Universidade de Lisboa
Faculdade de Farmácia

Formulation and characterization of Locust Bean Gum-based Energy Hydrogels supplemented with *Chlorella vulgaris*

Joana Gonçalves de Almeida

Dissertation supervised by Professor Isabel Alexandra Caldeira Ribeiro and co-supervised by Professor Ana Francisca Campos Simão Bettencourt

Food Quality and Health

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Abstract

In recent times, microalgae have gained relevance due to high content in bioactive compounds as well as nutritional value. Microalgae can grow under a diverse set of environmental conditions. Various growth systems have been used to isolate and cultivate microalgae, in the laboratory, outdoors and in small or large-scale production. In recent years, several products containing microalgae powder and biomass have been developed, namely pastas, emulsions, gelled desserts and biscuits.

Energy gels have been gaining relevance in the market, due to a growing tendency of the population to practice physical exercise. As a result, the main goal of this research was to create energy hydrogels supplemented with *Chlorella vulgaris*.

This research was divided into two parts, one focused on the internship carried out at the company Allmicroalgae, addressing the production processes of microalgae, and the second part was about the optimization and characterization of hydrogels supplemented with *Chlorella vulgaris* this one focused on a stability evaluation, nutritional determination, sensorial analysis, and antioxidant properties.

The results of the optimization showed that in relation to the chosen thickening agent (Locust Bean Gum) at low shear rates it exhibits Newtonian behavior. The stability evaluation showed that a preserving agent was needed in the final formulation. After the stability evaluation, the final formulations were settled with 0.6% of LBG, 50% maltodextrin, 0.1% of potassium sorbate, 0.2% of NaCl, 0.25% of citric acid and lemon flavor. To study the effect that Honey *C. vulgaris* had on the product, two gel formulations were created with 4 % HC thinking about the nutritional value of the product and with 1.5% HC this last one with a more subtle taste, thinking about the acceptability by the consumers.

Regarding the addition of microalgae in the hydrogels, their addition improved the nutritional value and mineral content of the product. Regarding the antioxidant activity, the microalgae extract has DPPH radical inhibition activity at different concentrations. For a 50% scavenging activity the extracts concentration of the extract was 2.437 mg/mL.

To conclude, energy gels enriched with Honey *Chlorella vulgaris* can be considered as an alternative way to include these health promoter microalgae in sports supplementation.

Keywords: Hydrogels; Honey *Chlorella vulgaris*; microalgae, locust bean gum, energy gels.

Resumo

Nos últimos anos, as microalgas, nomeadamente a espécie *Chlorella vulgaris* têm ganho uma grande relevância devido ao seu alto teor em compostos bioativos assim como valor nutricional. As microalgas podem crescer sob um conjunto diversificado de condições ambientais e vários sistemas de crescimento têm sido utilizados para isolar e cultivar microalgas. As microalgas podem ser cultivadas em laboratório, ou ao ar livre, e em produção em pequena ou grande escala. Nos últimos anos, foram desenvolvidos vários produtos contendo microalgas em pó e biomassa, nomeadamente massas, emulsões, sobremesas gelificadas e bolachas. A riqueza de compostos bioativos nas microalgas pode contribuir para o desenvolvimento de uma indústria alimentar baseada em algas, focada na produção e utilização de microalgas para produtos alimentícios funcionais inovadores. Os géis energéticos têm vindo a ganhar relevância no mercado, devido a uma tendência crescente da população em aderir a um estilo de vida saudável e praticar exercício físico.

Como resultado, o objetivo principal desta pesquisa foi criar hidrogéis energéticos suplementados com *Chlorella vulgaris.* Esta pesquisa foi dividida em duas partes, sendo uma focada no estágio realizado na empresa Allmicroalgae, abordando os processos de produção e controlo de qualidade das microalgas e a segunda parte sobre a otimização e caracterização destes hidrogéis, esta última focando-se numa avaliação de estabilidade, análise sensorial, avaliação nutricional e determinação da atividade antioxidante.

A produção de géis energéticos ricos em nutrientes e eletrólitos requer um componente que conecta a água e forma um colóide chamado hidrocolóide. O hidrocoloide escolhido foi a goma de alfarroba (GA). A GA mostrou alta solubilidade, dispersibilidade, viscosidade e biocompatibilidade com as formulações de gel. Os resultados deste trabalho demostraram que à medida que a concentração de GA aumenta, sua viscosidade aumenta consequentemente. A GA em baixas taxas de cisalhamento apresenta um comportamento Newtoniano e, concluiu-se que uma concentração de 0,6% (p/v) de GA seria a melhor percentagem para as formulações em comparação com determinações de viscosidades de géis energéticos vendidos no mercado.

A incorporação de microalgas em produtos alimentares enfrenta vários desafios principalmente devido às suas características organoléticas, incluindo forte cor, sabor e

odor. Os atributos sensoriais dos alimentos estão diretamente ligados à aceitação do consumidor, de modo que estratégias alternativas para melhorar a qualidade organolética dos alimentos contendo microalgas incluem a extração dos compostos alvo com a remoção da clorofila. Esse foi o principal motivo da escolha da Honey *Chlorella vulgaris* em pó para as formulações em gel. Honey *Chlorella vulgaris* é uma estirpe otimizada e selecionada da *Chlorella vulgaris* que faz com que esta tenha um menor teor de clorofila. Tornando-se mais agradável para o consumo sendo assim possível adicionar uma quantidade maior e consequentemente aumentar o valor nutricional do produto. Assim, em primeiro lugar, foi escolhida uma formulação de 4% Honey *Chlorella vulgaris*, após provar a formulação foi decidido adicionar outra formulação com menor percentagem desta microalga (1.5% (p/v)) enquanto todos os outros componentes se mantiveram nas mesmas quantidades.

O Carboidrato escolhido foi a maltodextrina que é um carboidrato complexo de fácil absorção, com pouco sabor. É muito popular entre os atletas porque é rapidamente absorvido pelo intestino, repondo os níveis de açúcar no sangue e proporcionando um boost de energia quase instantâneo.

Os resultados da avaliação da estabilidade mostraram que os géis mantidos em temperatura ambiente na segunda semana ganharam um odor atípico e apresentaram contaminação bacteriana nos ensaios microbiológicos, indicando que já não se encontravam aptos para consumo. Com estes resultados percebeu-se que era mais benéfico para a conservação do produto adicionar um agente conservante à sua formulação final de forma a prolongara a sua vida útil.

Após a avaliação da estabilidade as formulações finais foram estabelecidas, sendo constituídas por 0,6% de goma de alfarroba (hidrocolóide), 50% de maltodextrina (carbohidrato), 0,1% de sorbato de potássio (conservante), 0,2% de NaCl (fonte de eletrólitos), 0,25% de ácido cítrico (controlo de pH) e sabor de limão para disfarçar o sabor das microalgas. Com estas percentagens estabelecidas e para estudar o efeito que a Honey *C. vulgaris* teve no produto, foram criadas duas formulações, a primeira com 4 % de Honey *C. vulgaris*, a pensar no valor nutricional do produto, e a segunda com 1,5 % desta microalga, para um sabor mais subtil a pensar na aceitabilidade do sabor pelos consumidores.

Em relação à análise sensorial, os participantes avaliaram as formulações até 6, sendo 1-nada agradável e 6- excelente, foram avaliadas as formulações finais e comparadas com um gel energético do mercado. As pontuações das duas formulações (1-6) em relação ao seu cheiro foram de 4 (1.5% HC) e de 3,30 (4% HC), devido ao aroma de limão adicionado, e pontuação foi maior no gel do mercado testado. Em relação à textura, as pontuações variaram entre 3,95 (4% HC) e 4,70 (gel comercial). As pontuações do sabor foram as que mais variaram, sendo que, em relação à formulação 4% HC o resultado foi negativo (2,85) e o a do gel comercial 4,10. Dos três géis avaliados os participantes preferiram o gel do mercado, contudo os resultados foram positivos para a formulação com menor percentagem de microalga (1,5% HC).

Em relação há avaliação nutricional, a percentagem média de matéria seca foi de 51,1 ± 0,1% em 1,5% HC e em 4% HC foi de 52,4 ± 0,1%. Em relação às proteínas, a percentagem média de proteína variou de 0,6 ± 0,0 % (1,5% HC) e 1,4 ± 0,0 % (4% HC). A percentagem média de açúcares foi de 50,8 ± 1,84% em 1,5% HC e 56,5 ± 0,7 em 4% HC. Em relação ao teor de lipídios o método não teve sensibilidade suficiente para medir quantidades tão pequenas.

Os resultados da avaliação nutricional foram os esperados de acordo com o rótulo dos componentes adicionados e mostraram que a adição das microalgas melhorou o conteúdo nutricional do produto, sendo que a formulação de 4% HC possuiu maior valor nutricional. Comparando estes resultados com os dos rótulos dos géis energéticos do mercado, conclui-se que o conteúdo nutricional de ambas as formulações se encaixa na categoria de géis energéticos para desportistas, garantindo uma formulação adequada para os atletas.

Em relação à atividade antioxidante os resultados mostraram atividade de inibição do radical DPPH no extrato de Honey *Chlorella vulgaris* em diferentes concentrações. Para uma atividade de eliminação de 50%, a concentração de extratos do extrato foi de 2,437 mg/mL. O método DPPH não foi compatível com as amostras devido aos altos níveis de açúcar que precipitaram com a adição de metanol.

Conseguimos concluir que ao aumentar a percentagem da microalga o produto tornou-se menos apelativo para o consumidor. Contudo deve-se ter em conta que suplementos para desportistas normalmente tem um paladar pouco apelativo, e quem

pratica desporto, consume estes produtos não pelo seu sabor, mas principalmente pelo seu efeito benéfico no organismo.

Conclui-se também que os géis energéticos enriquecidos com Honey *Chlorella vulgaris* podem ser considerados como uma forma alternativa de incluir esta microalga promotora de saúde em suplementos para desportistas. No entanto, é necessário melhorar os desenvolvimentos tecnológicos que possibilitem o aumento dos níveis de incorporação e o desenvolvimento de outros alimentos enriquecidos com esta espécie de microalga.

Para trabalhos futuros, seria interessante experimentar diferentes aromas para ver qual disfarça melhor o sabor da microalga. Isso porque o aroma e sabor de muitas microalgas são considerados um dos principais problemas em relação à sua aplicação em produtos alimentares especialmente para aqueles que nunca provaram produtos à base de algas.

Palavras-chave: Hidrogéis, Honey *Chlorella vulgaris***,** microalgas, goma de alfarroba, géis energéticos.

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Abbreviations

EDTA	Complexation with ethylenediamine tetraacetic acid
EFSA	European Food Safety Authority
EU	European Union
FDA	Food and Drug Administration
FP	Flat Panels
GI	Glycemic Index
ICP-AES	Inductively coupled plasma - atomic emission spectrometry.
ICP-MS	Inductively coupled plasma mass spectrometry
LBG	Locust bean gum
MAS	Molecular absorption spectrophotometry method
MIC	Minimum Inhibitory Concentration
OD	Optical density
PBR	Photobioreactor
PID	Proportional Integral Derivative controller
RT	Room temperature
ROS	Reactive Oxygen Species
SD	Standard Deviation
TPC	Total phenolic compounds
TBS	Trypto-casein soy broth
Trolox	6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
Titr	Titration.
VanMolPhos	Vanadomolybdophosphoric acid method.

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Chapter 1. Introduction

1.1 Introduction of new foods on the market

With the growth of the world population, and the consequent increase in the demand for animal protein, achieving a suitable diet is a global concern and the need for new sources of nutrients that are easy and fast to produce is a demand. From a public health perspective, new sources of nutrients can help ensure food security and decrease the incidence of cardiovascular disease related to unhealthy foods. In addition, seeking for alternatives that contribute to the reduction of greenhouse gas emissions associated with animal farming, prevent the depletion of the oceans and reduce the stress on land availability are also a concern (Ncube et al., 2018 and Mitra & Mishra, 2019).

In recent years, several alternatives to animal proteins intended for human consumption and animal feed production have emerged, being some examples the insects, algae and in vitro meat. The latter is still in an experimental phase and there are still not enough studies to allow its commercialization in Europe (Moura et al., 2022).

Insect consumption, known as entomophagy, is part of the diet of at least 2 billion people in Asia, Latin America and Africa. Among the most consumed species are caterpillars, termites, grasshoppers, crickets, bed bugs and mealworms (van der Weele et al., 2019). In Western culture, the consumption of insects is still under accepted, and the introduction of species must be evaluated for risks and authorized by the European Commission, with the evaluation of several species taking place in the European Union. The European Food Safety Authority (EFSA) published in January 2021 a scientific opinion on the first completed evaluation of a proposal for an insect-derived food product, specifically on dehydrated Tenebrio larva as a Novel Food under the Regulation (EU) No. 2015/2283. Entomophagy has several nutritional benefits, Nutrient contents varies among species, metamorphic state, and preparation methods, but in general, insects

provide adequate amounts of energy and protein, meeting human amino acid requirements, and are rich in unsaturated fatty acids, minerals, and vitamins. However, according to available EFSA assessments, several insect species can cause allergies or cross-allergies, especially for those that suffer from shellfish allergy, so consumers should be "clearly informed on labeling and marketing that a food contains insects and of which species” (van der Weele et al., 2019).

Another promising option to alleviate this stress on the need for protein is the introduction of algae in human nutrition. Algae are simple, plant-like organisms capable of harnessing sunlight to convert carbon dioxide into sugars and oxygen during the process of photosynthesis. They can be divided into microalgae and seaweed. Microalgae are single-cell organisms that can grow under a diverse set of environmental conditions, whereas macroalgae are more complex. Seaweeds are associated with many health benefits, including lowering blood pressure and preventing strokes, but most importantly, they are a valuable source of protein. The most common varieties of edible seaweed include Nori, Wakame, Kombu, Dulse, and Carrageen (Bleakley & Hayes, 2017).

Algae have great potential as an alternative to animal protein and can be consumed directly as whole or used in food and feed supplements, however microalgae are consumed normally as a supplement or incorporated in food products. Algae and microalgae have gained relevance in the vegetarian and vegan communities and studies have shown that they are beneficial to health as they can be used to enhance the nutritional value of food, due to their well-balanced chemical composition. Moreover, they are consumed as a source of highly valuable molecules such as polyunsaturated fatty acids, pigments, antioxidants, pharmaceuticals and other biologically active compounds. The application of microalgal biomass and/or metabolites is an interesting and innovative approach for the development of healthier food products (Craddock et al., 2017).

The introduction of "novel foods" for human consumption in the European Union has always raised concerns. According to this regulation, the EU included some microalgae in the list of foods authorized in the EU market, namely *Aphanizomenon flos-aquae, Arthrospira platensis, Chlorella luteoviridis, Chlorella pirenoidosa , Chlorella vulgaris, Odontella aurita, Tetraselmis chuii* and *Haematococcus pluvialis* (European Parliament & Council of the European Union, 1997) and other microalgae species were successively approved as food or food ingredients after complying with the regulatory procedures for novel foods (Vigani et al., 2015).

1.2 Microalgae as functional foods

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that have a simple cellular structure, their growth requires light, carbon dioxide, water and nutrients, especially phosphorus and nitrogen (Becker, 2007). Due to their great adaptability, they are able to grow in different environments, not competing for the same resources as land plants used in agriculture. In addition, they present a growth rate that is 5 to10 times higher than that of conventional food crops. They are promising organisms for a wide range of biotechnological applications, including biodiesel production (Chisti, 2007), wastewater bioremediation (Mulbry & Wilkie, 2001) and dietary supplements for animal and human nutrition (Barkia et al., 2019).

Microalgae are mainly composed of lipids, proteins and carbohydrates and, currently, about 40,000 species are described, being the main commercially cultivated *Dunaliella salina* to obtain carotene, *Haematococcus pluvialis* to obtain astaxanthin and species of the genus *Chlorella* and *Arthrospira* for addition to natural foods, the last one mainly because it has high concentrations of protein, as we can see in Table 1.1. In addition to bioactive compounds, such as linoleic acid and polysaccharides, as described in Table 1.2 (Becker, 2007).

The recognized nutritional value of microalgae biomass has promoted their use as a protein supplement and as a nutraceutical. Due to their nutritional value, their biomass is commercialized, usually in the form of capsules and as a supplement for human or animal food. *Spirulina, Chlorella, Dunaliella* and *Scenedesmus*, when correctly processed, have a mild/pleasant flavor, which facilitates their commercialization, and can be incorporated into various types of human foods. For instance, *Spirulina* and *Chlorella* are marketed as a natural food or food supplement and are found in various formulations: powder, tablets, capsules or extracts.

Table 1.1: Composition of different microalgal species in percentage of dry biomass matter.

Microalgae	Composition (%dry matter)			Ref.
	Protein	Carbohydrates	Lipids	
Aphanizomenon flos-aquae	62	23	3	(Becker, 2007)
Arthrospira maxima	60-71	13-16	6-7	(Becker, 2007)
Arthrospira platensis	46-43	8-14	4-9	(Becker, 2007)
Chlamydomonas rheinhardii	48	17	21	(Becker, 2007)
Chlorella pyrenoidosa	57	26	2	(Becker, 2007)
Chlorella vulgaris	51-58	12-17	14-22	(Becker, 2007)
Dunaliella salina	57	32	6	(Sui & Harvey, 2021)
Euglena gracilis	39-61	14-18	14-20	(Becker, 2007)
***Haematococcus pluvialis* green stage**	29-45	15-17	20-25	(Shah et al., 2016)
***Haematococcus pluvialis* red stage**	17-25	36-40	32-37	(Shah et al., 2016)
Porphyridium cruentum	28-39	40-57	9-14	(Becker, 2007)
Spirogyra sp.	6-20	33-64	11-21	Becker, 2007)

Table 1.2: Bioactive compounds present in different microalgae species.

Microalgae	Bioactive compounds	Reference
Arthrospira maxima	Peptides; linoleic acid	(Mary Leema et al., 2010)
Arthrospira platensis	Phycocyanin	(Mary Leema et al., 2010)
Chlorella. pyrenoidosa	Peptides; sulfated polysaccharides	(X. Wang & Zhang, 2013)
Chlorella stigmatophora	Polysaccharides	(Guzmán et al., 2003)
Chlorella vulgaris	Phenols	(Arumugam et al., 2020)
Crypthecodinium cohnii	Docosahexaenoic acid (DHA)	(Ganuza et al., 2008)
Dunaliella salina	Carotenoids: β-carotene	(Sui & Harvey, 2021)
Haematococcus pluvialis	Carotenoids: Astaxanthin	(Mularczyk et al., 2020)
Muriellopsis sp.	Carotenoids: Lutein	(Del Campo et al., 2000)
Nannochloropsis oculata	Peptides; Eicosapentaenoic acid (EPA)	(Cha et al., 2011)
Porphyridium purpureum	Arachidonic acid (AA)	(Tannin-Spitz et al., 2005)
Scenedesmus sp.	Carotenoids: Lutein	(Del Campo et al., 2000)

1.3 Antioxidant and antimicrobial properties of microalgae

Currently there is a worldwide interest in finding new sources of safe antioxidants from natural resources to prevent oxidative spoilage of foods and to minimize oxidative damage to living cells. Components with antioxidant activities can be found in several species of microalgae, such as carotenoids, vitamins and phenolics (Shanab et al., 2012).

There are several studies that evaluate antioxidant activity in microalgae and some of these will be mentioned below. Lee et al., (2003) observed that the species *C. ellipsoidea* showed antioxidant activity in mice by exhibiting attenuating effects on oxidative stress and suppressing the activation of inflammatory mediators in peritoneal macrophages and in the liver. (Saranya and coworkers 2014) evaluated the antioxidant activity in *Chaetoceros calcitrans*, *C. salina* and *Isochrysis galbana* through five antioxidant assays (total antioxidant activity, DPPH radical scavenging activity, radical scavenging activity oxide, hydrogen peroxide radical scavenging activity and ferric reducing power) and total phenolic and carotenoid content were also measured. The results showed that the maximum total antioxidant activity was observed in *Isochrysis galbana*. It was also shown that the total phenolic and carotenoid content played a direct vital role in the total antioxidant activity.

In a study by Goiris et al.,(2012) 32 samples of microalgae biomass were selected for their antioxidant capacity through three antioxidant assays (antioxidant capacity, hydrogen peroxide radical scavenging activity and ferric reducing power) and the total phenolic and carotenoid content were measured. It was found that industrially cultivated samples of *Tetraselmis suecica, Botryococcus braunii, Neochloris oleoabundans, Isochrysis sp., C. vulgaris* and *Phaeodactylum tricornutum* had the highest antioxidant capacities. It was also concluded that carotenoids and phenolic compounds contributed significantly to the antioxidant capacity of microalgae.

Regarding the antimicrobial activity of microalgae, that has been attributed to compounds belonging to several chemical classes, including indoles, terpenes, acetogenins, phenols, fatty acids and volatile halogenated hydrocarbons, a large number of microalgae extracts and/or extracellular products have proven to be antibacterial, antifungal, antiprotozoal, and antiplasmodic agents (Amaro et al., 2011).

In a study by Kokou et al., (2012) the microalgae *C. minutissima, Tetraselmis chuii, Nannochloropsis sp., Arthrospira platensis* and *Isochrysis sp*. were tested for their ability to inhibit the growth of six bacterial strains of Vibrio as well as investigated for the influence of light on their antibacterial activity. The results showed that all microalgae cultures inhibited the growth of bacteria and that their antibacterial activity was not influenced by the presence or absence of light.

In a study by Viana Marques (2019), the antimicrobial activity of extracts of *C. vulgaris, Arthrospira platensis* and *Dunaliella tertiolecta* was evaluated against various bacterial strains causing goat (19 strains) and bovine (16 strains) mastitis, using the microdilution method. in broth. Extracts were obtained using a sodium acetate buffer, water and a chloroform-methanol mixture. The three microalgae extracts showed antimicrobial properties, the lowest minimum inhibitory concentration (MIC) value was 25 μg mL^{-1} for *C. vulgaris* and *Dunaliella tertiolecta* against *Staphylococcus sp*. of bovine mastitis and 25 μg mL^{-1} using extracts of *A. platensis*, which mainly inhibited the growth of *Staphylococcus sp*. of goat mastitis.

To conclude, the results of these studies demonstrate the potential of microalgae as both new sources of antioxidants and antimicrobials, and with special interest for the pharmaceutical, food, and veterinary industries.

1.4 Microalgae in the food market and new foods containing microalgae

Microalgae are a vast biological resource, representing one of the most promising sources for new products and applications (Y. Wang et al., 2021). Some microalgae such as *Chlorella* and *Spirulina* have been used for many centuries as nutrient-rich food in Asia, Africa and South America. However, large-scale commercial production of microalgae only started in the early 1960s (Japan) (Liang et al., 2004) being microalgae currently marketed primarily as dietary supplements, commonly sold in the form of pills, capsules or liquids. Microalgal tablets gained popularity among consumers because they are rich in proteins, vitamins, polysaccharides, polyunsaturated fatty acids (PUFAs), microelements and edible fiber. Although microalgae such as *Spirulina* and *Chlorella* are mainly used in the production of microalgal tablets (or capsule) and extract, they are also used in the processing of common foods, and in recent years diversification of other

microalgal products must be encouraged to ensure the development of microalgal biotechnology (Caporgno & Mathys, 2018).

To date, there are some foods produced with the addition of *Spirulina* and *Chlorella* powder, extracts, pigment of microalgae, and vitamins. (Gouveia et al., 2009) Among the application of microalgae in common food processing, the largest quantity of microalgae is being used in instant noodles or general noodles (M et al., 2017). Those microalgal noodles are produced with flour, *Chlorella* and *Spirulina*. Additionally, microalgae can also be added to many drinks to strengthen their nutritional value. Actually, a series of microalgal drinks such as microalgal health drink, microalgal sour milk, microalgal green tea have been developed. (Liang et al., 2004) In order to keep the clear color or homogeneity of these drinks, microalgae must be used in the form of microalgal extract (green algae extract, *Spirulina* extract or phycocyanin).

There is an increasingly growing market for food products with added microalgae, such as pasta, cookies, bread, snacks, chocolate bars, gummies, yogurts, drink mixes, soft drinks either, both as a nutritional supplement or as a source of natural food coloring, as we can see in Table 1.3. Biotechnological exploitation of microalgae resources for human nutrition purposes is restricted to a few species, due to strict food safety regulations, commercial factors, market demand, and specific preparation (Pulz & Gross, 2004). Foods supplemented with microalgae can be sensorially varied, namely in terms of flavor, color and texture. For example, various microalgae when processed correctly have a more pleasant taste and can be well incorporated into many types of food, adding not only nutritional value but also new, unique and appealing flavors. (Uribe-Wandurraga et al., 2019). In recent years, some research has been carried out on the development of a range of new healthy and attractive foods, prepared from microalgae biomass, rich in carotenoids and polyunsaturated fatty acids with antioxidant effect and other beneficial properties. In Table 1.3 there are some examples of food products containing microalgae as well as the quantity added and their benefits in each food product.

Table 1.3: Microalgae incorporation in different food products.

Product	Microalgae	Addition % (W/W)	Benefit	Ref.
Bread	*A. fusiformis*	1 and 3	Nutritional properties (proteins and mineral content)	(García-Segovia et al., 2017)
Bread	*A. platensis*	11	Techno-functional properties and nutritional properties (proteins and mineral content)	(García-Segovia et al., 2017)
Biscuits	*A. platensis*	1.63, 3, 5, 7, 8.36	Nutritional properties	(Fradinho et al., 2015)
Biscuits	*I. galbana*	1 and 3	Techno-functional properties and nutritional properties (ω-3 PUFAs)	(Şahin, 2019)
Cookies	*C. vulgaris*	0.5, 1, 2, and 4	Coloring agent	(Fradinho et al., 2015)
Vegetarian food gels	*C. vulgaris, H. pluvialis,*	0.75	Techno-functional and nutritional properties (antioxidative activity, ω-3 PUFAs)	(Ana Paula Batista et al., 2012)
Vegetarian food gels	*A. maxima and D. vlkianum*	0.1	Techno-functional and nutritional properties (ω-3 PUFAs).	(A. P. Batista et al., 2011)
Pasta	A. platensis	5, 10, and 20	Techno-functional properties and nutritional properties (antioxidative activity)	(Fradique et al., 2010)

Chapter 2. Aim of the thesis

The development of hydrogels is a trend in the food industry, and energy gels are gaining relevance in the food market because there is an increased tendency of the population to adhere to a healthy lifestyle and practice exercise. Microalgae especially *Chlorella vulgaris* have had through the years a great public interest due their rich nutritional and polyphenolic content and respective association with interesting bioactive properties. The richness of compounds in microalgae can contribute to develop an algal-based food industry, focused on producing and utilizing microalgae for innovative functional food products.

As a result, the main goal of this research was to create and optimize energy hydrogels supplemented with *Chlorella vulgaris.* Therefore, the specific goals of this study were:

I. To follow step by step the production and growth assessment of microalgae in the company Allmicroalgae;
II. To perform a sensorial analysis of the hydrogel formulations;
III. To evaluate the nutritional content of the hydrogel formulations;
IV. To evaluate the antioxidant properties of the hydrogels as well as the *Chlorella vulgaris* extract.

This thesis is organized in 5 key chapters. In chapter 1 the introduction is presented, where microalgae as functional foods and in food industry are addressed, as well as their antioxidant properties. Chapter 2 covers the research aims of this thesis. In chapter 3, microalgae production and quality control are explored, as the result of an internship at a microalgae production company. In chapter 4: The use of *Chlorella vulgaris* on energy hydrogel formulations is explored namely with a stability evaluation, nutritional determination, sensorial analysis, and antioxidant properties. Lastly, in chapter 5 the thesis conclusions are revealed as well as future perspectives for this work.

Chapter 3. Microalgae production and quality control

3.1 Introduction

According to taxonomic and biochemical studies, microalgae can present different types of metabolisms; they can be autotrophic, heterotrophic, and mixotrophic as we can see explained in Figure 3.1. Autotrophic microalgae use photosynthesis to capture sunlight and fix the inorganic carbon from atmospheric CO_2 which is then assimilated in the form of reserve materials such as carbohydrates, lipids, and proteins. Moreover, some species are heterotrophic requiring an external source of organic carbon as nutrients to turn them into their building blocks. If they can obtain their energy and carbon sources both from photosynthesis and from an exogenous organic source of carbon they are called mixotrophic. The main sources of carbon and energy for the heterotrophic growth of microalgae are carbohydrates and organic acids, especially glucose, acetate and glycerol (Wei et al., 2009).

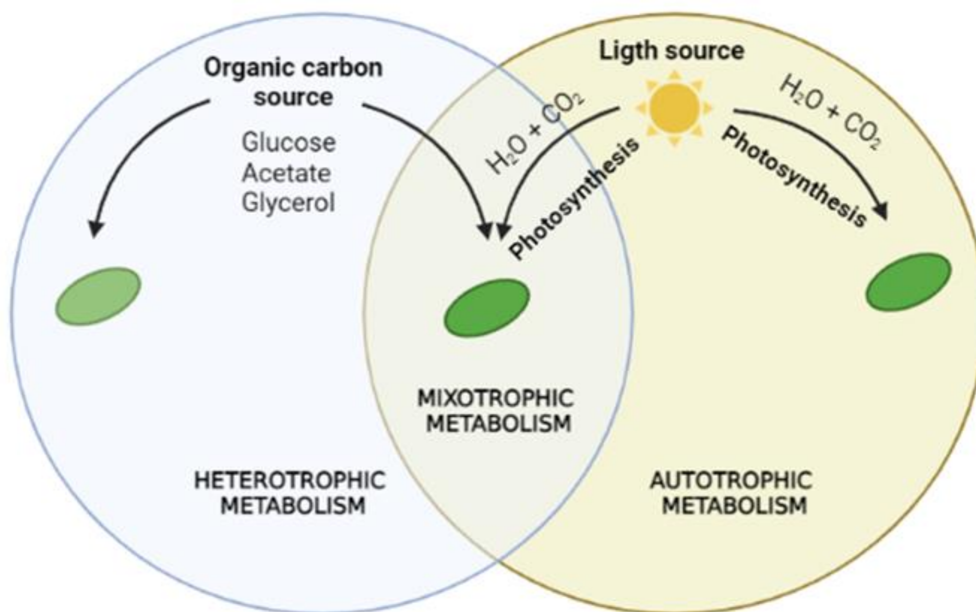


Figure 3.1: Scheme of the three types of metabolism of microalgae, created on Biorender.com.

Various growth systems have been used in order to isolate and cultivate microalgae in the laboratory, as well as outdoors, on small or large scale production. For the cultivation of microalgae open and closed systems are used. Open systems can be artificial ponds or containers, most commonly raceways. (Tan et al., 2020). Closed systems can be photobioreactors (PBR) such as flat panels, tubulars or fermentation reactors. They are robust cultivation systems which employ the effective cultivation techniques thus providing all the essential elements i.e., light, nutrients, temperature and mixing for the healthy growth of microalgae. There are different types of PBRs based on their configuration and operation modes but the most common are flat panels (FP), tubular, and column PBRs. Although most microalgae are autotrophic organisms, several strains have retained the capacity to grow heterotrophically, cultivated in fermentation reactors, using appropriate organic carbon sources (Ahmad et al., 2021). The heterotrophic production of microalgae has consistently improved growth performance and cell concentrations compared to photoautotrophic production. On the other hand, heterotrophic cultivation of microalgae can increase the production costs, due to the need of an organic carbon source, and lead to lower protein and pigment contents (Barros et al., 2019).

To learn more about the growth of microalgae I took an internship at Allmicroalgae for 1 month and a half, where I followed the different stages of cultivation of microalgae and their quality control. I also produced several culture media for heterotrophic cultivation, whose detailed composition is considered as trade secret/confidencial, and was omitted. Some of the activities done in the company will be described in this chapter.

3.2 Materials and methods

3.2.1 Chemicals

Test kit NH_4/NH_3 from Sera (Sera, Heinsberg, Germany), Ammonium, ethanol, hydrochloric acid and distilled water.

3.2.2 Production of microalgae

At Allmicroalgae both auto- and heterotrophic production pathways are commonly used. The present work focused on heterotrophic production because this is the method used to obtain Honey *Chlorella vulgaris*, which was used later on for the gels.

I also learned the autotrophic scale up, that is used to produce various microalgae species. These two ways of cultivation are summarized below.

3.2.2.1 Heterotrophic scale-up

The heterotrophic culture of Honey *Chlorella* was done in fermenters. The inoculum was kept in a master cell bank, where the strains were stored by cryopreservation. When needed, a vial is thawed and cultivation can start. When high concentrations were reached, a starting inoculum was prepared in a small volume, which was later used to inoculate a 7 L bench-top bioreactor (New Brunswick BioFlor/CelliGenr115; Eppendorf AG, Hamburg, Germany) with 5 L working volume. The bioreactor was operated in a fed-batch mode, being supplemented with a glucose solution as the carbon source, an antifoaming agent, and an ammonia solution (24% w/w) as a source of nitrogen and as a pH controller. A proportional Integral Derivative controller (PID) was responsible to maintain the parameters at the set-point. After 2-3 days depending on the growth of the culture, it was transferred to the industrial reactors where the culture medium was also added, firstly a 200L fermentation reactor and then a 5000L fermentation reactor (Figure 3.2).

Figure 3.2: Heterotrophic growth, an example of a a) 200 L fermentation reactor and b) 5000L fermentation reactors. Photos taken at Allmicroalgae company.

3.2.2.2 Autotrophic scale-up

On a laboratory scale, the inoculum was kept in 5L flasks under controlled conditions and when they reach the ideal concentrations, they were later used to inoculate 1000L flat panels. After reaching the ideal concentrations in the flat panels, they were finally transferred to the tubular industrial scale reactors.

The final steps for achieving the final product were harvesting and concentration, where the live culture is filtered to separate the solid biomass from the liquid phase, pasteurization, applied to reduce the number of any existing microorganisms, (on industrial fermenters this step is not necessary because the biomass is already axenic) centrifugation, where concentrated biomass is centrifuged to separate even more liquid from the alga. Finally, biomass can be sold as microalgae paste or as a fine powder after spray dried. All these production steps are summarized in Figure 3.3.

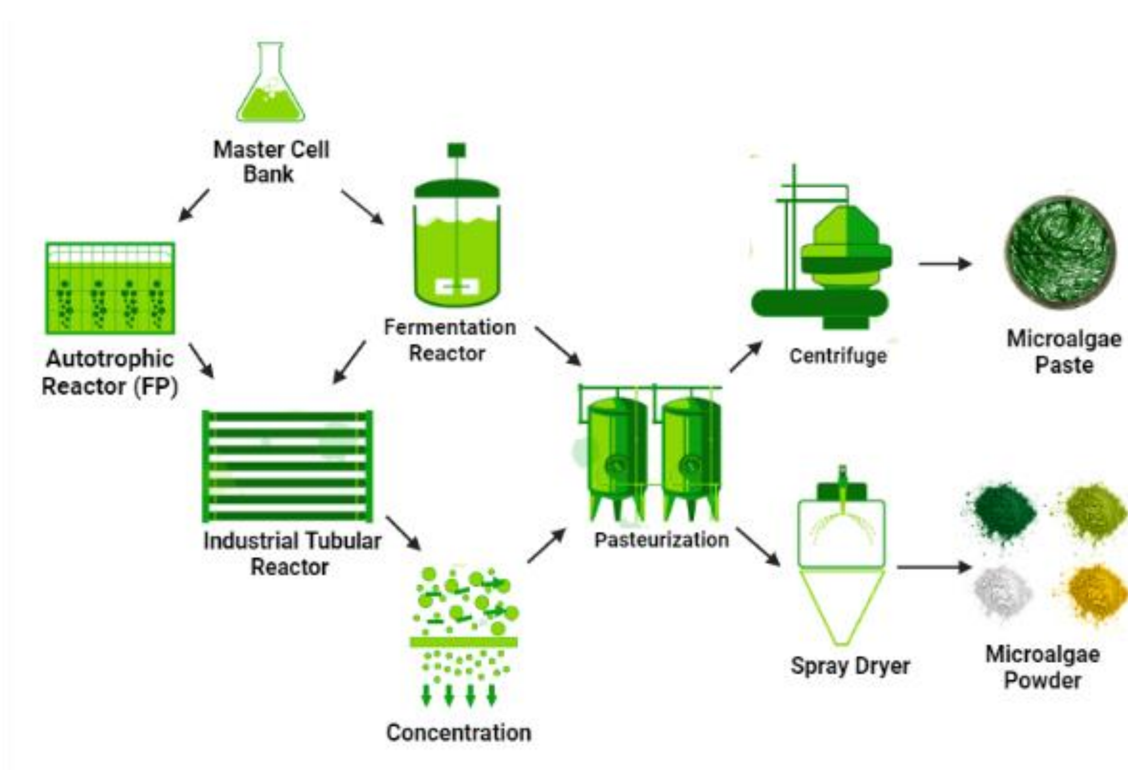


Figure 3.3: Production steps of microalgae in the company Allmicroalgae from the master cell bank to the final product. All the figures were taken from the company website: allmicroalgae.com.

3.2.3 Growth assessment of microalgae

3.2.3.1 Optical density (OD)

Cell growth was determined everyday by measuring the optical density (OD) of the culture, using a Genesys 10S UVVIS spectrophotometer (Thermo Scientific, Massachusetts, USA). These measurements were done directly in the fresh samples or dilutions were performed before, allowing the absorbance value not to exceed the maximum value of 1, maintaining the linearity of the Beer-Lambert law.

3.2.3.2 Optical microscopy

Morphology of cultured microalgae was observed two times a week by optical microscopy (Zeiss Axio Scope A1 Oberkochen, Germany).

3.2.3.3 pH

The pH the culture samples was measured everyday with PH/EC Combo HANNA Waterproof digital meter.

3.2.3.4 Ammonium Content

Ammonium (NH_4^+) content was determined using the test kit NH_4/NH_3 from Sera (Sera, Heinsberg, Germany), according to the manufacturer´s instructions. The result of the reaction was read at 697 nm (Genesys™ 10SUV-VIS spectrophotometer, Thermo Fisher Scientific™, USA) and compared to a calibration curve of ammonium nitrate.

3.2.3.5 Nitrates

Nitrates were determined according to Pereira et al., (2021). Briefly, the collected supernatant was diluted and hydrochloric acid was added at 30 mM. The absorbance of samples was measured spectrophotometrically (4251/50, Zuzi, Seville, Spain) at 220 and 275 nm. The organic matter interference was corrected by subtracting twice the absorbance read at 275 nm from the reading at 220 nm. The final absorbance was compared to a sodium nitrate calibration curve.

3.2.4 Results and discution

3.2.4.1 Production of microalgae in the Allmicroalgae company

In relation to the production of microalgae in Allmicroalgae, I followed the various steps of the cultivation, mainly in the FP and tubular PBRs and also in the fermenter reactors used for the heterotrophic growth. It was possible to learn that the growth in fermentation reactors is tightly controlled, since the microalgae that have a heterotrophic growth compete directly for the organic nutrients with other microorganisms such as bacteria. The control of the heterotrophic cultivation is monitored with a proportional Integral Derivative controller (PID) so the methods that I learned (except OD) were only applied to the autotrophic cultivation (FP and tubulars). Some of the culture samples that I collected from the 2000L fermentation reactor are shown in the Figure 3.4 b) and c) to determine the OD, they are grown in the dark to avoid pigment production as we can see in the covered 7 L bench-top bioreactor (Figure 3.4 a)).

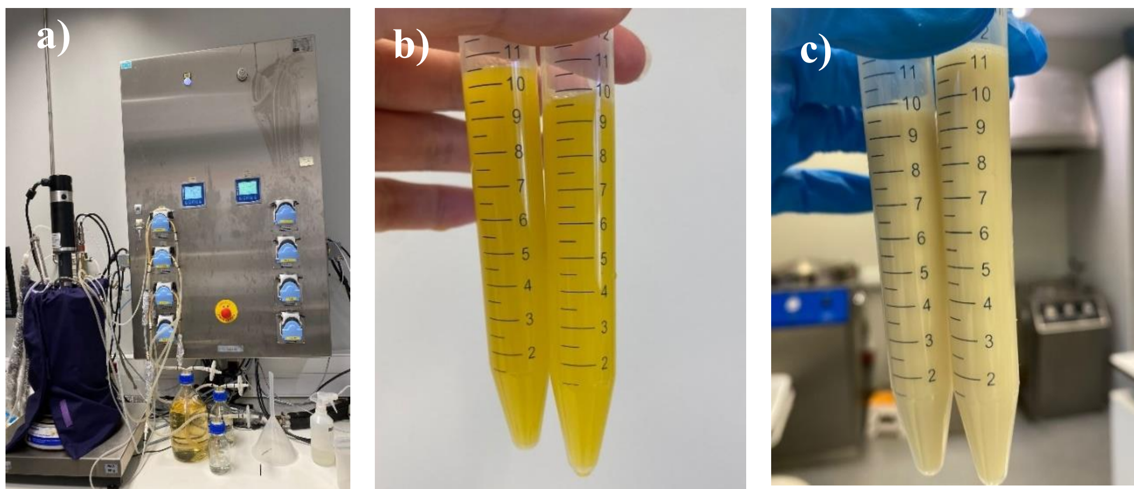


Figure 3.4: Heterotrophic growth, an example of a 7 L bench-top bioreactor a), and samples of the cultures that were growing in the industrial fermentation reactors, being b) Honey C. vulgaris and c) White C. vulgaris. These samples were collected for OD measurements.

Optical density (OD) was used as an indirect measurement of culture growth, since light absorbance can be directly related to cell density, through appropriate calibration curves (optical density vs. number of cells or dry weight).

OD and algal biomass depend on factors such as irradiance and nutrient availability, the physiological state of the microalgae as well as the production conditions (Sui & Harvey, 2021).

To control growth conditions of the microalgae that were cultivated at FP and tubulars the pH was measured every day. The pH measurements ranged from 7 to 10, depending on the microalgae species. The pH of microalgal cultures rises gradually during the day due to the uptake of inorganic carbon by microalgae. Higher pH limits the availability of CO_2, thus, inhibiting cell growth (Tejido-Nuñez et al., 2020). On the other hand, algal cultivation at high pH can suppress undesired biological contaminants. When the pH was higher than normal the CO_2 in the reactor was adjusted either manually (in FP) or automatically (in the tubular reactors), decreasing the pH to the normal range. Since microalgae consume carbon for their photosynthesis the CO_2 available will decrease in the culture and the pH will rise (Mat Aron et al., 2021).

The measurement of the ammonia and nitrate levels in the culture were the most effective way to determine if the culture medium needed to be added to the PBRs to promote the continuous growth of the culture. The culture medium needs to be maintained at a certain concentration, and the quantity to add back to the reactor is calculated having in consideration that concentration and the size of the reactor.

In relation to the culture medium, it was possible to learn that the correct maintenance of microalgae in culture depends on the appropriate choice of the medium, trying to mimic as much as possible the natural environment where the strains come from. Suitable medium for growing strains is quite complex, using various compounds to enrich them. Nitrogen (N) and phosphorus (P) are constituent elements of cell structure and nucleic acids, being essential for the growth of strains and becoming limiting when in low concentrations (Colusse et al., 2020). Moreover the culture medium can still have the addition of vitamins that some microalgae cannot produce, like vitamin B_{12} (Tandon et al., 2017).

With the microscopic observation of the cultures (Figure 3.5), the presence of some microorganisms could be detected in the early stages e.g., amoebas, ciliates and micro flagellates that are harmful to cultures because of the competition between them and the microalgal species for nutrients and space. Some of those microorganisms can even ingest the microalgae destroying the growing culture. This contamination causes

major implications to the development of algal mass culture systems, with respect to productivity, sustainability and commercial viability (Day et al., 2017). By the time an algal culture is being heavily contaminated the effects can be obvious by eye as a change in color occurs. Changes in coloration and optical density, are unlikely to be sufficiently sensitive to detect the early stages of infection. Therefore, by the time the contamination has been detected it may be too late to apply any strategies and be best solution is to discard all the culture (Day et al., 2017).

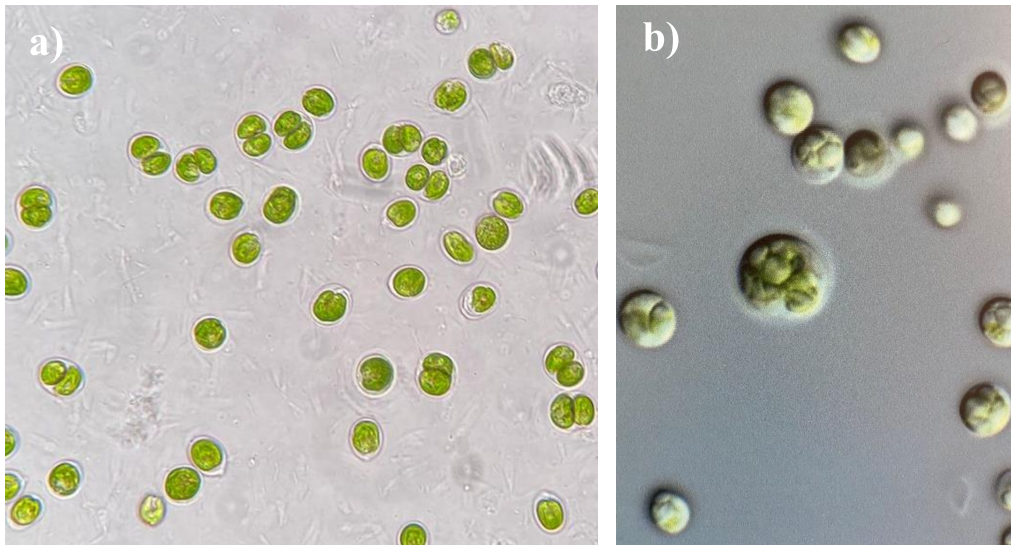


Figure 3.5: Optical microscopic view of a Healthy culture of Chlorella vulgaris showing its usual morphology in to different ampliations a) x100 and b) x 1000.

Allmicroalgae also supplied the microalgae powder that was used in this work to formulate the energy hydrogel formulations described in chapter 4.

Chapter 4: Exploring the use of Chrorella sp. hydrogel formulations for energy gels

4.1 Introduction

4.1.1 Energy gels

A gel is an intermediate between a solid and a liquid that has elastic (solid) and flow (liquid) characteristics (Geremias-Andrade et al., 2016). The gelation mechanism depends on the nature of the gelling agents and the conditions of gel formation, such as temperature, presence of ions, pH, concentration of gelling agents among others (Cao & Mezzenga, 2020). Various foods and supplements are sold in the form of gels and examples include jams, jellies, confectionery, desserts, quick-curing gels, and fruit and vegetable based products (Cao & Mezzenga, 2020). Invariably, one or more gelling agents are used to achieve the desired parameters, the gelling agents frequently used in these products are generally approved food hydrocolloids (Geremias-Andrade et al., 2016). These hydrocolloids are derived from natural sources including a range of polysaccharides and proteins that are today widely used in a variety of industrial sectors to perform a number of functions, such as the thickening and gelling of aqueous dispersions, emulsions and dispersions, stabilization of foams, suspension of material particles and inhibition or decrease syneresis as well as increase the water retention Examples of hydrocolloid components include carrageenan, cellulose derivate (carboxymethyl cellulose), gelatin, chitosan, locust bean gum, pectin, and starch.

Energy gels are products designed to help athletes replenish their energy, nutrient, fluid, and electrolyte levels after exercise. They are usually rich in carbohydrates, an efficient source of energy, as seen in Table 4.1. Energy gels also serve to maintain body balance, improve performance, and contain electrolytes that can improve performance when consumed before or during exercise with a certain intensity. The formulation of sports drinks should refer to the fulfillment of athlete's specific energy, nutrient, fluid, and electrolyte needs, based on the exercise phase (before, during, or after exercise) in Table 4.1 is showed the composition of some commercial energy gels.

Table 4.1: Composition of some energy gels presently sold in the market. Note: All information was taken from the label provided by the website that sells each product.

Product Name	Brand	Type of gel	Carbon hydrates (%)	Protein (%)	salt	Energy value (Kcal)
Recovery Gel Lemon flavored	Biotech USA	Recovery Gel	29	5.33	0.08	137
Recovery Plus Elite™	My Protein	Recovery Gel	28	29	0.19	204
Energy Gel Orange flavored	Biotech USA	Energy Gel	30	2.67	0.54	148
Energy Gel Bio	226ers	Energy Gel	73	0.25	3.75	298
Energy Gel Elite	My Protein	Energy Gel	50	0	0.39	201
Fuel Gel Long Platinose	NutriSport	Energy Gel	54	2	0.17	216
Sprint Gel Instant Energy	NuriSport	Energy Gel	57	0.02	0.03	228

Note: Information available at: https://www.sprintersports.com/pt/; https://www.myprotein.pt/, https://www.decathlon.pt/.

Nowadays there is no energy gel on the market that contains *Chlorella Vulgaris* in its formulation however these microalgae have some great health benefits that would be a great addition to this type of products. Some of these benefits are described below.

4.1.2 Chlorella supplementation

Chlorella is a genus of unicellular green algae, from the Phylum Chlorophyta and there are three species that are cataloged as Novel Food: *Chlorella pyrenoidosa, Chlorella luteovirdis* and *Chlorella vulgaris*, the latter being one of the most commercialized microalgae worldwide. Chlorella genus is widely distributed in freshwater, marine and terrestrial environments, has high photosynthetic capacity and ability to grow rapidly in autotrophic, mixotropic, and heterotrophic conditions (Ru et al., 2020). All these characteristics made it one of the first microalgae considered for large-scale cultivation

and commercial production. These species have a spherical shape with a diameter of 2 to 10 μm and a cellulose cell wall that varies in thickness and composition depending on the growth conditions (Hynstova et al., 2018).

Chlorella vulgaris has health benefits such as helping with disorders such as gastric ulcers, wounds, constipation, anemia, hypertension, and diabetes. (Goiris et al., 2012) (Ru et al., 2020) (Moreira et al., 2018). A recent analysis of *Chlorella* supplementation and cardiovascular risk factors in clinical populations concluded that this microalga presented antihypertensive properties (Fallah et al., 2018) and exhibited vasodilatory potential whereby reductions in arterial stiffness were observed after supplementation periods of 6 g/day for 28 days (Otsuki et al., 2015) (Kim et al., 2013). Employing the same dose and period of supplementation, *Chlorella* supplementation led to significant increases in peak oxygen consumption in young men (Umemoto & Otsuki, 2014), with vasodilation being attributed to one of the possible mechanisms. High bioavailability constituents found in *Chlorella* such as carotenoids (lutein, β-carotene and zeaxanthin), polyunsaturated fatty acids (PUFA), linoleic acid and water-soluble fibers bind to digested fat, increases plasma LDL-C (low-density lipoprotein) elimination, and reduces the absorption of sterols (cholesterol) from the intestine (Fallah et al., 2018) (Serra et al., 2021). A preventive action against atherosclerosis and hypercholesterolemia by glycolipids and phospholipids is also attributed to *Chlorella* intake, as are antitumor actions by glycoproteins, peptides and nucleotides (Yamaguchi, 1997). Another important substance in *Chlorella* is beta1,3-glucan, which is an active immunostimulator, a free-radical scavenger and a reducer of blood lipids (Spolaore et al., 2006).

Honey *Chlorella* is an optimized strain of *chlorella vulgaris*, that is named after its yellow coloration since this strain has low levels of chlorophyll. The lack of chlorophyll makes possible the addition of larger amounts of Honey *Chlorella* to food products for not having an unpleasant taste such as standard *Chlorella vulgaris* (Galante, 2018). With all these properties and characteristics, Honey *chlorella* was the microalga chosen to add in energy hydrogel formulations.

4.2 Materials and Methods

4.2.1 Chemicals

The following chemicals were used: DPPH (2,2-diphenyl-1-picrylhydrazyl), ethanol absolute anhydrous (for analysis-ACS-Reag. Ph.Eur.-Reag. USP) and n-Hexane from Carlo Erba reagents (Rodano, Italy); Maltodextrin from MyProtein (England) bought in Celeiro; Lemon flavor from Condi (Portugal) acquired from Continente (Portugal), and NaCl from Continente (Portugal); Potassium hexacyanoferrate (II) from Chem-Lab (Belgium); 2,6-Dichloroindophenol sodium salt hydrate Powder, D-Glucose anhydrous, Hydrochloric acid S.G. and Sodium hydrogen carbonate from Ficher Scientific U.K.; Locust bean Gum supplied by Industrial Farense, Lda; Potassium sorbate supplied by Lusifar - Químico Comercial, Lda (Portugal); Methanol from Millipore Corporation (Germany); Citric acid from Naturitas (Portugal); Kjeldahl tablets, L(+)-Ascorbic Acid, Phenol crystallized (detached crystals), Silicone antifoaming liquid and technical grade Zinc Acetate 2- hydrate from Panreac Applichem (Barcelona, ES); Folin & Ciocalteu's phenol reagent, sodium carbonate anhydrous, crystal violet and trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) from Sigma-aldaich (St. Louis, USA); Sulphuric acid 95% from VWR Chemicals (France) and Water was purified by Milli-Q purification system.

For the medium preparation: Bacteriological Agar (type E), Peptone, Yeast Extract, and Trypto-casein soy broth (TSB) from Bioakar Diagnostics (Allonne, France).

Microalgae: Honey *Chlorella vulgaris* spray-dried powder supplied from Allmicroalgae - Natural Products, S.A.

4.2.2 Otimization of the gel formulation

4.2.2.1 Viscosity determinations

All the viscosity determinations were performed by using the AMETEK Brookfield viscometer. This was done by dipping the spindle into the gel preparation assessing the viscosity for different rotation speeds. All samples were measured in 50 mL plastic tubes in an incubator at 25°C. Then viscosity and shear stress determinations were made for each sample measured.

Viscosity determination of LBG

For the formulation of gels containing Honey Chlorella, the first step was to choose the thickening agent, locust bean gum (LBG) was chosen to enhance a product produced nationally. Different LBG aqueous solutions were prepared, in deionized water, initially with concentrations of 0.4%, 0.6% and 1% to evaluate the obtained viscosity, these percentages were chosen according to the limits establish by EFSA (EFSA et al., 2017) and the solutions were prepared according to the literature, (Dakia et al., 2008) (Coviello et al., 2007) by being heated up at 80ºC for 30 min, under stirring to reach the maximum solubility of the LBG as its seen in the scheme of the Figure 4.1.

Viscosity determination of commercial energy gel products

Viscosity determination of two commercial energy gel product was performed to use as a baseline to choose the ideal percentage of the LBG to use in the gel formulations. The two commercial gels chosen were the most and the least thick of the gels purchased.

4.2.2.2 Addiction of Honey Chlorella

After deciding the concentration of LBG and maltodextrin, the addition of chlorella Honey to the produced hydrogel formulations was then performed by incorporating the chlorella powder already in the hydrogel solution (Figure 4.1) after heating and cooling it. Concentrations of 2%, 4% and 6% (w/v) were tested.

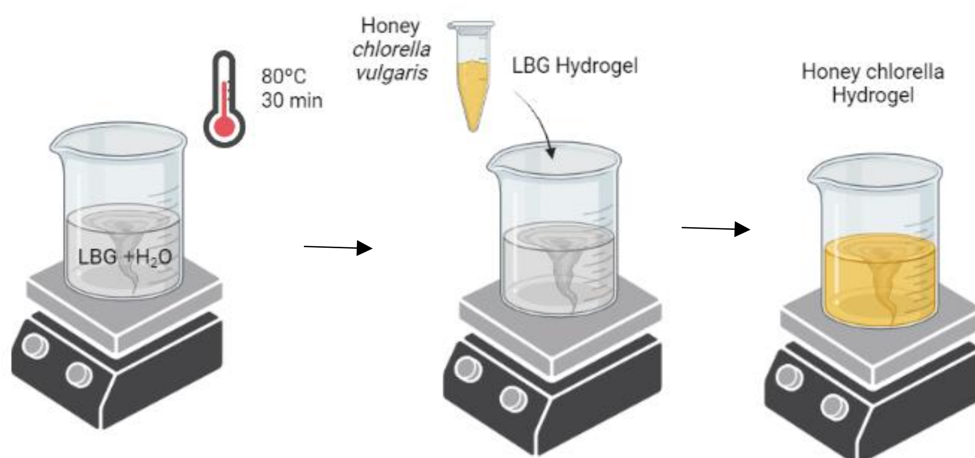


Figure 4.1: Scheme of the LBG hydrogel preparation and incorporation of Honey chlorella vulgaris in the hydrogel. Created in Biorender.com.

4.2.2.3 Stability evaluation

For the stability evaluation the gels were kept at 2 different storage conditions: room temperature (RT) and refrigerated (4 ºC) and were covered in aluminum foil to avoid light. Weekly measurements were performed for two weeks (Control, week 1 and week 2) where organoleptic properties were evaluated by observing the changes in color, odor and overall state. Microbiological assays were also carried out spreading the gels in plaques with Tryptic Soy Agar (TSA) and Glucose Peptone Yeast Extract Agar (GPYA) medium and letting them incubate at 37 ºC for 48 h to detect if there was any bacterial or fungi growth and therefore, to determine whether the addition of a preservative would be necessary. pH and viscosity determinations were also carried out to further see how the different storage conditions affected the gels. All these measurements are summarized in Figure 4.2.

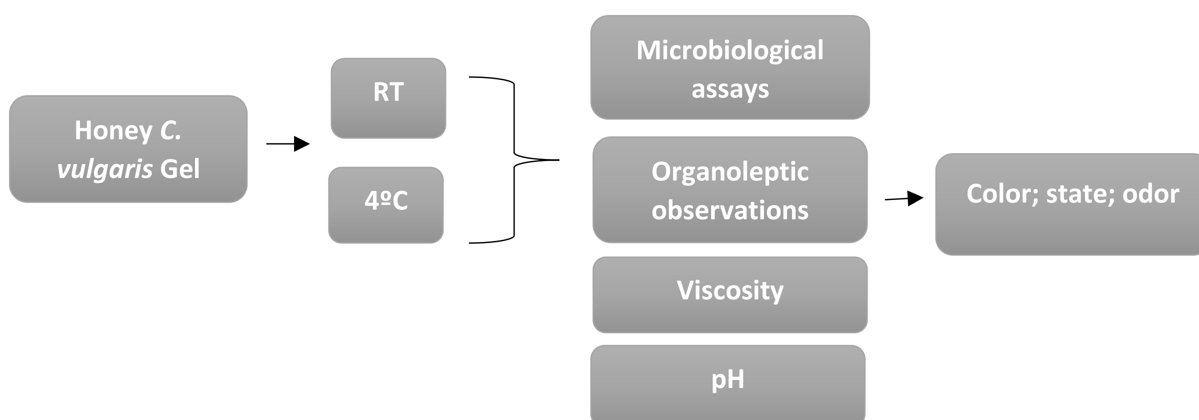


Figure 4.2: Scheme of the stability evaluation of the Honey C. vulgaris hydrogels.

4.2.3 Sensory analysis

A sensorial analysis study of the gels (one from the market and the two formulations 1.5% and 4%) was carried out in order to evaluate the impact of microalgal biomass incorporation on the sensorial performance. An untrained panel of 20 individuals tested gels, for four sensory attributes: color, odor, flavor and texture on a structure and discontinuous scale, ranging from 1 to 6. The panel consisted of 7 men and 14 women aged between 19 and 62 years. Each gel formulation was made and served immediately in plastic cups. Samples were served in random order to the panelists who were asked to taste samples and rate the attributes with score 1 being not pleasant at all, 2 somewhat pleasant, 3 fair, 4 pleasant, 5 quite pleasant and 6 excellent. Panelists were also asked to select what words would describe each sensory attribute. In relation to taste they could choose sweet, sour, salty, bitter, and umami, in relation to the smell, citrus, fragrant, woody, chemical, sweet, lemon, rotten, and spicy and finally in relation to the texture, viscous, thick, thin, sticky, velvety, gummy, elastic, and soft. The participants could choose more than one word to describe each attribute (Annex 1).

4.2.4 pH Determination

The pH was determined by using digital pH meter & ion-meter (Crison Ph Meter Glp 22). The glass electrode was calibrated with two solutions with the pH of 4 and 7 determined for the equipment (pH of 4.00 and 7.00). The solutions were then measured. The calculated sensibility was 95.5 %, the asymmetry potential was 10.5 mV, the slope was 55.9 mV/pH and the temperature was 22 ºC.

4.2.5 Nutritional Value of the Honey *Chlorella* gels

4.2.5.1 Samples preparation

Three samples of 100 g were tested: **Blank**: the formulation of the gel with all components except the microalga (blank formulation); **1.5%HC**: formulation of the gel containing 1.5% of Chlorella Honey; and **4% HC**: formulation of the gel containing 4% of Honey *C. vulgaris*. The goal of these analyzes was to compare the nutritional content

of the gel with chlorella and without *Chlorella* in order to explore its nutritional benefits and establish the label of the final product, quantifying all its components.

4.2.5.2 Determination of dry residue and Humidity %

The dry residue (DR) content of the samples was determined by the oven drying method (ISO, 1997). Porcelain capsules with sand were first placed to dry in the air circulation oven (air circulating oven (J.P. Selecta, Barcelona, ES)) at 100 ºC for 1 h. After being cooled in the desiccator, the capsules were weighed with the sand (Areia do Mar Lavada, fine grain José Manuel dos Santos, LDA) and glass rod on a digital scale (Mettler Toledo AB204-S classic precision balance), then 3 g of the samples were weight in the capsules, which were then placed to dry in the oven at 100 ºC for approximately 5 h. Finally, after cooling in the desiccator (Borosilicate glass desiccators), the capsules with the dry sample were weighed again.

The dry residue content of the samples was calculated by Equation (1):

$$\text{DR (g/100 g):} = \frac{(\text{C-A})}{(\text{B-A})} \times 100 \qquad \textbf{(Equation 1)}$$

Where, A= weight of the porcelain capsule + glass rod (g); B = weight of the porcelain dish + glass rod + fresh sample (g); C= weight of the capsule + glass rod + sample after drying (g).

The amount of water was calculated by Equation (2):

$$\text{Humidity (g/100 g)} = 100 - \text{DR} \qquad \textbf{(Equation 2)}$$

Samples were analyzed in triplicate and results are presented as mean ± standard deviation.

4.2.5.3 Determination of Ash %

The total ash of the gel samples was determined by gravimetric method at 550 ºC, according to the method ISO 936:1998 (ISO, 1998). Where 25 g of sample were first weighed in porcelain capsules, previously heated in a 103 °C air circulation oven (air circulating oven (J.P. Selecta, Barcelona, ES)) and weighed on a digital scale (Mettler Toledo AB204-S classic precision balance), after that the sample was heated on a hot plaque (Hot plaque Combimax mod. 230 A J.P. Selecta, Barcelona, ES) to remove the moisture. After that, the samples were carbonized with the aid of a gas bottle, and after they stopped releasing fumes, they were placed in a muffle furnace (Heraeus Muffle furnace MR 170E. 1000 °C. 220 V. 3 kW) at 550 °C for one hour. After incineration, the ashes were kept in the desiccator (Borosilicate glass desiccators) until reaching room temperature. The ashes were then dissolved in 5 mL of sulfuric acid and homogenized with a glass rod, the solution was then poured into a 100 mL flask and water was added to it to make 100 mL. The samples were then placed in tubes to be sent to EPAL to analyze their mineral content (Section 4.2.5.9).

4.2.5.4 Determination of the protein content

The total nitrogen content in the sample was determined by the Kjeldahl method (Official methods of Analysis, 2000). In a Kjeldahl tube were placed 5 g of sample, 20 mL of distilled H_2O, 20 mL of H_2SO_4 and 4 g of $CuSO_4$ and a few drops of antifoam solution. The sample was digested in a digestion device (P Selecta Bloc Digest 6) which heats up to 380 °C in several heating cycles. Subsequently, it was placed in a distillation device with 50 mL of boric acid + indicator. Then, 50 mL of boric acid indicator solution was added to the distillate. Finally, ammonium borate stock solution was titrated with H_2SO_4 (0.1 N), and total protein was calculated by multiplying total nitrogen (N) by the conventional conversion factor of 6.25 (Jones, 1931).

$$\%\mathrm{N} = \frac{0.14 \times (V_1 - V_0)}{m} \quad \textbf{(Equation 3)}$$

Where V_1 = Volume of H_2SO_4 0.1N spent in the titration in each sample; V_0 = Volume H_2SO_4 0.1N spent on blank titration; m = mass of samples (g).

4.2.5.6 Determination of lipids

For the determination of lipids, the extraction flasks were first dried with the boiling controllers in the air circulating oven (air circulating oven J.P. Selecta, Barcelona, ES) regulated at 103 ºC ± 2 ºC. In Erlenmeyer flasks, 10 g of sample were weighed (Mettler Toledo AB204-S classic precision balance) to which 150 mL of H_2O, 50 mL of 4N hydrochloric acid (100 mL of concentrated hydrochloric acid + 200 mL of H_2O) were added. Then the Erlenmeyer's were placed on hot plaques (Hot plaque Combimax mod. 230 A J.P. Selecta, Barcelona, ES) and allowed to boil for 15 min. After boiling, the solutions were filtered on a filter paper, the flasks were washed 3 times and the papers were placed in petri dishes to be dried in the oven at 103 °C ± 2 °C until well dried. After cooling, the papers were placed in extraction thimbles (Filter-LAB cellulose extraction thimbles) which were placed in the extraction flasks, filled with the solvent, in this case n-hexane, and covered with cotton. After that they were placed in a manual extractor Soxhlet for 4 h until the extraction was complete.

4.2.5.7 Total carbohydrates

The carbohydrate total of the gel samples was determined by calculation, i.e. subtracting from the dry matter value the amounts of, protein, and ash content (Shafakatullah and Krishnamoorthy, 2014).

The total carbohydrates were calculated by Equation (4):

$$\text{Carbohydrates (g/100 g)} = DM - P - A - F \quad \textbf{(Equation 4)}$$

Where DM is total dry matter (%), P is protein content (%), A is ash content (%), and F is total fat or lipid content (%).

4.2.5.8 Determination of total and reducing sugars by the phenol sulfuric acid method (PSA)

To determine the total and reducing sugars present in the sample, the phenol sulfuric acid method (PSA) (Suzanne et. al, 2010), was used, which consists of the dehydration of sugars in a concentrated acid medium and consequent formation of a complex with phenol. According to this method, simple or complex sugars and their

derivatives, when treated with phenol and concentrated sulfuric acid, turn the solution into a yellow-orange coloration, keeping this color stable. The colored sample absorbance is measured in a spectrophotometer and compared to a calibration curve, in order to present reach the concentration of total sugars.

For this method, firstly, several solutions were prepared: potassium ferrocyanide solution with a concentration of 172 g/L; zinc acetate solution with a concentration of 360 g/L, 5 % phenolic solution (w/v) and finally the D-glucose standard solution, 100 mg/L. The D-glucose control standard solution was also prepared from the last solution, 3 mg/L.

For sample preparation, 5 g were diluted by adding hot water and transferred to 100 mL flasks, filling the rest with distilled water. After dilution, proteins were precipitated in 100 mL flasks, each flask took 20 mL of the diluted sample, 2 mL of the potassium ferrocyanide solution and 2 mL of the zinc acetate solution, completing the volume with demineralized water. The solution was then filtered through filter paper.

After protein precipitation, the inversion was performed, where 50 mL of the precipitate was transferred to a 200 mL flask, 0.5 mL of concentrated HCl was added and, after stirring, it was placed in a water bath for 30 min, allowed to cool and with distilled water the flask was made up to volume and stirred.

In 10 mL glass tubes, 1 mL of each sample diluted 1:5 previously and 1 mL of the phenol solution were measured. 5 mL of concentrated sulfuric acid was added and allowed to cool in an ice bath. The ascorbic acid calibration curve was prepared according to the procedure described above, replacing the sample with the standard, using a concentration range that varied from 0-100 mg glucose/L After cooling, the solutions were read in the spectrophotometer with the absorbances at 490 mm against white.

The glucose concentration in the samples (mg/100 g) after treatment, total sugars was calculated using Equation 5:

$$\text{mg glucose/100 g} = 2000 \times (C/m) \qquad \textbf{(Equation 5)}$$

Where C= concentration obtained directly through the calibration line (mg/L) m= mass of sample for analysis (g).

4.2.5.9. Mineral Content

All these analysis of the mineral content of the formulations were done at EPAL and all the methodologies described below were done according to Caetano et al., (2021).

The trace minerals were determined by inductively coupled plasma–mass spectrometry (ICP-MS), some metals were also determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES), also referred to as inductively coupled plasma optical emission spectrometry (ICP-OES), namely barium, potassium, sodium, , and manganese. Mineral stock solutions at 1000 mg/L for calibration (Specpure, Alfa Aesar) were used. The quantification of cobalt, cadmium, molybdenum, nickel, selenium, silver, tin, arsenic, beryllium, thallium, and vanadium was carried out by ICPMS using a Analytik Jena Plasma Quant MS quadrupole ICP-MS, equipped with a standard quartz concentric nebulizer (0,4 mL/min), a quartz Scott spray chamber and a CETAC ASX-520 autosampler. Cadmium, cobalt, nickel, and vanadium were read in collision/reaction mode using helium with a flux of 120 mL/min. Two certified reference materials (CRM) were used for quality control on two natural waters, TM-35 (trace metal fortified sample, Environmental and Climate Change Canada) and SRM 1640a, (National Institute of Standards & Technology). Quantification of, barium, potassium, and manganese was carried out by ICP-OES using a Thermo iCAP 76000 DUO ICP-OES equipped with a standard quartz concentric nebulizer (35PSI), a quartz cyclonic spray chamber and a CETAC ASX-560 autosampler. The analysis was performed with a RF power of 1300 W, nebulizer gas pressure between 180 kPa to 250 kPa, integration time for axial UV of 25 s, axial visible region of 25 s, and radial visible region of 10 s. According to mineral working range, appropriated dilutions were performed to obtained two target standard controls with concentrations equivalent to the first and the last concentration level of linear range.

Some macrominerals were also determined by titrimetric and molecular absorption spectrophotometric methods. The calcium content was determined by complexation volumetric analysis. Results were expressed in mg/100 g. The magnesium content was also determined by complexation volumetric analysis. Considering

phosphorus occurs in organic compounds, a digestion or calcination method must be performed to oxidize organic matter effectively to release phosphorus. After that, the colorimetric determination of total phosphorus in the ashes was applied. Ten phosphorus standards solutions with concentrations between 0.03 and 0.50 mg/L were prepared in ultra-pure water for the calibration curve. The mass of iron, in milligrams, present in a 15 g sample of each sample was determined by the orange-red color of the complex between iron (II) and o-phenantroline.

4.2.6 Total phenolic content determination and antioxidant activity of the Honey *Chlorella* extract and hydrogel formulations

4.2.6.1 Samples preparation

Determination of the phenolic compounds was performed on the Honey *Chlorella* extract and hydrogel formulations, the determination of antioxidant activity was only performed on the extract.

4.2.6.2 Extraction method

The extraction of the active compounds from Honey *C. vulgaris* powder was performed from an adaptation on the method described by Severo et al., (2021). Firstly, an ethanol/ H_2O 50:50 solution was made and 0.01 mL of HCl (0.01% V/V HCl) was added to the solution, then 3 g of the powder was weighed, and 10 mL of the previously prepared solvent was added and homogenize for 1 min. After that, the samples were sonicated (J.P. Selecta) for 40 min with occasional shaking and vortexed for 2 min. The resulting suspensions were centrifuged at 40000 rpm for 8 min. Samples were extracted twice and the solvent was evaporated from the extracts in a rotary evaporator (IKA HB 10 digital) at 40 ºC. Finally, the resulting concentrates were dried overnight using a vacuum pump (Vacuubrand) and stored at -20 ºC until use for analysis.

The extraction yield of the extraction method used was determined according to the following formula:

$$\text{Extraction Yield (\%)} = \frac{DS\ (g)}{IS\ (g)} \times 100 \qquad \textbf{(Equation 7)}$$

Where, DS= dried sample extract mass (g); IS = Initial sample mass (g).

4.2.6.3 Total phenolic content determination of the extract and gels

The total phenolic content was determined based on a previously described method with slightly modifications (Severo et al., 2021). A Gallic acid (GA) stock solution (1 mg/mL) was first prepared in methanol/distilled water (8:2 V/V). A 0.1 mg/mL GA solution was prepared by dilution from the stock solution in methanol/distilled water (8:2 V/V) and used to obtain different GA solutions for a calibration curve with concentrations ranging from 0.002 to 0.01 mg/mL. These solutions were prepared by adding 0.2, 0.4, 0.6, 0.8 and 1 mL of the 0.1 mg/mL GA solution to a 10 mL volumetric flask and complete the volume with distilled water to a 96-well microplate, it was added 262.5 µL of each calibration curve solution and of each extracted sample. For the reaction, 7 µL of Folin & Ciocalteu's reagent was added and after 3 min, wells were supplemented with 30 µL sodium carbonate aqueous solution (35% w/V).

The microplate remained in the dark for further 60 min and then the absorbance was measured at 725 nm with a microplate absorbance reader (SPECTROstar Omega from BMG Labtech). For this analysis a blank was also prepared by replacing samples with distilled water. All the measurements were carried out in triplicate. The total phenolic content was calculated with the linear function obtained from the calibration curve and the results were expressed as mg of Gallic acid equivalents (GA eq)/ g of extract.

4.2.6.4 DPPH radical scavenging activity of the extract

The free radical scavenging activity was measured against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. In the presence of hydrogen donors DPPH protonates, changing its purple color to yellow and allowing to quantity the reaction by measuring

the absorbance in a 96-well plate, 180 µL of a DPPH solution (0.06 mg/mL) was added to 20 µL of each sample dilution. The plaque was incubated in the dark for 1h min at room temperature, the absorbance was measured at 517 nm (SPECTROSTAR Omega microplate reader). Trolox was used as positive control and different Trolox solutions with concentrations ranging from 0.01 to 0.4 mg/mL were prepared from a trolox stock solution (0.13 mg/mL). The negative control was prepared with 200 µL of the solvent and the blank with 20 µL of the solvent and 180 µL of the DPPH solution. All solutions were prepared in methanol/distilled water (8:2 V/V) and the assays were performed in triplicate. Samples were also measured in the absence of DPPH radical and the value deducted from the absorbance measured with the DPPH radical.

The following equation was used to calculate the % DPPH quenched:

$$\text{\% DPPH quenched} = \left(\frac{A_{Blank} - A_{Sample}}{A_{Blank}}\right) \times 100 \qquad \textbf{(Equation 8)}$$

Where A_{blank} represents the absorbance of the blank and A_{sample} represents the absorbance of the sample.

4.2.6.5 Statistical analysis

The statistical analysis regarding the sensorial evaluation, between the different samples was performed by applying the unpaired Student's t-test. The statistical significance was set at p values <0.05 with a 95% confidence interval. Statistical analysis was performed using GraphPad Prism 5 software v.5.03 (GraphPad Software, San Diego, California, USA).

4.3 Results and discussion

4.3.1 Optimization of the gel formulations

The first step to choose the optimal percentage of the thickening agent (locust beam gum (LBG)) to use in the gels, was to make a visual evaluation of the thickness of the chosen percentages (0.4 %, 0.6 % and 1 % w/v). These results showed that as the concentration of LBG increases, thickness increases accordingly (Figure 4.3). Studies have shown that LBG can be used at very low concentrations giving high viscosities at concentrations below 1% (Dakia et al., 2008 and Lv et al., 2019). However, at the concentration of 0.4% the gel did not present the thickness intended for the final product.

It was then decided to measure only the viscosity of the gels at 0.6 % and 1 % concentration of LBG to further evaluate the best concentration to choose. The results of this viscosity measurements are in the Figure 4.4, and show that in relation to the gels behavior, the gel at 0.6% behaves like a Newtonian fluid as well as the two commercial energy gels. A Newtonian fluid is defined as a fluid whose viscosity does not change with the rate of deformation or shear strain (Campus, 2004). Studies show that LBG in an aqueous phase exhibits a non-Newtonian flow, i.e., shear thinning or pseudoplastic steady flow behavior at high shear rates, while at low shear rates it exhibits a Newtonian flow (Dakia et al., 2008) these results are in accordance with the results obtained in this work. This is due to the fact that new polymeric chains start to form after the breakage of older ones upon low shear force, so the equilibrium between them is maintained hence the viscosity of LBG remains constant (Dakia et al., 2008). The gel at 1% behaved like a shear-thinning fluid where the viscosity decreased as the shear rate increased. The gel at 0.6% LBG showed lower viscosity values than those of the gel with 1% and in all shear strain range. For instance at 0.1 s^{-1} the 0.6 % LBG gel had a viscosity of 0.46 Pa.s while the gel at 1% presented a viscosity of 3.47 Pa.s. The viscosity of the commercial energy gels A and B (Figure 4.6) presented values at 0.10 s-of 0.25 Pa.s and 1.96 Pa.s for gel A and B respectively.

Comparing the results from Figure 4.4 with the results for commercial energy gels (Figure 4.6), it was concluded that a concentration of 0.6% LBG would be the best choice for the intended hydrogel formulation.

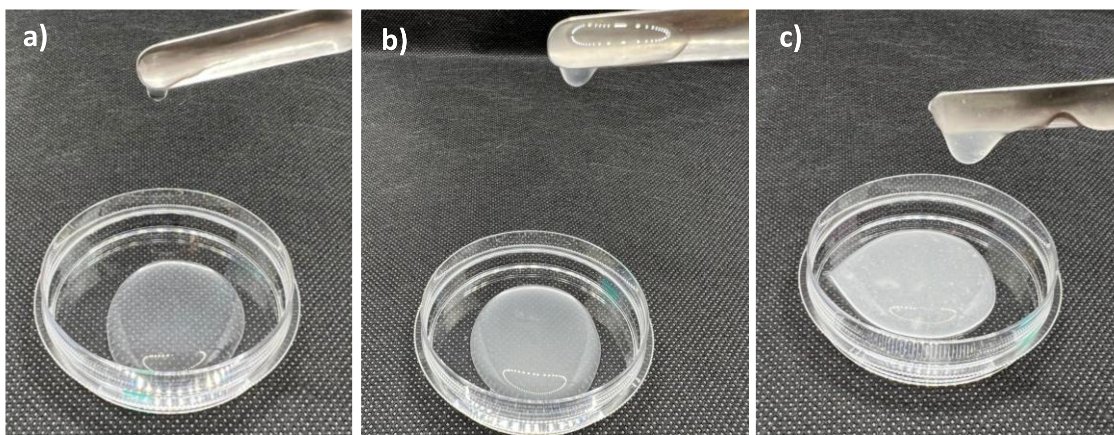


Figure 4.3: Different hydrogel formulations, a) locust bean gum hydrogel 0.4% (w/v), b) locust bean gum hydrogel 0.6% (w/v) and c) locust bean gum hydrogel 1% (w/v).

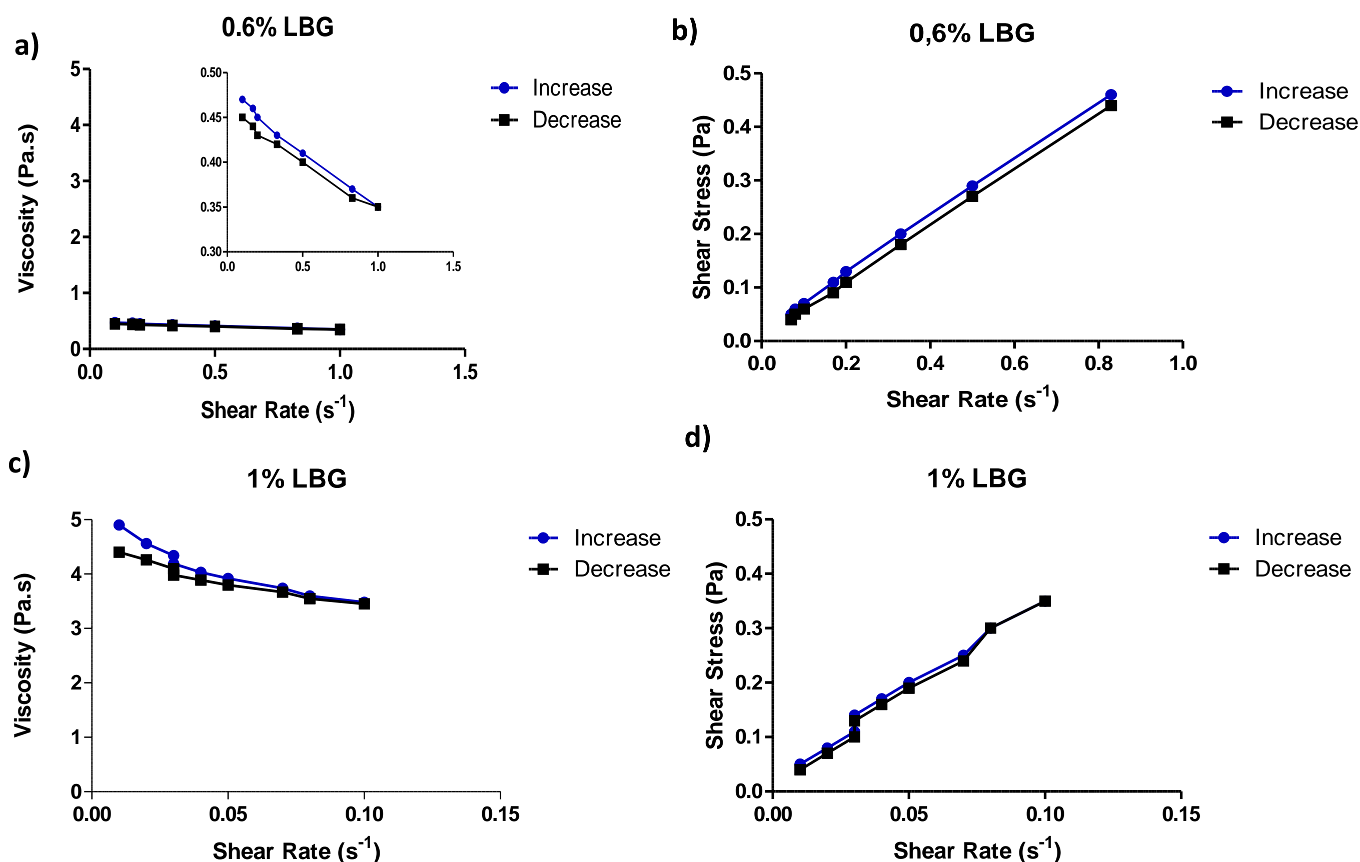


Figure 4.4: Viscosity vs shear rate and shear stress vs shear rate graphs from hydrogel formulations being a) Viscosity vs shear rate graph of 0.6% (w/v) LBG hydrogel, b) Shear stress vs shear rate graph of 0.6% (w/v) LBG hydrogel, c) Viscosity vs shear rate graph of 1% (w/v) LBG hydrogel and d) Shear stress vs shear rate graph of 1% (w/v) LBG hydrogel.

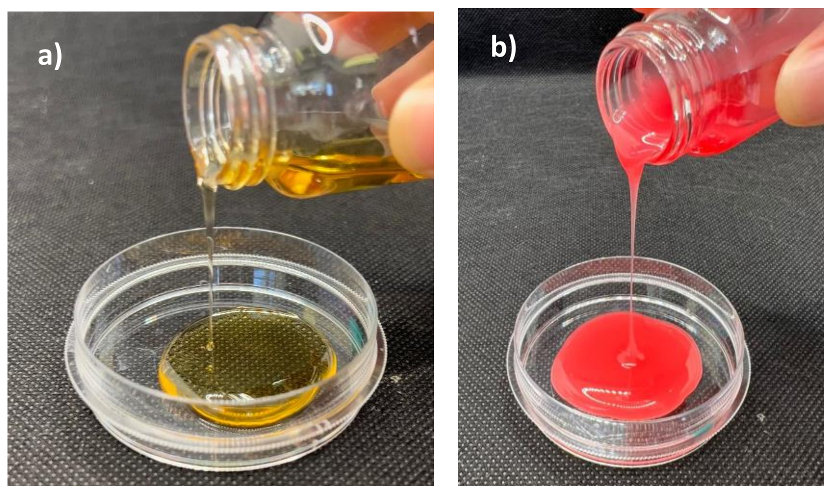


Figure 4.5: Commercial energy gel formulations a) Commercial energy gel A, b) commercial energy gel B.

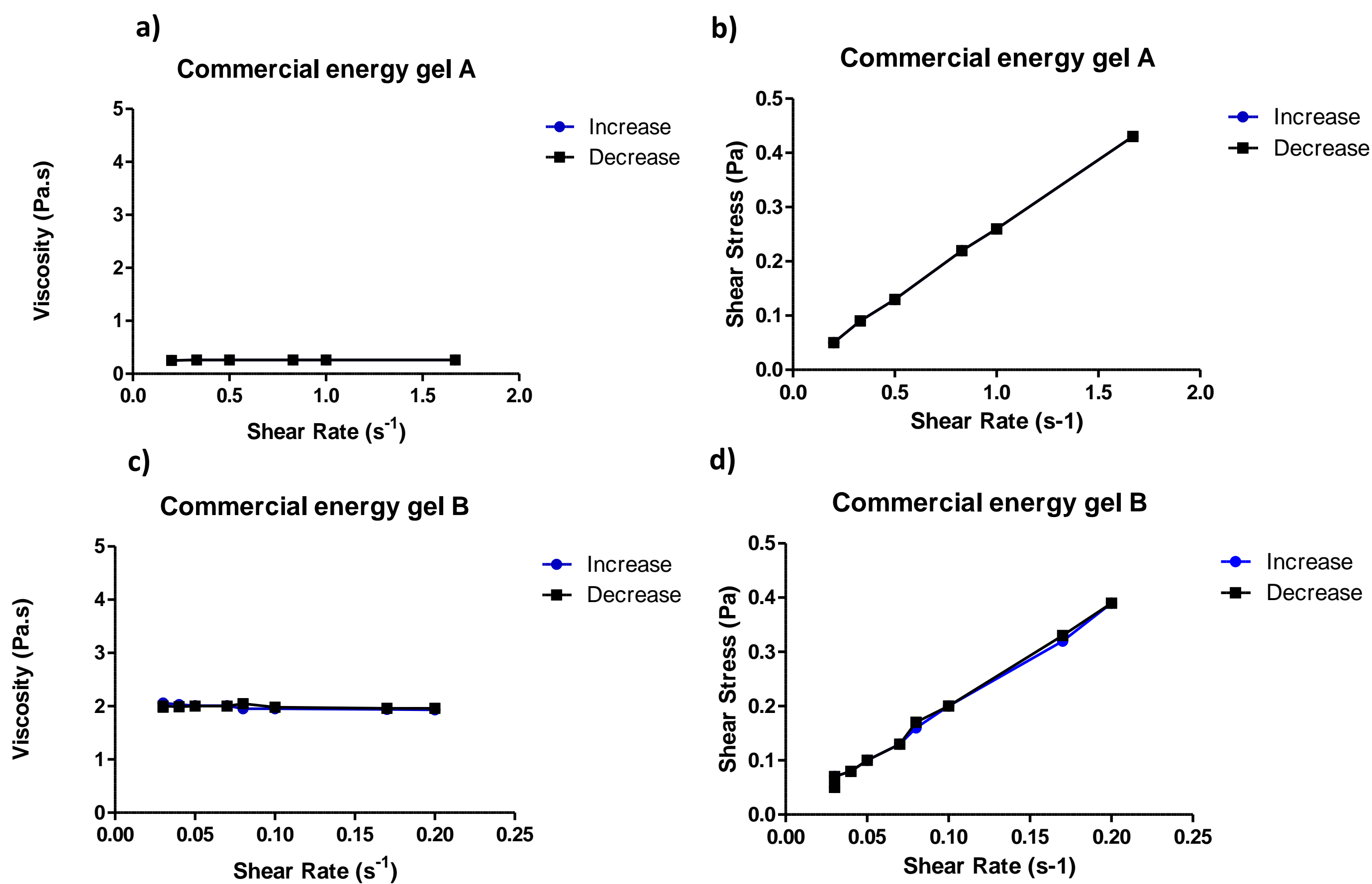


Figure 4.6: Viscosity vs shear rate and shear stress vs shear rate graphs from two commercial energy gels being a) Viscosity vs shear rate graph of commercial energy gel A, b) Shear Stress vs shear rate graph of commercial energy gel A, c) Viscosity vs shear rate graph of commercial energy gel B and c) shear stress vs shear rate graph of commercial energy gel B.

After deciding the ideal percentage of LBG to use, the next step was to choose the main source of carbohydrates. In a gel for athletes, a source of carbohydrates is essential to give the energy boost that athletes need during physical exercise. Carbohydrate ingestion at frequent intervals, is essential to maintain plasma glucose concentrations and support performance, this last one through a number of mechanisms including glycogen preservation, increased total carbohydrate oxidation rates (CHO_{TOT}), lowered subjective perception of fatigue and prevention of hypoglycemia (Roberts et al., 2014).

The Carbohydrate chosen was maltodextrin that is an easily absorbed complex carbohydrate with little taste or sweetness. Maltodextrin has high glycemic Index of 90-110. The Glycemic Index (GI) is the measure of the glycemic response (the extent to which blood glucose levels rise) obtained after the ingestion of a carbohydrate-containing food (Malfatti et al., 2011). The percentage chosen was 50% (w/v) based on the Table 4.8 that shows the composition of some energy gels presently sold in the market and according to (Malfatti et al., 2011). After deciding the carbohydrate and percentage to use and having a hydrogel formulation with 50% of maltodextrin, 0.6% of LBG, the Honey *C. vulgaris* was also added.

Regarding the addition of Honey *C. vulgaris* in the LBG gels, the organoleptic parameters namely their color, odor, homogeneity showed that with the increase of the percentage of this microalga, the color was more intense (Figure 4.7), as well as the odor. In order for this powder to incorporate well into the gel, it needed to be stirred very well. During this phase the focus was that the nutritional value of the final formulation, so a percentage of 4% w/v of honey *C. vulgaris* was chosen. This decision also took into account the percentages of microalgae usually used in food products that varied between 0.5% and 7% (w/v) as we can see in the Table 1.3.

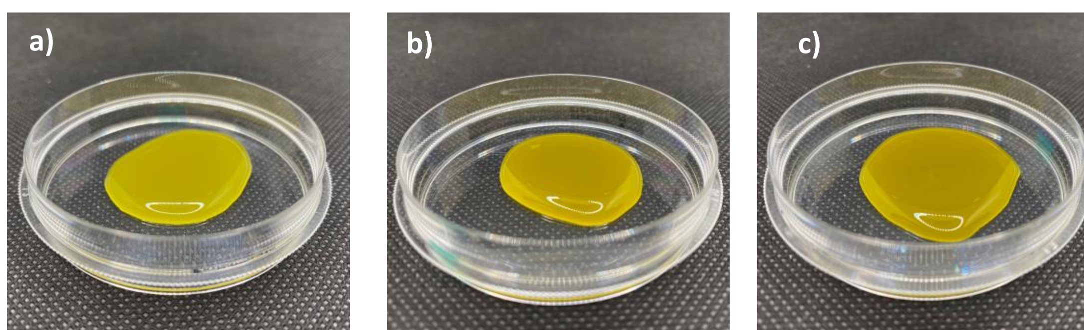


Figure 4.7: Honey Chlorella vulgaris in hydrogels a) LBG hydrogel with 2% (w/v) of Honey C. vulgaris, b) LBG hydrogel with 4% (w/v) of Honey C. vulgaris and c) LBG hydrogel with 6% (w/v) of Honey C. vulgaris.

4.3.1.1 Stability evaluation of the gels

In this phase of the study the gels had only the microalga, thickness agent and the carbohydrate source, and to see if there was a need of a preserving agent a stability assessment was made taking into count two storage conditions, at 4 ºC and at room temperature (RT). These results are shown on the Table 4.2, Table 4.3 and Table 4.4. It is possible to see that in relation to color, the gel kept in the refrigerator always kept the same color in the first two weeks while the gel kept at room temperature became browner and less vibrant green. Regarding the odor, the gel kept in the fridge kept its typical smell after 2 weeks, while the gel kept at room temperature in the second week gained an atypical odor indicating that it was no longer be good for consumption (Table 4.4). Both the gels kept in the fridge and the gel kept out, over time, became less homogeneous, with a slight sedimentation of the microalgae being noted at the bottom of the container, a solution to this problem is that the final product would have to have a note saying to shake well before consumption.

The pH values of the fresh formulations were 6.29 ± 0.02. The behavior during storage varied on the gels depending on the storage condition. The gels stored at RT after two weeks had a pH of 5.14 ± 0.03 and the gels stored at 4 ºC had a pH of 5.65 ± 0.02. Viscosity values remained constant for all formulations over the observation period as it is possible to observe in Figure 4.9.

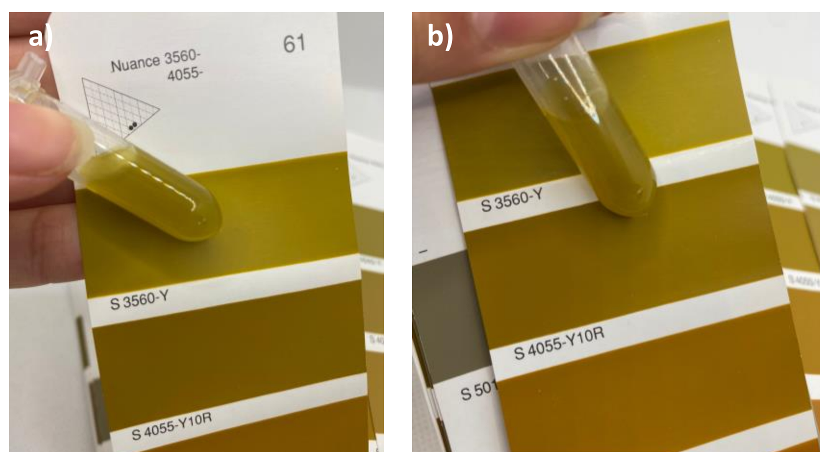


Figure 4.8: Comparison of the coloration of the gels on a color scale being a) Comparison of the gel stored at 4ºC for 2 weeks with a color scale, and b) Comparison of the gel stored at RT (room temperature) for 2 weeks with a color scale (NCS – Natural Colour System®).

Table 4.2: Results of the organoleptic evaluation as well as homogeneity and pH on freshly made hydrogels (Control).

Evaluated properties	Formulation
State **Color** **Odor**	Gel S 3560-Y Typical
Homogeneity	Homogeneous
pH	6.29 ± 0.02

Table 4.3: Results of the organoleptic evaluation as well as homogeneity and pH on hydrogels 1 week of storage.

Evaluated properties	Formulation and conditions	
	Room T	Fridge
State **Color** **Odor**	Gel Between S 3560-Y and S 4055-Y Typical	Gel S 3560-Y Typical
Homogeneity	Heterogeneous	Heterogeneous
pH	5.35 ± 0.01	5.73 ± 0.03

Table 4.4: Results of the organoleptic evaluation as well as homogeneity and pH on hydrogels 2 weeks of storage.

Evaluated properties	Formulation and conditions	
	Room T	Fridge
State **Color** **Odor**	Gel S 4055-Y Atypical	Gel S 3560-Y Typical
Homogeneity	Heterogeneous	Heterogeneous
pH	5.14 ± 0.03	5.65 ± 0.02

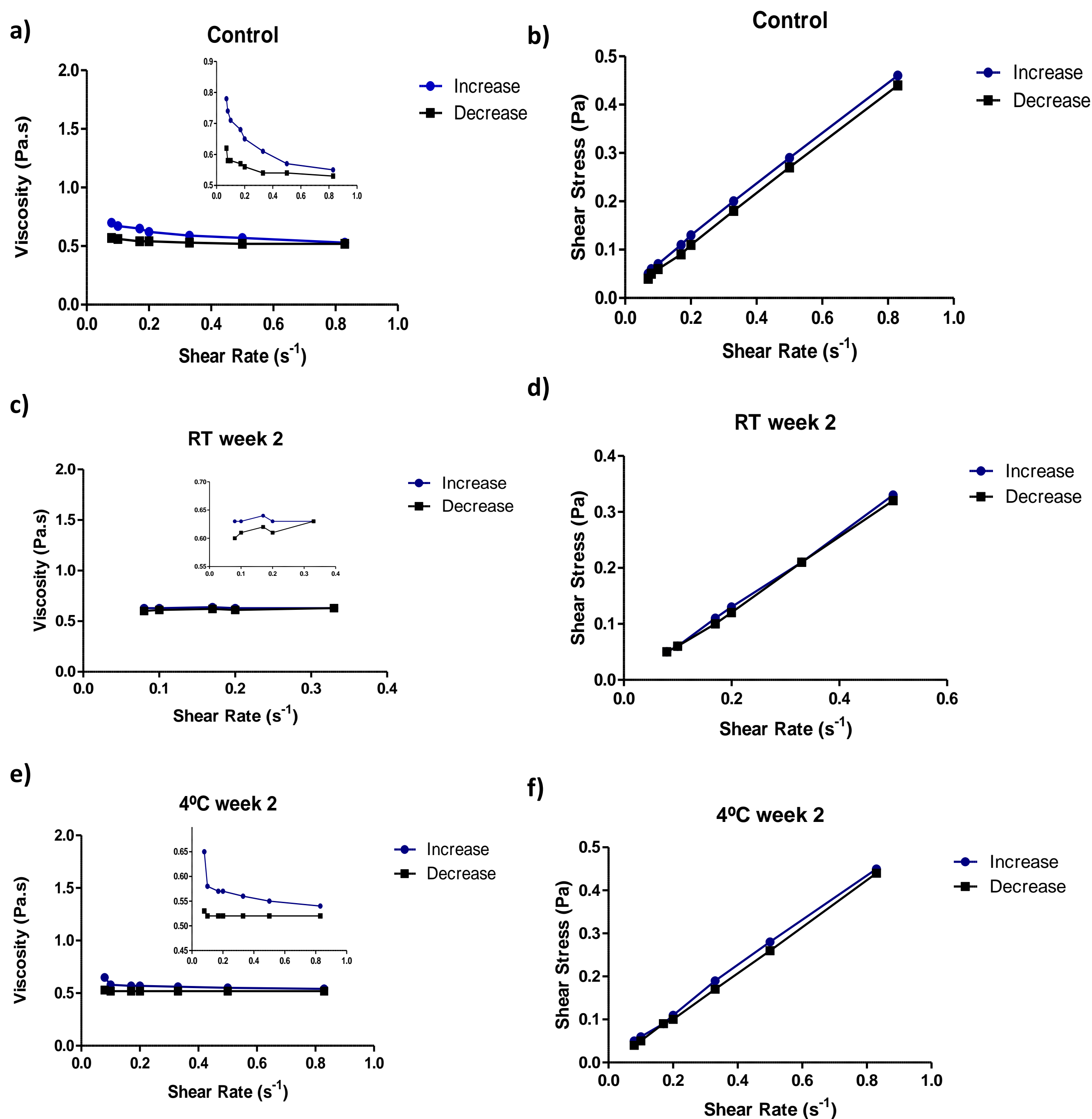


Figure 4.9: Viscosity vs shear rate and shear stress vs shear rate graphs from gels stored in different conditions being a) Viscosity vs shear rate graph of control (freshly made gel), b) Shear Stress vs shear rate graph of control (freshly made gel), c) Viscosity vs shear rate graph of RT (room temperature) gel after 2 weeks of storage d) shear stress vs shear rate graph of RT (room temperature) gel after 2 weeks of storage, d) Viscosity vs shear rate graph of 4 ºC gel after 2 weeks of storage e) shear stress vs shear rate graph of 4 ºC gel after 2 weeks of storage.

Microbiological assays were performed on the freshly made gels and weekly on the same gels stored at RT and 4 ºC. These tests were carried on TSA plates to detect the growth of bacteria and on GPYA plates to detect the growth of fungi, to see if they were good for consumption.

These results showed that regarding the control (freshly made gels), only 1 colony of bacteria was counted, which was probably the result of contamination in the handling of the plates and there was no fungal contamination. After 1 week the gels stored at 4 ºC did not show any type of bacterial or fungal contamination, as well as the tests carried out 2 weeks after storage at 4 ºC (Figure 4.10). This demonstrated conservation of the gels, with no sign of contamination. However, the gels stored outside the refrigerator (RT), 1 week later already had colonies of bacteria but not fungi. These results demonstrated that gel stored at RT spoiled more quickly than gel stored at 4ºC, and that it was most beneficial for the product to add a preserving agent to its final formulation to prolong its shelf life. The cause of the spoilage of the gel formulations can be the result of a succession of enzymatic reactions originating from spoiling microorganisms or from the food matrix itself. It can also be associated with non-enzymatic reactions, physical or chemical damage (Sevindik & Uysal, 2021).

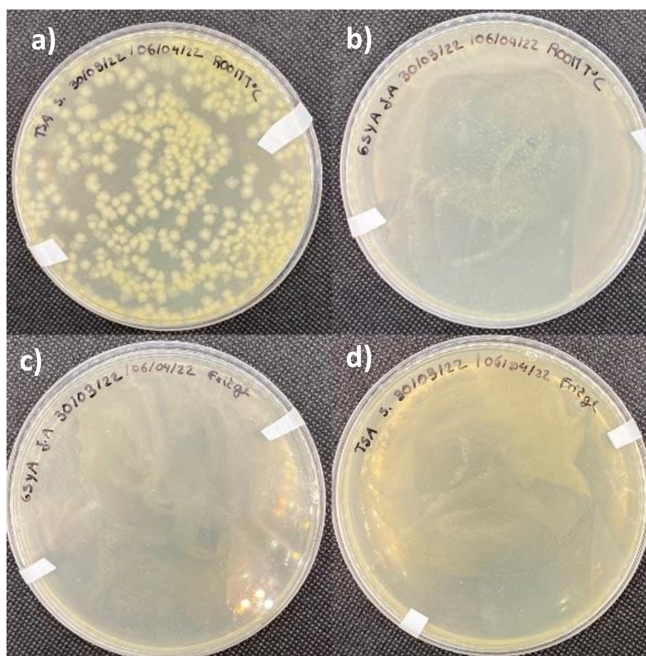


Figure 4.10: Results of the microbiology essays of the gels 2 weeks after being given storage conditions (4ºC and Room Temperature) being a) Bacterial growth and b), c) and d) clean plates showing no signs of fungi or bacterial growth

4.3.2 Final formulations

After the preliminary studies mentioned above, and with the percentages of the thickening agent (LBG), microalgae (honey *C. vulgaris)* and carbohydrates (maltodextrin) chosen, the last components were added to the final formulations. Those components were, a preserving agent (potassium sorbate), a source of electrolytes (NaCl), a pH balancer (citric acid) and an aroma (lemon flavor).

The potassium sorbate was chosen to extend the shelf life of the final formulations, and it was chosen because its widely used in food, with no known adverse effects (Younes et al., 2019). The percentage chosen was 0.1% because potassium sorbate according to the literature is widely used at 0.025 to 0.1 % levels in different food products like cheeses, dips, yogurt, sour cream, bread, cakes, pies and fillings, baking mixes, doughs, icings, fudges, toppings (CFNP, 2002). NaCl was also added at 0.1 % the addition of sodium is intended to improve fluid and energy absorption, because glucose transport from the intestine into the blood is dependent on sodium (Mora-Rodriguez & Hamouti, 2012), so having NaCl on the formulation plus the salt present in the microalgae enhances the functional aspect of the formulations. Citric acid was also added to balance the pH, the addition of honey *C. vulgaris* increases the pH of the formulation so it needs a component to lower the pH back to improve the tasting and acceptability of the product the percentage chosen was 0.25%. Finally, a few drops of lemon flavoring were added to the formulation to disguise the taste of the honey *C. vulgaris* and leaving it the most pleasant in terms of taste for consumers, this flavor was chosen due to the green/yellowish color of the product.

With this percentages established and to study the effect that honey *C. vulgaris* had on the product two gel formulations were created: the first with the 4 % of honey *C. vulgaris,* thinking about the nutritional value of the product, and one formulation with 1.5% of this microalga, with a more subtle taste thinking about the acceptability of the consumers. And finally, a blank formulation with no microalgae to compare with the other two. (Figure 4.11) The composition of each gel formulation including the blank is shown in the table below (Table 4.5).

Figure 4.11: From left to right: Blank, 1.5% HC and 4% HC

Table 4.5: Percentage of the components in the three gel formulations (w/v).

Formulation	Components (%)						
	Honey *Chlorella Vulgaris*	Maltodextrin	LBG	Citric Acid	Potassium Sorbate	NaCl	H_2O
Blank	0.0	50	0.6	0.25	0.1	0.2	49.0
1.5% HC	1.5	50	0.6	0.25	0.1	0.2	47.4
4% HC	4.0	50	0.6	0.25	0.1	0.2	44.9

4.3.3 Sensorial analysis

A sensory evaluation consists of measuring human sensory reactions to products. This includes measuring and analyzing the visual appearance, smell (aroma), taste, texture/mouthfeel and sound of what they are analyzing (Drake, 2021). The sensorial evaluation results are presented on the Tables 4.6 and 4.7 and in the Figures 4.12 and 4.13. and show that in relation to the smell, the scores of the two Honey *chlorella* formulations were practically the same, and that is due to the lemon flavoring added that was the main source of smell in the formulations. This score was higher on the tested commercial gel. The scores of the texture of the three gels varied between 3.95 (4% HC) and 4.70 (Commercial gel). The scores of the taste varied the most, with the 4% HC having a negative score (2.85) and the commercial gel having a score of 4.10. The gel with 4% of honey *chlorella* had the lowest scores, and that indicates that with the increase of the microalgae the taste, and smell of the product is less acceptable by the consumers.

Table 4.6: Sensorial Evaluation of the two gel formulas, 1.5% Honey chlorella and 4% Honey chlorella and a control commercial energy gel.

Sensorial Evaluation (1-6)	**Formulation**		
	1.5% HC	4% HC	Commercial Energy Gel
Smell	4.00 ± 1.2	3.30 ±1.6	5.60 ±0.4
Taste	3.65 ± 1.6	2.85 ± 0.8	4.10 ± 1.4
Texture	4.00 ± 0.8	3.80 ± 0.3	5.10 ± 0.5

Note: with score 1 being not pleasant at all, 2 somewhat pleasant, 3 fair, 4 pleasant, 5 quite pleasant and 6 excellent.

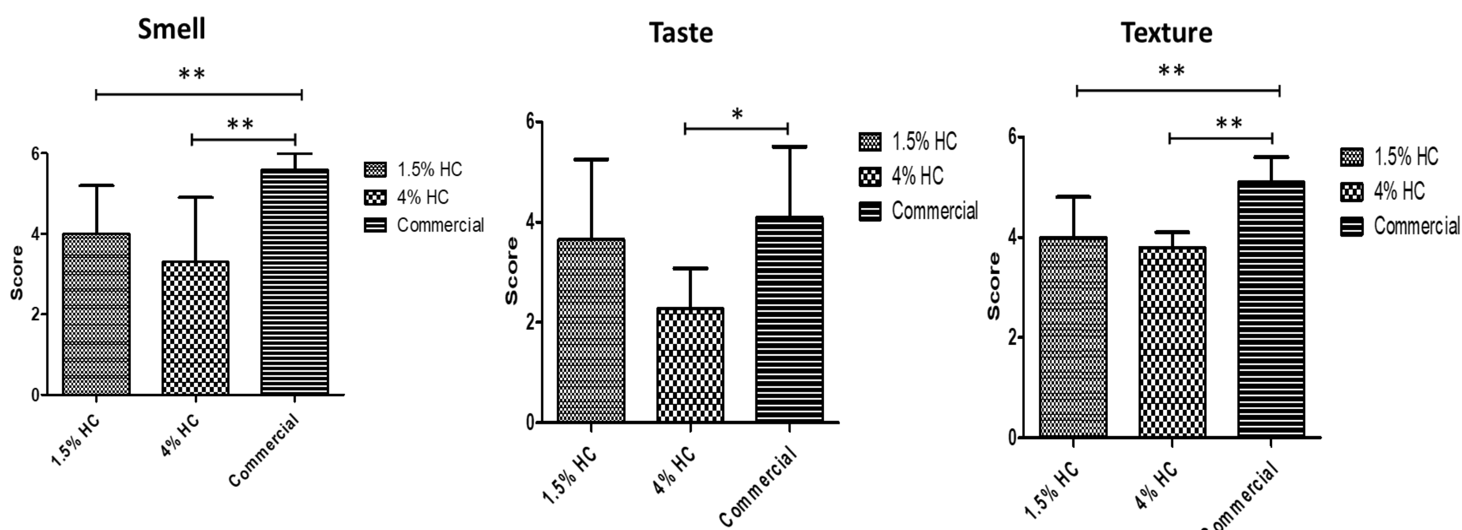


*Figure 4.12: Taste, texture, and smell score averages of the two gel formulas, 1.5% Honey chlorella and 4% Honey chlorella and a control commercial energy gel. Statistical parameters: *p< 0.005, **p < 0.0001.*

In regard to the description of the sensorial attributes, panelists quickly identify the aroma of the 1.5% HC and 4% HC gels, and the majority described the smell as citric, and lemon some described also as woody, sweet and chemical the last one being unpleasant. The commercial gel was described as sweet and fragrant and that is because the aroma was tropical. In relation to the texture people described the commercial gel as mainly thin and the 1.5% and 4% as mainly viscous, and sticky, and that is possibly because of the LBG. In relation to the taste, the commercial gel was described for the majority as acid and bitter. The 1.5% HC was described mainly as sweet, and some people also described it as acid and metallic, and the 4% HC was described mainly as metallic

and acid and sweet. The sweet taste was from the maltodextrin and the acid was from the citric acid added, the metallic taste was provided from the Honey *C. vulgaris* and that explained why it was more intense in the formulation with high concentration of this microalga. This is a downside of increasing the percentage of the microalga, because the gel with higher percentage was the least accepted by the panelists however the one with the highest nutritional value. Smell, taste, and texture are important quality characteristics and major factors affecting sensory perception and consumer acceptance of foods so sensory analysis of food products is an important contribution to their possible future commercialization (Ruiz-Capillas & Herrero, 2021). However, it is known that supplements for athletes usually have an unappealing taste, and those who practice exercise consume these products not for their taste but for their effect on the body (Ruiz-Capillas & Herrero, 2021).

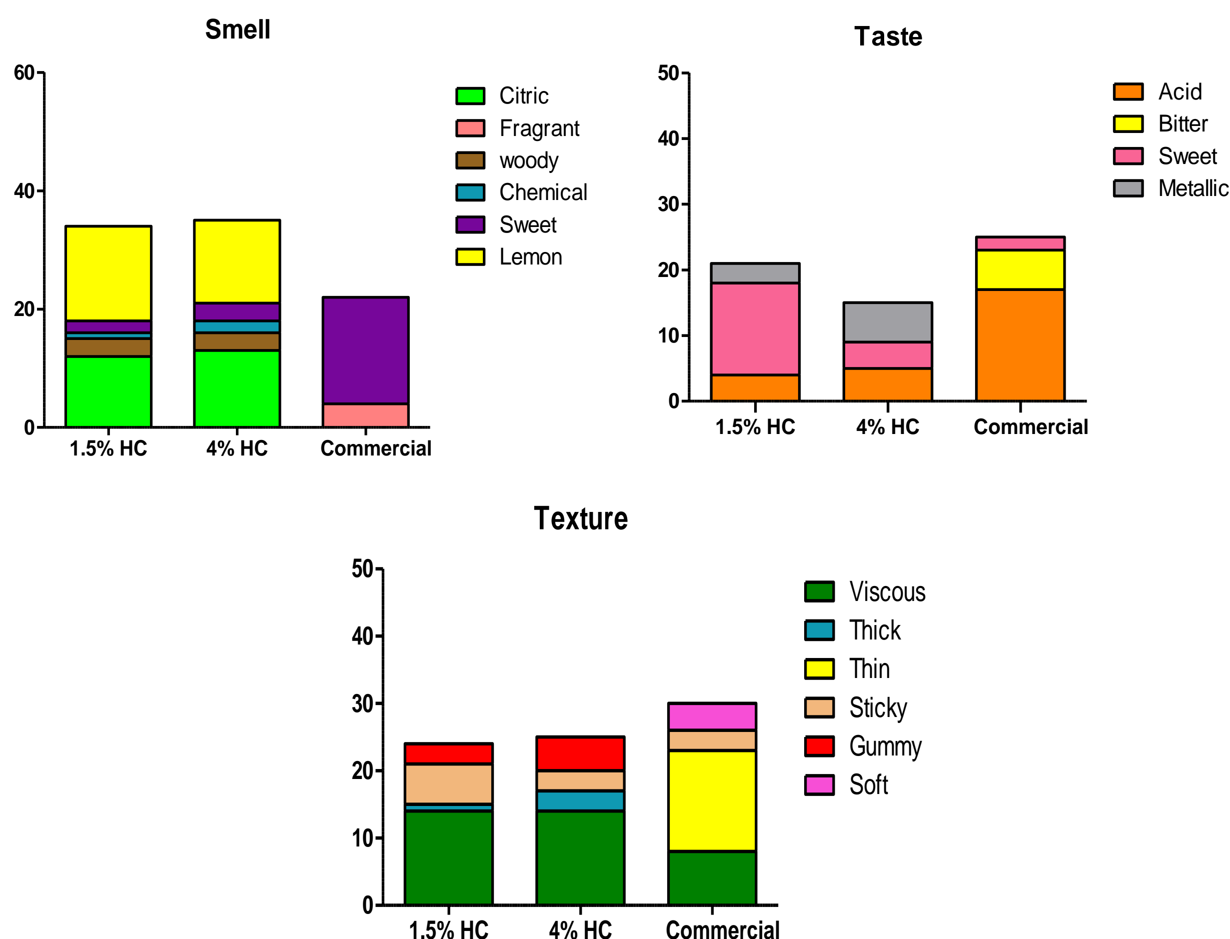


Figure 4.13: Taste, texture, and smell characterization of the two gel formulas, 1.5% Honey chlorella and 4% Honey chlorella and a control commercial energy gel.

4.3.4 pH

The pH of the formulations varied between 3.97 ± 0.02 (Blank) and 4.61 ± 0.01 (4%HC), these results show that the addition of the microalgae increases the pH of the gel, as expected, normal pH of honey *chlorella vulgaris* biomass varies between 7-10 (Uribe-Wandurraga et al., 2019). LBG properties are generally unaffected by pH, salts, or heat processing because it is non-ionic (Adedeji et al., 2022). Comparing these results with the pH results shown before optimization (Table 4.1), the pH decreased from 6.29 ± 0.02 to 4.61 ± 0.01 (4%HC), the reason for this change was the addition of acid citric acid in the formulation to lower the pH improving the taste of the final product. The pH value affects many phenomena and processes such as, growth and mortality of microorganisms, germinating or inactivation of bacterial spores and chemical reactions such as the Maillard reaction. Thus, knowledge of pH effects and its control during processing is necessary to produce safe, high-quality and value-added products (Andrés-Bello et al., 2013).

Table 4.7: pH of the gel formulations. Data are presented as mean value ± standard deviation (SD).

	Blank	**1.5% HC**	**4%HC**
pH	3.97 ± 0.02	4.30 ± 0.02	4.61 ± 0.01

4.3.5 Nutricional Evaluation

Nutritional Evaluation in food helps in understanding about the carbohydrates, proteins, fiber, sugar, etc. (P. Wang et al., 2022). To evaluate the nutritional value and differences of the final formulations determinations were caried out on the three gel samples 1.5% HC and 4% HC and Blank.

Firstly, dry matter was determined, the dry matter includes carbohydrates, fats, proteins, vitamins, minerals, and antioxidants. The mean percentage of dry matter was 51.1 ± 0.1 % in 1.5% HC and in 4% HC was 52.4 ± 0.1 %. As expected, the gel formulation with more honey *C. vulgaris* (4% HC) gave a higher dry matter value. Using the dry matter value, the water content was also determined (Table 4.8). The water content varied between 49.8 ± 0.18 (Blank) and 47.7 ± 0.1 (4% HC).

In relation to the proteins, the mean percentage of protein varied from 0.6 ± 0.0 % (1.5% HC) and 1.4 ± 0.0 % (4% HC). As the microalga is the source of protein in the gels, these results were the expected because according to the label, 100 g of powder Honey *C. vulgaris* contains 32.5 g of protein, which means that in 1.5 g contains 0.49 g of protein, and in 4 g contains 1.3 g of protein (Allmashop by Almicroalgae). LBG also contains protein but in a small amount contributing only with approximately 0.0036g of protein to the formulations (Calculated based on the label).

The mean percentage of sugars was 50.8 ± 1.84 % (1.5% HC) and 56.5 ± 0.7 (4% HC), In the formulation the main sugar source was the maltodextrin, and the amount on the gel formulations was 50% (w/v). Thus, as expected, all three formulations had a sugar value close to 50%, nevertheless, since Honey *C. vulgaris* contributed to an increase in the sugar content, as it contains 55% of carbohydrates of which 2.5% of sugars according to the label of the product, the gel formulation with more microalgae (4% HC) had a higher sugar content. Concerning lipid detection, analyzed samples were below the detection limit of the technique/equipment, and were therefore considered negligible. According to the Honey *C. vulgaris* label, this ingredient presents (6-13 g/100 g) of lipids, of which 25-35 % are polyunsaturated fatty acids, including omega-3 and omga-6 PUFAs. However, at the percentage of inclusion considered, it was not possible to detect.

Table 4.8: Proximate composition and sugar content in the three gels, gel formulation without honey chlorella vulgaris (Blanc), gel formulation with 1.5% of Honey chlorella vulgaris (1.5% HC) and gel formulation with 4% honey chlorella vulgaris (4% HC).

	Blank	**1.5% HC**	**4% HC**
Dry matter (%)	50.2 ± 0.2	51.1 ± 0.1	52.4 ± 0.1
Humidity (%)	49.8 ± 0.2	48.9 ± 0.1	47.7 ± 0.1
Protein (%)	Undetectable	$0.6 \pm 1.9 \times 10^{-2}$	$1.4 \pm 2.4 \times 10^{-2}$
Ash (%)	$1.2 \times 10^{-3} \pm 1.3 \times 10^{-4}$	$2.5 \times 10^{-3} \pm 3.39 \times 10^{-4}$	$3.1 \times 10^{-3} \pm 5.2 \times 10^{-4}$
Sugar (%)	49.5 ± 1.0	50.8 ± 1.8	56.5 ± 0.7
Carbohydrate (%) By difference	50.2 ± 0.2	50.5 ± .0.1	50.9 ± 0.3

Note: Mean ± S.D. of three determinations.

Energy gels are mostly composed of water, carbohydrates, and sodium, and may also contain amino acids and vitamins. Regarding carbohydrates (source of energy), in the gels on the market they ranged from 50% to 73% while in the formulations it ranged from 50.5 (1.5% HC) to 51% (4% HC), thus fitting the desired nutritional profile. In relation to protein content, which is important for muscle recovery, in the marketed gels it ranged from 0% to 2.67% and in the formulations, it ranged from 0.6 ± 0.0 (1.5% HC) and 1.4 ± 0.0 (4% HC). With these results we conclude that both formulations fit the nutritional profile of an energy gel plus all the other benefits from taking *Chlorella vulgaris*.

4.3.5.1 Minerals

The ash content is a measure of the total amount of minerals present within a food, where the mineral content is a measure of the amount of specific inorganic components present within a food, such as Na, Ca, P K, among others. (Tokuşoglu & Ünal, 2003) The mean of total ash was $1.2 \times 10^{-3} \pm 0.0$ % (Blank), $2.5 \times 10^{-3} \pm 0.0$ % (1.5% HC) and o $3.1 \times 10^{-3} \pm 0.0$ % (4% HC), and it increased with the percentage of microalgae added with the ashes.

From the analysis of the minerals present in the three gel formulations it was possible to quantify minerals included into different groups: i) macrominerals; ii) trace minerals, essential nutrients; iii) trace minerals, nonnutritive; and iv) trace minerals, toxic minerals. Table 4.9 summarizes all the results obtained, as well as the type of method used in each analysis. The macrominerals showed the highest concentrations in the gel formulation with more percentage of microalgae (4% HC), namely calcium, sodium, and magnesium.

Among the nutritious trace minerals, in the blank formulation, , magnesium and iron, stand out, 8.11 mg/100 g for iron and 0.41 mg/100 g for magnesium. In the 4% HC formulation the same trace minerals stand out, with concentrations between 17.64 mg/100 g for iron and 2.56 mg/100 g for magnesium. These results show that the addition of the microalgae enhanced the nutritional content of the product for these trace minerals.

For minerals without nutritional value, barium showed the highest concentrations in all 3 gel formulations. The blank presented 11.64 mg/100 g and the 4% HC presented 11.12 mg/100 g. The remaining elements are present in low concentrations or even in residual concentrations.

Table 4.9: Mineral content in the three gels, gel formulation without honey chlorella vulgaris (Blank), gel formulation with 1.5% of Honey chlorella vulgaris (1.5% HC) and gel formulation with 4% honey Chlorella vulgaris (4% HC).

Group		Mineral	Units	Blank	1.5% HC	4% HC	Method
Macrominerals		Calcium	mg/100 g	2.03	6.17	13.10	Titr. EDTA
		Magnesium	mg/100 g	0.41	1.21	2.56	Titr. EDTA
		Phosphorus	mg/100 g	0.01	0.03	0.07	MAS: VanMolPhos
		Potassium	mg/100 g	0.41	1.21	2.55	ICP-AES
		Sodium	mg/100 g	2.03	6.13	12.97	ICP-AES
Trace minerals or trace elements	Essential nutrient	Cadmium	µg/100 g	0.22	0.38	1.24	ICP-MS
		Cobalt	µg/100 g	0.20	0.20	0.20	ICP-MS
		Iron	mg/100 g	8.11	11.68	17.64	ICP-MS
		Magnesium	mg/100 g	0.41	1.21	2.56	ICP-MS
		Molybdenum	µg/100 g	1.79	1.54	1.24	ICP-MS
		Nickel	µg/100 g	4.41	5.74	7.96	ICP-MS
		Selenium	µg/100 g	0.81	0.81	0.82	ICP-MS
	Non-nutritious	Silver	µg/100 g	0.20	0.20	0.20	ICP-MS
		Tin	µg/100 g	0.20	0.20	0.20	ICP-MS
		Arsenic	µg/100 g	0.20	0.20	0.20	ICP-MS
	Toxic	Barium	µg/100 g	11.64	11.45	11.12	ICP-AES
		Beryllium	µg/100 g	0.20	0.20	0.20	ICP-MS
		Thallium	µg/100 g	0.20	0.20	0.20	ICP-MS
		Vanadium	µg/100 g	0.50	0.50	0.50	ICP-MS

Note: EDTA: complexation with ethylenediamine tetraacetic acid; ICP-AES: inductively coupled; plasma - atomic emission spectrometry; ICP-MS: inductively coupled plasma mass spectrometry; MAS: molecular absorption spectrophotometry method; Titr: titration; VanMolPhos: vanadomolybdophosphoric acid method;

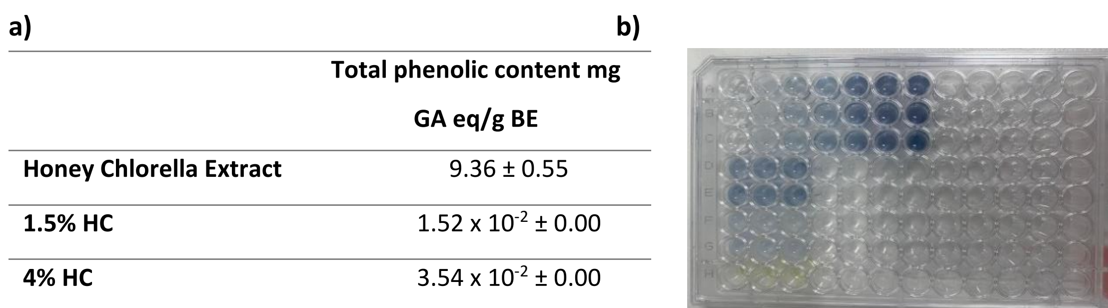
The obtained results show that the addition of the honey chlorella vulgaris enhanced the nutritional value of the gel formulation. This microalga represents a valuable source of nearly all essential vitamins (e.g., A, B1, B2, B6, B12, C, E, nicotinate, biotin folic acid and pantothenic acid) and a balanced mineral content (e.g., Na, K, Ca, Mg, Fe, and trace minerals) (Becker, 2004). The vitamin content is amenable to manipulation by varying the culture conditions as well as by strain selection or genetic engineering. However, vitamins cell content fluctuates with environmental factors, the harvesting treatment and the biomass drying methods (Brown et al., 1999, Borowitzka, 1988).

4.3.6 Evaluation antioxidant activity of extracts and obtained formulations

The extraction yield percentage of the honey *chlorella vulgaris* powder was 8.23% (m/m). These results are in accordance with studies that use a similar methodology like a study from Agregán et al., (2018) that obtained an extraction yield of 7.78 % and 7.14 % from *Chlorella vulgaris.*

Numerous studies have demonstrated the antioxidant properties of algae extracts rich in phenolic compounds. The results of the total phenolic content (TPC) were 9.36 ± 0.55 mg GA eq/g BE for the extract (Figure 4.14) and are in accordance with the literature. In a study from Andriopoulos et al., 2022 using methanol as the solvent the TPC of *Chlorella minutissima* at the early stationary was 9.04 ± 0.68 mg GA eq/g BE. In the formulations the TPC was 1.52 x 10-2 ± 0.00 mg GA eq/g BE for the 1.5% HC and 3.54 x 10-2 ± 0.00 mg GA eq/g BE for the 4% HC, these results shown that the TPC increased with the increasing of the percentage of microalgae.

Variation in the phenolic content measured by Folin–Ciocalteu in the literature might stem from actual differences in the phenolic content of different species of microalgae and the effect of different growth conditions, however, it might also be related to the choice of solvents, extraction time, and temperature (Andriopoulos et al., 2022).



a) **b)**

	Total phenolic content mg GA eq/g BE
Honey Chlorella Extract	9.36 ± 0.55
1.5% HC	1.52×10^{-2} ± 0.00
4% HC	3.54×10^{-2} ± 0.00

Figure 4.14: (a) Total phenolic content (mg Gallic Acid equivalents/g Honey chlorella vulgaris extract and 1.5% HC and 4% HC); (b) Example of a 96 well-plaque prepared for the total phenolic content evaluation by Folin & Ciocalteu's method after 1 h in the dark at room temperature.

Since phenolic compounds are known for their antioxidant activity, the different extracts antioxidant activity was assessed through the DPPH radical scavenging method. The DPPH radical scavenging activity assay allows the assessment of bioactive compounds capacity to scavenge the radical DPPH. The results (Figure 4.15 b)) showed that the extract DPPH radical inhibition activity at different concentrations. For a 50% scavenging activity the extracts concentration of the extract was 2.437 mg/mL. The DPPH method was not compatible with the samples due to the high sugar levels that precipitated the methanol.

A source of antioxidants is a value addition to the formulation as oxidation can affect the sensory attributes, nutritional value, texture properties, and shelf-life stability of food by decomposition of, vitamins, unsaturated essential fatty acids, and pigments such as anthocyanin, carotenoid, and myoglobin.

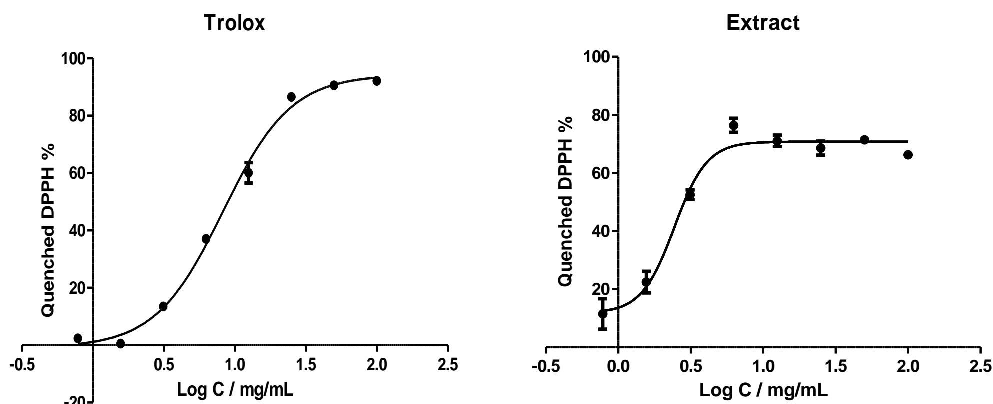


Figure 4.15: DPPH scavenging profile regarding Honey chlorella extract: (a) positive control Trolox; (b) Honey chlorella vulgaris extract.

Chapter 5: Conclusions and Future perspectives

Microalgae are an important biological resource, representing one of the most promising sources for the development of new food products and applications. Nowadays, due to consumers awareness of the direct link between food and health, the utilization of microalgae in functional foods is growing.

Microalgae incorporation into foods could lead to potential benefits for human health due to the presence of bioactive compounds in some microalgae species namely *Chlorella vulgaris*. For example, antioxidative, antihypertensive, immunomodulatory, anticancerogenic, hepato-protective, and anticoagulant activities have been attributed to microalgae bioactive compounds.

The incorporation of microalgae in food products faces challenges mainly due to their organoleptic characteristics, including a strong color, taste and odor. Sensory attributes of foods are directly linked to the consumer acceptance so alternative strategies to improve the organoleptic qualities of food containing microalgae have included the extraction of the target compounds with the consequent removal of chlorophyll. That was the main reason for choosing Honey *Chlorella Vulgaris* for the gel formulations, a strain of *Chlorella vulgaris* with lower chlorophyll content. That way it would be possible to add a larger quantity and increase the nutritional value of the product. So firstly, a formulation of 4% Honey *Chlorella vulgaris* was chosen, but after tasting the formulation it was decided to add another formulation with lower percentage (1.5% (w/v)) while all the other components were kept in the same quantities.

Producing energy hydrogels that are dense in energy, nutrients, and electrolytes, requires a component that can bind water and form a colloid called a hydrocolloid. LBG showed high solubility, dispersibility, viscosity, and biocompatibility with the gel formulations. This work has demonstrated the suitability of LBG in improving gel properties. The results of this work showed that as the concentration of LBG increases in the gel, its viscosity increases consequently. LBG at low shear rates exhibits a Newtonian flow and it was concluded that a concentration of 0.6% (w/v) of LBG would be a suitable

percentage for the formulations comparing with other energy gels sold on the market.

The Carbohydrate chosen was maltodextrin. Maltodextrin is very popular among athletes because it is quickly absorbed by the intestine being absorbed as rapidly as glucose, quickly replenishing blood sugar levels, and providing an almost instantaneous boost.

The stability results showed that the gels kept at room temperature in the second week gained an atypical odor indicating that it was no longer be good for consumption. The gels stored at room temperature, after 1 week already had colonies of bacteria. These results demonstrated that gel stored at room temperature spoiled more quickly than gel stored at 4ºC, and that it was most beneficial for the product to add a preserving agent to its final formulation to prolong its shelf life.

After the stability evaluation, the final formulations were settled with 0.6% of LBG, 50% maltodextrin, 0.1% of potassium sorbate (the preserving agent), 0.2% of NaCl (source of electrolyte), 0.25% of citric acid (pH balancer) and lemon flavor to disguise the taste of the microalgae. With these percentages established and to study the effect that honey *C. vulgaris* had on the product, two gel formulations were created: the first with the 4 % of Honey *C. vulgaris* thinking about the nutritional value of the product, and one formulation with 1.5% of microalgae with a more subtle taste thinking about the acceptability of the consumers.

The sensorial analysis showed that in relation to the smell, the scores (1-6) of the two Honey *chlorella* formulations were 4 (1.5% HC) and 3.30 (4% HC) , and that the main source of smell in the formulations was due to the added lemon flavoring. This score was higher on the tested commercial gel. The scores of the texture of the three gels varied between 3.95 (4% HC) and 4.70 (Commercial gel). The scores of the taste varied the most, with the 4% HC having a not so favorable score (2.85) and the commercial gel having a score of 4.10. Among all three gel samples, in relation to all parameters evaluated, there was a preference for the commercial gel, with a significant difference when comparing to the 4% HC. In relation to the sensorial attributes, Honey *C. vulgaris* provided a metallic taste and that explained why it was more intense in the formulation with higher concentration of this microalgae. In conclusion, the gel with higher percentage (4% HC) was the least accepted by the panelists despite being the one with the most nutritional value.

The results of the nutritional evaluation were the expected, according to the label of the components added, and showed that the addition of the microalgae compared to a formulation with no microalgae (Blank) enhanced the nutritional content of the product, with the formulation of 4% HC having a higher nutritional and mineral content than the Blank and the formulation with 1.5%.

Regarding the antioxidant activity the results showed extract DPPH radical inhibition activity at different concentrations. For a 50% scavenging activity the extracts concentration of the extract was 2.437 mg/mL. The DPPH method was not compatible with the samples due to the high sugar levels that precipitated with the methanol.

To conclude, energy gels enriched with Honey *Chlorella vulgaris* can be considered as an alternative way to include this health promoter microalga in sports supplementation. The optimization of the formulations resulted in 1.5% and 4% Honey *Chlorella* formulation incorporation, sufficient to produce a consumer acceptable energy gel enriched with microalgae. However, it is necessary to look for solutions that make it possible to increase the levels of incorporation of microalgae and thus not affect the taste of food in a negative way.

For future work, it would be interesting to use microalgae paste instead of spray-dried powder, due to the higher content of antioxidants and some nutrients. It would also be interesting to experiment with different aromas to see which best disguises the microalgae flavor. This is because the fishy aroma and taste of many microalgae is considered to be one of the main issues regarding the application of microalgae in food, especially for those who have not been exposed to algae-based products before. Finally, it would be interesting to carry out a sensory analysis only on a panel made up of athletes to determine their acceptability, because it is known that supplements for athletes tend to have an unappetizing taste, and those who practice exercise and take sport supplements have a more tolerable taste then the general population.

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Annex 1

Inquérito destinado a praticantes de exercício físico: Prova de Géis energéticos para desportistas contendo *Chlorella*

As microalgas são um alimento rico em proteínas, sais minerais e vitaminas do complexo B, com bioatividade demonstrada e potencial benefício na prevenção de doenças. Sendo o pó e cápsulas os produtos de microalgas mais populares, a diversificação de outros produtos saudáveis e sustentáveis deve ser incentivada.

O presente questionário insere-se na minha tese de mestrado em Qualidade Alimentar e saúde na Faculdade de Farmácia da Universidade de Lisboa e tem como objetivo a realização de provas sensoriais a géis energéticos contendo *Chlorella vulgaris*.

Agradeço que complete o seguinte questionário com todas as informações solicitadas, as quais serão mantidas confidenciais.

Nome:____

Contacto:_______________; **e-mail**_______________________________________

Grupo Etário: ☐ < 20 ☐ 20 – 25 ☐ 26 – 35 ☐ 36 – 45 ☐ 46 - 55 ☐ 56 – 65 ☐ > 65

Sexo: F ☐ M ☐

Em média quantas vezes pratica exercício por semana? ☐ 1-2 ☐ 3-4 ☐ 5-6 ☐ >7

Costuma ingerir suplementos para desportistas? Sim ☐ Não ☐

Se sim qual(is):

____

____

____

Sabe o que são géis energéticos para desportistas? Sim ☐ Não ☐

Se sim já provou? Sim ☐ Não ☐

Sofre de alguma das seguintes patologias:

Intolerância a algum alimento/ingrediente: Sim ☐ Não ☐

Em caso afirmativo, refira qual(is): ______________________

Alergia a algum alimento/ingrediente Sim ☐ Não ☐

Em caso afirmativo, refira qual(is): ______________________

Consome produtos à base de microalgas? Sim ☐ Não ☐

Do produto oferecido para provar classifique de 1 a 6: (sendo 1 nada agradável, 2 pouco agradável, 3 razoável, 4 agradável, 5 bastante agradável, 6 excelente)

O cheiro: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐

O sabor: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐

A textura: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐

Assinale com um X como descreveria o produto? (Pode colocar X em várias opções)

Cheiro		Sabor		Textura	
Cítrico		Ácido		Viscoso	
Perfumado		Amargo		Espesso	
Amadeirado		Salgado		Fino	
Químico		Doce		Adesivo	
Doce		Umami		Aveludado	
Limão		Metálico		Gomoso	
Podre		----------------		Suave	
Picante		----------------		Elástico	

Na sua opinião existe algum fator que melhoraria no produto? **Sim** ☐ **Não** ☐

Em caso afirmativo, refira qual(is) e como?

____

____

____

Compraria este produto? **Sim** ☐ **Não** ☐

Por fim, estes produtos despertaram-lhe interesse em querer consumir mais produtos contendo microalgas, algo que ainda é recente na nossa indústria alimentar?

Sim ☐ **Não** ☐

Observações:____

____

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____

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Obrigado pela sua disponibilidade e colaboração!