth is occurring, will create great pressure in the food chain systems, already seen for example in the demand for poultry in Southeast Asia, that recorded an astounding increase of 725% between 2000 and 2030 (Henchion, et al., 2017). On the other hand, in developed countries but especially in Europe, there has been a change in consumption patterns with sustainable and healthy foods gaining space, standing out the nutritionally and protein-rich foods (Mendes, et al., 2022). This expansion can be attributed to an aging population and increased consumer awareness of preventive healthcare, but also to the rising awareness in consumers regarding the role of protein in a healthy diet: muscle development and maintenance, satiety and weight management, hunger stimulation in hunger-suppressed individuals as the elderly individuals, and glucose control for persons with type 2 diabetes (Henchion, et al., 2017; Gannon, et al., 2003). Thus, even though with less expression in some countries, proteins are desired transversally, so its supply is a critical factor in food security, putting it under great stress (Probst, et al., 2015).

Animal proteins account for about 40% of global protein consumption. Its consumption has increased significantly in recent decades and is expected to increase to 944 million tonnes by 2050, resulting in concerns for sustainability and food security since meat consumption is associated with a high environmental footprint (Henchion, et al., 2017; Probst, et al., 2015). Firstly, livestock production results in higher levels of greenhouse gases release (12% of total emissions). Secondly, it is highly inefficient, requiring up to 15 kg of plants for 1 kg of animal product. This intensifies the pressure on land, resulting constraints on water availability, energy, and chemical inputs (fertilizers), and in terrestrial biodiversity loss (Henchion, et al., 2017; Probst, et al., 2015). Finally, there are recent concerns about the growth in antibiotic resistance and ethical issues about intensive animal production, since some crops could be used directly as food (competition) (Henchion, et al., 2017).

The strategies to bypass the growing global protein demand involve technological advancements in the agri-food system and shifts in consumption patterns, and lead to alternative protein sources namely pulses, insects, algae, and *in vitro* meat. In fact, the consumption of alternative protein sources is predicted to grow 9% annually, resulting in a third of the total protein consumption by 2054. Their success depends on proving food safety, production costs, nutritional qualities, scalability, and consumer acceptance (Henchion, et al., 2017; Probst, et al., 2015).

Beside pulses, algae are one of the most captivating alternatives, since they already established on the market. In fact, global algae protein market size doubled in the past years, reaching 928 million euros worth in 2023, and is projected to account for about 18% of the alternative protein market in 2054 (Mendes, et al., 2022; Probst, et al., 2015). Besides applications as food and feed, algae have a value as ingredients for cosmetic, pharmaceutical as well as in polymer manufacture. They do not require arable land and can be used as bio-sequesters of carbon dioxide. Because of all that, they have particular interest since the multiple applications allow the maximization of its potential in a biorefinery concept (Martins, et al., 2021).

Particularly interesting to close the “protein gap” as an alternative to animal proteins for human consumption and animal feed production are protein-rich microalgae. One of the two microalgae used primarily in the European Union for human consumption is *Arthrospira* spp. (*Spirulina* spp.) (Henchion, et al., 2017), highly correlated to protein and pigment–protein complexes production, Figure 1. Besides the high protein concentration, *Spirulina* has also

significant carotenoid and phycocyanin content, making it attractive alongside the already mentioned microalgae applications and easy harvesting process.

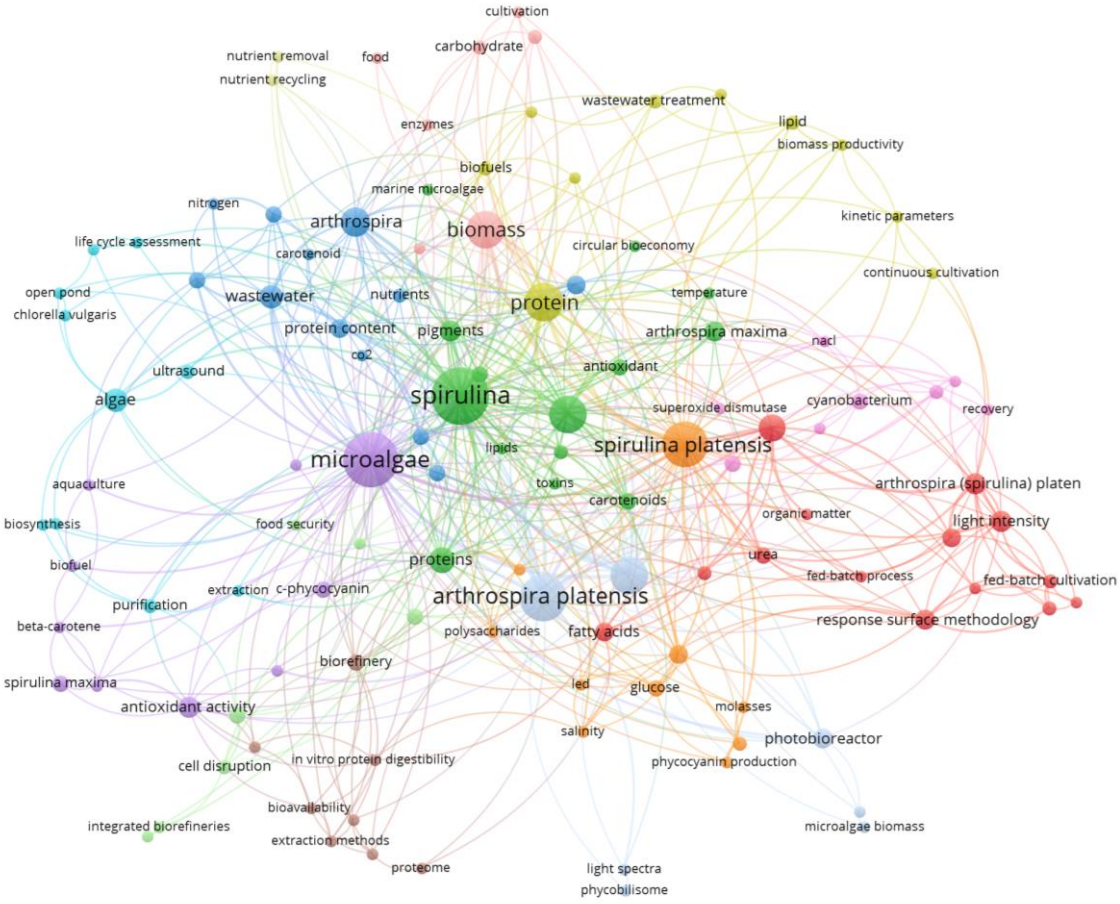


Figure 1 - *Spirulina*'s conceptual map. Created with VOSviewer version 1.6.20

In conclusion, addressing the escalating demand for protein amidst a growing global population necessitates a multifaceted approach. While acknowledging the slow pace of cultural shifts in consumption patterns, it's crucial to recognize that, in striving for a sustainable future, equilibrium among diverse protein sources is vital. *Spirulina*, emerging as a promising alternative, requires optimization in production processes to enhance its contribution to the nutritional enhancement of food products.

1.2. Research Goals

The overall framework of this study was to produce a culture of *Spirulina* microalgae and to valorise it in a biorefinery context, making the most of its biotechnological potential in a sustainable way. The work focused on the extraction of high valuable compounds, C-phycocyanin and proteins, and then in the repurposing of the residual biomass.

This main goal can be divided into several sub-goals, translated into Figure 2.

1. Production and harvesting of *Spirulina*;

2. Extraction and purification of valuable compounds (proteins and phycocyanin) for integration into food;

3. Production and application of an organic corrective with residual biomass;

4. Production and characterization of functional foods.

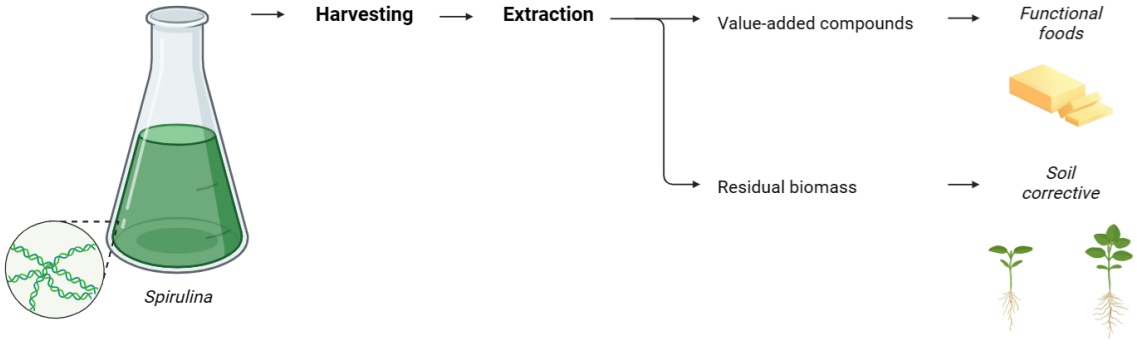


Figure 2. *Spirulina*'s processing into functional foods and soil corrective

1.3.Thesis structure

The current section (1 - Introduction) defines the framework of the thesis, and the relevance of the study as well as the main objectives and the thesis outline.

- Section 2 – State of the Art

Represents the knowledge gathered up to now on *Spirulina*: its characteristics and valorisation as an integral part of food, animal feed, fertilizer, an energy vector, and other applications.

- Section 3 – Methodology

Refers to the materials and the analytical and technical methods employed to achieve the objectives of this thesis. Methods described in the literature were mentioned, whereas other techniques and operational procedures were extensively explored.

- Section 4 – Results and Discussion

Comprises all the experimental results obtained during this study and can be divided in three distinct parts: characterization of the biomass; application as a functional ingredient and application as a soil corrective.

Results of *Spirulina*'s application as a functional ingredient were presented as a poster at the 8th Congress of the International Society of Applied Phycology (16-21 June, Porto, Portugal).

- Section 5 – Conclusions and future perspectives

Refers to the main results achieved in the developed work and proposes what should be explored in the future to complement the current thesis.

2. State of the Art

2.1. Spirulina

Spirulina is a blue-green cyanobacteria, with historical and ecological significance. As a cyanobacteria, it is associated with the periods of origin of plants and has played a crucial role in creating the present-day oxygen-rich environment (Varia, et al., 2022; Chittora, et al., 2020). Furthermore, it is most widely distributed in Central America and has been used as food for centuries (European Algae Biomass Association, 2021). With scientific name *Arthorspira*, recently, *Spirulina* became more known worldwide in the health food industry as a protein and vitamin supplement (Habib, et al., 2008).

In history, *Spirulina*'s use is associated with several ancestral cultures, namely in the 9th century, during the Kanem Empire (when it was called "Dihé") (Mendes, et al., 2022; Ali & Saleh, 2012), and in the 14th century with the Aztec people ("tecuitlatl", meaning stone's excrement) (Food and Drug Administration, 2002; Tan, et al., 2020). The first detailed study of *Spirulina*'s growth requirements and physiology was done by Claude Zarrouk, resulting in his Ph.D. thesis (Zarrouk, 1966) and in the first large-scale production plant on lake Texcoco, Sosa Texcoco Co. (early 1970s) (European Algae Biomass Association, 2021). Later, it was recognized by the International Association of Applied Microbiology as a "wonderful future food source" and by the United Nations, at the World Food Conference of 1974, as "the best food for the future" (Mendes, et al., 2022). In 1986, the Report of Conseil Superieur D'Hygiene Publique De France authorized the use of blue colouring extract by water from *Spirulina* (phycocyanin pigment) for food (European Algae Biomass Association, 2021).

Now, *Spirulina* is consumed and commercialized in many different countries (Ali & Saleh, 2012) as powder, tablets and capsules or as a liquid (Vantage Market Research, 2023). The global market of algae was valued at 19300 million euros (2022) and is projected to reach 42000 million euros by 2030 (Mendes, et al., 2022), while *Spirulina*'s market is expected to increase from 500 million euros in 2022 to 1030 million euros by 2030 (Vantage Market Research, 2023). Currently, it is estimated that 5% of the global *Spirulina* production comes from the European union (European Algae Biomass Association, 2021), distributed across around 200 companies, Figure 3 (Mendes, et al., 2022; Araújo, et al., 2021). Some of the most relevant producers are Cabassi & Giuriati (Italy), ECHLORIAL (France), DÖHLER GmbH (Germany), Necton and Allmicroalgae – Natural Products (Portugal) and Givaudan International (Switzerland) (Vantage Market Research, 2023; Probst, et al., 2015). Some of the best-known worldwide *Spirulina* producing companies are: Earthrise Farms, Algenol Biofuels, Cyanotech and DDW (USA), Hainan DIC Microalgae, Dongtai City Bio-Engineering and Prolgae Supplies (China), Marugappa Chettir Research Center and E.I.D. Parry (India), Genix (Cuba), DIC Corporation (Japan), Pond Technologies (Canada) and Solarium Biotechnology (Chile) (Ali & Saleh, 2012; Vantage Market Research, 2023).

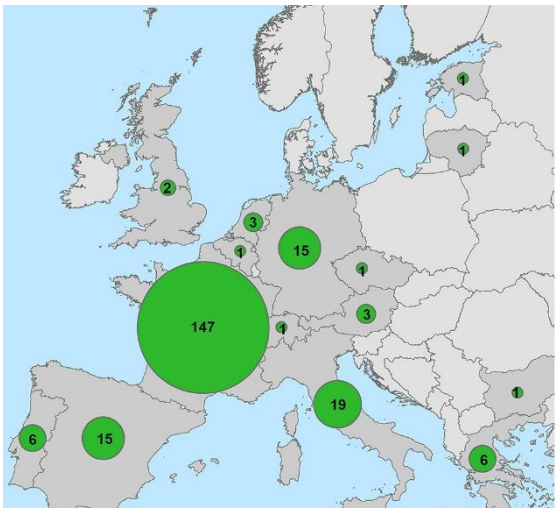


Figure 3 - Distribution of *Spirulina*'s production companies in Europe (Araújo, et al., 2021)

2.1.1. Composition and Morphology

Spirulina is a small (diameter between 3-12 µm) prokaryotic cyanobacteria, generally unicellular and often grow in large colonies (Mendes, et al., 2022). It has its name from its morphology,

since as it has a spiral or helical filaments, the main feature of the genus (Martins, et al., 2021; Habib, et al., 2008). However, this shape can morph spontaneously, depending on pH and nutrient conditions and others (Chittora, et al., 2020), as shown in Figure 4.

Arthrospira platensis, *Arthrospira maxima*, and *Arthrospira fusiformis* are three of the species of *Spirulina* that have been use and studied for their richness from a biochemical and nutritional point of view, being considered a sustainable source of natural high-value bioactive compounds (Varia, et al., 2022; Ali & Saleh, 2012). They are rich in proteins, carbohydrates, and lipids, but also in minerals, fatty acids, carotenoids and vitamins, Table 1 (Tan, et al., 2020).

Table 1 - Biochemical composition of *Spirulina*

	Reference	(Food and Drug Administration, 2002)	(Tan, et al., 2020)
Proximate composition (%)	Lipids	9.25 ± 0.09	11.32 ± 0.65
	Proteins	53.31 ± 0.67	56.39 ± 0.22
	Dietary Fibre		3.08 ± 0.38
	Carbohydrates	23.38 ± 0.07	14.82 ± 0.20
	Dry matter	77.54 ± 2.48	
	Ash		7.74 ± 0.09
	Moisture		9.73 ± 0.03
Minerals (mg/g)	Potassium	17.00 ±0.01	30.82 ± 0.31
	Phosphorous	9.70 ± 0.09	10.39 ± 0.22
	Calcium	2.70 ± 0.04	0.99 ± 0.00
	Magnesium	3.20 ± 0.03	3.40 ± 0.01
	Iron	0.53 ± 0.01	0.36 ± 0.00
	Manganese	0.02 ± 0.00	0.02 ± 0.00
	Zinc	0.02 ± 0.00	0.02 ± 0.00
Phytopigments (mg/g)	Chlorophyll a	7.44 ± 0.07	1.00 – 20.00
	Chlorophyll b	6.41 ± 012	
	C-phycocyanins	18.25 ± 1.81	28.51 – 103.00
	Allophycocyanins	5.34±0.14	8.32–25.02
	Phycoerythrin	3.47±0.23	4.25–10.28

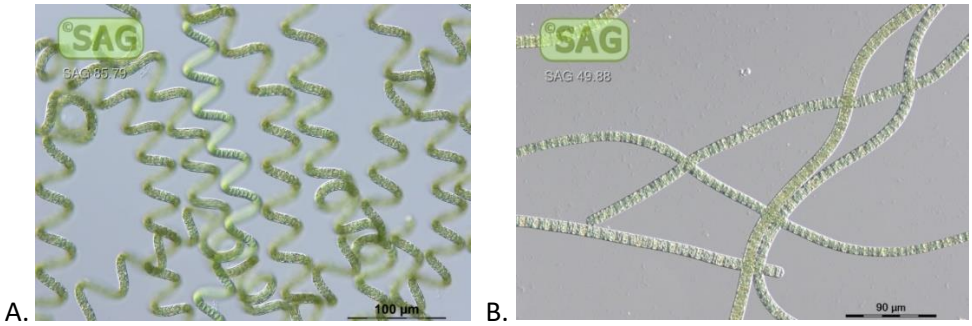


Figure 4 – *Spirulina* with (A.) and without (B.) its characteristic shape (Göttingen University, s.d.)

2.1.2. Cultivation

2.1.2.1. Production Systems

Production systems can be open or closed, differentiated by direct contact between the crop and the atmosphere (open systems) or by its absence (closed systems). Each of these systems can have different configurations: naturally occurring (lakes), circular or raceway ponds, and tubular, flat or tank photobioreactors, respectively, Figure 5 (Peralta, 2019; Habib, et al., 2008).

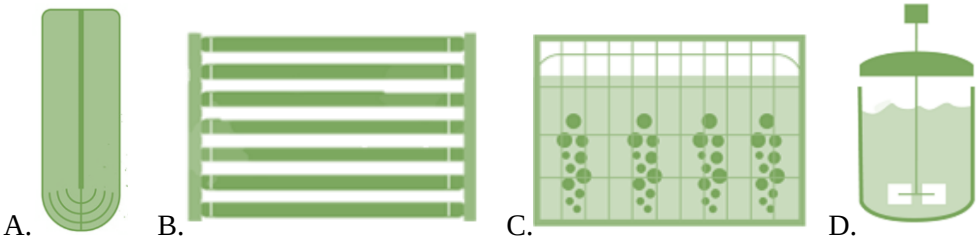


Figure 5 - Representative scheme of a raceway pond (A.), a tubular photobioreactor (B.), a flat photobioreactor (C.) and a stirred tank photobioreactor (D.). Adapted from (ALLMICROALGAE, s.d.)

Open pond cultivation has been one of the oldest ways to cultivate microalgae and is the most common system regarding S*pirulina*'s production (83%) (Araújo, et al., 2021), due to its relatively cheap construction, maintenance, and operation cost (low energy demand since has direct exposure to the sun and low oxygen build-up). It is also easy to clean and scale up (Tan, et al., 2020; Peralta, 2019), however, in comparison to other systems, open pond cultivation has low biomass yield, high evaporation losses, and insufficient mixing rate and light supply (restricted penetrability of light implying shallow ponds). Open system cultivation is also

associated with increased risk of contamination, but cultivation of *Spirulina* is feasible due to its high tolerance to harsh environmental condition (Thevarajah, et al., 2022).

Closed systems are those where algae are grown in an enclosed environment and the inputs, such as temperature, illumination and pH, can be precisely controlled. These transparent tanks, also known as photobioreactors, allow higher productivity and homogeneity of algae biomass, lower risk of contamination and demand less space comparing to open systems, due to their higher areal productivity. They do not depend on environmental factors, however, the biofilm formation during cultivation restricts uniform light distribution, hindering higher productivity. In addition, these systems are costly due to the construction and electric power requirement for operation and monitorization (Bhatnagar, et al., 2024).

A comparative analysis by Tredici (2004) stated that despite lower productivity in open systems (10-15 g/m²/day), the lower operational costs can make them economically viable for large-scale production compared to closed systems, which achieve higher productivity (25-30 g/m²/day) but at higher costs. These productivities were consistent with other studies (Vonshak & Richmond, 1988; Wang, et al., 2019). A recent solution to overcome these drawbacks are hybrid systems, integrating both systems in two-stages: maximize the biomass productivity in the first stage and enhancing the accumulation of biocompounds during the second stage (Thevarajah, et al., 2022). Additionally, modifications to the systems produce improvements to the productivities. Sirikulrat et al. (2021) demonstrated that the addition of a transparent light-scattering column to a raceway pond, in order to enhance the luminousness, increased the productivity by 48.3% to 22 g/m^3/day when compared to a conventional pond. Kubar et al. (2022) introduced a zigzag-flow column photobioreactor, that allowed the increase in biomass production by 28.8% as compared to conventional photobioreactor, slightly higher than the 17% increase obtained by Carlozzi and Torzillo (1996) with a curved tubular photo bioreactor.

2.1.2.2. Cultivation Conditions

Spirulina is generally found in a diverse environment, most commonly in tropical and subtropical regions in warm bodies of water with high carbonate/bicarbonate content, elevated pH (8.5-11), and salinity (> 30 g/L) (Silva, 2018; Ali & Saleh, 2012; Chittora, et al., 2020). It can grow photoautotrophically (using light), heterotrophically (utilizing only organic compounds,

i.e. glucose, as carbon and energy source) and mixotrophically (performing photosynthesis as the main energy source, though both organic compounds and carbon dioxide are essential) (Mata, et al., 2012; Chojnacka & Noworyta, 2004). A combination of these strategies can be used, for example in two-stages cultivation, in order to enhance biomass or biocompounds production (Santos, et al., 2016). In addition to light and carbon dioxide and/or an organic substrate, *Spirulina* requires a balanced supply of nutrients from the culture media for optimal growth and metabolism.

For industrial-scale biomass production, it is also crucial to understand and optimize these key factors influencing the crop's dynamics and growth (light, temperature, pH, nutrient availability, and carbon source), since the manipulation of these conditions can change its growth characteristics and chemical composition (Peralta, 2019). Culture media composition is one of the most influencing factors, and adding the fact that the media contributes the most to the cost of the process, it is commonly studied (Santos, et al., 2016; Soni, et al., 2019). The most simple and accessible media is seawater, however, Zarrouk medium, Aiba & Ogawa (A&O) medium and BG11 broth are also frequently used and studied, having similar composition (Table 2).

Table 2 - Comparison between three medium compositions (major ingredients)

Medium	Zarrouk ($g\ L^{-1}$)	A&O ($g\ L^{-1}$)	BG11 ($g\ L^{-1}$)
$NaHCO_3$	16.8	13.61	
$NaNO_3$	2.5	2.5	1.5
NaCl	1.0	1.0	
K_2SO_4	1.0	1.0	
K_2HPO_4	0.5	0.5	0.04
$MgSO_4.7H_2O$	0.2	0.2	0.075
$FeSO_4.7H_2O$	0.01	0.01	0.006
$CaCl_2.2H_2O$	0.04	0.04	0.036
EDTA	0.08	0.08	0.02
Na_2CO_3		4.03	0.001
Citric acid			0.006
Reference	(Dineshkumar, et al., 2016)	(Santos, et al., 2016)	(Dineshkumar, et al., 2016)

With increased sustainability concerns, and taking advantage of the high adaptability of *Spirulina*, alternative low-cost media are becoming the focus of new studies. Besides modifications of synthetic media and some other less known, such as the Kosaric, CFTRI, OFERR and Schlosser's media (Lim, et al., 2021; Ragaza, et al., 2020), *Spirulina* cultivation in wastewaters and vinasses, among others, has become more frequent due to lower cost (advantageous for industrial production) (Ragaza, et al., 2020) that would potentially enhance economic viability and sustainability (Thevarajah, et al., 2022).

For example, Reham et al. used a vermicompost-based culture medium, Gami et al. studied a fertilizer medium and Vijayarasa et al. (2021) used fishpond grain-soaked and poultry wastewater (Lim, et al., 2021). Cardoso et al. (2021) obtained a biomass production of 1.23 g L^{-1} with 32.3% in protein content in an aquaculture wastewater medium, slightly higher than Djaghoubi et al. (2015) using municipal wastewater (1.10 g L^{-1} and 28.16%, respectively). Mata et al. (2020) combined wastewater from the desalination process and Zarrouk's medium (25%), achieving similar biomass productivities. Santos et al. (2016) using sugarcane vinasse in an autotrophic and heterotrophic cyclic cultivation obtained a biomass concentration of 0.61 g L^{-1} but a higher protein content (77.3%), revealing a same trend as Pereira et al. (2022) using brewing residues: lower biomass concentration and higher protein content (0.9 g L^{-1} and 51.8%). Despite these positive results, there is still a concern about contamination of toxins, heavy metals, microorganisms, etc. in the biomass cultivated in these waste resources and, consequently, in the end products such as food, feed and pharmaceuticals (Rzymski, et al., 2015). The use of recycled medium is another option that has shown promising results, since it is able to reduce the cost of the cultivation medium by utilizing the non-consumed nutrients alongside with little nutrient supplementation (Thevarajah, et al., 2022; IEA Bioenergy, 2017).

Regarding illumination, solar irradiation is the light source used in outdoor cultivation. In general, 30% of indirect sunlight is recommended to avoid overheating the culture media and the photoinhibition. It is important to note that although sunlight is a natural resource, its availability varies with geographic region, seasonal changes, and climatic conditions (Thevarajah, et al., 2022). For closed systems, the most common light source is light-emitting diodes (LEDs) for their energy efficiency, longevity, and ability to provide specific wavelengths conducive to photosynthesis. In fact, recent studies have highlighted the efficacy of red and blue wavelengths, in promoting both biomass and protein production (blue enhance biomass

growth, while red increases the production of phycocyanin). Moreover, maintaining a light intensity within the range of 100-200 µmol m^{-2} s^{-1}, using a photoperiod of 12:12 h or 16:8 h light-dark cycle, has been found optimal for indoor Spirulina cultivation, ensuring sufficient light without causing photoinhibition (Bhat, et al., 2023; Stunda-Zujeva, et al., 2023).

2.1.3. Harvesting

Harvesting, the collection of the microalgae biomass the culture media, is a crucial step in the processing and makes up to 30% of the total production cost (Tan, et al., 2020).

Selection of an appropriate harvesting method depends on the end product value and properties (must not hinder the recovery of the product of interest), but also on the strain and media features (final moisture level, salt concentrations, cell damage, density and size) (Barros, et al., 2015). Harvesting methods are divided into mechanical, chemical, biological or electrical based methods, singularly or in a combination to obtain a greater separation rate at lower costs (Morais, et al., 2020; Barros, et al., 2015). Physical or mechanical processes, such as centrifugation, are the most reliable and the most commonly used, however, they require intensive use of energy. Chemical methods have high recovery rate, however there may be contamination, which can affect the end product. On the other hand, biological approaches such as bio- or auto-flocculation, are emerging and can lead to further reduction of operational costs (Nazari, et al., 2021).

2.1.3.1. Sedimentation

Sedimentation is the most simple and inexpensive method, since it only requires gravity. It is often employed in *Spirulina* harvesting due to the algae's morphology and capacity to bioflocculate. However, when comparing to other methods, it has low separation efficiency and is time-consuming, leading to the possibility of biomass deterioration (Barros, et al., 2015). Therefore, it is often employed after other method.

2.1.3.2. Centrifugation

Centrifugation is based on sedimentation but with enhanced gravitational force to increase the rate of sedimentation. Therefore, it is a fast method, with high recovery efficiencies. It is widely used since it has capacity for industrial processes and is suitable for almost all microalgae species (Grima, et al., 2004; Barros, et al., 2015). On the other hand, it is expensive and has high energy requirements, and there is possibility of cell damage due to high shear forces, so not recommended for some applications.

2.1.3.3. Filtration

It works by passing the media trough a filter cloth (canvas, nylon, metal or glass fibre) with application of suction (or vacuum) or pressure differences. Filtration has the advantages of continuous operation and high recovery efficiencies, however, is only suitable for harvesting fairly large microalgae, including *Spirulina*. Additionally, it can have high costs for membrane replacement (due to clogging) and pumping (Grima, et al., 2004; Barros, et al., 2015).

2.1.3.4. Flocculation-coagulation

Flocculation and coagulation are two processes, usually combined, based on the addition of cationic polymers to the medium, combining particles in suspension onto larger aggregates followed by the agglomeration of these into larger flocs. The effective particle size is increased, thus significantly reducing its energy demand and is generally followed by gravity sedimentation (Barros, et al., 2015).

Although these processes are simple and fast, with no energy requirements and inexpensive, the coagulants cost represent a significant portion of the overall process. Additionally, common flocculants may be toxic to biomass. Therefore, the use of naturally available flocculants, or biological alternatives, has been studied.

Bioflocculation refers to the use of biopolymers secreted by bacteria. This option is inexpensive and non-toxic, however, co-culture may result in microbiological contamination, interfering with food or feed applications of microalgal biomass (Barros, et al., 2015).

2.1.4. Preserving

Biomass processing may follow two routes according to the moisture requirements for application: dry or wet biomass. To preserve biomass for applications in which high levels of moisture is allowed, periodic transfer (serial subculture) is applied (Morais, et al., 2020; Foo, et al., 2023). This is a simple and easy method but is laborious and there is the possibility of genetic drifting and contamination over time. However, for the majority of applications, and since *Spirulina* is very perishable as its high moisture content contributes to its degradation, other methods of preservation are applied, namely lyophilization and cryopreservation. These methods facilitate the prolonged storage and easy transport from local microalgae production sites to processing plants, however, they are not energy-efficient and require special equipment (Arguelles, 2020; Silva, et al., 2019).

2.1.5. Applications

As mentioned before, *Spirulina* has a vast biotechnological potential as a source of compounds such as dyes, antioxidants, emulsifiers, and gelling agents used for various applications in the food, cosmetics, pharmaceutical, environmental and biofuel production areas, Figure 6 (Peralta, 2019). These applications might be employing some isolates or the whole biomass.

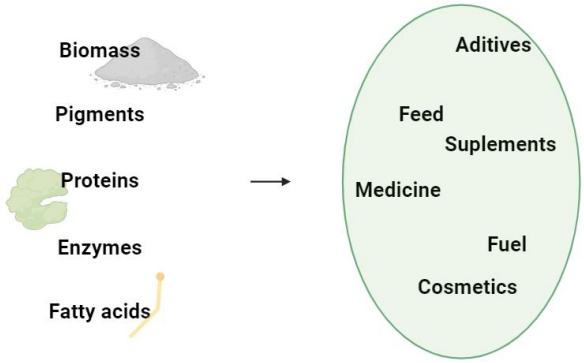


Figure 6 - *Spirulina*'s products and applications. Adapted from (Silva, 2018)

Newer applications also included in these areas are related to fibres and bioplastics, employed as food packaging and wound healing membranes, for example (Moreira, et al., 2019; Nematollahi, et al., 2020).

2.1.5.1. Food and Nutraceuticals

Around 75% of *Spirulina* production is directed to food and food supplements and nutraceuticals (Araújo, et al., 2021). Dry biomass of *Spirulina* is commercialized as powder or in capsules, or alternatively is incorporated into the food products to enhance its nutritional value or provide healthy properties (European Algae Biomass Association, 2021). The high digestibility of cyanobacteria cells, due to the lack of cellulose, unlike most algae, facilitates their use for human consumption (Tomaselli, 2004).

In Portugal, the most known form of *Spirulina* goods is as supplements. Some products with *Spirulina* incorporation sold in the supermarkets are mostly juices, smoothies and yoghurts, Figure 7, possibly because these products are already coloured, and consumers demand for healthy and natural flavours or ingredients (Lafarga, 2019).

Figure 7 - Products containing *Spirulina*: pasta, Sol Natural (A.); energetic bar, EOS Nutrisolutions (B.); smoothie, Innocent (C.); nectar juice, Continente (D.); E. candy, Tic Tac (E.); biscuits, Próvida (F.); yogurt, Continente (G.)

Additionally, other products are being formulated and tested, as described in the literature. Some of them are listed in Table 3.

Table 3 - Products formulated with *Spirulina*'s biomass or extracts, and tests subjected.

Product	*Spirulina* content	Evaluations	Reference
Cereal snack	5%	Product texture and characteristics Nutritional characterization Sensory analysis	(Letras, et al., 2022)
Cookies	Antioxidant extract	Product texture and characteristics Nutritional characterization	(Vieira, et al., 2020)
Vegan biscuits	20% (microencapsulated)	Product texture Nutritional characterization Sensory analysis	(Silva, et al., 2021)
Chocolate biscuits	1%	Product texture and characteristics Nutritional characterization Sensory analysis	(Morais, et al., 2006)
Biscuits	0.5%	Nutritional characterization Sensory analysis	(Barakat, et al., 2016)
Biscuits	4%	Product texture and characteristics Nutritional characterization Sensory analysis	(Gun, et al., 2022)
Croissant	1%	Product texture Nutritional characterization Microbiological analysis Sensory analysis	(Massoud, et al., 2016)
Doughnut	5.41%	Product texture Nutritional characterization Sensory analysis	(Rabelo, et al., 2013)
Gluten-free bread	4%	Product texture and nutritional characterization Sensory analysis	(Figueira, et al., 2011)
Ice cream	0.25% phycocyanin extract	Product texture and characteristics Antioxidant activity	(Amarante, et al., 2020)

Product	*Spirulina* content	Evaluations	Reference
Ice cream	1%	Product characterization Sensory analysis	(Faresin, et al., 2022)
Beer	5 % (w/w malt)	Process parameters Nutritional characterization Sensory analysis	(Beisler & Sandmann, 2022)
Detox	0.8 g	Product characteristics Nutritional characterization *In vitro* digestibility	(Paiva, et al., 2024)
Soup	0.5% (w/w)	Product characteristics Nutritional characterization Sensory analysis	(Lafarga, et al., 2019)
Energy gels	0.50% biomass	Product texture and nutritional characterization Sensory analysis	(Moreira, et al., 2018)
Snack	2.5%	Nutritional characterization Microbiological analysis Sensory evaluation	(Morsy, et al., 2014)
Yogurt	0.25%	Product characteristics Nutritional characterization Microbiological analysis Sensory evaluation	(Barkallah, et al., 2017)
Yogurt	1%	Product characteristics Nutritional characterization Cytotoxicity evaluation	(Silva, et al., 2019)
Cheese	0.75%	Nutritional characterization Microbiological analysis Sensory evaluation	(Ismail, et al., 2023)
Cheese	3% (with 3% *Chlorella vulgaris* and 1% curcumin)	Nutritional characterization Cell viability assay Sensory analysis	(Jalili, et al., 2024)

Some of the most common challenges in the formulation of these new products and in the following industrialization and commercialization are related to health safety, due to nonbiological and biological contaminants, the regulation (legal approval for consumption and commercialization) and the consumer's perception of colour, odour, flavour, and texture. Furthermore, there may be some production constraints, related to the scale-up of the process and its operational costs (Mendes, et al., 2022). However, the positive nutritional and sensorial results achieved reveal promising opportunities for the reformulation of other products, that could be welcomed by the consumers and of particular interest to market niches such as vegan, health-conscious, or sustainable-conscious consumers.

2.1.5.2. Animal feed and Aquaculture

Although *Spirulina* can be processed and consumed directly, with its well-balanced nutritional components already mentioned, it is also desirable as an animal dietary feed supplement. Including *Spirulina* as a protein source in animal feed culminates in a nutritional end product beneficial for human growth and development (Ragaza, et al., 2020).

Cereal grains and soybean meal are the main feedstuffs used in swine and poultry feeding. However, they can be used for human consumption directly (competition) and they may have sustainability issues, since they are grown mostly in America and transported over large distances (Martins, et al., 2021). *Spirulina* can be cultivated free of arable land, therefore using alternative resources, important to ensure a sustainable development of food production systems to meet growing global protein demands. Broiler feeding trials with *Spirulina* showed that it can be successfully incorporated into poultry diets, with additional amino acid supplementation. However, some studies show no influence on growth and meat quality traits, while others reveal improvements in meat quality, such as increased water-holding capacity and decreased off-flavours. Other contested claims are its effect on improving fatty acid composition (PUFA levels) and effects on lipid peroxidation. The exception of this inconsistency is the deeper colour pigmentation obtained, that can have negative impacts on some consumers. Similarly, *Spirulina* can be included in swine feed, with appropriate amino acid supplementation, without disadvantaging animal nutrition or product quality (Martins, et al., 2021; Altmann & Rosenau, 2022).

This cyanobacterium has also been the subject of studies in aquaculture as a fishmeal replacer or as a functional feed additive to enhance the health, quality, and stress resistance of the organism. The viability of replacing fishmeal with *Spirulina* are highly dependent on each species of fish and its trophic level, affecting negatively more carnivorous fish species than omnivorous in terms of growth performance and feed conversion ratio. The best growth performances ranged from 40 to 60% replacement levels, while complete fishmeal replacement only was acceptable in some carps (Ragaza, et al., 2020; Altmann & Rosenau, 2022).

Thus, *Spirulina* can be incorporated into animal diets without forfeiting productivity and quality, even though more studies are required. Despite increased interest, there are still limitations for use in commercial rations, being one of the biggest the production cost. However, with an optimized *Spirulina* production, achieving high production capacities, small space requirement and low energy and water consumption (Ragaza, et al., 2020; Altmann & Rosenau, 2022).

2.1.5.3. Cosmetics and Pharmaceuticals

Spirulina has gained significant attention in the cosmetic and pharmaceutical industries due to its robust antioxidant, anti-inflammatory, antimicrobial properties and others, mainly related to the high phycocyanin content, Figure 8 (Thevarajah, et al., 2022; Fernandes, et al., 2023).

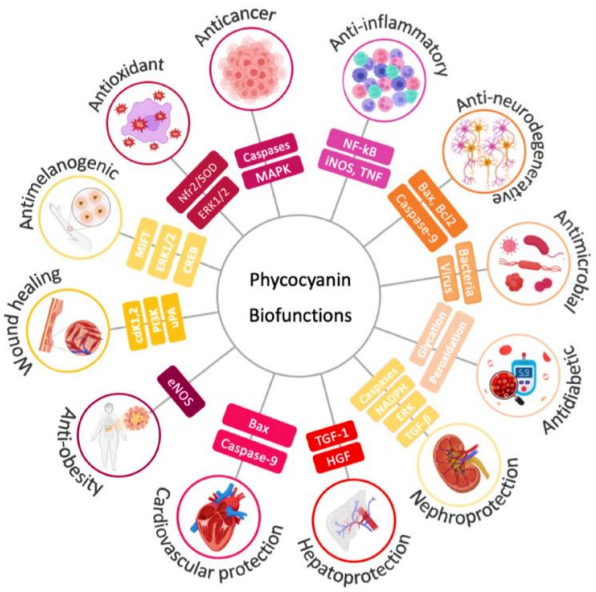


Figure 8 - Biological functions of phycocyanin. From (Fernandes, et al., 2023)

In addition, *Spirulina*'s content of essential fatty acids, vitamins (particularly E), and phenolic compounds contributes to its anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines (Jung, et al., 2016). Moreover, it exhibits notable antiviral and antimicrobial activities against a range of pathogenic virus (Mader, et al., 2016; McKinley, et al., 2024), bacteria and fungi (Chakraborty, et al., 2015; Abdel-Moneim, et al., 2022), which is particularly beneficial for treatment of infections, foodborne diseases and skincare applications.

The therapeutic potential of *Spirulina*'s bioactive compounds extends into the scope of chronic disease management. The antioxidant properties are particularly beneficial in the context of diabetes, where oxidative stress plays a significant role in disease progression. Studies have demonstrated that *Spirulina* supplementation can improve glycemic control and blood lipid profiles associated with diabetes complications (Hatami, et al., 2021; Parikh & Iyer, 2001). Furthermore, *Spirulina* has shown promise in obesity management by enhancing lipid metabolism, reducing body fat, and suppressing appetite, thereby lowering the risk of cardiovascular diseases (Moradi, et al., 2019). The anti-inflammatory properties are also beneficial in managing cardiovascular diseases, as they help reduce arterial inflammation and improve endothelial function, thereby lowering the risk of atherosclerosis and related complications (Prete, et al., 2024; Wu, et al., 2016).

These therapeutic benefits suggest that *Spirulina* could be an effective adjunct in the prevention and treatment of metabolic and cardiovascular disorders, potentially offering a natural, multi-targeted approach to improving human health, besides being a valuable ingredient in cosmetics for skin rejuvenation, wound healing, and overall enhancement of skin health.

2.1.5.4. Agri-environmental applications

In the face of growing environmental challenges, new processes for sustainable agriculture and environmental protection are more critical than ever. Current practices, heavily dependent on synthetic fertilizers and pesticides, intensive cultivation and over irrigation, lead to the overuse, exploitation and pollution of land and water resources (Singh, et al., 2016). Algae inoculation or application of its extracts have beneficial effects in improving soil and water quality, but also on growth, stress (biotic and abiotic) adaptation and pest control, boosting crop yields and resilience (Oosten, et al., 2017; Ammar, et al., 2022). Additionally, it can be also applied to

agricultural and livestock wastewater treatment, adding another front of acting. With minimal resource requirements and a capacity for carbon sequestration, *Spirulina* is a key player in advancing eco-friendly and sustainable industrial agro-environmental practices.

Nitrogen is a key nutrient for plant development since it is required to protein synthesis and enzyme function. It is usually supplemented with the addition of fertilizer, however, their excessive and long-term usage has resulted in several ecosystem imbalances (Ammar, et al., 2022). Firstly, the use and production of these synthetic fertilizers, plant-protection chemicals or pesticides, and plant-growth hormones, fossil-fuel based, releases greenhouse gases such as NO_2 and CO_2 (Ammar, et al., 2022; Varia, et al., 2022). Additionally, from the overuse, resistance to several insecticides available was developed by some common pests (Sharanappa, et al., 2024) and nutrient leaching causes eutrophication of freshwater and marine environments. Soil properties such as water and nutrient holding-capacity, as a result of organic matter loss, are also negatively impacted (Alobwede, et al., 2019). The need for reduction in chemical products in modern agriculture is strongly increasing the interest in organic based products, called biosolutions (biopesticides, biostimulants, biofertilizers, among others) (Lanzotti, et al., 2022). These are non-nutrient based solutions, that contain bioactive compounds or living microorganisms that enhance crop performance, seed germination, blooming, and fruit production, increase resistance to stress, and optimize nutrient utilization efficiency, or affect plagues (Orlina, et al., 2023; Varia, et al., 2022; El-Sayed, et al., 2024).

Spirulina possesses considerable quantities of vitamins and minerals and trace elements, which could be sequestrated to contribute to crop micronutrient uptake and, because it participates directly in the assimilation of atmospheric carbon dioxide, is also a major organic matter source (Ammar, et al., 2022; Thevarajah, et al., 2022; Alobwede, et al., 2019). Furthermore, the excretion of exopolysaccharides into the soil improves fertility, seed germination, plant growth, yield, and nutritional value of crops (Ammar, et al., 2022). In fact, according to Wuang et al. (2016), the germination of Chinese cabbage (*Brassica rapa Chinensis*) and Kai-Lan (*Brassica oleracea alboglabra*) was improved upon application of biofertilizer produced from *Spirulina platensis* cultivated in aquaculture wastewater. Furthermore, *Spirulina* biofertilizers have increased the growths of bayam red (*Amaranthus gangeticus*), arugula (*Eruca sativa*), pak choy (*Brassica rapa*) and red beet (*Beta vulgaris*) (Wuang, et al., 2016; Ammar, et al., 2022), and biomass micro- and macro-elements content in radish (*Raphanus sativus*) when compared to chemical fertilizers (Godlewska, et al., 2019). Agnol et al. (2021) tested carbon quantum dots,

nanoparticles easily obtained from *Spirulina* biomass pyrolysis, that increased overall growth of lentil seedlings when applied at 0.1 mg/mL concentration. On the other hand, the biopesticide properties are less studied. *Spirulina* extracts demonstrated biocontrol properties against *Bruchidius incarnatus* (beetle) and *Spodoptera frugiperda* (fall armyworm) increasing the mortality of larvae and deformities and decreasing adult longevity, number of eggs, larval and pupal duration and weight (Sharanappa, et al., 2024). Additionally, the application of a phycocyanin-rich Spirulina extract impacted microbial communities, promoting diversity but reducing the abundance of several pathogens from the Gammaproteobacteria class (Varia, et al., 2022).

On the other hand, *Spirulina* can also be used for bioremediation. Besides the nutrient excess, especially N and P that lead to eutrophication, those contaminated water resources mentioned earlier may contain heavy metals provenient from agriculture but also mining, energy generation and fuel manufacturing, for example (Ammar, et al., 2022). Despite important biochemical and physiological functions in living organisms that some heavy metals have, the majority are toxic even at low concentrations and bio accumulate, threatening both crop output and human health (Cepoi, et al., 2020). Biosorption using microalgae is an efficient, rapid, safe, and economical method, since they don't require treatment and possess high metal ion binding capacity on the cell wall (Moubayed & Al-Houri, 2022). In fact, several studies demonstrated Spirulina's capabilities of removal and/or accumulation of nutrients N and P, chromium, cobalt (82%), cadmium, copper (90.6%) and mercury (Peres, et al., 2018; Moubayed & Al-Houri, 2022). *Spirulina* also enhances other plants bioremediatory effects by increasing growth and eventually the residual metal content in leaves and stems, or by chelating the metals and avoiding their leaching before plant growth (Musio, et al., 2022).

2.1.5.5. Energy

The use of microalgae for biofuel production has been the focus of several research and industrial efforts over the last decades as an alternative to conventional fuels, due to its price increment and depletion, global warming, and population growth (Thevarajah, et al., 2022). The benefits of the use of microalgae include the fact that they do not compete for arable land with food and feed crops. Additionally, these valuable compounds can be obtained in several phases of the biomass life cycle: as a whole or residual biomass, as extractable compounds or even as secondary products of photosynthesis (Araújo, et al., 2021).

Firstly, the relative high content of carbohydrates in *Spirulina* (starch and cellulose) can lead to high quality bioethanol. Even though lipids are present in lower concentrations, they have been extracted to produce biodiesel (Pradana, et al., 2020). Additionally, the biomass resultant of the valuable compound extraction (or primary biomass) can be converted into biogas through anaerobic digestion, which can be upgraded to biomethane and utilized in natural gas grids or as a vehicle fuel. It can also be used in combined heat and power systems, where its combustion or gasification cogenerates heat and electricity. Other thermochemical processes, such as hydrothermal liquefaction, also allow for the conversion of Spirulina into bio-oil, which can be refined into biofuels (IEA Bioenergy, 2017). Finally, *Spirulina* holds promise for energy generation via photobiological processes with microbial fuel cells. Protons originating from organic matter biodegradation can lead to the production of hydrogen gas (Hasnaoui, et al., 2020), or form water after bonding with synthesized oxygen (Longtin, et al., 2021), while generating energy.

These diverse applications position *Spirulina* as a versatile and sustainable source of energy However, biofuel production has not been extensively explored since further technological developments are needed to upscale the production volumes and reduce the production costs (Araújo, et al., 2021)

2.2. Sustainability

Sustainable development is, by definition, the development that meets the needs of the present without compromising the ability of future generations to meet their own needs (World Commission on Environment and Development, 1987). However, considering the critical conditions of the environment in the present days, we must consider also recovering the excessive damage provoked by previous generations. This development comprises all fronts of action, which means that needs to be based on reliable, affordable, economically viable, socially acceptable and environmentally friendly energy services and resources (Ghatak, 2011).

Applying the sustainable development premises to industrial processes leads to the full exploitation of the raw materials, generating a diversified spectrum of products to offset the overall production costs and improve the sustainability of the overall process (Thevarajah, et al., 2022). This concept is related to the term biorefinery, that refers to co-production of transportation biofuels, bioenergy and/or marketable chemicals from renewable biomass sources (Ghatak, 2011).

In fact, biomass, where microalgae like *Spirulina* is included, is unexplored to its maximum potential since only the energy potential is generally recognized. As biorefineries can improve the sustainability of biomass, especially in the economic front (economically viable and competitive), by diversifying the product portfolio and the combinations of various feedstocks and processing technologies, harnessing and appropriate utilization of biomass becomes indispensable for a circular bioeconomy (Thevarajah, et al., 2022; Ghatak, 2011).

2.2.1. Environmental, Social and Economic Impact Assessment

During the conceptualization and design of biorefineries, it is important to evaluate the project regarding its contribution to the sustainable development. There are several tools available and studied that cover one or more dimensions of sustainability (economic, environmental, and social).

2.2.1.1. Footprints

The footprint methods provide rules for conducting a reliable and transparent assessment of specific environmental impacts of products and organisations throughout its life cycle. There are different types of footprints, each related to a natural resource.

The Ecological Footprint measures the use of bioproductive space, in hectare- equivalents, as land and water area required to produce the resources consumed and absorb the waste generated (Nautiyal & Goel, 2021). The Water Footprint measures the consumption and contamination of freshwater resources in cubic metres per year that are used to produce a good or service, including both direct and indirect water use (for example in supply chains). The Carbon Footprint measures the total amount of greenhouse gases emitted directly or indirectly by a product or process, in carbon dioxide (CO_2)-equivalents per unit of time or product. Recently, the nitrogen footprint was introduced to measure the release/consumption rate of nitrogen in the environment (Ercin & Hoekstra, 2012; Chapagain, 2017).

2.2.1.2. Life Cycle Assessment (LCA)

The LCA and the footprint methodologies differ in terms of the impacts assessed, having the LCA a broader scope of analysis and therefore being the preferable tool to assess the environmental dimension (Toro & Alzate, 2023). LCA is also associated with all stages of a product's life cycle, from raw material extraction (cradle) to disposal (grave), but covers various environmental indicators such as global warming potential, resource depletion, acidification, and eutrophication.

A variation that includes the social and socio-economic impacts along the life cycle of a product or process is the social-LCA (Bhatnagar, et al., 2024). It assesses factors like labour conditions, human rights, and community well-being, providing a more holistic view of sustainability that extends beyond environmental factors. However, due to the lack of quantitative information, it is less employed.

2.2.1.3. Techno-Economic Analysis (TEA)

TEA evaluates the economic feasibility of a technology or process by analysing its technical and financial aspects. It involves calculating costs, revenues, and potential risks associated with production processes, often employing indicators such as the Return on Investment, the payback period and the Net present value (Silva, et al., 2020). TEA is critical for determining whether new technologies can be scaled up from research to industrial use in a financially viable manner.

3. Materials and Methods

3.1. Microalgae Production and Characterization

3.1.1. Cyanobacteria Cultivation Media and Conditions

Inoculums of *Arthrospira maxima* (SAG Strain Number 49.88) and *Arthrospira platensis* (SAG Strain Number 85.79) were obtained from the Culture Collection of Algae at the University of Göttingen, Germany, Figure 4 B. and 4.A, respectively. According to the supplier, *A. maxima* filaments were no longer spiral, and both inoculums have bacterial or other types of contamination.

Cultures were scaled-up and maintained in A&O medium (Santos, et al., 2016), exposed to 2000 lux (30 µmol photons m^{-2} s^{-1}) measured with a light meter (Lutron, model LX-1102) with a 12:12 h photoperiod of light:dark cycle under agitation with regular aquarium pumps (Boyu, model S-4000B).

Growth tests were run with A&O medium, as well as with cheese whey. Previous experiments performed by Athanasiadou et al. (2023) showed that the concentration of 10% (v/v) cheese whey exhibited the best performance and that a concentration of 20% (v/v) appear to inhibit the growth. Therefore, Spirulina was grown until the microorganisms reached the stationary phase, with a photoperiod of 12:12 h, in 500 mL flasks with 10% and 20% cheese whey, provided by Prados de Melgaço®, in duplicate. A control flask, with A&O medium, was also used. Growth was monitored by measuring the optical density at 730 nm (Santos, et al., 2019) using a spectrophotometer (Shimadzu, model UV-1900i). Heterotrophic (glucose-fed) and mixotrophic

growth were not evaluated since it outputs a slower maximum specific growth rate (Lee, 2004; Santos, et al., 2016).

3.1.2. Concentration Measurement

The biomass concentration as dry weight was measured gravimetrically in an analytical balance (Mettler, model AT201) by obtaining the weight difference of fibre glass membranes (Prat Dumas, 1.2 µm porosity) before and after filtering the cell suspensions and drying of the retained cells. The filters with the obtained biomass were washed with distilled water for salt removal and then were dried at 105 °C (Neytech Vulcan, model A-550) to a constant weight.

3.1.3. Harvesting and Preservation

Biomass was harvested by filtration on nylon net filter and washed with distilled water for salt removal. Then, samples were frozen at -80 ºC, lyophilized (VirTis, model 6KBTEL-85), and stored at −4 °C until use (Santos, et al., 2019).

3.1.4. Composition Analysis

The macronutrient composition (proteins, lipids, carbohydrates and ashes) and energy value of the lyophilized biomass were evaluated preferably according to methods described in the literature. Additionally, the evaluation of some bioactive properties was performed (phycocyanin, antioxidant activity). The cheese whey wastewater, culture media residues and phycocyanin extraction residue were analysed for total N (Shimadzu, model TNM-1) and pH (Consort multi-parameter analyser, model C1010). The chemical oxygen demand (COD) was also measured on cheese whey wastewater.

3.1.4.1. Protein content

The protein content was estimated by the Kjeldahl method by conversion of total nitrogen, with some changes. Briefly, 0,250 g of the sample was added to each Kjeldahl digestion tube, followed by 12 mL of concentrated sulfuric acid, 2 Kjeldahl tabs (Thompson&Capper, Kjeltabs CK, 3.9 g), 4 anti-bumping spheres, and 3 mL of 35% hydrogen peroxide. The digestion was performed with heating ramps: 90 min to 180 ºC, 30 min to 240 ºC, 15 min to 320 ºC and then 90 min to 380 ºC. After digestion, distillation was performed with a semi-automatic distiller (Raypa, model DNP-1500-MP). To each digestion tube, 75 mL of demineralized water was added. Then, in the distiller, 100 ml of 40% NaOH was added to neutralize the acid and convert ammonium ions to ammonia gas. Distillation was performed for 4 min and the distillate was capture in a 4% boric acid solution containing the mixed indicator. Finally, the collected distillate was titrated with a standard 0.1 N HCl solution until the endpoint is reached (colour change of the indicator from green/blue to pink/orange). The Kjeldahl Nitrogen content, %N, was obtained with the equation (1):

$$\%N = (V_{titration} - V_{blank}) * 1.401/ m \qquad (1)$$

where, $V_{titration}$ stands for the volume of the standardized acid used for the titration of the samples (mL), V_{blank} is the volume of the standardized acid used for the titration of the blank (mL), and m is the weight of the sample (g).

The protein content, %Prot, was obtained by multiplying the total nitrogen in biomass by 4.22 (Santos, et al., 2016).

3.1.4.2. Lipid content

Lipids were quantified according to the Bligh and Dyer (B&D) method described by Breil et al. (Breil, et al., 2017). 500 mg lyophilized *Spirulina* were mixed with 3.2 mL of distilled water, 4 mL of chloroform and 8 mL of methanol to reach 1:2:0.8 parts chloroform:methanol:water (v/v/v) and homogenized in the vortex (Scientific Industries Vortex Genie, model 2) for 10 min. Then, chloroform and water containing 0.85% of KCl were added to get a final ratio of 2:2:1.8 chloroform:methanol:water (v/v/v), forming the biphasic system. For a clear separation of the phases and the cell debris, the mixture was centrifugated at 4000 rpm for 10 min. After

centrifugation, the lower chloroform phase was collected and let evaporate in a water bath at 50 ºC (Raypa) (Branco-Vieira, et al., 2018). To evaluate the total lipids content, %Lip, by gravimetry, the dry residue was weighed, and yields were determined with the equation (2), where $m_{residues}$ is the mass (g) of the residues after solvent evaporation, $V_{organic\ phase}$ and $V_{residues}$ are the volumes (mL) of the collected phase and of the residues after solvent evaporation, and *m* the mass (g) of the lyophilised biomass:

$$\%Lip = (m_{residues} * V_{organic\ phase}) / (m * V_{residues}) * 100 \qquad (2)$$

Additionally, a greener approach to the classical B&D method was tested, denominated green B&D ($B\&D_G$), using the ethanol–ethyl acetate–water ternary system instead of methanol-chloroform-water, in the same proportions (Breil, et al., 2017). The comparison of differences among the methods was evaluated with one-way ANOVA with Fisher's Least Significant Difference test post hoc test at the 0.05 probability level.

3.1.4.3. Ash content

Ashes content was estimated by dry biomass calcination in muffle furnace. Firstly, biomass was obtained by vacuum filtration with glass fibber membranes (1.2 µ, Ø 47 mm, Prat Dumas), previously washed, dried and weighed. Ashes were obtained by incinerating the membranes with the biomass in the muffle furnace (Vulcan, model A-550) at 450 ± 50 ºC for 1 h, repeating until constant weight. The ashes weight was calculated from the subtraction between the final weight (after cooled in a desiccator) and the initial weight of the membrane (Santos, et al., 2016).

3.1.4.4. Carbohydrate content

Carbohydrates, %CH, were calculated by difference, according to the equation 3 (Silva, 2018):

$$\%CH = 100 - \%Lip - \%Prot - \%Ashes \qquad (3)$$

where $\%Ashes$ is the percentage of ash content of the sample.

3.1.4.5. Total Energy

Total energy, E (kcal/100 g dry weigh), was estimated difference, following the equation 4 (Silva, 2018):

$$E = 4 * \%Prot + 4 * \%CH + 9 * \%Lip \quad (4)$$

3.1.4.6. Phycocyanin

The freezing–thawing method was adapted from Santos et. al (2019). Briefly, 5 mL of distilled water and 10 mg of lyophilised biomass were mixed and underwent a cycle of freezing for 24h and defrosting. Then, the extracts were centrifuged at 2720 g (4000 rpm) for 20 min (Nahita blue Medibas+, model 2741) and had their absorbances measured in a light-free environment at 615, 652 and 280 nm (A_{615}, A_{652} and A_{280}, respectively). The extraction with two cycles was also studied. Phycocyanin concentration in the extract ($C - PC_E$; mg·mL^{-1}), yield as the phycocyanin content in the biomass ($C - PC$; mg·g^{-1}), and the extract purity (P_E) were determined with the equations 5, 6 and 7 respectively, being *V* the solvent volume (mL) and *m* the mass (g) of the lyophilised biomass:

$$C - PC_E = (A_{615} - 0.474 * A_{652})/5.34 \quad (5)$$

$$C - PC = (C - PC_E * V_{solvent})/m \quad (6)$$

$$P_E = A_{615}/A_{280} \quad (7)$$

The comparison of differences among the methods was evaluated with one-way ANOVA with Fisher's Least Significant Difference test post hoc test at the 0.05 probability level.

3.2. Sensorial analysis of Butter Supplemented with *Spirulina*

3.2.1. Production of a Butter Supplemented with *Spirulina*

Butter commercially available (Primor®) was softened at room temperature and then was mixed with *Spirulina* powder commercially available (Naturitas®, to ensure consumer's safety), until uniform appearance was achieved. Three *Spirulina* concentrations were used: 1%, 2% and 5%.

3.2.2. Tests of Acceptance of a Butter Supplemented with *Spirulina*

Firstly, to avoid conducting a complex and time-consuming 3 product evaluation, that could drive away volunteer tasters, a preliminary screening study was conducted to determine the best accepted sample.

Three research questions were created following the idealization of the product:

- How open are consumers to new products?
- Does *Spirulina* influence the product's sensorial properties? If it does, is it positively?
- Is the product generally accepted and what can be upgraded?

To address these questions, an in-person survey following a sample tasting was conducted (Annex A). Four tastings were performed between April 13th and May 2nd at Clube de Natação de Valongo, Escola Secundária de Paços de Ferreira, and Instituto Superior de Engenharia do Porto, involving a total of 111 participants. The dissemination methods included emails, direct interaction, and a poster (Annex B).

The first section of the questionnaire surveyed sociodemographic characteristics (gender and age), consumer habits (butter consumption frequency and protein supplementation) and behavioural attributes, identified in the literature as influential in consumers' attitudes towards *Spirulina* (neophobia and adherence to a vegan/vegetarian diet) (Fantechi, et al., 2023). The last section utilised a discrete choice methodology and had questions related to the product's attributes: visual appearance, smell, flavour, texture, overall acceptance and buying interest. In each one, participants were presented with three options to choose from. The questionnaire concluded with a comments box where suggestions for improvement or other comments could be made.

3.3. Organic Corrective Production and Application

The use of biofertilizers make germination more effective, increasing the number of germinated seeds, by increasing the supply or availability of primary nutrients and/or growth stimulus. On the other hand, phytotoxicity is the delay of seed germination, inhibition of plant growth, or any adverse effects on plants caused by specific substances (Orlina, et al., 2023).

Biocompounds can induce any of these effects on crops, depending on their concentration. The effect at certain conditions is evaluated by conducting germination or growth tests. In order to achieve a biorefinery concept in this study, some of the residues with a potential biocompound content were evaluated. Three treatments at 100, 50 and 25% of initial solution concentration of residual culture media, collected after biomass harvesting, water-extract of residual cell debris from phycocyanin extraction, and distilled water as growth control (negative) were made, Table 4.

Table 4 - Treatment symbology's and their explanation

Treatment	Description
Control	Distilled water
CB100	Culture residual medium
CB50	Culture residual medium with 50% concentration
CB25	Culture residual medium with 25% concentration
FB	Solubilized cell debris from phycocyanin extraction

Since lettuce seeds are commonly used in bioassays for their high sensitivity (Orlina, et al., 2023), 15 mL of each treatment were applied to a petri-dish with cotton as the support, where 10 seeds of *Lactuca sativa* were placed.

The evaluation of the development of the radicle and the hypocotyl (structures represented at Figure 9), representative indicators of the capacity of establishment and development of the plant (Chan-Keb, et al., 2018), was performed after 7 days of exposure. Other response variable analysed was seed germination percentage, 3 and 7 days after exposure.

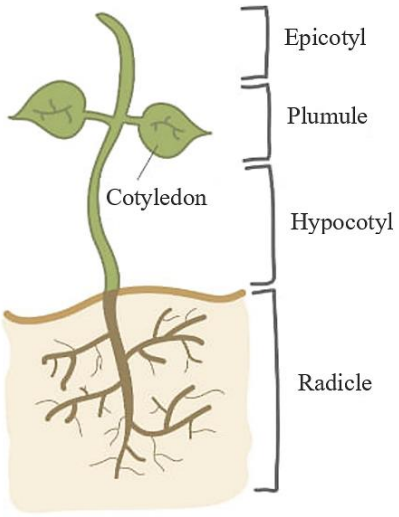


Figure 9 - Seedling physiology. Adapted from (Grover, s.d.).

Results for the radicle and hypocotyl elongation are expressed as the mean ± standard deviation from the ten replicates and the comparison of differences among different treatments was evaluated with one-way ANOVA with Fisher's Least Significant Difference test post hoc test at the 0.05 probability level (Choe, et al., 2024).

Additionally, the treatment's potential as a fertilizer were also assessed using lettuce in a pot experiment (Alvarenga, et al., 2023). Pots with 0.5 L were used, with twelve replicates per treatment. Lettuce was seeded (three plants per pot) on a non-sterile organic growing media (NUTRIMAIS, Universal). All pots were routinely watered with deionized water, while only the treated pots received a top-dressing fertilization 50 mL of CB100 treatment, applied 3 days after fist germination. The experiment was maintained without controlled conditions.

4. Results and Discussion

4.1.*Spirulina* Productivity and Characterization

4.1.1. Cultivation

In the production of stock cultures, it was possible to observe a faster and more homogeneous growth of *A. maxima* as compared to *A. platensis* cultures. Additionally, it was notorious that the latter followed two tendencies: bottom deposition and superficial aggregation, Figure 10. Samples from these two portions were analysed under a microscope, but no morphological difference was detected that could explain this difference in behaviour (e.g. increased crowding).

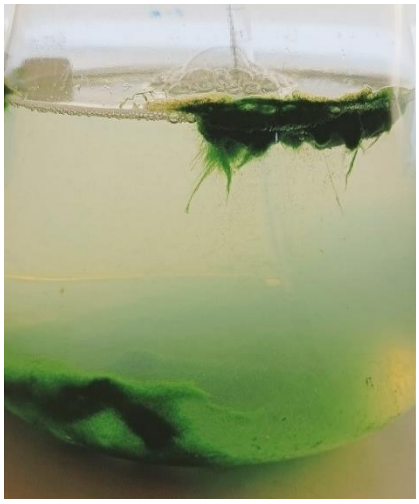


Figure 10 - Culture of *A. platensis* showing superficial agglomeration and bottom deposition

Therefore, due to the higher productivity, further studies were performed with *A. maxima* only, hereinafter referred to simply as *Spirulina*.

4.1.1.1. Cheese Whey Growth Test

Whey, the aqueous portion formed during milk coagulation, is the main by-product of the cheese manufacturing industry, consisting of lactose (4.5–5% w/v), soluble proteins (0.6–0.8% w/v), lipids (0.4–0.5% w/v), mineral salts, lactic and citric acids and B group vitamins (Amado, et al., 2016). This byproduct needs to be processed and treated, otherwise can pose significant environmental risks due to very high concentration of organic matter. Cultivating microorganisms represents a promising strategy, leveraging the nutrient-rich profile of whey, not only curbing its unregulated disposal, and mitigating its environmental footprint, but also enhancing the value of resulting products (Amado, et al., 2016; Athanasiadou, et al., 2023). Since the characteristics of the cheese whey are highly dependent on the type of milk used and consequently on the origin place of production, a simple assessment was performed, and the results are presented on the Table 5:

Table 5 - Characteristics of the cheese whey

Parameter	**Measurement**	(Athanasiadou, et al., 2023)	(Macedo, et al., 2021)	**Unit**
COD	69 ± 1	62 * 10^{-3}	50–120	g/L
pH	4.3	3.62	5.68 ± 0.72	
Total N	822 ± 5	-	820 ± 50	mg/L

The composition of the cheese may vary depending on several factors, including the quality, technology of the manufacturing process and milk composition, also variable with the environmental conditions and diet, water intake, stress, and the animal's health (Pereira, et al., 2019). Therefore, it is usual for chemical properties to differ. Obtained pH and total N content was similar to other references. The value for COD was in the range indicated by Macedo et al. (2021). The value obtained by Athanasiadou et al. (2023) is significantly lower, possibly due to

the removal of the precipitated material by centrifugation and filtration (only accounts for the soluble COD).

Before starting the growth test, biomass concentration of the stock culture was determined, obtaining approximately 0.50 g/L. After assembling the system, the test growth started with similar conditions as those used to produce the stock cultures (light and aeration). After 7 days, the cultures growing in the medium with 20% cheese whey stopped showing visual evolution, Figure 11, and in terms of optical density, so a microscopic analysis of all cultures was made, Figure 12:

Figure 11 - Cultures visual comparison at day 0 (A.) and day 7 (B.). From left to right: two flasks with 20% cheese whey, two flasks with 10% cheese whey and two flasks with A&O medium

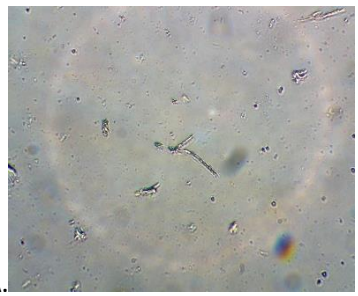
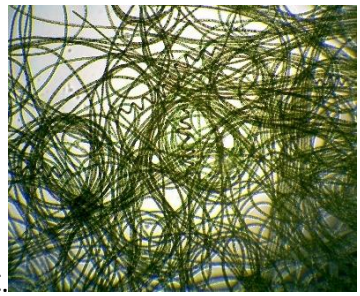
A.  B.  C. 

Figure 12 - Microscopy analysis of the cultures grown with 20% cheese whey (A.), 10% cheese whey (B.) and A&O medium (C.), respectively, at day 7 (x100 amplification)

As expected, there were no cells in the sample taken for microscopic observation, so the growth of the cultures in the 20% cheese whey medium stopped. It was also detected that the cultures

growing in the A&O medium had two different types of morphology: the linear strands identical to the figures of the culture provider and observed previously, and the spiral strands characteristic of the specie. A possible justification for this is the return to its characteristic morphology for being under ideal growth conditions since cross contamination with the other strain (*A. platensis*) was not possible.

The optical density measurements are represented in the graphic of Figure 13. The calibration curve (concentration in terms of absorbance) for the growth in the A&O medium is present in Annex 1.

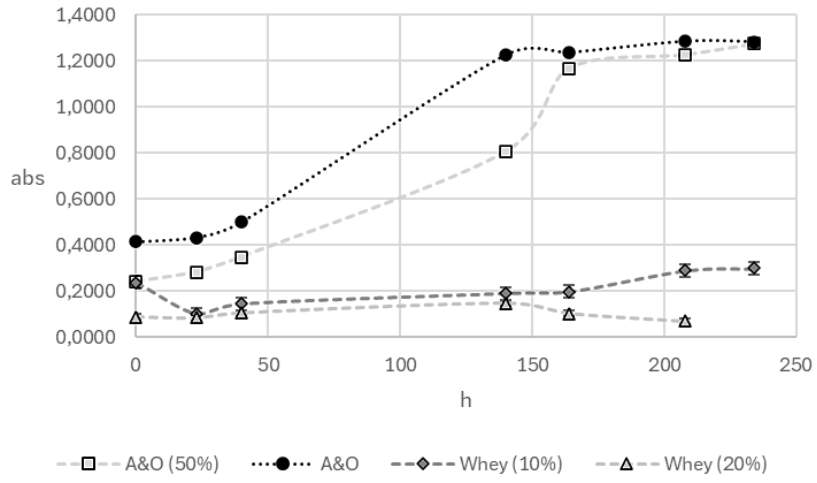


Figure 13 - Plot of the absorbance at 730 nm against the time of culture growth

Since the cultures growth (in 10% cheese whey and in A&O medium) appeared to be stabilized, the test was finished after 10 days (243 h). While the stationary phase with the A&O medium started at around 150 h, with the 10% cheese whey it only started after the 200-h mark, around 9 days. This time frame was shorter than the indicated in the literature by Masotti et al., 14 days (Masotti, et al., 2017), possibly due to an easier adaptation for having the same light and aeration conditions as those stock cultures were subjected.

At the end of the test growth, biomass concentration was once again measured gravimetrically, and the concentration is summarized in Table 6:

Table 6 - Final concentration of the cultures

A&O (50%)	A&O	Whey (10%)
1.24 g/L	1.20 g/L	0.16 g/L

As perceived by the results in Figure 11, both cultures in the A&O medium reached approximately the same concentration. The culture growing in the cheese whey was significantly less dense, reaching only 0.16 g/L. Athanasiadou et al. achieved a biomass production of 1.06 g/L in 14 days, also with a concentration of 10% (v/v) in cheese whey (Athanasiadou, et al., 2023). This low productivity compared to the literature value maybe due to some differences in the whey composition or in the methodology. In fact, the author clarified the whey in order to reduce the light obstruction by the precipitated material. Therefore, the trial was performed again with the filtered cheese whey with concentrations of 5 and 10% (v/v). No different results were achieved. It was hypothesized the possibility of bacterial contamination due to the intense orange colour and the turbidity formed.

Pereira et al. (2019) obtained the highest biomass yield of 2.98 g/L after 17 days of mixotrophic cultivation in Zarrouk media with 5.0% cheese whey, stating that heterotrophic cultivation was inappropriate possibly due to some growth inhibiting factors present in the whey. Therefore, a new test with corrected pH, as well as the combination of culture media and whey, was performed and its results are shown in Figure 14:

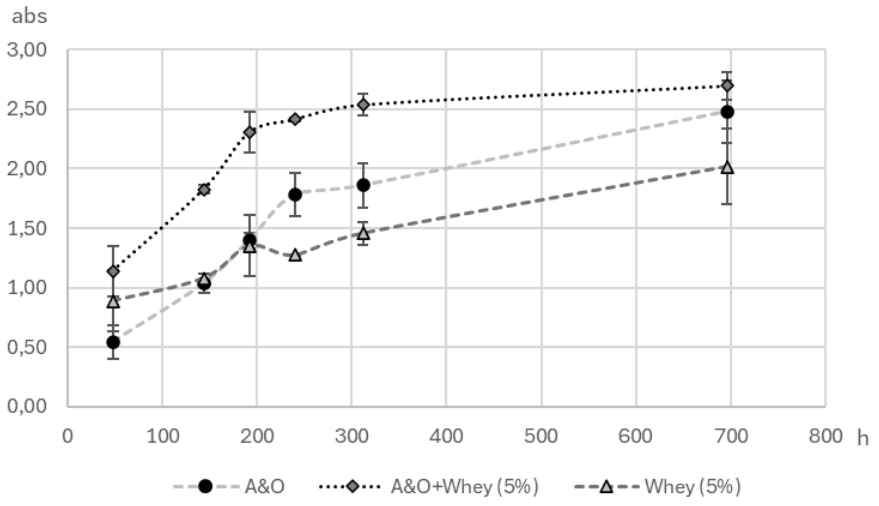


Figure 14 - Plot of the absorbance at 730 nm against the time of culture growth for the second test

As predicted, the combination of culture media and whey resulted in highest absorbance and, consequently, the highest biomass concentration. With the dry weight determination, it was possible to conclude that the biomass reached a concentration of 2.03 g/L, 1.71 g/L and 1.43 g/L with 5% cheese whey and A&O culture media, A&O culture media, and 5% cheese whey, respectively. These results are far more favourable than those previously obtained, showing the importance of pH on the *Spirulina* growth and the potential of the use of the cheese whey to boost *Spirulina* productivity.

4.1.2. Nutritional characterization

Table 7 summarizes the nutritional composition of *Spirulina* cultured in this study.

Table 7 - Chemical and nutritional composition of *Spirulina maxima* cultured in this study

	Composition (per 100 g dry *Spirulina*)
Proteins (g)	38.40 ± 0.00
Lipids (g)	6.91 ± 3.91
Carbohydrates (g)	47.96
Ash (g)	6.73 ± 0.53
Moisture (g)	7.10 ± 0.82
Energy (kcal)	407.62

In addition to carbohydrates and proteins, which represent about 47.96 and 38.40 g of its composition respectively, *Spirulina* cultured has other compounds of interest, namely lipids and ashes (approximately 7 g each).

These results are somewhat different from other studies conducted on *Spirulina maxima*. Silva et al. (2021) obtained significantly higher protein content (80.00 g) and lower carbohydrate content (0.69 g), similar lipid content (7.63 g) and a slightly higher ash content (11.67 g). On the other hand, Rodrigues et al. (2018) obtained also higher protein content and lower

carbohydrate content (66.7 and 1.6, respectively), but a much lower lipid content (0.8 %) and a much higher ash content (34.0 %). Both studies had similar culture conditions when compared to this work, except for the lower light intensity (30 vs 100 and 120 µmol photons m^{-2} s^{-1}), and possibly the age of the culture.

In fact, the lower protein and higher carbohydrate content compared to those references can be attributed to the reduced light intensity during cultivation. In these conditions, *Spirulina* tends to store more energy in the form of carbohydrates, as a survival mechanism, rather than utilizing it for protein production. Proteins require more energy and nitrogen, and low light conditions affect both the energy available and the efficiency of nitrogen assimilation, leading to a reduction in protein synthesis (Junique, et al., 2021; Niangoran, et al., 2021).

4.1.2.1. Lipid extraction – classical vs. green approach

Industrially, solid–liquid extractions employing volatile solvents are the most common methods to recover total lipids from biomass due to their simplicity, high lipid selectivity and solubility, affordability, and relatively easy scaling up (Melo, et al., 2021). The Bligh and Dyer method, using a combination of chloroform, methanol and water, has become one of the most recommended methods for determining total lipid in biological samples, since is thought to have recovery yields of ≥ 95%. One of its advantages is the formation of a biphasic system from the proportions of the solvents, but also the ability to efficiently extract both neutral lipids and polar lipids by using solvent mixtures of polar and non-polar solvents (Jesus & Filho, 2020). However, due to their high volatility and toxicity, organic solvents are dangerous to human health and environmental health (Jesus & Filho, 2020). In fact, there are legislative restrictions on the use of chlorinated solvents, especially chloroform (Melo, et al., 2021; Jesus & Filho, 2020). Additionally, these compounds are often fossil derived solvents, creating additional barriers from the environmental protection point of view. Therefore, these disadvantages are driving a demand for biocompatible, less or nontoxic bio-sourced solvents, fitted in the green chemistry principles, securing the balance between efficiency and greenness (Mussagy, et al., 2020).

Green solvents are bio-based solvents, partially or fully derived from renewable sources, either organic or inorganic. They can also be of fossil origin if the criteria of green chemistry are

satisfied. This class includes organic solvents, supercritical CO_2 and ionic liquids (Jesus & Filho, 2020). Taking profit from the advantages of the Bligh and Dyer method, several studies proposed the change of chloroform and methanol for similar greener methods, namely ethyl acetate and ethanol. In fact, ethyl acetate, ethanol and water ternary mixtures allowed to obtain similar or even higher recovery yields than the Bligh and Dyer method in other biological samples, besides the better environmental performance (Mussagy, et al., 2020; Lin, et al., 2004). Additionally, this mixture can be reused up to three consecutive extractive cycles, ensuring high extraction efficiency yields, decreasing even more the carbon footprint of the process (Mussagy, et al., 2020).

In this study, lipids were extracted and quantified using the Bligh and Dyer method (with chloroform, methanol and water) and a greener approach using ethyl acetate, ethanol and water, as described by Breil et al. (2017). However, it was not possible to obtain the phase separation in the green approach as in the classical one, even though the same proportions of solvents were used.

To compare both systems, the experimental points, one for each, were represented in the respective ternary system, shown in Figure 15. The differences in the solvents density changed the systems relative composition (in %w). Simultaneously, as the solubility curve (that separates the monophasic region above from the diphasic region below) has a lower profile, the experimental point of the $B\&D_G$ system was located in the monophasic region. To produce a shift to the biphasic region, additional water containing 0.85% of KCl was added, reaching a final ratio of 2:2:5.1 ethyl acetate:ethanol:water (v/v/v). After phase separation and collection, lipid content was determined and is expressed in Table 8.

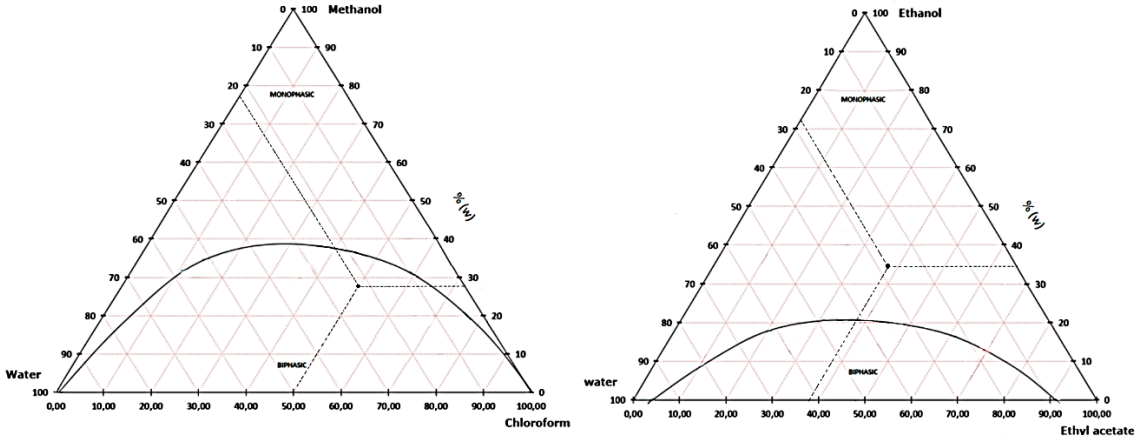


Figure 15 - Ternary systems of B&D (Methanol/Chloroform/Water) and B&DG (Ethanol/Ethyl Acetate/Water) with experimental points represented. Adapted from (Breil, et al., 2017).

Table 8 - Comparison between lipid content of *Spirulina* obtained with B&D (Methanol/Chloroform/Water) and B&DG (Ethanol/Ethyl Acetate/Water) methods

B&D (%)	**B&D$_G$ (%)**
6.90 ± 3.91	5.39 ± 2.81

As mentioned previously, lipid content obtained with the classical Bligh and Dyer method was 6.9%, while the greener alternative yielded only 5.4%. However, the ANOVA test showed no significant differences between methods. This means that this change in the solvents can be made without compromising selectivity and efficiency of the method, but with better environmental performance.

4.1.2.2. Phycocyanin

Phycocyanin is a photosynthetic pigment soluble in water and can be easily extracted, namely through the commonly used freeze/thaw method. In this, the ice crystals formed in the freeze lead to the rupture of the cell and subsequent release of the pigment into the extraction medium in thawing (Silva, 2018).

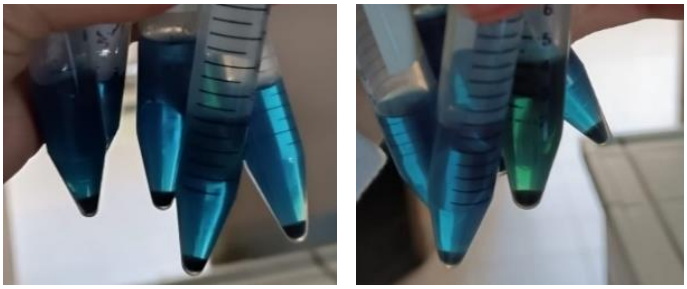


Figure 16 - Phycocyanin extracts after centrifugation

The results of phycocyanin concentration in biomass, C-PC (mg/g), and the extract purity, PE, obtained in the current work are present in the Table 9.

Table 9 - Phycocyanin content extracted, per gram of dry biomass weight, and purity of the extracts obtained by freeze–thawing with one and two cycles.

	1 cycle	2 cycles
C-PC (mg/g dw)	109.3 ± 2.2	112.0 ± 0.8
P_E	0.62 ± 0.00	0.85 ± 0.00

The extracted amounts of phycocyanin ranged between 93.5 and 121.10 mg/g biomass, however, both were disregarded for being too discrepant (also had visual discrepancy - green colour solution in Figure 16). The purity obtained was slightly higher with the 2-cycle approach, just as the yield, however, only significant differences were found in the purity parameter according to the ANOVA test.

Tan et al. (2020), extracted 172.84 ± 0.37mg/g of phycocyanin using a similar protocol (water as a solvent, 0.5% biomass/solvent ratio, one freeze–thaw cycle at -80 ºC for 2h and 25 ºC for 24h). The higher values could be explained with longer time of thawing, essential to ensure the complete breakage of the cells. According to the same authors, the purity of phycocyanin is usually classified as low purity (value of 0.7, usually utilized by the cosmetic and food industries as bio colorants), as reactive grade (purity of 3.9) or analytical grade (purity above 4.0). Their results, food grade, were consistent with the obtained with two cycles of extraction (0.85).

Santos, et al. (2020) used freeze-thawing extraction as a conventional reference methodology to provide the expected maximum extractable amount of phycocyanin from *Spirulina (A. platensis)*, obtaining 41.90 ± 0.23 mg/g of dry weight. This yield was significantly lower than the obtained (Table 9). This discrepancy may be due to the different *Spirulina* specie, or a significantly lower biomass/water ratio used (25 times more biomass was used by Santos, et al. for the same amount of water). Other differences in the procedure, as the freezing/thawing times (frozen for 4 h, thawed for 1.5 h, repeated four times for each sample) or measures at different absorbances (620 nm instead of 615 nm; 650 nm instead of 652 nm) might also have affected the result.

Additionally, since Pinto et al. (2022) stated that phycocyanin extraction does not require prior drying, another study was performed to compare the extraction efficiency of phycocyanin from fresh biomass and lyophilized biomass. Approximately, 100 mL of concentrated culture was filtered and then the 2-cycle extraction method was applied. Similar phycocyanin concentration in the extract was obtained, but the phycocyanin concentration in the biomass and the extract purity were considerably lower, Table 10.

Table 10 - Phycocyanin content extracted, per g of fresh and dry biomass, and purity of the extracts obtained by a two-cycle freeze–thawing extraction

	2 cycles
C-PC (mg/g biomass)	3.51 ± 0.8
C-PC (mg/g dw)	3.78
$\mathbf{P_E}$	0.46 ± 0.3

Even with the conversion of fresh weight to dry weight using the moisture content of 7.10% obtained previously, the value obtained was significantly lower. The higher biomass to water ratio, even considering the water present in the cells, might have influenced the extraction capacity.

4.2.*Spirulina* as a functional ingredient in butter

4.2.1. Product characterization

The product utilized, following the trial test, contained 2% of lyophilized *Spirulina* (w/w). The spirulina content heavily influenced its colour (Figure 17), while the texture remained unaltered, still resembling regular butter. Nutritional content was estimated and is summarized in Table 11.

Figure 17 - Produced butter enriched with *Spirulina* in a ceramic cup

Table 11 - Comparison of the nutritional parameters between the formulated butter enriched with 2% of *Spirulina* (w/w) and the commercialised Primor sem sal®

	Enriched butter (100 g)	Commercial butter (100 g)
Energy (kJ)	3052	3085
Fat (g)	81	83
of which saturates (g)	55	56
Carbohydrate (g)	1.3	1.1
of which sugars (g)	0.8	0.8
Protein (g)	2.0	0.6
Salt (g)	0.06	0.05
Fibre (g)	0.16	-

Briefly, the new formulated product has slightly less energy and fat content, but more carbohydrates and fibre. On the other hand, it is possible to conclude that the protein content increased drastically, around 227%. However, the product itself cannot be denominated as a "source" or as a "high protein" product since it contains less than 12% or 20% of the energy value provided by protein, respectively, according to the EC 1924/2006 Regulation (European Comission, 2006).

4.2.2. Sensorial analysis

The final sample comprised 111 individuals aged between 12 and 63 from Porto, of which 65% were female and 35% were male, with prevalence of 45-54 aged females (23%) (Figure 18).

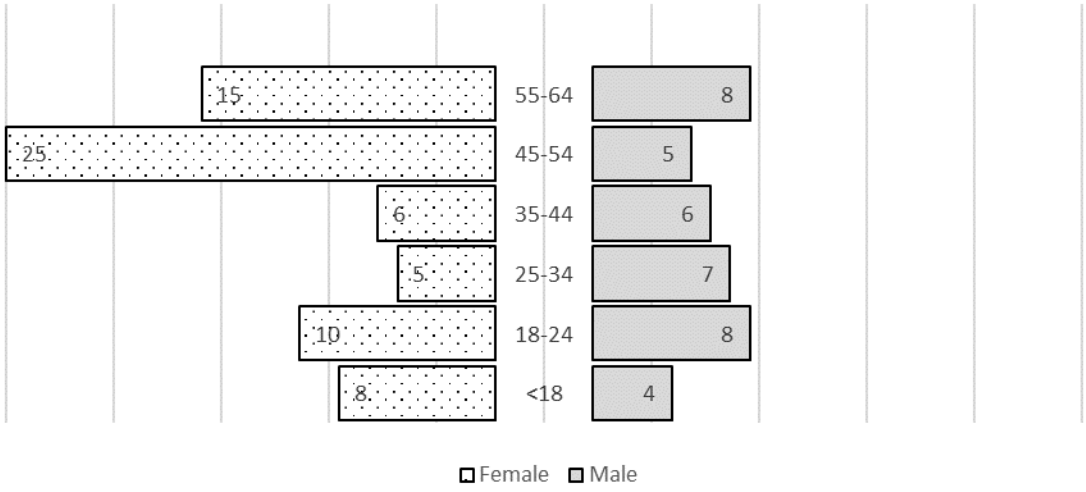


Figure 18 - Demographic structure of the inquired population

Regarding the habit tracking, 68% of the inquiries admitted consuming butter daily or weekly, Figure 19, with only 2% revealing never eating butter. Less significantly, only 2% were on a vegan or vegetarian diet and 17% regularly takes protein supplements, so no conclusions can be taken correlating these habits to the bigger or smaller acceptability of the new product. On the other hand, 55% of the inquires revealed being **v**ery willing to try new products ("Explorer"), against the 3% that are **r**eluctant to try new products and prefer sticking to familiar foods ("Conservative"), contradicting hypotheses of neophobia pointed out as a barrier to the entry of new products (Fantechi, et al., 2023; Grahl, et al., 2020). However, it is possible that those

more hesitant did not take part in the taste test, influencing the results. Additionally, most of the inquiries revealed that they did not know spirulina or its properties, in line with other studies (Lucas, et al., 2023; Lafarga, et al., 2021).

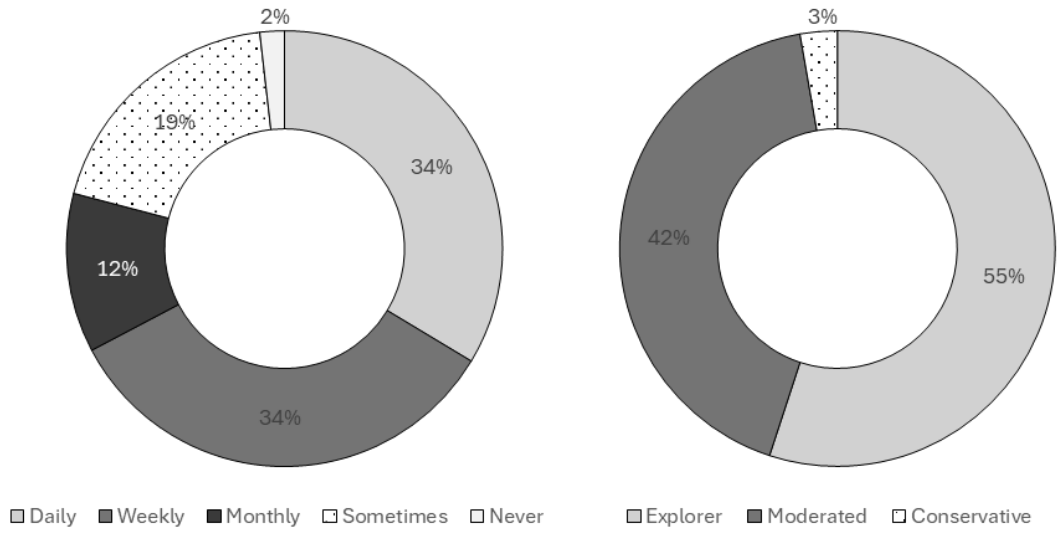


Figure 19 – Tracking consumer's habits regarding butter consumption and openness to new products

After tasting the product, participants were asked to objectively characterize the intensity of the aroma and flavour, from weak to strong (Figure 20). Then, those same characteristics were evaluated on terms of "likeness", from "unpleasant" to "pleasant", as well as the colour, texture and globally (Figure 21).

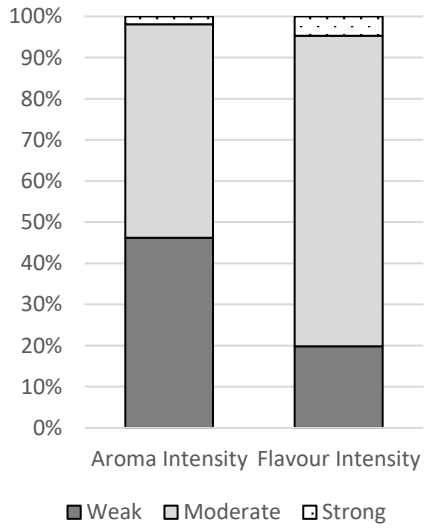


Figure 20 -Aroma and flavour intensity of *Spirulina* perceived in the enriched butter

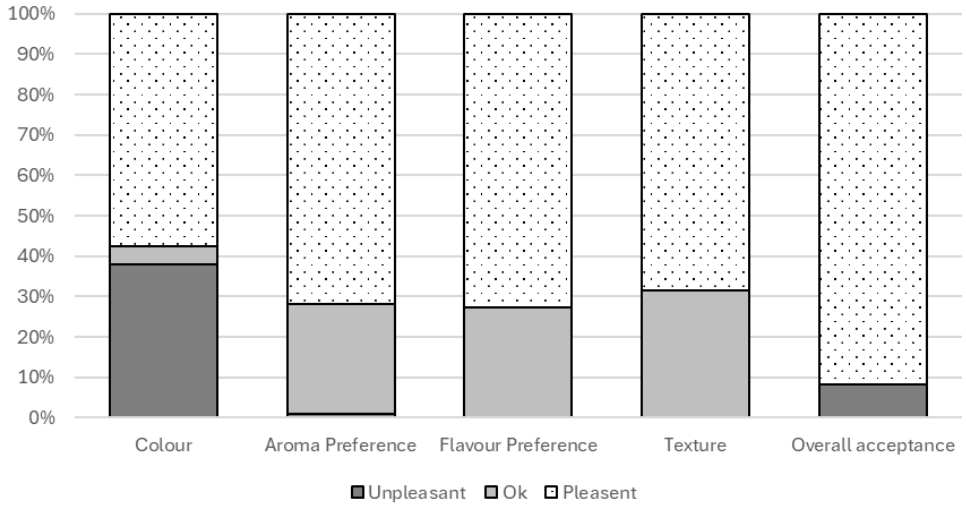


Figure 21 -Evaluation of the colour, aroma, flavour and texture of the enriched butter according to individual preferences

Spirulina's presence was more noticeable on the flavour rather than in the aroma. In the first, the most common answer was "moderate", with 74%, while in the latter there was an equilibrium between "moderate" and "weak". However, both had similar acceptance levels, with more than 70% of the participants finding those characteristics pleasant. The most impacted aspect of the product was the visual appearance. Effectively, the bluish-green colour of the product, characteristic of *Spirulina* but not common in the diary segment, gathered the most "unpleasant" votes, with 76%, accounting for 38% in that question. Finally, the overall acceptance of 92% confirms the potential of *Spirulina* as a food supplement, specifically as an enrichment ingredient in butter. The additional comments made at the end of the questionnaire, regarding the lack of salt in the product, might indicate that the global acceptance could be easily increased if regular butter was used instead of the unsalted one. Some comments were also made regarding increasing the intensity of the *Spirulina*'s flavour in the butter, proving once again that the food industry is subjective and not everyone can be pleased with the same product, suggesting that several products with different *Spirulina* and salt concentrations will gather more potential consumers.

Lastly, tasters were asked if they would buy the products instead of the traditional butter, if it was at an "fair" price, given its benefits. The majority, over 60%, would buy, with only 3% answering that they would not buy the new product (Figure 22).

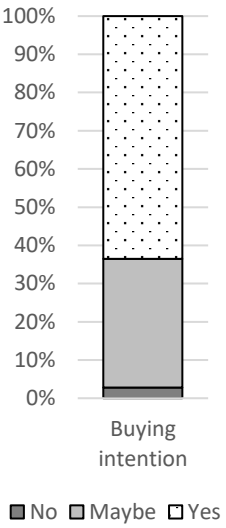


Figure 22 -Hypothetical buying intention of the enriched butter

To summarize, despite the lack of knowledge about *Spirulina*, the surveyed community still engaged in the sensory analysis test, showing the low effect of neophobia, and revealing the opportunity for new products to enter more traditional markets. Furthermore, with the right marketing strategies that educate consumers on the benefits of *Spirulina,* initial hesitations could be mitigated, and greater acceptance could be fostered.

Additionally, *Spirulina* influences the product's sensorial properties, yet consumers still found these to be pleasant, except for the colour. Overall, the product was generally accepted, and introducing a line of different products could further increase its acceptance, indicating its potential as a promising market product. A future step is to evaluate the production and the market price through economic projection studies and determine if the community finds it a "fair price", essential in determining the product's overall feasibility and long-term success in the marketplace.

4.3.*Spirulina* as a Soil Corrective

4.3.1. Treatment comparison

The treatments described in Table 4 were analysed in terms of pH and total N content. Obtained data is present in Table 12:

Table 12 - Total Nitrogen content (mg/L) and pH of each treatment applied as soil corrector.

Treatment	**pH**	**Total N** (mg/L)
Control	**7**	0
CB100	9	204 ± 3
CB50	9	110.8 ± 0.8
CB25	9	59.4 ± 0.3
FB	6	27.6 ± 0.0

All the treatments produced from dilutions of the culture media had the same pH value, 9, and a total N content inversely proportional to the dilution factor: CB100 had the highest N content, 204 ± 3 mg/L, 2.4 times higher than the CB25. The FB treatment had the lowest pH value, 6, and also the lowest N content, 27.62 mg/L.

4.3.2. Germination Test

Following the application of the treatments to the lettuce seeds, the first data collected was the number of germinated seeds (Figure 23).

Firstly, it is possible to observe that the CB100 and CB50 solutions inhibited totally seed germination and growth. Additionally, between the first and second observation periods, there were no new seeds germinating in the control, unlike CB25 and FB. At the end of the germination test, it was possible to conclude that the CB25 and the FB treatments had the highest performance. The inhibition provoked by the CB100 and CB50 might be explained with

the mineral richness of the solutions, since exposing seedlings to elevated nutrient concentrations at this stage can harm their development (Orlina, et al., 2023).

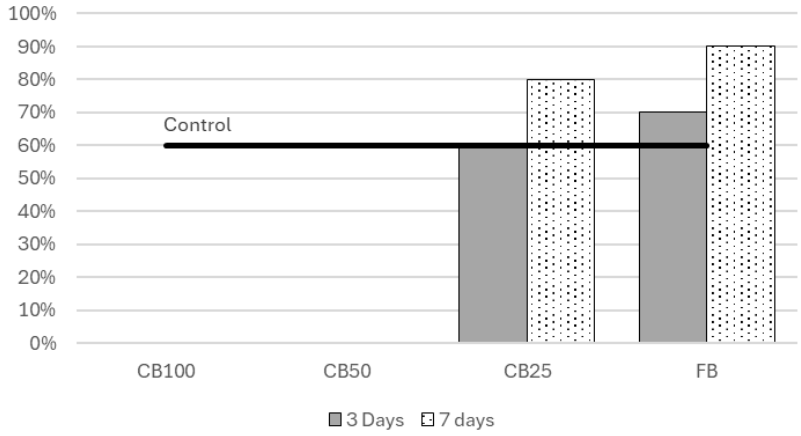


Figure 23 – Germination percentage in the different treatments comparing to the control group, 3 and 7 days after seedling.

After seven days of observation, lettuce seedlings were carefully separated from the cotton bed, Figure 24, and the radicle and hypocotyl in each sample replicate was measured.

Figure 24 - Elongation alterations observed on seeds of *Lactuca sativa* exposed to different treatments.

As shown in Figure 25, the FB treatment produced the longest radicle length, with a mean of 2.75 ± 1.86 cm, but also the biggest hypocotyl length, 0.68 ± 0.22 cm. However, hypocotyl length was not significantly different between treatments. Control and CB25 did not have significant difference in radicle length mean also.

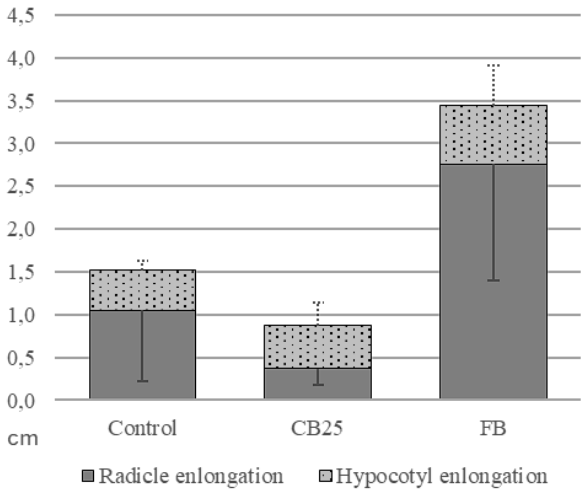


Figure 25 - Radicle (solid) and hypocotyl (dotted) elongation of lettuces versus treatment. Error bars represent standard deviation.

In fact, lettuce grow better with a pH ranging from 6 to 7 (Orlina, et al., 2023; Maluin, et al., 2021), and a higher level of pH during seed germination results in many disorders and metabolic changes, leading for example to significantly lower relative radicle length (Orlina, et al., 2023). Therefore, this higher performance of the FB treatment might be due to its pH. Additionally, nitrogen is an essential element required for successful plant growth, since it is involved in plant photosynthesis and is a key constituent of proteins, amino acids, nucleic acids, enzymes, hormones, and chlorophyll. Additionally, it functions as a signalling substance that regulates root growth, canopy development and lifespan, and is involved with plant defence and stress response mechanisms (Valenzuela, 2024). However, it is known that high N concentrations (≥ 30 mg L^{-1}) negatively impact root growth and integrity (Hoque, et al., 2007). This aspect might also have influenced the higher performance of FB in comparison to the CB25 treatment, since it has the lowest N content, and the only one below the referred N-inhibition limit.

For future studies, it might be important to consider even higher dilution rates of the culture media (or fragmented application). Additionally, adding a buffer to lower the pH must be taken into account for application in lettuce or other plants with similar pH preferences, or specify this biofertilizer to plants that prefer alkaline soils.

4.3.3. Soil test

It was not possible to obtain results from the soil test experiment due to several external factors related to the uncontrolled conditions maintained.

4.4.*Spirulina*'s Biorefinery

In Europe the *Spirulina* sector is still immature and thus relatively small. Its future growth relies on technical innovations to upscale the production, while reducing the production costs. The biorefinery approach, especially the concept of "high-value product first" followed by valorisation of other metabolites, is currently being investigated as a mean to increase the environmental sustainability and economic feasibility (cost offsetting) of existing conventional industrial processes (Araújo, et al., 2021). *Spirulina* biorefinery holds particular promise due to the rapid growth, nutrient density, and low environmental impact at a large scale but also due to its ability to accumulate phycocyanin along with other commercially valuable metabolites (Thevarajah, et al., 2022).

From the obtained results and the state-of-the art revision, it is known that *Spirulina* can be cultivated using industrial waste streams as nutrient sources, such as cheese whey or wastewater, effectively reducing resource consumption while providing a sustainable solution to waste disposal and reducing the environmental impacts of the one of the highest contribution stages (Ye, et al., 2018). Additionally, recirculating the growth medium further optimizes water and nutrient use, cutting down on the costs and on the environmental footprint of cultivation (water demand can be reduced up to 84%) (Costa, et al., 2019). When it comes to harvesting, one of the more expensive steps in microalgae processing, *Spirulina*'s filamentous morphology facilitates cell recovery (up to 98%) from the culture medium, as the cells can be collected by simple filtration (Costa, et al., 2019). The ability to extract valuable compounds, such as phycocyanin and lipids, using environmentally friendly methods offers significant economic and environmental benefits. Besides, the accumulation of considerable amounts of other valuable bio-compounds, such as carbohydrates and fatty acids could also be advantageous. However, the decision on what to extract, how and in what order must be carefully studied. Chaiklahan, et al. (2018) performed an economic analysis that showed that the investment for a stepwise extraction of lipids and polysaccharides after phycocyanin extraction from *Spirulina* was unfeasible. Even though phycocyanin is the most valuable compound, and therefore should be extracted first to avoid deterioration, studies demonstrated that the phycocyanin content after liquid–liquid extraction of the polar phase remains approximately the same (Hilali, et al., 2024), suggesting an inversion in the extraction order, Figure 26.

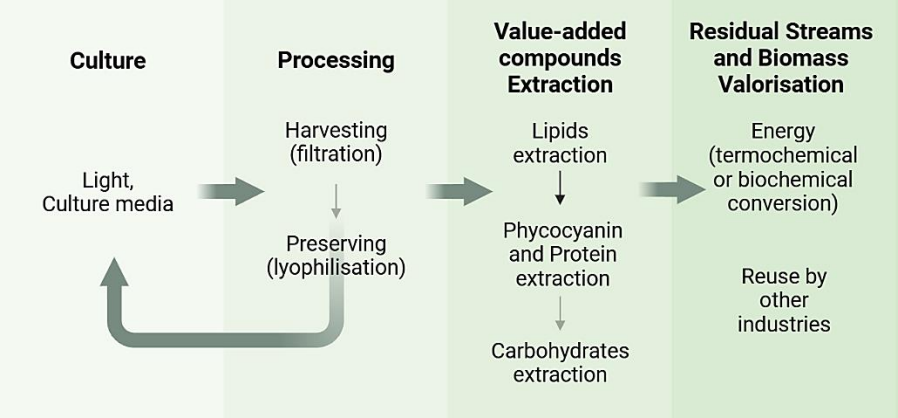


Figure 26 -Proposed approach of *Spirulina* processing for value maximization

Moreover, the redirection of processing residues—particularly phycocyanin extraction waste—into other applications like soil amendments or animal feed aligns with a zero-waste philosophy, ensuring that every part of the biomass is utilized. Nonetheless, biorefinery of the residual biomass involves additional capital and operational costs, requiring feasibility evaluation (Thevarajah, et al., 2022). In fact, the use of microalgal residue of phycocyanin extraction as animal feed (protein source) has low value, and therefore has little interest for a biorefinery (Chaiklahan, et al., 2018). Even though a tecno-economical assessment was not performed, due to the low price of commercially available N fertilizers, redirecting the residual biomass to fertilization applications would also not be feasible. Ultimately, the redirection of the residual biomass into bioenergy conversion is the most secure alternative, since those technologies are well-established, scalable, and relatively simple to integrate into existing biorefinery models. A simplified scheme of a possible complete biorefinery model is present in Figure 27.

As seen before, even with key opportunities for the *Spirulina* biorefinery such as its versatility, alignment with health, wellness and sustainability and support from the political agenda in terms of a regulatory perspective, there are several critical challenges. The biggest challenge is related to technical aspects in the culture and processing, particularly optimization and viability. Quality control is also a raising concern, since open ponds and/or with waste resources as a culture media make cultivation vulnerable to contamination by pollutants, heavy metals, harmful bacteria or other microalgae species.

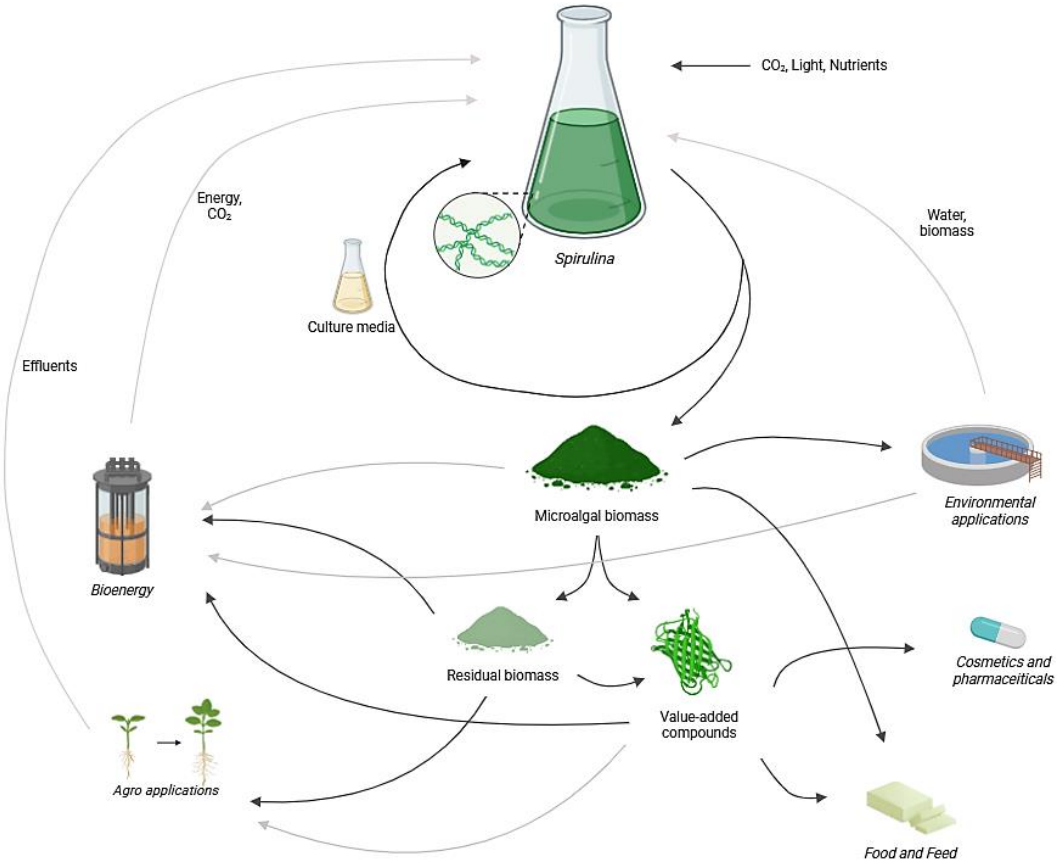


Figure 27 – Proposed global *Spirulina*-based biorefinery

Studies on gene manipulation of *Spirulina* to overcome the challenges associated with lower biomass yields and simultaneous metabolite synthesis and abiotic stress tolerance (fluctuations in cultivation parameters, intolerance to pollutants and contaminants) are scarcely available. However, as they have been successful in other microalgal species, they could be applied to modify *Spirulina* strains, contributing to a reduction in overall production costs (Thevarajah, et al., 2022). Also scarce, but with future potential, is the application of artificial intelligence-based models to further optimize and control all variables related to the *Spirulina* biorefinery functioning, assuring that the best possible conditions are maintained (Li, et al., 2024).

Despite the potential of *Spirulina* biorefineries, several challenges such as high costs and technological barriers still remain and must be addressed. However, technological advancements offer the opportunity to overcome current bottlenecks. These future trends, combined with policy support and investment, hold the potential to transform not only *Spirulina* but also other bioresources' biorefineries into cornerstones of the circular bioeconomy.

5. Conclusions and Future Perspectives

In an era defined by resource depletion, climate change, and the need for sustainable alternatives to conventional fuels and materials, the concept of a biorefinery has emerged as a key strategy for the bioeconomy. Therefore, the objective of this thesis was to maximize the biotechnological potential of *Spirulina* through its application in multiple sectors, including food, agriculture, and energy, within a sustainable biorefinery framework.

Firstly, *Spirulina* cultures were progressively scaled up and characterized, revealing high content of several value-added compounds such as proteins (38.4%), carbohydrates (48.0%) and phycocyanin (112.0 mg/g). It was possible to produce *Spirulina* in diluted cheese whey wastewater, but the biggest yield was achieved by its mixture with culture media, 4.32 g/L. Additionally, two green methods were successfully used to extract phycocyanin and lipids. For the case of phycocyanin, only water was used, with the freeze-thawing method, achieving similar results to those present in the literature. In the case of lipids, water, ethanol and ethyl acetate were used to obtain similar results to the traditional method, 5.4% and 6.9% respectively (the method has not been applied on *Spirulina* before).

Two applications of *Spirulina* or its residues were tested: functional foods and soil corrective. For the food application, butter was studied since it is a popular product in a typical Portuguese household (68% consume it daily or weekly), but also because it does not need confection after production and has a long shelf life. The sensorial analysis indicated that the butter supplemented with 2% *Spirulina* had high acceptance, with 92% of participants rating it favourably in terms of the overall product. Even though *Spirulina* enriches the butter in protein content and its nutritional content in general, it is not enough to be promoted as a “source of proteins”. Therefore, for future studies, new ways to incorporate the microalgae in food must

be explored, in a way that it allows higher concentrations without compromising sensorial properties. Also, when performing the sensorial test, a direct comparison with competing products (unaltered) must be made in order to have a reference point to produce more reliable answers. Regarding the agricultural application, *Spirulina* residual biomass after phycocyanin extraction acted as a soil corrective and improved lettuce germination rates to 150% and enhanced plant growth metrics compared to untreated support. However, due to the uncontrolled environmental conditions, the scalability of the test to soil applications was not possible. The highest dilution of the residual culture media also had positive influence on the germination, but less expressive and only after 7 days (33% more germination that in the control). Besides, it would have been interesting to evaluate the biostimulant potential of those solutions in aspects other than plant growth, namely the influence on the nutritional composition. These results demonstrate that *Spirulina* not only holds promise as a source of value-added compounds, but also as a bioresource for producing functional food products and organic fertilizers, offering eco-friendly alternatives in food and agriculture.

However, it is also important to acknowledge the limitations of this study regarding the final goal that was the idealisation of a biorefinery. While some of the experimental results were promising, the scalability but specially the economic feasibility of industrial-scale application of the green extraction methods and residual biomass repurpose as soil corrective require further investigation. Therefore, in conclusion, this thesis highlights *Spirulina* as a versatile bioresource with the potential to address sustainability challenges across various industries and, in the future, play a critical role in advancing a more circular and sustainable economy.

Bibliography

Abdel-Moneim, A.-M.et al., 2022. Antioxidant and antimicrobial activities of *Spirulina platensis* extracts and biogenic selenium nanoparticles against selected pathogenic bacteria and fungi. *Saudi Journal of Biological Sciences,* Volume 29, p. 1197–1209.

Agnol, L. et al., 2021. Green synthesis of *Spirulina*-based carbon dots for stimulating agricultural plant growth. *Sustainable Materials and Technologies,* 30(e00347).

Ali, S. & Saleh, A., 2012. *Spirulina* - An overview. *International Journal of Pharmacy and Pharmaceutical Sciences,* 4(3), pp. 9-15.

ALLMICROALGAE, s.d. *O Nosso Processo.* [Online] Available at: https://www.allmicroalgae.com/pt-pt/o-nosso-processo-spirulina/ [Acedido em 28 june 2024].

Alobwede, E., Leake, J. & Pandhal, J., 2019. Circular economy fertilization: Testing micro and macro algal species as soil improvers and nutrient sources for crop production in greenhouse and field conditions. *Geoderma,* Volume 334, pp. 113-123.

Altmann, B. & Rosenau, S., 2022. *Spirulina* as Animal Feed: Opportunities and Challenges. *Foods,* 11(7).

Alvarenga, P. et al., 2023. Evaluation of the fertilizer potential of *Chlorella vulgaris* and *Scenedesmus obliquus* grown in agricultural drainage water from maize fields. *Science of The Total Environment,* 861(160670).

Amado, I., Vázquez, J., Pastrana, L. & Teixeira, J., 2016. Cheese whey: A cost-effective alternative for hyaluronic acid production by *Streptococcus zooepidemicus*. *Food Chemistry,* Volume 198, p. 54–61.

Amarante, M., Braga, A., Sala, L. & Kalil, S., 2020. Colour stability and antioxidant activity of C-phycocyanin-added ice creams after in vitro digestion. *Food Research International,* Volume 137.

Ammar, E. et al., 2022. Algae as Bio-fertilizers: Between current situation and future prospective. *Saudi Journal of Biological Sciences,* Volume 29, p. 3083–3096.

Araújo, R. et al., 2021. Current Status of the Algae Production Industry in Europe: An Emerging Sector of the Blue Bioeconomy. *Frontiers in Marine Science,* 7(626389).

Arguelles, E., 2020. Maintenance and Preservation of Microalgal Cultures. Em: *Methods in Microalgal Studies.* s.l.:Philippine Science Letters and University of the Philippines Los Baños, pp. 53-60.

Arias, A., Feijoo, G. & Moreira, M., 2023. Biorefineries as a driver for sustainability: Key aspects, actual development and future prospects. *Journal of Cleaner Production,* 418(137925).

Athanasiadou, V. et al., 2023. Evaluation of *Arthrospira (Spirulina) platensis* growth on cheese whey in the context of circular economy. *Sustainable Chemistry and Pharmacy,* 34(101173).

Barakat, E., Kewaisny, N. & Salama, A., 2016. Chemical and Nutritional Evaluation of Fortified Biscuits with Dried *Spirulina* Algae. *Journal of Food and Dairy Sciences,* 7(3), pp. 167-177.

Barkallah, M. et al., 2017. Effect of *Spirulina platensis* fortification on physicochemical, textural, antioxidant and sensory properties of yogurt during fermentation and storage. *LWT,* Volume 84, pp. 323-330.

Barros, A., Gonçalves, A., Simões, M. & Pires, J., 2015. Harvesting techniques applied to microalgae: a review. *Renewable and Sustainable Energy Reviews,* Issue 41, pp. 1489-1500.

Beisler, N. & Sandmann, M., 2022. Integration of *Arthrospira platensis* (spirulina) into the brewing process to develop new beers with unique sensory properties. *Frontiers in Sustainable Food Systems,* Volume 6.

Bhatnagar, A., Härri, A., Levänen, J., & Niinimäki, K. (2024). Exploring the role of social life cycle assessment in transition to circular economy: A systematic review. *Resources, Conservation and Recycling, 207*(107702). doi:10.1016/j.resconrec.2024.107702

Bhatnagar, P. et al., 2024. Algae: A promising and sustainable protein-rich food ingredient for bakery and dairy products. *Food Chemistry,* 441(138322).

Bhat, O., Unpaprom, Y. & Ramaraj, R., 2023. *Spirulina* Cultivation Under Different light-emitting Diodes for Boosting Biomass and Protein Production. *Molecular Biotechnology.*

Branco-Vieira, M. et al., 2018. Biochemical characterization of *Phaeodactylum tricornutum* for microalgae-based biorefinery. *Energy Procedia,* Volume 153, pp. 466-470.

Breil, C. et al., 2017. “Bligh and Dyer” and Folch Methods for Solid–Liquid–Liquid Extraction of Lipids from Microorganisms. Comprehension of Solvatation Mechanisms and towards Substitution with Alternative Solvents. *International Journal of Molecular Sciences,* 18(4).

Cardoso, L. et al., 2021. Scaling-up production of *Spirulina* sp. LEB18 grown in aquaculture wastewater. *Aquaculture,* 544(737045).

Carlozzi, P. & Torzillo, G., 1996. Productivity of *Spirulina* in a strongly curved outdoor tubular photobioreactor. *Application Microbiology Biotechnology,* Volume 45, pp. 18-23.

Cepoi, L. et al., 2020. Growth and heavy metals accumulation by *Spirulina platensis* biomass from multicomponent copper containing synthetic effluents during repeated cultivation cycles. *Ecological Engineering,* 142(105637).

Chaiklahan, R. et al., 2018. Stepwise extraction of high-value chemicals from *Arthrospira (Spirulina)* and an economic feasibility study. *Biotechnology Reports,* 20(e00280).

Chakraborty, B., Jayaswal, R. & Pankaj, P., 2015. Antimicrobial Activity of *Spirulina platensis* Extract Against Gram Positive and Gram Negative Bacteria - A Comparative Study. *International Journal of Current Pharmaceutical Review and Research,* 6(4), pp. 212-214.

Chan-Keb, C. et al., 2018. Acute toxicity of water and aqueous extract of soils from Champotón river in *Lactuca sativa* L.. *Toxicology Reports,* Volume 5, pp. 593-597.

Chapagain, A. (2017). Water Footprint: State of the Art: What, Why, and How? Em *Encyclopedia of Sustainable Technologies* (pp. 153-163). doi:10.1016/B978-0-12-409548-9.10164-2

Chittora, D. et al., 2020. Cyanobacteria as a source of biofertilizers for sustainable agriculture. *Biochemistry and Biophysics Reports,* 22(100737).

Choe, H. et al., 2024. Morpho-physiological, biochemical, and molecular responses of lettuce (*Lactuca sativa* L.) seedlings to chlortetracycline stress. *Environmental and Experimental Botany,* Volume 219.

Chojnacka, K. & Noworyta, A., 2004. Evaluation of *Spirulina* sp. growth in photoautotrophic, heterotrophic and mixotrophic cultures. *Enzyme and Microbial Technology,* 34(5), pp. 461-465.

Costa, J. et al., 2019. Operational and economic aspects of *Spirulina*-based biorefinery. *Bioresource Technology,* 292(121946).

Dineshkumar, R., Narendran, R. & Sampathkumar, P., 2016. Cultivation of *Spirulina platensis* in different selective media. *Indian Journal of Geo Marine Sciences,* 45(12), pp. 1749-1754.

Djaghoubi, A. et al., 2015. Growth and Nitrogen Removal Efficiency as Protein Content of *Spirulina* from Tertiary Municipal Wastewater in Ouargla (Algerian Bas-Sahara). *Energy Procedia,* Volume 74, pp. 1402-1409.

El-Sayed, H., Hassan, A., Barakat, K. & Ghonam, H., 2024. Improvement of growth and biochemical constituents of *Rosmarinus officinali*s by fermented *Spirulina maxim*a biofertilizer. *Plant Physiology and Biochemistry,* 208(108452).

Ercin, E. & Hoekstra, A., 2012. *Carbon and Water Footprints: Concepts, Methodologies and Policy Responses,* s.l.: s.n.

European Algae Biomass Association, 2021. *Algae as Novel Food in Europe,* s.l.: s.n.

European Comission, 2006. *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,* s.l.: s.n.

Fantechi, T., Contini, C. & Casini, L., 2023. Pasta goes green: Consumer preferences for S*pirulina*-enriched pasta in Italy. *Algal Research,* 75(103275).

Faresin, L., Devos, R., Reinehr, C. & Colla, L., 2022. Development of ice cream with reduction of sugar and fat by the addition of inulin, *Spirulina platensis* or phycocyanin. *International Journal of Gastronomy and Food Science,* 27(100445).

Fernandes, R. et al., 2023. Exploring the Benefits of Phycocyanin: From *Spirulina* Cultivation to Its Widespread Applications. *Pharmaceuticals,* 16(592).

Figueira, F., Crizel, T., Silva, C. & Mellado, M., 2011. Pão sem gluten enriquecido com a microalga *Spirulina platensis*. *Brazilian Journal of Food Technology,* 14(4).

Food and Drug Administration, 2002. *GRAS Notice 101: Spirulina,* s.l.: s.n.

Foo, S., Mok, C., Ho, S. & Khong, N., 2023. Microalgal culture preservation: Progress, trends and future developments. *Algal Research,* 71(103007).

Gannon, M. et al., 2003. An increase in dietary protein improves the blood glucose response in persons with type 2 diabetes. *The American Journal of Clinical Nutrition,* 78(4), pp. 734-741.

Ghatak, H., 2011. Biorefineries from the perspective of sustainability: Feedstocks, products, and processes. *Renewable and Sustainable Energy Reviews,* Volume 15, pp. 4042-4052.

Godlewska, K. et al., 2019. Potential applications of cyanobacteria: *Spirulina platensis* fltrates and homogenates in agriculture. *World Journal of Microbiology and Biotechnology,* 35(80).

Göttingen University, s.d. *SAG Culture Collection.* [Online] Available at: https://uni-goettingen.de/ [Acedido em 2024].

Grahl, S., Strack, M., Mensching, A. & Mörlein, D., 2020. Alternative protein sources in Western diets: Food product development and consumer acceptance of *spirulina*-filled pasta. *Food Quality and Preference,* 84(103933).

Grima, E., Fernández, F. & Medina, A., 2004. Downstream Processing of Cell-mass and Products. Em: *Handbook of Microalgal Culture: Biotechnology and Applied Phycology.* s.l.:Blackwell Publishing, pp. 215-251.

Grover, J., s.d. *Difference between Radicle and Plumule: Definition, Structure.* [Online] Available at: https://collegedunia.com/exams/difference-between-radicle-and-plumule-biology-articleid-2070 [Acedido em 11 july 2024].

Gun, D., Celekli, A., Bozkurt, H. & Kaya, S., 2022. Optimization of biscuit enrichment with the incorporation of *Arthrospira platensis*: nutritional and sensory approach. *Journal of Applied Phycology,* Volume 34, p. 1555–1563.

Habib, A., Parvin, M., Hasan, M. & Huntington, T., 2008. A review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals and fish, s.l.: s.n.

Hasnaoui, S. et al., 2020. Enhancement of bio-hydrogen generation by *spirulina* via an electrochemical photo-bioreactor (EPBR). 45(11), pp. 6231-6242.

Hatami, E. et al., 2021. The effect of *spirulina* on type 2 diabetes: a systematic review and meta-analysis. *Journal of Diabetes & Metabolic Disorders,* Volume 20, pp. 883-892.

Henchion, M. et al., 2017. Future Protein Supply and Demand: Strategies and Factors Influencing a Sustainable Equilibrium. *Foods,* 6(53).

Hilali, S. et al., 2024. NaDES-based biorefinery of *Spirulina* (*Arthrospira platensis*): A new path for sustainable high value-added metabolites. *Separation and Purification Technology,* 329(125123).

Hoque, M., Ajwa, H. & Smith, R., 2007. Nitrite and Ammonium Toxicity on Lettuce Grown under Hydroponics. *Communications in Soil Science and Plant Analysis,* Volume 39, pp. 207-216.

IEA Bioenergy, 2017. *State of Technology Review – Algae Bioenergy.* s.l.:s.n.

Ismail, H. et al., 2023. Functionalization of Ricotta cheese with powder of S*pirulina platensis*: physicochemical, sensory, and microbiological properties. *INTERNATIONAL JOURNAL OF FOOD PROPERTIES,* 26(1), pp. 1968-1983.

Jalili, S. et al., 2024. Optimizing *Spirulina platensis*, *Chlorella vulgaris* microalgae and curcumin application in functional cheese production and investigating its physicochemical properties and sensory evaluation by RSM. *Journal of Food Measurement and Characterization,* Volume 18, p. 1144–1157.

Jardin, P., 2015. Plant biostimulants: Definition, concept, main categories and regulation. *Scientia Horticulturae*.

Jesus, S. & Filho, R., 2020. Recent advances in lipid extraction using green solvents. *Renewable and Sustainable Energy Reviews,* 133(110289).

Jung, S. et al., 2016. Spirulina-PCL Nanofiber Wound Dressing to Improve Cutaneous Wound Healing by Enhancing Antioxidative Mechanism. *Journal of Nanomaterials,* 2016(6135727).

Junique, L. et al., 2021. Determination by response surface methodology of optimal protein and phycocyanin productivity conditions in *Arthrospira* (*Spirulina*) *platensis* under different combinations of photoperiod variation and lighting intensity. *Bioresource Technology Reports,* 15(100763).

Kubar, A. et al., 2022. Developing a Zigzag-baffled column photobioreactor to increase mass-transfer, CO2 fixation and biomass yield during *A. platensis* cultivation. *Journal of CO2 Utilization,* 63(102126).

Lafarga, T., 2019. Effect of microalgal biomass incorporation into foods: Nutritional and sensorial attributes of the end products. *Algal Research,* Volume 41.

Lafarga, T. et al., 2021. Consumer knowledge and attitudes towards microalgae as food: The case of Spain. *Algal Research,* 54(102174).

Lafarga, T. et al., 2019. Effect of microalgae incorporation on the physicochemical, nutritional, and sensorial properties of an innovative broccoli soup. *LWT,* Volume 111, pp. 167-174.

Lanzotti, V. et al., 2022. NMR Metabolomics and Chemometrics of Lettuce, *Lactuca sativa* L., under Different Foliar Organic Fertilization Treatments. *Plants,* 11(2164).

Lee, Y.-K., 2004. Algal Nutrition: Heterotrophic Carbon Nutrition. Em: A. Richmond, ed. *Handbook of Microalgal Culture: Biotechnology and Applied Phycology.* s.l.:Blackwell, pp. 116-124.

Letras, P. et al., 2022. 3D printed gluten-free cereal snack with incorporation of *Spirulina* (*Arthrospira platensis*) and/or *Chlorella vulgaris*. *Algal Research,* Volume 68.

Li, H. et al., 2024. Machine learning-assisted optimization of food-grade *Spirulina* cultivation in seawater-based media: From laboratory to large-scale production. *Journal of Environmental Management,* 369(122279).

Lim, H. et al., 2021. Perspective of *Spirulina* culture with wastewater into a sustainable circular bioeconomy. *Environmental Pollution,* Volume 284.

Lin, J.-H., Liu, L.-Y., Yang, M.-H. & Lee, M.-H., 2004. Ethyl acetate/ethyl alcohol mixtures as an alternative to folch reagent for extracting animal lipids. *Journal of Agricultural and Food Chemistry,* 52(16), p. 4984–4986.

Longtin, N. et al., 2021. Analysis of *Spirulina platensis* microalgal fuel cell. *Journal of Power Sources,* 486(229290).

Lucas, B., Costa, J. & Brunner, T., 2023. Attitudes of consumers toward *Spirulina* and açaí and their use as a food ingredient. *LWT - Food Science and Technology,* 178(114600).

Macedo, A., Azedo, D., Duarte, E. & Pereira, C., 2021. Valorization of Goat Cheese Whey through an Integrated Process of Ultrafiltration and Nanofiltration. *Membranes,* 11(7).

Mader, J. et al., 2016. Calcium spirulan derived from *Spirulina platensis* inhibits herpes simplex virus 1 attachment to human keratinocytes and protects against *herpes labialis*. *Journal of Allergy and Clinical Immunology,* Volume 137, pp. 197-203.

Maluin, F. et al., 2021. Some Emerging Opportunities of Nanotechnology Development for Soilless and Microgreen Farming. *Agronomy,* 11(1213).

Martins, C. et al., 2021. Using Microalgae as a Sustainable Feed Resource to Enhance Quality and Nutritional Value of Pork and Poultry Meat. *Foods,* 10(12).

Masotti, F., Cattaneo, S., Stuknyté, M. & Noni, I., 2017. Technological tools to include whey proteins in cheese: Current status and perspectives. *Trends in Food Science & Technology,* Volume 64, pp. 102-114.

Massoud, R., Khosravi-Darani, K., Nakhsaz, F. & Varga, L., 2016. Evaluation of Physicochemical, Microbiological and Sensory Properties of Croissants Fortified with *Arthrospira platensis (Spirulina)*. *Czech Journal of Food Sciences,* 34(4), pp. 350-355.

Mata, S. et al., 2020. *Spirulina* sp. LEB 18 cultivation in a raceway-type bioreactor using wastewater from desalination process: Production of carbohydrate-rich biomass. *Bioresource Technology,* 311(123495).

Mata, T., Martins, A. & Caetano, N., 2012. Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews,* Volume 14, pp. 217-232.

McKinley, L. et al., 2024. Antiviral potential of *Spirulina* in individuals with human immunodeficiency virus or Hepatis C virus infections: A systematic review and meta-analysis. *Clinical Nutrition ESPEN,* Volume 63, pp. 440-446.

Melo, T. et al., 2021. Ethanol Extraction of Polar Lipids from *Nannochloropsis oceanica* for Food, Feed, and Biotechnology Applications Evaluated Using Lipidomic Approaches. *Marine Drugs,* 19(11).

Mendes, M. et al., 2022. Algae as Food in Europe: An Overview of Species Diversity and Their Application. *Foods,* 11(1871).

Moradi, S. et al., 2019. Effects of *Spirulina* supplementation on obesity: A systematic review and meta-analysis of randomized clinical trials. *Complementary Therapies in Medicine,* 47(102211).

Morais, M., Miranda, M. & Costa, J., 2006. Biscoitos de chocolate enriquecidos com *Spirulina platensis*: Caracterìsticas físico-químicas, sensoriais e digestibilidade. *Alimentos e Nutrição Araraquara,* 17(3), pp. 323-328.

Morais, W. et al., 2020. Microalgae for biotechnological applications: Cultivation, harvesting and biomass processing. *Aquaculture,* 528(735562).

Moreira, B. et al., 2018. Microalgal biotechnology for development of energy gel in sports. *International Food Research Journal ,* 25(5), pp. 1942-1947.

Moreira, J. et al., 2019. Antioxidant ultrafine fibers developed with microalga compounds using a free surface electrospinning. *Food Hydrocolloids,* Volume 93, pp. 131-136.

Morsy, M. et al., 2014. Production and evaluation of some extruded food products using S*pirulina* algae. *Annals of Agricultural Science Moshtohor Journal,* 52(4), p. 495–510.

Moubayed, N. & Al-Houri, H., 2022. Characterization of adsorption ability of *Spirulina platensis* for copper ions removal from aqueous solutions. *Desalination and Water Treatment,* Volume 250, pp. 118-125.

Musio, B. et al., 2022. A spectroscopic study to assess heavy metals absorption by a combined hemp/spirulina system from contaminated soil. *Environmental Advances,* 7(100144).

Mussagy, C. et al., 2020. Integrative platform for the selective recovery of intracellular carotenoids and lipids from *Rhodotorula glutinis* CCT-2186 yeast using mixtures of bio-based solvents. *Green Chemistry,* Volume 22, p. 8478–8494.

Nautiyal, H., & Goel, V. (2021). Sustainability assessment: Metrics and methods. Em *Methods in Sustainability Science* (pp. 27-46). doi:10.1016/B978-0-12-823987-2.00017-9

Nazari, M., Rigueto, C., Rempel, A. & Colla, L., 2021. Harvesting of *Spirulina platensis* using an eco-friendly fungal bioflocculant produced from agro-industrial by-products. *Bioresource Technology,* 322(124525).

Nematollahi, M. et al., 2020. Effect of organic carbon source and nutrient depletion on the simultaneous production of a high value bioplastic and a specialty pigment by *Arthrospira platensis*. *Algal Research,* 47(101844).

Niangoran, N., Buso, D., Zissis, G. & Prudhomme, T., 2021. Influence of light intensity and photoperiod on energy efficiency of biomass and pigment production of *Spirulina (Arthrospira platensis)*. *OCL,* 28(37).

Oosten, M. et al., 2017. The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. *Chemical and Biological Technologies in Agriculture*, 4(5).

Orlina, E., Besana, N., Seneris, G. & Capina, K., 2023. Phytotoxicity Assessment of Biofertilizer Produced from Bioreactor Composting Technology Using Lettuce (*Lactuca sativa* L.) Seeds. *Open Journal of Ecology,* Volume 13, pp. 257-270.

Paiva, K. et al., 2024. Investigating the impact of pasteurization on the bioactive-rich potential of *psyllium* and *spirulina* in a concentrated beverage. *Measurement: Food,* 13(100129).

Parikh, P. & Iyer, U., 2001. Role of *Spirulina* in the Control of Glycemia and Lipidemia in Type 2 Diabetes Mellitus. *Journal of Medicinal Food,* 4(4), pp. 193-199.

Peralta, Â., 2019. *Produção e monitorização de microalgas,* s.l.: s.n.

Pereira, L. et al., 2022. Evaluation of protein content and antimicrobial activity of biomass from *Spirulina* cultivated with residues from the brewing process. *Journal of Chemical Technology & Biotechnology,* 97(1), pp. 160-166.

Pereira, M. et al., 2019. Mixotrophic cultivation of *Spirulina platensis* in dairy wastewater: Effects on the production of biomass, biochemical composition and antioxidant capacity. *PLoS ONE,* 14(10).

Peres, E. et al., 2018. Treatment of leachates containing cobalt by adsorption on *Spirulina* sp. and activated charcoal. *Journal of Environmental Chemical Engineering,* Volume 6, pp. 677-685.

Pinto, L. et al., 2022. Lipid and phycocyanin extractions from *Spirulina* and economic assessment. *The Journal of Supercritical Fluids,* 184(105567).

Pradana, Y. et al., 2020. Advancing biodiesel production from microalgae *Spirulina* sp. by a simultaneous extraction–transesterification process using palm oil as a co-solvent of methanol. *Open Chemistry,* 18(1), pp. 833-842.

Prete, V. et al., 2024. Beneficial Effects of *Spirulina* Supplementation in the Management of Cardiovascular Diseases. *Nutrients,* 16(642).

Probst, L., Frideres, L. & Pedersen, B. A. F., 2015. *Sustainable, Safe and Nutritious Food: New nutrient sources,* s.l.: s.n.

Rabelo, S. et al., 2013. Development of cassava doughnuts enriched with *Spirulina platensis* biomass. *Brazilian Journal of Food Technology,* 16(1), pp. 42-51.

Ragaza, J. et al., 2020. A review on *Spirulina*: alternative media for cultivation and nutritive value as an aquafeed. *Reviews in Aquaculture,* Volume 12, pp. 2371-2395.

Rodrigues, L., Valle, A. & D'Elia, E., 2018. Biomass of Microalgae *Spirulina Maxima* as a Corrosion Inhibitor for 1020 Carbon Steel in Acidic Solution. *International Journal of Electrochemical Science,* Volume 13, pp. 6169-6189.

Rzymski, P. et al., 2015. The multidisciplinary approach to safety and toxicity assessment of microalgae-based food supplements following clinical cases of poisoning. *Harmful Algae,* Volume 46, pp. 34-42.

Santos, P. et al., 2020. Influence of thermal and electrical effects of ohmic heating on C-phycocyanin properties and biocompounds recovery from *Spirulina platensis*. *LWT- Food Science and Technology,* 128(109491).

Santos, R., Araújo, O., Medeiros, J. & Chaloub, R., 2016. Cultivation of *Spirulina maxima* in medium supplemented with sugarcane vinasse. *Bioresource Technology,* Volume 204, pp. 38-48.

Santos, R., Corrêa, P., Dantas, F. & Teixeira, C., 2019. Evaluation of the co-production of total carotenoids, C-phycocyanin and polyhydroxyalkanoates by *Arthrospira platensis*. *Bioresource Technology Reports,* 7(100226).

Sharanappa, C.H. et al., 2024. Investigation on the insecticidal activities of cyanobacterial extracts as an alternative source for the management of fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae). *Heliyon,* 10(e29060).

Silva, A., Brazinha, C., Costa, L. & Caetano, N., 2020. Techno-economic assessment of a *Synechocystis* based biorefinery through process optimization. *Energy Reports,* 6(1), pp. 509-514.

Silva, N. et al., 2019. Dehydration of microalgae Spirulina platensis in a rotary drum with inert bed. *Powder Technology,* Volume 351, pp. 178-185.

Silva, S., 2018. *Spirulina platensis como fonte de ingredientes funcionais e testes de incorporação da sua forma livre e microencapsulada em iogurtes,* s.l.: s.n.

Silva, S. et al., 2019. Spray-dried *Spirulina platensis* as an effective ingredient to improve yogurt formulations: Testing different encapsulating solutions. *Journal of Functional Foods,* Issue 103427.

Silva, S., Valle, A. & Perrone, D., 2021. Microencapsulated *Spirulina maxima* biomass as an ingredient for the production of nutritionally enriched and sensorially well-accepted vegan biscuits. *LWT,* Volume 142.

Singh, J., Kumar, A., Rai, A. & Singh, D., 2016. Cyanobacteria: A Precious Bio-resource in Agriculture, Ecosystem, and Environmental Sustainability. *Frontiers in Microbiology,* 7(529).

Sirikulrat, K., Pekkoh, J. & Pumas, C., 2021. Illumination system for growth and net energy ratio enhancement of *Arthrospira (Spirulina) platensis* outdoor cultivation in deep raceway pond. *Bioresource Technology Reports,* 14(100661).

Soni, R., Sudhakar, K. & Rana, R., 2019. Comparative study on the growth performance of *Spirulina platensis* on modifying culture media. *Energy Reports,* Volume 5, pp. 327-336.

Stunda-Zujeva, A., Ende, S., Berele, M. & Beyer, A., 2023. Developing an economically efficient LED light regime for *Arthrospira platensis* cultivation. *Journal of Applied Phycology,* Volume 35, p. 2599–2606.

Tan, H. et al., 2020. Optimization of the Freezing-Thawing Method for Extracting Phycobiliproteins from *Arthrospira* sp.. *Molecules,* 25(3894).

Tan, J. et al., 2020. A review on microalgae cultivation and harvesting, and their biomass extraction processing using ionic liquids. *Bioengineered,* 11(1), pp. 116-129.

Thevarajah, B. et al., 2022. Large-scale production of *Spirulina*-based proteins and c-phycocyanin: A biorefinery approach. *Biochemical Engineering Journal,* 185(108541).

Tomaselli, L., 2004. The Microalgal Cell. Em: A. Richmond, ed. *Handbook of Microalgal Culture: Biotechnology and Applied Phycology.* s.l.:Blackwell, pp. 3-19.

Toro, J. & Alzate, C., 2023. Sustainability of Biorefineries: Challenges and Perspectives. *Energies,* 16(3786).

Tredici, M., 2004. Mass Production of Microalgae: Photobioreactors. Em: A. Richmond, ed. *Handbook of Microalgal Culture: Biotechnology and Applied Phycology.* s.l.:Blackwell, pp. 178-214.

Valenzuela, H., 2024. Optimizing the Nitrogen Use Efficiency in Vegetable Crops. *Nitrogen,* 5(1), pp. 106-143.

Vantage Market Research, 2023. *Spirulina Market - Global Industry Assessment & Forecast,* s.l.: s.n.

Varia, J., Kamaleson, C. & Lerer, L., 2022. Biostimulation with phycocyanin-rich *Spirulina* extract in hydroponic vertical farming. *Scientia Horticulturae,* 209(111042).

Vieira, M. et al., 2020. 3D printed functional cookies fortified with *Arthrospira platensis*: Evaluation of its antioxidant potential and physical-chemical characterization. *Food Hydrocolloids,* Volume 107.

Vijayarasa, J., Pakeerathan, K., Thiruchchelvan, N. & Mikunthan, G., 2021. Bio-Remediation of Agro-Based Industries' Wastewater and Mass Production of *Spirulina (Spirulina platensis* (Gomont) Geitler 1925). *Biology and Life Sciences Forum,* 3(1), pp. 1-24.

Vonshak, A. & Richmond, A., 1988. Mass Production of the Blue-green Alga *Spirulina*: An Overview. *Biomass,* Issue 30, pp. 233-247.

Wang, J. et al., 2019. Field study on attached cultivation of *Arthrospira (Spirulina)* with carbon dioxide as carbon source. *Bioresource Technology,* Volume 283, pp. 270-276.

Weightman, R., 2014. Nitrogen recomendations for optimizing yield and quality of baby leaf lettuce, s.l.: s.n.

World Commission on Environment and Development. (1987). *Our common future.* United Nations. Oxford University Press.

Wuang, S., Khin, M., Chua, P. & Luo, Y., 2016. Use of *Spirulina* biomass produced from treatment of aquaculture wastewater as agricultural fertilizers. *Algal Research,* Volume 15, pp. 59-64.

Wu, Q. et al., 2016. The antioxidant, immunomodulatory, and anti-inflammatory activities of *Spirulina*: an overview. *Archives of Toxicology,* Volume 90, p. 1817–1840.

Ye, C. et al., 2018. Life cycle assessment of industrial scale production of *Spirulina* tablets. *Algal Research,* Volume 34, pp. 154-163.

Zarrouk, C., 1966. Contribution to the Study of a Cyanophycea: Influence of Various Physical and Chemical Factors on the Growth and Photosynthesis of *Spirulina Maxima,* s.l.: s.n.

Annexes

Index

A. Growth curve

To monitor *Spirulina* growth, a calibration curve was established, correlating optical density measurements at 730 nm with dry biomass weight. The initial obtained curve, with the equation A.1, did not showed good linearity (R^2 = 0.9415).

$$Concentration = 1.81 * abs - 0.5604 \qquad (A.1)$$

Therefore, the two different approaches were made. Firstly, by removing the point with the highest concentration and absorbance, a curve with good linearity (R^2 = 0.9905) is produced, Figure 28, and new equation is obtained, A.2.

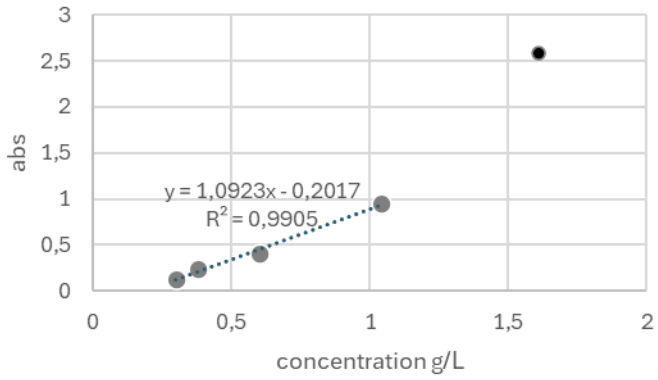


Figure 28 - Plot of the absorbance against the biomass concentration for low concentration cultures

$$Concentration = 1.0923 * abs - 0.2017 \qquad (A.2)$$

However, it would not be possible to determine the concentrations of the most concentrated test cultures without extrapolating results. Therefore, a polynomial approach was performed, as shown in Figure 29.

The obtained equation for the calibration curve is expressed below (A.3).

$$Concentration = 1.2666 * \ abs^2 - 0.5975 * abs + \ 0.2507 \qquad (A.3)$$

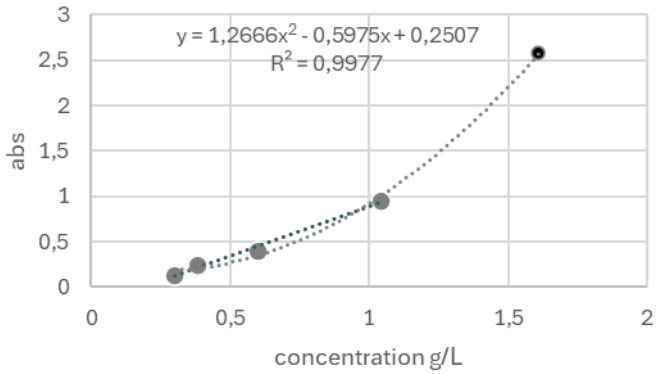


Figure 29 - Plot of the absorbance against the biomass concentration for high concentration cultures

B. Survey

Questionário

Avaliação organolética na caracterização de produtos de consumo

Manteiga enriquecida com *Spirulina*

PARTE 1 - Caracterização do provador

1. Idade
   - ☐ <18 anos
   - ☐ 18-24 anos
   - ☐ 25-34 anos
   - ☐ 35-44 anos
   - ☐ 45-54 anos
   - ☐ 55-64 anos
   - ☐ >65 anos

2. Género
   - ☐ Feminino
   - ☐ Masculino
   - ☐ Outro:

3. Quão confortável está em relação a novos produtos?
   - ☐ Muito conservador (só consumo produtos que já conheço)
   - ☐ Moderado
   - ☐ Explorador (estou à vontade para experimentar novos produtos)

4. Com que frequência consome manteiga?
   - ☐ Diariamente
   - ☐ Algumas vezes por semana
   - ☐ Algumas vezes por mês
   - ☐ Raramente
   - ☐ Nunca

5. Com que frequência consome margarina (tipo Planta)?
   - ☐ Diariamente
   - ☐ Algumas vezes por semana
   - ☐ Algumas vezes por mês
   - ☐ Raramente
   - ☐ Nunca

6. Segue alguma dieta específica (vegetariana, vegana, sem glúten, etc.)?
   - ☐ Sim (Qual?)
   - ☐ Não

7. Toma suplementos proteicos?
   - ☐ Sim
   - ☐ Não

PARTE 2 - Prova

Aparência Visual – observe o produto

1. Cor da manteiga:
   - ☐ Apelativa
   - ☐ Desagradável

Cheiro/Aroma – cheire o produto

1. Intensidade do aroma do suplemento na manteiga:
   - ☐ Fraco
   - ☐ Moderado
   - ☐ Forte

2. Aroma geral da manteiga:
   - ☐ Agradável
   - ☐ Aceitável
   - ☐ Desagradável

Sabor – prove o produto

1. Intensidade do sabor do suplemento na manteiga:
   - ☐ Fraco
   - ☐ Moderado
   - ☐ Forte

2. Sabor geral da manteiga:
   - ☐ Agradável (o suplemento complementa)
   - ☐ Aceitável
   - ☐ Desagradável (o suplemento interfere)

Textura

1. Sensação na boca ao comer a manteiga
   - ☐ Agradável
   - ☐ Normal (sem influência)
   - ☐ Desagradável

Aceitação Global

1. Gostaria de consumir essa manteiga novamente?
   - ☐ Não
   - ☐ Sim

2. Se este produto estivesse a um preço que considera adequado, ponderava comprar?
   - ☐ Não
   - ☐ Talvez
   - ☐ Sim

Comentários Adicionais

1. Existe alguma outra observação ou sugestão que gostaria de fornecer?

C. Poster

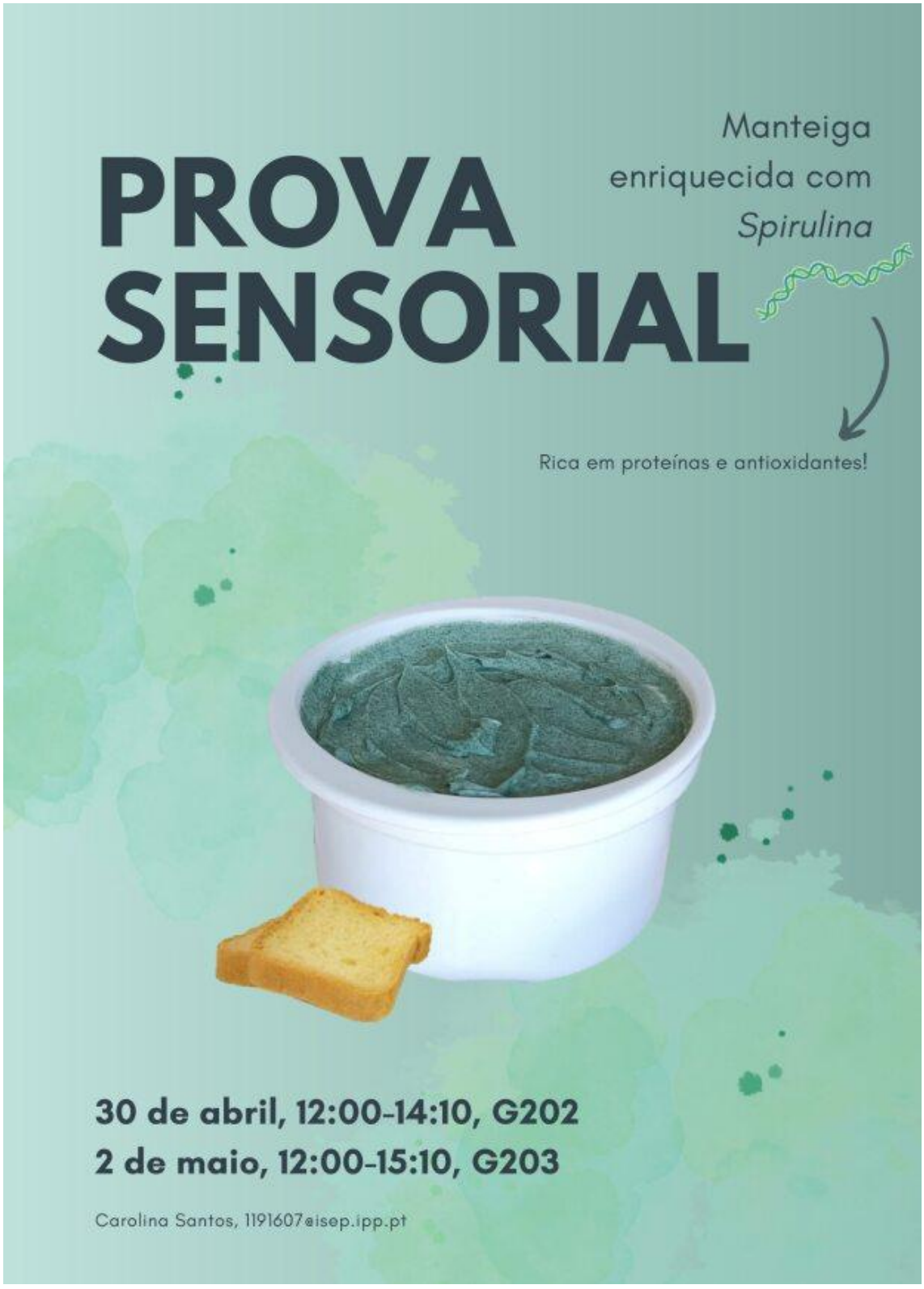


Figure 30 - Poster publicising the sensory analysis test

D. Other products

Other products were fortified with lyophilised *Spirulina*, however, were not subjected to any test. Both products, pancakes (Figure 30) and fresh pasta (Figure 31), during confection, maintained rheological product characteristics.

Figure 31 - Pancakes fortified with 2% *Spirulina*

Figure 32 - Cooked fresh pasta fortified with 5% *Spirulina*