Development of microalgae jelly gummies and characterization of its structural properties

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Dissertation for the Master's Degree in Food Science and Engineering

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Preface and Acknowledgement

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Resumo

Nos últimos anos, o sector alimentar tem evoluído em prol de satisfazer o consumidor, não só a nível de acessibilidade de uma maior diversidade de produtos alimentares, mas também a nível nutricional e funcional. Esta dissertação está inserida na Agenda mobilizadora PRR no. C644915664-00000026 - Pacto da Bioeconomia Azul, nomeadamente no projeto WP5 Algae Vertical – SP4A: Sea to fork, fundada pela "Next Generation EU European Fund", o Plano de Recuperação e Resiliência (PRR), e financiada pela Sumol+Compal. Atendendo ao aumento da preocupação com as questões ambientais e respondendo ao desafio da escassez global de recursos terrestres, a União Europeia tem criado planos com base na Biotecnologia Azul, utilizando o mar como principal recurso promovendo a sustentabilidade, aumentando a produtividade e reduzindo custos.

Este estudo teve como principal objetivo o desenvolvimento de gomas funcionais com microalgas promovendo benefícios para a saúde do consumidor. O trabalho consistiu no desenvolvimento de gomas com diferentes níveis de incorporação de *Chlorella vulgaris* e de Spirulina (0%, 0,5%, 1,0% e 2,0%).

Avaliou-se a textura, composição química, perfil nutricional, potencial antioxidante, cor das gomas, bem como a sua apreciação sensorial. A análise sensorial foi realizada recorrendo a um painel não treinado de 30 provadores, com o intuito de avaliar a qualidade em termos de textura, sabor, cor e aroma. Avaliou-se ainda a preferência dos provadores quanto às gomas com diferentes níveis de incorporação das microalgas.

O estudo físico-químico revelou um aumento no teor de minerais, hidratos de carbono e compostos fenólicos com o aumento da concentração de microalga. A nível de textura com o aumento de microalga, verificou-se uma diminuição na firmeza e resiliência do produto e pouca ou nenhuma influencia na coesividade e elasticidade. Relativamente à coloração do produto verifica-se uma grande influencia por parte da adição das diferentes microalgas, com uma perda de pigmentos para um armazenamento das gomas à temperatura ambiente durante dois meses. A avaliação sensorial revelou uma grande aceitabilidade das mesmas, apesar da avaliação menos positiva ao nível de sabor, para gomas com maiores níveis de incorporação das microalgas. De acordo com o estudo microbiológico realizado, as gomas enriquecidas com microalgas apresentam potencial atividade antimicrobiana, possibilitando uma maior *shelf-life* das mesmas.

Palavras-chave: Gomas, microalgas, compostos bioativos, Spirulina, *Chlorella vulgaris.*

Abstract

In recent years, the food sector has clearly evolved to answer consumer's needs not only in terms of food accessibility but also providing nutritional satisfying foods. Also, the growing challenges of food scarcity and sustainability have driven the search for innovative and efficient food sources and the European Union has created plans based on Blue Biotechnology, using the sea as the main resource promoting sustainability, increasing productivity and reducing costs. In this context, microalgae have emerged as a promising solution due to their high nutritional value, rapid growth, and low environmental footprint. This work is included in the PRR mobilization agenda no. C644915664-00000026 - Blue Bioeconomy Pact, namely in the WP5 Algae Vertical project – SP4A: Sea to fork, founded by the "Next Generation EU European Fund", the Recovery and Resilience Plan (PRR), and financed by Sumol+Compal.

In this study, the main goal was to develop fortified macroalgae-based gummies with different levels of *Chlorella vulgaris* and Spirulina incorporation (0%, 0.5%, 1.0% e 2.0%) and access their nutritional profile, as well as potential health benefits. Gummies were characterized in terms of texture, chemical composition, colour, and bioactive potential, namely, antioxidant activity and total phenolic content. Sensory analysis was carried out by an untrained panel of 30 tasters in order to evaluate the sensory quality of the microalgae-enriched gummies, in comparison to a control gum. The preference between different concentration levels was also evaluated, revealing a high acceptability of the developed gummies.

The physicochemical study revealed an increase in the content of minerals, carbohydrates and phenolic compounds with the increase in the concentration of microalgae. In terms of texture, with the increase in microalgae, there was a reduction in firmness and resilience of the product and little or no influence on cohesiveness and elasticity. Regarding the colour of the product, the addition of the different microalgae greatly influenced the product colour, with a loss of pigments when the gums were stored at room temperature for two months. The sensory evaluation revealed a high acceptability, despite the less positive evaluation in terms of flavor, for gums with higher levels of microalgae incorporation. According to the microbiological study carried out, the gums enriched with microalgae have potential antimicrobial activity, allowing them to have a longer shelf life.

Keywords: Gummies, microalgae, bioactive properties, Spirulina*, Chlorella vulgaris.*

Resumo estendido

A consciencialização da população sobre as consequências e benefícios para a saúde física e mental fornecidos pelos alimentos que ingerimos, tem aumentado, gerando uma maior procura por alimentos ricos em compostos com a capacidade de prevenir diversas doenças relacionadas com uma alimentação desequilibrada. Alimentos funcionais podem ser descritos, de forma geral, como alimentos ou ingredientes alimentares que proporcionam benefícios à saúde humana, por meio da incorporação de compostos bioativos, como vitaminas, minerais, fibras alimentares ou probióticos [1, 2, 3]. Os alimentos funcionais enquadram-se em diversas categorias, incluindo probióticos, prebióticos, fibras alimentares, peptídeos bioativos, antioxidantes e produtos fortificados ou enriquecidos. Alimentos modificados ou enriquecidos com esses componentes oferecem benefícios fisiológicos, potencialmente reduzindo os riscos de doenças ou auxiliando em funções de saúde [4]. Surge então como uma abordagem inovadora para melhorar o perfil nutricional e adicionar propriedades funcionais aos alimentos básicos, a fortificação com microalgas. Este processo tem sido desenvolvido, apresentando um número considerável de estudos em inovação alimentar que relatam diversos impactos positivos aquando da adição de microalgas em termos nutricionais, sensoriais e de textura [5].

Esta dissertação está inserida na Agenda mobilizadora PRR no. C644915664-00000026 - Pacto da Bioeconomia Azul, nomeadamente no projeto WP5 Algae Vertical – SP4A: Sea to fork, fundada pela “Next Generation EU European Fund”, o Plano de Recuperação e Resiliência (PRR), e financiada pela Sumol+Compal. Um aumento da preocupação com as questões ambientais surge como consequência ao desafio da escassez global de recursos terrestres. Por consequência, a União Europeia tem vindo a desenvolver planos com base na Biotecnologia Azul, utilizando o mar como principal recurso promovendo a sustentabilidade, aumentando a produtividade e reduzindo custos.

Este estudo teve como principal objetivo o desenvolvimento de gomas funcionais com microalgas promovendo benefícios para a saúde do consumidor. Os experimentos incluíram o desenvolvivento de gomas fortificadas com *C. vulgaris* e com spirulina, utilizando gelatina suína como agente gelificante. Ao longo do processo de confeção das gomas, diversas formulações foram desenvolvidas com diferentes combinações de dois tipos de microalgas até atingir um produto final com as características desejadas a nivel de textura, aparência, consistência e sabor (avaliação qualitativa básica feita apenas pelos desenvolvedores). Após a seleção da formulação controlo (0% microalga) para as gomas, uma amostra controlo foi preparada com 30 g de gelatina sem a incorporação de microalgas, e seis formulações de gomas enriquecidas com microalgas foram desenvolvidas usando spirulina e *C. vulgaris*, incorporando 0,5%, 1,0% e 2,0% do peso seco total da goma, resultando num total de sete amostras distintas. A quantidade de microalgas adicionada substituíu a mesma quantidade removida de gelatina.

Relativamente à preparação das diferentes formulas o processo produtivo realizou-se da seguinte forma: o agente gelificante (gelatina suína) foi hidratado com água e deixado em repouso por 30 minutos. Enquanto isso, uma mistura de açúcar e água foi aquecida usando uma placa de aquecimento, até atingir uma temperatura entre 115-120 ºC. A formulação permaneceu em banho-maria, a uma temperatura aproximada de 60 °C, ate dissolução completa dos restantes ingredientes da fórmula, obtendo uma mistura homogenea. Essa mistura foi deixada em repouso, e a espuma formada foi removida com o uso de uma colher, evitando a formação de bolhas nas gomas, o que é visto como um defeito de produção. Após a remoção da espuma e equanto a mistura ainda estava quente, a formulação foi transferida para bandejas de molde de silicone previamente oleadas, resultando em gomas de aproximadamente 5 g/cada. Essas bandejas permaneceram em temperatura ambiente por cerca de 30 minutos até o resfriamento para atingir o equilíbrio, sendo então colocadas na geladeira (acima de 4 °C) por 24 horas. Em seguida, foram retiradas dos moldes e armazenadas em um recipiente fechado, que permaneceu na geladeira até posterior análise.

As gomas foram avaliadas quanto à sua textura, composição química, perfil nutricional, potencial antioxidante, cor, apreciação sensorial e estabilidade microbiológica. A avaliação físico-química realizada nas gomas permitiu tomar conhecimento quanto ao teor em proteínas, lípidos, hidratos de carbono, humidade, cinza e minerais presentes nas gomas desenvolvidas. Para avaliar o potencial antioxidante das gomas, foram utilizadas duas metodologias: os ensaios in vitro de DPPH (2,2-difenil-1-picril-hidrazil) e FRAP (capacidade de redução de ferro do plasma). O método DPPH segue um princípio simples, no qual um radical sintético é criado e a capacidade de eliminar ou neutralizar esse radical é monitorizada por meio de um espectrofotômetro UV/visível [6]. Por outro lado, o ensaio FRAP é baseado na capacidade de uma substância reduzir Fe(III) em Fe(II) [7]. De modo a congregar informação aos resultados dos antioxidantes procedeu-se à determinação do conteúdo total de compostos fenólicos, cujo método utilizado foi Folin-Ciocalteu modificado [8]. A avaliação da cor foi realizada na superfície das gomas utilizando um colorímetro portátil CR-400, com uma repetição de 15 medições. De modo a estudar as caracteristicas de textura, foram realizados dois testes, com auxilio de um texturometro; um teste de prefuração e um teste de compressão em 8 gomas de cada fórmula, com volume médio de 5 mm^3. A análise sensorial foi realizada recorrendo a um painel não treinado de 30 provadores, com o intuito de avaliar a qualidade em termos de textura, sabor, cor e aroma. Avaliou-se ainda a preferência dos provadores quanto às gomas com diferentes níveis de incorporação das microalgas, revelando uma grande aceitabilidade das mesmas, apesar da avaliação menos positiva ao nível de sabor para gomas com maiores níveis de incorporação das microalgas. O crescimento dos microrganismos foi supervisionado e contado, e os resultados foram analisados. O processo de inoculação foi realizado em três momentos: no dia seguinte à produção (T0) e em amostras mantidas em temperatura ambiente, coletadas após um mês (T1) e com dois meses de armazenamento (T2). Para determinar a existência de diferenças significativas entre as médias ajustadas dos mínimos quadrados, utilizou-se a análise de variância (ANOVA) utilizando o teste do método de Tukey-Kramer.

Os resultados da composição quimica e actividade antioxidante demonstraram grandes vantagens ralativamente à adição de microalga nas gomas, aumentando o teor em minerais e compostos fenolicos. Estes resultados foram muito positivos, especialmente porque se esperava uma perda de compostos bioativos durante a produção das gomas devido às altas temperaturas necessárias para ativar as propriedades gelificantes, o que poderia se tornar um fator determinante para a colocação no mercado. No caso da analise de textura verificou-se uma diminuição na dureza, resiliencia e adesividade com o aumento de microalga, com exceção dos resultados relativos as gomas de *C.vulgaris*, cuja dureza aumentou para baixas concentrações de microalga. No entanto, os valores de elasticidade e coesividade nao demostraram grande alteração. Mesmo com algumas alterações nas características de textura, o principal fator para a rejeição do produto, referido pelo painel durante a análise sensorial, foi o sabor das gomas, que piora com o aumento do nível de incorporação de microalgas. De acordo com o estudo microbiológico realizado, nas gomas enriquecidas com microalgas apresentam potencial atividade antimicrobiana, possibilitando uma maior *shelf-life* das mesmas. Os ensaios microbiologicos mostraram uma presença baixa, mas ainda assim, contaminação de microrganismos como mofo e leveduras nas gomas, bem como algumas alterações de cor. A carga de microrganismos teve tendencia a diminuir, ao longo do tempo e com o aumento de concentração de microalga, no entanto seria necessario tomar algumas pedidas para controlar o cerscimento de microrganismos nas mesmas, como por exemplo, a criação de uma camada protetora, ou mesmo a alteração das condições de armazenamento para um produto congelado, ou a embalagem que impede a luz de atingir o produto, evitando a perda de cor.

Palavras-chave: Gomas, microalgas, compostos bioativos, Spirulina, *Chlorella vulgaris.*

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a_w – water activity

AAT – total antioxidant activity

CAGR - Compound Annual Growth Rate;

Cv – *Chlorella vulgaris*;

DPPH – 1,1-difenil-2-picrilhidrazil;

EFSA - European Food Safety Authority;

EU - European Union;

FRAP - Ferric-Reducing Ability of Plasma;

GMO's - Genetic Modified Organisms;

PUFA – Polyunsaturated Fatty Acids;

RSA – Radical Scavenging Activity;

RDV - Recommended Daily Value;

SD – standard deviation

Sp – Spirulina;

TPC – Total Phenolic Content

ΔE – Colour difference between two samples

1. **Introduction**

This work was developed to obtain a Master's degree in Food Engineering and is part of the WP5 Algae Vertical - SP4A: Sea to fork, in partnership with the “Eco-Novel Food and Feed” group from LEAF – Linking Landscape, Environment, Agriculture and Food Research Center of Instituto Superior de Agronomia, University of Lisbon.

This project aims to develop innovative microalgae-based food products, with a potential positive impact on human health and this dissertation focused on the development of innovative functional jelly gummies with the incorporation of two different strains of microalgae, *Chlorella vulgaris* and *Limnospira platensis* (Spirulina), providing benefits to human health.

Different formulations were characterized in terms of texture, colour, nutritional profile (moisture, ash, protein, fat, carbohydrates and minerals), antioxidant potential, antimicrobial activity, and consumer acceptability.

The relevance of this project lies in the increasing need to find new sources of ingredients, namely protein sources. Microalgae-enriched food products are in line with that requirement, being positioned as a pivotal element in the future of nutrition. In recent years, consumer preferences have shifted due to heightened awareness and knowledge of functional, nutritious, and sustainable food options. Microalgae exemplify this trend and are regarded as a hallmark of modern dietary innovation. These unicellular aquatic microorganisms, with over 50,000 classified species, are sustainable due to their rapid growth, ease of cultivation, and non-reliance on arable land [9]. Moreover, they play a crucial environmental role, capturing significant amounts of carbon dioxide daily and producing 50% of the Earth's oxygen. During this process, various species of microalgae synthesize a broad spectrum of nutrients, including proteins, fats, carbohydrates, vitamins, antioxidants, pigments, minerals, and other compounds essential to human health [9].

Among the most recognized microalgae are the genera *Chlorella* and Spirulina. Both are rich in proteins, vitamins, pigments, fatty acids, sterols, and other valuable compounds, making them highly attractive for food industry applications. *Chlorella* is a eukaryotic microorganism, while Spirulina (a cyanobacterium) is prokaryotic.

Despite their nutritional potential, the commercial exploitation of *Chlorella* and Spirulina remains limited. Nonetheless, they stand out as exceptional sources for functional foods, nutraceuticals, and dietary supplements. The introduction of bioactive compounds in gummy candies presents several challenges, particularly in maintaining the stability, taste, texture, and appearance of the final product, as certain bioactive compounds, such as vitamins and probiotics, are heat sensitive, and may degrade during gummy processing and storage with light, and moisture exposure.

To ensure that functional gummies provide the intended health benefits it is necessary to develop formulations that avoid any loss of the desired compounds due to degradation. These compounds often offer a strong or unpleasant flavour that can impact negatively the gummy taste, and may also affect the gummy's structural integrity, by altering formulations to accommodate bioactive compounds [10, 11].

The following work is divided into 5 chapters: the first chapter contains an introduction to the work and the proposed main goals; the second chapter presents a literature review on the main topics; the third chapter is focused on the materials and methods used during the development of the work; the fourth chapter is dedicated to the results and their discussion; the fifth chapter encompasses all the conclusions and suggestion of future work.

2. Literature review

2.1. Microalgae

Scientific studies estimate a number between 30,000 to more than 1 million algae species [12], where microalgae represent the great majority of elements of this group, with considerable variety of characteristics, existing in oceans, seas, rivers and even ice. They can also be found living at the bottom of the ocean (benthonic), attached to stones (epilithic), on sand (epipelic), on other algae or plants (epiphytic), or animals (epizoic). In Portugal, a high incidence of these algae species occurs in the region of Cabo de São Vicente due to the warm and up-welling waters, rich in nutrients, allowing an easy growth of microalgae [4]. On the other hand, microalgae can be produced using photobioreactors, a controlled environment that uses light and nutrients for the algae growth, as is the case with the microalgae used in the development of the present project.

Microalgae are unicellular microorganisms lower than 2.0 mm of diameter [13], able of capturing light and use their energy to perform photosynthesis, converting CO_2 into sugar and oxygen. These microorganisms, responsible for about half of the annual oxygen production [14], are the basis of marine food chain due to their ability to convert inorganic matter to organic matter and bioactive compounds. Thus, it is expected that incorporating microalgae into food products will enhance their nutritional value and oxidation resistance.

2.1.1. Taxonomy

Microalgae used to be classified according to their colour, but recently the current classification system is based according to the type of pigments available, chemical nature and cell wall constituents [9]. Morphological characteristics, such as occurrence of flagellate cells, structure of the flagella, scheme and path of nuclear and cell division, presence of an envelope of endoplasmic reticulum around the chloroplast, and possible connection between the endoplasmic reticulum and the nuclear membrane, are also considered in the classification (Table 1) [9, 15].

Table 1 - Taxonomy classification of some algae groups, and identification of the most common species [15].

Group	Kingdom	Phylum
Prokaryotic	Bacteria	*Cyanophyta* (Cianobacteria and Prochlorophyta)
Eukaryotic	Plantae	*Chlorophyta* (Green algae)
		Rhodophyta (Red algae)
		Glaucophyta
		Charophyta
	Chromista	*Haptophyta*
		Cryptophyta
		Ochrophyta (Golden algae and Diatoms)
		Cercozoa
	Protozoa	*Myzozoa*
		Euglenozoa

Prokariot

Cianobacteria (*Cyanophyta* and *Prochlorophyta*) are prokariotic microorganisms grouped in the Kingdom Bacteria. *Cyanophyceae* contain phycocyanin and phycoerythrin, which are compounds that mask the chlorophyll pigmentation by providing a blue tone to it, and so the name "blue-green algae". They are able to produce chlorophyll a, and the main storage product is glycogen (a-1,4-linked glucan). The *Prochlorophyta*, besides producing chlorophyll a, it can also synthesize chlorophyll b and carry a starch-like polysaccharide as the main storage compound [9].

Surrounded by a firm or amorphous mucilage, their cells can have a unicellular or filamentous organization and DNA diffuse through the cytoplasm. Filamentous cells can be differentiated into heterocysts and akinets, which allow these micro algae to fixate nitrogen and grow in such hostile environments dating their existence to 3.5 billion years ago at the start of life as we know [14].

Cyanobacteria are able of producing toxins, such as microsystin, which may become a concern for human health. However, there are species like *Arthrospira platensis* (spirulina), a filamentous gram-negative cyanobacteria, used as a source of feed and fine chemicals, completely safe to human health.

Eukaryotes

Eukaryote autotrophic microorganisms are divided and grouped into three Kingdoms: Plantae, with four *phyla*, Chromista, also with four *phyla*, and Protozoa, with two *phyla*. Their photosynthetic apparatus is organized in chloroplasts, which contain alternating layers of lipoprotein membranes (thylakoids) and an aqueous phase, the stroma. The three most abundant eukaryote algae classes are the diatoms (*Bacillariophyceae*), the green algae (*Chlorophyceae*), and the golden algae (*Chrysophyceae*).

Green algae can be macroscopic or microscopic organisms with a great morphology variability, including coccoid, unicellular or colonial flagellates, multicellular or multinucleated filaments and their main storage compounds are, starch (a-1,4-linked glucan) and oil. Green algae species such as *Haematococcus pluvialis,* are an important source of astaxanthin; *Dunaliella* spp. are an excellent source of β-carotene and *Chlorella vulgaris* has a value as a food product or ingredient [9].

Red algae or *Rhodophyta* lacks flagellate cells. The cell walls are composed of cellulose and xylan, polysaccharidic mucilages like agar or carrageenans (used in hundreds of food products including ice creams, beer, pâté’s, shampoo, soy milk, and pet foods) [9]. These algae possess chlorophyll a and d, phycobiliproteins (phycoerythrin and phycocyanin), and floridean starch (a-1,4-linked glucan) as storage products. *Porphyra umbilicalis* is one of the most consumed red seaweeds around the world, also known as Nori [9].

Representing the largest group of phytoplankton with more than 100,000 species [16], diatoms contain polymerized silica in their cell walls, and they often accumulate lipids and a storage polysaccharide named chrysolaminarin (b-1,3-linked glucan). Their colour comes from the mixture of the green colour of chlorophylls a, c_1 and c_2 with brown and yellow pigmentation of fucoxanthin and β-carotene. Diatoms are mainly used in aquaculture due to their significant amounts of PUFAs [9].

The *Chrysophyta* or golden algae are similar to diatoms in terms of pigmentation, oil and carbohydrates production. These microorganisms are distinguished by the presence of a nucleus and separate organelles for photosynthesis and respiration [16].

2.1.2. Chlorella vulgaris

Chlorella species are microorganisms with a wide range of physiological, biochemical and morphological characteristics which can be placed in four groups: spherical, ellipsoidal, spherical or ellipsoidal, and globular to subspherical cells [9]. Their reproduction is asexual, and occurs with the increment of cell size, followed by the division of the chloroplast until four cells are formed. Then the daughter cell wall matures and divides into four new cells, which are expelled with the rupture or dissolution of the parental walls.

C. vulgaris is a spherical microorganism with a size that varies from 2 to 10 μm [17]. They contain a cell wall with approximately 2 nm of thickness (when mature can reach 21 nm), a mitochondrion made up of double membranes, proteins and phospholipids, and a single chloroplast, composed of phospholipids, where starch granules can be synthesized. When cells are in state of death their inner colour becomes darker (figure 1).

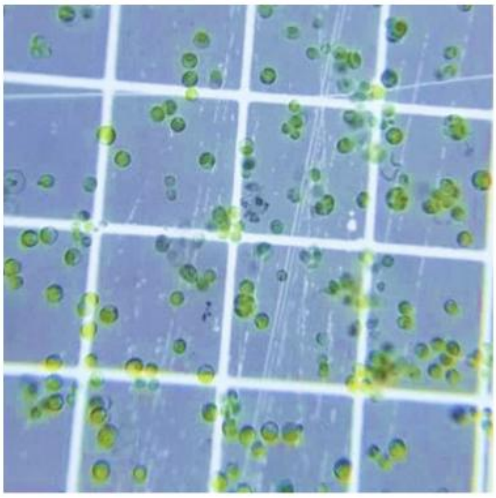


Figure 1 – *C. vulgaris* under the microscopic [17]

Chlorella vulgaris has an important nutritional value due to their ability to synthesize essential and non-essential nutrients, such as proteins, lipids and carbohydrates, as well as some pigments resulting from secondary metabolisms. The chemical and nutritional composition may vary according to the growing conditions. However, previous studies have established values of its constitution, which can be described in Table 2.

Table 2 - Chemical composition of *C. vulgaris* biomass according to two different studies.

Source	Components (%)							Reference
	Moisture	Dry Matter	Proteins	Carbohydrates	Lipids	Fiber	Ash	
Study 1	5.83	94.17	51.45	11.86	12.18	9.18	9.50	*C. vulgaris*, grown in a 1200 L Zigzag Photobioreactor washed using tap water [18]
Study 2	7.04	92,96	51	13.4	12.1	-	7.2	*C. vulgaris* grown in Net House Photobioreactor using sugarcane press mud waste [19]

The variation in cell composition differs according to the culture conditions under which the microalgae species is grown. For example, *Chlorella* spp*., Botryococcus braunii,* and *D. salina*, classified as *Chlorophyceae* species, show a typical biochemical composition, under favorable environmental conditions, of 30–50% proteins, 20–40% carbohydrate and 8–15% fatty acids. These three species, have the capacity to accumulate up to 80% of fatty acids, 80% of hydrocarbons, and 40% of glycerol, respectively, on the basis of the dry weight [9].

Due to a wide variety of microalgae species, many analyses of the chemical composition have been published with estimated data collected from different studies. Table 3 contains an overview of estimated data for *C. vulgaris* compared with some other microalgae species and other conventional foods.

Table 3 - General composition of different algae strains and animal origin food sources (% of dry matter) [9]:

Commodity	Protein	Carbohydrates	Lipids
Chlorella vulgaris	51-58	12-17	14-22
Anabaena cylindrica	43-56	25-30	4-7
Chlamydomonas rheinhardii	48	17	21
Dunaliella salina	57	32	6
Spirulina maxima	60-71	13-16	6-7
Synechococcus sp.	63	15	11
Meat	43	1	34
Milk	26	38	28

C. vulgaris possesses a total protein content between 42-58% of dry biomass weight. [17]. According to several studies, *C. vulgaris* contains a large amount of essential aminoacids, especially glutamic acid ($C_5H_9NO_4$) and aspartic acid ($C_4H_7NO_4$), representing a proximately concentration of 10% of the total daily protein intake [20]. L-aspartate is a glycogenic amino acid, with the capacity to promote energy via its metabolism during Krebs cycle and it's associated with the nervous system function, more precisely, related to memory and cognitive process [21]. Glutamic Acid ($C_5H_9NO_4$) is the most widespread neurotransmitter in brain function, and a precursor of the inhibitory neurotransmitter GABA, promoting a reduction of neuronal excitability, as well as glutamine, a potential mediator of hyperammonemic neurotoxicity. Also, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine are the main essential aminoacids identified in this microalgae. These aminoacids are not synthesized by humans so, incorporating these microalgae into foods is an excellent approach to increase other foods nutritional value.

Many microalgae species have a wide variability regarding lipid profile, with a high concentration of long-chain fatty acids, with most of them carrying 18 carbon atoms. *C. vulgaris* lipids constitution can vary from 5 to 40% of the total biomass dry weight, reaching up to 58% when grown under unfavorable environment conditions [20]. Around 70% corresponds to saturated fatty acids (SFA), 16% corresponds to monounsaturated fatty acids (MUFA) and finally 8% corresponds to PUFA [20]. Glycolipids, waxes, hydrocarbons, phospholipids and fatty acids are synthesized in the chloroplasts and are directed to different organelle cell walls, such as the mitochondria and the chloroplast itself.

Omega-3 fatty acids – EPA and DHA

C. vulgaris cell walls contain cellulose, which glycosidic bond is linked via β-(1→3), forming a bioactive compound called b-1,3-glucan [9]. This polysaccharide has an immunostimulator activity, that helps reduce lipids from blood and has a free radical scavenging function, leading to an increment of its studies and research related to human health and clinical effects, such as, anti-tumor effects.

2.1.3. *Arthrospira Platensis* (spirulina)

A. platensis is a cyanobacteria found in alkaline, brackish and saline waters, with the capacity to form massive blooms. Arranged in an open helix enclosed in a thin mucilaginous sheath (Figure 2). Composed of multi-layers of glucan and peptidoglycan polymers, the central region contains a number of carboxysomes, ribosomes, cylindrical bodies, and lipid droplet. The cell periphery contains gas vacuoles and several other sub-cellular components. *A. platensis* reproduction appends with the fragmentation of a mature trichome into a number of shorter segments. The destruction of multiple cells results in segments of a few cells, that later, increase in length by binary fission assuming the helical shape [9].

Figure 2 - Microscopic image of a living *A. platensis* in helical cell form [22].

Spirulina contains over 100 nutrients, making it one of the most complete food sources in the world [23]. Its cell walls are composed of protein, carbohydrates and fatty acids. Spirulina´s chemical compositions, just like any other microalgae, depend on the growth conditions, more precisely with cultivation period in relation to sunlight period. In Table 4 we can observe the chemical composition of spirulina estimated in 3 different studies:

Table 4 - Chemical composition and physical properties of spirulina powder (g/100 g sample, on dry weight basis) from 3 different studies:

Source	**Components (%)**							**Reference**
	Moisture	**Dry Matter**	**Proteins**	**Carbohydrates**	**Lipids**	**Fiber**	**Ash**	
Study 1	6.98	93.02	56.79	13.60	8.33	4.25	10.05	*A. platensis* from National Research Center, Cairo [1]
Study 2	5.37	94.63	61.57	3.47	7.19	7.93	7.10	*A. platensis* from Aquaculture Research Center at Arab Academy for Science, Technology & Maritime [24]
Study 3	11.20	88.80	62.60	6.06	6.70	-	14.90	*A. platensis* grown in a autotrophic photobioreactor and inoculated in a raceway open pond (Allmicroalgae SA,, Portugal)

Spirulina shows a high proportion of essential amino acids, with approximately 60% of its total dry weight. It is also an excellent source of phycobiliproteins, a coloured and water-soluble biliprotein which encompasses allophycocyanin, phycocyanin and phycoerythrin. These pigments, along with chlorophyll a, β-carotene, flavonoids and other pigments, contribute to an antioxidant activity up to 39.2% [25]. *A. platensis* is the richest algal source of gamma-linolenic acid (GLA) with values around 24.58 mg/100 g of dry biomass [25], an omega-6 fatty acid with the ability to decrease low-density lipoproteins in hypercholesteremic patients, and showing good results in atopic eczema treatments. It is also extremely rich in minerals, such as calcium and iron, and vitamins, offering exceptional health benefits, supporting child growth, preventing blindness and eye diseases and helping in cases of general weakness. Rich in vitamin E (60.0 mg/100g of dry biomass) [26] and vitamin B12 (193 µg/10 g of dry biomass), it becomes an excellent alternative to people who follows vegan diets that lack vitamin B12 [27].

Recently, spirulina has been classified as a super-food, more potent than any other food source, such as plants, grains or herbs. Spirulina turns out to be a great source of vitamin, with a wide range of use in food and feed industry, due to its nutritional value and the compilation of all its benefits described above.

2.1.4. Bioactive compounds

Bioactive compounds can be defined as chemical compounds that can exert a specific physiological or metabolic function on human body, besides their nutritional one, providing targeted health benefits [28].

Bioactive compounds include a wide variety of compounds with the ability to provide functionality to foods, such as non-digestible carbohydrates (soluble and insoluble fibers), antioxidants (such as polyphenols, carotenoids, tocopherols, tocotrienols, phytosterols, isoflavones, organosulfur compounds), plant steroids and phytoestrogens [1].Microalgae can biosynthesize, metabolize, accumulate and secrete a great diversity of metabolites, many of which are valuable substances with potential applications in food, pharmaceutical and cosmetics industries [29].

Pigments

Besides chlorophylls (representing 1 to 2% of the biomass dry weight), carotenoids are also usually present. With colours ranging from yellow to red, they are isoprenoid polyene pigments derived from lycopene [30]. β-carotene, essentially present in the chloroplasts of some microalgae species, has high demand in the market as a natural food colouring agent and it can be used in soft-drinks, cheeses and butter or margarines, among several other food products [7].

Different pigments as astaxanthin, canthaxanthin, lutein, pheophytin a and pheophytin b and violoxanthin are also common in some macroalgae species as *C. vulgaris.* These pigments can be extracted through different classical approaches such as maceration or Soxhlet extraction, or innovative ones, as ultrasound-assisted extraction or microwave, using different solvents [9]. Their main interest comes from their antioxidant activity [9]. Some studies also suggest the presence of polyphenols, such as luteolin [13], which is reported in literature having the potential to inhibit cytokine expression, nuclear factor kappa B (NFkB) and TLR4 signaling at micromolar concentrations in immune cells, including mast cells [13]. Due to their antioxidant potential, high sustainable production and renewable nature, microalgae-derived pigments are expected to surpass synthetics as well as other natural pigment sources [31].

Astaxanthin

One of the most important antioxidant activity pigments found in *A. platensis* is astaxanthin. This is a rosy-coloured pigment accumulated in lipid vesicles outside the chloroplast. Under stress, β-carotene is converted into astaxanthin trough β-carotene oxygenase (BCO1) and β-carotene hydroxylase (BCH) [19]. This pigment is water-soluble and possesses an antioxidant activity 10 times higher than other carotenoids such as zeaxanthin, lutein, canthaxanthin and β -carotene, and over 500 times greater than α-tocopherol [9]. Studies have confirmed the presence of this component in *A. platensis*, revealing effective radical scavenging and singlet oxygen quenching properties, enhancing the immune system, and preventing diseases like cancer, diabetes, high blood pressure, and protecting skin from the damaging effects of UV radiation [9].

Phycobiliproteins

Phycobiliproteins are a deep coloured water-soluble fluorescent pigments, a major component of photosynthetic light-harvesting pigments named phycobilisomes [31]. The main natural resources of phycobiliproteins are cyanobacterium Spirulina for phycocyanin (blue) and rhodophyte *Porphyridium* for phycoerythrin (red).

Phycocyanin is currently used as a natural colouring, in chewing gums, candies, dairy products, jellies, ice creams and soft drinks, in countries like Japan and China. A selective number of investigations revealed some pharmacological properties of phycocyanin including, antioxidant, anti-inflammatory, neuroprotective and hepatoprotective effects [30].

2.1.5. Nutritional and functional properties of microalgae

The production and interest of algae for the food and feed market has increased in the last 70 years, partially due to their nutritional composition [16]. Algae contain lipids, carbohydrates and proteins, making them an important food source for the growing population. Microalgae are also rich in bioactive components such as PUFA, pigments, vitamins and antioxidants, which can be an element that promotes consumers health. Besides, algae are used as food, feed and fertilizers for centuries, especially in Asia. In fact, during the first half of the 20th century, a Japanese philosopher, named George Ohsawa, popularized the notion of a macrobiotic lifestyle movement in Europe, introducing to Westerns diet, macroalgae like Nori (*Porphyra* sp.) and Wakame (*Undaria pinnatifida*), amongst many other algae species [32]. A search for biologically active substances from algae began during the 1950s, post that the commercialization of microalgae species started with *Chlorella* species in the 1960's followed by spirulina in the 1970's [16]. Beyond basic nutrition, microalgae possess functional properties that offer a range of health benefits, aligning with current consumer demand for functional foods. Some key health-promoting properties include antioxidant potential, anti-inflammatory effects, immune system support, lipid metabolism and cardiovascular health and potential anticancer properties.

The European Union has recently created strategies for the development of the European Bioeconomy, which stands in the use of biological resources to cover the demand of energy, industrial and food and feed sectors [33]. Due to its vast applications in different fields, algae become an illustrious resource that can respond to the concerns with resource management [33].

Microalgae's nutrient density and health benefits have made them popular ingredients in functional foods and dietary supplements such as smoothies, snack bars, and beverages [21]. However, an emerging interest in incorporating microalgae into more diverse products is emerging, namely in the development of confectioneries like gummies. Due to their natural pigments, microalgae also contribute as colouring agents to foods, adding visual appeal along with nutritional benefits.

However, challenges exist in incorporating microalgae in food products. Their characteristic taste and odor can be challenging to mask, particularly in formulations where taste is a key factor. Advances in microencapsulation and flavour masking techniques offer promising solutions for integrating microalgae into various foods without compromising sensory qualities [34].

2.1.6. Previous studies on food fortification with microalgae

Due to their rich nutritional composition and bioactive properties, microalgae have been successfully integrated into diverse food matrices, from snacks to dairy products [35]. Emerged as an innovative approach to enhance the nutritional profile and add functional properties to staple foods, the fortification with microalgae have been developed presenting a considerable number of studies in food innovation and the impacts of microalgae addiction nutritional, sensory and texture wise [5]. Microalgae such as spirulina and *C. vulgaris* have been added to dairy products, including yogurts, cheese and milk to increase protein, essential fatty acids, and antioxidants content [31]. Bakery products are generally well-suited for fortification with microalgae due to their stable matrix but the high temperatures involved in baking, which can inactivate unwanted microbial content, without significantly affecting the bioactive content provided by microalgae [31]. The snack and beverage industries have also shown interest in microalgae as a functional ingredient, developing protein-rich and antioxidant-enriched products [26]. And emerging as a rehearse area, the microalgae incorporation into confectionery products, including gummy candies, chocolates, and energy bars, offer an attractive delivery system for microalgae due to their pleasant taste, texture, and consumer appeal. Studies on microalgae-fortified gummy candies have demonstrated potential use as a delivery system for bioactive compounds in food products. Microalgae such as spirulina can add nutrients like protein, vitamins, and antioxidants to gummy candies, achieving a balance between nutrient enhancement [35]. However, adding microalgae can compromise consumer's acceptance, sensory wise, as high levels of microalgae may alter the colour and candies flavour profile. Encapsulation and flavour-masking techniques have been explored in order to improve the sensory properties and, at the same time retaining the health benefits provided by the fortified candies [5]. Consumer's perception plays a significant role in the success of microalgae-fortified foods. While the health benefits of microalgae are widely recognized and become one of the keys to improve consumer acceptance, their sensory characteristics—such as taste, colour, and odor—can become the most important factor affecting consumer's acceptance. Consumers tend to respond positively to microalgae-fortified foods when the algae concentration is quite low and when the product aligns with consumer preferences for natural, plant-based ingredients [21, 35].

The encapsulation of microalgae properties in gummy formulation is an innovative way of bringing these properties and their benefits to the consumer, with a recent growing interest for the food science community. Although it is a topic with an innovative character, some studies have recently emerged developing gum formulations with the addition of microalgae, which are analyzed in terms of their physicochemical properties, texture, pigments, coloring and sensory properties, and have shown a considerable potential as a functional confectionary product aimed at health-conscious consumers. A group of scientist from Turkey have developed gummy formulations with the incorporation of *Porphyridium cruentum*, *Dunaliella salina*, and *Chlorela vulgaris*, with concertation ratios from 0.10 to 0.50 g/100g of each algae [36]. This study concluded a significant influence in the gummy physicochemical and textural properties by the added microalgae. An enhancement of the pigment concentration with the increment of microalgae. In terms of texture, favorable changes in parameters such as cohesiveness and chewiness. And according to the sensory tests some differences were noticeable, for the enriched gummies compared to control, in terms of colour, aroma and odor, with flavor, aroma and visual representing the biggest limitations in consumer's acceptance.

Another study with gummy candies enriched with açaí and spirulina contributed to strength the speculations about the effect of microalgae potential for the increment of protein, lipids, mineral and phenolic compounds concentration, compared to control gummies [37].

2.2. Functional foods and market trends

There is no universally accepted concept for "functional foods, as each country and culture have their own regulatory frameworks. This concept was first developed in Japan in the 1980's with the escalating health care costs, and latter resulting in the creation of a regulatory system, by the Ministry of Health and Welfare, to approve certain foods with health benefits in hopes of improving the health of the aging population [5].

Functional foods can be generally described as food or food ingredient that provide health benefits, such as enhancing immune function, supporting gut health, or offering anti-inflammatory effects, through the incorporation of bioactive compounds such as vitamins, minerals, dietary fiber, or probiotics [5, 6, 26]. Functional foods fall under multiple categories, including probiotics, prebiotics, dietary fiber, bioactive peptides, antioxidants, and fortified or enriched products. Modified or enhanced food with such components offer additional physiological benefits, potentially reducing disease risks or supporting health functions [38].

The scientific evidence and research efforts have led to an increase of global interest in this specific food category leading to a shift in consumer priorities, driven by greater awareness of health and wellness. Consumers are recently in pursuit over preventive health solutions through diet, giving rise to the functional food industry, with a potential market expansion aligned with development of new products and discover of new health benefits provided by these food sector [38]. The functional food market has shown consistent and relevant growth over the past two decades. Recent reports show an increment for the global functional food market size, estimated at over $300 billion, in 2023, with projections indicating continued growth driven by evolving consumer preferences and technological advancements in food processing and bioactive delivery [24]. Fortified dairy products, functional beverages, dietary supplements and confectioneries such as gummy candies are some of the products with major interest to explore among many other included in this market [39]. The global gummy market, encompassing both conventional and functional gummies, has reached a multimillionaire-dollar valuation and is projected to continue expanding [24]. North America and Europe represent the largest markets, with significant growth occurring in Asia-Pacific regions, where urbanization and disposable income are rising. Analysts predict a compound annual growth rate (CAGR) for the gummy market of around 5-8% over the next few years. Due to the appealing characteristics of functional gummies as alternatives to traditional supplements, the functional gummies market shows and even stronger and expansive trajectory [24].

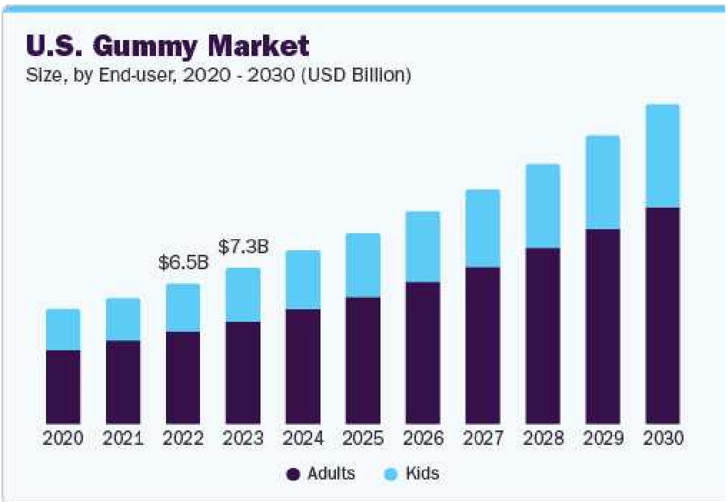


Figure 3 - Collected data and estimated variation from 2020 to 2030, of Gummy Market size by end-user [24].

North America region has shown big results in terms of functional and fortified gummy products, contributing 37.4% of the global revenues in 2023 [24]. Asia and the Pacific gummy market is also expected to expand at a CAGR of 12.9% from 2024 to 2030 [24], with regional gummies producers responding to consumer’s demand for new healthy alternatives whose flavours can be appealing to the local population.

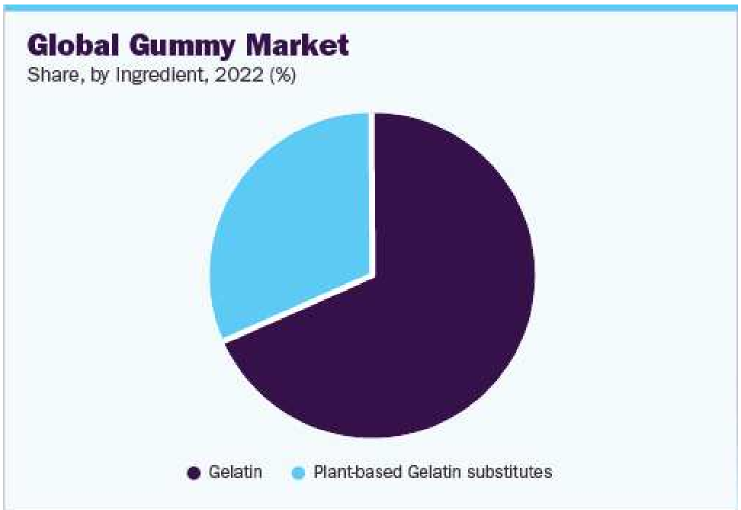


Figure 4 - Percentage of Market demand shared by gelling agent (gelatine vs plant based substitutes) [24].

Market studies highlight distinct preferences within the gummy segment, showing strong age- and demographic-based variations. For instance, younger favour gummies with vibrant colour, fruity flavours, and sweet-sour profiles, while older consumers prioritize products with clean labels, natural ingredients, and lower sugar content [38]. This has led to an innovation need and creation of formulation, ingredient sourcing, and production technologies to respond to specific demographic needs, like sugar-free or low-calorie options, influencing the jelly gummy market.

Due to a higher interest on natural plant-based ingredients, particularly searched among health-conscious and ethically motivated consumers, the gummy market players are introducing vegan or plant-based gummy products that use pectin, agar or any other plant-based gelling agent in place of gelatine [40]. The increase of demand for natural products, the convenience and On-the-Go Products, personalized nutrition and targeted health benefits and the growing Interest in immune-boosting are current trends responsible for foods shape the functional food market [40].

The functional confectionery, particularly gummy candies, market growth benefits from the gummies appealing texture, ease of consumption, and versatility as a delivery vehicle for various bioactive compounds, that consequently are used to deliver a range of nutrients, such as vitamins, minerals, and plant-based compounds [40]. The younger consumers, who find traditional pills and tablets unappealing, are especially attracted by the candy-like appearance and chewable format of gummies making them a favorable option for delivering bioactive compounds.

2.3. Jelly Gummies

Functional gummies are now occupying a distinct “niche” in the health and wellness market with the addition of new products incorporating probiotics, adaptogens, and plant extracts, including microalgae and macroalgae [41]. Microalgae, for example, offers a promising solution, by providing a nutrient-dense, sustainable ingredient that aligns with consumer interest in plant-based functional foods.

Although the functional gummy market presents a promising future, the regulatory constraints, stability of bioactive properties during processing, and consumer perception of added health ingredients in traditionally indulgent foods, represent some of several challenges faced by the functional food sector [41]. Being so, the continued research for new products and technological advances will be essential to optimize the stability and sensory properties of functional ingredients in products like gummies, expanding market opportunities.

Some market players are already seizing the opportunity and exploring the functional market (table 5) bringing to consumers a vast diversity of new gummy products with different properties and health related functions (Figure 5).

Figure 5 - Functional gummy brands found in stores with different properties and functions.

Table 5 - Example of key gummy market players. Left: jelly gummy companies. Right: functional jelly gummy companies [31].

Jelly Gummy Companies	Functional Jelly Gummy Companies
Albanese Confectionery Group	JellProcaps Group
Arcor SAIC	Santa Cruz Nutritionals
Ferrara Candy Company	Amapharm
Haribo GmbH & Co. KG	Herbaland Naturals
Jelly Belly Candy Company	

2.3.1. Jelly gummies ingrediente list

When analyzing the ingredients of leading commercial gummy brands, some common components include glucose syrup, sugar, gelatine, corn starch, vegetable oil, acidity regulators, artificial fruit flavours, emulsifiers, glycerin, and artificial colourants. Gelatine and sugar are two key components that directly impact the texture and mouthfeel of the gummy [26]. Altering their concentrations, or substituting them with alternative ingredients, can significantly affect the texture. For example, reducing sugar content may lead to a firmer texture, which could be less appealing to consumers familiar with a softer and more elastic gummy. Additionally, gelatine, a protein derived from collagen, is essential in giving gummies their characteristic bounciness. Substituting it with a plant-based ingredient, such as agar or pectin, can change the texture and may lack the traditional chewiness and resilience [42]. Sugar alternatives, like stevia, erythritol, and fiber-based sweeteners, are also under consideration to lower calorie content and reduce glycemic impact, though each of these may affect the sweetness and texture in different ways [26, 42].

Based in all the previously presented contra-positions, the growing consumer demand for products that are low in sugar, free from artificial additives, and made with natural or plant-based ingredients becomes one the industry's main challenges in producing gummies creating healthier, more natural formulations without compromising texture and/or flavour. In Figure 6 it's possible to observe the ingredients list of one of the most popular brands of gummy candies in the world.

Figure 6 - Haribo Gummy Bears Ingredients List [43]

Glucose syrup

Glucose syrup is made by breaking down glucose (also known as sugar) molecules in starchy foods such as wheat or corn through hydrolysis, a chemical reaction that essentially results in a concentrated, sweet liquid with a very high glucose content [43]. This ingredient is the one used in the largest quantity for gummy production and one of the three most important for favour and texture control as a sweetener and thickener.

Sugar:

Sugar is a general term for soluble, sweet-tasting carbohydrates, many of which are used in food. Excessive sugar consumption can lead to a number of health problems, including an increased risk of diseases such as type 2 diabetes, obesity, cardiovascular disease and dental problems. One serving (13 gummy bears) contains 14 grams of sugar, or three and a half sugar cubes. That may not be an exaggeration, but it can certainly add up, especially if you eat more than 13 gummy bears in one sitting (which happens to most people) [43].

Gelatine

Gelatine consists of an amphoteric polymer derived from collagen by alkaline, acidic or thermal hydrolysis [44]. These treatments cause the disruption of the structural organization of collagen, leading to the loss of its conformation to varying degrees.

Commercially, there are two types of gelatine, Type A and Type B. Type A cationic gelatine, results from the partial acid hydrolysis of collagen. During this treatment, the amide groups of glutamine and asparagine are converted to carboxyl groups, resulting in a shift in the isoelectric point of the protein to higher values (pI = 7–9) [44]. type B Anionic gelatine, is derived from the alkaline treatment of collagen. The consequent increase in carboxyl groups makes gelatine type B negatively charged, with a lower isoelectric point (pI = 4.7–5.5) [44].

Another important physical property of gelatine is its viscosity, which depends on concentration, temperature, and pH. In fact, viscosity has been found to increase with polymer concentration and decrease with temperature and pH. Thermal stability of gelatine is another important parameter that is influenced by several parameters such as polymer concentration, molecular weight distribution, and Bloom value. However, the melting point of gelatine is generally found in the range of 28–31 °C for mammalian-derived gelatine and in the range of 11–28 °C for fish-derived gelatine [44].

Although, some consumers are now moving towards plant-based foods, diets in order to achieve a healthier lifestyle, it is impossible to deny the important role of the gelatine usage in these type of candy products and its potential as a carrier for many types of bioactive compounds and its ability to tune and control the release kinetics of selected drugs [43].

2.3.2. Jelly gummies production equipment

The production of jelly gummies has a long-standing history, primarily due to the simplicity and popularity of its basic process. Traditionally, the gummy production begins by mixing gelatine or any other gelling agent, sugar, flavourings, and colourings, and placing this mixture to heat until it becomes a homogeneous liquid. Right away this product is poured into molds to cool and set for a few hours until it reaches the signature chewy texture that defines a classic gummy [45].

As the food industry continues to innovate in response to health trends, the gummy production process is evolving. New formulations are tested to maintain the traditional gummy experience while meeting health-conscious consumer demands. Plant-based alternatives to gelatine, such as pectin, carrageenan, or konjac, are gaining popularity to cater to the vegan and vegetarian markets, though they often require careful formulation adjustments to achieve a similar chewiness.

2.3.2.1. Large scale production systems

The quality and safety of gummy manufacture is heavily reliant on a well-structured and controlled process. The use of the right equipment and proper manipulation prevents the appearance of malformations and inconsistencies in the final product. A large-scale production of jelly gummies is separated into the following stages: mixing and cooking, molding, cooling and demolding, and coating. Sometimes, gummies need to be placed in a drying room, with temperatures not exceeding 26ºC [46], to remove excess moisture, reducing the risk of bacterial growth and spoilage risk.

1º - Mixing and cooking

To begin the gummy production, ingredients are added to a cooking vessel (Figure 7), according to the intended purpose, and heated until the mixture presents a translucent appearance without sugar crystals. The humidity levels must be controlled and maintained between 35-40% [46] to avoid drying or stickiness, ensuring a production time of 6-8 hours. Using a cooking vessel in this stage is essential, considering the integrated monitored cooking and mixing system along with scales, which allows a higher automation and process control. The figure below represents one example of these machinery structure types:

Figure 7 - JellyCook™ batch cooker. 50kg/hour ServoForm™ Mini depositor.

2º - Molding

Using a depositing unit, the mixture is dosed and dropped into the selected molds to set and be sent to the cooling stage. There is a wide variety of molds used in gummy manufacture, such as corn starch molding systems, silicone molds, Teflon aluminum molds, polycarbonate molds and plastic molds. The corn starch technique is summarized by placing powdered corn starch in a metal tray, then molds are pressed over the powder leaving the marks on the tray where the mixture will be poured. In large-scale production, this technique results in an increment of expenses, by adding more inputs into the production line. Being so, silicone and plastic molds are preferred. In these cases, oil needs to be added into the molds to prevent the gummies from stuck, therefore some depositors allow the attachment of an auto oil spraying system to facilitate the demolding process. In Figure 8, an example of a starch-free molding depository unit with a maximum output of 1900 kg/h and a maximum speed of 55 stroke/minute, capable of producing solid, striped and layered products in either 2D or 3D formats:

Figure 8 - Baker Perkins‘ ServoForm™ Jelly depositors (3D/2D). 1900 kg/h.

3º step - Cooling and Demolding

After the molding step, the molds filled with the gummy mixture are forwarded to a cooling conveyor, where low temperatures are reached until the right texture is achieved. Inside the conveyor is incorporated a brush removal system to remove the solid gummies, dropping them into a polyurethane coating conveyor.

4º step - Coating

The coating involves adding a thin layer of sugar, carnauba wax, bee wax or vegetable oil, providing texture and flavour effects, while creating a protective layer around the gummy increasing shelf-life, preventing stickiness and controlling moisture levels. These processes can be executed with the use of sugar polishing pans, sugar standing drums or oil coating drums, where a spraying system drizzles the liquid or powdered coating on top of the gummies, which keep rotating inside the drum to cover evenly all the production. Figure 9 represents a continuous gummy production system with the capacity to produce up to 240,000 units per hour, perfect for a large-scale gummy production:

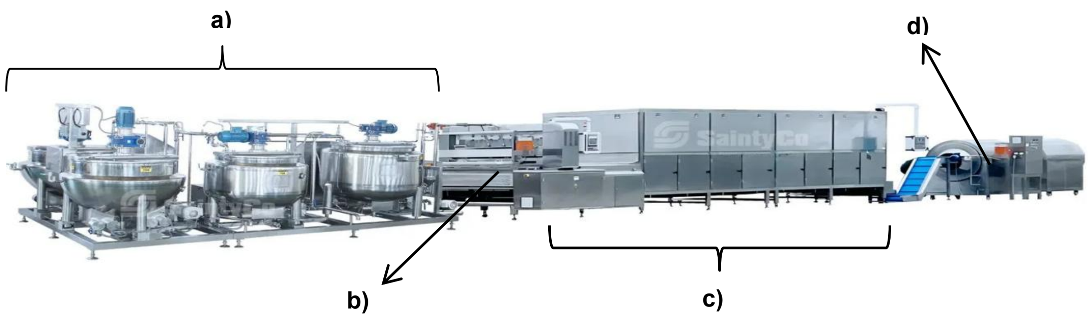


Figure 9 - G-600 Gummy Production Line: Semi continuous cooking system for both pectin/gelatine gummy (a); Depositing system king system with an auto mold lubrication system (b); Cooling tunnel (c); Coating system (d)

3. Materials and Methods

3.1. Ingredients, sourcing and microalgae selection criteria

Ultrapure water was obtained from Synergy®Water Purification System (Merck Millipore, Burlington, VT, USA). Methanol, DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), acetic acid, sodium acetate, TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), hydrochloric acid, iron (III) chloride hexahydrate, Folin–Ciocalteu reagent, sodium carbonate, gallic acid, sodium nitrate (III), aluminum chloride hexahydrate, sodium hydroxide, quercetin dihydrate, were purchased from MilliporeSigma (SaintLouis, MO, USA).

For the development of functional gummies, two different microalgae strains were used: Spirulina (Lot: PA03734), produced at December 6 of 2023 and distributed by the retail company M.C.H, S.A, with an expiration date of December 5 of 2025 (nutrition composition - Appendix I) and *C.vulgaris* (Lot: AKEY_0095_3), provided by Pagarete Microalgae Solutions, which expiration date is on May of 2026 (nutritional table -Appendix I). Pork gelatine, glucose syrup and citric acid were provided by Sosa Ingredients (Navarcles, Barcelona, Spain).

3.2. Formulation methods for gummies

Throughout the gummy development process, several formulations were developed with different combinations of the two microalgae strains with other ingredients until it was reached a final product with the desired texture, appearance, consistency and flavour characteristics (basic qualitative evaluation made only by the developers). After selecting the final basic formulation for the gummies, 1 batch of control gummies were prepared using 30 g of gelatine without the incorporation of microalgae, and six formulations of microalgae-enriched gummies were developed using both spirulina and *C. vulgaris,* incorporating 0.5%, 1.0% and 2.0% of total gummy dry weight. The amount of microalgae added was substituted for the same amount of gelatine used. Table 6 contains the list of ingredients for the different prepared formulas.

Table 6 – List of Ingredients and total weighing used in gummy development: control,; spirulina formulas with the exact concentration; and C. vulgaris with the exact concentrations:

Ingredients	Control	*Sprirulina*			*C. vulgaris*		
	0.0%	0.5%	1.0%	2.0%	0.5%	1.0%	2.0%
Glucose syrup	100 g	100 g	100 g	100 g	100 g	100 g	100 g
Sugar	80 g	80 g	80 g	80 g	80 g	80 g	80 g
Water	100 g	96.5	93	76	96.5	93	76
Gelatine	30 g	28.5 g	27 g	24 g	28.5 g	27 g	24 g
Citric acid	5 g	5 g	5 g	5 g	5 g	5 g	5 g
Spirulina	-	1.5 g	3 g	6 g	-	-	-
C. vulgaris	-	-	-	-	1.5 g	3 g	6 g
Total	315 g	311.5	308	291	311.5	308	291

3.2.1. Jellie Gummy formulation

Experiments included fortified gummies with *C. vulgaris* and with spirulina, using pork gelatine as gelling agent. Figure 10 shows the process for the gummy preparation, which is summarized as follows: the gelling agent (pork gelatine) was hydrated with water and left to set for 30 minutes. Meanwhile, a mixture of sugar and water was heated using a heating plate, until it reached a temperature between 115-120 ºC. The formulation remained in a water bath, reaching a temperature of 60°C, long enough for the complete dissolution of the rest of the formula ingredients. This mixture was left to set, and the formed foam was removed with the use of a spoon, preventing the formation of bubbles in the gummies, which is seen as a production defect. After complete homogenization, the formulation was transferred to previously oiled silicone mold trays resulting in gummies of approximately 5 g/each. These trays remained at room temperature for about 30 min until cooling to achieve an equilibrium, and after that they were placed in the refrigerator (over 4°C) for 24 h. Afterwards, they were removed from the moulds and stored in a closed container that was kept in the refrigerator until further analysis. The flowchart bellow, expresses the complete production process with all the involved stages, inputs and outputs:

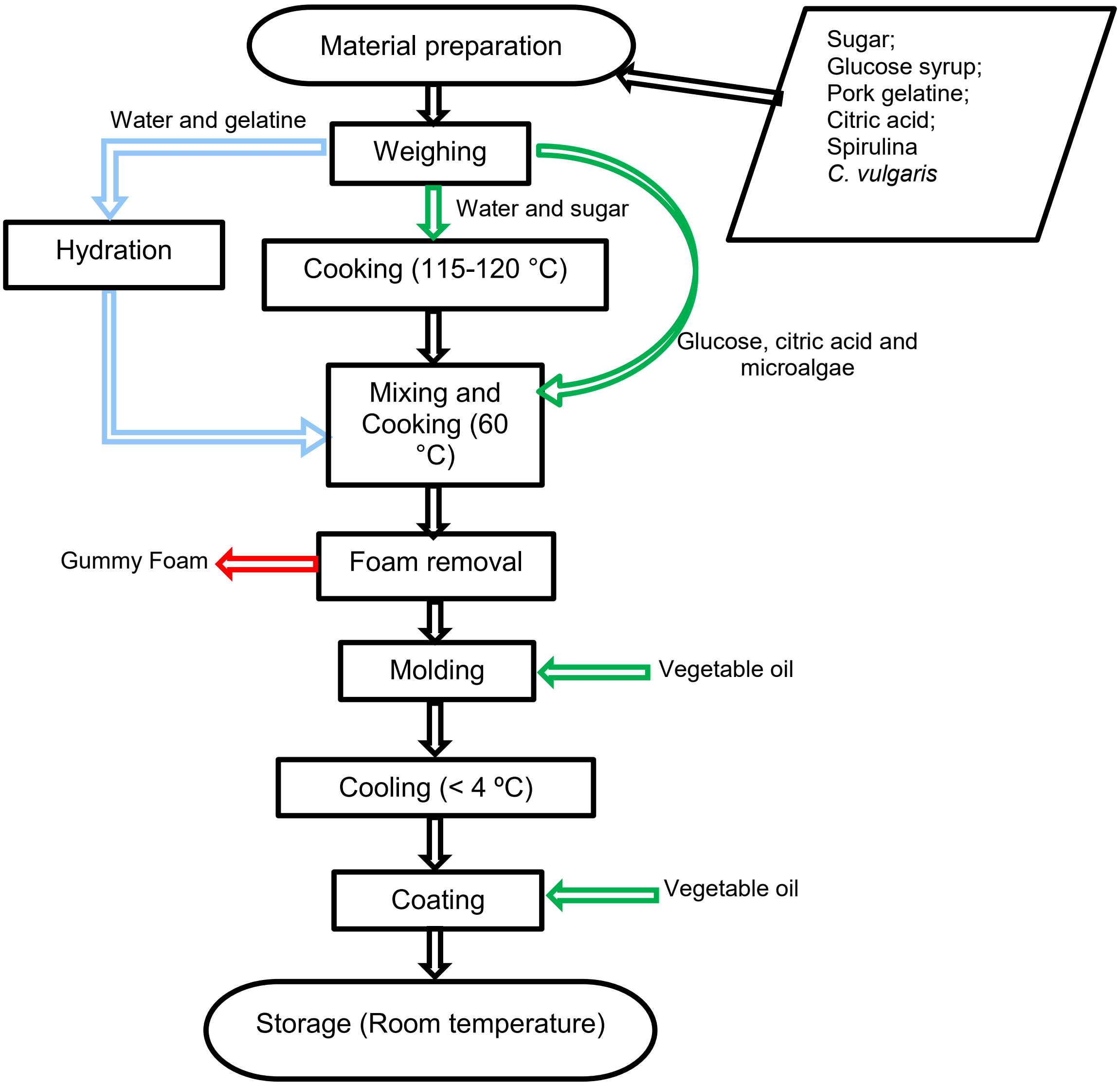


Figure 10 - Gummy jellies production flowchart with its inputs (→), outputs (→) and complementary processes (→).

3.3. Nutritional composition

The proximate composition included the determination of moisture, ash, minerals, protein, total fat, and carbohydrate content. The determination of each parameter was performed in triplicate, and the data were presented as mean ± SD. The moisture content was measured by weight difference after 24 h inside an oven at 100°. The total ash content was determined by incineration at 500°C in a muffle furnace. Fat content was determined following the Portuguese standard method NP4168. The protein content (N × 6.25) was estimated by the combustion method DUMAS, using a Vario EL elemental analyser (Elementar, Langenselbold, Germany). The analysis of minerals was carried out using ICP-OES. The carbohydrate content was determined by the difference between the protein, fat, ash, and moisture contents. All chemical and microbiological analysis methodologies were performed in accordance with Analytic Excellent International (AOAC) [47].

3.4. Bioactive properties

The ability to protect an organism against oxidative stress, preventing or delaying the arising of a vast number of diseases and increasing life quality, is one of the biggest benefits provided by antioxidants. To completely characterize a compound as antioxidant it is necessary to support the results on several methodologies. To evaluate the antioxidant potential of gummy jellies two methodologies were followed: the DPPH (2,2-difenil-1-picril-hidrazil) and FRAP (Ferric-Reducing Ability of Plasma) *in vitro* assays. DPPH method follows a simple principle where a synthetic radical is created and the capacity to eliminate or neutralize this radical is monitored using a UV/visible spectrophotometer [3], while FRAP assay, is based on the ability of a substance to reduce Fe(III) into Fe(II) [7]. The Folin–Ciocalteu method was used to assess the total phenolic content, an indicator of the antioxidant potential. All the bioactive properties were evaluated in gummies extracts prepared as follow: 5 g of each gummy where cut into tiny crumbles, suspended in 50.5 mL of an acidic solution (with 40 mL of methanol, 10 mL of water and 0.5 mL of HCl) and placed in a stomacher until it reached a homogeneity. The mixture was left in a rotating shaker and set to agitate overnight. Before each assessment, the samples were placed in an ultrasound bath, for at least 15 minutes, to facilitate the extraction of the desired compounds.

3.4.1. DPPH assay

The DPPH assay involved the use of 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH, Sigma-Aldrich, ≥99.0% purity, St. Louis, MO, USA), a nitrogen-centred free radical that changes colour from purple to yellow when reduced by a radical scavenger, forming DPPH-H [46]. Calibration was performed using several aqueous solutions of Trolox at known concentrations ranging from 0 to 1000 µmol L^{-1}, yielding a linear calibration curve ($y = 577.67x + 2.9753$, $r^2 = 0.9978$). The results were reported as milligrams of Trolox equivalents per 100 g of dry weight (mg Trolox/100 g dw).

For the assay, different dilutions of the initial extract and a Trolox stock solution (Sigma-Aldrich, ≥97.0% purity, St. Louis, MO, USA) at 0.2 mg/mL in methanol were prepared as in Table 7. Each 100 µL aliquot of the methanolic solutions was mixed with 3.9 mL of DPPH solution in methanol (24 µg/mL). The blank consisted of 100 µL of methanol added to 3.9 mL of DPPH solution. Following a 40 min incubation at room temperature in the dark, absorbance was recorded at 515 nm. The radical-scavenging activity of each sample was calculated by the DPPH inhibition percentage as follows: $I\% = [(Abs_A - Abs_{sample})/Abs_A] \times 100$, where Abs_A was the absorbance of the blank and Abs_{sample} was the absorbance in the presence of the extract at different concentrations.

Table 7 - Trolox solution concentration values for each point of the calibration curve, with volume values of Trolox (µL) per methanol (µL).

Point	[Trolox] (µM)	Volume of Trolox (µL)	Volume of methanol (µL)	Total volume (µL)
1	0	0	2000	2000
2	75	150	1850	2000
3	150	300	1700	2000
4	300	600	1400	2000
5	500	1000	1000	2000
6	600	1200	800	2000
7	700	1400	600	2000
8	1000	2000	0	2000

3.4.2. FRAP assay

The FRAP reagent [48] was prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine; Sigma-Aldrich, ≥98.0% purity, St. Louis, MO, USA) solution, and 20.0 mM $FeCl_3 \cdot 6H_2O$ solution in a 10:1:1 (v/v) ratio. An aliquot (90 µL) of either the extract or the standard was added to 3 mL of the FRAP reagent, and the mixture was incubated at 37 °C for 30 min. Absorbance was then measured at 593 nm against a blank consisting of distilled water. Calibration was performed using several aqueous solutions of Trolox at known concentrations ranging from 0 to 1000 µmol L^{-1}, yielding a linear calibration curve ($y = 14.356x + 0.0581$, $r^2 = 0.9976$). Results were expressed as µmol Trolox equivalents per gram of dry mass (µg Trolox/100 g dw).

3.4.3. Total Phenolic Content (TPC)

The total phenolic content in the microalgae extracts was assessed using a modified Folin–Ciocalteu method [8], which relies on the reduction of an ammonium molybdate complex by phenolics, producing blue-coloured reaction products. Gallic acid (GA) was used as a standard, and the experiment was performed in triplicate.

For the assay, a 150 µL aliquot of each extract, or the GA standard, was combined with 140 µL of Folin–Ciocalteu reagent (diluted 1:10 v/v) and 2.4 mL of deionized water and vortexed. After 3 min at room temperature, 300 µL of sodium carbonate solution (7.5% w/v) was added, vortexed again, and the mixture was incubated at room temperature for 2 h in the dark. The absorbance of the resulting blue solutions was measured at 725 nm against a blank containing only deionized water, using a UV-160A Recording Spectrophotometer (Shimadzu, Kyoto, Japan).

The same procedure was applied to prepare the GA calibration curve (absorbance vs. [GA] mg/L), using seven separately prepared stock solutions of GA at concentrations of 0, 50, 100, 150, 200, 250, and 350 mg/L ($y = 33.632x + 0.0135$, $r^2 = 0.9906$). Absorbance was measured in triplicate at 725 nm, using methanol as a blank. The total phenolic content was then expressed as mg of GA equivalent per gram of dry weight (mg GAE/g dw).

3.5. Colour evaluation

The colour evaluation was performed on the gummies surface using the portable CR-400 colorimeter. The results were expressed according to the CIEL*a*b* system, which chromatic parameters are: Luminosity (L*), with values between 0 and 100; the red-green axis (a*) with, +a (redder colour) for positive values and -a (greener colour) for negative values; and the yellow-blue axis (b*), with +b (yellow-er colour) for positive values and -b (bluer colour) for negative values.

This colour determination test consisted in the analysis of the L*, a* and b* parameters, over preservation time for each sample. Each sample was measured 15 times, with two preservation conditions: A (the day after production) and B (two months later, preserved in room temperature).

3.6. Texture Analysis

Texture is one of the main criteria that consumers use to judge a product acceptability. By biting any food product, a physical sensation is produced inside the mouth, allowing to have a basal argument to determine that specific food's quality [27, 49].

Based in the fact that sweet gummies can be made with different ingredients (e.g., sugar free, vegan, with fruit incorporation) and formulas, these products present a wide range of textural characteristics from soft to hard and to stick-to-your-teeth. However, gummies can be often described as a sweet and chewing product with a high capacity to return to its initial state after suffering from most types of deformation activities. To study these characteristics, a puncture and a TPA test were performed on 8 gummies of each formula, with an average volume of 5 mm^3, in two separate moments: after being removed from fridge storage and one day after production.

The puncture test was performed in a texturometer TA.XT.Plus from Stable Micro Systems (UK) with a capacity of 5 kg.f. A 2 mm diameter drilling probe was attached and was performed 1 perforation in the middle of each gummy, with the following test conditions: pre-test speed of 1 mm/s; test speed of 5 mm/s; post-test speeds of 5 mm/s; perforation distance of 75% strain; and a trigger force of 0,05 N. The measured properties resulted from this test were: Firmness (N) = Maximum peak force; Adhesiveness (N.s) = Negative area and Stickiness (N) = Absolute negative force.

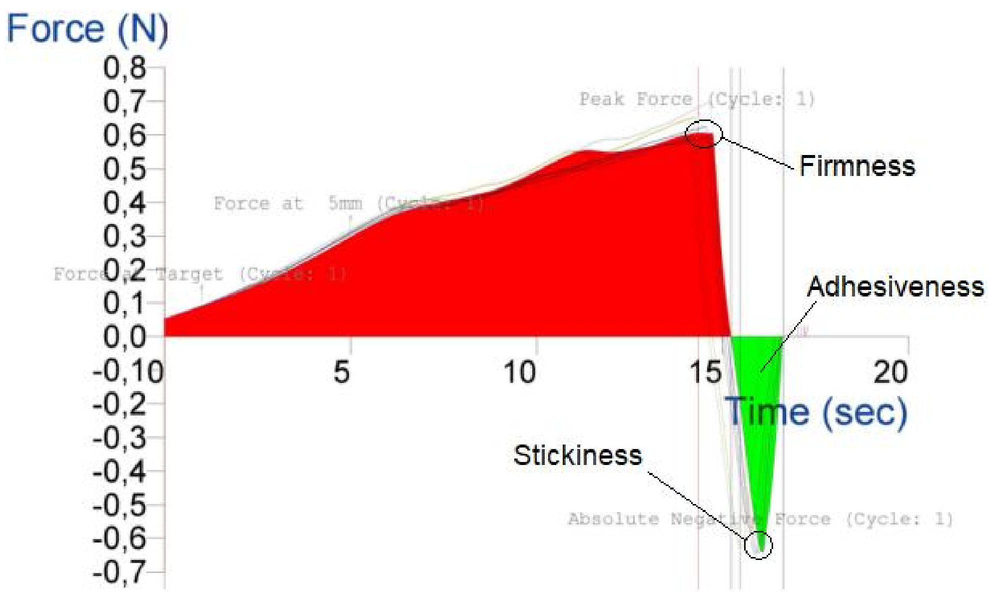


Figure 11 - Example of a texture curve that resulted from a puncture test with a p/2 probe on gummies with 1% spirulina. Red: Positive area; Green: Negative area.

For the TPA test, the same 8 gummies with the same temperature exposition conditions were placed in the same texturometer TA.XT.Plus from Stable Micro Systems (UK) with a capacity of 30 kg.f, and using a cylindrical probe with a 60 mm base diameter, was performed a two cycle compression test, spaced by a 5 second interval. The test conditions were: pre-test speed of 1 mm/s; test speed of 1 mm/s; post-test speeds of 5 mm/s; the compression distance was 75% strain; and the trigger force was 0,03 N. With the TPA test hardness, elasticity, resilience and chewiness values obtained using the following equations [27], related to figure 12.

$$\text{Hardness (N)} = \text{F1} \quad (5)$$

$$\text{Elasticity (\%)} = \frac{\text{T2}}{\text{T1}} \text{ x } 100 \quad (6)$$

$$\text{Resilience (\%)} = \frac{\text{A5}}{\text{A4}} \text{ x } 100 \quad (7)$$

$$\text{Cohesiveness (\%)} = \frac{\text{A2}}{\text{A1}} \text{ x } 100 \quad (8)$$

$$\text{Chewiness (N)} = \text{F1 x } \frac{\text{T2}}{\text{T1}} \text{ x } \frac{\text{A2}}{\text{A1}} \quad (9)$$

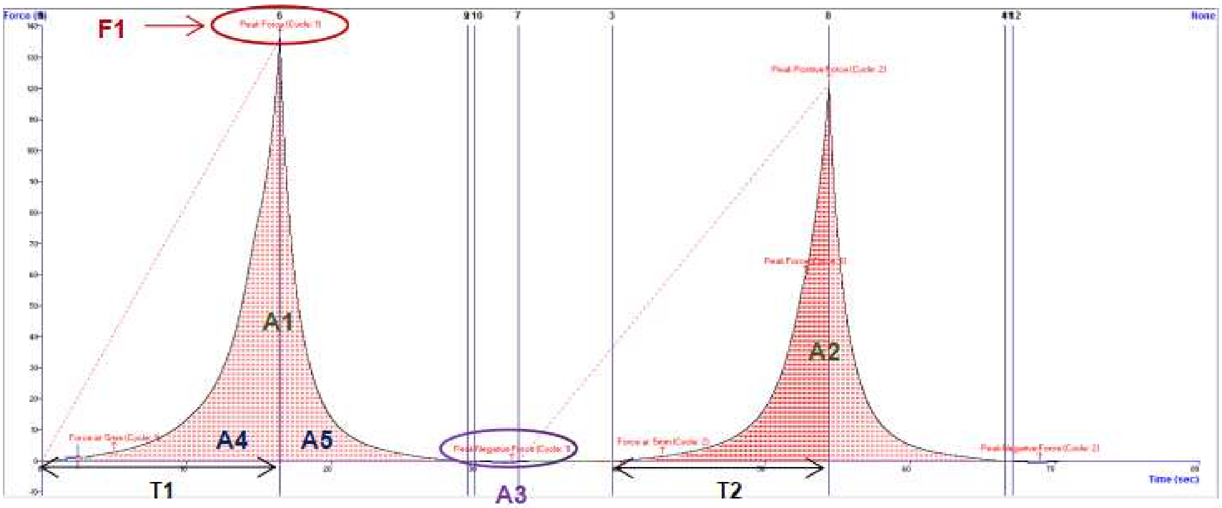


Figure 12 - Example of one texture curve resulting from a TPA test with a P/60 probe, on a 0.5% *C. vulgaris* gummy. F1: Peak positive force; A1: Positive area (Cycle: 1); A2: Positive area (Cycle: 2); A3: Peak negative force (Cycle: 1); A4: Area to positive peak (Cycle: 1); T1: Peak positive distance (Cycle: 1); T1: Peak positive distance (Cycle: 2);

3.7. Sensory evaluation

Sensory analyses were performed by a group pf 30 untrained panellists, inside a proper room with separated cabins to reduce panellist's interactions as much as possible. The samples were introduced in the booth trough a sliding window in front of the desk along with the respective test sheets, which can be found in Appendix II. The analyses were separate into two different days, one for the spirulina-enriched gummies and a second on for the *C. vulgaris*-enriched ones. Each sample was identified with one letter and two numbers, and displayed in concentration order according to Tables 8 and 9:

Table 8 - Displayed order and respective identifications for spirulina-enriched gummies sensory analysis.

1st Test (spirulina-enriched gummies)		
Order	Code	Microalgae concentration
1st	P	0.0%
2nd	A65	0.5%
3rd	B24	1.0%
4th	C44	2.0%

Table 9 - Displayed order and respective identifications for *C. vulgaris*-enriched gummies sensory analysis.

2nd Test (*C. vulgaris*-enriched gummies)		
Order	Code	Microalgae concentration
1st	P	0%
2nd	D51	0.5%
3rd	E39	1%
4th	F98	2%

The first part of the test consisted in the perception of different organoleptic characteristic between the control and the different microalgae-enriched gummies with different levels of incorporation (0.5%, 1.0% and 2.0%), using a multiple-comparison test, and at the end was performed a preference test, where the panellists had to ordinate the microalgae samples according to preference order. Integrity, colour, texture, scent and flavour, were the characteristics evaluated in the developed gummies, with a scale rate from “much more intact” to “much less intact” compared to control, for integrity, and “much more pleasant” to “much less pleasant” compared to control for the remaining characteristics.

Concerning the preference test, only the microalgae-enriched gummies were analysed and rated in ascending order of preference, from 3rd to 1st, placing the worst in 3rd place and the best in 1st.

3.8. Microbiology study

After a market study, it was verified that gummy products can be found in most stores, close to chocolates and other sweets. These products are commonly stored at room temperature and can reach a shelf-life of nearly one year, packaged inside polyethylene bags or polypropylene packages. Aiming to verify the storage capacity and longevity of jelly gummies manufactured in a homemade or a small-scale production, a microbiology study was performed for all the samples, according to Analytic Excellent International (AOAC) [47], and results were collected over two months.

Samples were prepared with 2 g of each gummy mixed with 20 mL of Ringer's solution (in duplicate for each gummy formulation) that was crushed for 5 minutes using a Stomacher MK1204 ITR until a homogenized appearance was obtained. Series of dilutions were prepared until 2-10 g/mL in Ringer solution. Then, 0.1 mL of each solution was spread at the surface of agar Sabouraud medium (in triplicate), and 3 plates with twice the concentration were also prepared, pouring 0.2 mL instead of 0.1 mL per plate. Plates were incubated at room temperature until visible growth was observed. The microorganisms' growth was monitored and counted, and the results were analysed. The inoculation process was performed three times: the day after production (T0) and in samples kept at room temperature collected after one month (T1) and with two months of storage (T2).

To identify a possible contamination source in the gummies, the main ingredients used in the formulations were also individually analysed (sugar, pork gelatine, citric acid, and glucose) by plating 0.1 mL of 1 g/mL solutions of each ingredient into the same media.

3.9. Statistical analyses

The statistical analysis of the results was performed in the software Microsoft Office Excel and Minitab Statistical Software 17.

To determine the existence of significant differences between adjusted least square means, was used an analysis of variance (ANOVA) using a Tukey–Kramer method test, also known as Tukey range test, with a confidence 95%. Values were considered significant when $p<0.05$.

4. Results and Discussion

4.1. Nutritional Composition

4.1.1. Moisture and Ash

The nutritional composition of the developed gummies showed a significant decrease in the moisture content, which may be due to the fact that the water content in the formulation process is reduced as the pork gelatine, previously hydrated, is also reduced (Figure 13). Although there is a tendency to decrease, the moisture content in the *C. vulgaris*-enriched gummies, the ones incorporated with 0.5% shows values of moisture close to control, and the spirulina-enriched gummies show no difference between each other with the level of microalgae incorporation for a static test ANOVA (p<0.005). In general, the gummies developed in this project show a moisture content of 30 to 36%.

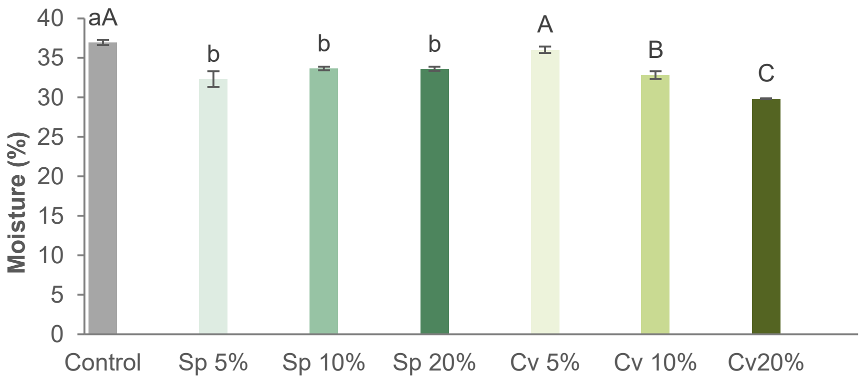


Figure 13 - Moisture content for the microalgae-enriched gummies. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

Regarding the ash content, which is very low (ranging from 0.2 to 0.3%), it is possible to observe a significant increase in the microalgae-enriched gummies with 2.0% of incorporation, when compared to the control (Figure 13). The same effect was observed in previous studies with the incorporation of spirulina in gluten-free pastas [50] and *C.vulgaris* in cookies [30]. This is a very positive result taking into account that, ash may be considered a sample pre-treatment step for analysing the presence of specific minerals in food ingredients [1].

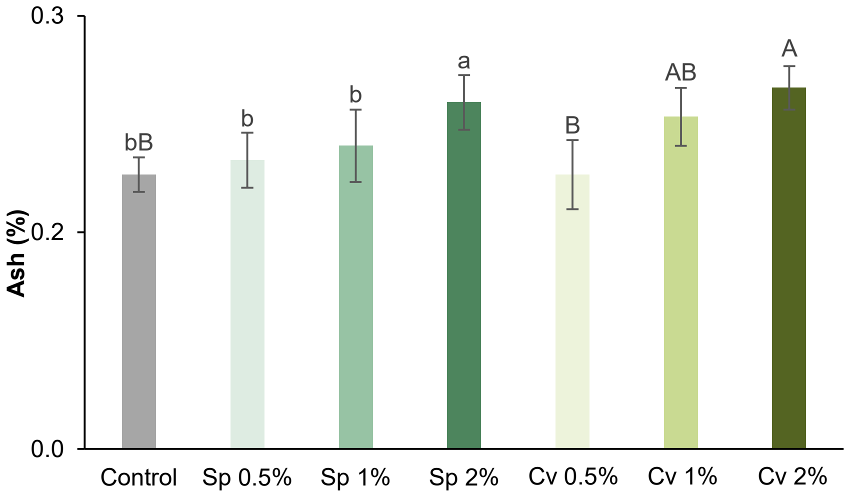


Figure 14 - Ash content for the microalgae-enriched gummies. Means resulted from triplicate assays. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

4.1.2. Protein

As observed in Figure 14, the protein content had a significant decrease (*p*<0.005) at the highest concentrations, because the added microalgae to the formulation, although a great source of protein, corresponded to a substitution in the amount of pork gelatine, therefore impacting in the final protein content. Therefore, by reducing pork gelatine content, the protein content in the gummies decreased from 6.77% to 6.03% in the spirulina samples and 6.78% to 6.36% in the *C.vulgaris* samples.

An analysis to the aminoacid concentration could be an important data to this study, as spirulina and *C.vulgaris* contain all essential aminoacids [51, 52] compared to gelatine, which is considered an incomplete protein for not possessing Tryptophan and Histidin [53].

The values of protein obtained varied from 6.0 g to 6.8 g of the total gummy weight, which is somewhat similar to what is found in the literature for gummies (6,90 g/100 g dw) [54]. Therefore, the microalgae-enriched gummies show a lower value but not a massive difference that prevents the substitution of microalgae for gelatine, as the essays resulted in very good results.

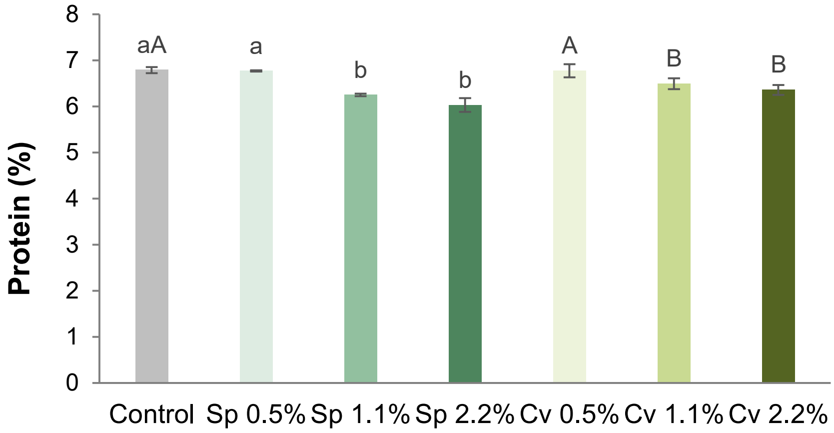


Figure 15 - Protein content (%) for microalgae-enriched gummies.Bars correspond to standard deviation. Different lowercase letters represent significant differences ($p<0.05$) between spirulina-enriched (Sp). Different uppercase letters represent significant differences ($p<0.05$) between *C. vulgaris*-enriched (Cv) and control gummies.

4.1.3. Lipids

The lipid content of the different prepared gummies showed to be considerably low, especially in *C. vulgaris*. This was kind of expected because *C. vulgaris* raw biomass show a fat content around 12.5% [51]; after being incorporated into a food matrix as a gummy in such low percentages of incorporation (0.5-2%), it is expected the fat content in the gummy to be considerably low. In fact, when added to cookies, *C. vulgaris* and spirulina presented the also considerably low results for an incorporation of 4% of microalgae [30]. In Figure 15 is easy to observe that an increase in the level of microalgae incorporation leads to an increase of fat content (from 0.01 to 0.14% in spirulina, and from 0.03 to 0.04% in *C.vulgaris*);

According to table 10, we can see that *C.vulgaris* samples do not show significant differences between each other and the control. The statistical test resulted in a p-value of 0.069, which in turn would result in the rejection of the null hypothesis. However, the p-value may be influenced by the sample size, which may lead to erroneous interpretations of the results [2]. In the case of gummies lipid content, the values are very low resulting in a higher p-value but the mean results don't show significant differences between *C.vulgaris* samples and control.

4.1.4. Carbohydrates

The carbohydrate content shows an unsurprising result, knowing that sugar is one of the main ingredients in gummy manufacture, exceeding 50% of the total gummy composition. The results on Table 10 show a carbohydrate content varying between 56% to 63% for the different microalgae formulas. It is important to highlight that the obtained value is an approximation to the real value since to determine it, it is necessary to know the fiber content and that component was not evaluated. For that reason, the real carbohydrate content can be slightly lower than the estimated, as this determination also encompasses the error of all previous determinations. However, taking into account the results presented in table 11 and the carbohydrate content in the raw microalgae strains used, present in Appendix I, and also the level of their incorporation, it is possible to conclude that increment in carbohydrate content of gummies is due to the presence of the two microalgae, *C. vulgaris a*nd spirulina. In the case of spirulina-enriched gummies, it is visible a lower influence of the level of microalgae incorporated in the gummy matrix, which is possibly due to the fact that spirulina as a lower carbohydrate content than *C. vulgaris* [48, 55]. The value obtained for the control is also in agreement with its higher moisture- concentration and the fact that it contains a greater amount of protein than the other formulas.

Table 10 - Estimated carbohydrate content obtained by the difference between total percentage and the results obtained during chemical proximal analysis. Carbohydrates results highlighted in red.

Components (%)	Control	Spirulina-enriched gummies			*C. vulgaris*-enriched gummies		
	0.0%	0.5%	1.0%	2.0%	0.5%	1.0%	2.0%
Moisture	36.94 ± 0.32	32.33 ± 0.99	33.64 ± 0.226	33.60 ± 0.269	36.04 ± 0.407	32.83 ± 0.487	29.84 ± 0.035
Ash	0.19 ± 0.01	0.20 ± 0.02	0.21 ± 0.025	0.24 ± 0.019	0.19 ± 0.024	0.23 ± 0.020	0.25 ± 0.015
Protein	6.79 ± 0.07	6.77 ± 0.01	6.25 ± 0.03	6.03 ± 0.15	6.78 ± 0.14	6.49 ± 0.12	6.36 ± 0.11
Lipids	0.01 ± 0.00	0.00 ± 0.00	0.06 ± 0.01	0.14 ± 0.03	0.02 ± 0.01	0.02 ± 0.00	0.04 ± 0.01
Total	43.92	39.30	40.17	40.01	43.03	39.57	36.49
Carbohydrates	**56.08**	**60.70**	**59.83**	**59.99**	**56.9**	**60.43**	**63.51**

4.1.5. Mineral profile

To understand the impact of microalgae incorporation into gummies, the mineral content was analyzed (Figure 16). Spirulina showed higher concentrations of Na, K, Mg, and S, while *C. vulgaris* was richer in Fe, Zn, Mn, Ca and P (Figure 16).

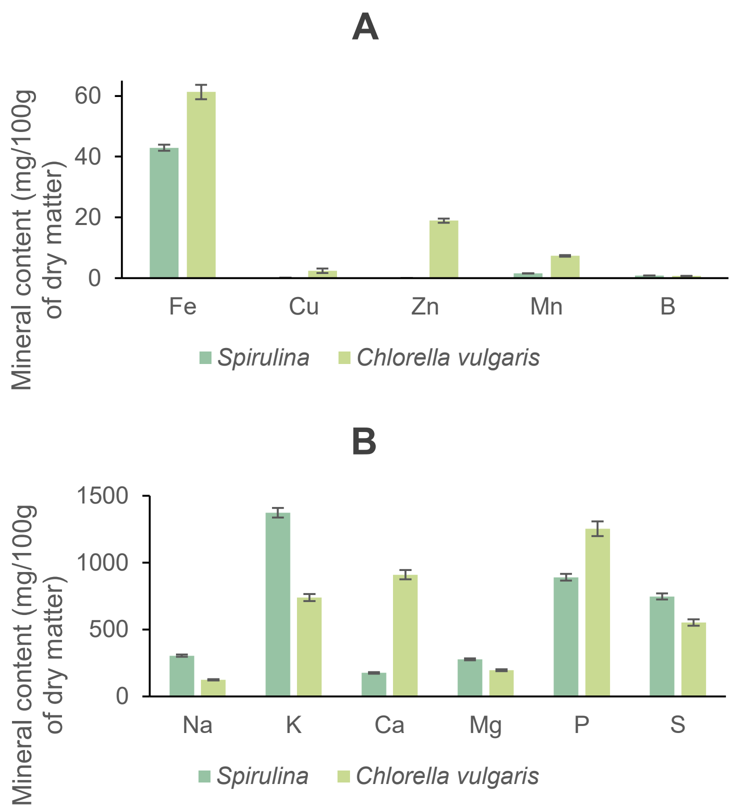


Figure 16 - Mineral content (mg/100 g of dw) in the raw microalgae used for gummy production (Spirulina and *C. vulgaris*). Figure A - Results for Fe, Cu, Zn, Mn and B; Figure B - Results for Na, K, Ca, Mg, P and S.

As expected, the mineral content in the developed gummies was significantly improved with the incorporation of microalgae in the formulation for the most part of the analyzed minerals.

In Figure 17, it is possible to observe a significant impact of the microalgae in the mineral content of the developed gummies, with the concentration levels for the *C. vulgaris*-enriched gummies of 2.0% reaching 40.9 mg/100g dw, and Ca, P and S reaching 39.3, 56.6 and 49.7 mg/100g dw respectively. These results are quite interesting taking into account that heat treatment could influence the mineral bioaccessibility it by protein denaturation and consequent enzymatic inactivation of crucial enzymes [56], whose effect could explain the lower results of Sulfur for higher concentration of spirulina.

This is in complete agreement with what is usually observed in food products incorporated with microalgae already described in literature. For example, when Incorporating *A. platensis* and *C. vulgaris* in 1.5-2.0% levels of incorporation in cookie formulations, they presented a greater accessibility of P, K, Ca, Mg, Fe, Zn, and Se for absorption in the body, compared with control cookies [56]. The same effect in the mineral profile content occurred when *C. vulgaris* was added to gluten free bread formulation, increasing the concentration of K, Ca, Mg, P, and S, compared to control [30].

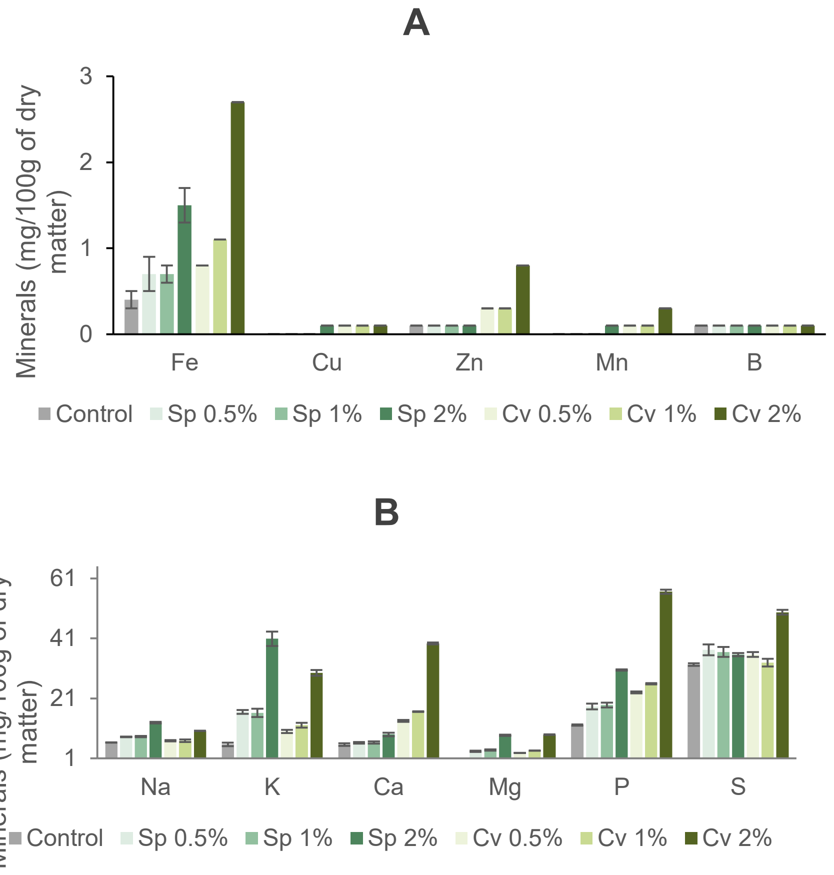


Figure 17 - Mineral profile (mg/100 g of dry matter) for the gummy formulas represented by different colours. Figure A - Results for Fe, Cu, Zn, Mn and B; Figure B - Results for Na, K, Ca, Mg, P and S.

According to the European Union Regulation N-1924/2006, Directive N-9090/494 (CE), when a food has 15% RDV in its composition, it is considered a source of the corresponding minerals. In cases where it has at least twice the content required for this nutritional declaration, 30% of the RDV, it is considered to have a high content of that specific mineral. The work developed here suggests that, despite being possible to reach the recommended daily value (RDV) for Fe and Mn in *C. vulgaris* formulation, it is necessary to consume 39 gummies of 2 g each to surpass 15% of the RDV values.

Table 11 - Mineral composition (mg/100 g dw) of microalgae-enriched gummies with 0.0%, 0.5%, 1.0% and 2.0% incorporation with *C. vulgaris* (Cv) and Spirulina (Sp).

Formulation	Na	Fe	Cu	Zn	Mn	B	K	Ca	Mg	P	S
Control	6.3 ± 0.1	0.4 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	5.7 ± 0.6	5.6 ± 0.4	0.8 ± 0.0	12.1 ± 0.2	32.3 ± 0.4
Sp 0.5%	8.2 ± 0.1	0.7 ± 0.2	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	16.5 ± 0.6	6.2 ± 0.3	3.4 ± 0.2	18.3 ± 1.0	37.2 ± 1.8
Sp 1.0%	8.3 ± 0.2	0.7 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	16.2 ± 1.4	6.3 ± 0.4	3.8 ± 0.2	18.8 ± 0.8	36.5 ± 1.7
Sp 2.0%	12.9 ± 0.3	1.5 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	40.9 ± 2.4	9.0 ± 0.6	8.7 ± 0.2	30.5 ± 0.2	35.6 ± 0.5
Chl 0.5%	6.9 ± 0.2	0.8 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	10 ± 0.5	13.5 ± 0.3	2.8 ± 0.0	23 ± 0.3	356 ± 0.8
Chl 1.0%	6.9 ± 0.4	1.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	12.1 ± 0.8	16.6 ± 0.1	3.6 ± 0.0	25.9 ± 0.2	32.9 ± 1.3
Chl 2.0%	10.2 ± 0.0	2.7 ± 0.0 **	0.1 ± 0.0	0.8 ± 0.0	0.3 ± 0.0 **	0.1 ± 0.0	29.5 ± 1.0	39.3 ± 0.3	8.9 ± 0.1	56.6 ± 0.7	49.7 ± 0.8
15% RDV * (mg)	900	2.1	0.2	1.5	0.3	NM	300	120	56.3	105.0	NM

* Recommended daily value (RDV) per European Community Regulation N,1924/2006, Directive N-9090/494 (CE). NM - not mentioned.

**Values that exceed the 15% of the recommended daily value (RDV)

4.2. Bioactive properties

Bioactive properties of the microalgae-enriched gummies were assessed through the determination of the AAT and of TPC, and the results are presented in figures 18-20. An increase in the level of incorporation of microalgae led to an enhancement in the antioxidant, with *C. vulgaris*-enriched gummies standing out as the gummies with a higher antioxidant potential.

This results are quite interesting because previous works report a quite low AAT for *C. vulgaris*, and with the levels of incorporation used, from 0.5-2.0%, we expected to have a AAT between 0.4-1.6 µmol Trolox/100g dw, which is higher than the AAT values reported for the raw microalgae (0.8 µmol Trolox/100g dw) when assayed by FRAP (Figure 18) [57].

Regarding the ferric reducing capacity (FRAP), sauces with 4% of spirulina incorporation also exhibited significantly greater activity up to 86.3% more compared to control [10]. In the case of the gelly gummies the antioxidant activity reached values of 35% and 170.35% more for the spirulina-enriched and *C. vulgaris*-enriched gummies with 2.0% incorporation, respectively.

It is worth mentioning that the control gummies also presented antioxidant potential in a lower extent, possibly due to some of the specific ingredients used in its confectionery, however, the AAT values were lower in the control, compared to those found in the gummies enriched with microalgae, demonstrating that the addition of this microalgae conferred a significant effect on increasing the antioxidant capacity of this product.

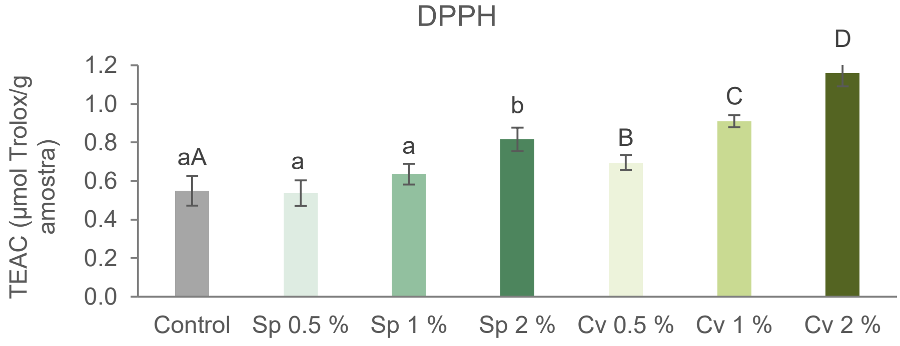


Figure 18 - Total Antioxidant Activity expressed in TEAC (µmol Trolox/g sample) for the spirulina-enriched and *C. vulgaris*-enriched gummies, with the DPPH (2,2-difenil-1-picril-hidrazil) assay. Bars correspond to standard deviation. Different lowercase letters represent significant differences ($p<0.05$) between spirulina-enriched (Sp). Different uppercase letters represent significant differences ($p<0.05$) between *C. vulgaris*-enriched (Cv) and control gummies.

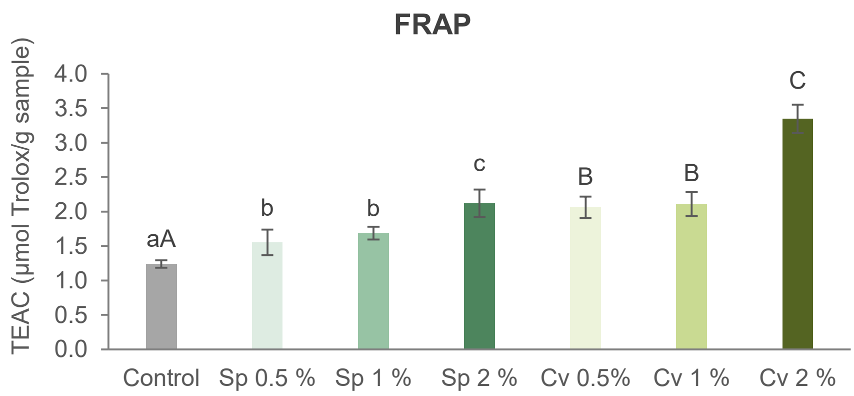


Figure 19 - Total Antioxidant Activity expressed in TEAC (µmol Trolox/g sample) for the spirulina-enriched and *C. vulgaris*-enriched gummies, with the FRAP (Ferric-Reducing Ability of Plasma) assay. Bars correspond to standard deviation. Different lowercase letters represent significant differences ($p<0.05$) between spirulina-enriched (Sp). Different uppercase letters represent significant differences ($p<0.05$) between *C. vulgaris*-enriched (Cv) and control gummies.

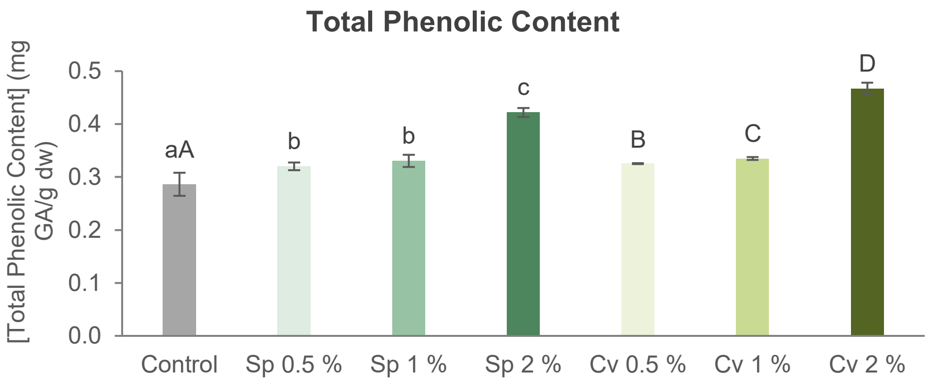


Figure 20 - Total Phenolic Content (TPC, mg GA/g dw) for the seven formulas of spirulina and *C. vulgaris* jelly gummies, with the Folin–Ciocalteu method. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

The total phenolic content (TPC) is interesting in all the prepared gummies, when compared to the TPC reported in literature for *C. vulgaris* and spirulina raw biomass (84 mg GAE/g dw and 4 mg GAE/g dw, respectively) [50, 65] and taking into consideration that only up to 2% of microalgae were added, resulting in a theoretical TPC between 0.1-1.6%. These differences in the TPC content can be due to several aspects, concerning the growth conditions of microalgae, the existence (or not) of a cellular disruption pre-treatment process [30].

It was possible to establish a direct relationship between the total phenolic content and the antioxidant capacity of the developed gummies. The gummies with added microalgae presented the highest levels of TPC, proportionally to the highest antioxidant activities, both using DPPH as well as FRAP.

4.3. Colour Determination

For the colour analysis, the mean results of L*, a* and b* obtained during colour measurement with the CR-400 colourimeter, allowed the confirmation of colour differences between different microalgae concentrations and its changes after two months of gummies storage at room temperature and protected from light. Figures 21 and 22 show the different spirulina-enriched and *C. vulgaris*-enriched gummies with different levels of microalgae incorporation, respectively, one day after production (A). Figures 23 and 24 show the same gummies but two months of storage (B).

spirulina-enriched gummies			
0.0%	0.5%	1.0%	2.0%

Figure 21 – Colour variation of control (0.0%) and spirulina-enriched gummies with different levels of incorporation (0.5-2.0%), one day after production (A).

C. vulgaris-enriched gummies			
0.0%	0.5%	1.0%	2.0%

Figure 22 - Colour variation of control (0.0%) and *C. vulgaris*-enriched gummies with different levels of incorporation (0.5-2.0%), one day after production (A).

spirulina-enriched gummies			
0.0%	0.5%	1.0%	2.0%

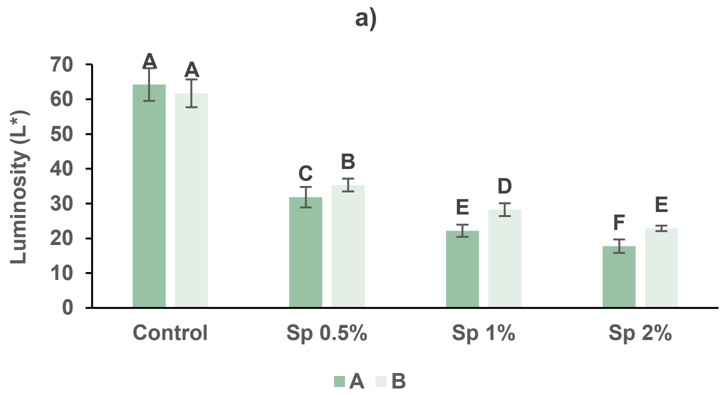
Figure 23 - Colour variation of control (0.0%) and spirulina-enriched gummies with different levels of incorporation (0.5-2.0%), two months after production (B).

C. vulgaris-enriched gummies			
0.0%	0.5%	1.0%	2.0%

Figure 24 - Colour variation of control (0.0%) and *C. vulgaris*-enriched gummies with different levels of incorporation (0.5-2.0%), two months after production (B).

Luminosity (L*)

With the addition of microalgae, gummies became darker and the L* (luminosity) decreases significantly, as it's possible to observe by the values of the microalgae-enriched gummies with 0.5% of incorporation, when compared to control (Figure 25 **(a)**) with an evident decrease of brightness for higher values of microalgae incorporation. Although, luminosity values are equal for the same microalgae concentration for both spirulina-enriched and *C. vulgaris*-enriched gummies one day after production (A). After two months of storage at room temperature (B), several changes were detected such as an increment of brightness for the majority of samples, mostly due to colour saturation. Over time, control gummies have proven to be the ones whose brightness did not change significantly (p<0.05), and therefore, changes were attributed to the presence of microalgae.



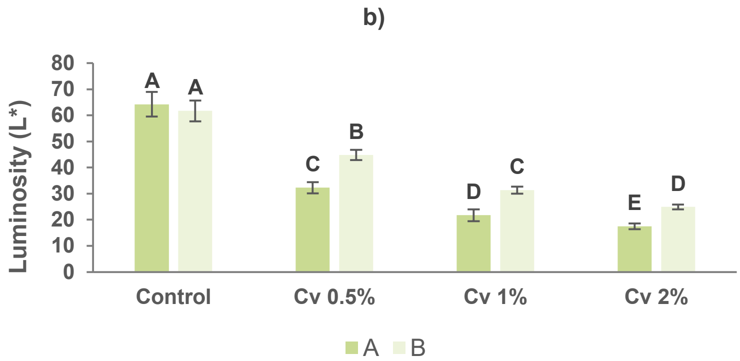
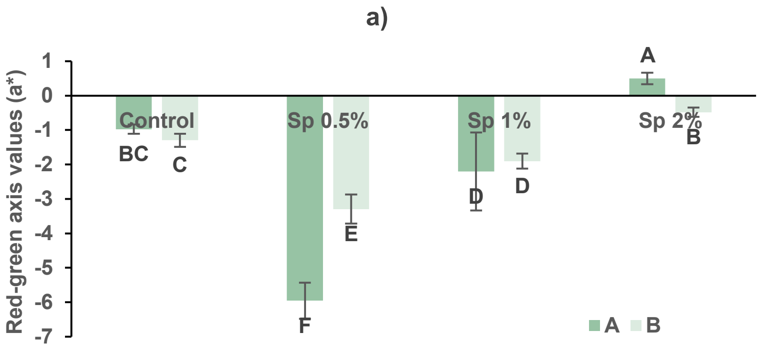


Figure 25 - Graph **a)** - Luminosity (L*) « for spirulina -enriched gummies (Sp), one day after production (A) and two months of storage (B); Graph **b)** - Luminosity (L*) for *C. vulgaris*-enriched gummies (Cv), one day after production (A) and two months after production (B); Bars correspond to standard deviation, different letters represent significant differences (p<0.05) between samples.

Red-green axis (a*) and yellow-blue axis (b*)

As expected, due to presence of chlorophyll in microalgae chemical composition, with the addiction of microalgae the values for a* (red-green axis) becomes more negative approaching the green range of the axis (Figure 26). Spirulina-enriched gummies with 0.5 and 1% of incorporation stand out as having a higher incidence of green in its colour profile, reaching saturation values as microalgae concentration increases. *C. vulgaris*-enriched gummies show higher values, mostly due to the presence of other pigments such as carotenoids, which provide colouring at a range from yellow to red, thus its positive values for higher concentration formulas. In the case of b* (blue-yellow axis) the results are distinct for the different added microalgae: *C. vulgaris* gummies present high values in the yellow range of the axis and spirulina have a more saturated values mostly derived from the presence of phycocyanin and phycoerythrin (blue toned pigment).



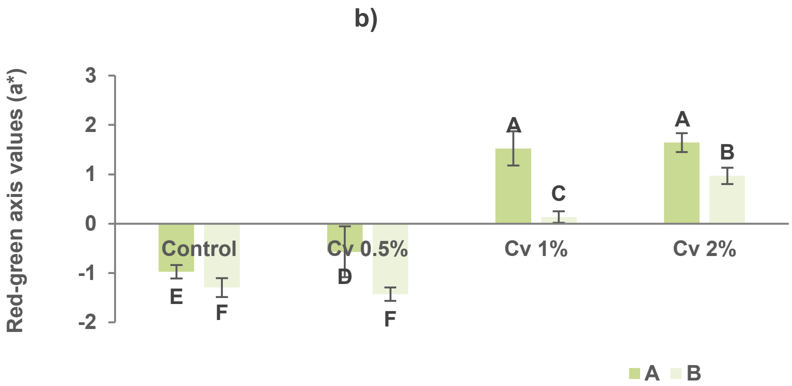


Figure 26 - Graph a) - Red-green colour axis (a*) values for spirulina-enriched gummies, one day after production (A) and two months after of storage (B); Graph b) - Red-green colour axis (a*) values for *C. vulgaris*-enriched gummies (Cv) gummies, one day after production (A) and two months of storage (B); Bars correspond to standard deviation, different letters represent significant differences ($p<0.05$) between samples.

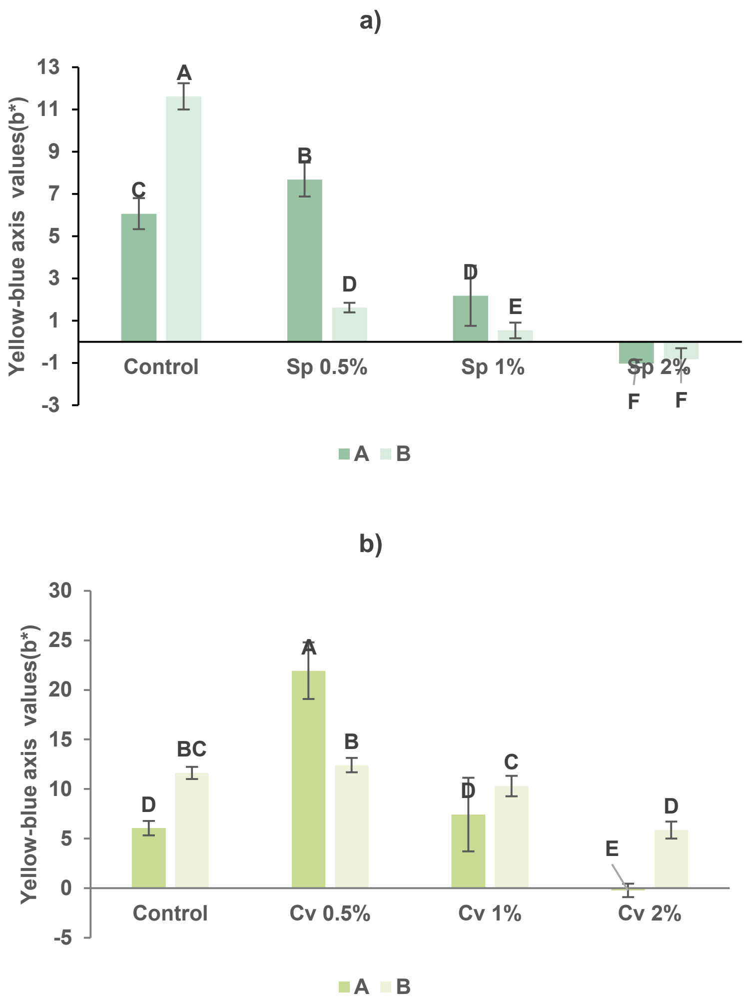


Figure 27 - Graph **a)** - Yellow-blue colour axis (a*) values for spirulina. Enriched gummies (Sp), one day after production (A) and two months of storage (B); Graph **b)** - Yellow-blue colour axis (a*) values for *C. vulgaris*-enriched gummies (Cv), one day after production (A) and two months of storage (B); Bars correspond to standard deviation, different letters represent significant differences (p<0.05) between samples.

After two months preserved at room temperature, inside polyethylene bags, the control gummies had a significant increase in yellowish tones, possibly due to oxidation processes. For spirulina-enriched and *C. vulgaris*-enriched gummies, the a* value (Red-green axis) increases significantly over time. This may occur due to chlorophyll degradation, leading to the formation of undesirable grey-brown compounds, which can also explain the increase of yellowish tones for *C. vulgaris* gummies with 1% and 2% of incorporation (Figure 27 **(b)**).

With the obtained results it is possible to confirm a significant colour loss over time for most of the microalgae-enriched gummies due to pigment degradation, in the preservation conditions. However, spirulina-enriched gummies with 1.0-2.0% of incorporation did not differ significantly, over time, in terms of a* and b* for spirulina-enriched gummies with 1.0% of incorporation and for *C. vulgaris*-enriched gummies with 2.0% of incorporation, suggesting a positive impact caused by the increase of microalgae concentration for colour stability, according to statistical analysis.

ΔE Determination

In addition to the analysis method above described the colour perception differences between two samples can be analysed by calculating ΔE. This determination allows a better understanding of the visual impact in terms of sensory perception, relating the values obtained for luminosity (L*), red-green axis (a*) and the yellow-blue axis (b*) in the following equation:

$$\Delta E = \sqrt{\Delta L *^2 + \Delta a *^2 + \Delta b^{*2}} \quad \textbf{(15)}$$

Were:

- ΔE - Colour perception difference between two samples;
- ΔL*, Δa* and Δb* - Colour difference between two samples for the respective parameter.

The values obtained for ΔE imply a significant colour difference between samples when ΔE ≥ 4 [58].

Table 12 - Colour difference (ΔE) obtained for each gummy formulation measured one day after production and preserved two months at room temperature with no light incidence

Samples	Control	Spirulina-enriched gummies			*C. vulgaris*-enriched gummies		
	0%	0.5%	10%	2.0%	0.5%	1.0%	2.0%
ΔE	8.8	8.3	6.8	5.3	16.2	10.6	9.6

A study on microalgae colour stability in rock hard candies, reported by João Germano (2014) presented distinct results for the Just Noticeable Difference values obtained for the jelly gummies [6]. In his study the candies were maintained in four different f storage conditions for 10 weeks. In the case of the hard candies preserved at room temperature with no light incidence, there was detected a significant difference of colour (ΔE = 4,00 / ΔE = 5,07) after 10 weeks, but mainly on samples with no microalgae concentration (control), concluding a positive influence of colour stability when microalgae is added to the product. Values of ΔE greater than 5 are found in previous studies with gluten-free bread with values of 77 for incorporating 4% of *T. chuii* [59]. In a previous study, a ΔE* value of 12.5 was obtained in bread wheat with addition of 1% C. vulgaris [60].

According to results of Table 12 all gummy samples have shown a significant difference, in terms of visual impact, over time. Also, the values were much higher compared to the hard candies reported previously [6], implying a lower pigment stability in this product compared to a similar product found in the candies sector. This occurrence may indicate a significant influence of the storage condition in colour change, for the jelly gummy products. Therefore, storage conditions must change, packaging wise, preserving in lower temperatures, or even by modifying the gummy chemical composition with the intent to prevent oxidation and pigment loss.

4.4. Texture Analysis

4.4.1. Puncture test

Regarding firmness test (strength at the highest peak) [27] a statistical test ANOVA (95% confidence) allowed to verify a high variability of firmness results between control, spirulina-enriched and *C. vulgaris*-enriched gummies (Figure 28). The different levels of spirulina incorporation had little to no impact on the gummies' structure, however, in the *C. vulgaris*-enriched gummies, they, become much harder with a 0.5% level of incorporation, followed by a decrease of hardness with the increment of algae concentration, but still higher than for the control gummies. The same behavior was observed when 4% of *C. vulgaris* was incorporated into bread [61].

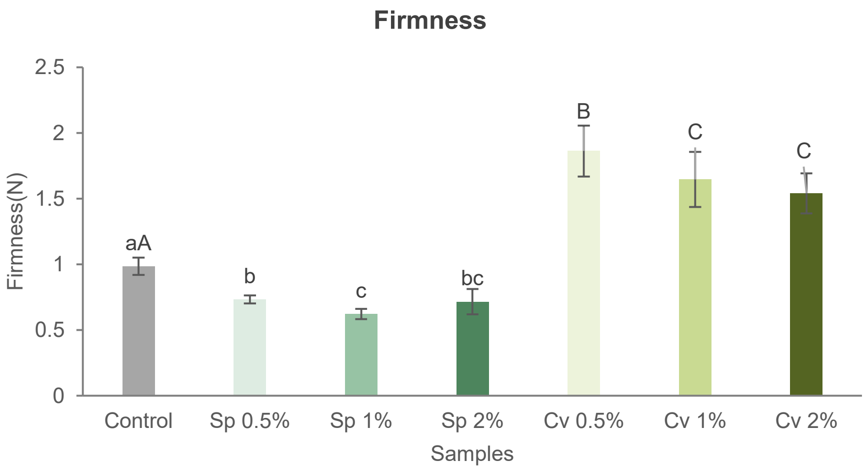


Figure 28 - Firmness (N) results of the prepared gummies, control, spirulina (Sp) and *C. vulgaris* (Cv), resulting from puncture test with a p/2 probe. Bars correspond to standard deviation. Different lowercase letters represent significant differences ($p<0.05$) between spirulina-enriched (Sp). Different

uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

Stickiness is the force necessary to overcome the attractive forces between the surface of the product and the surface of the material (the probe) with which the product comes in contact [39]. Figure 29 exhibits the results obtained in the puncture test for stickiness, corresponding to the lowest value of force (absolute negative force) applied during the puncture test. According to results, two samples stand out: *C. vulgaris*-enriched gummies with 0.5% and 1.0% of incorporation, showing a significantly higher stickiness values when compared to the rest of the gummies (p<0.005). The *C. vulgaris*-enriched gummies with 2.0% of incorporation approached control stickiness values. The spirulina-enriched gummies do not share a significant statistical difference between each other, meaning that, changing spirulina level of incorporation in gummies will not change significantly this texture characteristics, at least for lower concentration formulas.

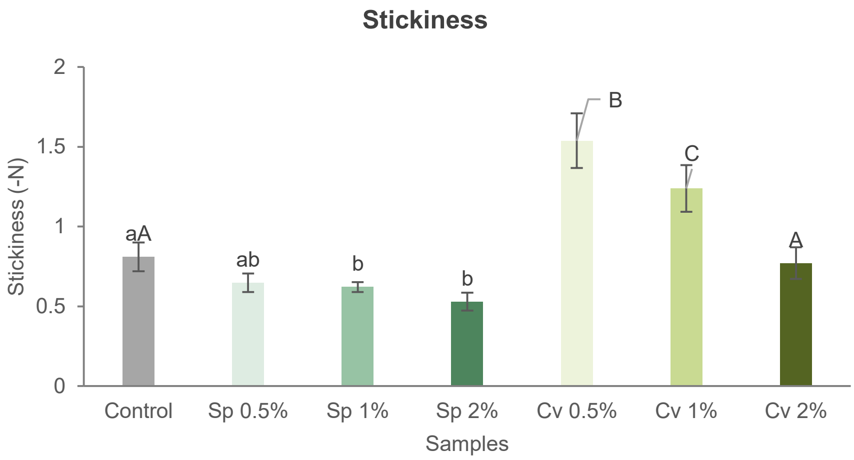


Figure 29 - Stickiness (N) results of the prepared gummies, control, spirulina (Sp) and *C. vulgaris* (Cv), resulting from puncture test with a p/2 probe. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

Adhesiveness is defined as the work required to overcome the sticky forces between the sample and the probe [49]. Adhesiveness (Figure 30) shows a similar trend to stickiness for a statistic test ANOVA, with 95% confidence, where *C. vulgaris*-enriched gummies with 0.5% and 1.0% of incorporation show higher adhesiveness value when compared to control, and spirulina-enriched gummies with 1.0% and 2.0% of incorporation showed a lower adhesiveness value compared to control. Gummies with 0.5% of spirulina incorporation and 2.0% of *C. vulgaris* incorporation show similar results of adhesiveness when compared to the control gummies.

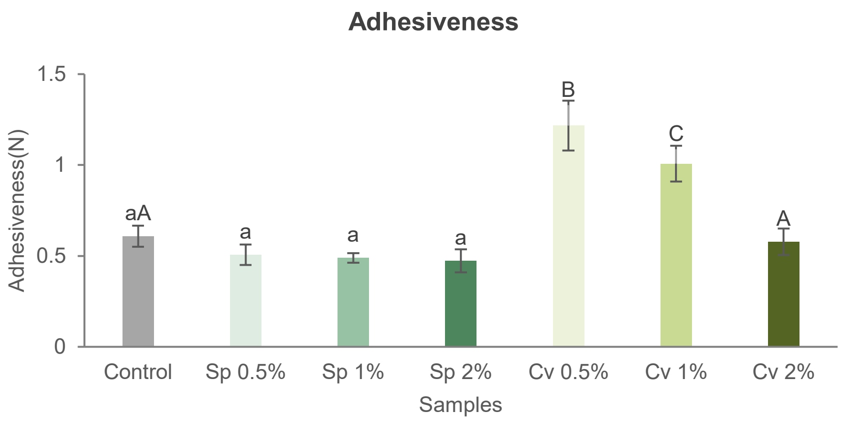


Figure 30 - Adhesiveness (N.s) results of the prepared gummies, control, spirulina (Sp) and C. vulgaris (Cv), resulting from puncture test with a p/2 probe.Bars correspond to standard deviation. Different lowercase letters represent significant differences ($p<0.05$) between spirulina-enriched (Sp). Different uppercase letters represent significant differences ($p<0.05$) between C. vulgaris-enriched (Cv) and control gummies.

4.4.2. TPA, or Texture Profile Analysis test results

Figure 31, shows the results for hardness, which represents the force necessary to cause deformation, replicating the sensation of a food being compressed between the teeth, tongue or mouth [27]. According to Figure 31, for a statistic analysis in 95% confidence ($p<0.005$), the *C. vulgaris*-enriched gummies with 2.0% of incorporation show significant higher values of hardness compared to the rest of the gummies, with a peak of 176.20 N, and the spirulina-enriched gummies with 2.0% of incorporation show a significant lower value.

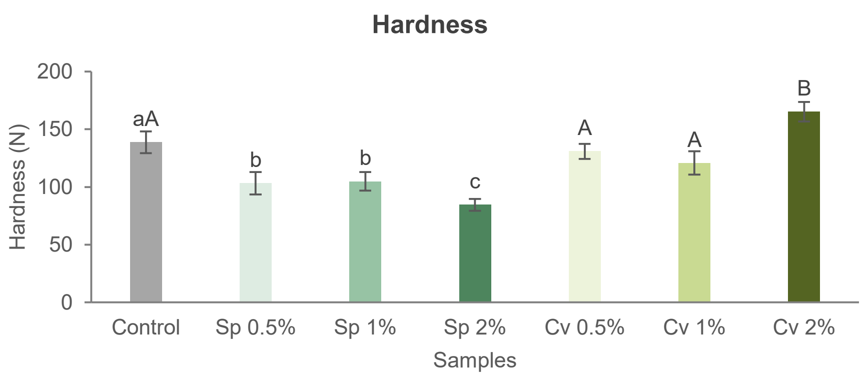


Figure 31 - Hardness(N) results of the prepared gummies, control, spirulina (Sp) and *C. vulgaris* (Cv), resulting from a TPA test with a p/60 probe. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

Resilience is the deformation energy per volume, in other words, the energy used when applying a force to a material without rupture [27]. In Figure 32, it is possible to observe a decrease in the resilience values with the incorporation of the microalgae which probably means that the energy used to apply force in the gummies decreases with the chance to reach significant different values (p<0,005).

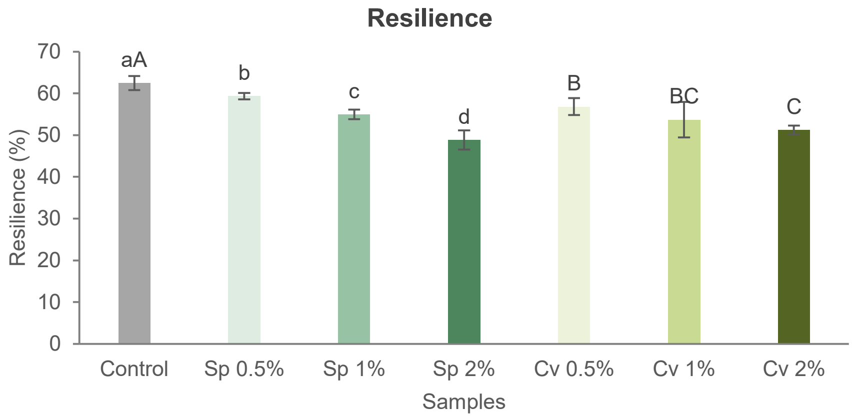


Figure 32 - Resilience (%)results of the prepared gummies, control, spirulina (Sp) and *C. vulgaris* (Cv), resulting from TPA test with a p/60 probe. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

Springiness, also known as elasticity (Figure 33), which stands for the capacity to recover shape after compression and measures the return speed to the initial state after removing the force that caused the deformation [27], showed high average values with no significant differences between samples, approximately 100% of springiness, which is an expected result for a product with a gel-like texture, like gummies.

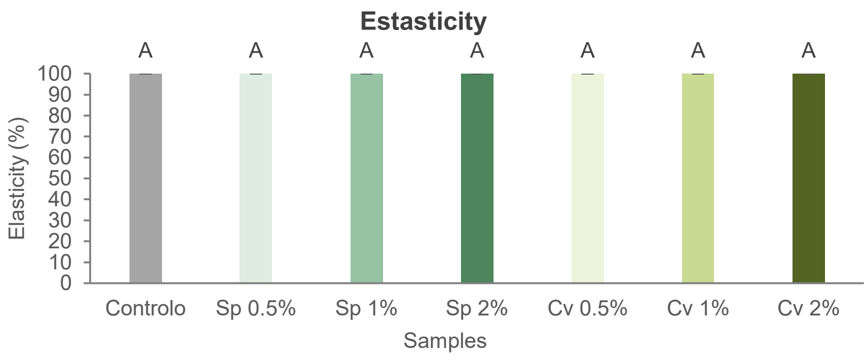


Figure 33 - Elasticity (%)results on the prepared gummies, control, spirulina (Sp) and *C. vulgaris* (Cv) resulting from a TPA test with a p/60 probe. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

Cohesiveness refers to the forces inside the food that keep the mass together and prevent it from disintegrating [27]. Figure 34 shows very homogeneous and high values of cohesiveness, with values ranging from 79% to 82%. The statistical analysis (p<0.05) with a level of significance α=0,05 confirms that there is no significant difference between means, therefore, the substitution of gelatine for microalgae does not affect significantly the results of cohesiveness. A textural study performed on commercial gummies obtained similar cohesiveness results from values to 0.86 to 0.93 for different brands found on the Market [27]. Another case with the addiction of *C.vulgaris* on gummy formula, shared the same absent effect on coheviveness and hardness increment [36].

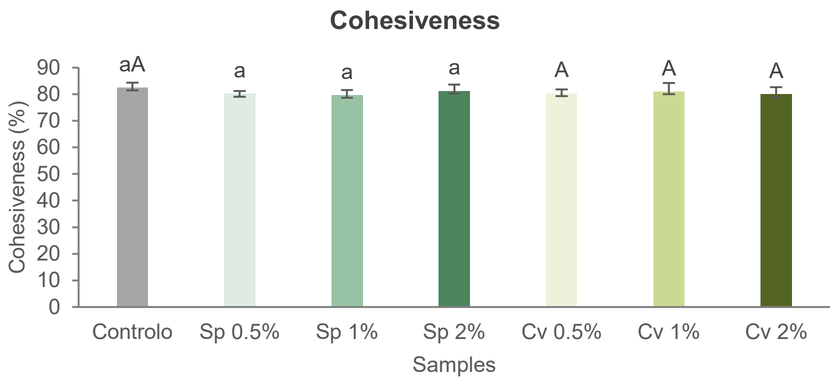


Figure 34 - Cohesiveness (%) results on the prepared gummies, control, spirulina (Sp) and *C. vulgaris* (Cv) resulting from a TPA test with a p/60 probe. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Cv) and control gummies.

Chewiness measures the energy needed to disintegrate a food to the point of being swallowed [27]. This property can be calculated after knowing the results of hardness, springiness and cohesiveness:

$$\text{Chewiness (N)} = \text{F1 x } \frac{\text{T2}}{\text{T1}} \text{ x } \frac{\text{A2}}{\text{A1}} \rightarrow \qquad \textbf{(9)}$$

→ Chewiness (N) = (Hardness) x (Springiness/100) x (Cohesiveness/100)

Due to the fact that springiness and cohesiveness do not show significant differences between samples, hardness becomes the biggest influence on chewing values, resulting in a similar graph (Figure 35) to hardness with the same type of variation between samples, where *C.vulgaris*-enriched gummies with 2.0% of incorporation are significantly harder to chew and the spirulina-enriched gummies with 2.0% of incorporation much easier. These texture differences may have been a decisive factor for the appreciation of the product during the sensory analysis.

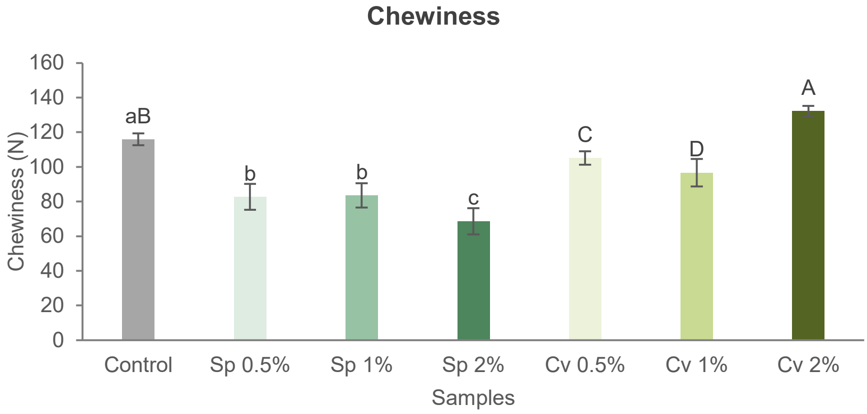


Figure 35 - Chewiness (N) results on the prepared gummies, control, spirulina (Sp) and *C. vulgaris* (Chl), resulting from a TPA test with a p/60 probe. Bars correspond to standard deviation. Different lowercase letters represent significant differences (p<0.05) between spirulina-enriched (Sp). Different uppercase letters represent significant differences (p<0.05) between *C. vulgaris*-enriched (Chl) and control gummies.

4.5. Sensory Analysis

The sensory analysis started by focusing on gummies integrity, and visual aspect, in relation to marks, cracks and shape. Due to de fact that the scale presented in the sensory test is numbered from 1 to 5, with lower values being associated with the best results (…more intact than control / more pleasant than control.). Therefore, the lower values of the accumulated frequency results observed in Figure 36, 37 and 38, represent the best results obtained during the tests.

According to the group of 30 panellists, spirulina-enriched gummies were slightly less intact than the control ones, by 40% of the panellists when referred to microalgae-enriched gummies with 0.5% of incorporation, by 26.7% for microalgae-enriched gummies with 1.0% of incorporation, and by 30% for microalgae-enriched gummies with 2.0% of incorporation. Although the spirulina-enriched gummies results showed higher score for “slightly less intact” and “no difference” in relation to control gummies, as the level of microalgae incorporation increases, so does the appreciation level, from 3.3% to 23.3% of the panellists stating that the gummies look much more intact when compared to control. For the *C. vulgaris*-enriched gummies, results were slightly better as the majority of panellists noticed no difference between the samples and the control, with some divided opinions between “less intact” and “no difference” for the microalgae-enriched gummies with 2.0% of incorporation.

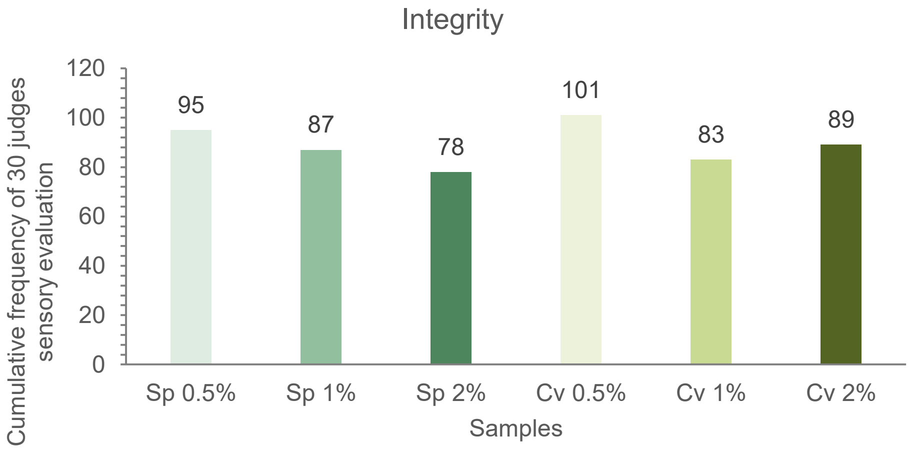


Figure 36 - Sensory test frequencies results, related to integrity parameter, performed on spirulina and *C. vulgaris* samples with 0.5%, 1% and 2% microalgae.

For the remaining characteristics, the analysis consisted of rating the microalgae-enriched gummies’ pleasantness compared to control. Colour wise, the results were very positive for all the microalgae-enriched gummies, as the majority of panellists showed a certain rejection towards the translucent appearance of the gummies with 0.0% microalgae (control). Another very positive aspect concerns the perceived scent since the majority of panellists did not notice a big difference between spirulina-enriched gummies regardless the level of incorporation (Figure 37), but, for *C. vulgaris* enriched gummies, the scent appreciation decreased slightly at higher levels of microalgae incorporation (Figure 38).

Texture and flavour were the main conditions for the rejection of gummies with higher levels of microalgae incorporation, with *C. vulgaris*-enriched gummies showing better results for texture, with 56.7%, 43.3% and 33.0% of the panellists detecting no difference in comparison to control gummies, for the 0.5%, 1.0% and 2.0% level of microalgae incorporation, respectively (Figure 38). The spirulina-enriched gummies with 2.0% of incorporation were much less appreciated in terms of flavour and texture by 36.7% of the panellists, which is in compliance with the fact that these gummies showed low firmness and chewiness. The opposite was observed for *C. vulgaris*-enriched gummies, with higher acceptability by consumers who find firmer and chewable jelly gummies more appealing.

The flavour of the gummies had an evident and significant rejection rate in microalgae-enriched gummies with higher levels of incorporation, with 40% of the panellists finding that *C. vulgaris*-enriched gummies with 2.0% of incorporation much less pleasant than the control gummies (Figure 38) The spirulina-enriched gummies with lower levels of incorporation obtained better flavour evaluation in comparison to control. This is quite common in other microalgae-based food products, such as the case of a gluten-free bread where a high level of addition of 2% affected negatively the flavour parameters during Sensory analysis [45].

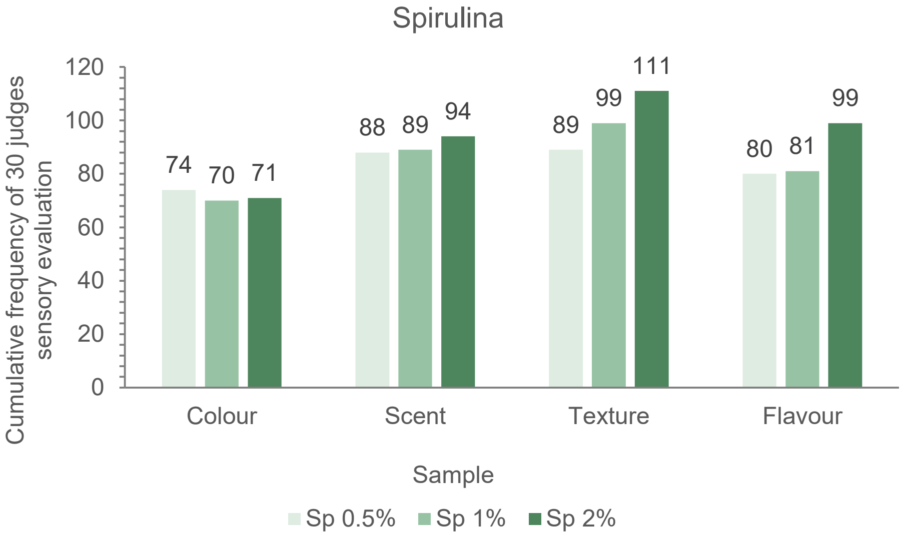


Figure 37 - Sensory test frequencies results related to spirulina samples (0.5%, 1% and 2% microalgae), for colour, scent, texture and flavour parameters.

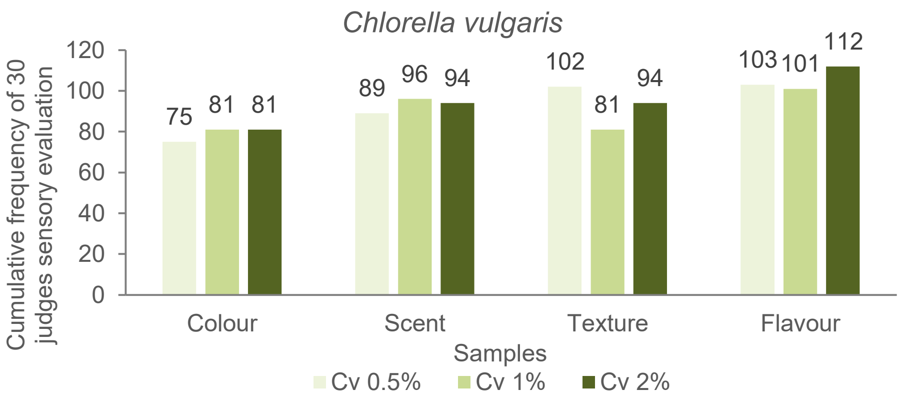


Figure 38 - Sensory test frequencies results related to *Chlorella vulgaris* samples (0.5%, 1% and 2% microalgae), for colour, scent, texture and flavour parameters.

The preference test showed a decreasing preference order with the increase level of microalgae incorporation, as it can be observed in Figures 38 and 39 for spirulina and *C. vulgaris*-enriched gummies, respectively. 17 of the 30 panellists placed the spirulina-enriched gummies with 0.5% of incorporation-as the more appreciated gummies (Figure 39 **(a)**), corresponding to 57% of the votes, and only 4 people placed this sample in the last place. When the level of microalgae incorporation reaches 2.0%, a total of 21 panellists (70%) placed these gummies in last place (Figure 39 **(c)**).

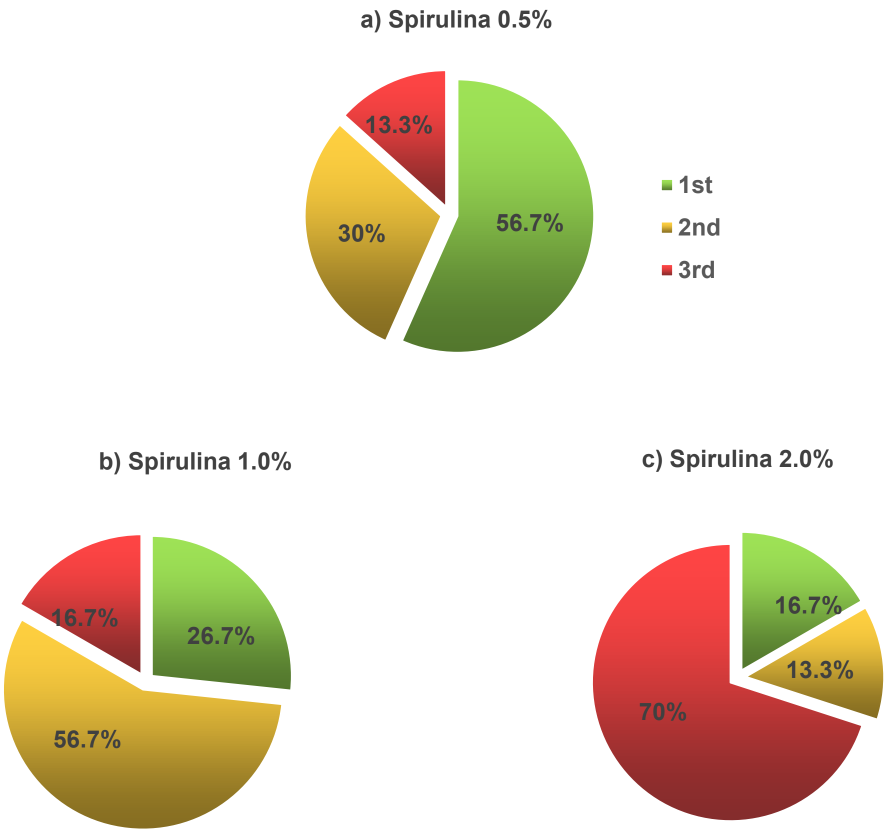


Figure 39 – Preference results on spirulina-enriched gummies, with a scale from 1st to 3rd, in percentage units. **(a)** – spirulina-enriched gummies with 0.5% of incorporation; **(b)** – spirulina-enriched gummies with 1.0% of incorporation; **(c)** - spirulina-enriched gummies with 2.0% of incorporation.

The same tendency was observed for the *C.vulgaris*-enriched gummies, as 60.0% of the panellists placed the gummies with lower level of microalgae incorporation in first place (Figure 40 **(a)**), corresponding to a total of 18 judges, and 63.3% placed the microalgae-enriched gummies with higher level of incorporation in third place (Figure 40 **(c)**).

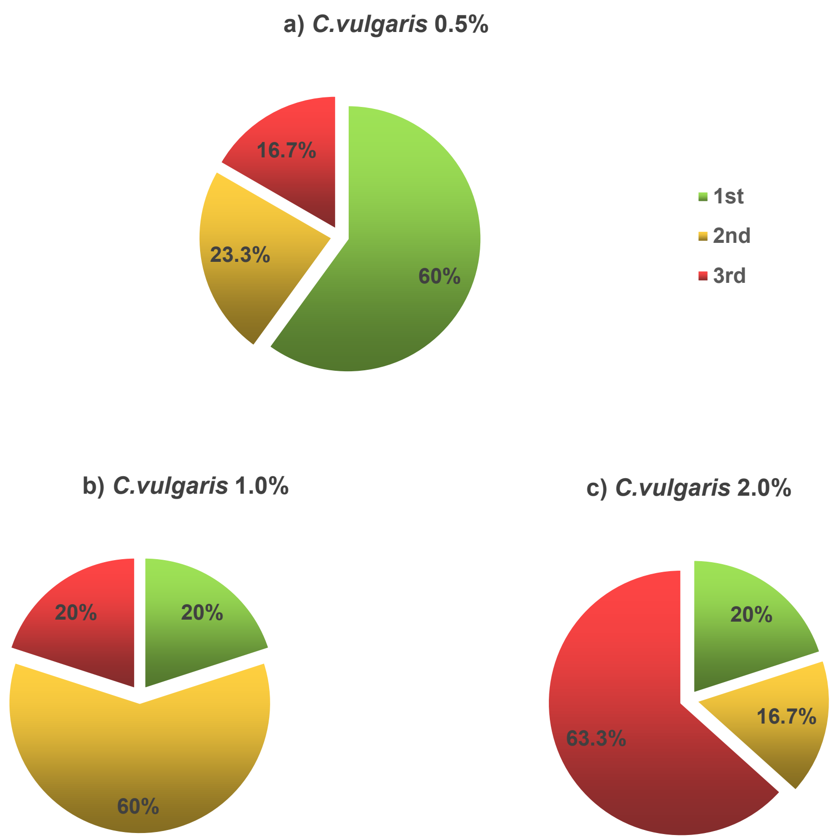


Figure 40 – Preference test results on *C. vulgaris*-enriched gummies, with a scale from 1^{st} to 3^{rd}, in percentage units. **(a)** – *C.vulgaris*-enriched gummies with 0.5% of incorporation; **(b)** – *C.vulgaris*-enriched gummies with 1.0% of incorporation; **(c)** - *C.vulgaris*-enriched gummies with 2.0% of incorporation.gummies.

In the comments section of the sensory analysis questionnaire, the spirulina-enriched gummies with 0.5% of incorporation had the highest acceptability and a very high buying intention rate. Concerning the spirulina-enriched gummies with 2.0% of incorporation, panelists reported that it was too dark in colour, but in terms of flavour it was quite pleasant. Panellists found that *C. vulgaris*-enriched gummies with 0.5% of incorporation, had the most appealing colour but a strange aftertaste. Finally, concerning the *C. vulgaris*-enriched gummies with incorporation of 2.0% of microalgae, panellists reported that it had a very intense fish after-taste feeling. These results are similar to the ones obtained from several sensory analysis reported for different microalgae-enriched food products, for example, in the case of cookies enriched with spirulina and *C.vulgaris* with incorporation rates between 2-6% [30].

It is important to highlight the fact that an incompatibility with the school season contributed to a low number of judges on the tasting panel (30 judges). This inconvenience regarding the number of judges can lead non reliable and inconclusive results. However, and according to the obtained results, it is possible to conclude that the incorporation of microalgae into gummies contributes positively to the appreciation of the product's colour. The different levels of incorporation had little or no impact on the odor of the gumnies. In terms of texture, little or no difference was detected. And the flavour offered by the different microalgae was the biggest contributor to the rejection of the product, especially in *C- vulgaris*-enriched gummies with 2.0% of incorporation.

4.6. Microbiology study

No evidence of yeast/fungi contamination in individual ingredient of the gummies' formulation was observed. As for the microalgae-enriched gummies, after 4 days of inoculation, some samples indicated signs of contamination with three different yeast species. This contamination was not exclusive to microalgae samples, has the control also presented signs of contamination. Surely, the contamination observed resulted from the contaminated environment while manipulating the products. This inconvenience made it impossible to study the gummies' shelf-life through one year of storage.

According to "Instituto Nacional de Saúde Dr. Ricardo Jorge", gummy products are included in the group 1B (fully cooked products manipulated after heat treatment), and due to its composition and low humidity, only yeast and molds could be detected over time, for maximum allowed values of 10^3 - ≤10^4 UFC/g, and $5x10^2$ – ≤10^{3} UFC/g, for yeast and molds respectively.

The results obtained for the gummies enriched with *C. vulgaris* and spirulina at T0 (inoculation one day after production), T1 (one month of storage) and T2 (two months of storage) with storage conditions at room temperature, are presented in Table 13.

Table 13 - Colony count (UFC/g) for each sample with three different inoculation dilutions (10^{-2}, 10^{-1} and 2 x volume of 10^{-1}) for T0 (inoculation one day after production) T1 (one month after storage) and T2 (two months after storage).

Period of time	Control	Spirulina-enriched gummies			*C. vulgaris*-enriched gummies		
	0%	0.5%	1.0%	2.0%	0.5%	1.0%	2.0%
T0	< 5 UFC/g	< 5 UFC/g	1,09 x 10^4 UFC/g	165 UFC/g	1,56 x 10^5 UFC/g	< 5 UFC/g	< 5 UFC/g
T1	4,85 x 10^4 UFC/g	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g

T2	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g	< 5 UFC/g

To obtain the results in Table 13, the petri plates with countable UFC's between 30-300 UFC/petri plate were selected, calculated according to the dilution factors and the conclusion drawn from this study is that, one day after production (T0), the samples without microalgae (control gummies) do nott show signs of contamination. On the other hand, the spirulina-enriched gummieswith 1.0% of incorporation presented a counting value of 1,09 x 10^4 UFC/g and the spirulina-enriched gummies with 2.0% of incorporation a counting value of 165 UFC/g. The *C. vulgaris*-enriched gummies with 0.5% of incorporation showed a counting value of 1,56 x 10^5 UFC/g. These results show that the incorporation of spirulina and *C. vulgaris* may be providing substrate to microorganism growth, reaching unsatisfactory results in the first days of storage. Nevertheless, the number of microorganisms decreases as soon as the concentration of microalgae increases to 2.0%, which means that over time, gummies showed up to be an unpleasant environment for microorganism's growth, mainly due to low humidity levels (Figure 41), as most of the samples presented a reduction of microbiota load over time. The incorporation of microalgae has showed great benefits reducing the microbiota load which significantly decrease with the increment of microalgae concentration (Figure 42). As for the control samples the contamination count reached values of 4,85 x 10^4 UFC/g. The counting values of the microalgae-enriched gummies decrease for an insignificant value after 2 months (T2) stored in room temperature. This study has proved that the addiction of microalgae contributes to enhance this product quality extending its shelf life. All the pictures and results collected in this study can be found on appendix III.

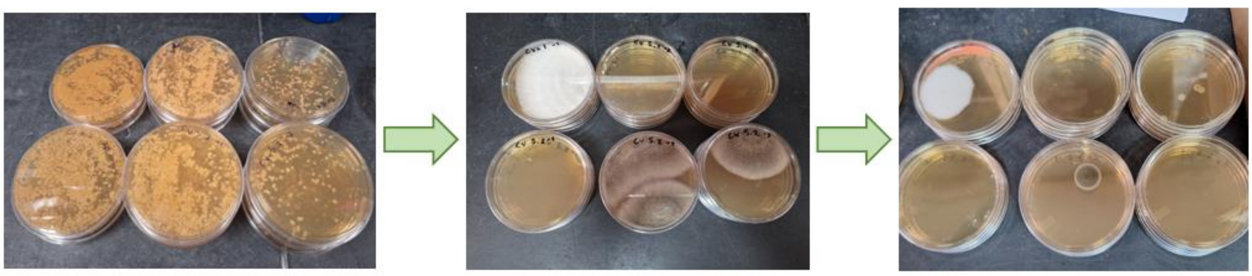


Figure 41 - Inoculation of *C. vulgaris*-enriched gummies with 0.5% of incorporation from T0 to T2 (over two months), in order form left to right.

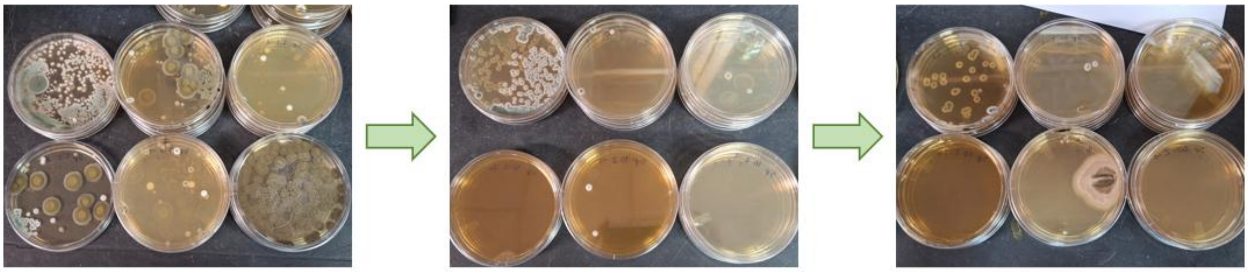


Figure 42 - Inoculation of spirulina-enriched gummies from T2 (after two months), in order of microalgae incorporation level (0.5%; 1.0% and 2.0%) enhancement form left to right.

According to some study, most fungi need humidity, at least 80%, to develop [62]. If the humidity is below this limit, toxins may not be produced, which is desirable. It should also be noted that pathogenic microorganisms cannot grow at water activity (a_w) values lower than 0.86 and that yeasts and molds cannot grow at a_w values lower than 0.62 [63]. A study carried out on commercial gummies indicated a_w values between 0.508 and 0.592 [18]. Considering that the final objective is to achieve a product with characteristics as close as possible to the commercial one, analysing the a_w of the microalgae-enriched gummies over time could be an important step to support the microbiology study results.

5. Conclusion

In summary, the results obtained during this project demonstrated a great potential in terms of the microalgae usage, to develop new functional food with a great nutritional profile and bioactive potential that could reach consumers in the near future.

In terms of nutritional profile, an increase in the mineral content was highly positive, with the clear increase in the amount of specific minerals. Concerning the bioactive properties of the developed gummies there was also an increment of the antioxidant potential in relation to a gummies with no microalgae (control). The antioxidant results were very positive especially since a loss of bioactive compounds was expected during gummy production due to the high temperatures needed to activate the gelling properties of the gelling agent, which could become a main conditioning factor to market placement. This proves that the addition of microalgae at the end of gummy production, at low cooking temperatures, proved to be a good method to prevent the loss of bioactive compounds.

The texture results were surprising, especially in terms of firmness, knowing that the used microalgae aren't one of the species with the highest protein concentration. However, and apparently, during the cooking process the present amino-acid chains react with components from other ingredients, resulting in a firmer texture. Even with some changes in texture characteristics, the main factor to reject the product referred by the panellists during sensory analysis, was gummies flavour, which worsens with the increase of microalgae incorporation level. It is important to note that the developed gummies do not have any added synthetic flavour other than the ingredients already described, making it possible to improve the results, flavour wise, by adding fruit flavours, fruit juices or even pulp to mask the microalgae sea flavour and increase consumer's acceptability for gummies with higher levels of microalgae incorporation.

The microbiology showed a low, but still, presence microorganisms like mold and yeasts, in the gummies, as well as some colour changes. The load of the microorganisms tends to decrease but to be able to achieve a shelf-life of over one year is necessary to apply some changes, whether in formulation, like adding potassium sorbate, a component with the capacity to inhibit mold and yeast growth, changing the oil coating process for bee wax, creating a protective coat, or even changing the storage conditions to a frozen product, or packaging that prevents light to reach the product and avoiding colour loss.

6. References

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8. Appendices

8.1. Appendix I - Used microalgae nutritional information

Table 14 - Nutritional informationof Spirulina powder from M.C.H, S.A (Lot: PA03734) produced at December 6 of 2023 expired at December 5 of 2025. **Dally Value (DV) (8400kj/2000 kcal); ***Nutrient reference value.

Nutritional Declaration		**Per 100 g**	**Per 2,5 g**	**%DV****
Energy		1600 kj 370 kcal	40 kj 9 kcal	<1
Fat		7,4 g	0,2 g	<1
of which saturated		4,1 g	0,1 g	<1
Carbohydrate		10 g	0,3 g	<1
of which sugars		1,8 g	<0,1 g	<1
Fiber		6,5 g	0,2 g	-
Proteins		70 g	1,8 g	4
Salt		2 g	0,05 g	1
Vitamins and minerals	Per 100 g	%NRV***		
Iron	86,8 mg	620	2,17 mg	16
Iodin	32 µg	21	0,8 µg	0,5

Table 15 - Nutritional table of spray-dried powdered *Chlorella vulgaris* provided by Pagarete Microalgae Solutions (Lot: AKEY_0095_3), expired on May of 2026. Values collected from the Certificate of Analysis 03-07-S09-COAL2023X16023-RO (Reported: 13/03/2023).

Parameters	**Results**	**Units**
Protein	54.9	%
Lipids	12.5	%
Carbohydrates	22.5	%
Moisture	4.2	%
Ash	6.0	%
Pigments / Carotenoids		
Total Carotenoids	0.50	%
Chlorophyll	2.3	%

8.2. Appendix II - Test Sheet used on Multiple-comparision test and Preference test during the sensory analysis

Test Sheet

Name:____

Age:_____

You will receive one standard sample (P) and three coded samples. Please evaluate the samples from left to right for the following characteristics and indicate the degree of difference between each sample and the standard one. Check with a cross on the following scales according to your perception.

Sample code:_________

.Integrity:

1. ( ) The sample appears much more intact than P, without any type of marks or cracks;
2. ( ) The sample appears slightly more intact than P;
3. ( ) There is no difference between the sample and P in relation to integrity;
4. ( ) The sample appears slightly less intact than P;
5. ( ) The sample appears much less intact than P, with several cracks and breaks;

Obs:____
____

.Colour:

1. ( ) The sample has a much more pleasant colour than P, without undesirable stains;
2. ( ) The sample has a slightly more pleasant colour than P;
3. ( ) The sample has a colour as pleasant as P;
4. ( ) The sample has a slightly less pleasant colour than P;

5. ( ) The sample has a much less pleasant colour than P, with the presence of several undesirable stains;

Obs:____

.Scent:

1. ( ) The sample has a much more pleasant scent than P;
2. ( ) The sample has a slightly more pleasant scent than P;
3. ( ) There is no difference between the sample and P in relation to scent;
4.( ) The sample has a slightly less pleasant scent than P;
5.( ) The sample has a much less pleasant scent than P;

Obs:____

.Texture (gum resistance during chewing processe)

1. ( ) The sample has a much more pleasant texture than P, melting in the mouth without leaving any gum residue;
2. ( ) The sample has a slightly more pleasant texture than P;
3. ( ) There is no difference between the sample and P in relation to texture;
4. ( ) The sample has a slightly less pleasant texture than P;
5. ( ) The sample has a much less pleasant texture than P, with gum residue remaining after tasting;

Obs:____

.Flavour

1. ( ) The sample has a much more pleasant flavour than P, with a pleasant after-taste remaining;
2. ( ) The sample has a slightly more pleasant flavour profile than P;
3. ( ) The sample and P do not differ in taste;
4. ( ) The sample has a slightly less pleasant flavour profile than P;
5. ( ) The sample has a much less pleasant flavour than P, with the appearance of an unpleasant after-taste;

Obs:____
____

After tasting each sample, right in ascending order, from left to right, according to preference:

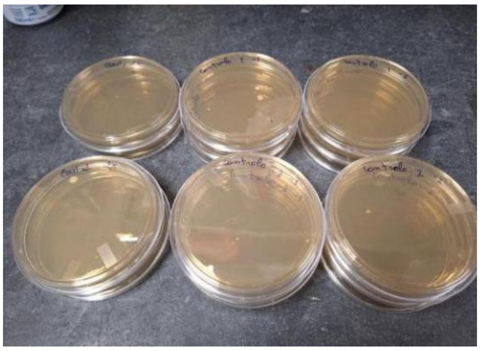
_______ < _______ < _______

Obs:____
____

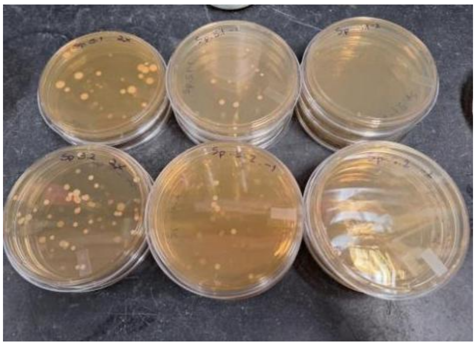
8.3. Appendix III - Images of the microbiology analysis results over time.

Table 16 - Microbiology results from inoculation one day after production (T0)

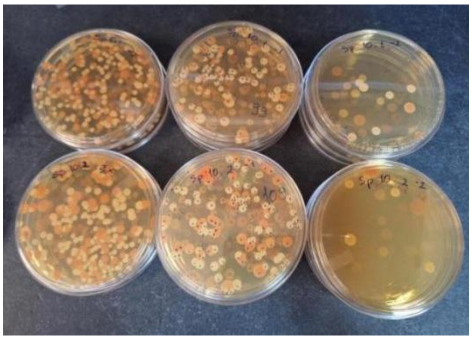
Control gummies



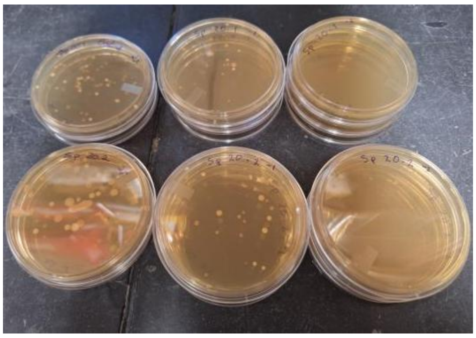
Spirulina-enriched gummies 0.5%



Spirulina-enriched gummies 1.0%

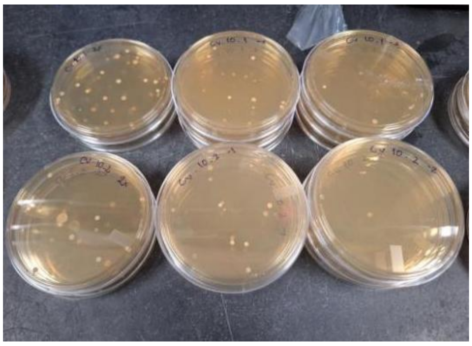


Spirulina-enriched gummies 2.0%



***C.* -enriched gummies**

***C. vulgaris*-enriched gummies 1.0%**



***C. vulgaris*-enriched gummies 2.0%**

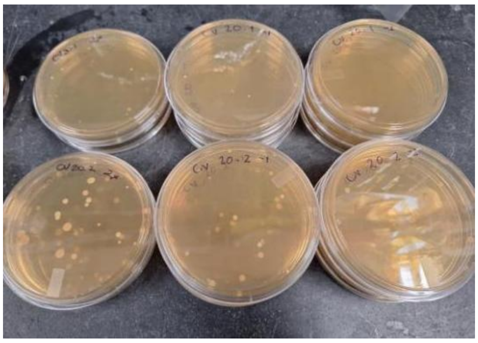
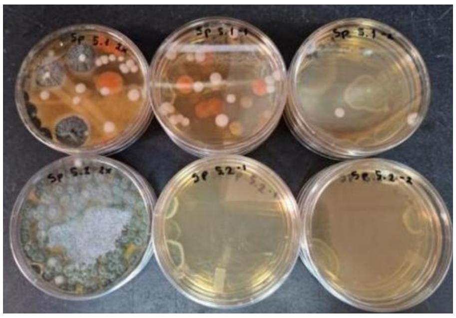


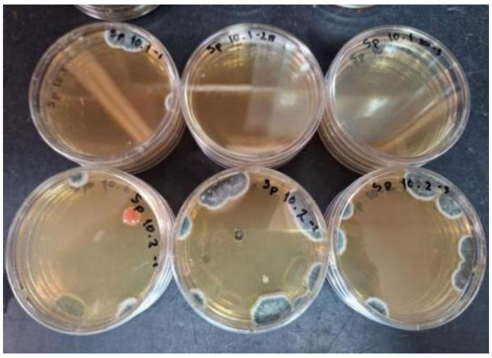
Table 17 - Microbiology results from inoculation one month after production (T1)

Control gummies

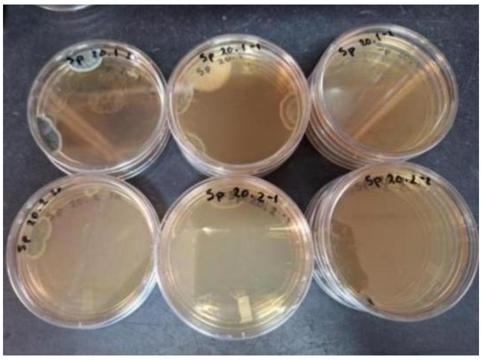
Spirulina-enriched gummies 0.5%



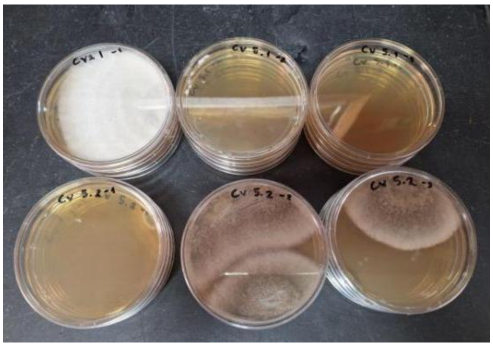
Spirulina-enriched gummies 1.0%



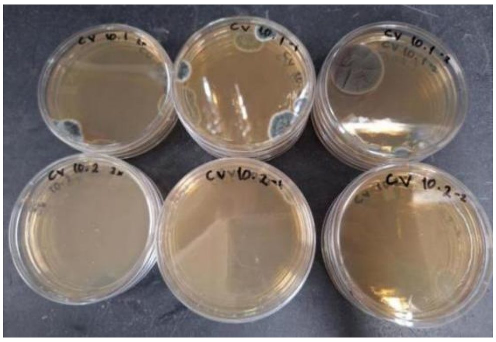
Spirulina-enriched gummies 2.0%



***C. vulgaris*-enriched gummies 0.5%**



***C. vulgaris*-enriched gummies 1.0%**



***C. vulgaris*-enriched gummies 2.0%**

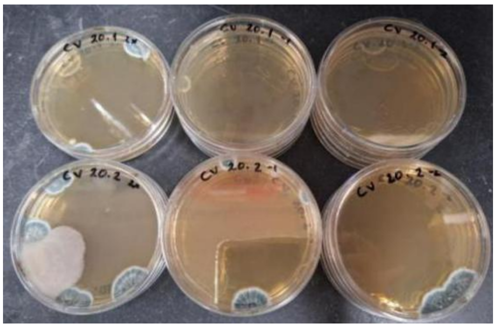
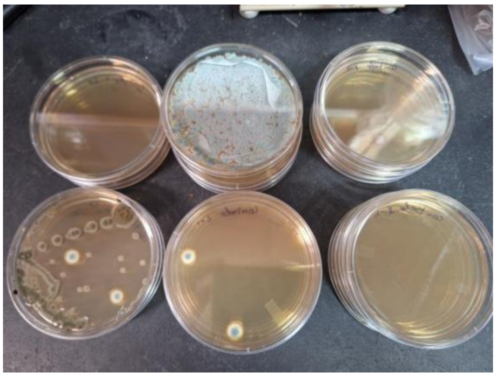


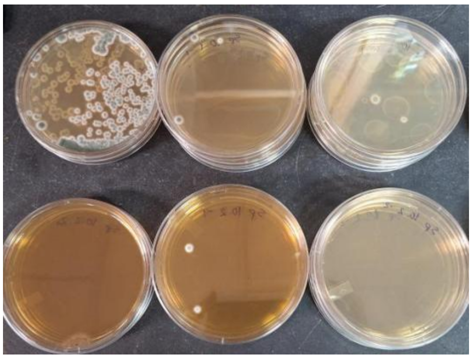
Table 18 - Microbiology results from inoculation two months after production (T2)

Control gummies

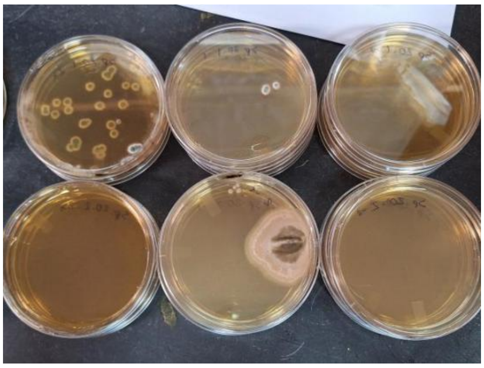


Spirulina-enriched gummies 0.5%

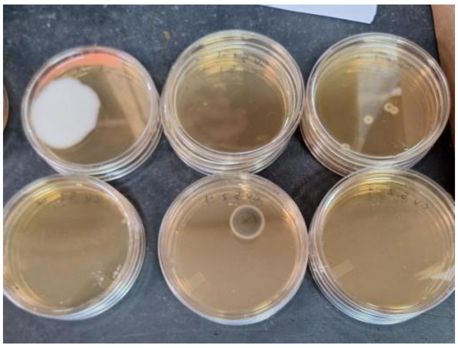
Spirulina-enriched gummies 1.0%



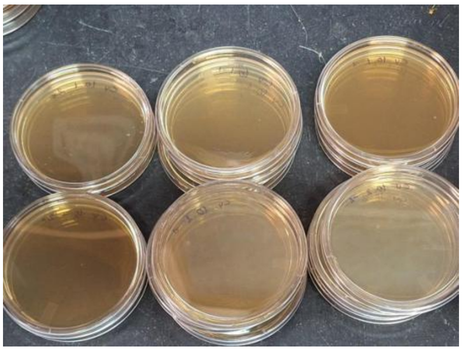
Spirulina-enriched gummies 2.0%



***C. vulgaris*-enriched gummies 0.5%**



***C. vulgaris*-enriched gummies 1.0%**



***C. vulgaris*-enriched gummies 2.0%**