CHARACTERIZATION OF *Spirulina* MICROALGAE AS A NEW ALTERNATIVE FOOD SOURCE

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Doctoral Dissertation
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Doctoral Dissertation

KARAKTERIZACIJA MIKROALGE *Spirulina* KOT NOVEGA ALTERNATIVNEGA VIRA PREHRANE

Doktorska disertacija

Supervisor: Prof. Dr. Nives Ogrinc

Co-Supervisor: Prof. Dr. Polona Jamnik

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To Miha, Ian and Nik,

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Abstract

A reduction in agricultural productivity associated with water scarcity and land degradation, combined with the growing world population and malnutrition, highlights the need for new alternative food sources. In this challenging field, *Spirulina* microalgae emerges as a promising candidate thanks to its high nutritional value and beneficial effect on human health. Unlike conventional foods, *Spirulina* also offers a more sustainable means of production. However, despite its potential, alternative products like *Spirulina* often suffer from a lack of quality and safety inspection, leading to fluctuations in their overall quality.

This thesis focuses on determining the quality, safety and authenticity of *Spirulina* products from the Slovenian market. In addition, the lactic acid fermentation of *Spirulina* biomass, considering its potential to improve the nutraceutical profile, is also investigated, as is the subsequent extraction of bioactive compounds using various solvents. The role of *Spirulina* biomass fermentation and the difference in antioxidant activity between water and ethanol extracts are investigated at the cellular and proteome level using yeast as a model organism. The *Spirulina* ethanol extracts are then further assessed at the molecular level (proteins, metabolites) to explore the role of lactic acid fermentation in compound transformation.

The first part of this doctoral thesis focuses on the quality and safety of commercial *Spirulina*-based dietary supplements in Slovenia. It involves investigating their amino acid, fatty acid and elemental composition and provides information on compliance with the declared nutrient values. In addition, an assessment of iron bioavailability in *Spirulina* products is presented. The findings confirm that *Spirulina* supplements are a valuable source of essential and non-essential amino acids and ω-6 but not ω-3 polyunsaturated fatty acids. They are also a rich dietary source of phosphorous (3.36–26.70% RDA), calcium (0.15–29.50% RDA), selenium (0.01–38.60% RDA) and potassium (0.50–7.69% RDA). However, an analysis of iron bioavailability suggests that, despite their high iron content (7.64–316.00% of RDA), only a small fraction (8.00–18.00%) is bioavailable. Moreover, the addition of additives to *Spirulina* products leads to significant variations in nutrient content and lower product quality. Additionally, inappropriate declarations were found in 86.70% of analyzed samples.

The second part of the thesis focuses on studying the impact of lactic acid fermentation on *Spirulina* biomass and the choice of solvent on the antioxidant properties of the final extract. The fermentation process was carried out using *Lactobacillus plantarum* bacterial culture. The study presents the nutritional composition of the biomass (including minerals, proteins, fats, carbohydrates, and dietary fibers) before and after fermentation. Additionally, it provides the total phenolic content and *in vitro* antioxidant activity of water and ethanol extracts from both non-fermented and fermented broth. The effect of *Spirulina* treatment *in vivo* is demonstrated by cellular antioxidant activity and lipid peroxidation, and cell response at a proteome level using the yeast *Saccharomyces cerevisiae*. The results show how fermentation increases protein bioavailability while reducing the fat content (from 6.26% before to 6.00% dwt after fermentation). Ethanol extracts also exhibit higher *in vitro* (30.00% higher) and intracellular (20.00–40.00% higher) antioxidant activity and lower intracellular oxidative lipid damage (from 13.80 before to 5.71 fluorescence/optical density after treatment). Additionally, a greater antioxidant efficiency of ethanol than water extracts and a lowering of cell stress response-related protein expression in yeast treated with fermented *Spirulina* ethanol extract is observed. The following proteome analysis of the fermented *Spirulina* ethanol extracts showed a decrease in protein content (from 847 to 490) after fermentation and consequent increase in amino acid content indicating a proteolytic activity during fermentation. Similarly, the lipid content decreased, while the lipid metabolites increased. The chlorophyll and carotenoid content lowered after fermentation.

The final part of the thesis explores combining elemental composition and stable isotope ratios to verify the country of origin of *Spirulina* and other algal products. This approach is also employed to determine the potential presence of undeclared ingredients in commercial products. The method successfully differentiated between Hawaiian, Italian and Portuguese products (100%), as well as a good separation of Chinese samples. However, the separation of Indian and Taiwanese samples (66.7%) was less successful.

To summarize, this thesis provides an insight into quality and safety of *Spirulina* products from the Slovenian market, and shows that differences in isotopic, elemental and nutrient composition reflect cultivation, processing methods and environmental conditions, and combined, provide a promising tool for determining the quality and authenticity of *Spirulina* and similar algal products. Finally, the discovery of essential role of lactic acid-fermented *Spirulina* in combating cell oxidative stress and its implications in metabolomics raises new questions offering new research possibilities.

Povzetek

Nižja kmetijska produktivnost, povezana z zmanjšano zalogo vode in degradacijo tal, skupaj z rastjo svetovne populacije in podhranjenosti nakazuje na potrebo po proizvodnji alternativnih živil. Na področju pridelave hrane je mikroalga *Spirulina* zaradi svoje visoke prehranske vrednosti in pozitivnega učinka na zdravje ljudi obetaven nov prehranski vir, ki v nasprotju s konvencionalnimi živili ponuja bolj trajnostno pridelavo. Vendar pa imajo alternativni izdelki, kot je mikroalga *Spirulina*, še vedno pomanjkljiv nadzor kakovosti in varnosti ter so pogosto podvrženi nihanju v kakovosti.

Doktorsko delo se osredotoča na ugotavljanje kakovosti, varnosti in pristnosti izdelkov spiruline s slovenskega trga, uporabo mlečnokislinske fermentacije biomase mikroalge *Spirulina* z namenom izboljšanja njenega prehranskega profila in funkcionalnih lastnosti ter ekstrakcijo njenih bioaktivnih spojin ob uporabi različnih topil. Vpliv fermentacije biomase mikroalge *Spirulina* in razliko v antioksidativni aktivnosti med vodnim in etanolnim izvlečkom smo raziskali na celični in proteomski ravni z uporabo kvasovk kot modelnega organizma. Etanolne izvlečke mikroalge *Spirulina* smo nato nadalje raziskali na molekularni ravni (proteini, metaboliti), da bi ugotovili vlogo mlečnokislinske fermentacije pri transformaciji spojin.

V prvem delu doktorske naloge obravnavamo kakovost in varnost komercialnih prehranskih dopolnil mikroalge *Spirulina* v Sloveniji. Prva raziskava opisuje aminokislinsko, maščobnokislinsko in elementarno sestavo izdelkov spiruline ter podaja informacije o skladnosti analiziranih vrednosti z deklariranimi vrednostmi hranil. Poleg tega predstavlja tudi oceno biološke dostopnosti železa v izdelkih spiruline. Raziskava potrjuje, da so prehranska dopolnila iz mikroalge *Spirulina* dragocen vir esencialnih in neesencialnih aminokislin ter polinenasičenih ω-6, ne pa tudi ω-3, maščobnih kislin. V analiziranih vzorcih je bila prav tako visoka vsebnost fosforja (3,36–26,70 % PDV), kalcija (0,15–29,50 % PDV), selena (0,01–38,60 % PDV) in kalija (0,50–7,69 % PDV); rezultati analize biološke dostopnosti železa pa kažejo, da je kljub visoki vsebnosti železa v vzorcih (7,64–316,00 % PDV) njegova absorpcija v organizmu možna le v manjši meri (8,00–18,00 %). Prisotnost aditivov v izdelkih spiruline se odraža v znatnih razlikah v vsebnosti hranil in nižji kakovosti izdelka; pri 86,70 % analiziranih vzorcev pa smo ugotovili neskladnost deklaracije z izmerjenimi vrednostmi.

Drugi del doktorske naloge se osredotoča na vpliv mlečnokislinske fermentacije biomase mikroalge *Spirulina* in izbire topila na antioksidativne lastnosti končnega ekstrakta. Fermentacijo biomase mikroalge *Spirulina* smo izvedli z uporabo kulture bakterij *Lactobacillus plantarum* ter analizirali hranilno vrednost biomase (minerali, proteini, maščobe, ogljikovi hidrati, prehranske vlaknine) pred in po fermentaciji, skupno vsebnost fenolov in *in vitro* antioksidativno aktivnost v vodnih in etanolnih ekstraktih nefermentirane in fermentirane mikroalge *Spirulina*. *In vivo* učinek tretiranja z mikroalgo *Spirulina* je predstavljen z analizo celične antioksidativne aktivnosti in lipidne peroksidacije ter odzivom celic na ravni proteoma z uporabo kvasovk *Saccharomyces cerevisiae*. Fermentacija mikroalge *Spirulina* se odraža v višji biološki dostopnosti proteinov in nižji vsebnosti maščob (od 6,26 % pred, do 6,00 % suhe mase po fermentaciji). Etanolni izvlečki

fermentirane biomase spiruline kažejo večjo *in vitro* (30,00 % večjo) in intracelularno (20,00–40,00 % večjo) antioksidativno aktivnost ter manjši obseg oksidativnih poškodb lipidov (od 13,80 pred, do 5,71 fluorescence/optične gostote po tretiranju) v primerjavi z drugimi vzorci. Ugotovili smo večjo antioksidativno učinkovitost etanolnih v primerjavi z vodnimi izvlečki mikroalge *Spirulina* ter znižanje vsebnosti proteinov, povezanih z odzivom celic na stres v kvasovkah, obdelanih z etanolnim izvlečkom fermentirane mikroalge *Spirulina*. Nadalje, analiza proteoma etanolnih izvlečkov spiruline je pokazala znižanje števila izraženih proteinov (z 847 na 490) po fermentaciji in posledično povišano vsebnost aminokislin, kar kaže na proteolitično aktivnost med fermentacijo. Zmanjšala se je tudi vsebnost lipidov, medtem ko se je vsebnost lipidnih metabolitov povečala. Vsebnost klorofila in karotenoidov se je po fermentaciji znižala.

Zadnji del naloge predstavlja možnosti uporabe kombinacije elementarne sestave in razmerij stabilnih izotopov za preverjanje geografskega porekla prehranskih dopolnil iz mikroalge *Spirulina* in drugih alg ter za ugotavljanje morebitne prisotnosti nedeklariranih snovi v komercialnih izdelkih mikroalge *Spirulina*. Dosegli smo zanesljivo diferenciacijo havajskih, italijanskih in portugalskih vzorcev (100 %) ter odlično diferenciacijo kitajskih vzorcev spiruline, medtem ko je bila ločba indijskih in tajvanskih vzorcev (66,7 %) nekoliko manj uspešna.

Če povzamemo, rezultati raziskav, zajetih v tem doktorskem delu, podajajo vpogled v kakovost in varnost izdelkov mikroalge *Spirulina* na slovenskem trgu ter kažejo na vpliv gojenja in proizvodnega procesa ter okoljskih pogojev na izotopsko, elementno in prehransko sestavo končnih izdelkov. Predstavljeni rezultati ponujajo obetavno metodo za preverjanje kakovosti, pristnosti in geografskega porekla teh in podobnih izdelkov v obliki analize razmerja stabilnih izotopov lahkih elementov v kombinaciji z analizo elementne sestave ter drugih hranilnih spojin. Novo odkrita vloga fermentirane mikroalge *Spirulina* v boju proti celičnemu oksidativnem stresu in vpliv mlečnokislinske fermentacije na transformacijo organskih molekul odpirata dodatna raziskovalna vprašanja in ponujata nove priložnosti za nadaljnje raziskave.

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Abbreviations

AIR	... Ambient Inhalable Reservoir
AFA	... *Aphanizomenon flos-aquae*
C20:4 ω-6	... arachidonic acid
BF_3	... boron trifluoride
C3	... C3 plants, Calvin cycle photosynthesis
C4	... C4 plants, Hatch-Slack cycle photosynthesis
$CaCl_2$	... calcium chloride
CO_2	... carbon dioxide
$\delta^{13}C$	... carbon isotopic composition ($^{13}C/^{12}C$)
CAM	... crassulacean acid metabolism, CAM plants
δ	... delta, relative isotopic composition in parts per thousand or "per mil" (‰)
DPPH	... 2,2-diphenyl-1-picrylhydrazyl
K_2HPO_4	... dipotassium phosphate
DA	... discriminant analysis
DHA	... docosahexaenoic acid
DWT	... dry weight
EPA	... eicosapentaenoic acid
E	... element
EA-IRMS	... elemental analyzer - isotope ratio mass spectrometer
ED-XRF	... energy dispersive X-ray fluorescence
EU	... European Union
FA	... fatty acid
FAME	... fatty acid methyl ester
F/OD	... fluorescence/optical density
FAO	... Food and Agriculture Organization of the United Nations
FDA	... Food and Drug Administration
GABA	... gamma(γ)-amino butyric acid
GLA	... gamma(γ)-linolenic acid
C18:3 ω-6	... gamma(γ)-linolenic acid
GC-FID	... gas chromatography - flame ionization detector
GC-MS	... gas chromatography - mass spectrometry
GRAS	... generally recognized as safe
C17:0	... heptadecanoic acid
HPLC	... high performance liquid chromatography
δ^2H	... hydrogen isotopic composition ($^2H/^1H$)
IF	... impact factor
ICP-MS	... inductively coupled plasma - mass spectrometry
ICP-OES	... inductively coupled plasma - optical emission spectroscopy
IS	... internal standard
IPS	... International Postgraduate School

IRMS	isotope ratio mass spectrometry
JSI	Jožef Stefan Institute
LAB	lactic acid bacteria
C18:2 ω-6c	linoleic acid
C18:3 ω-3	linolenic acid
LC-MS	liquid chromatography - mass spectrometry
LDL	low-density lipoprotein cholesterol
$MgSO_4$	magnesium sulphate
i	atomic mass number of the heavier isotope of an element (E)
j	atomic mass number of the lighter isotope of an element (E)
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid
C14:0	myristic acid
NCBI	National Center for Biotechnology Information
NADPH	nicotinamide adenine dinucleotide phosphate
$\delta^{15}N$	nitrogen isotopic composition ($^{15}N/^{14}N$)
NMR	nuclear magnetic resonance
C18:1 ω-9c/9t	oleic/elaidic acid
ω	omega
OPLS-DA	orthogonal partial least squares discriminant analysis
O_2	oxygen
$\delta^{18}O$	oxygen isotopic composition ($^{18}O/^{16}O$)
C16:0	palmitic acid
C16:1 ω-7	palmitoleic acid
PUFA	polyunsaturated fatty acid
K_2SO_4	potassium sulphate
PCA	principal component analysis
QPS	qualified presumption of safety
Rs	ratio between the sample heavier and lighter isotope
Rr	ratio between the reference material's heavier and lighter isotope
ROS	reactive oxygen species
RDA	recommended dietary allowance
SFA	saturated fatty acid
ARRS	Slovenian Research Agency
$NaHCO_3$	sodium bicarbonate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
$NaNO_3$	sodium nitrate
SPE	solid phase extraction
C18:0	stearic acid
C18:4 ω-3	stearidonic acid
SO_2	sulfur dioxide
$\delta^{34}S$	sulfur isotopic composition ($^{34}S/^{32}S$)
TOF	time of flight
TFA	total fatty acids
TPC	total phenolic content
2D-DIGE	two-dimensional differential gel electrophoresis
2-DE	two-dimensional gel electrophoresis
UHPLC	ultra-high-performance liquid chromatography
UFA	unsaturated fatty acid
V-CDT	Vienna Cañon Diablo Troilite
VPDB	Vienna Pee Dee Belemnite

VSMOW	. . .	Vienna Standard Mean Ocean Water
WHO	. . .	World Health Organization
XRF	. . .	X-ray fluorescence

Chapter 1

Introduction

The rapid economic development in the 20th century has profoundly impacted human nutrition. Malnutrition has continued to spread fast, despite the unprecedented wealth and surplus of food stocks on the global level. The World Health Organization (WHO) estimates that approximately half of humanity suffers from malnutrition. Many over-nourished, some normally nourished, and virtually all under-nourished suffer from vitamin and mineral deficiencies. Over half of the world's diseases are caused by different micronutrient deficiencies, overeating and hunger. In addition, the hungry perform well below their potential, while the overweight spend decades in later life crippled with diseases, which are at least partly attributable to overeating. Accordingly, the problem of malnutrition not only affects the quality of life of individuals but is slowing down or, in some cases, even reversing societal development (ACC/SCN, 2000; Gardner & Halweil, 2000; World Health Organization, 1998).

The problem is made worse by the fact that the world population is projected to reach 9.8 billion by 2050, resulting in a substantial increase in the demand for food. According to various studies (Tilman et al., 2011; United Nations, 2022), food production must be doubled during the same period to meet the rising demand effectively. However, increasing water scarcity and reduced land productivity diminish agricultural yields (FAO, 2009; Rhoades et al., 1992). Although the intensive use of croplands and land clearings may result in higher crop production, these agricultural practices have significant environmental impacts. They threaten biodiversity, contribute to the fragmentation of habitats, and result in high greenhouse gas emissions. Also, they harm terrestrial, freshwater, and marine ecosystems due to the excessive use of pesticides and fertilizers (Beddington, 2011; Burney et al., 2010; Dirzo & Raven, 2003; Godfray et al., 2010; Vitousek et al., 1997). Population growth, the increased demand for food, declining agricultural productivity, ongoing malnutrition, and the challenges posed by climate change underscore the urgent need for the sustainable production of alternative food products (FAO, 2009; Gardner & Halweil, 2000).

One solution is to harness the potential of algae, which serve as a rich source of nutritional and functional compounds (Sotiroudis & Sotiroudis, 2013). Algae are a diverse group of simple, non-flowering, predominantly aquatic plants that possess chlorophyll but lack true leaves, roots, stems, and vascular tissue (R. E. Lee, 2018). They are divided into macro- and microalgae, which differ according to their size and content of organelles. Benthic, multicellular algae, which can reach several meters in length, are macroalgae, while microalgae are eukaryotic microscopic unicellular phytoplankton. Cyanobacteria, a polyphyletic group of organisms, are a prokaryotic group of microalgae that also exhibit remarkable biodiversity and can exist in both unicellular and multicellular forms. For this reason, classifying cyanobacteria as microalgae or bacteria still presents a challenge among taxonomists (Hachicha et al., 2022; Palinska & Surosz, 2014; Thajuddin & Subramanian, 2005).

Cyanobacteria possess an intermediate structure between plants (due to oxygenic photosynthesis) and bacteria (due to the absence of chloroplasts, mitochondria and a membrane-bound nucleus). They contain essential photosynthetic pigments such as chlorophyll a, carotenoids, phycobilisomes (especially phycoerythrin and phycocyanin), and xanthophylls, which serve as light collectors (Garcia-Pichel, 2009; Sandesh Suresh et al., 2019) and the presence of chlorophyll a and phycocyanin pigments is why cyanobacteria are also called blue-green algae. These pigments are present in the membrane vesicles, thylakoids (Thajuddin & Subramanian, 2005). Cyanobacteria are also one of the oldest known organisms, with their presence dating back nearly 3.5 billion years, to the Archean eon. They are believed to have played a crucial role in oxygenating Earth's atmosphere and are considered the photosynthetic ancestors of chloroplasts in plants and algae. Notably, cyanobacteria possess the unique ability to carry out both aerobic respiration and photosynthesis concurrently, as well as fix CO_2 and, in certain cases, nitrogen (Fournier et al., 2021; Garcia-Pichel, 2009; Vermaas, 2001; Whitton, 1992).

Arthrospira spp., the focus of this doctoral thesis, is one of the most important microalgal biomasses produced, belonging to the group of prokaryotic microalgae – cyanobacteria (de Morais et al., 2014; Soni et al., 2017). *Arthrospira* possesses a unique organization of cylindrical filaments – rows of cells referred to as trichomes, in a left-hand open helix across its entire length, from which it gets its name *Arthrospira.* The trichome spiral shape is specific for the *Arthrospira* genus, but the size (3–4 μm) and length (50–500 μm) of the helix vary among species. Also, a considerable variation in the degree of helicity may be noticed among different strains and within the same strain (J. Costa & Morais, 2013; Thajuddin & Subramanian, 2005). In the literature, there is also confusion regarding whether *Arthrospira* is a unicellular or a multicellular organism (Chamorro et al., 2002; Dartsch, 2008; Matufi & Choopani, 2020; Soni et al., 2017; Usharani et al., 2012). However, unlike other bacteria, which also possess a spiral shape and are unicellular, helical filaments of *Arthrospira* microalgae are multicellular, consisting of cells bound in a single row (Figure 1.1). The growth of *Arthrospira* occurs through cell division, leading to elongation. However, it uses fragmentation to reproduce, which means that when a part of a filament breaks off, a new filament is initiated (Ciferri, 1983; Tomaselli, 2002).

Figure 1.1: Morphological structure of *Spirulina.* (Sili et al., 2012).

The *Arthrospira* genus, unlike other cyanobacteria, generally cannot fix atmospheric nitrogen (Fujisawa et al., 2010). This ability has only been attributed to one related species (*Spirulina labyrinthyformis*) isolated from a hot spring (Carvalho et al., 2012). The surface of *Arthrospira* is smooth, has no cellulose covering, and is easily digested by simple enzyme systems. *Arthrospira* microalgae are photosynthetic and, thus, autotrophic. The blue pigment phycocyanin is its primary photosynthetic pigment, together with chlorophyll a and carotenoids (S. K. Ali & Saleh, 2012; Capelli & Cysewski, 2010; Habib et al., 2008).

There is some controversy surrounding the name of this cyanobacteria. In 1892, the aseptate form of this blue-green microalgae was attributed to the *Spirulina* genus, while the septal form was attributed to the *Arthrospira* genus. Later, in 1932, the members of the two genera were reunified under the designation *Spirulina*, due to the similar helical morphology and without considering the presence of the septum. In 1989 the two forms of microalgae were again classified separately into two genera: *Arthrospira* and *Spirulina*, a classification which is currently accepted in the scientific community (Sánchez et al., 2003; Tomaselli et al., 1996). Therefore, the cyanobacteria that make up the commercial products, more often known as *Spirulina*, are members of the genus *Arthrospira*. Nevertheless, these names are now commonly used interchangeably due to the extensive historical use and marketing of *A. platensis* and *A. maxima*, as *Spirulina*. *Spirulina* is also the name that the scientific community commonly uses when referring to various species of *Arthrospira* microalgae. For this reason, according to the Food and Agriculture Organization (FAO), *Arthrospira* species can be referred to as *Spirulina* (Habib et al., 2008). In this thesis, *Spirulina* refers to the genus *Arthrospira*.

The most interesting *Spirulina* species used in food and supplement production are *S. platensis* and *S. maxima*. *S. platensis* is more widely distributed and mainly found in Africa, Asia and South America, while *S. maxima* is confined chiefly to California and Central America. The beginnings of the *S. platensis* use in food are considered in the Kanem area of Africa, with the center in Lake Chad, where the first recorded use of *Spirulina* as a foodstuff was in 1940, while the first recorded use of *S. maxima* was in 1521, when it was harvested from Lake Texcoco (today's area of Mexico City) (Belay, 2007; Tomaselli, 2002). Among the many algae-based products, it is also possible to come across the commercial name *Spirulina pacifica*, which is the *S. platensis* strain pacifica (Nicoletti, 2016; Ötleş & Pire, 2001; Savranoglu & Tumer, 2013; L.-C. Wu & Ho, 2007). The biological classification of the most commonly used *Arthrospira* species, *A. platensis*, is presented in Table 1.1.

Table 1.1: Biological classification of *Arthrospira platensis*. NCBI Taxonomy ID: txid1154, txid118562 (Schoch et al., 2020).

Taxonomic rank	**Scientific name**
Domain	Bacteria - Prokaryota
Kingdom	Eubacteria
Phylum	Cyanobacteria
Class	Cyanophyceae
Sub-class	Oscillatoriophycideae
Order	Oscillatoriales
Family	Microcoleaceae
Genus	*Arthrospira*
Species	*A. platensis*

Spirulina is a highly nutritious food which contains various phytonutrients, nutraceuticals, antioxidants and prebiotics. Additionally, it is a good source of protein, containing all of the essential, as well as most of the non-essential amino acids, omega-6 fatty acids, minerals, vitamins, and pigments (Bashir et al., 2016; Chopra & Bishnoi, 2008; Chu et al., 2010; Liestianty et al., 2019; Pulz & Gross, 2004; Vo et al., 2015). Various studies have proven *Spirulina*'s effectiveness in the treatment of many pathological conditions, such as certain types of cancer, hyperlipidemia, cardiovascular diseases and hepatotoxicity (Czerwonka et al., 2018; Gad et al., 2011; Habib et al., 2008; Nagaoka et al., 2005). Due to its high nutritional value and digestibility, it has been designated by the

FAO as a high-quality dietary supplement and food product (Pelizer et al., 2003). After thorough research was conducted to address its safety and quality, the Food and Drug Administration (FDA) awarded *Spirulina* GRAS status (Generally Regarded As Safe) for its use in food products (Belay, 2007; U.S. Food and Drug Administration, 2012). *Spirulina* is the most widely consumed microalga in the US and EU (Boukid & Castellari, 2021).

In the EU, microalgae are regulated under the EU Novel Foods Regulation 2015/2283. Here, novel foods are defined as food that humans in the EU had not consumed to a significant degree before the 15th of May, 1997. This regulation ensures that food products commercialized in the EU are adequately labelled and safe for consumption (European Commission, 2023b). However, *Spirulina* products and proteins derived from *Spirulina* can be commercialized in the EU as food without regulation due to its long history of consumption. Up-to-date information regarding *Spirulina*'s status as a novel food is available in the EU Novel Food Catalogue (European Commission, 2023a).

1.1 Production of *Spirulina*

Due to their small size (5 to 500 µm), microalgae cannot be harvested from the environment and must be cultivated in controlled industrial facilities. Efficient cultivation of microalgae requires meeting specific physiological needs, such as an energy source (light and temperature), a carbon source (CO_2 supply), a nutrient source (various minerals) and the removal of toxic metabolites (excess oxygen). In addition, the production of microalgae (Figure 1.2) involves several key steps, including culturing system (vessel) design, culturing medium and operational conditions selection and optimization, biomass harvesting and drying (Brányiková & Lucáková, 2021; Lafarga et al., 2021).

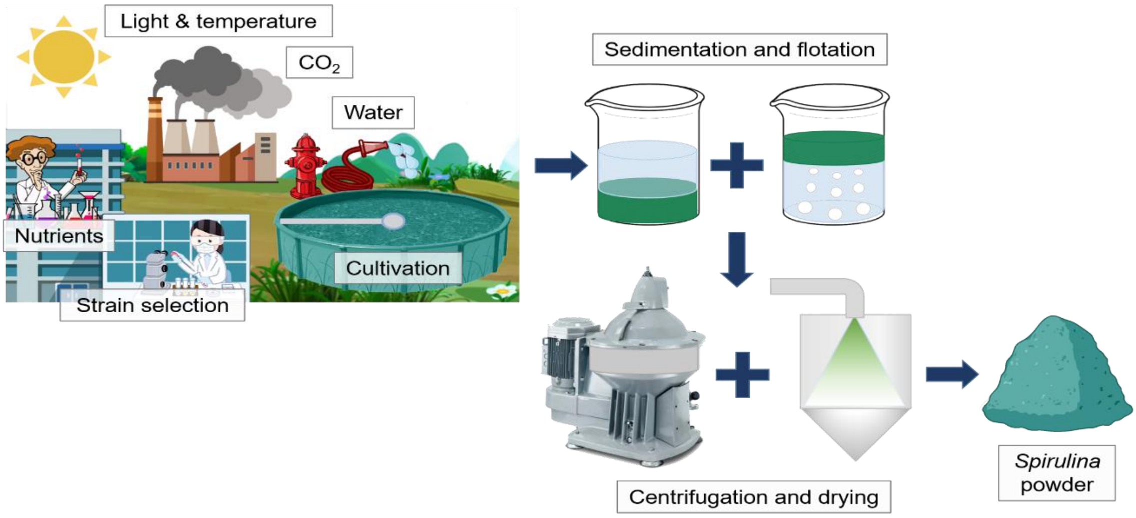


Figure 1.2: *Spirulina* powder production scheme.

1.1.1 Culturing conditions

Production of the *Spirulina* microalgal biomass demands specific growth conditions and production techniques. An alkaline pH of 8.5 to 11.0 is required for growth, with optimal pH values from 9.0 to 10.0 (Ciferri, 1983; Ragaza et al., 2020). The optimal temperature range for cultivation is from 30 to 35 °C regarding culture density and protein productivity (Colla et al., 2007; Ogbonda et al., 2007). During cultivation, however, the temperatures can rise to 39 °C without affecting its photosynthetic ability and drop to 15 °C for growth to still succeed. Low overnight temperatures (25 °C) can result in a loss in biomass, reduction of β-carotene concentration, and diversion of the culture photosynthetic activity from the synthesis of carbohydrates to the synthesis of protein (Habib et al., 2008).

Spirulina microalgae can be found in nature in freshwater, thermal springs, swamps, brackish water, seawater and alkaline salt water, with salt concentrations higher than 30 g/L and a pH of 8.5–11.0. The optimal salt concentration for *Spirulina* cultivation is between 20 and 70 g/L (Habib et al., 2008). Salt deficiency or excess in the culturing medium leads to osmotic stress in the cells, resulting in the acceleration or deceleration of carbohydrate, fat and protein production (Mogale, 2016).

Light quality and intensity are among the most important parameters when producing *Spirulina*. Sunlight is preferred, but artificial illumination is also an option. Low light intensities will result in biomass with a high concentration of cultured cells, rich in proteins and pigments, but with a lower growth rate due to the self-shading effect of the microalgae. Alternatively, high light intensities result in a higher growth rate (AlFadhly, Alhelfi, Altemimi, Verma, & Cacciola, 2022; Lafarga et al., 2021; Mogale, 2016; Soni et al., 2017).

In order to homogenize and maintain the suspension of the filaments, i.e., to prevent clumping, enable an even distribution of nutrients and prevent thermal stratification, it is necessary to provide sufficient agitation and aeration to the culturing medium. It also enables a homogenous distribution of carbon dioxide and eliminates inhibitory chemicals like oxygen from the medium. Inadequate ventilation will reduce energy efficiency, biomass growth, and production (Delrue et al., 2017; Soni et al., 2017).

Zarrouk's medium has been used as a well-established standard and optimal medium for various *Spirulina* species production. Zarrouk's medium remains the only conventional medium used in large-scale commercial *Spirulina* cultivation. Its composition, which provides an effective nutritional supplementation, includes sodium bicarbonate ($NaHCO_3$), sodium nitrate ($NaNO_3$), magnesium sulphate ($MgSO_4$), potassium sulphate (K_2SO_4), calcium chloride ($CaCl_2$), and dipotassium phosphate (K_2HPO_4) (Madkour et al., 2012; Pandey et al., 2010). However, as this medium includes the use of some expensive chemicals, various alternatives have been tried, such as growth media using food co-products (Barrocal et al., 2010; Coca et al., 2015), commercial fertilizers (Gómez et al., 2021; Madkour et al., 2012; Raoof et al., 2006), seawater (Mary Leema et al., 2010) and wastewater (Y. Chang et al., 2013; Phang et al., 2000; Zhai et al., 2017). These mediums provide a lower cost for *Spirulina* production, but the use of the final products may be limited. Namely, *Spirulina* produced in a medium containing wastewater cannot be added to food products or be used as a food supplement due to the varying content of nutrients and the possible presence of toxins in the medium and final product (Lafarga et al., 2021; Lim et al., 2021). For this reason, modified Zarrouk's medium variants have also been studied using cheaper nitrogen sources (Carvalho et al., 2004; Rodrigues et al., 2011).

1.1.2 Cultivation system

Spirulina can be cultivated in an open or closed system (Figure 1.3) (Singh & Sharma, 2012). The open raceway ponds consist of a pond and a separator delineating the culture's circulation. Circulation of the culture fluid is enabled by a paddle wheel that provides constant agitation for an even distribution of nutrients, oxygen and *Spirulina* fibers. The standard depth of the raceway ponds is from 20 to 30 cm (Murphy et al., 2015). While open cultivation systems are easier to construct and operate, demand a lower financial input (Ugwu et al., 2008) and are more cost-effective, as they require limited equipment and low maintenance (Borowitzka & Moheimani, 2013), some negative aspects remain to be addressed. For example, in this type of production system, the culture is directly exposed to the external environment, meaning that parameters such as seasonal and temperature fluctuations, light intensity and low light utilization by the algae, pH, nutrient and dissolved oxygen concentrations, water loss due to evaporation and loss of carbon due to its diffusion to the atmosphere must be considered when building an open system (Borowitzka & Moheimani, 2013; Cuaresma et al., 2011). For instance, lower water aeration reduces biomass production compared to a closed system (Cuaresma et al., 2011). Another issue is culture contamination by fauna and toxin-producing algae (Singh & Sharma, 2012). In addition to artificial ponds and containers, natural waters such as ponds, lakes and lagoons are categorized under this production technique. Circular ponds, raceway ponds and tanks are the most commonly used open production systems (Ugwu et al., 2008). *Spirulina* cultivation for commercial purposes is performed mainly in raceway ponds (Figure 1.3b), which are used to mimic the optimal growth conditions of the natural alkaline and saline lakes in tropical and subtropical regions (Lafarga et al., 2021; Ragaza et al., 2020).

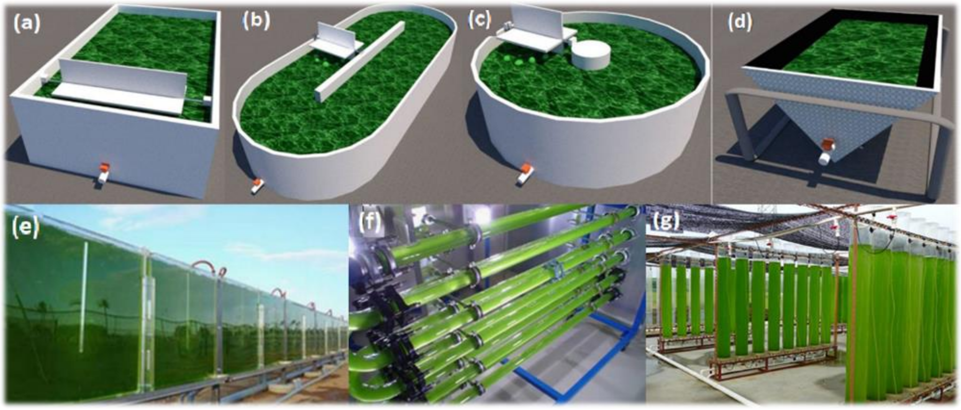


Figure 1.3: Types of photobioreactor systems for *Spirulina* production. Open systems: (a) rectangular open, (b) raceway, (c) circular and (d) V-shaped pond; closed systems: (e) flat-plate, (f) tubular and (g) vertical column photobioreactor (Brányiková & Lucáková, 2021; dos Santos et al., 2021; Huo et al., 2018).

Closed production systems are essentially photobioreactors. Despite the higher construction and operation costs, there are several advantages to this type of cultivation system. First, these are enclosed culture vessels, which are designed for controlled cultivation of microalgal biomass. In closed systems, there is no direct exchange of contaminants or gasses with the environment, and parameters such as light intensity, temperature, pH level, carbon dioxide and water supply are highly controlled, while aeration, gas exchange and culture density (0.6 to 1.2 g_{dwt}/L) are predetermined (Hase et al., 2000; Torzillo et al., 1986). Due to a highly controlled environment, they enable a higher cell concentration development (up to 90% higher) and cultivation of only one microalgal culture due to the probability of contamination (Tsoglin et al., 1996). Types of closed photobioreactors include tubular photobioreactors, flat plates and vertical columns, with tubular photobioreactors being the most commonly used closed systems for commercial *Spirulina* production (Ragaza et al., 2020; Soni et al., 2017).

Additional solutions regarding microalgae production are also available, such as systems combining open and closed systems in so-called hybrid systems, which combine the advantages and minimize the disadvantages of both systems. Here, five to seven times higher productivity is achieved compared to an open cultivation system while providing better control of variable culturing parameters and reduced energy consumption. However, hybrid systems require substantial land area and complex technical handling (Soni et al., 2017; Zittelli et al., 2013).

1.1.3 Harvesting and drying

Another crucial point in *Spirulina* production is harvesting and drying, which impact the final product quality through possible contamination and changes in the biomass's nutrient content and organoleptic properties (Desmorieux & Hernandez, 2004; Lafarga et al., 2021). *Spirulina* should be harvested in the early morning due to the higher percentage of proteins in the biomass. Different methods are available and used by producers for microalgae collection, e.g., ultrasonic vibration, flotation, filtration, and centrifugation (AlFadhly, Alhelfi, Altemimi, Verma, & Cacciola, 2022). However, centrifugation remains the most commonly used technique for harvesting biomass (Dassey & Theegala, 2013). Combining techniques, such as an initial pre-concentration of the biomass using a flotation or sedimentation process, followed by centrifugation, can also reduce production costs (Lafarga et al., 2021). Also, because *Spirulina* is larger than other microalgae, the centrifugation process may be excluded since 80% of the biomass can be recovered using flotation (S. -G. Kim et al., 2005).

The drying method used in *Spirulina* production depends on the intended use of the final product, as different drying techniques affect the biomass quality to a different extent. The main drying technique large producers use is spray drying, while small producers usually prefer the convective drying technique. Solar drying and lyophilization (freeze-drying) have also been assessed but are less frequently used. Although the solar drying method is the most cost-effective, there remains a high risk of biomass contamination. Lyophilization, on the other hand, offers high-quality dried biomass production, but the process is too expensive to be incorporated into large-scale microalgae production (AlFadhly, Alhelfi, Altemimi, Verma, & Cacciola, 2022; Desmorieux & Hernandez, 2004; Lafarga et al., 2021; Shekarabi et al., 2019).

1.1.4 Environmental impact of *Spirulina* production

Production of conventional crops results in high water and land use, deforestation, soil erosion, and water contamination, and the use of fossil fuels adds to high atmospheric CO_2 levels, where a 2.2% increase is predicted between the years 2015 and 2040 (Belay, 2007; Shao et al., 2019; Soni et al., 2017). *Spirulina* microalgae production could help reduce the negative impacts on the environment, as it can be cultivated in arid lands, which are not suitable for other agricultural means, and the land area and amount of water needed for its production are lower (on a nutrient content basis) compared to other crops (25% water consumption compared to soy protein, 17% compared to maize protein and 2% compared to beef protein production). The culturing medium used in *Spirulina* production can also be recycled after harvesting, reducing freshwater and nutrient consumption. This recycling process also contributes to decreased environmental pollution caused by the emission of nitrogen oxide, ammonia, phosphate, and nitrate from fertilizers into the environment. Additionally, no waste is produced, as all biomass produced is adequate for food, feed or other commercial or industrial use (Belay, 2007; Habib et al., 2008; Soni et al., 2016; Ye et al., 2018).

Spirulina biomass doubles in 4–5 days due to its high photosynthetic activity, and no biocides are needed in its production since the high alkalinity of the cultivation medium inhibits the growth of other contaminating algae (Belay, 2007). Also, *Spirulina* production has a lower negative impact on the environment than food products containing the same amount of protein and β-carotene since it requires less energy and freshwater due to the possibility of using alkaline or brackish water in its production and reuse of the growth medium. Additionally, more protein is produced per unit area (20 times higher than soy, 40 times higher than corn, and 200 times higher than beef), and there is no competition for production land (Habib et al., 2008; Hu et al., 2008; Ye et al., 2018; Zhu et al., 2014).

Microalgae are also interesting because of their high photosynthetic efficiency, which amounts to 10–20% *vs* 3–6% in plants (Duran Quintero et al., 2021; Sudhakar & Mamat, 2019). Due to their successful CO_2 sequestration and copious oxygen production, microalgae are considered a future effective tool to help reduce the greenhouse effect. *Spirulina* consumes approximately 1.8 kg of CO_2 per kg of dry biomass (0.36-1.78 kg CO_2 per kg of fresh biomass) and converts it to O_2 and biomass. As it shows an excellent prospect to contribute to CO_2 reduction in the atmosphere, the possibility of using flue gas as a source of inorganic carbon in *Spirulina* cultivation would be economical and positive for the environment (Doucha et al., 2005; Duarte et al., 2017).

1.2 Overview of *Spirulina*'s Nutritional Quality

Spirulina has gained the interest of the scientific community and industry as a food product for daily nutrition due to its high content of various macro- and micronutrients and bioactive compounds, which have a positive impact on human health and vitality and have been experimentally proven to be effective in the treatment of various pathological conditions (Chopra & Bishnoi, 2008; Gutiérrez-Salmeán et al., 2015; Pulz & Gross, 2004).

1.2.1 Proteins

Spirulina microalgae has a high protein content ranging from 60 to 70% of dry weight. Such amounts make *Spirulina* exceptional among food protein sources, superior to meat, eggs, milk, and grains (Table 1.2). Even the best plant protein sources (e.g. soya) contain only up to 40% of protein (AlFadhly, Alhelfi, Altemimi, Verma, Cacciola, et al., 2022). In the literature, *Spirulina* protein values on a dry basis are compared to the protein values of other foods on a wet basis. This fact might seem misleading since *Spirulina* protein content on a wet basis is considerably lower – 7.2% (E. G. Oliveira et al., 2009). However, if we consider that *Spirulina* is mainly consumed in dry form (powder, tablet, capsules), while other foods are mainly consumed fresh, such a comparison makes sense. The protein content fluctuates by 10 to 15% depending on the harvest time, where the highest content of protein was achieved when *Spirulina* was harvested early in the morning (AlFadhly, Alhelfi, Altemimi, Verma, & Cacciola, 2022; Ragaza et al., 2020).

The proteins of *Spirulina* microalgae are complete from a qualitative point of view, containing all the essential and most of the non-essential amino acids. The essential amino acids constitute 39–47% of the total protein mass (AlFadhly, Alhelfi, Altemimi, Verma, Cacciola, et al., 2022; Ragaza et al., 2020), and their proportions can be compared to those of meat, eggs and milk; but are superior to those in legumes and other plant sources. Additionally, *Spirulina* proteins are easy to digest (83–90%) due to cellulose-free cell walls, which consist of mucopolysaccharides and simple absorbable sugars (AlFadhly, Alhelfi, Altemimi, Verma, Cacciola, et al., 2022; Lupatini et al., 2017; Muys et al., 2019; Ragaza et al., 2020).

Table 1.2: Protein content of *Spirulina* and other foods. Protein values are given for the most commonly used form (dry/fresh).

Food product	**Protein content** (%)	**Reference**
Spirulina (dry)	55.0–70.0	(Aouir et al., 2017; Campanella et al., 1999; E. G. Oliveira et al., 2009; Saranraj & Sivasakthi, 2014)
Beef (fresh)	17.1–20.7	(Biel et al., 2019; Hall & Schönfeldt, 2013)
Pork (fresh)	18.2–21.9	(Heber et al., 2010; J. Ma et al., 2019)
Chicken (fresh)	21.0–24.0	(Negrão et al., 2005; Taşkıran et al., 2020)
Fish (fresh)	8.6–24.0	(Aberoumand, 2012; Azam et al., 2004; Usydus et al., 2011)
Cow milk (fresh)	3.28–3.95	(Palmquist & Moser, 1981; Vanga & Raghavan, 2018; Yasmin et al., 2020)
White cheese (fresh)	10.9–23.6	(Jaoude et al., 2010; Yaman et al., 2022)
Hard cheese (fresh)	21.5–25.7	(Popović-Vranješ et al., 2016)
Eggs (fresh)	11.7–12.6	(AlFadhly, Alhelfi, Altemimi, Verma, Cacciola, et al., 2022; Kusum et al., 2018; Lesnierowski & Stangierski, 2018)
Soybean (fresh)	35.4–40.3	(Ciabotti et al., 2016; Karr-Lilienthal et al., 2006)
Beans (fresh)	19.0–25.2	(Barreto et al., 2021; J. Liu et al., 2022)

Spirulina is an excellent source of phycobiliprotein, mainly C-phycocyanin (20% of *Spirulina* protein fraction) and allophycocyanin in a ratio of around 10:1. These proteins are used in various applications, such as nutritional ingredients, biomedical markers, and natural food and cosmetics colorings. They are believed to be responsible for most of *Spirulina*'s positive health effects, where they act as therapeutic agents in diseases induced by oxidative stress (Bhat & Madyastha, 2001; Grover et al., 2021; Lupatini et al., 2017; Patil et al., 2008). C-phycocyanin contains approximately 4.7% of phycocyanobilin (6.7 mg/g of *Spirulina*), a homolog of biliverdin, which is reduced to phycocyanorubin (bilirubin homolog) in mammalian cells. The latter strongly inhibits the activity of the NADPH oxidase enzyme complex and, in that way, prevents and helps in the treatment of diseases caused by NADPH oxidase overactivity, such as cardiovascular disease, certain cancer types, allergies, diabetes, Alzheimer's and Parkinson's disease, rheumatoid arthritis and others (McCarty, 2007).

Additionally, *Spirulina* proteins can be hydrolyzed to bioactive peptides. These have been shown to possess various beneficial biological activities such as antioxidative, antiviral, anticoagulant, antidiabetic, antihypertensive, anti-obesity and antiproliferative, and have received much attention. They present a valuable source for the development of foods and pharmaceuticals with added value (Czerwonka et al., 2018; Gad et al., 2011; Joventino et al., 2012; Nagaoka et al., 2005; Vo et al., 2013; Z. Wang & Zhang, 2017).

1.2.1.1 Amino acids

The proteins from *Spirulina* contain all of the essential amino acids – histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, which constitute about 45% of the total protein content in *Spirulina*; as well as the non-essential amino acids – alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, proline, serine and tyrosine. The content of sulfur-containing amino acids methionine and cysteine is somewhat poorer (Andrade et al., 2018; Muys et al., 2019; Ragaza et al., 2020; Vo et al., 2015), but due to the high *Spirulina* amino acid bioavailability, which amounts from 85.1% in dry *Spirulina* to 86.6% in fresh *Spirulina*, also the content of underrepresented amino acids is still notable (Shioji et al., 2021). The amino acid composition in *Spirulina* microalgae is balanced and in proportion to that recommended by FAO. As such, it can be compared to conventional protein sources (meat, milk, eggs) and is superior to vegetable proteins (Andrade et al., 2018; Lupatini et al., 2017; Muys et al., 2019; Ragaza et al., 2020). Table 1.3 presents the amino acid composition of *Spirulina*.

Table 1.3: *Spirulina* amino acid composition. Ranges of amino acid values (mg/g_{dwt}), as presented in the literature (Andrade et al., 2018; Bashir et al., 2016; Campanella et al., 1999; Dewi et al., 2016; Z.-Y. Li et al., 2007).

Non-essential	**Content** (mg/g_{dwt})	**Essential**	**Content** (mg/g_{dwt})
Alanine	33.4–58.0	Histidine	6.00–14.9
Arginine	4.00–51.0	Isoleucine	20.6–42.0
Aspartic acid	49.8–64.0	Leucine	34.4–61.7
Cysteine	4.00–6.40	Lysine	24.5–36.0
Glutamic acid	83.1–101	Methionine	12.8–17.1
Glycine	28.9–43.0	Phenylalanine	20.8–35.2
Proline	21.0–27.0	Threonine	29.0–33.8
Serine	23.0–38.6	Tryptophan	8.50–11.0
Tyrosine	23.9–30.7	Valine	25.3–60.0

The protein and amino acid content and profile in *Spirulina* are affected by climatic conditions in open production systems and culturing conditions in closed production systems (Carcea et al., 2015; M. A. C. L. de Oliveira et al., 1999; Muys et al., 2019). In this respect, the cultivation temperature is one of the most important parameters. For example, high culturing temperatures of (40 °C) result in a lower protein content than when grown at optimal temperature (*S. platensis*: 25–30 °C and *S. maxima*: 30–35 °C). Also, *S. platensis* is more affected by high temperatures than *S. maxima* due to its lower optimal growth temperatures (M. A. C. L. de Oliveira et al., 1999). *Spirulina*'s highest essential amino acid content was observed at 30 °C and pH 9.0, and the lowest at 25 °C and pH 8.5 (Ogbonda et al., 2007). All the amino acid profiles were similar when different nitrogen sources (nitrate, nitrite, ammonium and urea) were used for cultivation; the highest amino acid content was obtained using urea and the lowest using ammonium. Also, different cultivation times might reflect a different amino acid content (A. Choi et al., 2003).

Drying techniques also affect the protein and, as a result, amino acid content. For example, infrared drying in spreading cylinders and convective drying techniques result in the highest protein losses, followed by thin-layer drying. Conversely, lyophilization consistently yields the best protein recoveries. Among the amino acids, methionine is particularly susceptible to changes during drying. For instance, the drum-drying technique has been shown to reduce methionine levels by ≥30% compared to the spray-drying technique (E. W. Becker & Venkataraman, 1984; Desmorieux & Hernandez, 2004).

Amino acid analysis of food products is divided into free (not bound in protein) and total (free plus protein–bound) amino acid analysis. While total amino acid analysis is used to determine the nutritional quality or total protein content of a food product (Otter, 2012; Zhao et al., 2023), free amino acid analysis is used to assess proteolytic activity in food processing, also to determine their contribution to overall acceptability of the food product, due to their role in the taste and flavor (Kowalska et al., 2022).

The free amino acid analysis includes protein precipitation and precipitated protein removal. The remaining free amino acids are then extracted, derivatized, and analyzed (Mustafa et al., 2007; Rutherfurd & Gilani, 2009). In contrast, the total amino acid analysis is somewhat more complex and varies in the execution of individual steps. Here, prior to the amino acid extraction, protein hydrolysis and, at the same time, free amino acids solubilization in an oxygen-free environment is required (Carcea et al., 2015; Rutherfurd & Gilani, 2009; Sharoba, 2014; Violi et al., 2020). Commonly used methods for amino acid separation and quantification include liquid (high performance liquid chromatography, HPLC) and gas chromatographic (gas chromatography–mass spectrometry, GC-MS), or other methods, such as capillary electrophoresis (Rutherfurd & Gilani, 2009; Violi et al., 2020).

In this study, the total amino acid content of the *Spirulina* commercial samples was determined using acid hydrolysis with the addition of scavenging agents. Amino acid extraction was then performed using solid phase extraction (SPE) and identification and quantification using GC-MS (Masten Rutar et al., 2022).

1.2.2 Lipids

The total lipid content in *Spirulina* on a dry weight basis ranges from 6 to 11% and consists of saponifiable (83%) and unsaponifiable (17%) fractions. The saponifiable fraction consists primarily of phosphatidyl glycerol (25.9%), monogalactosyl, digalactosyl (23%) and sulfoquinovosyl diglycerides (5%), undefined phospholipids (4.6%) and triglycerides (0.3%). The unsaponifiable fraction consists mainly of sterols, pigments, terpene alcohols and paraffin. The cholesterol content is less than 0.1 mg/100 g of dried *Spirulina* (Falquet, 1997; Marzieh Hosseini et al., 2013; Muys et al., 2019; E. G. Oliveira et al., 2009; Ragaza et al., 2020).

1.2.2.1 Fatty acids

The analysis of *Spirulina*'s fatty acid content reveals the presence of several saturated fatty acids (SFA), including myristic (C14:0), palmitic (C16:0), heptadecanoic (C17:0) and stearic (C18:0). Additionally, significant amounts of omega-6 unsaturated fatty acids (UFA) are detected, particularly palmitoleic (C16:1), oleic (C18:1), linoleic acid (C18:2), gamma (γ) linolenic (GLA; C18:3 ω-6) and arachidonic (C20:4 ω-6) fatty acid. The content of omega-3 unsaturated fatty acids, however, varies among studies. Some reports indicate the presence of α-linolenic (C18:3 ω-3), stearidonic (C18:4 ω-3), eicosapentaenoic (EPA; C20:5 ω-3) and docosahexaenoic (DHA; C22:6 ω-3) polyunsaturated fatty acids (PUFA), while others suggest either trace amounts or no detection of omega-3 fatty acids (AlFadhly, Alhelfi, Altemimi, Verma, Cacciola, et al., 2022; Aouir et al., 2017; Falquet, 1997; Liestianty et al., 2019; Ötleş & Pire, 2001; Sharoba, 2014; Tokuşoglu & Ünal, 2003). The fatty acid composition of *Spirulina* is presented in Table 1.4.

Table 1.4: *Spirulina* fatty acid composition. Ranges of fatty acid values (% of total fatty acids (TFA)), as reported in the literature (Bensehaila et al., 2015; Olguín et al., 2001; Ötleş & Pire, 2001; Sharoba, 2014; Tokuşoglu & Ünal, 2003).

Fatty acid	**Content** (% TFA[a])	**Fatty acid**[b]	**Content** (% TFA)
Butyric (C4:0)	ND–1.01	Palmitoleic (C16:1)	0.52–6.38
Caproic (C6:0)	ND–0.95	Margaric (C17:0)	ND–0.45
Caprylic (C8:0)	3.65–4.0	Heptadecenoic (C17:1)	ND–0.27
Undecylic (C11:0)	0.58–1.57	Stearic (C18:0)	0.95–8.82
Undecylenic (C11:1)	0.89–1.65	Oleic (C18:1, ω-9)	0.33–35.7
Lauric (C12:0)	ND–7.60	Linoleic (C18:2, ω-6)	9.43–18.0
Lauroleic (C12:1)	ND–0.52	γ-linolenic (C18:3, ω-6)	3.64–31.2
Tridecylic (C13:0)	0.72–0.95	α-linolenic (C18:3, ω-3)	ND–2.40
Myristic (C14:0)	ND–3.60	Eicosadienoic (C20:2, ω-6)	ND–1.01
Myristoleic (C14:1)	0.30–0.53	Arachidonic (C20:4, ω-6)	0.34–0.41
Pentadecylic (C15:0)	ND–1.53	EPA (C20:5, ω-3)	ND–2.91
Pentadecenoic (C15:1)	1.26–3.16	Erucic (C22:1, n-9)	ND–5.33
Palmitic (C16:0)	25.0–46.1	DHA (C22:6, ω-3)	ND–3.51

[a] TFA: Total fatty acids; [b] Fatty acid: EPA (eicosapentaenoic), DHA (docosahexaenoic). ND: not detected.

One of the most important fatty acids found in *Spirulina* is the γ-linolenic fatty acid (GLA), and, *Spirulina* is believed to be the most abundant natural source of GLA. Before *Spirulina*, the main source was the evening primrose (*Oenothera biennis*), which contains 8-12% of GLA in total fatty acids (TFA). The GLA content in *Spirulina* can reach up to 31.7% of TFA and 1.4% of *Spirulina* dry mass (Belay et al., 1993; Tanticharoen et al., 1994). GLA is a precursor for anti-inflammatory eicosanoid prostaglandin biosynthesis in the human body, which has an important anti-inflammatory and anti-proliferative function (Timoszuk et al., 2018). GLA has also been shown to reduce plasma low-density lipoprotein cholesterol (LDL) and triacylglycerol levels and, therefore, can aid in treating cardiovascular diseases (Dasgupta & Bhattacharyya, 2007). Additionally, it has a role in the treatment of other pathological conditions, such as Parkinson's disease, atopic eczema, arthritis, diabetes, and multiple sclerosis; it has also been investigated as a tumor suppressant (Ahmed et al., 2009; Raja et al., 2016; F. Wang et al., 2015). The GLA content in *Spirulina* is affected by the temperature, carbon source, light intensity, aeration and salinity stress conditions during cultivation (Choopani et al., 2022; Ronda & Lele, 2008).

Environmental conditions during cultivation and the growth phase influence the ratio and content of the fatty acids in the final product (Carcea et al., 2015; Cohen et al., 1987, 1993; Olguín et al., 2001). For example, cyanobacteria react rapidly to changes in external conditions and climate. Thus, the fatty acid composition of *Spirulina* has been shown to vary between production season and year. Moreover, the variation in the ratio between the SFA and UFA is in agreement with the metabolic needs of cyanobacteria, such as membrane fluidity, which adapts to changing external conditions (Carcea et al., 2015; M. A. C. L. de Oliveira et al., 1999). In certain blue-green algae and *Spirulina*, lipid composition is modified at lower temperatures to increase the UFA levels and improve membrane fluidity. Conversely, an increased SFA content is noticeable with increasing cultivation temperatures (de Jesus et al., 2018; Renaud et al., 2002; Y.-Z. Wang et al., 2016). Also, different *Spirulina* strains respond differently to temperature during growth. Thus, the highest γ-linolenic FA synthesis occurs between 35 and 40 °C in *S. maxima*, while in *S. platensis*, it is at 30 °C (M. A. C. L. de Oliveira et al., 1999). Another factor influencing γ-linolenic fatty acid content is salinity, where higher values were found in samples with prolonged exposure to salinity stress (Bhakar et al., 2013). Ocean acidification is also a factor affecting microalgae's fatty acid composition. This acidification results from increased atmospheric CO_2 and changes in coastal processes, which result in a lower content of essential fatty acids in the microalgal biomass (Rossoll et al., 2012). Therefore, it is essential to control growth conditions since this is the only way to achieve a stable fatty acid composition (Carcea et al., 2015).

Processing techniques, especially drying, also affect the total lipid yield and fatty acid composition. Notably, lyophilization (freeze-drying) gives the best results regarding nutrient preservation compared to spray drying, oven drying or sun drying. Thus, the highest monounsaturated fatty acid (MUFA) and PUFA levels in microalgal products are observed in lyophilized samples. However, sun-dried products contain the highest levels of saturated fatty acids due to an accelerated oxidation process (Shekarabi et al., 2019).

Fatty acid analysis of food products is divided into free (hydrolysis products of triglycerides) and total (free plus bound) fatty acid analysis. The purpose of the total fatty acid analysis is to determine the nutritional quality of a food product. Alternatively, the amount of free fatty acids can be used to assess product quality, stability and degradation during storage (Medeiros Vicentini-Polette et al., 2021; H. Park et al., 2021). Free fatty acid content is commonly determined by the acidity index (mg KOH/g), which gives information about the free fatty acid content but does not provide identification of the individual fatty acids. Up to now, no standardized methodology has been available for free fatty acid analysis without the occurrence of triacylglycerol breakdown (Medeiros Vicentini-Polette et al., 2021).

Total fatty acid analysis of food samples requires several steps: lipid extraction and derivatization to fatty acid methyl esters (FAME), extraction, identification and quantification (Ruiz-Rodriguez et al., 2010). The lipid extraction efficiency depends on the solvent and the lipid’s polarity (Señoráns & Luna, 2012; Servaes et al., 2015). Various well-established methods for lipid extraction are available, with the Soxhlet and Folch methods being the most common (Hewavitharana et al., 2020; Señoráns & Luna, 2012). The simultaneous extraction and esterification of fatty acids in foods are also possible using the BF_3 method, which is widely used due to short reaction time and efficient derivatization (J. Chang et al., 2016; Eze et al., 2015; Hewavitharana et al., 2020). The most commonly prepared fatty acid derivatives are FAME; their identification and content are determined by measuring their retention times and chromatographic peak areas and by comparison to an authentic standard (Al-Dhabi & Arasu, 2016; Carcea et al., 2015; Ötleş & Pire, 2001; Shantha & Napolitano, 1992; Sharoba, 2014). GC-MS is the method of choice for analyzing fatty acids, due to better selectivity, efficiency and cost compared to other techniques, such as liquid chromatography–mass spectrometry (LC-MS) and GC coupled to a flame ionization detector (GC-FID) (Hewavitharana et al., 2020).

In this thesis, total fatty acids in *Spirulina* samples were extracted and then derivatized using the BF_3 method, saponified and extracted. The obtained FAME were analyzed by GC-MS (Masten Rutar et al., 2022).

1.2.3 Elements

Spirulina is a valuable mineral source; however, its elemental composition depends significantly on the growth and processing conditions during harvesting and drying. The most essential inorganic nutrients in *Spirulina* are iron (Fe), calcium (Ca) and phosphorus (P). In addition, it also contains selenium (Se), potassium (K), magnesium (Mg), chromium (Cr), manganese (Mn), copper (Cu), sodium (Na), zinc (Zn), molybdenum (Mo) and boron

(B), among others (Campanella et al., 1999; Liestianty et al., 2019; Muys et al., 2019; Rahim et al., 2021; Tokuşoglu & Ünal, 2003; Usharani et al., 2012).

The iron content in *Spirulina* is ten times greater than other Fe-rich food sources, which is especially important for people who consume smaller amounts of meat and those with iron deficiency and higher iron requirements (pregnant women and children). Additionally, no phytates or oxalates are present in *Spirulina*, which would inhibit its bioavailability and absorption, as happens in some vegetable iron sources. Therefore, the iron present is expected to be highly available for absorption in the human intestine (AlFadhly, Alhelfi, Altemimi, Verma, Cacciola, et al., 2022; Gutiérrez-Salmeán et al., 2015). *Spirulina* also raises iron blood levels in rats more efficiently than casein or wheat gluten (P. E. Johnson & Shubert, 1986; Kapoor & Mehta, 1998). Iron from *Spirulina* has been reported to be more than twice as absorbable as iron from vegetables and meat sources. A 60% higher Fe absorption level was determined when *Spirulina* was compared to other Fe supplements, e.g., iron sulfate (Puyfoulhoux et al., 2001). However, the latest scientific evidence shows that iron bioavailability studies performed on rats do not correlate well with a human model. Namely, rats absorb both ferrous and ferric iron equally, while ferrous iron is preferred in humans. Also, heme and non-heme iron bioavailability are similar in rats, while heme iron is preferred in humans. In the case of low iron status, humans can increase the non-heme iron absorption up to 40%. The absorption is twice as high in rats and in contrast to humans, rats can also synthesize their ascorbic acid and possess an intestinal phytase activity, aiding iron absorption (Wienk et al., 1999). The iron content in commercial *Spirulina* products has been shown to vary greatly, mainly attributed to the iron content in the *Spirulina* growth medium. Also, the available reports on its bioavailability are contradictive, as Fe bioavailability can also be limited by high amounts of P and Ca, as well as the presence of tannic acid (Campanella et al., 1999; Isani et al., 2022; Peng et al., 2005; Puyfoulhoux et al., 2001).

Phosphorus, Ca and Mg (their proportion in *Spirulina* is similar to that of milk) are known to lower the risk for bone decalcification and consequently contribute to bone health (AlFadhly, Alhelfi, Altemimi, Verma, Cacciola, et al., 2022; Craig & Mangels, 2009; Liestianty et al., 2019). A low Na/K ratio decreases the risk of developing hypertension and cardiovascular disease (AlFadhly, Alhelfi, Altemimi, Verma, Cacciola, et al., 2022; Campanella et al., 1999; Iwahori et al., 2017). Also, the recommended daily amount of *Spirulina* microalgae can provide a substantial amount of Cu, Cr, Zn, Mn and Se elements (Campanella et al., 1999; Liestianty et al., 2019; Rahim et al., 2021; Ramírez-Rodrigues et al., 2021; Tokuşoglu & Ünal, 2003).

As the elemental composition of the culturing medium can influence the elemental content of *Spirulina*, it has the potential to be utilized as a nutritionally enriched natural source of minerals, as various minerals can be incorporated into its cultivation medium. Namely, *Spirulina* mineral content increased when their concentration was increased in the growth medium, and *vice versa* (Cases et al., 2001; Frontasyeva et al., 2009; Sukumaran et al., 2014). The alkalinity of the growth medium also affects mineral assimilation, as, at a higher pH, higher assimilation of metal ions occurs (Dmytryk et al., 2014). Moreover, processing conditions and techniques also influence the mineral composition, e.g., changes in mineral levels have been reported after the flaking process in *Spirulina* flake production, drying methods and excipient addition to the final product (Mohapatra et al., 2021; Sadowska & Świderski, 2020; Sistani et al., 2001; Sreeramaiah & Goudar, 2012).

Sample preparation of food samples commonly relies on microwave digestion as a preferred technique for sample preparation. This method is widely used due to its ability to reduce sample preparation time, contamination, and acid consumption compared to other digestion techniques (Carcea et al., 2015; Dolan & Capar, 2002; Drivelos & Georgiou, 2012; Krejčová et al., 2012). The elemental content in food samples is determined by comparing the spectra of the sample with a reference material. Commonly used methods for elemental analysis include inductively coupled plasma – optical emission spectroscopy (ICP-OES) and inductively coupled plasma – mass spectrometry (ICP-MS) (Carcea et al., 2015; Dolan & Capar, 2002; Drivelos & Georgiou, 2012; Krejčová et al., 2012). While the ICP-OES enables a rapid multi-element analysis, the ICP-MS method offers a wide linear dynamic range, high sensitivity and low limits of detection and is the optimal choice for a multi-element analysis (J. S. Becker, 2005; Drivelos & Georgiou, 2012; Krejčová et al., 2012). X-ray fluorescence (XRF) is another popular method for multi-elemental analysis, which was also used in our study (Masten Rutar et al., 2022; Masten Rutar, Strojnik, et al., 2023) and does not require any sample preparation providing direct analysis of solid samples. Nevertheless, despite its practicality, this method is limited by low sensitivity for high-mass elements (Drivelos & Georgiou, 2012).

1.2.3.1 Toxic trace elements

Spirulina is a highly effective accumulator of trace elements, which is advantageous when accumulated elements are essential for human health and well-being. However, it can threaten consumer health by accumulating toxic trace elements (Al-Dhabi & Arasu, 2016). When *Spirulina* is grown in an open environment, it can become contaminated with cadmium (Cd), arsenic (As), lead (Pb), aluminum (Al) and mercury (Hg), as these elements are commonly found in agricultural areas as trace contaminants originating from fertilizer and pesticide products, and can contaminate the culturing medium through environmental factors (wind, precipitation). Additional possible sources of contamination are also atmospheric pollution and construction materials. Therefore, the agricultural, but also pedoclimatic conditions contribute to their presence in the final products (Al-Dhabi, 2013; Belay, 2007; Gumbo & Nesamvuni, 2017; Jung et al., 2019; Muys et al., 2019). The chemicals used in *Spirulina* cultivation are also a potential source of contaminants (Rzymski et al., 2019). For this reason, no herbicides or pesticides should be used (Belay, 2007; D. V. Moreno et al., 2007), and it is advised only to use *Spirulina* produced under standardized and controlled conditions (Jung et al., 2019). When a closed culturing environment is used for cultivation, increased values of toxic elements are rarely confirmed (Al-Dhabi, 2013; Sharoba, 2014). Nevertheless, constant product monitoring is necessary due to *Spirulina*'s affinity to accumulate toxic trace metals (Al-Dhabi & Arasu, 2016; Belay, 2007).

In this study, trace-elemental composition was analyzed using ICP-MS (Masten Rutar et al., 2022).

1.2.4 Bioactive compounds

Besides the nutrients described previously, *Spirulina* is a good source of other compounds, such as pigments, polysaccharides, vitamins and antioxidants. Its blue-green color comes from phycocyanin (blue) and chlorophyll (green) pigments. It is also rich in carotenoids (yellow, orange and red). A list of pigments in *Spirulina* includes beta-carotene, chlorophyll-a, C-phycocyanin and allophycocyanin, zeaxanthin, xanthophyll, oscillaxanthin, canthaxanthin, 3-hydroxyechinenone, echinenone, myxoxanthophyll, diatoxanthin and beta-cryptoxanthin (Habib et al., 2008). Phycobiliproteins, lutein and β-carotene, have the highest market demand. Carotenoids are a source of flavorings and colorants for food and feed and of pro-vitamin A in dietary supplements. In the health market, natural β-carotene is preferred due to its mixed composition of cis- and trans-isomers. Both isomers have important anticancer activity, but the cis-isomer is rarely found in synthetically produced pigments (Downham & Collins, 2000; Mary Leema et al., 2010).

Additionally, these pigments have antioxidative and anti-inflammatory properties (W. S. Park et al., 2018). Phycobiliproteins, only found in cyanobacteria, have a wide range of possible commercial and pharmaceutical applications. These applications include substituting toxic synthetic pigments in the cosmetic industry, as well as finding utility in the food and drug industries and biomedical research (Glazer, 1994; Mary Leema et al., 2010; J. Moreno et al., 1995). Phycobiliproteins have also been shown to possess antioxidant, hepatoprotective, antiviral, neuroprotective, anticancer and immunostimulatory properties (Fernández-Rojas et al., 2014; Ramos et al., 2011; Romay et al., 2003).

Various studies have shown that *Spirulina* is a source of B vitamins: thiamin (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5) and folic acid (B_9). Pyridoxine (B_6) and biotin (B_7) were only found in small amounts. Also, high amounts of vitamin E (α-tocopherol) were found, comparable to that in wheat germ (Andrade et al., 2018; Grosshagauer et al., 2020; Liestianty et al., 2019; Ragaza et al., 2020; Rahim et al., 2021; Shao et al., 2019). B vitamins aid in detoxifying the body, intestinal functioning, stimulating the immune system and cell regeneration. Vitamin E, on the other hand, has a role in preventing oxidative lipid damage, coronary disease, atherosclerosis and multiple sclerosis (Andrade et al., 2018; S. Johnson, 2000).

Many *Spirulina* producers claim that their products are a rich source of B_{12} (cyanocobalamin) for vegetarians. Also, similar claims can be found in the recently published scientific material (Liestianty et al., 2019; Ragaza et al., 2020; Shao et al., 2019). However, 72.6–97.9% is present in pseudo-B_{12} form, where the lower ligand is replaced by adenine, and it is not bioavailable for humans, meaning *Spirulina* is not a good source of this vitamin (van den Oever & Mayer, 2022; Watanabe et al., 1999).

Phenolic compounds (phloroglucinol, p-coumaric, caffeic, chlorogenic and ferulic acid) and polysaccharides (immulina, rhamnose and mannose) are also important constituents of *Spirulina* microalgae. While the phenolics possess antioxidant activity and the polysaccharides provide an immunostimulatory activity, both act as anti-cancer compounds (Andrade et al., 2018; Ragaza et al., 2020).

The components in *Spirulina* microalgae with the main antioxidative properties are PUFA, phycocyanin, vitamin E, carotenoids (especially β-carotene) and phenolic compounds. Among them, GLA, phenolic compounds and phycocyanin are responsible for most of its therapeutic properties: anti-inflammatory, anti-microbial, anti-cancer, anti-obesity, anti-diabetes and anti-toxicity (Andrade et al., 2018; Ragaza et al., 2020; Shao et al., 2019). Among the mentioned bioactive compounds, the present study included assessment of total phenolic content, carotenoid and chlorophyll pigment analysis and PUFA analysis, including GLA (Jamnik et al., 2022; Masten Rutar et al., 2022; Masten Rutar, Vrhovšek, et al., 2023).

1.3 New Approaches in Improving and Determining Food Bioactivity

With a growing world population the demand to meet the population caloric needs has increased. For this reason, significant advancements have been made in large-scale crop production to enhance the hardiness and yield of crops. These improvements have involved intensive fertilization, irrigation, pesticide usage, and plant breeding. However, this has led to the loss of crop nutritional quality (diversity and nutrient content) and nutrient bioavailability. As a result, despite meeting caloric requirements, the crops produced today do not meet the micronutrient demand (Scharff et al., 2022). In order to address the nutrient deficiency faced by the growing global population, it is essential to focus on improving the nutritional quality of food. With changing food habits and urbanization, there is a growing interest in so-called "smart" foods with higher nutrition per bite, i.e., food products that are a good nutrient source and possess beneficial physiological activities (Nayak et al., 2021). Given this, several different techniques have been used to improve the nutritional and bioactive quality of food, including crop breeding techniques, seeds and pulses germination, application of microorganisms to plant growing medium (bacteria or fungi) and microbial processes (fermentation). Among them, lactic acid fermentation of substrates of plant, animal and algal origin has attracted much attention and has shown promising results (Chiellini et al., 2023; Gaikwad et al., 2020; Irfan & Datta, 2017; Nayak et al., 2021; Scharff et al., 2022).

1.3.1 Lactic acid fermentation as a way of increasing nutritional and functional properties of microalgae

Fermentation is described as a process where chemical changes occur in the organic substrate, in the form of incomplete oxidation of organic compounds, as a response to microbial enzyme activity – and the fermentative microorganisms have an essential role in this process (Chojnacka, 2010; Limón et al., 2015). Throughout history, lactic acid fermentation has been used to prolong the shelf life of food products. Today, it is also applied to improve their bioactivity to achieve beneficial health effects and reduce the antinutritional factors' activity. Fermentation of food products has also been shown to improve nutrient digestibility and reduce allergenicity (Limón et al., 2015; Sanjukta & Rai, 2016).

Lactic acid bacteria (LAB) have been used in food fermentation for centuries. They can be divided into two groups: the homofermentative LAB, which convert a glucose molecule into two lactic acid molecules via glycolysis (the Embden-Meyerhof pathway), and the heterofermentative, which convert a molecule of glucose into lactic acid, ethanol and CO_2 (Wee et al., 2006). Among LAB, *Lactobacillus* is one of the most important genera. They are a group of anaerobic (but aerotolerant) Gram-positive bacteria which produce L-(+)-lactic acid. Within this genus, *Lactobacillus plantarum* is the most versatile homofermentative species with many favorable properties and has gained the status GRAS and QPS (Qualified Presumption of Safety). Thus, it is widely used for raw food processing and industrial fermentation (EFSA, 2021; Elagöz et al., 1996; Guidone et al., 2014; B. Liu et al., 2010; Ray & Joshi, 2015).

The application of LA to improve the functional (nutraceutical) properties of food is relatively recent and a result of LAB's ability to use enzymatic hydrolysis to degrade the cyanobacterial and plant cell walls and convert organic macromolecules (proteins, lipids, phenolic compounds and polysaccharides). The latter produces smaller compounds with high immunomodulatory, anti-inflammatory and antioxidant functions (de Marco Castro et al., 2019; Limón et al., 2015; Sanjukta & Rai, 2016). Besides the enhanced content and change in the profile of bioactive molecules, lactic acid fermentation also enhances their bioavailability (Septembre-Malaterre et al., 2018). During fermentation, *L. plantarum* is involved in the production of bioactive peptides (with immunomodulatory, antimicrobial and ACE-inhibition activity), antioxidants (pyrogallol and hydroxytyrosol), flavoring compounds (4-vinyl phenol), vitamins (B_2, B_9 and B_{12}), and exopolysaccharides (with antioxidant, immunomodulatory, cholesterol-lowering and antitumor function). Finally, among many other health-promoting properties, it also helps to improve iron bioavailability by degrading complex iron-binding organic molecules, such as gallic acid (Behera et al., 2018; S.-J. Lee et al., 2015; Ricci et al., 2010; Rodríguez et al., 2009).

Recently, interest in the fermentation of micro-, macroalgal and cyanobacterial biomass has grown in an attempt to enhance the nutraceutical properties of these bioactive-compound-rich organisms. The first reported algal lactic acid fermentation was performed in 1998 on *Ulva* spp., where a microbial consortium was used, including *L. brevis* (Uchida & Miyoshi, 2012). Since then, the most commonly used LAB for algal fermentation is *L. plantarum*, although other LAB species, among them *L. fermentum*, *L. casei*, *L. paracasei*, *L. brevis*, *L. rhamnosus*, *L. pentosus*, *L. delbrueckii*, *L. kefir*, *L. acidophilus*, *L. salivarius*, *L. mesenteroides*, and *S. thermophilus*, have been applied in various studies (Pérez-Alva et al., 2022). Lactic acid fermentation resulted in higher content of vitamins B_1 and B_9 (Uchida et al., 2017) and γ-amino butyric acid (GABA) (Cha et al., 2011), higher antioxidant and anticoagulant activity (Kaga et al., 2021; Shobharani et al., 2013; Taniguchi et al., 2019), antimicrobial activity (Q.-D. An et al., 2009; Martelli et al., 2020), immunomodulatory (Taniguchi et al., 2019), ACE-inhibitory activity (S.-J. Lee et al., 2015), and anti-inflammatory activity (W.-W. Lee et al., 2011; Lin et al., 2016; Mun et al., 2017). Additionally, suppression or improvement of certain pathological conditions was noticed *in vitro* and *in vivo* when fermented algal material was administered: inflammatory bowel disease (Nemoto et al., 2017), obesity (Y.-M. Kim & Jang, 2018), hepatotoxicity (Cha et al., 2011; Kang et al., 2011), hypertension (Kaewsahnguan et al., 2021; Uchida et al., 2017), γ-ray irradiation (W. Lee et al., 2013), diabetes- and ageing-related glycation (Eda et al., 2016; Kaga et al., 2021; Taniguchi et al., 2019). The results of these studies indicate the possibility of developing added-value food products by including lactic acid-fermented microalgae in their composition (Han et al., 2015; Kaga et al., 2021; S.-J. Lee et al., 2015; Uchida & Miyoshi, 2012).

1.3.1.1 Overview of *Spirulina* lactic acid fermentation case studies

Along with other microalgae, *Spirulina* has also been a subject of lactic acid fermentation studies due to its rich nutritional and bioactive compound composition. In a study by de Marco Castro et al. (2019), fresh *Spirulina* biomass was fermented by *L. plantarum* for 72 hours. After 24-hour fermentation, the highest DPPH scavenging activity was determined, while the highest total phenolic content, C-phycocyanin content, oxygen radical antioxidant capacity and ferric-reducing antioxidant potency were noticed after 36 hours of fermentation. Protein fragmentation and methionine content increased linearly with the fermentation time, demonstrating a release of smaller peptides from parent proteins (de Marco Castro et al., 2019). Similarly, the study of Bao et al. (2018) showed that after fermentation by *L. plantarum* and *Bacillus subtilis*, more than 30% of the protein was hydrolyzed, which resulted in the polypeptide content increase in the fermented *Spirulina* biomass by 16–19% and free essential amino acid increase by 6–19%, as compared to the non-fermented *Spirulina* biomass. The authors found that *Spirulina* lactic acid fermentation increases protein bioavailability (Bao et al., 2018). In their following study, they also showed (by determining the *in vitro* Th1/Th2 immunomodulatory potential and proliferation of the splenic lymphocytes) that *Spirulina* fermentation by mixed probiotics (*L. plantarum* and *B. subtilis*) also results in an enhanced immunostimulatory activity (J. An et al., 2020).

Choi et al. (2018) performed *Spirulina* fermentation using *L. plantarum*. Their study showed a significantly higher β-carotene extraction yield in the fermented *Spirulina*, which resulted in a highly enhanced neuroprotective effect. This effect was attributed to oxidative stress suppression by inducing the brain BDNF/p-CREB expression antioxidative pathway. Also, the results showed that pure β-carotene (as a single component) was less effective than when analyzed as a part of the *Spirulina* extract due to the synergistic effects with the components of the extract (W. Y. Choi, Kang, Heo, et al., 2018). The same authors also managed to connect the synergistic antioxidant effects of β-carotene and other bioactive compounds found in *L. plantarum* fermented *Spirulina* extract with its high cognitive-enhancing effects in the hippocampus *in vivo*, on mice with scopolamine-induced dementia (W. Y. Choi, Kang, & Lee, 2018).

Niccolai et al. (2019) showed that after lactic acid fermentation, *Spirulina* digestibility only grew by 4.4%. However, its antioxidant activity increased by 79% and total phenolic content by 320% (Niccolai et al., 2019). Lactic acid fermentation using mixed lactic acid bacterial strains significantly increased protein content, amino acid content and the essential- to total amino acid ratio in fermented *Spirulina*. While the non-fermented *Spirulina* powder addition to the bacterial growth medium has been shown to increase the probiotic lactic acid bacterial growth and inhibit the growth of the harmful pathogenic microorganisms (human opportunistic pathogens), the LAB *Spirulina* fermentation has been shown to even further boost its antibacterial activity. These results suggest that regular *Spirulina* (especially fermented) consumption would improve the intestinal LAB profile and, at the same time, inhibit the growth of harmful pathogenic bacteria. This would improve intestinal absorption and reduce malnutrition (Bhowmik et al., 2009; Parada et al., 1998; Yu et al., 2020).

Liu et al. also used mixed LAB strains for *Spirulina* fermentation. The fermented *Spirulina* showed a significantly higher DPPH radical and nitric oxide scavenging activity, UV-protective and anti-inflammatory activity than the non-fermented sample. Also, the fermented sample showed a higher total phenolic content, suggesting the microalgal cell wall degradation by the LAB and the consequent release of the smaller polyphenols. However, the rise in the phycocyanobilin content of the fermented samples points to the transformation of phycocyanin to phycocyanobilin. The authors suggest the potential use of lactic acid-fermented *Spirulina* as an efficient antioxidative agent in skin-care products to protect against the formation of free radicals in the skin induced by UV radiation (J.-G. Liu et al., 2011).

For bioactive compound extraction from *Spirulina* microalgae, different extraction mediums were used due to the different solubility properties of the active compounds (Czerwonka et al., 2018; Koh et al., 2017; Ojima et al., 2019; Putri et al., 2020; Shukla et al., 2009; L.-C. Wu et al., 2005).

When fermenting *Spirulina*, the formation of biogenic amines such as putrescine, tryptamine, cadaverine, tyramine, spermine, histamine, and spermidine is possible. High concentrations of these compounds in fermented *Spirulina* might be produced, as their synthesis mechanism is similar to that of some bioactive compounds (gamma-aminobutyric acid (GABA), L-glutamic acid), and their presence, in sufficiently high concentrations, could have toxic effects on the human body (Tolpeznikaite et al., 2023).

1.3.2 Functionality assessment on a molecular and cellular level

Analysis of a bioactive compound activity can be carried out using chemical-based or cellular-based assays. However, the activity assessed by chemical-based assays does not entirely reflect the *in vivo* behavior of the sample. For this reason, the effectiveness of bioactive compounds should be estimated in a more biologically relevant environment. Although animal or human models are more adequate for such analyses, they are time-consuming and expensive. As alternative intermediate testing models, cell-based assays are used for assessing bioactivity on a cellular level and enable an analysis of cell morphology, function, as well as physiological and chemical properties (Rinschen et al., 2019; D.-P. Xu et al., 2017). Also, cell-based assays can lead to a better understanding of the effects of antioxidant activity, given that lipid, protein, nucleic acid, and other cell components' oxidative damage and defense systems are fundamentally similar at all cellular organization levels (Cheli & Baldi, 2011; Sigler et al., 1999).

Additionally, assessment of nutritional compound bioactivity can be performed using specific ‘omics’ techniques, which provide a comprehensive view of the activity at the molecular level. These techniques are used primarily in genome (genomics), mRNA (transcriptomics), proteome (proteomics) and metabolome (metabolomics) analyses. The application of these techniques is increasing among food scientists for studying the molecular basis of positive physiological effects of bioactive compounds (Horgan & Kenny, 2011). Conversely, ‘omics’ techniques allow the monitoring of the fermentation process and provide information regarding the role of microorganisms and their metabolic activity in the complexity of fermented foods (Rizo et al., 2020).

The metabolomic approach extends the principles of genomics and transcriptomics, allowing qualitative and quantitative assessment of metabolites in a biological system. In fermented food profiling, it is used for representative metabolite analysis, to understand the fermented products’ nutritional, functional, sensory, and safety properties (Gao et al., 2021). Among the omics techniques, this thesis focuses on metabolomic and proteomic approaches for investigating of *Spirulina* and its bioactivity using a cell model.

1.3.2.1 Metabolomic approach

Metabolomics investigates small endogenous and exogenous molecular compounds (metabolites) produced at any point in an organism’s lifecycle, substrates or products of chemical reactions due to cellular metabolism. In metabolomic research, the activity of the metabolic network is directly affected, resulting in the production of metabolites, whose analysis then gives information regarding the biological activity and status of the analyzed system. In this way, it provides an insight into various cellular physiology aspects (Cajka & Fiehn, 2016; Guma et al., 2016; C. H. Johnson et al., 2016; X. Liu & Locasale, 2017; Saxena & Schlegel, 2015; Wishart, 2016; Zamboni et al., 2015). In a non-target metabolome analysis, a large number of unknown metabolites can be profiled, and relative differences between two conditions or across a population can be observed (Barkal et al., 2016; Denzel et al., 2014; Doroghazi et al., 2014; Jang et al., 2016; C. H. Johnson et al., 2015; Shin et al., 2014; Wen et al., 2014). In a semi-target metabolome analysis, high numbers of metabolites are identified and then quantified, which means gathering a large mass of data in a single analysis, which would otherwise be obtained using several biochemical analyses (Breitling et al., 2006; Gika et al., 2016; X. Liu & Locasale, 2017; Shin et al., 2014). Furthermore, in target metabolome analysis, testing of a specific hypothesis provides more profound insight, as absolute concentrations of metabolites are determined, and the conversion rates of one metabolite into another can be obtained (Alves et al., 2015; Bennett et al., 2008; Jain et al., 2012; Link et al., 2015; J. O. Park et al., 2016; Shlomi et al., 2008).

Nuclear magnetic resonance (NMR) and MS methods are popular and effective tools for metabolite composition analysis. In semi-target or non-target metabolomic analysis, MS is more commonly used due to its higher sensitivity and throughput and ability to analyze a high number of metabolites in a complex sample. NMR is a more quantitatively accurate method, as the number of atomic nuclei corresponds to the number of metabolites in the analyzed sample. The addition of an internal standard (IS) before extraction can help overcome this limitation when using MS. Simplicity and accuracy are the main advantages of metabolite quantification using an IS due to simultaneous metabolite and IS preparation and analysis. Metabolite concentrations are obtained by assessing the difference in signal intensity between IS and metabolite. For separation of the analyzed molecules, LC and MS are used (Dunn et al., 2011; Godejohann, 2007; Lu et al., 2017; Patti et al., 2012; Sud et al., 2016; Zamboni et al., 2015). Many software options are available for analysis of the raw MS data. Metabolite identification and characterization are performed by comparing the gathered features with the features of known metabolites (Fahy et al., 2009; Horai et al., 2010; Kanehisa et al., 2014; X. Liu & Locasale, 2017; Smith et al., 2005; Wishart et al., 2013).

1.3.2.2 Proteomic approach

Proteomics can be considered the primary method for biological system characterization, as the levels of proteins (which are the effectors of biological function) depend on the mRNA levels and the post-translational regulation (Cox & Mann, 2007). Analysis of the proteome enables insight into the cell's functional and structural information and information regarding the cell stress/drug response mechanisms (Aslam et al., 2017). In particular, proteomics enables the study of proteins directly involved in biological activities. It provides a unique possibility for investigating cell physiology by identifying proteins that change their expression levels qualitatively and quantitatively per changing environmental conditions (Siciliano & Mazzeo, 2015). Applying the proteomic approach in nutritional research is vital for identifying and differential quantifying protein biomarkers, as the bioactive compounds in ingested food affect the gene and protein expression (Tewari et al., 2015). Cell proteome is characterized by protein post-translational modifications, localization, turnover and interactions at a particular time and under specific conditions (Krishna & Wold, 1993).

For protein sample analysis, different techniques of gel electrophoresis can be used (two-dimensional gel electrophoresis (2-DE), two-dimensional differential gel electrophoresis (2D-DIGE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)) (Aslam et al., 2017). The complex protein matrix assessment with high sensitivity is enabled by LC-MS, which is among the most commonly used techniques in proteome analysis (Altelaar & Heck, 2012; Aslam et al., 2017; Y. H. Lee et al., 2010; Schmidt et al., 2014; Wiśniewski & Mann, 2012). In order to assist in handling and storing the massive amount of proteomics data, bioinformatic tools have been established, including proteome repositories and pathway databases, which offer analysis of the protein domain, protein structure prediction, protein-protein interaction, pathway analysis, protein metabolism, signaling and MS data analysis (Aslam et al., 2017).

1.3.2.3 Role of yeast in bioactivity studies

The bioactivity of functional compounds can be studied at the cellular and molecular level using simple organisms like yeast. Yeast cells enable the study of rudimental cellular processes in eukaryotes (i.e. cellular metabolic pathways or response to stress conditions) due to the preservation of the basic cellular processes among eukaryotes. Therefore, the studies of the cell processes in yeast can offer insight into the equivalent processes in other eukaryotes.

Yeast *Saccharomyces cerevisiae* is among the most studied eukaryotes. When in the stationary phase, yeast *S. cerevisiae* has several common characteristics with the inactive mammalian cells: the characteristically condensed (G_0) chromosomes, reduced translation and increased autophagy rates (Dickson & Brown, 1998; Fuge et al., 1994; Piñon, 1978; Pringle, 1981). When *S. cerevisiae* is in the prolonged stationary phase (G_0), most of its cell energy comes from mitochondrial respiration (the mitochondrial respiratory chain is the primary source of reactive oxygen species (ROS) in yeast) and over time, cell damage accumulates. Yeast is a referable model for oxidative stress-related damage and ageing-related damage research. In addition, yeast cells have been shown to have identical mechanisms of antioxidative defense as the higher eukaryotes. As these characteristics are common to both yeast and multicellular organisms, the results obtained in the former are referable also to the latter (V. Costa & Moradas-Ferreira, 2001; Gralla & Kosman, 1992; Longo et al., 1996; Moradas-Ferreira et al., 1996). Research performed primarily on component parts and yeast can give information applicable to human counterparts since the homologs found in the human genome (across the whole proteome) correlate to 46% of the proteome in yeast (D. Ma, 2001). Research on proteins and metabolic pathways is more straightforward in yeast cells than in more complex organisms, owing to easy RNA-level manipulations and viable expressions of proteins in yeast. Yeast can form colonies in solid growth mediums or grow in a liquid medium in a dispersed form. It has a short life cycle and does not require an expensive medium for culturing. The presented features give yeast many technical advantages over human cells in research (Guthrie & Fink, 1991).

In the present study, fresh *Spirulina* biomass was fermented by *L. plantarum* and after, water and ethanol extracts of fermented and non-fermented *Spirulina* broth were prepared. The extracts were then used to treat the yeast *S. cerevisiae* culture. The study included nutrient and microbiological analysis of the fermented and non-fermented *Spirulina* broth, total phenolic content and antioxidant activity analysis of all the *Spirulina* extracts and additional proteome and metabolome profiling of the ethanolic fermented and non-fermented *Spirulina* extracts. Finally, the treated yeast cells were analyzed at the cellular (cell oxidation and lipid peroxidation level) and proteome levels to assess the impact of the *Spirulina* extracts on yeast (Jamnik et al., 2022; Masten Rutar et al., 2021; Masten Rutar, Vrhovšek, et al., 2023).

1.4 Determining the Authenticity and Origin of *Spirulina* Products

Maintaining a tightly controlled environment is essential for the production of *Spirulina* to ensure consistent product composition and quality. However, this level of control often leads to higher production costs for *Spirulina* products, which unfortunately makes it an attractive target for intentional adulterations. In order to lower the *Spirulina* production cost, the addition of lower quality products (possibly even produced in a different geographical area), and at the same time, incorrect labelling of product ingredients, composition and origin, is not uncommon. For example, commonly found adulterants in *Spirulina* products are mung bean powder and flour, which, compared to *Spirulina*, require a lower-cost production but also have a much lower content of protein (Gallardo-Velázquez et al., 2009; Muys et al., 2019; D. Wu et al., 2011). Pedoclimatic conditions and agrotechnical measures highly impact the product's nutrient and toxic compound composition and differ according to geographical region. Therefore, the geographical production location also impacts the products' quality and safety, and it requires constant monitoring (Drivelos & Georgiou, 2012; Muys et al., 2019).

Discovering adulterations is vital in food product analysis, both from an ethical and safety point of view. The undeclared ingredients might threaten consumer health if the added substances are health-threatening. In food supplements containing natural products as active ingredients (also *Spirulina*), and with declared health benefits, such as lowering hypertension, weight loss, preventing diabetes mellitus, and alleviating arthritic symptoms, another form of adulteration has been detected (Cole & Fetrow, 2003; Moreira et al., 2016). In these products, undeclared pharmaceuticals (antihypertensive and diuretic drugs) were added in the past to enhance their efficiency and popularity among consumers. In this type of adulteration, a risk of drug overdose and interference is present for consumers already using certain prescription drugs (Firenzuoli et al., 2010; Liang et al., 2006). Therefore, regular control and inspection of these products can establish consumers' confidence that

Spirulina products are safe to consume and reduce the health risks of consuming adulterated products (De Carvalho et al., 2012; Moreira et al., 2016; D. Wu et al., 2011).

Authentication of food products also means verifying the products' compliance with the producer's declaration, including the production method, the geographical, species or genetic origin, and the processing methods (Aung & Chang, 2014). Various techniques have been used for determining the authenticity of food products: molecular techniques (genomics and proteomics), vibrational and fluorescence spectroscopy, nuclear magnetic resonance (NMR), immunological techniques, chromatographic techniques and mass spectrometry without chromatography, sensory analysis, elemental and isotopic techniques (Danezis et al., 2016). Molecular techniques (nucleotide and protein-based) are used for species identification and detection. These methods are used for mislabeling and false description identification of foods (M. E. Ali et al., 2014; Danezis et al., 2016; Sforza et al., 2011; Sun et al., 2014).

Vibrational spectroscopy presents an inexpensive, non-destructive and fast technique for food quality and authenticity assessment, where at specific frequencies, the samples absorb some of the infrared (IR) radiation, which produces a spectral 'fingerprint' of the sample. These techniques typically provide only qualitative identification since the limits of detection by vibrational techniques are high. In contrast, fluorescence spectroscopy provides a simple, non-destructive and inexpensive method with low limits of detection (Cozzolino, 2012; Danezis et al., 2016; Poulli et al., 2007). One of the best methods for obtaining high-quality molecular spectroscopic and structural data is NMR analysis, which provides precise analysis of complex food matrices, where the amount of a target metabolite can be determined. It also requires only minimal preparation of the sample. NMR also offers the possibility for a comprehensive metabolic food profile collection, which is helpful in the authenticity analysis (Danezis et al., 2016; Longobardi et al., 2013). Further, immunoassays are used, which rely on a specific interaction between the antigen and its cognate antibody. The most commonly used method is enzyme-linked immunosorbent assay (ELISA) (Asensio et al., 2008; Gajewski & Hsieh, 2009; Nielsen, 2010). Chromatographic techniques enable fast and reliable identification of similar chemical compounds in a complex matrix of food products. Namely, authentication is based on determining unique compound markers and minimal differences among the food compound profiles. High chemical food complexity and a high request for food quality and authenticity among consumers demanded using chromatographic techniques with high resolution (LC, GC) coupled with MS. The most commonly used methods include LC-MS/MS, GC-MS/MS or LC-time-of-flight-MS (LC-TOF-MS) (Cserháti et al., 2005; Di Stefano et al., 2012; Ibáñez et al., 2013). Somewhat less used methods include the MS not coupled to chromatography and sensory analysis (Danezis et al., 2016; Dreisewerd, 2014; X. Li et al., 2015; Luykx & van Ruth, 2008; Ouyang et al., 2014; Strike et al., 1999).

Chemometric and bioinformatic statistical methods are fundamental to obtaining relevant chemical information from large and complex datasets for food authentication studies. Also, standard certified reference materials must provide reliable measurement results and accurate data processing to interpret the analytical data using these tools effectively. Natural variability of the sample composition must be considered, and adequate sample handling and storage conditions must be provided. Also, the methods and the gathered results must be comparable with other compatible studies (Danezis et al., 2016). In food product authentication, chemometrics must be combined with adequate mathematical tools and database infrastructure (Bertacchini et al., 2013). In commercial food product authentication and geographical origin studies, a comparison of the gathered data with a reference data set is required; therefore, product reference databases are essential for data interpretation. Such databases must contain comprehensive, reliable, and standardized information regarding food origin, including the geographical origin of production, method of production and processing, species (subspecies), and other critical information. The samples that make up the database have to be sufficiently representative, and for commodities, which are prone to seasonal variability, yearly databanks should also be established (Camin et al., 2017; Danezis et al., 2016).

Currently, the primary methods applied in food authentication are the molecular techniques and techniques coupled to chromatography, which account for almost half of the published research. In descending order, these are followed by isotopic, vibrational and fluorescence spectroscopy, elemental and NMR techniques (Danezis et al., 2016). In this thesis, GC-MS was used to determine the amino acid and fatty acid composition. In addition, the elemental techniques ICP-MS and ED-XRF were used both (i) alone to determine the product composition and (ii) combined with the stable isotopic approach to discriminate between samples of different origins. Isotopic techniques for food authentication are described separately in further detail below.

1.4.1 Stable isotope ratio analysis

Isotopes are atoms of the same element with the same atomic number but different atomic mass, i.e. a different number of neutrons. Based on their stability, isotopes are divided into stable isotopes, which do not decay, and radioactive isotopes, which do decay. In food authenticity studies, mainly stable isotope ratio analysis is used, as stable isotope ratio values of food products differ according to geographical origin, climatic conditions, geology and soil pedology of the production region (Danezis et al., 2016; Meier-Augenstein, 2018; van Leeuwen et al., 2014). Regarding atomic mass, stable isotopes are separated into isotopes of heavy and light (bio-elements) elements. The most commonly analyzed heavy element isotopic ratio is Sr ($^{87}Sr/^{86}Sr$), while the most commonly determined light element isotopic ratios are those of C ($^{13}C/^{12}C$), N ($^{15}N/^{14}N$), S ($^{34}S/^{32}S$), H ($^{2}H/^{1}H$) and O ($^{18}O/^{16}O$) (Alexandre, 2020; Danezis et al., 2016).

Given that stable isotopes of an element have the same electronic structure, they have similar reactivity. However, different isotopes of an element tend to show very different abundance in nature (with the lighter isotope being predominant) due to a process of fractionation, where depletion or enrichment of an isotope from its mean natural abundance occurs (van Leeuwen et al., 2014), and different physico-chemical factors can lead to changes in the heavy-to-light isotope ratio of a specific element (Kelly et al., 2005). Analysis of isotope ratios at natural abundance levels requires high precision, as the changes in the isotopic ratios are commonly noticed at the third or fourth significant figure. The required precision is achieved by comparing the stable isotope ratio in the analyzed sample to the values determined in a suitable reference material. The δ-notation (‰) is then used to report sample isotopic abundance relative to the isotopic abundance of the reference material according to the equation (1.1) (Brand et al., 2014):

$$\delta\, ^{i/j}E = \frac{^{i/j}R_s - {}^{i/j}R_r}{^{i/j}R_r} \tag{1.1}$$

Here, i denotes the highest and j the lowest atomic mass number of the element (E) (O, H, C, S, N), and R stands for the isotope ratio between the heavier and the lighter isotope of the element (E) ($^{18}O/^{16}O$, $^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{34}S/^{32}S$, $^{15}N/^{14}N$) in the measured samples (s) or reference material (r). The delta values are small numbers, frequently presented in per mil (‰). The $\delta^{18}O$ and $\delta^{2}H$ values are expressed relative to the Vienna-Standard Mean Ocean Water (VSMOW) standard, the $\delta^{13}C$ values relative to Vienna-Pee Dee Belemnite (V-PDB), the $\delta^{34}S$ values relative to Vienna Cañon Diablo Troilite standard (V-CDT) and finally, $\delta^{15}N$ values are reported relative to Ambient Inhalable Reservoir (AIR) standard.

The stable isotope ratio of light elements is commonly determined using the isotope ratio mass spectrometry (IRMS) technique. Here, the analytes must be converted to a simple gas before the ionization, comparable to the original sample by isotopic composition. Usually, δ^{13}C, δ^{15}N, δ^{34}S, δ^{2}H and δ^{18}O analyses are performed on CO_2, N_2, SO_2, H_2, and CO, respectively. For the analysis of the isotopes of light elements, IRMS is commonly interfaced with an elemental analyzer (EA) (thermal conversion EA (TC) or continuous flow EA (CF)), equilibration devices, pyrolizer, HPLC or GC (Danezis et al., 2016; Drivelos & Georgiou, 2012; Meier-Augenstein, 1999).

Carbon and nitrogen isotope ratio measurements provide information on the primary producer 'type' and diet, regional climate and agricultural practices used in production (organic or conventional). Oxygen and hydrogen isotopic data are related to the water isotopic composition in the production region, while the sulfur isotopic composition is strongly affected by anthropogenic pollution, geology and volcanism, and distance from the sea. Hydrogen, oxygen and sulfur isotope ratio analysis in this way provides information on the geographical origin of the product. This method can also determine production year and detect counterfeits (Drivelos & Georgiou, 2012; Kelly et al., 2005; Meier-Augenstein, 2002).

Hydrogen (^{2}H/^{1}H) and oxygen (^{18}O/^{16}O) stable isotope ratios depend on the latitude and are valuable for determining geographical origin. It works because seawater evaporation causes isotopic fractionation and a decrease in the heavy isotopomer concentration in the cloud water relative to the seawater (with the δ^{2}H and δ^{18}O values of approximately 0‰). More water evaporation, condensation and precipitation occur as the clouds move further from the equatorial area and inland, and, at the same time, the heavy isotope values of hydrogen and oxygen continue to decrease. Finally, the groundwater consists of this meteoric water, and its hydrogen and oxygen isotope ratio is gradually changing from the coastal to the inland regions. Therefore, the groundwater in warmer climatic regions and closer to the equator has a more positive δ^{2}H and δ^{18}O values, while in the cooler climates, the δ^{2}H and δ^{18}O groundwater values are more negative (Alexandre, 2020; Fogel & Cifuentes, 1993; Kelly et al., 2005). The result is a distinct water isotopic composition between different regions, and the analyzed meteoric water δ^{2}H and δ^{18}O values will indicate where the source precipitation occurred. Variation in the water δ^{2}H and δ^{18}O values in particular areas proportionally correlates to their values in organic compounds of the food products originating from the area in question (Alexandre, 2020; Fogel & Cifuentes, 1993; Kelly et al., 2005). In microalgae-based food products, microalgal hydrogen and oxygen isotopic fractionation also influence the final δ^{2}H and δ^{18}O values. High hydrogen isotopic fractionation has been observed in microalgae during hydrogen uptake from the growth medium and assimilation to carbohydrates (-100 to -120‰) and then further during lipid and protein biosynthesis for an additional -30 to -60‰. The process of hydrogen isotopic fractionation has been shown to depend on the type and intensity of lighting. The δ^{2}H values obtained for different microalgae cultivated in changing lighting conditions ranged from -214 to -143‰ (Estep & Hoering, 1981). The microalgal oxygen isotopic fractionation, on the other hand, ranges from 19.4 to 25.8‰, which points to the importance of the photosynthetic cycle in this process (Kiddon et al., 1993).

The carbon isotope ratio is related to different fixation processes of atmospheric and dissolved carbon in aquatic environments in plants and certain microorganisms, where the heavier ^{13}C and the lighter ^{12}C isotopes are differentiated. Other factors, such as concentrations of the local atmospheric and water dissolved CO_2, species and growth rate of the primary producer, growth temperature, light intensity, aridity and salinity, as well as fertilization, also affect the carbon isotope ratio since they affect the CO_2 uptake (O'Leary, 1993; Spangenberg & Ogrinc, 2001; van Leeuwen et al., 2014; J. Xu et al., 2012).

Many aquatic photosynthetic organisms can uptake CO_2 and HCO_3^- from the surrounding media due to CO_2-limiting conditions. Cyanobacteria and algae evolved various mechanisms to accumulate inorganic carbon, and a great diversity of photosynthetic pathways can be found among the organisms in the aquatic environment – the C3, C4 and CAM pathways (Keeley, 1999; J. Xu et al., 2012). In the C3 pathway, a 3-phosphoglycerate (3-PGA, a so-called C3 body) is formed. This process, the Calvin cycle, is the most common form of CO_2 fixation. The primary producers using this process are known as C3 organisms. Those using the C4 CO_2 fixation pathway, on the other hand, use a Hatch-Slack cycle, where a C4-dicarboxylic acid (oxaloacetic acid) is produced. The primary producers (and their products) of these two processes differ in the abundance of the ^{13}C isotope. Those using the C3 pathway incorporate less ^{13}C than those using the C4 pathway, which are enriched in the ^{13}C isotope. Therefore, the material originating from C3 organisms has 10 to 15‰ lower $\delta^{13}C$ values (ranging from -35 to -22‰) compared to the material originating from the C4 organisms (-17 to -10‰). Aquatic organisms also use the crassulacean acid metabolism (CAM), which means they can use the C3 and the C4 pathway for CO_2 fixation and have intermediate $\delta^{13}C$ values (-27 to -11‰). In algae and cyanobacteria, the C3 carbon fixation cycle is believed to be predominant, despite the growing evidence regarding the development of the C4 pathway in some marine algae, due to stress exposure related to low CO_2 concentrations (Collister et al., 1994; Meier-Augenstein, 1999; O'Leary, 1988; J. Xu et al., 2012).

The carbon fixation pathway in *Spirulina* microalgae is similar to that of the C3 organisms, as *Spirulina* uses the Calvin cycle for its carbon uptake. Although the $\delta^{13}C$ values have never been determined previously in *Spirulina*, the freshwater phytoplankton (a part of which is also *Spirulina*) carbon isotopic ratio analysis showed similar $\delta^{13}C$ values to that of C3 plants (Cloern et al., 2002; Cogne et al., 2003; Q. Li et al., 2016). However, as *Spirulina* is mainly cultivated in controlled bioreactor systems and not in the natural environment, additional factors are expected to influence the $\delta^{13}C$ values, such as significant air pollution, type of production system and nutrients used. As $\delta^{13}C$ values of CO_2 are higher in open systems (> -29‰) compared to closed systems (-32‰ to -29‰), also the $\delta^{13}C$ values in microalgal biomass behave correspondingly. In the case of microalgae grown in open systems, we expect more positive $\delta^{13}C$ values, while the values of those grown in closed systems should be more negative (West et al., 2009). Additionally, different nutrient mediums (mineral additions or fertilizers) and excipients are used in *Spirulina* production, which is expected to influence the carbon isotopic composition of the final product notably. The carbon isotope ratio analysis can be used for *Spirulina* dietary supplements' authenticity assessment to detect foreign and undeclared plant material addition. This way, product adulteration with plants using a different CO_2 fixation pathway can be detected (van Leeuwen et al., 2014).

Atmospheric nitrogen fixation occurs during different biological or abiotic processes. The N_2 from the atmosphere is converted to nitrite or nitrate and, finally, ammonia, which is then fixed into organic matter. This process results from bacterial activity, natural physical processes in which high temperatures are reached (lightning, fires), and human activity, including using fertilizers. This step is followed by the nitrogen assimilation process (ammonia incorporation), in which nitrogen fixation and assimilation cause nitrogen isotopic fractionations. In the case of microalgae production, the main factor influencing $\delta^{15}N$ value is the use of fertilizers. While in synthetic fertilizers (synthesized following the Haber-Bosch process), the $\delta^{15}N$ values range from -6‰ to 6‰, the $\delta^{15}N$ values in the organic fertilizers range from 0.6‰ to 36.7‰, with manure values between 10‰ and 25‰ (Bateman & Kelly, 2007; Fogel & Cifuentes, 1993; van Leeuwen et al., 2014).

The $\delta^{15}N$ values determined in non-nitrogen fixing plankton have been determined to range between -3 and 18‰, and for marine blue-green algae, from -2 to 4‰ (Fogel &

Cifuentes, 1993). However, a wide range of fertilizers is used in commercial microalgae cultivation. Accordingly, where the synthetic fertilizers are used, lower δ^{15}N values can be detected, as the δ^{15}N values of these fertilizers are close to the atmospheric δ^{15}N value, which is approximately 0‰. When organic fertilizers (mainly animal waste) are used, higher δ^{15}N values are expected. Mixtures and varying amounts of different fertilizers result in a varying nitrogen isotopic composition of the final product (Bateman & Kelly, 2007; Thorsen et al., 2019). Consequently, nitrogen isotope ratio analysis can be applied in *Spirulina* product authenticity studies to discriminate between synthetic and organic fertilizer use, i.e., between organic and conventional production (Fogel & Cifuentes, 1993).

Compared to hydrogen, oxygen, carbon and nitrogen, the isotopic ratio of sulfur is less commonly used. Sulfur assimilation includes its incorporation into cysteine after assimilatory sulphate reduction to sulfite (Fogel & Cifuentes, 1993; Thode, 1991). Two main factors control the isotopic composition of sulfur of a component in the ecosystem – the source isotopic composition and the isotopic discrimination of sulfur during various transformation processes (Krivachy Tanz et al., 2015; Mitchell et al., 1998). The most important sources of sulfur in crops are sulfate from the bedrock and SO_2 from the atmosphere. Additionally, up to 100 km inland from the ocean, seawater influences the δ^{34}S values of crops. This effect is due to sea spray, a marine water aerosol with δ^{34}S values of approximately 21.0‰, which resemble the very uniform δ^{34}S values of the seawater (Krivachy Tanz et al., 2015; Thode, 1991). The δ^{34}S values of marine sulfate are highest near the sea and continue to decrease over 100 km inland to approximately 6‰ (Chukhrov et al., 1980; Wadleigh et al., 1996; Wadleigh & Blake, 1999). In addition to the natural sources, anthropogenic sources of sulfur must also be taken into account. For example, fossil fuel combustion can influence atmospheric δ^{34}S values, even when the sources are distant. Significant differences in the sulfur isotopic ratio values in precipitation can be due to anthropogenic activity, which is responsible for the additional contribution of sulfur, with a different isotopic composition, to that of the atmosphere (Mitchell et al., 1998).

The isotopic composition of sulfur in algal products is mainly affected by the δ^{34}S values of meteoric water and growth medium, local pollution and geological features (bedrock δ^{34}S values), as only minimal isotopic fractionation occurs during its assimilation by microalgae. Compared to the dissolved sulfate in the surrounding water, a total sulfur ^{34}S depletion in algae can be observed by only 1–2‰ (Bai & Wang, 2014; Ishii, 1953; Thode, 1991; Trust & Fry, 1992; Y. Wu et al., 2021; Yau et al., 2022). Algae cultivation using seawater could produce higher δ^{34}S values in the product due to higher δ^{34}S seawater values (17–21‰) (Moncreiff & Sullivan, 2001; Tostevin et al., 2014). An extensive range of δ^{34}S values, which differ according to cultivation location (and in accordance with previously described factors), is the basis for geographical origin discrimination among products (Krivachy Tanz et al., 2015).

Multi-isotope ratio analysis and multi-elemental composition analysis have proven valuable tools in providing information on the geographical origin of food and beverages. Studies have also shown that the best results come from combining both authentication techniques (Drivelos & Georgiou, 2012). Analyzing stable isotope ratios and elemental composition in local (regional) products of premium quality to build a database of authentic products is the best method for verifying geographical origin. Namely, the analyzed samples can then be compared to the data determined in authentic samples, and in that way, their authenticity can be assessed (Kelly et al., 2005).

The present study analyzed the stable isotopic composition of light elements (C, N, S, H and O) by IRMS in commercial *Spirulina* products and combined the results with the products' elemental composition. Statistical analysis of the data, including principal component analysis (PCA), discriminant analysis (DA), and orthogonal partial least squares discriminant analysis (OPLS-DA), was performed to classify *Spirulina* samples according to their composition and geographical region of production (Kejžar et al., 2021; Masten Rutar, Strojnik, et al., 2023). However, this study was carried out with the awareness that a reliable *Spirulina* product database has not yet been established. Therefore, the authenticity of the samples could not be reliably verified, and the results presented in this thesis serve as a basis for further investigations.

Chapter 2

Aims and Hypothesis

An increase in global population, coupled with fresh water scarcity, land degradation, climate change, and interest in sustainable food production (FAO, 2009; Rhoades et al., 1992), as well as the rise in malnutrition among the population, provide compelling reasons to initiate unconventional food production (Gardner & Halweil, 2000). A promising alternative food source is microalgae, which are highly nutritious and beneficial for human health (Sotiroudis & Sotiroudis, 2013). Among these, *Spirulina* is one of the most important algal products, belonging to prokaryotic microalgae, cyanobacteria. The *Spirulina*-based product market is expected to grow with an 18.1% compound annual growth rate by 2028 (Soni et al., 2017; Sotiroudis & Sotiroudis, 2013). However, it is important to note that *Spirulina*'s nutritional composition can be influenced by various factors such as cultivation methods, commercialization processes (including processing and packaging techniques), and transport, which can impact its nutritional and toxicological properties (Kejžar et al., 2021). Additionally, the supplement market faces challenges such as mislabeling of ingredients and product origin, as well as the substitution of ingredients with lower-quality materials. Consequently, regular inspection of *Spirulina* products is necessary to ensure their quality and authenticity (De Carvalho et al., 2012; Gallardo-Velázquez et al., 2009; Moreira et al., 2016; D. Wu et al., 2011).

With these objectives in mind, my aim was to evaluate the nutritional quality, safety, and authenticity of *Spirulina* dietary supplements available in the Slovenian market as well as explore the functional value of fresh *Spirulina* biomass. The primary focus was on analyzing the elemental, amino acid and fatty acid composition of *Spirulina* products to assess their nutritional quality and verify their compliance with the label information provided. Additionally, the safety of these commercial products was assessed, considering *Spirulina*'s ability to accumulate essential and non-essential elements, as well as toxic trace elements (Al-Dhabi & Arasu, 2016).

A further aim was to investigate the impact of fermentation on the antioxidant activity of *Spirulina* biomass extracts. Previous studies have shown that fermentation can enhance antioxidant activity by releasing phenolic compounds from plant-based foodstuffs and increasing the content of naturally occurring antioxidants (Đorđević et al., 2010; Katina et al., 2007; Othman et al., 2009). Here, my aim was also to investigate the role of different extraction solvents on *Spirulina* bioactivity, since different solvents have been used in prior research to prepare *Spirulina* extracts due to the varying solubility of bioactive compounds (Chu et al., 2010; Czerwonka et al., 2018; Putri et al., 2020; Santoyo et al., 2006; L.-C. Wu et al., 2005). This work was followed by assessing the antioxidant effect of fermented and non-fermented *Spirulina* biomass water and ethanol extracts on the yeast cells at the cellular and proteome level. Yeast was chosen as a model organism, as it shares homology with the human genome, allowing for insights applicable to human counterparts (D. Ma, 2001).

Lastly, I aimed to explore how determining the multi-isotope (δ^{34}S, δ^{2}H, δ^{13}C, δ^{18}O, δ^{15}N) and multi-elemental composition, in conjunction with multivariate statistical methods such as OPLS-DA, can be utilized to verify the origin and authenticity of *Spirulina* products from the Slovenian market.

In light of these objectives, the following hypotheses were examined:

- *Spirulina* (*Arthrospira* spp.) products from the Slovenian market are highly nutritional and rich in amino acids, fatty acids and essential elements.
- *Spirulina* products available in physical and online stores in Slovenia are safe for consumption, and their composition is consistent with the declared values.
- *Spirulina* biomass lactic acid fermentation and subsequent bioactive compounds' extraction in ethanol result in significantly enhanced antioxidant activity in treated model organisms (yeast cells), reflected at the cellular and molecular level.
- The geographical origin of *Spirulina* products can be differentiated by combining the results of their stable isotope ratio and multi-elemental composition analyses.
- The presence of adulterants in *Spirulina* products can be detected using elemental composition combined with stable isotope ratio analysis results and amino acid and fatty acid compositional data.

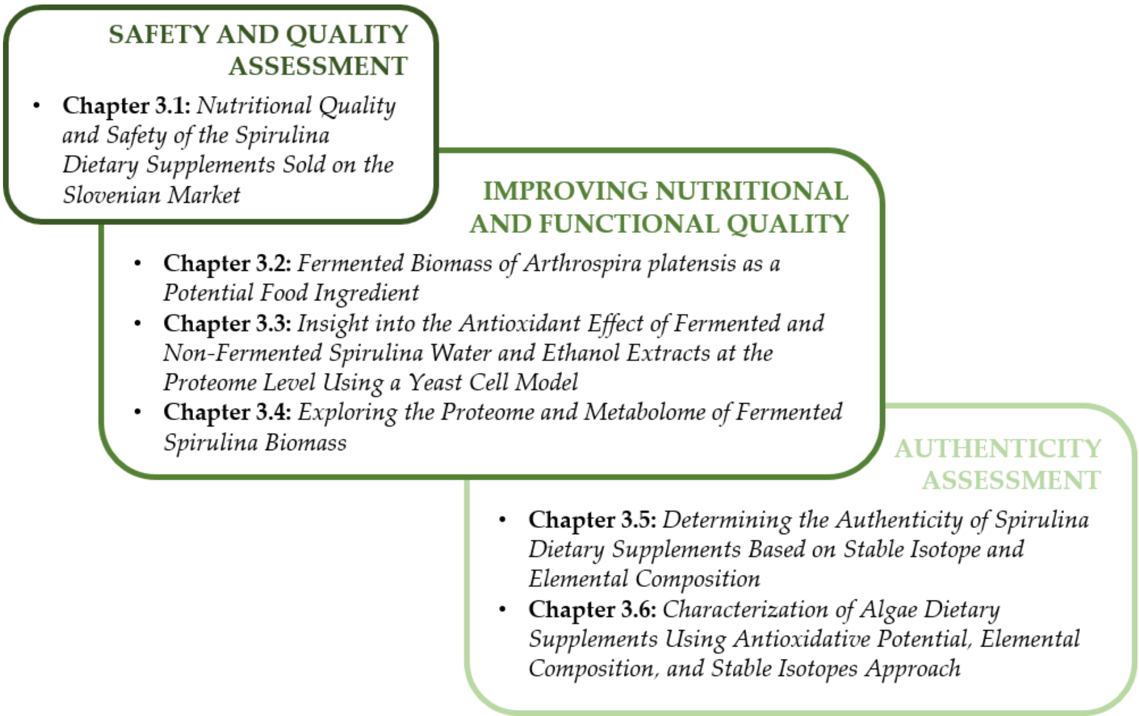
2.1 Structure of the Scientific Part of the Thesis

To assess the proposed hypotheses, the scientific part of this doctoral thesis is divided into three separate but interconnected sections (Figure 1.4).

The first section addresses the quality and safety of commercial *Spirulina* dietary supplements in Slovenia. Here, nutritional composition and compliance with the declared values of the *Spirulina* products are determined, and iron bioavailability is assessed. The results are presented in a scientific article titled *Nutritional Quality and Safety of the Spirulina Dietary Supplements Sold on the Slovenian Market* (Masten Rutar et al., 2022).

In the second part, the effect of lactic acid fermentation by *L. plantarum* on *Spirulina* fresh biomass nutritional quality and bioactive properties is assessed at the cellular and molecular level, using a proteomic and metabolomic approach. The results are presented in three scientific articles titled (i) *Fermented Biomass of Arthrospira Platensis as a Potential Food Ingredient* (Jamnik et al., 2022), (ii) *Insight Into the Antioxidant Effect of Fermented and Non-fermented Spirulina Water and Ethanol Extracts at the Proteome Level Using a Yeast Cell Model* (Masten Rutar et al., 2021), and (iii) *Exploring the Proteome and Metabolome of Fermented Spirulina Biomass* (Masten Rutar, Vrhovšek, et al., 2023).

The third part examines the possibility of combined elemental and stable isotope ratio composition application to assess the authenticity of the Slovenian market's commercial *Spirulina* and other algal products regarding their geographical origin and composition. Two scientific articles are presented regarding this topic: (i) *Determining the Authenticity of Spirulina Dietary Supplements Based on Stable Isotope and Elemental Composition* (Masten Rutar, Strojnik, et al., 2023) and (ii) *Characterization of Algae Dietary Supplements Using Antioxidative Potential, Elemental Composition, and Stable Isotopes Approach* (Kejžar et al., 2021).



SAFETY AND QUALITY ASSESSMENT

- **Chapter 3.1:** *Nutritional Quality and Safety of the Spirulina Dietary Supplements Sold on the Slovenian Market*

IMPROVING NUTRITIONAL AND FUNCTIONAL QUALITY

- **Chapter 3.2:** *Fermented Biomass of Arthrospira platensis as a Potential Food Ingredient*
- **Chapter 3.3:** *Insight into the Antioxidant Effect of Fermented and Non-Fermented Spirulina Water and Ethanol Extracts at the Proteome Level Using a Yeast Cell Model*
- **Chapter 3.4:** *Exploring the Proteome and Metabolome of Fermented Spirulina Biomass*

AUTHENTICITY ASSESSMENT

- **Chapter 3.5:** *Determining the Authenticity of Spirulina Dietary Supplements Based on Stable Isotope and Elemental Composition*
- **Chapter 3.6:** *Characterization of Algae Dietary Supplements Using Antioxidative Potential, Elemental Composition, and Stable Isotopes Approach*

Figure 2.1: Scheme of the research topics presented in this doctoral dissertation.

Chapter 3

Publications

3.1 Scientific Paper: “Nutritional Quality and Safety of the *Spirulina* Dietary Supplements Sold on the Slovenian Market”

In this chapter, I present the paper entitled “Nutritional Quality and Safety of the *Spirulina* Dietary Supplements Sold on the Slovenian Market” by Jasmina Masten Rutar, Marta Jagodic Hudobivnik, Marijan Nečemer, Katarina Vogel Mikuš, Iztok Arčon and Nives Ogrinc. My contribution included sample collection, preparation, and performing amino acid and fatty acid analyses using GC-MS. I was also responsible for experimental design, statistical analysis and interpretation of the data and preparation of the manuscript. This paper was published in the journal Foods (impact factor (IF) 5.2) in 2022. It characterizes commercially available *Spirulina* dietary supplements based on their elemental (Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Zn, Br, Rb, Se and Sr), toxic trace elemental (As, Cd, Hg and Pb), amino acid and fatty acid content as well as examines the compliance with their nutrient declaration. Iron speciation was also researched to determine iron bioavailability in *Spirulina* products.

It aimed to assess the nutritional quality, safety and authenticity of *Spirulina* products available on the Slovenian market. Therefore, 46 *Spirulina* products were collected in fresh and dry form (capsules, flakes, tablets or powder) in physical or online stores. The products were declared to originate in Hawaii, Italy, Japan, Portugal, Taiwan, India, European Union (EU), outside the EU, China, or had no declared origin. Elemental analyses were performed using ED-XRF and ICP-MS, while the amino acid and fatty acid profiles were obtained using GC-MS.

The *Spirulina* dietary supplements were found to be rich in essential and non-essential amino acids as well as ω-6 polyunsaturated fatty acids, iron (up to 316% of Recommended Dietary Allowance (RDA)), calcium (up to 29.5% RDA), phosphorous (up to 26.7% RDA), potassium (up to 7.69% RDA) and selenium (up to 38.6% RDA) when consumed within suggested amounts. However, iron bioavailability has proven poor due to the prevalence (82–92%) of the less bioavailable Fe^{3+} (ferric) form. The content of toxic trace elements is well below the maximum allowed levels in food supplements and therefore does not pose a risk. However, 86.7% of the products had inappropriate declarations, which is a cause for concern and could undermine consumer confidence.

According to high deviation in elemental, amino- and fatty acid composition among analyzed samples, in specific cases, adulteration was suspected, which is also indicated by the principal component analysis (PCA), where most samples form four distinct clusters with specific characteristics, while the samples with different composition stand out. Elemental, amino acid and fatty acid composition variability in *Spirulina* products could be attributed to several factors, including product composition and presence of excipients, production and processing techniques and *Spirulina* strain used in cultivation. This study shows that combining *Spirulina* product nutrient composition analysis with multivariate statistical analyses offers a valuable method for determining authenticity.

The results obtained in this research were presented as a scientific conference poster presentation at the 2nd Food Chemistry Conference – Shaping the Future of Food Quality, Safety, Nutrition and Health in Seville, Spain, 17th to 19th September 2019, and at the 9th International Symposium on Recent Advances in Food Analysis in Prague, Czech Republic, 5th to 8th November 2019. Additionally, results were presented as an oral presentation at the IMEKO Foods conference entitled "Food on a global market – opportunities and threats", 7th – 9th November 2022, in Dubrovnik, Croatia.

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Article

Nutritional Quality and Safety of the *Spirulina* Dietary Supplements Sold on the Slovenian Market

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Abstract: The microalgae *Spirulina* may be a popular dietary supplement rich in essential nutrients and vitamins, but oversight of the supplement industry, in general, remains limited, and increasing incidents of adulteration, misbranding, and undeclared ingredients together with misleading claims create potential risks. In response, this study characterized the elemental, amino acid and fatty acid content of commercially available *Spirulina* supplements in Slovenia using EDXRF, ICP-MS and GC-MS and compared the results with their nutritional declaration. The gathered data confirm that *Spirulina* supplements are a good source of calcium (0.15 to 29.5% of RDA), phosphorous (3.36–26.7% of RDA), potassium (0.5 to 7.69% of RDA) and selenium (0.01 to 38.6% of RDA) when consumed within recommended amounts. However, although iron contents were relatively high (7.64 to 316% of RDA), the actual bioavailability of iron was much lower since it was mainly present as the ferric cation. This study also confirms that pure *Spirulina* supplements are a good source of essential and non-essential amino acids, and ω-6 but not ω-3 polyunsaturated fatty acids. The presence of additives resulted in significant variation in nutrient content and, in some instances, lower product quality. Moreover, a high proportion (86.7%) of inappropriate declarations regarding the elemental content was observed. Overall, the study conclusions underline the need for a stricter control system for *Spirulina*-based supplements.

Keywords: *Spirulina*; microalgae; cyanobacteria; elements; toxic elements; amino acids; fatty acids; authenticity; safety; quality

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1. Introduction

The challenges associated with living sustainability, keeping food production costs down while meeting increasing food demand, has meant that sourcing alternative lipid, protein, pigment and polymer sources has become a global trend. In this context, microalgae rich in functional nutrients that positively affect human health is an excellent example of an alternative nutrient source [1–5]. *Spirulina,* or correctly *Arthrospira* spp., is one of the most important microalgal groups currently produced and contains macro- and micronutrients such as high-quality proteins, minerals, vitamins, fatty acids, polysaccharides and other bioactive compounds [6–9].

Spirulina is a multicellular filamentous cyanobacterium (blue-green microalgae) with nitrogen-fixing symbiotic bacteria. Its multicellular cylindrical trichomes are typically arranged along its entire length in a left-handed open helix, and its surface is without covering and smooth. It is a photosynthetic autotroph with phycocyanin as its primary photosynthetic pigment [10,11]. *Spirulina* is an excellent source of iron, calcium and phosphorous, pigments (carotenoids, c-phycocyanin, chlorophyll-a), and vitamins (vitamin E, vitamin B12). It is also a rich source of digestible proteins (up to 70% of its protein content), polysaccharides and lipids and has a well-balanced amino acid profile. It is also regarded as a good source of essential fatty acids, including ω-6 linoleic and γ-linolenic fatty acid, as well as ω-3 fatty acids such as α-linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [9,12,13]. It is suggested that this high nutritional value could positively influence the treatment of several pathological conditions such as certain cancers, hepatotoxicity, cardiovascular disease and hyperlipidemia, among others [14–17]. The lack of cellulose in its cell wall and the absence of phytates and oxalates means that nutrient assimilation in the human gut is high, making it popular among consumers [18].

The *Spirulina* and *Spirulina*-based product market is expected to show continued and rapid growth until 2028 with a compound annual growth rate (CAGR) of 18.1%. Most microalgae-based commercial products are produced in Asia or Australia, while European companies account for approximately 5% of the global food/feed microalgae market [4]. The demand for *Spirulina* products is attributed to increasing health awareness and vegetarianism, malnutrition, dietary supplement intake, and the demand for natural colorings. At present, most production is directed towards food, supplements and nutraceuticals, which together account for 75% of the reported uses [4]. *Spirulina* supplements are segmented into powder, the most popular form, as well as flakes, capsules, tablets, frozen *Spirulina* and phycocyanin extract, and are promoted by producers and suppliers as a health food [19].

The cultivation and commercialization, such as processing and packaging methods and transport of *Spirulina,* can change its chemical composition, affecting its nutritional quality and toxicological properties [20]. Studies have shown the presence of cadmium, mercury, arsenic and lead in *Spirulina* products in anomalous quantities due to pesticide or fertilizer use adjacent to *Spirulina* cultivation areas [21]. Such contamination is of concern because dietary supplements are not strictly regulated or inspected to the same extent as other food products and pharmaceuticals. Further increased market demand and high cost and complexity of *Spirulina* culturing encourage the adulteration of these products with inferior cheaper materials/ingredients, e.g., flour and mung bean powder, resulting in economic losses and potentially putting consumers at risk [22,23].

Combatting adulteration, increasing consumer trust and guaranteeing the quality and safety of *Spirulina* products available on the market requires that products are regularly monitored [23–25]. This study focused on the characterization of commercial *Spirulina* supplements sold on the Slovenian market, including elemental, toxic elemental, amino acid, and fatty acid composition, and their compliance with their product declaration and identifying possible adulterations. Furthermore, the bioavailability of Fe was estimated for the first time.

2. Materials and Methods

2.1. Samples

Forty-six *Spirulina* dietary supplements were purchased from different health food stores/supermarkets and online stores in Slovenia (Table 1). The samples were collected over two months. Forty-four supplements (95.7%) were labelled as *Spirulina* spp., while two samples (4.35%) were mixed with other algae (*Chlorella*, *Lithothamnium*) and plant-based nutritional supplements, e.g., wheatgrass and barley grass. Among the *Spirulina*-only samples, 34 samples (73.9%) were labelled as pure *Spirulina*, while the remaining ten (21.7%) also contained additives. The samples were either fresh or in powder, tablet, or capsule form originating from Italy, Portugal, Japan, China, India, Taiwan, and Hawaii.

Table 1. List of *Spirulina* supplements purchased from the Slovenian market, including their origin, product content and form as declared on the product label. The samples are clustered according to the declared country of origin.

Sample	Form	Declared Origin	Declared Product Content
S1	Tablets	Japan	*Spirulina*, edible scallop shell powder, edible refined processing fat
S4	Tablets	Hawaii	*Spirulina pacifica*, silicon dioxide, chicory inulin, magnesium stearate
S26	Tablets	Hawaii	*Spirulina pacifica*, silicon dioxide
S7	Tablets	India	*Spirulina platensis*
S19	Tablets	India	*Spirulina platensis*, calcium carbonate, micro-crystalline cellulose, stearic acid, croscarmellose sodium, silica
S34	Tablets	India	*Spirulina platensis*
S38	Tablets	India	*Spirulina platensis*
S9	Powder	Mongolia-China	Wheatgrass, barley grass, *Spirulina*, *Chlorella*
S8	Tablets	China	*Spirulina platensis*
S10	Powder	China	*Spirulina*
S11	Powder	China	*Spirulina platensis*
S12	Powder	China	*Spirulina*
S13	Tablets	China	*Spirulina*
S17	Powder	China	*Spirulina platensis*
S23	Tablets	China	*Spirulina*
S24	Powder	China	*Spirulina*
S25	Tablets	China	*Spirulina*
S27	Powder	China	*Spirulina*
S31	Powder	China	*Spirulina platensis*
S32	Tablets	China	*Spirulina*
S33	Powder	China	*Spirulina*
S41	Powder	China	*Spirulina platensis*
S43	Powder	China	*Spirulina*
S3	Powder	Outside EU	*Spirulina platensis*
S5	Powder	Outside EU	*Spirulina*
S6	Powder	Outside EU	*Spirulina platensis*
S16	Powder	Outside EU	*Spirulina*
S35	Tablets	Outside EU	*Spirulina*
S36	Powder	Outside EU	*Spirulina*
S14	Tablets	Taiwan	*Spirulina platensis*
S15	Powder	Taiwan	*Spirulina platensis*
S40	Tablets	Taiwan	*Spirulina platensis*
S29	Tablets	Portugal	*Spirulina platensis*, silicon dioxide, magnesium stearate
S30	Tablets	Portugal	*Spirulina platensis*, silicon dioxide, magnesium stearate
S44	Flakes	Italy	*Spirulina platensis*
S46	Fresh	Italy	*Spirulina platensis*
S18	Capsules	EU	*Spirulina, Chlorella, Lithothamnium*
S2	Capsules	NS [1]	*Spirulina pacifica*, magnesium stearate
S20	Tablets	NS	*Spirulina*, silicon dioxide, magnesium stearate
S21	Tablets	NS	*Spirulina*
S22	Tablets	NS	*Spirulina platensis*, maltodextrin, silicon dioxide, magnesium stearate, hydroxypropyl methyl cellulose
S28	Tablets	NS	*Spirulina*
S37	Tablets	NS	*Spirulina maxima*, corn maltodextrin, magnesium stearate
S39	Tablets	NS	*Spirulina*
S42	Capsules	NS	*Spirulina*
S45	Powder	NS [1]	*Spirulina*

[1] NS—Not Specified.

2.2. Sample Preparation

Fresh samples were freeze-dried and ground to obtain a fine powder, the tablets were also finely ground, and the capsules were opened and the contents used for analysis. The samples were stored in plastic containers and kept refrigerated (4 °C) until analysis.

Samples were analyzed in duplicate in small batches. All analyses were performed within one to three months after collection.

2.3. Macro-Elemental Analysis by X-ray Fluorescence Spectrometry

The macro-elemental composition (Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Zn, Br, Rb and Sr) was determined non-destructively using Energy Dispersive X-Ray Fluorescence Spectrometry (EDXRF). Pellets (0.5–1.0 g) were prepared using a pellet die and a hydraulic press, and the disc radioisotope excitation sources Fe-55 (25 mCi, Eckert & Ziegler, Berlin, Germany) and Cd-109 (20 mCi, Eckert & Ziegler, Berlin, Germany) used for fluorescence excitation. Fluorescence was measured using the EDXRF spectrometer with an XR-100 SDD detector (Amptek, Bedford, MA, USA), a PX5 digital pulse processor (Amptek, Bedford, MA, USA), and a PC-based, multichannel analyzer software package (DPPMCA). In Fe-55 mode, the spectrometer was equipped with a vacuum chamber to measure light elements (Si, P, S, and Cl), and in Cd-109 mode, in the air for K, Ca, Ti, Mn, Fe, Zn, Br, Rb and Sr. The energy resolution was 125 eV at 5.9 keV. X-ray spectra were analyzed using AXIL Spectral Analysis software. Quantification was performed using the Quantitative Analysis of Environmental Samples (QAES) software developed in-house [26,27]. The estimated uncertainty budget of the EDXRF analysis was 11%. The method was validated by analyzing NIST 1547 (peach leaves) and NIST 1573a (tomato leaves).

2.4. Iron Speciation by Fe K-Edge X-ray Absorption Near Edge Structure (XANES)

Powdered *Spirulina* samples were pressed into pellets (0.1–0.3 g), fixed on Teflon holders with Fe free scotch tape and mounted on an LN_2 cooled stage. X-ray absorption spectra were obtained in fluorescence detection mode using an unfocused CLÆSS beamline at the ALBA synchrotron facility (ALBA, Barcelona, Spain). A pair of horizontal and vertical slits allowed the reduction of the beam size on the sample to about 5 mm × 1 mm, illuminating a major part of the pellet. A silicon (Si 111) double crystal monochromator was used with 1 eV resolution at the Fe K-edge (7112 eV). The samples were inserted between the first and the second ionization cell at 45° relative to the beam. An SDD fluorescence detector, positioned at 90° to the beam, was used to measure the intensity of the Fe-Kα fluorescence radiation. The fluorescence spectra were recorded as the ratio of the fluorescence detector signal and the signal of the incident photon beam from the first ionization chamber with an integration time of 4 s/step. The absorption spectra were measured within the interval −150 eV to 350 eV relative to the Fe K-edge. In the XANES region, equidistant energy steps of 0.2 eV were used and 1 eV steps elsewhere. Three replicates were measured to check scan reproducibility and improve the signal-to-noise ratio. No evidence of Fe K-edge shifts in consecutive scans of the samples was observed due to the absorbed dose of ionizing radiation. The monochromator's exact energy was calibrated against a 5 μm thick Fe metal foil. The first inflection point in the XANES spectrum of Fe metal was at 7112 eV, while the absolute energy reproducibility was ± 0.03 eV or better. The Fe K-edge XANES spectra were analyzed using the IFEFFIT software package ATHENA [28].

The bioavailability of Fe was estimated by measuring the relative amounts of Fe^{2+} (ferrous iron) and Fe^{3+} (ferric iron). For this, we used the linear combination fit (LCF) method as described in [28,29]. The relative amounts of each Fe cation (Fe^{2+} and Fe^{3+}) in the sample were determined based on a linear combination fit of the XANES spectrum of the sample, that of the Fe reference compound with known valence states of Fe.

2.5. Toxic Trace Element and Se Analysis by Inductively Coupled Plasma-Mass Spectrometry

Inductively coupled plasma-mass spectrometry (ICP-MS) was used to measure Se, As, Cd, Hg and Pb levels in commercial *Spirulina* samples. Powdered samples (0.05–0.1 g) were weighed into Teflon vials, followed by 2 mL of 65% HNO_3 (Suprapur®, Merck, Darmstadt, Germany). Samples S10, S20, S31, S40, S41, S46 and S47 were prepared in duplicate. The samples were then digested in an UltraWave closed vessel microwave digestion system (Milestone, Sorisole (BG), Italy) at 1500 W and 100 bar maximum pressure. The temperature program was as follows: ramped to 240 °C in 20 min, held for 15 min and then cooled to 40 °C. The digests were then quantitatively transferred to 10 mL polyethylene graduated vials and filled to the mark with MilliQ water. The samples were then filtered through hydrophilic syringe filters (Millipore Millex-HV, Merck, Darmstadt, Germany) (0.45 µm) and diluted in a 1:10 ratio. The reference material BCR-414 (plankton trace elements) and blank samples (HNO_3) were prepared similarly.

The samples were then analyzed using a triple quadrupole instrument ICP-QQQ (Agilent 8800, Santa Clara, CA, USA) in 1:10 dilution for Se, As, Cd and Hg and 1:100 dilution for Pb. A dilution of 1:100 was used for Hg in the reference material. Calibration curves were prepared for Se, As, Cd and Pb using MULTI XVI (ICP Multi-Element Standard Solution XVI CertiPUR®, Merck, Darmstadt, Germany) in 5% HNO_3. The following concentrations were prepared: 0, 0.1, 0.5, 1, 5, 10, 50, 100, 250 and 1000 ng/mL. The Hg calibration curve was prepared using the NIST 3133 reference material (RM) in a 5% HNO_3 solution at concentrations of 0, 0.1, 0.5, 1, 5 and 10 ng/mL.

2.6. Fatty Acid Analysis by Gas Chromatography-Mass Spectrometry Method

The analysis of fatty acids in *Spirulina* samples was determined using Gas Chromatography-Mass Spectrometry (GC-MS).

2.6.1. Fatty Acid Extraction and Esterification

Powdered *Spirulina* samples (150 mg) were weighed directly into screw-cap vials, and 500 µL of dichloromethane and 3 mL of 0.5 M sodium hydroxide in methanol were added for total lipid extraction. Samples were then purged with nitrogen and heated for 10 min at 90 °C. Once cool, 3 mL of BF_3-MeOH was added to generate the fatty acids methyl esters (FAMEs) and purged with nitrogen. The samples were heated for 10 min at 90 °C. Once cool, the FAMEs were extracted using 1.5 mL of hexane. The hexane phase was transferred directly into a GC vial and stored at −20 °C. All the samples were prepared in triplicate.

2.6.2. FAME Analysis

Analysis was carried out using a 7890B GC and 5977A Series GC/MSD (Agilent, Santa Clara, CA, USA). Separation was achieved on a 30 m × 0.25 mm × 0.25 µm VF-WAXms capillary column (Agilent J&W, Santa Clara, CA, USA). The injection volume was 1 µL, with a split ratio of 10:1. The carrier gas was helium maintained at a 1.5 mL/min constant flow. The injector temperature was 280 °C and the detector temperature 350 °C. The temperature program was as follows: initial column temperature set at 50 °C for 1 min then programmed to 170 °C at 15 °C/min and held for 5 min, then from 170 °C to 200 °C at 3 °C/min, held 5 min, and from 200 °C to 230 °C at 5 °C/min, and held 17 min.

A standard Supelco 37 component FAME Mix in dichloromethane (Bellefonte, PA, USA) was used for identification and quantification. Compounds were identified based on a comparison of retention times with authentic compounds. With each set of samples, blank samples and the FAME Mix standard were analyzed to verify the stability of the analytical system. The results are expressed as the weight percent of an individual fatty acid to the total fatty acid (TFA) content calculated from the peak area using the appropriate correction factors [30].

2.7. Amino Acid Analysis by Gas Chromatography-Mass Spectrometry

The amino acid composition was determined using Gas Chromatography-Mass Spectrometry (GC-MS).

2.7.1. Liquid Phase Hydrolysis

The total amino acid extraction was based on hot protein hydrolysis and simultaneous free amino acid solubilization. Before the analysis, the hydrolysis micro-reaction vessels (5 mL, heavy-wall borosilicate glass, 20 mm × 65 mm, screw top, with a solid phenolic cap) were cleaned by pyrolysis at 500 °C for 6 h and left to cool overnight. The hydrolyzing agent (6N HCl) was prepared fresh from a 30% hydrochloric acid solution. The samples (15 mg) were weighed directly into the reaction vials and the hydrolyzing agent (1 mL) containing 4% thioglycolic acid, which acts as a reducing agent to prevent amino acid oxidation. After, 1% phenol was added to prevent halogenation of the tyrosine. Oxidation was prevented by purging the samples with N_2 (5 min). The vials were then sealed and heated at 110 °C for 24 h.

2.7.2. Amino Acid Derivatization

For derivatization, a commercial EZ:faast Amino Acid Hydrolysate kit (Phenomenex, Torrance, CA, USA) was used. The procedure was as follows: 355 µL of the Na_2CO_3 solution was added to the hydrolysate sample (100 µL) to obtain a pH of 2–2.5. To this was added 20 µL of the norvaline internal standard solution (0.2 mM) and 100 µL 10% n-propanol. The samples were then extracted using solid-phase extraction and the amino acids eluted with 200 µL freshly prepared eluting medium (sodium hydroxide:n-propanol in 3-picoline, 3:2, v/v). Further, the amino acids were derivatized in a mixture of chloroform and propyl chloroformate (50 µL). The amino acids were then extracted into the organic chloroform layer by repeated emulsification and allowing the reactions to proceed for 1 min in between vortexing. Iso-octane (100 µL) was then added, and the mixture was emulsified for an additional 5 s and allowed to react for 1 min. The organic layer was then transferred into a GC vial and reduced to dryness (N_2). The amino acid derivatives were then reconstituted in a solution (100 µL) of iso-octane:chloroform (80:20, v/v). All samples were prepared in duplicate.

2.7.3. Gas Chromatography-Mass Spectrometry Method for Amino Acid Analysis

Amino acid analysis was performed using a 7890B GC and 5977A Series GC/MSD (Agilent, Santa Clara, CA, USA). Separation was achieved on a 10 m × 0.25 mm × 0.15 mm ZB-AAA GC column provided in the EZ:faast kit together with a FocusLiner®. The split ratio was 15:1, and the injector temperature was 250 °C. The injection volume was 1.5 µL. Helium was used as the carrier gas at a 1.5 mL/min flow rate. The temperature program was 110 °C to 320 °C at 30 °C/min. The detector temperature was set to 310 °C. Calibration curves were prepared for individual amino acids at concentrations of 50, 100 and 200 nmol/mL using the standard mixture (SD) provided. The amino acid standard mixture consisted of 200 nmoles/mL of each amino acid: alanine (ALA), glutamic acid (GLU), hydroxylysine (HLY), leucine (LEU), phenylalanine (PHE), threonine (THR), valine (VAL), aspartic acid (ASP), glycine (GLY), hydroxyproline (HYP), lysine (LYS), proline (PRO), tryptophan (TRP), cystine (C-C), histidine (HIS), isoleucine (ILE), methionine (Met), serine (SER) and tyrosine (TYR). Each calibrant was prepared in triplicate. From then on, the SD solutions were treated following the same procedure as the samples. Individual amino acids were identified by comparing peak retention times with known amino acids in the standard. The amino acid content results are expressed in mg/g of sample dry weight (dwt).

2.8. Statistical Analysis

Statistical analysis was performed using XLSTAT software (Addinsoft, Long Island, NY, USA, 2019). First, basic statistical methods were used for data analysis (median and quartiles, minimum, maximum, average). Principal component analysis (PCA) was applied further to identify characteristic parameters to discriminate samples based on their macro and trace-elemental composition, amino acid and fatty acid composition. The results are presented as biplots, simultaneous variables and as PCA plots.

3. Results and Discussion

3.1. Elemental Composition

The results are presented in Table 2. The elemental content in the supplements was as follows: Se < Rb < Br < Ti < Zn < Sr < Mn < Fe < Ca < Cl < Si < S < P < K. We specifically focused on elements Fe, Ca, K, Se and P since *Spirulina* is promoted as a rich source of these elements [13,15,31].

Table 2. Macro-element composition of *Spirulina* supplements available on the Slovenian market.

S. No. [1]	Si (g/kg)	P (g/kg)	S (g/kg)	Cl (g/kg)	K (g/kg)	Ca (g/kg)	Ti (mg/kg)	Mn (mg/kg)	Fe (g/kg)	Zn (mg/kg)	Se (ug/g)	Br (mg/kg)	Rb (mg/kg)	Sr (mg/kg)
S1	1.57	10.5	7.53	0.66	16.1	8.18	18.2	43.4	0.81	14.0	0.06	1.29	1.55	37.9
S2	7.69	11.4	7.53	3.07	14.8	1.32	56.9	159	3.29	43.6	0.59	11.2	7.47	10.0
S3	1.16	10.6	8.64	3.18	15.4	0.82	5.45	29.4	0.49	8.33	0.12	1.91	1.67	17.8
S4	13.5	10.0	7.57	5.77	17.1	2.20	46.5	128	3.48	52.7	0.42	16.5	11.9	24.3
S5	1.34	12.4	9.04	3.78	20.8	0.74	8.77	22.1	0.48	14.6	0.06	1.77	1.23	25.1
S6	21.7	12.7	6.47	0.09	11.0	1.28	9.31	54.9	0.90	13.9	0.02	0.50	4.12	10.2
S7	10.7	11.8	7.77	0.60	14.3	5.10	3.96	32.8	0.37	10.4	0.03	0.47	0.81	22.1
S8	16.0	11.5	7.53	1.03	14.7	2.04	60.6	35.0	1.39	16.0	0.40	0.91	0.91	27.3
S9	5.21	5.06	3.14	2.61	19.7	2.83	10.4	43.0	0.44	19.1	0.03	10.8	3.75	12.7
S10	2.34	14.1	9.38	2.11	18.5	3.09	35.5	36.6	1.68	18.5	0.37	1.34	1.58	34.7
S11	1.42	12.9	8.29	0.48	15.2	1.20	4.42	26.5	0.57	11.1	0.10	1.19	1.07	28.2
S12	1.63	13.9	8.42	1.77	16.8	5.24	12.3	38.2	1.38	33.0	0.15	1.39	1.48	31.8
S13	16.6	12.6	7.79	1.97	15.5	5.39	14.8	34.4	1.79	33.6	0.14	1.47	0.92	31.1
S14	7.94	12.2	7.54	0.19	13.6	1.00	9.91	34.9	0.69	16.5	2.70	0.57	2.23	7.41
S15	1.43	11.9	7.32	0.21	13.7	0.89	5.07	33.3	0.66	15.7	2.63	0.48	1.60	6.82
S16	1.59	14.7	7.50	0.52	14.3	2.78	6.08	36.3	0.65	17.5	0.07	0.91	0.50	12.7
S17	1.61	13.5	8.64	2.21	16.6	5.34	12.3	30.9	1.74	34.9	0.13	1.88	1.20	32.3
S18	2.74	12.6	6.17	0.60	8.63	63.5	43.1	47.1	0.75	11.1	0.11	9.11	2.55	478
S19	19.4	12.2	9.29	2.55	18.4	28.0	15.9	30.3	0.56	9.74	0.10	2.26	1.24	27.6
S20	16.8	12.1	8.39	3.04	16.3	2.43	47.5	29.1	1.13	23.0	0.92	2.00	1.91	32.2
S21	14.7	9.77	7.30	1.94	16.2	1.35	28.2	150	0.69	22.4	0.07	3.09	2.97	9.66
S22	1.85	6.82	3.60	0.55	7.40	1.37	11.1	21.1	0.39	7.69	0.07	1.24	1.06	8.00
S23	15.1	11.2	7.12	1.12	14.2	2.03	65.8	28.3	1.39	14.0	0.44	0.88	0.78	28.0
S24	1.40	9.27	6.05	0.87	12.5	2.45	19.0	88.3	0.77	15.4	0.06	1.04	1.16	11.2
S25	15.4	9.61	8.32	2.68	17.3	1.02	9.37	51.5	0.60	10.0	0.08	1.60	1.43	18.7
S26	15.0	10.9	7.91	5.63	17.5	2.28	42.3	185	3.09	35.5	0.55	17.4	9.96	14.1
S27	1.79	11.2	8.30	2.07	16.8	0.80	8.61	22.8	0.72	15.6	0.08	1.27	1.04	22.3
S28	4.91	10.3	8.66	3.47	17.4	0.72	2.58	26.2	0.47	7.27	0.11	2.54	2.44	15.4
S29	15.6	10.9	7.24	1.67	15.6	1.62	35.7	192	1.14	20.9	0.07	2.71	2.06	15.6
S30	15.1	10.1	6.99	1.63	15.2	1.50	34.5	195	1.12	21.7	0.07	3.21	1.33	15.2
S31	1.53	11.4	6.72	1.36	14.7	1.67	13.0	178	0.78	22.0	0.10	1.82	2.85	7.85
S32	16.1	10.2	8.15	2.62	14.2	2.54	39.4	35.9	1.39	22.9	1.23	1.86	2.30	35.5
S33	1.06	10.2	7.99	2.18	14.9	1.00	5.84	28.0	0.64	5.42	0.06	1.62	1.55	29.7
S34	16.9	10.9	8.09	4.34	15.9	0.91	7.82	27.8	0.52	11.3	0.28	4.47	0.57	31.2
S35	7.52	10.1	7.85	2.20	14.4	0.96	6.85	26.5	0.63	7.18	0.11	2.38	1.27	26.3
S36	1.97	11.4	7.10	2.58	14.9	2.17	24.7	34.5	1.01	17.0	0.21	6.74	1.23	55.0
S37	6.43	6.16	3.88	0.91	5.83	0.75	12.8	19.3	0.28	6.02	0.07	1.67	0.55	9.75
S38	7.56	10.3	8.04	0.30	9.00	4.93	2.81	24.9	0.41	13.3	0.02	0.67	1.33	26.1
S39	12.1	9.93	6.71	1.76	12.7	0.97	8.88	14.7	0.44	13.1	0.14	1.86	0.99	19.8
S40	8.49	10.2	8.06	0.34	8.84	5.52	4.39	28.3	0.42	14.7	0.03	0.85	1.09	29.3
S41	0.94	8.64	7.18	5.03	16.4	0.52	3.58	18.6	0.38	10.3	0.05	2.93	2.42	15.9
S42	1.07	10.1	7.72	4.70	15.1	0.69	8.89	29.1	0.55	9.91	0.13	3.78	1.21	23.7
S43	1.34	10.1	9.91	5.34	15.7	1.42	3.16	23.2	0.41	2.30	0.26	5.51	1.34	71.8
S44	0.78	8.56	6.63	2.75	20.6	3.45	10.4	84.9	0.69	24.9	0.00	8.00	6.55	86.2
S45	1.02	11.0	7.87	1.13	14.3	1.26	3.18	27.4	0.45	18.1	0.10	1.43	1.47	22.0
S46	0.68	6.64	7.38	5.36	26.9	0.46	5.36	32.9	0.93	7.59	0.00	7.07	4.21	4.39

[1] Sample number.

Although the recommended daily intake (RDI) varies, the majority of the producers recommend 3–10 g of *Spirulina* supplement intake per day, regardless of the product form (powder, capsule or tablet). As well as the RDI, the data are further evaluated using Dietary Reference Values (DRV) such as Population Reference Intake (PRI), which represents the level of nutrient intake adequate for all people in a population group, or Adequate Intake (AI), which is used when a PRI cannot be determined. Where possible, the elemental composition was also evaluated in terms of Tolerable Upper Intake Level (UL), i.e., the maximum daily intake of a nutrient from all sources unlikely to pose adverse health effects on humans [32]. The data are presented as the median value and interquartile range (IR, in parentheses).

Given the recommended daily dose, the minimal intake of Ca is 1.38 to 4.60 mg Ca/day, the maximal Ca intake is 84.0 to 280 mg Ca/day, and a median value of 4.38 (2.92–7.8)–14.6 (9.72–26.0) mg Ca/day. The PRI for Ca is 950 mg/day [33]. Therefore, the values are within the recommended PRI values of 0.15% to 29.5%. This variability can be attributed to the differences in supplement formulation and the presence of additives. For example, values were found in S19, which contains calcium carbonate, and S1, which contains calcium carbonate in the form of edible scallop shell powder [34]. The lowest values were in S41, S42 and S46. The highest amount of Ca was in S18, a mixed sample containing *Spirulina*, *Chlorella* and *Lithothamnium* algae (191–635 mg/day for 3 g to 10 g of supplement/day, respectively). This value is likely due to *Lithothamnium calcareum* (*L. calcareum*), a seaweed that crystallizes calcium carbonate in its cell walls [35]. However, the UL of 2500 mg/day is unlikely to be exceeded by including *Spirulina* supplements in the diet [36].

The minimal and maximal P intake based on RDI was 18.5–61.6 and 44.1–147 mg/day, respectively. The median value was 32.7 (30.3–35.9)–109 (101–120) mg P/day. The P content represents 3.36–26.7% of the AI (550 mg/day) [33]. The lowest value among all samples was in S9, a mixed sample containing wheatgrass, barley grass, *Chlorella* and *Spirulina*, i.e., 15.2 (for 3 g of supplement/day)–50.6 mg/day (for 10 g of supplement/day). This most likely results from the lower amount of *Spirulina* and *Chlorella* algae in the supplement, especially since *Spirulina* and *Chlorella* typically contain much higher phosphorous content than cereal grasses [13,15,37]. Like Ca, if the *Spirulina* supplement consumption remains within the RDI, the UL determined for P (3000 mg/day) [36] is unlikely to be exceeded.

The minimal intake of K varies from 17.5 to 58.3 mg/day and maximal from 80.7 to 269 mg/day, with a medium intake of 45.6 (42.8–50.0)–152 (143–167) mg/day. This amount would account for 0.5–7.69% of the AI value (3500 mg/day) for an adult [33]. Three of the highest K values were in S5, S44 and S46, where S44 and S46 originate from Italy. The lowest values were measured in S37 and S22, which contained additives and did not declare an origin. No UL was set for K consumption.

The daily intake of Fe (mg Fe/day) was from 0.84–2.81 (minimal value) and 10.4–34.8 (maximal value) with a median of 2.07 (1.47–3.40)–6.89 (4.90–11.3). Such amounts account for between 5.25 to 218% of the PRI for females (16 mg/day) and 7.64 to 316% for males (11 mg/day) [33]. The high deviation observed among the samples is due to the high amount of Fe in S4 and S26 from Hawaii and S2 with no declared origin. High Fe values likely result from a high concentration of Fe in the growth medium. The Fe content in the *Spirulina* microalgae has been proven to reflect that in the growth medium [38,39].

Iron bioavailability in *Spirulina* was determined by analyzing the relative ferrous and ferric iron amounts using XANES analysis. The results are presented in Table 3.

Table 3. Relative amounts of Fe^{3+} and Fe^{2+} cations as determined by LCF analysis of Fe K-edge XANES spectra of the *Spirulina* samples.

Sample	Fe^{3+} (%)	Fe^{2+} (%)
S4	92	8
S17	87	13
S22	85	15
S41	82	18
S46	88	12

The results show that most iron (82–92%) is present as Fe^{3+}, which means that the bioavailability of Fe from the supplements is low, as only a small amount of iron is available in a more bioavailable ferrous form, Fe^{2+} [40–42]. This finding also means that promoting *Spirulina* as a rich source of dietary iron should be reconsidered.

The minimal and maximal Se intake was 0.01 to 0.04 and 8.10 to 27.0 µg/day, respectively, with the median being 0.30 (0.20–0.80)–1.01 (0.66–2.67) µg Se/day. The amounts of Se also varied significantly, but S14, S15 and S32 stand out due to their high Se value. The former two are from Taiwan, and the latter is from China. High Se values in these samples are believed to be due to higher amounts of Se in the growth medium. However, Se from these types of samples has been shown to have a lower bioavailability than classical sources such as selenomethionine and inorganic Se salts. In addition, Se from *Spirulina* is metabolized differently due to its chemical form [43]. The lowest Se values were determined in pure *Spirulina* samples from Italy (S44 and S46). The Se accounts for 0.01% to 38.6% of the recommended AI of 70 µg/day [33]. The UL for Se is 300 µg/day [36] but is unlikely to be exceeded by adding daily *Spirulina* supplements to a regular diet.

Determined values of all elements are similar to those previously reported in the literature [13,15,20,44]. However, the significant variability observed in the amounts of certain elements is likely related to the growth medium used and intentional enrichment. It has been shown that micronutrients in the growth medium significantly improve uptake and accumulation of the macro- and micronutrients [39,43,45]. The growth medium pH can also affect *Spirulina* mineral assimilation, i.e., metal ion assimilation increases at higher pH [46]. According to the data, *Spirulina* food supplements are a good source of iron, calcium and phosphorous and can provide substantial amounts of potassium and selenium. However, the intake of specific nutrients is product dependent and depends on the amount of supplement consumed, since the recommended daily consumption values differ among producers.

The high iron content in *Spirulina* supplements is significant for those who consume, for example, fewer foods of animal origin and therefore have a lower iron intake in their diet. In addition to containing high amounts of iron, *Spirulina* also does not contain phytates or oxalates that would cause iron chelation, making *Spirulina* iron highly available for absorption in the human intestine [15,47]. However, as this study has shown, more research is needed to assess the actual iron bioavailability from *Spirulina* due to the predominance of the ferric (Fe^{3+}) iron form.

Compliance with Their Nutrient Declaration

Measured elemental values were compared to the values declared on the products. Table 4 lists the 15 products that provided information on the content of Fe, Mn, Ca, Zn and P together with the degree of deviation (%) from the declared values. Iron had the most declarations (32.6% of the products), followed by Mn (13.0%), Ca and Zn (8.70%), P (4.3%) and finally K and Se (2.17%). The maximum permissible deviation of mineral content in food supplements is from −20% to 45% [48]. An excessive negative deviation from the declared Fe content was found in S5, S24 and S28 and a positive deviation in S4, S8, S10, S13, S14, S15, S17 and S36. The Mn content deviated positively in S4 and negatively in S12, S13 and S17, while S28 had an insufficient Ca content. The Zn content was low in S17, S23 and S24, while the P content was high in S12. In the case of K, the measured content in S17 was within the declared limits (+10.7%), while Se was much lower (−95.8%) than the declared value. However, several producers state that mineral levels can deviate due to seasonal fluctuations. In addition, even though some values are high, they remain below the UL and are unlikely to pose a risk to human health. However, the proportion of inappropriate declarations (86.7%) is a cause for concern and could undermine consumer confidence, and supports the need for regular monitoring and improved quality control.

3.2. Toxic Trace Element Content

The content of Cd, Hg and Pb was evaluated according to the maximum allowed European Commission levels [49]. In contrast, the As content was evaluated according to benchmark dose lower confidence limit ($BMDL_{01}$) for cancers of the lung, skin and bladder, and skin lesions determined by The Scientific Panel on Contaminants in the Food Chain (CONTAM) [50]. Toxic trace element content and their maximum allowed values are presented in Table 5.

The maximum measured values of Cd and Pb were 226 μg/kg dwt and 1320 μg/kg dwt, respectively, and did not exceed the maximum allowed value in food supplements. The daily $BMDL_{01}$ values were calculated based on the average male (87 kg) and female (68 kg) body weight in the Slovenian population [51]. The $BMDL_{01}$ values for As range between 0.3 and 8 μg/kg b.w. per day. Based on an RDI of 3–10 g/day, the maximum daily intake from consuming *Spirulina* was 8.11–27.0 μg/day and did not exceed the upper $BMDL_{01}$ value for all of the supplements tested. In the case of Hg, S39 exceeded the maximum value of 100 μg/kg dwt by 1% (101 μg/kg dwt). All others were below the maximum allowed Hg value in food supplements. The samples' median value (interquartile range) was 5.57 (3.57–7.63) μg/kg dwt. The lowest values of As, Cd and Pb were found in

S46 produced in Italy, while the lowest Hg value was measured in S18, a mixed sample containing *Spirulina*, *Chlorella* and *Lithothamnium* algae. The measured values of elements Cd, Hg and As are comparable to literature values [6,21,52,53]. Pb values are also consistent with those determined by Al-Homaidan [53] but are lower than in commercial samples tested by Hsu et al. and Campanella et al., which ranged between 5600–15,200 µg/kg dwt, and 30.8% of the *Spirulina* samples tested by Rzymski et al., which ranged from 3500 µg/kg dwt to 5000 µg/kg dwt. The authors attributed the high values to local contamination, greater propensity toward Pb assimilation by microalgae and natural background levels [6,52,54,55].

Table 4. Compliance with *Spirulina* declared nutrient values (% deviation).

S. No. [1]	**Fe (g/kg)**		**Mn (mg/kg)**		**Ca (g/kg)**		**Zn (mg/kg)**		**P (g/kg)**	
	DV [2]	**MV** [3] **(Deviation (%))**	**DV**	**MV (Deviation (%))**	**DV**	**MV (Deviation (%))**	**DV**	**MV (Deviation (%))**	**DV**	**MV (Deviation (%))**
S4	1.7	3.48 (+105)	67.0	128 (+91.1)	-	-	-	-	-	-
S5	1.04	0.48 (−53.7)	-	-	-	-	15	14.6 (−2.67)	-	-
S8	0.78	1.39 (+78.2)	-	-	-	-	-	-	-	-
S10	0.07	1.68 (+2453)	-	-	3.33	3.09 (−7.07)	-	-	-	-
S12	1.23	1.38 (+12.2)	67	38.2 (−43.0)	-	-	-	-	9.33	13.9 (+49.0)
S13	0.07	1.79 (+2457)	-	-	3.33	5.39 (+61.9)	-	-	-	-
S14	0.40	0.69 (+72.0)	40	34.9 (−12.8)	-	-	-	-	-	-
S15	0.40	0.69 (+64.5)	40	33.3 (−16.8)	-	-	-	-	-	-
S17	0.30	1.74 (+480)	30	30.9 (+3.00)	1.20	5.34 (+345)	180	34.9 (−80.6)	12.0	13.5 (+12.5)
S23	1.23	1.39 (+13.0)	-	-	-	-	360	14.0 (−96.1)	-	-
S24	1.23	0.77 (−37.6)	-	-	-	-	360	15.4 (−95.7)	-	-
S26	2.30	3.09 (+34.4)	130	185 (+42.3)	-	-	-	-	-	-
S28	0.6	0.47 (−22.3)	-	-	6.67	0.72 (−89.2)	-	-	-	-
S35	0.62	0.63 (+1.29)	-	-	-	-	-	-	-	-
S36	0.62	1.01 (+62.9)	-	-	-	-	-	-	-	-

[1] Sample number; [2] declared Value (DV); [3] measured Value (MV): the maximum permissible deviation (% deviation) according to European Commission is is from −20% to +45% [48].

Table 5. Toxic trace element content in *Spirulina* supplements.

Element	**ML** [1]**/BMDL$_{01}$** [2]	**Min**	**Max**	**Median (IR** [3]**)**
Cd (µg/kg dwt)	3000	1.22	226	22.6 (13.6–53.9)
Hg (µg/kg dwt)	100	0.84	101	5.57 (3.57–7.63)
Pb (µg/kg dwt)	3000	47.4	1320	355 (167–611)
As (ug/day)	Male (87 kg) 26.1–696 Female (68 kg) 20.4–544			
RDI [4] 3 g		0.04	8.11	0.82 (0.49–1.10)
RDI [4] 10 g		0.15	27.0	2.72 (1.63–3.66)

[1] Maximum allowed level by European Commission [49]; [2] benchmark dose lower confidence limit for cancers of the lung, skin and bladder, and skin lesions determined by The Scientific Panel on Contaminants in the Food Chain (CONTAM) Panel [50]; [3] interquartile range; [4] recommended daily *Spirulina* intake.

Based on the data from the present study, *Spirulina* supplements do not contribute significantly to the intake of toxic trace elements and do not pose a serious risk. Nevertheless, *Spirulina* is an effective accumulator of trace elements which is an advantage when it accumulates elements essential for human health but a disadvantage for toxic elements [21]. *Spirulina* is also a potential source of Pb, Hg, Cd, and As in open production systems, where pedoclimatic conditions and agricultural practices could contribute to their presence in higher values and again supports the need for regular monitoring [56–58]. In contrast, this is not an issue when grown in controlled (closed) environments [21].

3.3. Amino Acid Content

Amino acids asparagine (ASN) and glutamine (GLN) were quantitatively converted to aspartate (ASP) and glutamate (GLU) during acid hydrolysis. Tryptophan (TRP) was lost during acid hydrolysis, and arginine (ARG) and cysteine (CYS) were not included in the GC-MS EZ:faast kit due to their thermal instability [59,60]. Again, the results (Table 6) are presented according to the RDI of 3–10 g. The measured values are compared to the daily recommended amounts [61] for the average male (87 kg) and female (68 kg) in Slovenia [51].

Table 6. Total amino acid content (mg/day) in *Spirulina* supplements, presented for minimal (3 g) and maximal (10 g) recommended daily intake.

Amino Acid	**ADR [1] (mg/Day)**		**Min (mg/Day)**		**Max (mg/Day)**		**Median (IR [2]) (mg/Day)**	
	Male (87 kg)	**Female (68 kg)**	**3 g**	**10 g**	**3 g**	**10 g**	**3 g**	**10 g**
ALA	-	-	62.5	208	148	493	124 (112–132)	414 (374–439)
GLY	-	-	46.9	156	102	341	89.9 (83.1–94.1)	300 (277–314)
VAL	2262	1768	153	509	369	1231	307 (279–325)	1022 (930–1083)
LEU	3393	2652	90.1	300	202	674	169 (160–182)	565 (532–606)
ILE	1740	1360	111	370	257	858	220 (203–231)	733 (677–771)
THR	1305	1020	74.1	247	177	589	149 (137–160)	497 (458–533)
SER	-	-	65.5	218	175	582	140 (127–154)	466 (423–513)
PRO	-	-	52.0	173	105	349	91.6 (85.3–93.9)	305 (284–313)
ASP	-	-	125	415	304	1012	255 (239–270)	850 (796–900)
MET	870	680	34.7	116	74.0	247	61.4 (56.3–66.2)	205 (188–221)
GLU	-	-	218	725	712	2374	498 (455–585)	1661 (1517–1949)
PHE [3]	2175	1700	63.3	211	121	403	109 (102–112)	362 (341–374)
LYS	2610	2040	90.9	303	377	1256	231 (199–280)	770 (662–934)
HIS	870	680	48.9	163	66.6	222	58.7 (56.3–62.4)	196 (188–208)
TYR [3]	2175	1700	52.6	175	119	397	104 (96.8–111)	347 (323–371)

[1] Adult daily requirement (WHO) [61]; [2] interquartile range; [3] combined recommendations (2175 mg/day total for males and 1700 mg/day total for females).

The amino acid values determined in this study are presented in Table S1 and are within the literature values, except for VAL and HIS, where the measured values were higher [6,62,63]. The VAL content ranged from 50.8–123 mg/g dwt in this study, while the previously reported values range from 35.8–60 mg/g dwt. The HIS content varied from 16.3–22.2 mg/g dwt, compared to 6.00–11.9 mg/g dwt reported in the literature (Figure 1; Table S1). The lowest content of all amino acids except MET was determined in S22, closely followed by S19 and S37. These samples contain *Spirulina* and excipients, especially S22 and S19, which have the most declared excipients among all the samples. It is known that a high excipient content affects the amino acid content due to the reduced amount of *Spirulina* [6]. Valine, ILE and HIS were present in the lowest amounts in all samples. The sample with the second-lowest content of all other analyzed amino acids was S9—a mixed sample of wheatgrass, barley grass, *Spirulina* and *Chlorella*. This result could also be due to the low microalgae content in the supplement, as cereal grasses are not as rich in protein as *Spirulina* and *Chlorella* [64,65]. These results suggest that, combined with elemental composition, the amino acid content could distinguish authentic samples from adulterated ones. The highest values of ALA, GLY, VAL, LEU, ILE, THR, SER, ASP and TYR were found in S36 and S40, PRO and LYS were highest in S6, PHE in S8, HIS in S28, MET in S39 and GLU in S46—all were declared as pure *Spirulina* products.

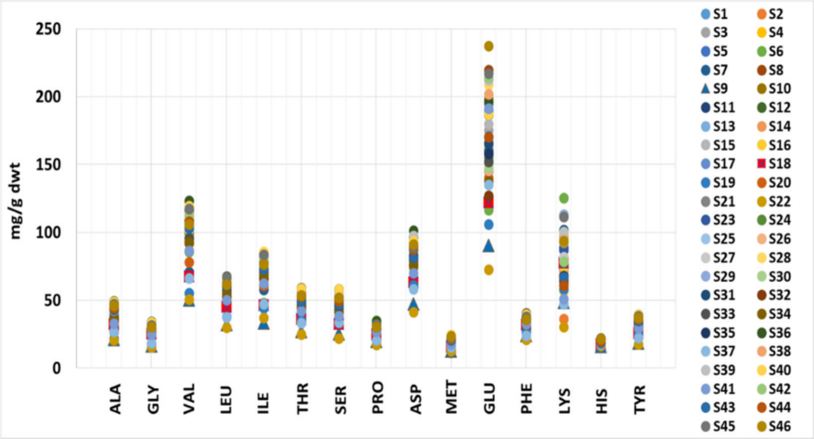

Figure 1. The amino acid content range in *Spirulina* supplements.

In addition to product composition, other factors affecting the amino acid content have been reported. For example, cells grown under stress conditions, including salinity stress, have a lower capacity for protein synthesis, which is seen in the lower protein content found in biomass grown in salinated water [66,67]. Differences also occur due to different cultivation times, light intensities, temperatures, and the growth medium's nutrient composition. For example, *Spirulina* grown in a urea growth medium has a higher amino acid content than others [62]. The drying processes used can also affect the amount of protein and, consequently, amino acid content. The highest protein losses are connected to convective and infrared drying in spreading cylinders, while freeze-drying gives the highest protein recoveries. In addition, thin layer drying results in higher protein recoveries than cylinder drying [68]. Drying at low temperatures (40–50 °C) does not affect the products' nutritional quality compared to the fresh *Spirulina* [69].

This study shows that the *Spirulina* supplements contained all the essential and non-essential amino acids measured. Coverage of daily requirements for adults [61] based on median values (%) was: TYR (males (M): 4.79–16.0, females (F): 6.13–20.4), LEU (M: 4.99–16.6, F: 6.39–21.3), PHE (M: 4.99–16.6, F: 6.38–21.3), HIS (M: 6.75–22.5, F: 8.63–28.8), MET (M: 7.06–23.5, F: 9.03–30.1), LYS (M: 8.85–29.5, F: 11.3–37.8), THR (M: 11.4–38.1, F: 14.6–48.7), ILE (M: 12.6–42.1, F: 16.2–53.9) and VAL (M: 13.6–45.2, F: 17.4–57.8). According to these results, the tested *Spirulina* supplements are a good source of essential and non-essential amino acids, as shown by other authors analyzing commercial *Spirulina* products [9,63,66]. However, despite that S22, S19 and S37 stand out because of their low amino acid content, overall, *Spirulina* food supplements would be reasonable when choosing high amino acid content products.

3.4. Fatty Acid Content

The amounts of individual fatty acids (FA) are presented in Tables 7 and S2. The results in Table 7 are presented as the median value (interquartile range in parentheses) of weight percent of individual fatty acids of total fatty acids (TFA). The most abundant FA was palmitic acid (16:0) followed by linoleic (C18:2 ω-6c), γ-linolenic (C18:3 ω-6), palmitoleic (C16:1 ω-7), stearic (C18:0) and oleic/elaidic acid (C18:1 ω-9c/9t). The amounts of fatty acids agree with published data [6,70], although levels of γ-linolenic, linoleic, palmitoleic and stearic acid vary [71–74]. These differences could be due to higher or lower content of saturated/unsaturated FA in the algal cells, which depends on metabolic needs, such as membrane fluidity, which depends on the growth conditions [75]. It is known that certain microalgae, including *Spirulina*, regulate their lipid composition at low temperatures to achieve better membrane fluidity, which results in increased levels of unsaturated FA.

Table 7. Fatty acid content (% of total fatty acid content) in *Spirulina* supplements.

Fatty Acid	Min	Max	Median (IR [1])
SFA [2]			
C14:0	0.23	0.63	0.35 (0.31–0.40)
C15:0	<LOD	0.22	<LOD
C16:0	18.4	45.9	41.8 (40.6–42.9)
C17:0	<LOD	0.42	0.33 (0.27–0.36)
C18:0	0.97	25.8	3.90 (1.70–5.46)
C22:0	<LOD	30.2	<LOD
MUFA [3]			
C14:1	<LOD	1.12	0.33 (0.09–0.41)
C16:1 ω-7	3.07	11.7	6.93 (5.96–7.94)
C17:1	<LOD	0.72	0.35 (0.31–0.42)
C18:1 ω-9c/9t	1.72	9.34	3.78 (3.24–4.38)
PUFA [4]			
C16:2 ω-4	<LOD	9.38	0.42 (0.36–0.49)
ω-6 PUFA			
C16:2 ω-6	<LOD	9.71	<LOD
LA, C18:2 ω-6c	11.1	31.0	22.0 (20.6–22.7)
GLA, C18:3 ω-6	<LOD	24.1	19.4 (16.7–20.3)
C20:3 ω-6	<LOD	0.82	<LOD (<LOD–0.33)
ω-3 PUFA			
C16:3 ω-3	<LOD	9.58	<LOD
C18:3 ω-3	<LOD	18.4	<LOD

[1] Interquartile range; [2] saturated fatty acids; [3] monounsaturated fatty acids; [4] polyunsaturated fatty acids; LOD—Limit of Detection.

High temperatures during growth also favor the formation of saturated FA [70,76,77], and the formation of certain fatty acids is more intensive at specific temperatures for certain strains, i.e., the highest γ-linolenic FA production in *Spirulina maxima* is between 35 and 40 °C. In contrast, in *Spirulina platensis*, the production of γ-linolenic is higher at 30 °C [78]. Culture age and growth medium salinity are also important, and prolonged exposure to high salinity stress results in higher values of γ-linolenic acid in different *Spirulina* strains [72]. As with amino acids, the drying process can affect lipid yield and total fatty acid content. Namely, the maximum monounsaturated and polyunsaturated FA levels are obtained in the freeze-dried samples, while the sun-dried samples contain higher amounts of saturated acids. In addition, freeze drying provides better nutrient preservation than other dehydration methods such as sun drying, oven drying or spray drying [79].

In most samples, only small amounts or no ω-3 fatty acids were found, making commercial *Spirulina* samples a good source of ω-6 (especially LA and GLA), but not ω-3 fatty acids, which was also confirmed by other studies [6,70,74,80].

Like elemental and amino acid composition, certain samples stood out regarding TFA content (Figure 2). According to median and associated IR values, anomalously high levels of C15:0 (0.22% of TFA), C16:2 ω-6 (9.71% of TFA), C16:2 ω-4 (9.38% of TFA), C16:3 ω-3 (9.58% of TFA) and C18:3 ω-3 (18.4% of TFA) were found in S6. Additionally, in the same sample, anomalously low levels of C16:0 (18.4% of TFA), C17:1 (< LOD of TFA) and C18:3 ω-6 (< LOD of TFA) were observed, suggesting possible adulteration. S19 contained unusually high levels of C18:0 (25.8% of TFA), and low values of C17:1 (< LOD of TFA), C18:2 ω-6c (12.1% of TFA) and C18:3 ω-6c (9.72% of TFA); likewise, S37 had lower amounts of C18:2 ω-6c (11.1% of TFA) and a marked deviation in its C22:0 content (30.2% of TFA). The presence of various excipients could explain these differences. However, for S37, there remains the possibility of adulteration since it contains high levels of C22:0 FA, which was otherwise detected only in S6 (0.27% of TFA, possibly adulterated), and in the mixed sample S9 (0.59% of TFA), which contains wheatgrass, barley grass, *Spirulina* and *Chlorella*. Sample S9 also stands out due to its high content of C14:0 (0.84% of TFA), C18:3 ω-3 (22.8% of TFA), C16:2 ω-6 (5.52% of TFA), and C16:3 ω-3 (5.27% of TFA) and low content of C16:1 ω-7 (2.75% of TFA), C16:0 (24.6% of TFA) and C18:3 ω-6 (5.69% of TFA).

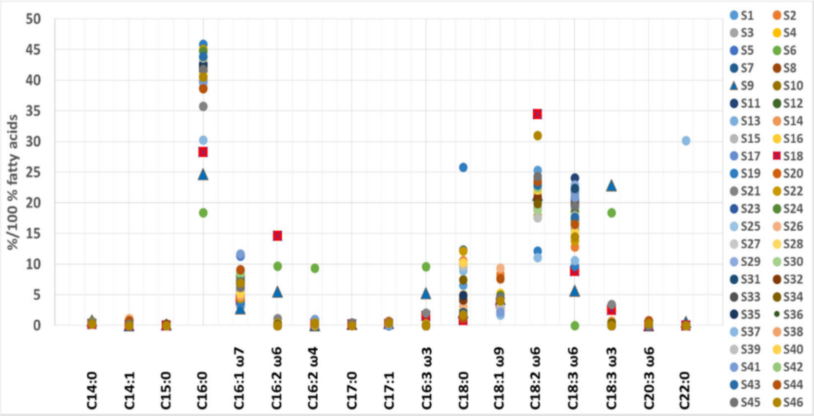

Figure 2. The fatty acid content range in *Spirulina*-based supplements.

In addition, S18 differed from supplements containing only *Spirulina*. This sample contains high amounts of C16:2 ω-6 (14.6% of TFA), C18:2 ω-6c (34.4% of TFA) and C18:3 ω-3 (2.52% of TFA). In contrast, the amounts of C16:0 (28.3% of TFA), C18:0 (0.88% of TFA) and C18:3 ω-6 (8.84% of TFA) were exceptionally low. Higher levels of C18:3 ω-3 (ALA, α-linolenic acid) in mixed samples likely result from the presence of *Chlorella*, which is known to contain large amounts of this FA. Alternatively, low GLA and palmitic acid levels point to a low *Spirulina* content in S9 and S18, as their high content is typical for pure *Spirulina* products [74]. The fatty acid distribution (% of TFA) in the analyzed samples is presented in Figure 2.

The data make it possible to distinguish between mixed samples and samples containing pure *Spirulina* based on the fatty acid composition. Such knowledge could help separate authentic from adulterated samples. In addition, the *Spirulina* supplements tested proved to be a good source of ω-6, but not ω-3 polyunsaturated fatty acids. However, since the FA content varied in samples, it would be sensible to choose pure *Spirulina* products for consumption with no added excipients from a trusted producer to guarantee optimal FA composition.

3.5. Principal Component Analysis (PCA) of Spirulina Samples from Slovenian Market Analysis Results

The data set of 46 *Spirulina* samples and 43 analyzed parameters (macro- and trace-elemental, amino acid and fatty acid composition data) was analyzed by PCA to identify the trends and examine the distribution of variables in the investigated samples (Figures 3 and 4). PC1 explained 33.0% and PC2 13.5% of the total variance. Four groups of samples can be identified (Figure 3), each represented by different variables. Most parameter vectors are directed towards the upper right quadrant of Figure 4. The positive trend is due to an increased amino acid composition as well as C16:0, C16:1 ω-7, C18:2 ω-6c and C18:3 ω-6 fatty acids. The blue group (Figure 3) has the highest content of these compounds, followed by the yellow group, while the red group has the lowest amount. Finally, ungrouped samples (S6, S9, S18, S19, S22 and S37), which have the lowest content of selected amino acids and fatty acids, are located on the left-hand side of the graph. A similar positive trend for elements Cl, Ti, Fe, Zn, Mn, Rb and Br and fatty acids C14:1 and

C18:1 ω-9c/C18:1 ω-9t is observed in the upper part of the second PCA graph (Figure 4). The samples from Hawaii (orange group) contained the highest amounts of these elements and fatty acids. Interestingly, the undeclared sample S2 falls in the same group, indicating that this *Spirulina* supplement might originate from Hawaii. Alternatively, ungrouped samples to the left of the vertical line of Figure 3 (S6, S9, S18, S19, S22 and S37) show a positive Ca, Sr and Cd and C14:0, C18:0 and C16:2 ω-4 trend (Figure 4).

Samples S9 and S18 were expected to stand out since they contain other plant or algae material, and their parameters were expected to differ from the samples containing pure *Spirulina*. In addition, S19 and S22 contain various excipients, affecting the nutrient composition. In contrast, close inspection of the data for S6 and S37 suggests possible adulteration since S6 is declared as pure *Spirulina* and S37 contains excipients, which did not affect our results. Overall statistical evaluation of the results supports our previous observations.

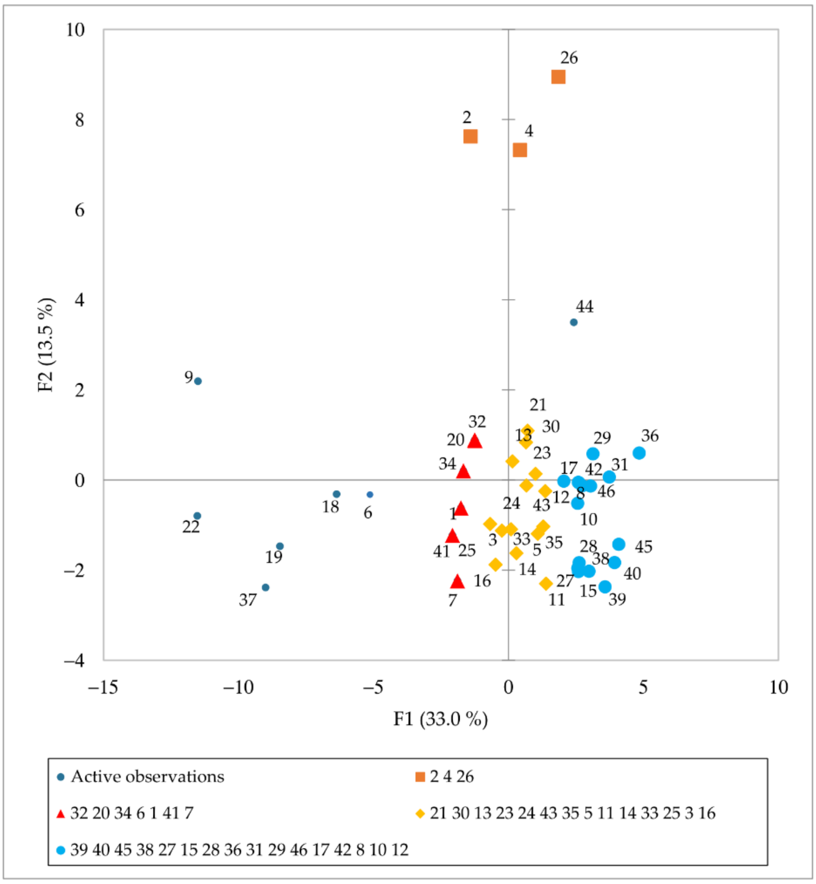

Figure 3. Principal Component Analysis score plot of *Spirulina* dietary supplements (n = 47) available in Slovenia.

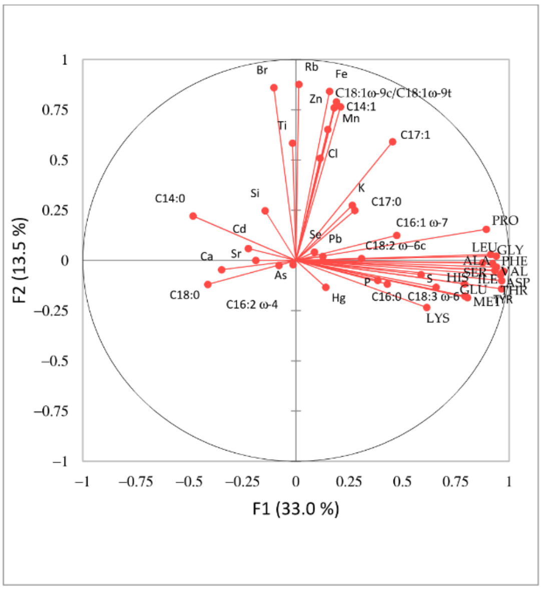

Figure 4. Principle Component Analysis variable loading plot for *Spirulina* dietary supplements (*n* = 47) available in Slovenia.

4. Conclusions

This study finds that when consumed in recommended amounts, the analyzed *Spirulina* supplements are a good source of calcium, phosphorous, potassium, and selenium, while toxic trace elements do not represent a serious health risk. They are also a good source of essential and non-essential amino acids as well as ω-6 polyunsaturated fatty acids. The study data also show that *Spirulina* contains low amounts of ω-3 polyunsaturated fatty acids, and although they contain high amounts of Fe, since it is mainly present as Fe^{3+}, the Fe is less bioavailable.

Therefore, a well-thought-out selection of *Spirulina* supplements would be advised and choosing pure *Spirulina* supplements is advisable, especially since pure *Spirulina* samples have a higher amino acid, γ-linolenic and linoleic fatty acid, P and Se content. Additionally, as different *Spirulina* products might have a different nutrient composition, the products should be chosen according to the specific nutrient needs of the individual. Supplements from Hawaii are rich in Fe, Zn, Mn, Cl, Ti, Rb and Br.

Notably, a high proportion (86.7%) of inappropriate declarations was found among the analyzed samples regarding the content of Fe, Mn, Ca, Zn, P, K and Se, which is a cause for concern. Deviations of more than 45% over the declared value could pose a risk to human health through excessive elemental intake. Such deviations can also undermine consumer confidence. Fortunately, in this case, UL levels were not exceeded.

This study also showed how adding algal or plant material supplements alters the elemental, amino acid and fatty acid composition. Such data could distinguish mixed products from those containing only *Spirulina* and therefore be valuable in authenticity studies. Multivariate analysis was able to discern these products from those containing only *Spirulina*. In addition, the amino acid, fatty acid and mineral composition data suggest that at least two samples were adulterated, since such differences in composition compared to pure *Spirulina* could not be explained by the addition of excipients.

Overall, *Spirulina* is a good source of nutrients. However, in the case of dietary supplements, regular monitoring and inspection are advised to identify adulterations and deviations from the declared content. In this way, potential hazards for consumer health can be avoided.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/foods11060849/s1, Table S1: Amino acid composition of *Spirulina* supplements available on the Slovenian market (mg/g dwt), Table S2: Fatty acid composition of *Spirulina* supplements available on the Slovenian market (% of total fatty acid content).

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Table S1. Amino acid composition of *Spirulina* supplements available on the Slovenian market (mg/g dwt).

Sample	ALA[1]	GLY[2]	VAL[3]	LEU[4]	ILE[5]	THR[6]	SER[7]	PRO[8]	ASP[9]	MET[10]	GLU[11]	PHE[12]	LYS[13]	HIS[14]	TYR[15]
S1	39.2	28.4	95.6	53.8	66.8	44.6	42.4	28.7	76.0	16.4	164	34.6	65.3	16.3	29.5
S2	38.0	27.8	91.7	54.4	65.2	43.9	41.5	28.4	74.0	18.0	167	34.5	36.3	17.2	29.1
S3	37.5	28.8	91.6	51.2	68.1	46.6	44.8	28.4	80.2	19.8	175	35.5	66.5	18.1	32.5
S4	42.0	30.7	98.6	57.6	70.0	47.5	43.9	31.9	79.5	14.7	158	36.8	56.9	18.9	32.1
S5	40.7	29.5	101	54.8	72.2	47.8	47.2	30.5	84.7	16.3	190	36.1	88.9	19.2	32.3
S6	38.2	31.9	85.8	49.8	48.0	44.3	37.8	34.9	74.9	11.6	116	37.6	126	19.9	28.3
S7	28.7	24.1	71.1	44.8	57.9	47.5	42.3	25.2	85.6	20.6	195	36.6	88.3	18.8	33.9
S8	44.8	33.3	104	58.7	75.0	54.8	52.5	31.3	92.5	19.5	220	40.3	77.5	19.6	37.0
S9	21.0	16.3	50.4	32.1	33.3	27.1	25.4	20.0	47.3	12.7	90.5	24.0	48.5	15.8	18.6
S10	42.5	32.1	102	53.5	73.9	54.8	52.5	30.8	91.9	18.6	199	39.1	76.6	19.2	36.6
S11	39.0	29.5	101	56.4	75.0	50.3	46.2	30.8	91.4	22.7	145	36.7	72.9	19.2	34.8
S12	46.1	33.1	109	58.4	78.9	49.1	41.5	31.3	88.5	21.4	187	35.6	68.5	18.3	36.4
S13	41.5	29.8	102	50.8	71.9	49.8	47.0	28.8	83.6	18.8	154	34.0	72.7	18.3	32.6
S14	47.3	32.2	116	54.8	79.4	45.3	38.1	30.7	80.6	19.3	151	34.0	70.7	18.8	34.5
S15	43.5	30.5	108	58.6	76.1	52.1	50.6	30.9	87.9	22.8	180	37.2	95.0	20.8	37.8
S16	38.5	29.8	91.5	51.2	68.0	45.0	36.9	27.5	83.9	20.7	162	36.0	75.0	19.0	37.4
S17	44.7	33.4	107	52.9	76.0	52.8	51.5	30.4	92.2	20.4	161	36.8	96.9	20.1	36.3
S18	32.6	25.4	67.5	45.0	46.6	36.4	32.7	24.9	63.4	15.9	122	32.0	77.9	17.7	25.8
S19	21.8	18.1	55.4	38.0	44.2	35.6	33.5	20.2	61.4	16.1	106	26.7	58.1	17.2	24.8
S20	33.1	27.3	78.1	51.9	60.9	45.8	42.5	26.0	78.9	19.3	140	34.1	61.5	19.2	32.1
S21	26.5	31.3	109	55.5	73.5	49.9	46.9	31.3	85.7	22.0	158	36.3	66.6	20.5	33.5
S22	20.8	15.6	50.8	30.0	37.0	24.7	21.8	17.3	41.5	12.5	72.5	21.1	30.3	16.3	17.5
S23	41.6	30.1	105	54.6	74.5	49.7	45.9	30.5	85.3	20.0	160	36.2	86.9	19.8	34.9
S24	41.3	29.1	106	57.5	73.1	48.3	46.2	30.6	82.8	20.6	155	33.8	78.2	21.2	32.4
S25	37.0	27.2	94.6	55.1	67.2	47.1	44.0	27.9	80.3	17.9	167	33.4	64.6	19.0	32.4
S26	38.5	29.3	94.9	56.5	67.9	50.3	47.6	33.4	83.4	21.5	146	34.7	92.4	20.8	33.3
S27	42.7	31.6	104	61.4	74.3	54.6	53.1	30.2	89.4	22.0	196	36.4	81.7	20.8	36.8
S28	38.4	29.3	102	60.4	75.7	53.6	51.3	29.8	90.4	22.8	209	37.2	97.6	22.2	38.4
S29	41.5	29.9	107	56.8	77.9	52.7	50.5	30.8	88.3	22.5	213	38.8	113	22.0	38.0
S30	36.4	27.2	96.0	56.7	69.2	50.4	48.0	28.5	85.3	19.6	148	35.2	93.8	20.4	34.8
S31	45.3	32.0	116	60.3	82.7	56.5	52.2	33.0	96.8	21.8	165	40.3	102	21.9	37.9
S32	35.4	25.7	92.5	53.2	66.2	45.5	42.1	27.8	76.1	19.9	127	32.2	64.0	19.0	30.8
S33	37.9	26.8	96.0	56.9	70.8	47.7	43.7	28.5	79.7	20.4	152	34.0	77.6	19.5	33.2
S34	36.7	26.3	93.1	54.9	68.4	44.3	42.1	27.8	76.5	20.2	138	32.2	66.7	18.3	30.5
S35	43.8	30.3	109	61.7	78.6	49.6	46.2	31.0	82.6	22.3	158	36.6	67.8	20.0	34.7
S36	49.3	34.0	123	67.4	84.4	58.9	58.2	34.6	101	23.2	197	39.1	93.0	21.8	38.4
S37	26.8	18.4	66.4	37.4	47.4	33.5	33.6	20.9	58.1	15.6	135	24.3	47.3	17.7	22.3
S38	43.7	30.1	113	63.0	80.7	55.5	53.2	31.2	94.3	23.3	202	38.3	97.6	21.8	38.4
S39	42.7	31.3	111	64.2	81.0	57.6	56.1	31.6	97.6	24.7	212	39.2	100	22.0	39.0
S40	48.8	33.6	119	66.9	85.8	58.6	58.2	32.2	93.9	24.4	187	39.5	93.3	21.1	39.7
S41	32.8	25.0	86.4	50.2	62.5	42.1	38.4	26.8	70.0	17.9	191	32.6	51.2	18.1	29.4
S42	44.6	30.6	112	63.0	79.1	53.2	51.0	31.4	89.8	21.2	214	37.7	78.4	20.5	36.7
S43	42.9	30.7	103	61.1	73.1	49.3	46.9	30.5	81.7	20.6	171	36.2	67.3	18.8	34.8
S44	44.4	30.3	108	62.5	76.8	52.6	49.5	31.5	87.3	21.5	170	37.3	60.9	19.6	36.2

S45	47.4	31.5	118	66.6	83.5	53.7	51.9	32.2	89.1	22.3	217	38.2	112	22.1	38.2
S46	45.9	30.3	106	61.6	76.3	53.7	51.8	30.5	91.1	23.6	237	36.0	93.6	21.7	37.2

[1] Alanine; [2] Glycine; [3] Valine; [4] Leucine; [5] Isoleucine; [6] Threonine; [7] Serine; [8] Proline; [9] Aspartate; [10] Methionine; [11] Glutamate; [12] Phenylalanine; [13] Lysine; [14] Histidine; [15] Tyrosine

Table S2. Fatty acid composition of *Spirulina* supplements available on the Slovenian market (% of total fatty acid content).

Sample	C14:0	C14:1	C15:0	C16:0	C16:1n7	C16:2n6	C16:2n4	C17:0	C17:1	C16:3n3	C18:0	C18:1n9c /C18:1n9t	C18:2n6c	C18:3n6	C18:3n3	C20:3n6	C22:0
S1	0.38	0.36	0.00	43.1	3.07	0.00	0.22	0.36	0.22	0.00	6.54	4.77	25.3	15.8	0.19	0.00	0.00
S2	0.52	1.10	0.00	40.9	5.58	0.00	0.00	0.39	0.55	0.00	10.6	8.35	19.1	12.8	0.35	0.00	0.00
S3	0.40	0.30	0.00	41.3	8.03	0.00	0.49	0.27	0.34	0.00	1.40	3.45	24.0	20.2	0.00	0.00	0.00
S4	0.37	0.46	0.00	42.0	7.58	0.00	0.42	0.25	0.45	0.00	2.64	5.24	21.0	20.1	0.00	0.00	0.00
S5	0.44	0.37	0.00	38.9	11.3	0.00	0.96	0.27	0.24	0.00	1.41	2.26	21.7	22.0	0.00	0.47	0.00
S6	0.59	0.00	0.22	18.4	5.56	9.71	9.38	0.35	0.00	9.58	4.13	4.36	22.0	0.00	18.4	0.00	0.27
S7	0.32	0.00	0.00	42.8	4.19	0.00	0.21	0.39	0.34	0.00	12.3	3.46	19.0	16.9	0.00	0.30	0.00
S8	0.36	0.45	0.00	41.7	5.85	0.00	0.39	0.37	0.31	0.00	4.52	3.66	21.5	20.5	0.00	0.24	0.00
S9	0.84	0.00	0.15	24.6	2.75	5.52	0.00	0.25	0.40	5.27	2.13	4.23	21.3	5.69	22.8	0.00	0.59
S10	0.29	0.12	0.00	42.1	7.88	0.00	0.51	0.27	0.31	0.00	1.71	3.55	22.3	21.0	0.00	0.00	0.00
S11	0.28	0.23	0.00	41.0	7.81	0.00	0.52	0.25	0.28	0.00	0.97	2.57	22.4	24.1	0.00	0.00	0.00
S12	0.33	0.33	0.00	42.6	6.66	0.00	0.41	0.35	0.36	0.00	1.76	3.74	22.7	20.9	0.00	0.00	0.00
S13	0.40	0.33	0.00	43.1	6.36	0.29	0.41	0.33	0.33	0.00	3.85	3.67	21.3	19.3	0.54	0.00	0.00
S14	0.35	0.14	0.00	41.4	5.49	0.00	0.40	0.27	0.32	0.00	9.56	2.52	21.1	18.5	0.00	0.00	0.00
S15	0.31	0.19	0.00	43.5	5.99	0.44	0.00	0.29	0.36	0.00	1.57	3.06	24.0	20.3	0.00	0.00	0.00
S16	0.36	0.00	0.00	44.8	6.25	0.00	0.37	0.00	0.31	0.00	1.98	4.33	21.9	19.7	0.00	0.00	0.00
S17	0.35	0.33	0.00	41.6	6.70	0.00	0.44	0.31	0.34	0.00	1.61	3.66	22.9	21.6	0.20	0.20	0.00
S18	0.24	0.08	0.00	28.3	4.15	14.6	0.20	0.12	0.23	1.56	0.88	3.66	34.4	8.84	2.52	0.00	0.00
S19	0.54	0.00	0.00	45.9	3.81	0.00	0.00	0.29	0.00	0.00	25.8	1.82	12.1	9.72	0.00	0.00	0.00
S20	0.35	0.41	0.00	42.1	6.94	0.00	0.44	0.33	0.35	0.00	4.32	4.01	20.8	19.7	0.00	0.24	0.00
S21	0.33	0.38	0.00	35.7	8.20	1.12	0.56	0.33	0.42	2.00	4.03	3.61	19.1	20.3	3.45	0.43	0.00
S22	0.41	0.33	0.00	45.1	4.44	0.42	0.00	0.33	0.29	0.35	12.1	3.88	17.9	13.8	0.70	0.00	0.00
S23	0.38	0.44	0.00	42.5	6.12	0.00	0.46	0.38	0.36	0.00	4.40	3.64	20.9	20.5	0.00	0.00	0.00
S24	0.34	0.41	0.00	44.7	6.54	0.00	0.44	0.40	0.41	0.00	2.47	4.88	20.7	18.6	0.00	0.00	0.00
S25	0.34	0.00	0.00	41.4	7.57	0.00	0.59	0.32	0.34	0.00	3.95	3.18	22.5	19.8	0.00	0.41	0.00
S26	0.40	1.12	0.00	38.8	7.96	0.00	0.39	0.42	0.72	0.00	3.12	9.34	22.5	14.6	0.67	0.00	0.00
S27	0.27	0.00	0.00	42.5	7.83	0.00	0.49	0.00	0.39	0.00	1.38	3.01	24.3	19.8	0.00	0.00	0.00
S28	0.30	0.00	0.00	43.3	6.41	0.00	0.38	0.27	0.35	0.00	5.09	3.93	22.2	17.9	0.00	0.00	0.00

S29	0.29	0.33	0.00	39.8	8.60	0.00	0.60	0.36	0.40	0.00	4.27	3.27	19.0	22.9	0.00	0.46	0.00
S30	0.28	0.34	0.00	40.3	8.69	0.00	0.55	0.33	0.37	0.00	4.49	3.25	18.8	22.3	0.00	0.42	0.00
S31	0.33	0.42	0.00	42.3	6.92	0.00	0.48	0.34	0.34	0.00	2.13	3.83	20.4	22.3	0.35	0.00	0.00
S32	0.37	0.42	0.21	41.6	7.27	0.00	0.44	0.28	0.34	0.00	4.19	4.03	20.9	19.9	0.00	0.00	0.00
S33	0.31	0.27	0.00	41.5	7.93	0.00	0.43	0.24	0.47	0.00	1.55	4.06	23.4	19.7	0.00	0.40	0.00
S34	0.63	0.69	0.00	39.8	8.14	0.21	0.48	0.37	0.31	0.00	7.50	3.00	19.9	17.3	0.45	0.72	0.00
S35	0.38	0.34	0.00	42.6	7.00	0.00	0.39	0.27	0.39	0.00	4.93	4.02	22.7	17.0	0.00	0.00	0.00
S36	0.50	0.77	0.00	42.1	8.01	0.00	0.48	0.37	0.37	0.00	1.92	4.55	22.4	18.5	0.00	0.00	0.00
S37	0.48	0.12	0.00	30.3	5.34	0.00	0.25	0.12	0.14	0.00	8.95	1.72	11.1	10.6	0.00	0.00	30.2
S38	0.31	0.00	0.00	40.6	4.87	0.00	0.26	0.40	0.50	0.00	9.88	4.27	22.3	16.4	0.00	0.29	0.00
S39	0.41	0.26	0.00	43.7	6.21	0.00	0.38	0.35	0.33	0.00	9.72	3.06	17.6	17.9	0.00	0.35	0.00
S40	0.34	0.00	0.00	41.0	5.23	0.00	0.27	0.39	0.51	0.00	10.3	4.43	22.1	15.3	0.00	0.30	0.00
S41	0.23	0.00	0.00	39.7	11.7	0.00	0.88	0.22	0.29	0.00	1.22	2.20	22.9	20.9	0.00	0.00	0.00
S42	0.51	0.53	0.00	43.9	8.37	0.00	0.49	0.33	0.44	0.00	1.66	4.43	22.5	16.8	0.00	0.35	0.00
S43	0.43	0.29	0.00	43.9	7.12	0.00	0.35	0.36	0.47	0.00	1.80	4.56	22.9	17.7	0.00	0.32	0.00
S44	0.31	0.80	0.00	38.7	9.13	0.00	0.41	0.27	0.64	0.00	1.25	7.66	23.5	16.5	0.00	0.82	0.00
S45	0.34	0.21	0.00	41.8	6.26	0.00	0.35	0.37	0.45	0.00	1.83	4.63	24.3	19.5	0.00	0.00	0.00
S46	0.26	0.00	0.00	40.6	6.99	0.00	0.38	0.00	0.41	0.00	1.69	4.00	31.0	14.5	0.00	0.34	0.00

3.2 Scientific Paper: "Fermented Biomass of *Arthrospira platensis* as a Potential Food Ingredient"

This chapter presents the paper entitled "Fermented Biomass of *Arthrospira platensis* as a Potential Food Ingredient" by Polona Jamnik, Nik Mahnič, Aleksandra Mrak, Lea Pogačnik, Barbara Jeršek, Alberto Niccolai, Jasmina Masten Rutar, Nives Ogrinc, Larisa Dušak, Blaž Ferjančič, Mojca Korošec, Ana Cerar, Borut Lazar, Urša Lovše, Tjaša Pungert, Primož Fabjan and Nataša Poklar Ulrih. The paper was published in Antioxidants (IF 7.0) in 2022 and describes the effect of lactic acid fermentation on *Arthrospira platensis* (*Spirulina platensis*) energy value and biomass nutritional composition, lipid peroxidation, intracellular antioxidative activity and microbiological safety. As part of this work, my responsibilities included sample preparation, fermentation, preparation of fermented and non-fermented *Spirulina* broth extracts and analysis of cellular antioxidant activity.

Lactic acid fermentation has been shown to enhance the functional and nutritional properties of foodstuffs and their shelf life and safety from a microbiological point of view. In this study, fresh *A. platensis* biomass was fermented for 72 hours by adding *Lactobacillus plantarum* culture suspension. Non-fermented and fermented *Spirulina* broth were analyzed for *L. plantarum* growth, and the concentration of lactic acid was determined at $t = 0$, 24, 48 and 72 hours of fermentation. In addition, nutritional composition analysis (crude protein, crude fat, total mineral, soluble and insoluble dietary fiber, and available carbohydrate content) was performed before and after fermentation. Analyses of total phenolic content and *in vitro* antioxidant activity were performed on water and ethanol extracts of fermented and non-fermented *Spirulina* broth. Finally, cellular antioxidant activity and lipid peroxidation were performed on yeast (*S. cerevisiae*) treated with water and ethanol extract of fermented and non-fermented *Spirulina* broth.

Spirulina broth fermentation resulted in an increased level of non-protein nitrogen, which indicates a higher protein bioavailability. Fat content, however, decreased, while the content of other nutrients remained the same. Notably, the study shows that *in vitro* and intracellular antioxidant activity of ethanol extracts of fermented *Spirulina* broth are higher than when the non-fermented broth is used. Furthermore, cells exposed to fermented broth ethanol extract showed less lipid damage caused by oxidation, indicating its protective role from oxidative stress. No pathogenic bacteria were detected in the fermented broth, which had a lower pH than non-fermented broth, indicating an improved shelf life. According to our results, fermented broth has the potential as a dietary supplement or a food ingredient.

A part of this research was presented from the 26th to the 31st of August 2018 at the XXII International Mass Spectrometry Conference, which was held in Florence, Italy and at the 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality, 1st to 3rd of April 2019, in Portorož, Slovenia, as a scientific conference poster presentation. Additionally, an oral presentation was given at the 13th Jožef Stefan International Postgraduate School Students' Conference and the 15th Young Researchers' Day of Chemistry, Material Science, Biochemistry and Environment on the 27th and 28th of May 2021 (online) and at the 2nd ISO-FOOD Symposium: ISO-FOOD From Food Source to Health, in Portorož, Slovenia, 24th – 26th April, 2023.

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Article

Fermented Biomass of *Arthrospira platensis* as a Potential Food Ingredient

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Abstract: Lactic acid fermentation (LAF) is known to improve nutritional properties and functionality and to extend the shelf life of foods. We studied the LAF of *Arthrospira platensis* as the sole substrate using *Lactobacillus plantarum* as the starter culture. Fermented (FB) and non-fermented broth (NFB) were analysed by means of pH, lactic acid bacteria (LAB) count, lactic acid concentration, microbiological safety, and nutritional composition. Additionally, water and ethanol extracts were prepared on which total phenolic content, DPPH radical scavenging activity, and cellular antioxidant activity were determined. The maximum increase in LAB count and lactic acid concentration and drop in pH was observed in the first 24 h of fermentation. Total phenolic content and DPPH radical scavenging activity of ethanol extracts increased after fermentation compared with NFB. Ethanol extracts of FB have been shown as a potential source of antioxidants, which efficiently lowered oxidation level in the cells of yeast *Saccharomyces cerevisiae*, as well as the oxidative damage of lipids. Additionally, the level of non-protein nitrogen increased, indicating higher protein bioavailability, and fat content decreased in comparison with NFB. No presence of pathogenic bacteria and low pH indicate enhancement of FB microbiological stability. Therefore, inclusion of fermented *A. platensis* into food products could lead to added-value foods based on microalgae.

Keywords: *Arthrospira platensis*; lactic acid fermentation; *Lactobacillus plantarum*; antioxidant activity; microbiological safety; nutritional composition

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1. Introduction

Lactic acid fermentation is known to improve the nutritional properties and functional value of food substrates and to enhance their shelf life and microbiological safety, as well as enhancing their sensory characteristics [1]. Both, the profile and amount of bioactive compounds is changed. Some molecules (bioactive peptides, polysaccharides, short-chain fatty acids) are generated, while antinutritional compounds and sugar content are decreased. Additionally, molecules with added biological value are generated after conversion of phenolic compounds. These transformations lead to improvements in the bioaccessibility and bioavailability of the food components, which is related to modification of their health-related properties [2]. As *Arthrospira platensis* (*Spirulina*) contains many functional bioactive constituents (e.g., long-chain polyunsaturated fatty acids, phenolic compounds, sterols, proteins, peptides, amino acids, vitamins, polysaccharides, pigments) with different activities [3], lactic acid fermentation might allow the preparation of food products based on microalgae with better nutritional and functional characteristics compared with the original non-fermented microalgal biomass. Uchida and Meyoshi [4] have already reported on lactic acid fermentation of microalgae (i.e., *Chlorella* spp., *Tetraselmis* spp., *Pavlova lutheri*, *Chaetoceros* spp., *Nannochloropsis* spp.) as well as macroalgae (i.e., seaweeds), and their studies have opened up the possibility of producing fermented foods from algae. A few studies have investigated *Spirulina* as a sole substrate for lactic acid fermentation. Niccolai et al. [5] investigated the use of lactic acid fermentation of *Spirulina* biomass as the sole substrate for the production of probiotic-based products. Their data showed that after 48 h of fermentation the concentration of *Lactobacillus plantarum* and lactic acid increased significantly. Additionally, the antioxidant activities *in vitro*, the total phenolic content, and digestibility increased after fermentation. Similarly, de Marco Castro et al. [6] established that the total phenolic content and antioxidant activity in vitro enhanced in fermented spirulina compared with untreated biomass. Additionally, protein fragmentation and free methionine content increased linearly with the fermentation time. Yu et al. [7] used three kinds of probiotic combinations (lactic acid bacteria, *Bacillus* strains, and their mixture) and showed different effects on *Spirulina* fermentation, in which the lactic acid bacteria and *Bacillus* strains showed positive effects in the context of flavour, nutrition, or bioactivity. Enhanced total antioxidant capacity and beta-carotene profile of *A. maxima* fermented by *L. plantarum* was thought to contribute to the apparent higher brain-derived neuroprotective factor compared with its untreated control [8]. Lactic acid fermentation of *Spirulina* was investigated also in terms of its use for cosmetic products, and it was shown that antioxidant, anti-inflammatory, and UV protective activities of fermented *Spirulina* increased compared with native *Spirulina* [9]. Higher levels of free polyphenols and phycocyanobilin were detected in fermented compared with non-fermented spirulina.

L. plantarum is extensively used as a starter culture as well as a probiotic microorganism in the food industry. The long history of *L. plantarum* strains application in food fermentation led to the design of added-value foods with improved nutritional and technological features [10].

To the best of our knowledge, no studies were found to compare *A. platensis* biomass as a sole substrate before and after fermentation with *L. plantarum* in the context of whole nutritional composition and energy value, microbiological safety, antioxidative activity in the cells, and lipid peroxidation. Additionally, two different extraction solvents were used to distinguish between polar and less polar antioxidants and to study the effect of lactic acid fermentation on their activity.

2. Materials and Methods

2.1. *Lactobacillus plantarum Inoculum Preparation*

Lactobacillus plantarum (LMG 6907) was obtained from the Institute of Dairy Science and Probiotics, Department of Animal Science, Biotechnical Faculty. A stock culture in 20% (*v/v*) glycerol was transferred into 20 mL of MRS broth (Merck, Darmstadt, Germany). Then overnight cultivation was performed on a rotary shaker (30 °C, 150 rpm). The overnight culture was centrifuged at 14,000× *g* 5 min and washed once with the physiological solution (0.9% (*w/v*) NaCl) to prepare the suspension for inoculation.

2.2. *Arthrospira platensis Cultivation*

Fresh *Arthrospira platensis* biomass was obtained from Severino Becagli algae farm (Grosseto, Italy) in collaboration with AlgEn, where it was cultivated in 500 m^2 ponds under controlled conditions (pH 10.6). Constant mixing was ensured by a paddle wheel. Greenhouses with nets prevented the access of insects to the ponds. The quality of the *A. platensis* was ensured by high-tech processes with critical control points in all phases of production.

2.3. Lactic Acid Fermentation

An amount of 10 g of fresh *A. platensis* biomass was mixed with 10 mL of physiological solution to get a broth (non-fermented broth–NFB) sampled immediately after preparation. NFB was inoculated with *L. plantarum* suspension (1% (*v/v*) inoculum), and the fermentation was carried out at 30 °C for 72 h. Samples of fermented broth (FB) were taken at t = 0 (immediately after inoculation), 24, 48, and 72 h. Samples were frozen for further analyses, except for microbiological analysis and *L. plantarum* growth determination, where samples were analysed immediately after sampling.

2.4. L. plantarum Growth Determination

Non-fermented and fermented (t = 0, 24, 48, and 72 h) broth was diluted according to Koch and appropriate dilutions were transferred on MRS agar (Merck, Darmstadt, Germany) containing cycloheximide (Sigma-Aldrich, St. Louis, MO, USA) in a concentration of 100 mg/L. Plates with inoculated MRSc agar were incubated in an anaerobic jar at 30 °C, 48 h. After incubation, the number of colonies was counted, and results are expressed as logarithm of the number of colony-forming units (CFU) per g of broth-log CFU/g.

2.5. Determination of Lactic Acid Concentration

Samples were collected from NFB and FB (t = 0, 24, 48, 72 h), diluted with water (1:1), and centrifuged at 4000× *g* for 15 min at 4 °C. The supernatant was then incubated at 90 °C for 10 min to denature proteins, centrifuged at 12,000× *g* for 10 min, and then used for lactic acid determination according to the method of Borshchevskaya et al. [11]. An amount of 25 µL of supernatant was mixed with 1 mL of 0.2% (*w/v*) $FeCl_3$ ($FeCl_3$, Sigma-Aldrich, St. Louis, MO, USA), and then absorbance at 390 nm was measured. The concentration of lactic acid was obtained from a calibration curve using lactic acid as standard (Sigma-Aldrich, St. Louis, MO, USA) and expressed as g lactic acid/L. Additionally, at each time point pH value was measured.

2.6. Determination of Nutritional Composition

The chemical composition of microalgae biomass before and after lactic acid fermentation was performed to determine the key changes in certain macronutrient contents arising from the fermentation. Total and non-protein nitrogen were determined by the Kjeldahl method (AOAC 981.10) [12]. The calculated difference on behalf of proteinic nitrogen was multiplied by a general conversion factor 6.25 to evaluate the amount of crude protein. Crude fat content was determined by the Weibull–Stoldt method (AOAC 963.15), total mineral content by dry ashing at 550 °C (AOAC 920.181), and soluble and insoluble fraction of dietary fibre by the enzymatic–gravimetric method (AOAC 941.43) [12]. The available carbohydrate content was calculated as the difference between the dry mass, and the content of analysed nutrients and ash. Nutritional value was calculated using energy factors: 17 kJ/g for crude protein and available carbohydrate; 37 kJ/g for crude fat; and 8 kJ/g for total dietary fibre [12]. All results are reported per 100 g of broth dry weight.

2.7. Microbiological Analysis

The microbiological quality and safety of NFB and FB after 24 h of fermentation was determined by microbiological analysis (yeast and moulds (YM), aerobic mesophilic bacteria (AMB), anaerobic mesophilic bacteria (ANMB), aerobic spore-forming bacteria (ASFB), anaerobic spore-forming bacteria (ANSFB), lactic acid bacteria (LAB), coliform bacteria (CC), *Escherichia coli, Staphylococcus aureus*, *Bacillus cereus*, *Clostridium perfringens*, *Salmonella* spp., and *Listeria monocytogenes*). Samples were prepared for quantitative microbiological analysis by addition 10 g of broth to 90 mL of physiological solution (0.9% (*w/v*) NaCl) and homogenized (Stomacher, 1 min at medium speed). The number of yeast and moulds and the number of bacteria were determined with the plate count method with appropriate medium and incubation conditions. Media were dichloran rose-bengal chloramphenicol agar (Oxoid CM, Hampshire, UK) with chloramphenicol supplement (Oxoid, SR0078, Basingstoke, UK) for YM; plate count agar (Oxoid, Basingstoke, UK) for AMB and ANMB; thioglycollate agar (Oxoid, Basingstoke, UK) with 20 g/L of agar for ASFB and ANSFB; De Man, Rogosa, Sharpe medium (Oxoid, Basingstoke, UK) with cycloheximide (Sigma-Aldrich, St. Louis, MO, USA, 100 mg/L) for LAB; violet red bile lactose agar (Oxoid, Basingstoke, UK) for CC; tryptone bile x-glucuronide medium (Oxoid, Basingstoke, UK) for *E. coli*; Baird–Parker medium (Oxoid, Basingstoke, UK) with Egg Yolk Tellurite Emulsion (Oxoid) for *Staph. aureus*; *Bacillus cereus* agar (Oxoid, Basingstoke, UK) with polymyxin B supplement (Oxoid, SR0099) for *B. cereus*; Sulfite polymyxin sulfadizine agar (Merck, Darmstadt, Germany) for *Cl. perfringens*. *Salmonella* spp. and *Listeria monocytogenes* were analysed in 10 g of broth (NFB and FB) after non-selective enrichment of sample (10 g) in Universal pre-enrichment broth (UPB, Oxoid, Basingstoke, UK) and 24 h incubation of suspension at 37 °C, following isolation on Rambach agar (Merck) for *Salmonella* spp. and on ALOA medium (Oxoid, Basingstoke, UK) with Chromogenic *Listeria* Selective Supplement (Oxoid, SR0226, Basingstoke, UK) and ISO Differential Supplement (Oxoid, SR0244) for *L. monocytogenes*. For *L. monocytogenes,* Fraser broth (FB, Oxoid, Basingstoke, UK) with selective supplement (Oxoid, SR0156, Basingstoke, UK) as the second enrichment was used as well (0.1 mL of UPB was transferred to 10 mLFB, incubated 24–48 h), followed by isolation on ALOA medium. Agar plates for bacteria were incubated at 37 °C for 24–48 h, and agar plates for yeast and moulds were incubated at 25 °C for 5–7 days. For ANMB, ANSFB, and LAB agar plates were incubated under anaerobic conditions. After incubation, the number of typical colonies was counted, and results are expressed as logarithm of the average number of CFU per g of NFB or FB (log CFU/g).

2.8. Preparation of NFB and FB Extracts for Antioxidant Activity Determination

To determine the total phenolic content and antioxidant activity of *A. platensis* biomass before and after fermentation, extracts from NFB and FB were prepared using two different solvents: water and 96% ethanol.

To obtain higher yields, a two-stage extraction was performed. For the first stage, 8 g of NFB or FB was weighted into 50 mL centrifuge tubes, to which 12 mL of extraction solvent (water or 96% ethanol) was added. The extraction was performed for half an hour in a water bath (40 °C) with shaking. Afterwards, the samples were centrifuged for 10 min (6000 rpm), and the supernatant was collected. For the second stage, the remaining sediment was extracted with another 12 mL of each solvent following the same procedure. Both supernatants were joined to obtain the final extract, which was stored in a freezer at −20 °C until being analysed (determination of total phenolic content and antioxidant activity).

Concentrated extracts were used to determine antioxidant activity in the cells. The water extracts were freeze-dried, while the ethanol extracts were first evaporated and then freeze-dried in order to obtain dry extracts. The mass yields of water extracts were 11.5% (NFB) and 7.6% (FB), whereas for the ethanol extracts they were lower, 4.3% (NFB) and 5.6% (FB). The dry extracts were then dissolved in water for water extracts or DMSO (Sigma-Aldrich, St. Louis, MO, USA) for ethanol extracts in order to obtain concentrated extracts with a concentration of dry extract equal to 50 mg/mL (water extracts) and 100 mg/mL (ethanol extracts).

2.9. Total Phenolic Content

To determine total phenolic content Folin–Ciocalteu reagent was used followed by a spectrophotometric quantification [13,14]. Briefly, the reaction mixtures for calibration curves (for each solvent individually) were prepared with 25 to 200 µL of the standard solution of gallic acid (0.45 mM in water or 96% ethanol) and an adequate amount of the corresponding solvent to obtain 725 µL. To this, 125 µL of Folin–Ciocalteu reagent (freshly diluted in water; 1:2) was added, followed by the addition of 125 µL 20% Na_2CO_3 (in water) after exactly 5 min. After mixing, the samples were kept in the dark at ambient temperature for 60 min to finish the reaction, followed by absorbance measurement against a blank sample (water or 96% ethanol) at 765 nm. To determine the total phenolic content in extracts, 10 µL of water extracts and 40 µL of ethanol extracts were analysed the same way. The results are expressed as equivalent of gallic acid (in mg) per dry weight of broth (in g). Gallic acid and Na_2CO_3 were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.10. Antioxidant Activity In Vitro

Antioxidant activity in vitro was evaluated using the $DPPH^{\bullet}$ radical scavenging method [15]. Briefly, calibration curves for each solvent (water and 96% ethanol) were prepared as follows: 5 to 50 µL of Trolox standard solution (1.11 mM in water or 96% ethanol) and an adequate amount of the corresponding solvent to obtain 50 µL was mixed with freshly prepared 0.11 mM $DPPH^{\bullet}$ (in methanol). The absorbance of the reaction mixture was measured after one hour at 550 nm instead of the usually used wavelength 517 nm to avoid the interferences in the coloured extracts. To determine antioxidant activity in extracts, 35 µL of water extracts and 50 µL of ethanol extracts were analysed the same way. The results are expressed as Trolox equivalent antioxidant capacity (TEAC) in mg per dry weight of broth (in g). Trolox and $DPPH^{\bullet}$ were from Sigma-Aldrich, St. Louis, MO, USA.

2.11. Cellular Antioxidant Activity (CAA) Assay

Cellular antioxidant activity was evaluated by measuring intracellular oxidation in the yeast *Saccharomyces cerevisiae* as a model organism [16]. The yeast *S. cerevisiae* was provided from the Culture Collection of Industrial Microorganisms (Biotechnical Faculty, Slovenia). It was grown in YEPD medium (Sigma-Aldrich, St. Louis, MO, USA) at 28 °C and 220 rpm until the stationary phase, then the cells were washed and suspended in PBS buffer [17]. Yeast cells were treated with water and ethanol extracts of NFB and FB in a concentration of 1.5 mg and 3 mg dry extract/mL of yeast suspension for 2 h. Yeast cells treated with the same volume of solvent used were considered as controls. After treatment, the 2 mL of cell suspension were centrifuged (14,000× *g*, 5 min) and washed twice with 50 mM potassium phosphate buffer (pH 7.8). The cell pellets were resuspended in 0.99 mL of 50 mM potassium phosphate buffer. After a 5 min incubation at 28 °C, H_2DCF diacetate (Sigma-Aldrich, St. Louis, MO, USA) was added to reach a final concentration of 10 µM and incubated for 30 min at 28 °C and 220 rpm. Then the fluorescence of the yeast suspension was measured by Safire II microplate reader (Tecan, Männedorf, Switzerland). The excitation wavelength of DCF was 488 nm, emission wavelength was 520 nm. The optical density of yeast suspension was measured at 650 nm.

Results are expressed as relative values of fluorescence/optical density to the corresponding control (yeast cells treated with water or DMSO).

2.12. Lipid Peroxidation

Yeast cells in the stationary phase were suspended in PBS buffer as described in Section 2.11. Cells were first exposed to ethanol extract of FB in the concentration of 1.5 mg dry extract/mL of yeast suspension for 2 h and then to 100 mM menadione for 1 h as an oxidative stress inductor. Control cells were exposed only to menadione (Sigma-Aldrich, St. Louis, MO, USA) without previous treatment with extracts. Lipid peroxidation was quantified by the determination of thiobarbituric acid (TBA)-reactive substances (TBARS) [18]. In both cases, cells were centrifuged (4000× *g*, 5 min) and washed once with PBS. To the sediment, a reagent containing 91.8 mM trichloroacetic acid (TCA, Merck, Darmstadt, Germany), 2.5 mM thiobarbituric acid (TBA, Merck, Darmstadt, Germany), 45.4 µM butylhydroxytoluene (BHT, Merck, Darmstadt, Germany), and 25 mM HCl (HCl, Merck, Darmstadt, Germany) was added. The cells were then disrupted by vortexing with 0.5 mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK, USA), twice for 4 min each time using a homogenizer (Bullet Blender Storm 24, Next Advance, Troy, New York, NY, USA) with 5 min interval for cooling the samples on ice. The cell homogenates were centrifuged at 13,000× *g* for 10 min. Supernatants were incubated at 90 °C (Thermomixer R, Eppendorf, Hamburg, Germany) for 30 min, and after cooling, 1-butanol was added. After centrifugation at 13,000× *g* for 10 min, 200 uL of upper butanolic phase was removed, and the fluorescence was measured, using a Varioskan™ LUX (Thermo Fisher, Waltham, MA, USA) microplate reader. The excitation and emission wavelengths were 515 nm and 555 nm, respectively. To normalize the fluorescence values, the optical density of the yeast suspension was measured at 650 nm. Results are expressed as relative values of fluorescence/optical density (F/OD) to the corresponding control.

2.13. Statistical Analysis

Fermentation of *A. platensis* biomass and consequently all analyses were conducted in at least three replicates. Data are expressed as means ± SD (standard deviation). Differences between non-fermented and fermented broths or treated and non-treated cells (cellular assays) were determined using the Student t-test and were considered statistically significant when $p < 0.05$.

3. Results

3.1. Lactic Acid Fermentation

Figure 1 presents lactic acid fermentation of *A. platensis* broth inoculated with *L. plantarum*, where pH, lactic acid (LA), and LAB growth were followed.

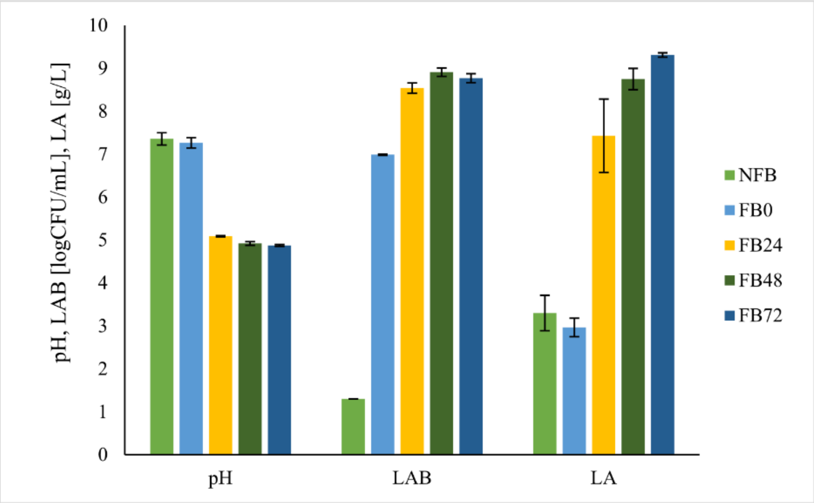

Figure 1. Determination of pH, LAB concentration (LAB), and lactic acid concentration (LA) in NFB and FB. Data represent mean values ± SD.

The greatest changes were observed in the first 24 h. pH decreased from 7.3 to 5.1 and remained constant in the next 48 h. The growth of *L. plantarum* was the most rapid in the first 24 h. At inoculation (t = 0), the cultivability was 7.0 log CFU/g and increased to 8.5 log CFU/g after 24 h; later values remained the same. Lactic acid reached a concentration of 9.3 g/L at 72 h; again, the rapid increase was observed in the first 24 h, where values reached 7.4 g/L.

3.2. Microbiological Quality and Safety

Microbiological quality of non-fermented and fermented broth is presented in Figure 2 and Table 1, with quantitative and qualitative results of different microorganisms in NFB and FB. NFB contained less than 4 log CFU/g of aerobic mesophilic bacteria (AMB) and also a very small number of anaerobic mesophilic bacteria (ANMB) and lactic acid bacteria, but all spore-forming bacteria were below 1–2 log CFU/g (Figure 2). After 24 h of lactic acid fermentation of spirulina broth, the number of mesophilic bacteria and spore-forming bacteria (aerobic and anaerobic) increased for an average of 3–5 log CFU/g. An increased number of lactic acid bacteria is expected, as *L. plantarum* suspension was added for fermentation.

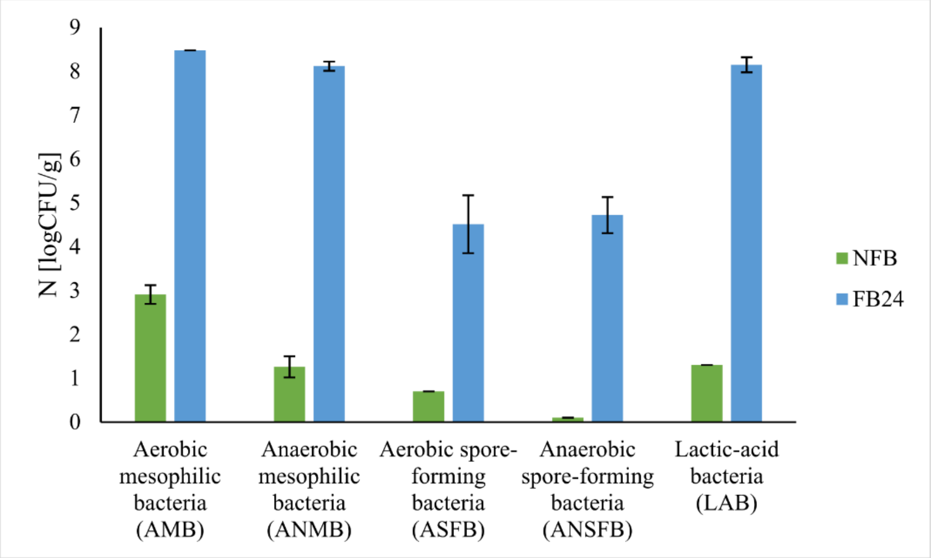

Figure 2. The average number (N [logCFU/g]) of different groups of microorganisms in NFB and FB ± SD.

Table 1. Pathogenic bacteria, yeast, and moulds in non-fermented (NFB) and fermented *A. platensis* broth (FB).

Microorganisms	N (CFU/g) in NFB	N (CFU/g) in FB
Coliform bacteria	<10	<10
Escherichia coli	<100	<100
Staphylococcus aureus	<100	<100
Bacillus cereus	<100	<100
Clostridium perfringens	<100	<100
Yeast	<10	<10
Moulds	<10	<10
Salmonella spp. *	Neg. in 10 g	Neg. in 10 g
L. monocytogenes *	Neg. in 10 g	Neg. in 10 g

Legend: N, average number [CFU/g], *, qualitative analysis.

Results of microbiological examination of *A. platensis* samples prior to and after lactic acid fermentation showed that samples did not contain pathogenic bacteria (Table 1). Yeast and moulds were found neither in NFB nor in FB after 24 h fermentation.

3.3. Nutritional Composition

Lactic acid fermentation affected the nutritional value of the microalgal biomass. Proteins are the most abundant nutrient. While there was no significant difference between the average contents of crude proteins in NFB and FB, a significant change could be observed in the ratio between non-protein nitrogen and total nitrogen in favour of non-protein nitrogen content after the fermentation. Significant differences may also be seen in the amount of crude fat, which was lower in the FB. The content of some components also changed after fermentation: lower contents of insoluble fibres and available carbohydrates and higher content of soluble dietary fibres and crude proteins were determined in FB; however, those differences from NFB were not statistically significant (Table 2). Calculated energy values of FB (1390 kJ/100 g DW) and NFB (1425 kJ/100 g DW) were similar.

Table 2. Nutritional composition of non-fermented (NFB) and fermented *A. platensis* broth (FB). Values are expressed as percentage of dry weight. Data represent mean values ± SD.

Component	NFB	FB
Crude protein	46.56 ± 2.43	47.37 ± 1.49
Total ash	12.65 ± 0.34	12.78 ± 0.08
Crude fat	**6.26 ± 0.04**	**6.00 ± 0.01**
Soluble dietary fibres	3.20 ± 0.46	4.02 ± 0.69
Insoluble dietary fibres	19.33 ± 0.70	17.79 ± 0.94
Total dietary fibres	22.53 ± 0.38	21.81 ± 0.72
Available carbohydrates	13.00 ± 0.74	11.05 ± 1.94
Non protein nitrogen/total nitrogen	**24.8% ± 1.5%**	**28.4% ± 1.1%**

Legend: Statistically significantly different values between FB and NFB are written in bold ($p < 0.05$).

3.4. Total Phenolic Content

Total phenolic content (TPC) of water extracts was in general higher compared with ethanol extracts in FB, as well as in NFB. After fermentation a 33% decrease in TPC was observed in water extracts, while ethanol extracts showed higher content (a 45% increase) compared with NFB (Figure 3).

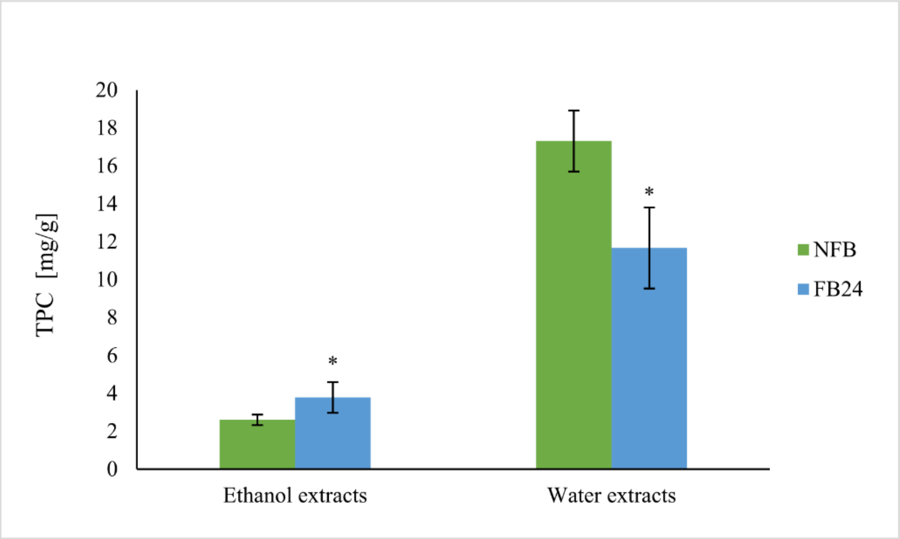

Figure 3. TPC of water and ethanol extracts of NFB and FB. Data represent mean values ± SD. Asterisk (*) indicates statistically significant difference between FB and NFB for particular extract ($p < 0.05$).

3.5. Antioxidant Activity

3.5.1. In Vitro

It is shown (Figure 4) that water extracts had higher ability to scavenge DPPH$^{\bullet}$ radicals than ethanol extracts. However, with 24 h fermentation, a 35% decrease in TEAC for water extracts was observed. In the case of ethanol extracts TEAC increased from 3.7 mg/g before fermentation to 5.3 mg/g after fermentation (a 30% increase).

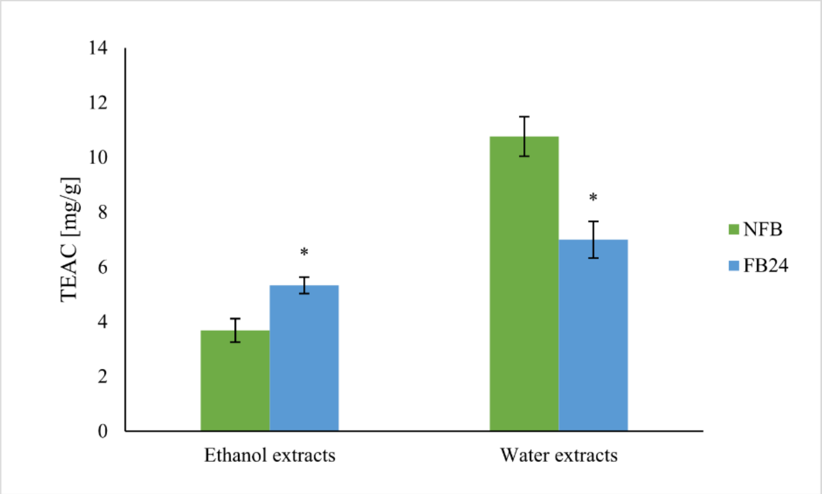

Figure 4. Trolox equivalent antioxidant activity (TEAC) of water and ethanol extracts of non-fermented broth (NFB) and fermented *A. platensis* broth (FB). Data represent mean values ± SD. Asterisk (*) indicates statistically significant difference between FB and NFB for particular extract ($p < 0.05$).

3.5.2. Cellular Antioxidant Activity

Cellular antioxidant activity was determined using yeast *Saccharomyces cerevisiae* as a model organism. Yeast cells were exposed to water and ethanol extracts of NFB and FB in concentration of 1.5 mg and 3 mg dry extract/mL of yeast suspension. Results are shown in Figure 5.

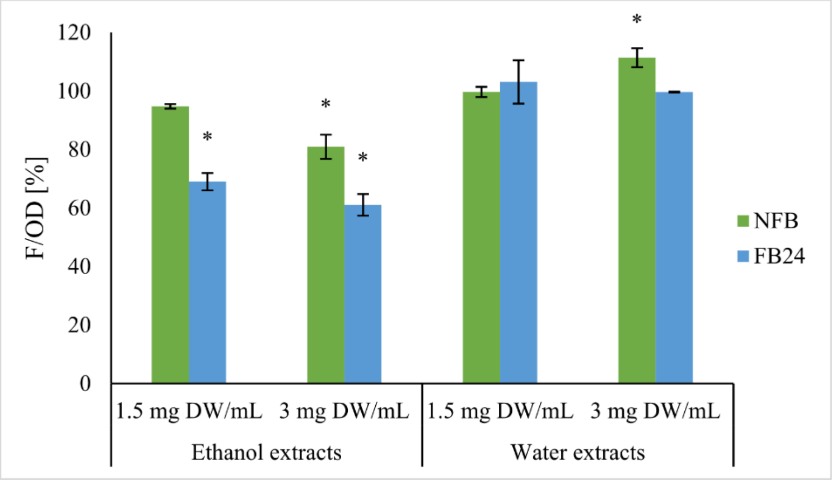

Figure 5. Determination of intracellular oxidation in yeast *Saccharomyces cerevisiae* exposed to water and ethanol extracts of NFB and FB in concentration of 1.5 and 3.0 mg DW/mL. Data represent mean relative values F/OD to the corresponding control set as 100%. Asterisk (*) indicates statistically significant difference between yeast cells treated with NFB or FB extract and control cells (where water or DMSO was added) for particular extract and concentration ($p < 0.05$).

No changes in intracellular oxidation level were observed when cells were treated with NFB, as well as FB water extracts at a lower concentration, while at a higher concentration a slight increase in oxidation level was observed for NFB water extract.

In contrast, a 20% and 40% decrease in intracellular oxidation was observed when cells were treated with NFB and FB ethanol extracts (3 mg DW/mL), respectively. Similarly, a lower concentration of FB ethanol extracts caused a decrease in cell oxidation level (30%), while no significant changes were observed when cells were exposed to NFB ethanol extract.

3.6. Lipid Peroxidation

The cells exposed to FB ethanol extract in a concentration of 1.5 mg DW/mL showed a reduced level of oxidation compared with the control, and lipid peroxidation was further investigated (Table 3). Results show that previous exposure of cells to FB extract decreased the level of oxidative damages caused by the treatment of the cells with menadione.

Table 3. Determination of lipid peroxidation. Data represent mean values of F/OD ± SD. Asterisk (*) indicates a statistically significant difference between yeast cells treated with FB extract followed by menadione and cells treated only with menadione ($p < 0.05$).

Condition	F/OD
Yeast cells	3.37 ± 0.54
Yeast cells + menadione (1 h)	13.8 ± 0.63
Yeast cells + ethanol extract of FB (2 h) + menadione (1 h)	5.71 ± 1.85 *

4. Discussion

Lactic acid fermentation of *A. platensis* has already been shown to improve its functional value, including antioxidant activity [5,6]. Thus, the aim of our work was to further evaluate the potential of fermented *A. platensis* biomass to be a component of food products or supplements.

The fermented biomass was investigated in the context of quality, safety, and bioactivity and compared with non-fermented biomass. Thus, determination of nutritional composition and microbiological characterization, as well as antioxidant activity assay, were performed.

Looking at lactic acid fermentation, the most significant changes regarding pH, concentration of lactic acid, and *L. plantarum* growth occurred during the first 24 h of fermentation at 30 °C with *L. plantarum* concentration of 8.5 log CFU/g, lactic acid concentration of 7.4 g/L, and pH value of 5. Niccolai et al. [5] showed that lyophilised *A. platensis* biomass enabled *L. plantarum* growth with a maximal bacterial concentration of 10.6 log CFU/mL at 48 h. Similarly, maximal concentration of lactic acid concentration (3.67 g/L) was reached after 48 h of fermentation at 37 °C and was 2-fold lower compared with our study at 24 h. Thus, in both cases, *A. platensis* biomass was shown to be a suitable substrate for *L. plantarum* growth and fermentation. *A. platensis* cell wall structure is similar to prokaryotic bacteria. It is composed of peptidoglycan. Lactic acid bacteria (LAB) during fermentation degrade cell walls of cyanobacteria by different peptidoglycan hydrolyses. This results in the release and degradation of complex organic molecules of the cells into simpler compounds [6,19], enabling their growth. On the other hand, the release of compounds and/or their metabolism by LAB can mean better functional value of fermented biomass compared with non-fermented. It is known that lactic acid fermentation of food substrates can improve the efficiency of their original bioactive compounds due to their release or transformation by LAB, and thus can enrich the substrate with their metabolites [2]. *A. platensis* has high nutritional value due to its content in proteins, essential amino acids, essential fatty acids, vitamins, minerals, and pigments [20,21], and thus we tested how lactic acid fermentation changed the nutritional composition of spirulina. The content of proteins, lipids, dietary fibres, carbohydrates, and non-protein nitrogen was determined.

Analysis showed that during lactic acid fermentation the substrate changed significantly in the content of non-protein nitrogen and crude fat. The non-protein nitrogen in microalgae derives from free amino acids, peptides, amines, amine oxides, and nucleotides [22]. The LAB proteolysis system combines the action of proteinases and peptidases, which efficiently break down proteins into small peptides and amino acids [23]. De Marco Castro et al. [6] found that peptides were released from proteins during lactic acid fermentation of *A. platensis* with *L. plantarum* and that free methionine content was increased as well. Due to protein hydrolysis during mixed fermentation of *A. platensis* by *L. plantarum* and *B. subtilis*, the polypeptide content was increased, proving that fermented *A. platensis* has greater protein bioavailability [23]. Similarly, Yu et al. [7] showed the increased content of amino acids and the ratio of essential amino acids to total amino acids in the fermented *Spirulina* compared with the non-fermented biomass. Additionally, the interest in protein hydrolysates has recently gained importance due to aiding digestive dysfunction and malnutrition [24]. The decrease in crude fat content found in our study is contrary to the findings of Dewi and Amalia [25], where the total fat content in *A. platensis* did not change during fermentation with *L. plantarum.* The discrepancies may be attributed to the different methods used for crude fat extraction and duration of fermentation, which was 24 h in our study, compared with 2–10 days [25]. On the other hand, crude fat contents decreased significantly in studies of bean flours fermentations with *L. plantarum*, where similar procedure for fat determination was used [26,27]. The content of insoluble dietary fibres tends to decrease with lactic acid fermentation. They cannot be digested or absorbed by humans and are insoluble in water. Organic acids and enzymes produced naturally by microorganisms decrease the molecular weight and thus improve their solubility, which may support our findings [28]. Bao et al. [23] found that lactic acid fermentation with *L. plantarum* did not affect the change of soluble polysaccharides in the microalga *A. platensis.* The concentration of polysaccharides is expected to strike a balance between the breakdown of polysaccharides and the formation of bacterial polysaccharides, which may explain the similar values of soluble and total dietary fibres in FB and NFB.

We further checked whether the change in nutritional composition was also reflected in antioxidant activity change. As can be seen from Figure 4, TEAC was higher in water compared with ethanol extracts. This means that hydrophilic antioxidants isolated from *A. platensis* have a higher ability to scavenge DPPH$^{\bullet}$ radical than less polar (ethanol soluble) antioxidants. However, with fermentation, the scavenging ability of water extracts decreased, while ethanol extracts showed higher antioxidant activity. Similar results were obtained for TPC, where the values in water extracts were again higher compared with the ethanol ones, and after fermentation, a decrease in TPC in water extracts and an increase in ethanol extracts were observed. Thus, these results indicate that polyphenols are very likely the most abundantly present antioxidants in *A. platensis*, although it is known that FC reagent used for TPC determination can react also with other nonphenolic compounds present in the extract besides polyphenols, which contribute to higher final content [29]. On the other hand, lactic acid fermentation was shown to be responsible for an increase in DPPH$^{\bullet}$ radical scavenging ability of polyphenols and/or others in 96% ethanol-soluble compounds. These results are in agreement with Curiel et al. [30], who showed that fermentation of medicinal plant myrtle (*Myrtus communis* L.) with *L. plantarum* caused an increase in the concentration of total phenols and antioxidant activity, mostly due to esterase activities of *L. plantarum*. In the presence of this enzyme, the hydrolysis of glycoside bond between a polyphenol and sugar moiety in glycosylated polyphenols occurs. It was already shown that polyphenol aglycones contain multiple hydroxyl groups and hence exhibit a higher antioxidant activity than their glycosides [31]. It was also shown that feruloyl esterases are present in *L. plantarum* strains and are responsible for metabolizing compounds that are abundantly present in fermented plant matrices (e.g., hydroxycinnamoyl esters) [32]. Thus, the increased amount of less polar aglycones (extracted by ethanol) in FB is very likely the reason for the increased antioxidant activity of ethanol extracts after fermentation.

Niccolai et al. [5] performed lactic acid fermentation of *Arthrospira* using *L. plantarum* and showed that after fermentation total phenolic content and antioxidant activity increased significantly (by 320% and 79%, respectively). Similarly, de Marco Castro et al. [6] established that the total phenolic content and antioxidant activity in vitro was enhanced in fermented spirulina compared with untreated biomass. In both cases, water or methanol was used as solvent for the preparation of extracts.

Previous studies [33–36] have already shown that it is not necessary that antioxidative activity is measured by chemical methods related to antioxidative activity in the cells (CAA). That is, CAA considers bioavailability, cellular uptake, distribution, and metabolism of compounds in the cell. Furthermore, at lower or higher concentrations of compounds, different mechanisms of antioxidative activity could be expressed. That is, antioxidants can directly react with free radicals or inhibit the activity or expression of enzymes related to free radical generation. On the other side, they can enhance the activity or expression of intracellular antioxidant enzymes [37]. We selected yeast *Saccharomyces cerevisiae* in the stationary phase as a model microorganism since such cells are physiologically closest to humans [38,39]. First, cell viability using the CFU method was determined to check any negative effects of extracts on yeast growth, and no changes in viability were observed when yeast cells were treated with water extracts, as well as being treated with ethanol extracts of fermented and non-fermented biomass at concentrations of 1.5 and 3 mg DW/mL (data not shown). Thus, these concentrations were further evaluated to measure antioxidant activity in the cells. In contrast with in vitro antioxidant assay, water extracts did not show a decrease in intracellular oxidation level compared with the control. No difference was observed between non-fermented and fermented broth extracts as well. Although NFB ethanol extracts significantly decreased intracellular oxidation level, an even greater decrease in intracellular oxidation was observed when FB ethanol extracts were used. Petelinc et al. [33] have similarly treated the yeast cells with propolis fractions of different polarities obtained using solid-phase extraction of crude propolis extract and eluted with 30–70% ethanol (EL30-EL70). Among them the greatest decrease in the intracellular oxidation level was observed in the cells treated with less polar EL70 eluate, followed by EL60 and EL50. On the other side, for EL30, they showed a trend of increased intracellular oxidation. Additionally, for the EL70, the cellular uptake of particular phenolic compounds was confirmed to the greatest extent, which might be responsible for the highest antioxidant activity in the cells.

In both cases, in vitro and cellular antioxidant activity of FB ethanol extracts was higher compared with NFB, indicating the role of lactic acid bacteria metabolism in the transformation of *A. platensis* compounds soluble in 96% ethanol and consequently higher bioactivity. Similarly, Li et al. [40] showed the enhancement of CAA of methanol extract of fermented apple juice compared with non-fermented and explained its enhancement as being due to the fact that bacterial metabolism, mainly deglycosylation and degallation activities of apple polyphenol compounds, releases free aglycones where higher number of hydroxyl groups or lower steric hindrance to hydroxyl groups can be found [41].

Further antioxidant activity of FB ethanol extract was confirmed by measuring lipid oxidative damages in yeast cells. Cells that were first exposed to FB ethanol extract and then to menadione as an oxidative stress inductor showed a lower level of oxidative lipid damage, indicating its protective role before oxidative stress compared with the cells exposed only to oxidative stress inductor. Using 96% ethanol as an extraction solvent, more non-polar compounds were extracted, whose further analyses are needed to determine their identity and also to establish which compounds have entered the cells and are responsible for such effect.

The results confirm the nutritional quality and antioxidant activity of fermented biomass, but microbiological safety is another important parameter that has to be evaluated before using it as a component of food products. It is known that lactic acid fermentation can enhance the shelf life of substrates and thus microbiological safety [1]. To evaluate the microbiological safety of fermented *A. platensis* biomass, analysis of different microorganisms was performed (Table 1, Figure 2). Results show that the number of mesophilic bacteria (aerobic, anaerobic) and spore-forming bacteria (aerobic and anaerobic), as well as lactic acid bacteria, increased in FB compared with NFB, which is expected since during fermentation nutrients become more available and bacteria that are already present on the substrate can grow. Further studies about *L. plantarum*'s ability to grow on plate count agar (PCA)—not just on MRS agar (results not shown), which is selective for lactic acid bacteria—explained a higher number of both total aerobic as well as total anaerobic mesophilic bacteria grown on PCA. Similarly, anaerobic spore-forming bacteria were identified in nutraceutical preparations of *A. platensis* for human consumption at a high number (10^5 CFU/tablet) [42]. Additionally, no presence of pathogenic bacteria, as well as yeasts and moulds in FB after 24 h fermentation, was detected, which is important if we use FB as a food ingredient or supplement. As contamination might occur also during harvest and post-harvest procedures, these results are important for obtaining microbiologically safe *A. platensis* biomass.

5. Conclusions

Fermented biomass of *A. platensis* has been shown as a potential source of antioxidants, which showed activity also in the cells, since reduced intracellular ROS level, as well as oxidative damages of lipids, was determined. Compared with non-fermented biomass, the level of non-protein nitrogen increased, indicating higher protein bioavailability, and fat content decreased, while the content of other nutrients remained the same. Additionally, fermented *A. platensis* showed no presence of pathogenic bacteria and has lower pH, indicating enhancement of its shelf life. Therefore, fermented *A. platensis* showed the potential to be used as a nutritional supplement or as an ingredient in food products.

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3.3 Scientific Paper: “Insight Into the Antioxidant Effect of Fermented and Non-Fermented *Spirulina* Water and Ethanol Extracts at the Proteome Level Using a Yeast Cell Model”

In this chapter, I present a paper with the title “Insight Into the Antioxidant Effect of Fermented and Non-Fermented *Spirulina* Water and Ethanol Extracts at the Proteome Level Using a Yeast Cell Model” by Jasmina Masten Rutar, Berta Cillero-Pastor, Ronny Mohren, Nataša Poklar Ulrih, Nives Ogrinc and Polona Jamnik, published in 2021 in Antioxidants (IF 7.675). My responsibilities included sample preparation and analysis, experimental design, statistical data analysis and interpretation of the results, and writing and preparing the manuscript for publication. This study aimed to evaluate the effect of *Spirulina* lactic acid fermentation and solvent choice for extraction of bioactive compounds, using a yeast cell model for *Spirulina* extract treatment application.

Fresh *Spirulina* samples were collected, lyophilized and then fermented using *L. plantarum* culture. Fermented and non-fermented *Spirulina* broth was extracted in water and ethanol. The *Spirulina* treatment was performed *in vivo* and assessed by analyzing the cell response at the proteome level using yeast (*S. cerevisiae*) as a model organism. Peptide separation and analysis were performed using a UHPLC coupled to a Q-Exactive HF Orbitrap mass spectrometer.

PCA analysis indicated the important effect of lactic acid fermentation and extraction solvent choice on yeast protein profile. A clear separation between the *Spirulina* water extract-treated cells and the *Spirulina* ethanol extract-treated cells and between non-fermented *Spirulina* and fermented *Spirulina* water or ethanol extract-treated cells was achieved. Differentiation between the water and ethanol extracts is due to the greater antioxidant efficiency of ethanol than water extracts. Furthermore, the essential role of fermentation in separating the yeast treated with fermented and non-fermented *Spirulina* extracts was evidenced by the lowering of the expression of the cell stress response-related proteins after fermentation. This points to yeast cells having a reduced need for inducing endogenous systems for maintaining homeostasis in the presence of exogenous antioxidants. Namely, a higher downregulation of proteins related to stress response was observed in yeast treated with fermented *Spirulina* ethanol extract compared to yeast treated with non-fermented *Spirulina* ethanol extract, while their abundance in yeast treated with fermented *Spirulina* water extract samples increased compared to yeast treated with non-fermented *Spirulina* water extract.

The results obtained in this research were presented as an oral presentation at the 13th Jožef Stefan International Postgraduate School Students' Conference and 15th Young Researchers' Day of Chemistry, Material Science, Biochemistry and Environment (CMBE day), which was held online on the 27th and 28th of May 2021 and at the 2nd ISO-FOOD Symposium: ISO-FOOD From Food Source to Health, in Portorož, Slovenia from 24th – 26th April 2023. The research work was also presented as a poster presentation at the XXII International Mass Spectrometry Conference, IMSC 2018 held from 26th to 31st August 2018, Florence, Italy, at the 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality in Portorož, Slovenia, April 1-3, 2019, and online, at the XXI EuroFoodChem conference, from 22nd to 24th November 2021.

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Insight into the Antioxidant Effect of Fermented and Non-Fermented *Spirulina* Water and Ethanol Extracts at the Proteome Level Using a Yeast Cell Model

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Abstract: *Spirulina* is rich in various antioxidants and nutraceuticals and it has proven to be effective in the treatment of various pathological conditions. This study explores the antioxidant effect of fermented and non-fermented *Spirulina* extracts on the proteome level using the yeast *Saccharomyces cerevisiae* as a model organism. Yeast cells were treated with fermented *Spirulina* water extract (SV), non-fermented *Spirulina* water extract (NFV), fermented *Spirulina* ethanol extract (SE), and non-fermented *Spirulina* ethanol extract (NFE). Cell lysates were prepared, and label-free quantitative proteome analysis was performed. In SV, when compared to NFV samples, the levels of most differentially expressed proteins were upregulated. Alternatively, SE compared to NFE samples showed a significant downregulation for the majority of the analyzed proteins involved in different cellular processes. Additionally, a higher downregulation of stress response related proteins was observed in SE compared to NFE samples, while their abundance in SV samples increased compared to NFV. This study provided a global view, on a proteome level, of how cells cope with exogenous antioxidants and remodel their cellular processes to maintain metabolic and redox balance. Furthermore, it combined for the first time the analysis of different extract effect, including the contribution of lactic acid fermentation to the cell activity.

Keywords: *Spirulina*; lactic acid fermentation; *Saccharomyces cerevisiae*; ethanol; proteome; antioxidant; stress response

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1. Introduction

Spirulina (*Arthrospira* spp.) is a *filamentous* cyanobacterium (blue-green algae) capable of photosynthesis. It is a very nutritious food containing various phytonutrients, antioxidants, nutraceuticals, and probiotics [1–3] and a good source of proteins (covering all essential and most non-essential amino acids) [4,5], vitamins, and minerals. It is especially rich in iron, gamma-linolenic fatty acid, B vitamins, and carotenoids. Due to its high content of nutrients, it has been proven experimentally to be effective in the treatment of many pathological conditions such as cardiovascular disease, hyperlipidemia, hepatotoxicity, and certain cancers, among others [6–9].

Fermentation of food products by lactic acid bacteria has been shown to improve the nutraceutical profile of foodstuffs. For example, lactic acid bacteria can degrade plant as well as cyanobacterial cell walls using enzymatic hydrolysis. This results in the production of molecules with high immunomodulatory, antioxidant, and anti-inflammatory biological activity by converting large organic compounds [10–12]. Lactic acid fermented *Spirulina* contains high amounts of polyphenols and phycocyanobilin and has higher protective activity, preventing cell damage by UVB radiation-induced oxidative stress. Furthermore, fermented *Spirulina* biomass has significantly higher antioxidant activity compared to non-fermented *Spirulina* biomass [13]. Studies have shown that after fermentation, the nutraceutical value of *Spirulina* evaluated through total phenolic and C-phycocyanin content, radical scavenging capacity, oxygen radical antioxidant capacity, protein fragmentation and ferric reducing antioxidant power, increases significantly, with the best results being achieved after 36 h of fermentation. After that time, the total antioxidant capacity diminishes, while the bioactive peptide, thermostable protein content and free methionine content increase, reaching a maximum at 72 h of active *Spirulina* biomass fermentation [10,14].

Different solvents (water, ethanol, methanol, DMSO, hexane, and petroleum ether) have been used to prepare *Spirulina* extracts for antioxidant, anticancer, antimicrobial, antiproliferative properties, protective effects against apoptotic cell death, and neuroprotective effects analysis due to differences in solubility of the various bioactive compounds [3,7,15–21]. For example, phycobiliproteins are soluble in water, whereas phenolic compounds and biologically active phytochemical substances such as sterols, tannins, flavonoids, reducing sugars, anthraquinone, chlorophyll-a, carotenoid pigments, and tocopherol have a higher solubility in alcohols. Ethanol and methanol extracts retain higher antiradical and antioxidant activity compared to water extracts, due to which it is possible to believe that alcohol-soluble components have the main antioxidant properties and are present in higher concentrations [15,16,22,23]. Conversely, *Spirulina* water extracts significantly reduce apoptotic cell death induced by free radicals [3].

Due to the feasible expression of proteins of any origin and easy RNA level manipulation, analysis of proteins and pathways is facilitated in yeast, whereas the same phenomena would be difficult to study in more complex organisms. Using yeast as a test organism also has many technical advantages over human cells. For instance, it has a fast life cycle and can grow in colonies on solid media or as dispersed cells in a liquid, and does not require expensive media [24]. Furthermore, apparent homologs can be identified in the human genome over the entire proteome, corresponding to 46% of the yeast proteome. Thus, yeast studies focused primarily on component parts can yield information that applies to the human counterparts [25], all of which makes yeast a model organism for researching basic human cellular processes, metabolic pathways, and cell stress response [26].

Our previous results have already shown the important role of lactic acid fermentation in enhancing the antioxidant effect of *Spirulina* when ethanol extracts were tested by measuring the intracellular oxidation level in the yeast *Saccharomyces cerevisiae*. Namely, a 19%- and 39%-decrease in intracellular oxidation level according to the control was observed when cells were treated with ethanol extracts of non-fermented and fermented *Spirulina*, respectively. In contrast, no changes in intracellular oxidation level were observed, when cells were exposed to water extracts of non-fermented as well as fermented *Spirulina* biomass. Additionally, total phenolic content (TPC) was determined, and it was higher in the water extracts of both non-fermented (17.31 mg equivalent of gallic acid/g DW) and fermented (11.67 mg eq. GA/g DW) *Spirulina* biomass compared to ethanol extracts. Thus, after fermentation a 33%-decrease of TPC was observed in water extracts, while ethanol extracts showed higher content (3.78 mg eq. GA/g DW) when compared to non-fermented *Spirulina* biomass extracts (2.60 mg eq. GA/g DW) [27]. In the present study, the difference between water and ethanol extracts and the role of lactic acid fermentation in the antioxidant activity of the extracts was further investigated at a proteome level using the same cell model organism. The yeast cells were in the stationary phase, which resemble cells of multicellular organisms in important aspects: (i) most of the energy comes from mitochondrial respiration, (ii) cells are in the G_0 phase, (iii) oxidative damages accumulate over time [28] and have the same defense mechanisms as higher eukaryotes [29,30].

The current study provides an in-depth insight into the lactic acid fermentation of *Spirulina* and solvent choice for bioactive compounds extraction by providing results of

Spirulina treatment effect on the proteome level of the yeast cells. To the best of our knowledge no studies have yet been published on the effect of *Spirulina* treatment on the yeast cell proteome level.

2. Materials and Methods

2.1. Lactic Acid Fermentation of Spirulina

Lactobacillus plantarum stock culture LMG 6907 (Institute of Dairy Science and Probiotics, Department of Animal Science, Biotechnical Faculty, Slovenia) in 20% glycerol (50 µL) was added to De Man, Rogosa, and Sharpe medium (20 mL) (MRS, Merck, Darmstadt, Germany) and incubated (150 rpm, 30 °C, overnight) on a rotary shaker until the late exponential phase was reached. The obtained broth was then centrifuged (14,000× g, 5 min) (2 mL) and washed twice with a physiological solution to obtain suspension for inoculation.

The freeze-dried *Spirulina* sample (2.47 g) was reconstituted to a total of 10 g by adding sterile ddH$_2$O. The reconstituted sample was then mixed with the physiological solution (10 mL), inoculated with the *L. plantarum* suspension (200 µL, 1% (v/v) inoculum) and allowed to ferment (30 °C, 24 h). The fermented samples were then stored at −20 °C.

2.2. Spirulina Extract Preparation

In order to determine the effect that *Spirulina* biomass has on yeast cells before and after fermentation, both the fermented and non-fermented *Spirulina* broth were extracted with water and ethanol (96%). Two-stage extraction was performed to obtain higher yields. In the first stage, the *Spirulina* broth (8 g) was mixed with 12 mL of the extraction solvent (water or ethanol) and placed in a water bath (40 °C, 30 min) with constant shaking. The samples were then centrifuged (6000 rpm, 10 min), after which the supernatant was collected. In the second stage, the sediment was again extracted with corresponding solvent (12 mL) by the same procedure and both supernatants were combined and stored at −20 °C.

The water extracts were then freeze-dried, while the ethanol extracts were evaporated under vacuum (GeneVac HT-4 Series II, Genevac Ltd., Ipswich, UK) and then freeze-dried. The extracts were then resuspended in water (for the water extracts) or DMSO (C_2H_6OS, Sigma Aldrich, Steinheim, Germany) (for the ethanol extracts) to obtain concentrated extracts, i.e., 50 mg dry extract/mL (water extracts) and 35 mg dry extract/mL (ethanol extracts).

2.3. Yeast Culture Preparation and Treating of Cells with Spirulina Extracts

The effect of *Spirulina* treatment in vivo was evaluated by analyzing the cell response on a proteome level using yeast *Saccharomyces cerevisiae* as a model organism. The yeast *Saccharomyces cerevisiae* was obtained from the Culture Collection of Industrial Microorganisms held by the Biotechnical Faculty of the University of Ljubljana (Slovenia). Yeast was cultivated in YEPD broth (Sigma Aldrich, St. Louis, MO, USA) (220 rpm, 28 °C) until the stationary phase was reached. The cells were then suspended in 50 mM potassium phosphate buffer (PBS, pH 7.8) [31].

Yeast cells were treated with water or ethanol extracts of fermented and non-fermented *Spirulina*, i.e., 3 mg dry water extract/mL or 2.1 mg dry ethanol extract/mL of yeast suspension. Controls were prepared by treating yeast cells using the same volume of each solvent. After treatment, the samples were incubated for 2 h (220 rpm, 28 °C). In this way, four sets of samples were prepared: yeast culture treated with (1) fermented *Spirulina* water extract (SV), (2) non-fermented *Spirulina* water extract (NFV), (3) fermented *Spirulina* ethanol extract (SE) and (4) non-fermented *Spirulina* ethanol extract (NFE), in addition to the water control sample (KV) and ethanol control sample (KE).

2.4. Preparation of Yeast Cell Lysates

The treated yeast culture broths were centrifuged (4000 rpm, 3 min). The supernatant was removed and the residual pellet was washed twice with 50 mM PBS and then frozen at −80 °C until extraction. Prior to extraction, the pellet was thawed, and ABC/urea buffer (300 µL) was added. Extraction was performed using three successive freeze-thaw cycles followed by two cycles of homogenization using zirconia/silica beads. The supernatant was then obtained by centrifugation (15,000× *g*, 4 °C, 30 min).

Protein concentration in the extracts was determined according to the method of Bradford [32] with bovine serum albumin (Sigma Aldrich, St. Louis, MO, USA) as the standard. Absorbance (595 nm) was measured using a Safire 2 microplate reader (Tecan, Männedorf, Switzerland) instrument, and the protein concentrations obtained from the standard calibration curve.

2.5. Protein Digestion to Peptides for LC-MS

Dithiothreitol (DTT, Sigma Aldrich, St. Louis, MO, USA) was used for reducing protein samples (20 mM, 45 min) and iodoacetamide (IAM, Sigma Aldrich, St. Louis, MO, USA) for alkylation (40 mM, 45 min). Alkylation was performed in the dark and the reaction was terminated using DTT (20 mM, 45 min). Protein digestion in thermoshaker followed (2 h, 37 °C, 750 rpm) with a freshly prepared enzyme mixture of Trypsin/Lys-C (Promega, San Luis Obispo, CA, USA). The enzyme mixture was added to the protein solution in a ratio of 1:25. Thereafter, the lysate was diluted with ABC buffer (50 mM) (Sigma Aldrich, St. Louis, MO, USA) to reach a Urea concentration of 1 M and further digested (overnight, 37 °C, 750 rpm). Formic acid, (FA, Biosolve, Valkenswaard, The Netherlands) was added at a final concentration of 1% to terminate the digestion.

2.6. LC-MS Analysis

Peptide separation was performed using an Ultimate 3000 Rapid Separation UHPLC system (Thermo Scientific, Dionex, Amsterdam, The Netherlands). The analytical column was a PepSep C18 (1.9 µm, 120 Å, ID 75 µm × 150 mm). Samples were desalted using an online C18 trapping column. Elution was performed using a linear gradient from 5% to 35% ACN with 0.1 FA in 90 min with a flow rate of 300 nL per minute. The UHPLC system coupled to a Q-Exactive HF Orbitrap mass spectrometer from Thermo Scientific was used for analysis. Data dependent acquisition was as follows: full MS scan from 250 to 1250 *m/z* at a resolution of 120,000. MS/MS scans of the top 15 most intense ions were followed at a resolution of 15,000.

2.7. MS Raw Data Analysis

Proteome Discoverer (PD) version 2.2 was used for protein identification and quantification by analyzing the data dependent acquisition spectra. The search engine Sequest was used in the PD software with the SwissProt database (SwissProt TaxID: 4932, Baker's yeast (*Saccharomyces cerevisiae*)). The following settings were used for the database search: trypsin as the enzyme, a maximum of 2 cleavages missed, 6 was the minimum peptide length, 10 ppm was the precursor mass tolerance, 0.02 Da was the fragment mass tolerance, modifications of methionine oxidation and protein N-terminus acetylation were dynamic, and modification of cysteine carbamidomethylation was static.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (1) partner repository with the dataset identifier PXD027102 (reviewer account details: username: reviewer_pxd027102@ebi.ac.uk, password: NaG26baj).

Default Label Free Quantitation (LFQ) settings in PD were used for protein quantitation. Briefly, peptide precursor intensities were used for peptide abundances, whereas the total peptide amount was used for normalization. For hypothesis testing, we used background-based ANOVA. The calculations of protein ratios were based on pairwise peptide ratios, and the protein differential abundancy is expressed in log2 fold change.

2.8. Bioinformatic Analysis of Data

Principal component analysis (PCA) and Volcano plot were prepared in Proteome Discoverer software using proteins (abundance) identified by PD with high confidence

(FDR < 5%) from two biological replicates. The UniProtKB database (https://www.uniprot.org/; 15 July 2021) was used to investigate the role of the proteins. Functional annotation analysis was performed using DAVID (https://david.ncifcrf.gov/; 8 July 2021) and QuickGO (https://www.ebi.ac.uk/QuickGO/; 8 July 2021) software and the Kyoto Encyclopedia of Genes and Genomes (KEGG) to integrate the GO categories (Biological Process, Molecular Function, Cellular Component). All identified protein sequences UniProt accession numbers, including identification and quantification data, are provided in the Supplementary Table S1.

3. Results and Discussion

3.1. Principal Component Analysis (PCA)

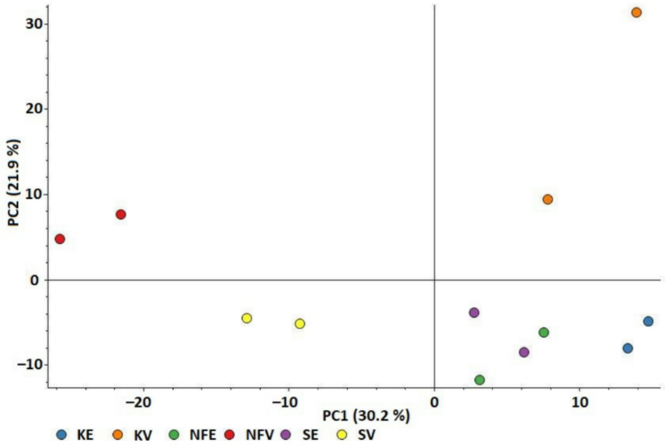
Principal component analysis (PCA) of the treated samples and controls was used to assess the similarity of proteome profiles (Figure 1) and demonstrate the relationship between yeast samples treated with fermented and non-fermented *Spirulina*, samples treated with water and ethanol extracts and between treated yeast samples and control samples. Two principal components explained the variance of the results. PCA showed the relationship between all samples: component 1 explained 30.2% and component 2 explained 21.9% (Figure 1a) and PCA showed samples without control samples: component 1 explained 39.1% and component 2 explained 18.6% (Figure 1b). The PCA plot shows a clear separation between the *Spirulina* water extract-treated cells and the *Spirulina* ethanol extract-treated cells (including control samples). In addition, a clear separation between the cells treated with *Spirulina* water extracts and water control sample and good separation between the cells treated with ethanol extracts and the ethanol control samples can be observed (Figure 1a). Furthermore, a clear separation between non-fermented *Spirulina*- and fermented *Spirulina* water extract-treated cells can be seen, whereas the separation between non-fermented *Spirulina*- and fermented *Spirulina* ethanol extract-treated cells is less apparent but still distinguishable. A very good separation of the *Spirulina* water extract-treated samples versus the *Spirulina* ethanol extract-treated samples was obtained (Figure 1b) regardless of whether non-fermented or fermented extracts were applied.

PCA analysis of samples indicated the important effect of extraction solvent and lactic acid fermentation on yeast protein profile. For this reason, we made a comparison of the quantitative protein profiles of cells treated with fermented and non-fermented *Spirulina* extracted using water and ethanol.

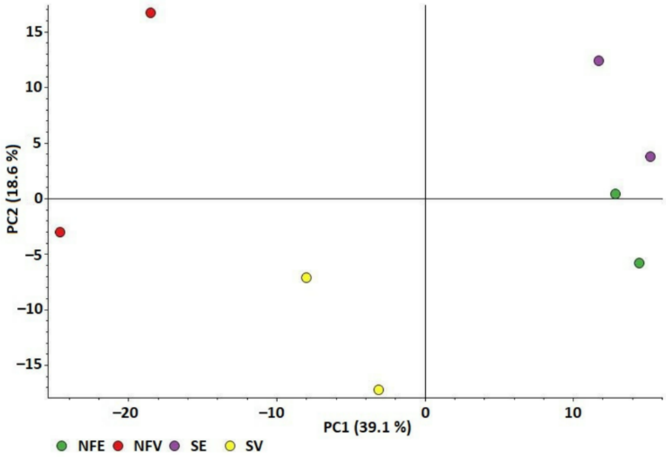
3.2. Quantification and Identification of Differentially Abundant Proteins

The quantitative proteomic analysis identified 641 proteins, of which 91 resulted as differentially abundant (p-value ≤ 0.05, log2 fold change $\geq |0.58|$) in SV compared to NFV samples. Among them, 71 proteins were upregulated and 20 proteins were downregulated (Figure 2a). Furthermore, 643 proteins were identified when comparing SE and NFE. Eighty proteins were differentially abundant (p-value ≤ 0.05, log2 fold change $\geq |0.58|$), of which 18 were upregulated and 62 were downregulated (Figure 2b). All identified proteins, including differentially abundant proteins, and data referring to PD identification and quantitation are presented in the Supplementary Table S1.

Conversely, when the proteomic profile of the yeast cells treated with ethanol extracts was analyzed, the opposite situation is observed. The SE samples, when compared to the NFE samples, showed a significant downregulation for the majority (77.5%) of the analyzed proteins involved in different yeast cell processes: genetic information processing (52.5%), carbohydrate metabolism (5.0%), transport and catabolism (3.75%), nucleotide metabolism (3.75%), amino acid metabolism (2.5%), metabolism of cofactors and vitamins (2.5%), and energy metabolism (1.25%). The remaining 22.5% significantly expressed proteins were upregulated in SE samples (Figure 3b) and were associated with genetic information processing (10.0%), energy metabolism (3.75%), carbohydrate metabolism (2.5%), and amino acid metabolism (2.5%).



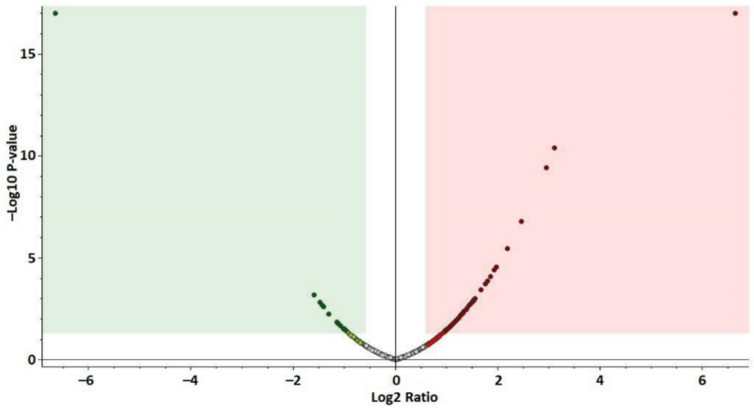
(**a**)



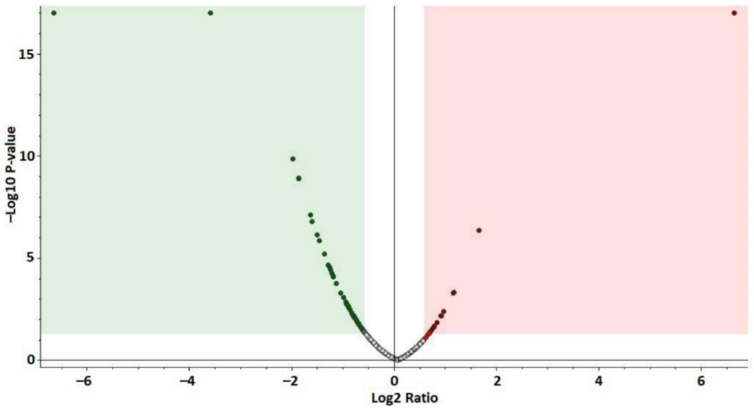
(**b**)

Figure 1. Principal Component Analysis (PCA) of protein abundances. Projection of four sets of samples: yeast treated with fermented *Spirulina* water extract (SV), non-fermented *Spirulina* water extract (NFV), fermented *Spirulina* ethanol extract (SE), and non-fermented *Spirulina* ethanol extract (NFE); and two sets of control samples: water control sample (KV) and ethanol control sample (KE). (**a**) PCA of all samples; (**b**) PCA of samples without control samples.

Further bioinformatic analysis was also performed to obtain more insight into the activity of the differentially abundant proteins. In SV, compared to NFV samples, significantly upregulated proteins (78%) are involved in different cellular processes. Among them, proteins involved in genetic information processing were the most abundant group (56%), followed by those involved in carbohydrate metabolism (6.6%), amino acid metabolism (5.5%), lipid metabolism (2.2%), transport and catabolism (1.1%), nucleotide metabolism (1.1%), and metabolism of cofactors and vitamins (1.1%). Twenty-two percent of significantly expressed proteins show downregulation in SV compared to NFV samples (Figure 3a). Among them are proteins involved in genetic information processing (6.6%), energy metabolism (2.2%), carbohydrate metabolism (1.1%), amino acid metabolism (1.1%), and metabolism of cofactors and vitamins (1.1%).



(**a**)

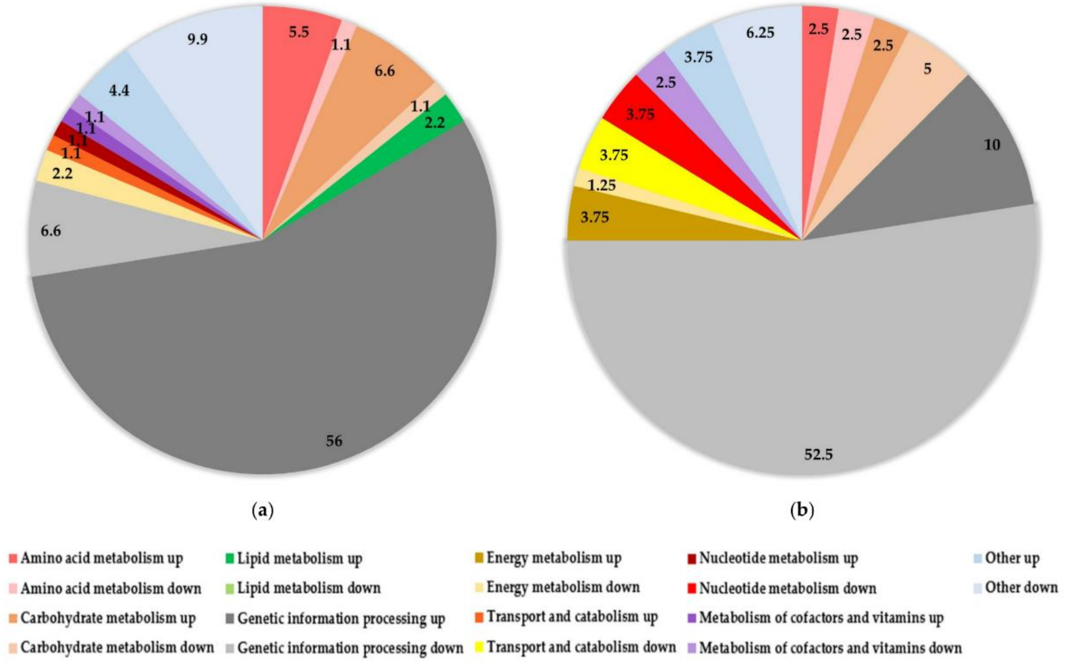


(**b**)

Figure 2. Volcano plots showing differentially abundant proteins in treated yeast samples. The −log10 (*p*-value) is plotted against the log2 fold change. Points above the horizontal line (non-axial) present proteins with significantly different abundances (*p*-value < 0.05). Points to the left of the vertical line (non-axial) present protein log2 fold changes lower than −0.58, and points to the right of the vertical line (non-axial) present protein log2 fold changes greater than 0.58. Significantly downregulated and upregulated proteins *p* value < 0.05) are plotted in the green (left) and red (right) fields, respectively. (**a**) Yeast treated with water extract of fermented *Spirulina* (SV) vs. yeast treated with water extract of non-fermented *Spirulina* (NFV). Fold changes of SV/NFV. (**b**) Yeast treated with ethanol extract of fermented *Spirulina* (SE) vs. yeast treated with the ethanol extract of non-fermented *Spirulina* (NFE). Fold changes of SE/NFE.

This turn in cell response when comparing water and ethanol extracts could result from differences in solubility of bioactive compounds from *Spirulina* in both solvents and also from fermentation effect on the bioactive compound release from *Spirulina* and their structural changes affecting their solubility in particular solvent.

Spirulina ethanol extracts have been determined to have a higher total phenolic and flavonoid content and higher concentrations of carotenoids and chlorophyll-a compared to water extracts. The compounds responsible for most of the antioxidant activity appear to be polar phenolic compounds, zeaxanthin and myxoxanthophyll-like compounds [16,23,33]. Water extracts, although having lower antioxidant activity, have also been shown to have a beneficial effect on cancer cell growth inhibition, a protective effect against apoptotic cell death due to free radicals, type 2 diabetes managing properties and help improve many other physiological disorders due to their antioxidant activity [3,7,34,35]. They also had higher concentrations of phycocyanin, free amino acids, carbohydrates, and total proteins compared to *Spirulina* ethanol extracts [23].



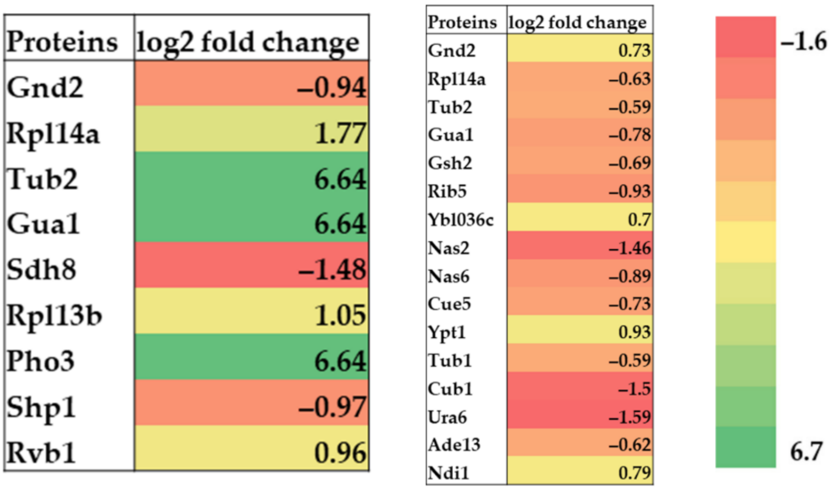
(**a**) (**b**)

Figure 3. Pie charts showing differentially abundant protein profiles in yeast treated with fermented *Spirulina* extract (S) compared to yeast treated with non-fermented *Spirulina* extract (NF). Results are expressed in percent (%) of significantly upregulated or downregulated proteins involved in different cellular processes. The darker shade of the same color represents the upregulated proteins involved in a specific cell process, and the lighter shade of the same color represents the downregulated proteins involved in the same cell process. (**a**) Yeast treated with water extracts (SV compared to NFV); (**b**) Yeast treated with ethanol extracts (SE compared to NFE).

Studies have shown that an increasing amount of ethanol in the extraction solvent up to 70% also increases antioxidant activity of the *Spirulina* extract, as measured by DPPH (2,2-Diphenyl-1-picrylhydrazyl hydrate) free radical scavenging assay, β-carotene bleaching method, ABTS$^{\bullet+}$ assay or PMRC method [16,22]. This points to the higher solubility of the antioxidative compounds in ethanol and the possibility that ethanol soluble components possess the main antioxidant properties [22,33]. Furthermore, ethanol extracts of different materials (*Spirulina*, green propolis, plants) showed superior antioxidant activity compared to the water extracts [16,22,36–38]. In addition, they benefit from ethanol GRAS (Generally Recognized as Safe) status, meaning it can be used as a safe solvent for the food industry [38].

Additionally, in our study, lactic acid bacteria presumably caused modifications of bioactive compounds present in *Spirulina*, which enhanced their ethanol solubility, bioavailability and bioactivity in fermented *Spirulina* extracts. Fermentation has already been shown to improve antioxidant activity by increasing the phenolic compound release from plant-based foods, and it is a suitable method for increasing the content of natural antioxidants. Moreover, during fermentation, the structural breakdown of the cell walls occurs due to the activity of bacterial enzymes. This result is reflected in liberating and/or inducing the synthesis of different bioactive compounds responsible for increasing the total phenols and antioxidant activity after fermentation and facilitating flavonoid extraction [39–41]. With protease activity of lactic acid bacteria, phycocyanin, an important antioxidative and anti-inflammatory compound in *Spirulina* can be converted to phycocyanobilin. Other important compounds are bioactive peptides, formed through protein hydrolysis and, together with phycocyanobilin, contribute significantly to fermented *Spirulina* bioactivity [13,42,43]. Structural changes in phytochemicals are another possible mechanism responsible for increasing the bioactivity of plant-based foods after fermentation. The presence of lactic acid bacteria in a fermentation process has been shown to contribute to simple phenolic conversion and depolymerization of high-molecular-weight phenolic compounds [41].

To better understand the difference in cell response and to connect it with the antioxidant effect previously determined when comparing the effects of fermented *Spirulina* extracts to non-fermented for particular solvent, we further focused on particular differentially abundant proteins, related to cell stress response and compared their log2 fold changes as an indicator of the degree of abundance (log2 fold changes greater than 0.58 indicate significantly more abundant proteins, and log2 fold changes lower than −0.58 indicate significantly less abundant proteins) (Figure 4). Namely, we used yeast cells in the stationary phase, where, in contrast to the exponential phase (where rapid growth and high biosynthetic activity lead to the dilution of non-repaired lipids or proteins by new and functional ones), the cells are permanently exposed to endogenously produced ROS. This results in accumulation of oxidative damage in the cells [25]. Cell treatment with exogenous antioxidants has already been shown to have effect on endogenous antioxidant defense systems [44].



Proteins	log2 fold change
Gnd2	–0.94
Rpl14a	1.77
Tub2	6.64
Gua1	6.64
Sdh8	–1.48
Rpl13b	1.05
Pho3	6.64
Shp1	–0.97
Rvb1	0.96

(**a**)

Proteins	log2 fold change
Gnd2	0.73
Rpl14a	–0.63
Tub2	–0.59
Gua1	–0.78
Gsh2	–0.69
Rib5	–0.93
Ybl036c	0.7
Nas2	–1.46
Nas6	–0.89
Cue5	–0.73
Ypt1	0.93
Tub1	–0.59
Cub1	–1.5
Ura6	–1.59
Ade13	–0.62
Ndi1	0.79

(**b**)

Figure 4. Heat map presenting differentially abundant proteins involved in stress response in yeast treated with fermented *Spirulina* extract (S) compared to yeast treated with non-fermented *Spirulina* extract (NF). Results are expressed as log2 fold change SV/NFV (**a**) and SE/NFE (**b**). Significantly more abundant proteins have log2 fold changes greater than 0.58, and significantly less abundant proteins have log2 fold changes lower than −0.58; *p*-value ≤ 0.05.

3.3. Stress Response Related Proteins

Six stress response related proteins showed upregulation, and three proteins were downregulated in SV samples compared to NFV samples (Figure 4a). Among them, proteins Pho3, Tub2, and Gua1 showed highly increased levels (6.64). Rpl14a and Rpl13b showed increased values of 1.77 and 1.05, respectively, and Rvb1 had a value of 0.96. Protein Sdh8 had the most decreased level (−1.48), followed by Shp1 (−0.97) and Gnd2 (−0.94). These data show a higher expression of stress response related proteins in yeast treated with fermented *Spirulina* water extract than yeast treated with non-fermented *Spirulina* water extract.

Differentially abundant proteins involved in the stress response of yeast cells treated with *Spirulina* ethanol extracts showed an opposite state. Here, 12 stress response related proteins showed downregulation and 4 proteins were upregulated in the SE samples compared to the NFE samples (Figure 4b). The proteins Ura6 (−1.59), Cub1 (−1.5) and Nas2 (−1.46) showed the highest downregulation, followed by Rib5 (−0.93), Nas6 (−0.89), Gua1 (−0.78), Cue5 (−0.73), Gsh2 (−0.69), Rpl14a (−0.63), Ade13 (−0.62), Tub1 and Tub2 (−0.59). The highest upregulation was noticed in Ypt1 (0.93), followed by Ndi1 (0.79), Gnd2 (0.73) and Ybl036c (0.70). Unlike water extract-treated samples, the data shows the lower expression of stress response related proteins in yeast treated with fermented *Spirulina* ethanol extract than yeast treated with non-fermented *Spirulina* ethanol extract.

The turn in stress response related activity showed as differential expression of stress response related proteins is also shown when comparing four differentially abundant proteins common to both sample groups. Proteins Rpl14a, Tub2 and Gua1, show increased levels in yeast treated with fermented *Spirulina* water extracts, whereas their levels were reduced when fermented *Spirulina* ethanol extracts were applied in comparison with corresponding extracts of non-fermented *Spirulina*. Alternatively, Gnd2 protein was downregulated in yeast treated with water extracts of fermented *Spirulina*, whereas its expression was upregulated in yeast treated with ethanol extracts of fermented *Spirulina* compared to corresponding extract of non-fermented *Spirulina*. The list and names of differentially abundant proteins involved in stress response are presented in Table 1.

Table 1. Stress response related proteins.

Gene Symbol	UniProt Accession No.	Protein Name	Stress Response Related Activity
GSH2	Q08220	Glutathione synthetase (Gsh2)	Glutathione biosynthesis
GND2	P53319	6-phosphogluconate dehydrogenase, decarboxylating 2 (Gnd2)	Glutathione metabolism
PHO3	P24031	Constitutive acid phosphatase (Pho3)	Thiamine, riboflavin metabolism
RIB5	P38145	Riboflavin synthase (Rib5)	Riboflavin biosynthesis
YBL036C	P38197	Pyridoxal phosphate homeostasis protein (Ybl036c)	Homeostatic regulation of the active form of vitamin B6
SDH8	P38345	Succinate dehydrogenase assembly factor 4, Mitochondrial (Sdh8)	Response to reactive oxygen species
RPL13B	P40212	60S ribosomal protein L13-B (Rpl13b)	Autophagy
RPL14A	P36105	60S ribosomal protein L14-A (Rpl14a)	Autophagy
CUE5	Q08412	Ubiquitin-binding protein CUE5 (Cue5)	Autophagy
YPT1	P01123	GTP-binding protein YPT1 (Ypt1)	Autophagy
SHP1	P34223	UBX domain-containing protein 1 (Shp1)	Autophagosome assembly, proteasome-mediated ubiquitin-dependent protein catabolic process, piecemeal microautophagy of the nucleus
TUB2	P02557	Tubulin beta chain (Tub2)	Phagosome function
TUB1	P09733	Tubulin alpha-1 chain (Tub1)	Phagosome function
RVB1	Q03940	RuvB-like protein 1 (Rvb1)	DNA damage, DNA repair

Table 1. *Cont.*

Gene Symbol	UniProt Accession No.	Protein Name	Stress Response Related Activity
CUB1	Q08977	Cu$^{(2+)}$ suppressing and bleomycin sensitive protein 1 (Cub1)	DNA repair and/or proteasome function
NAS2	P40555	Probable 26S proteasome regulatory subunit p27 (Nas2)	Proteasome regulatory complex assembly
NAS6	P50086	Probable 26S proteasome regulatory subunit p28 (Nas6)	Proteasome regulatory complex assembly
GUA1	P38625	GMP synthase (glutamine-hydrolyzing) (Gua1)	Purine biosynthesis
ADE13	Q05911	Adenylosuccinate lyase (Ade13)	Purine biosynthesis, AMP, IMP biosynthesis
URA6	P15700	Uridylate kinase (Ura6)	Pyrimidine biosynthesis
NDI1	P32340	Rotenone-insensitive NADH-ubiquinone oxidoreductase, mitochondrial (Ndi1)	Positive regulation of the apoptotic process

A decline in the abundance of Gnd2 protein could result in a higher intracellular oxidation level in SV compared to NFV since this protein is involved indirectly in glutathione metabolism through NADPH production, necessary for maintaining high reduced glutathione (GSH) levels for efficient oxidative stress response [45,46]. Without NADPH, the glutathione oxidized form (GSSG) cannot be reduced to the active form (GSH), causing its levels to drop, which indicates greater cellular oxidative stress [47,48].

Furthermore, upregulation of proteins Rpl14a and Rpl13b might be due to a greater need for degradation of intracellular constituents in SV than NFV samples because these proteins are involved in autophagy. Autophagy is a central part of the integrated stress response mechanism, induced by different stress stimuli: nutrient and energy stress, endoplasmic reticulum stress, pathogen-associated molecular patterns, danger-associated molecular patterns, redox stress, hypoxia, and damage to mitochondria [49,50], which points to a more intense stress response related outcome in the yeast treated with fermented as compared to non-fermented *Spirulina* water extracts.

Additionally, the possibility of DNA damage occurrence and the need for nucleotide base synthesis was indicated in the treated yeast cells since a higher abundance of the DNA damage/DNA repair associated protein Rvb1 was detected in SV compared to NFV samples, as well as upregulation of the Gua1 protein involved in purine biosynthesis. Rvb1 protein has been shown in previous studies to be required for DNA repair and, in higher eukaryotes, it modulates cellular signaling, response to stress and apoptosis and DNA damage [51,52].

An increase in the abundance of Pho3 protein involved in riboflavin metabolism to flavin adenine dinucleotide (FAD) might also indicate a potentially higher oxidation level in SV compared to NFV samples. FAD is a coenzyme with an essential role as an electron acceptor/donor in oxidoreductases such as glutathione peroxidase in *S. cerevisiae*, a FAD-dependent enzyme protecting cells from oxidative stress [53]. Its induction in the treated yeast cells could be related to a higher need for antioxidative action to combat ROS produced in the cell endogenously and cell treatment with *Spirulina* extract.

In contrast to water extract-treated samples, in SE compared to NFE samples, upregulation of the protein involved in glutathione metabolism (Gnd2) indicates a higher NADPH production and consequently a more efficient GSSG reduction to its active form GSH. The result is that GSH predominates in the cells, indicating lower cellular oxidative stress [42,43]. Due to the latter, presumably the need for glutathione biosynthesis also declined, which is seen in our results as downregulation of the Gsh2 protein, relevant for glutathione biosynthesis. It has been suggested in the study of Izawa et al. [54]

that *S. cerevisiae* cells adapt to oxidative stress by de novo synthesis of glutathione and glutathione recycling activities, activities which are likely to be reversed when oxidative stress recedes.

Moreover, downregulation of proteins Rpl14a and Cue5 in SE compared to NFE samples might propose a lower occurrence of damage leading to a reduced need for degradation of intracellular components since these proteins are involved in autophagy. This could also be indicated by the downregulation of Nas2, Nas6, and Cub1 proteins associated with the proteasome function, suggesting reduced protein damage and consequently less need for damaged protein degradation in the treated yeast.

Lower abundance of protein Cub 1 which, besides its proteasome function, is also involved in DNA repair, the downregulation of Gua1 and Ade13 proteins associated with purine biosynthesis and Ura6 protein involved in pyrimidine biosynthesis in SE compared to NFE samples might indicate a reduced need for damaged DNA repair and consequently less need for nucleotide base synthesis in the treated yeast cells. Additionally, Rib5, a riboflavin biosynthesis involved protein, was downregulated in SE compared to NFE samples. Riboflavin is an antioxidant nutrient that has antioxidant activity as a component of the glutathione redox cycle or independently, by mechanisms such as converting reduced riboflavin to its oxidized form [55]. A study by Walther and Wendland showed that the synthesis of increased amounts of riboflavin might be connected to oxidative stress exposure in a specific fungus [53]. From this, we might presume that a lower riboflavin biosynthesis is connected to lower oxidative stress exposure.

Downregulation of above mentioned proteins might be a result of a high antioxidant effect of fermented *Spirulina* ethanol extracts. Different studies have shown enhancement of the antioxidant activity of *Spirulina* extracts after fermentation compared to non-fermented *Spirulina* analyzed by scavenging of nitric oxide and the DPPH assay [13,14,56].

4. Conclusions

A proteomic approach was used to analyze protein expression alterations in yeast cells treated with fermented or non-fermented *Spirulina* water and ethanol extracts. The results provide a better insight into the chosen extraction solvent effect and the effect of lactic acid fermentation on the antioxidant effect of *Spirulina* previously determined at the cellular level. Proteome analysis showed significant separation between the yeast cells treated with fermented and non-fermented *Spirulina* and between the yeast cells treated with ethanol and water extracts. The results indicated a greater antioxidant efficiency of ethanol than water extracts when comparing fermented to non-fermented *Spirulina* and the essential role of fermentation shown as the lowering of cell stress response related proteins expression. Namely, cells have a reduced need to induce endogenous systems for homeostasis maintenance when they cope with exogenous antioxidants. Further studies, to give an in-depth insight into the *Spirulina* extract effect on the subcellular proteome, are still needed in order to fully understand *Spirulina* bioactive compound mechanism of action. Additionally, our results showed that this kind of approach offers a great potential to study bioactive compounds' mechanism of action also from other natural sources at a proteome level using a simple eukaryotic cell model.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox10091366/s1, Table S1: Proteome Discoverer protein identification and quantification data.

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3.4 Scientific Paper: "Exploring the Proteome and Metabolome of Fermented *Spirulina* Biomass"

This chapter describes the paper (manuscript in preparation) by the authors Jasmina Masten Rutar, Urška Vrhovšek, Nataša Poklar Ulrih, Isabelle Fournier, Nives Ogrinc, Polona Jamnik and Nina Ogrinc entitled "Exploring the Proteome and Metabolome of Fermented *Spirulina* Biomass", to be published in Food Research International (IF 7.425) in 2024. My contribution to this work included sample preparation, fermentation, data analysis, interpretation of the results, and manuscript preparation. The study focuses on characterization of the lactic acid fermented *Spirulina* biomass by exploring protein expression and metabolite production.

Spirulina prokaryotic microalgae – cyanobacteria, are rich in bioactive compounds with high bioactive potential. Lactic acid fermentation enhances their bioactive efficiency further, as it has been shown to improve nutritional and functional properties and extend the shelf life of foods. This study assesses the role of lactic acid fermentation in the changing protein and metabolic profile of *Spirulina* biomass. For this study, fresh *Spirulina* biomass was obtained. A part of the biomass was subdued to a 24-hour lactic acid fermentation using *Lactobacillus plantarum* to obtain a fermented *Spirulina* biomass. After, ethanolic extracts of the fermented and non-fermented biomass were prepared. Proteomic and metabolomic approaches were then applied to study the *Spirulina* biomass extracts to gain a deeper insight into the processes that occur during fermentation. The total number of identified proteins after fermentation decreased from 847 to 490 due to their proteolytic degradation into bioactive peptides and amino acids. String protein interaction network database revealed that the over-expressed proteins in fermented *Spirulina* extract were involved in photosynthesis, bacterial secretion system and pentose phosphate pathway, while the proteins involved in cellular metabolic and biosynthetic processes and antioxidant activity were under-expressed. In addition, metabolome profiling showed that while the non-fermented and fermented *Spirulina* extracts possessed some qualitative similarities, the fermented *Spirulina* extract had poorer chlorophyll and carotenoid content compared to the non-fermented *Spirulina* extract, as presented by the UPLC-DAD analysis. Conversely, the amino acid content notably increased after fermentation, which hints at the proteolytic activity during fermentation. Further, the SpiderMass MS analysis and consequent PCA-LDA assessment showed a significant separation of the fermented and non-fermented *Spirulina* extracts based ob their lipid composition. While the non-fermented *Spirulina* extracts were distinguished by higher lipid compound content, the fermented extracts showed higher content of lipid metabolites.

In summary, the study shows the essential role of lactic acid fermentation in metabolite transformation. It also shows how the use of the omic platforms allows us to establish the role of microorganisms and their metabolic potential during fermentation and, simultaneously, observe how the microorganisms adapt to the changing conditions during the process to ensure the safety and quality of fermented foods.

Article

Exploring the proteome and metabolome of fermented Spirulina biomass

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Abstract: Abstract.

Keywords: Spirulina; *Arthrospira platensis*, lactic acid fermentation, *Lactobacillus plantarum*, carotenoids, chlorophyll, proteome, metabolome

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1. Introduction

Microalgae are an important source of nutrients and functional compounds [1]. Spirulina (*Arthrospira platensis*) is one of the most prominent microalgal biomasses produced due to its rich protein and amino acid composition, containing all the essential and most of the non-essential amino acids, and minerals phosphorus, calcium, selenium and potassium. Also, Spirulina is an excellent source of various bioactive compounds, such as polysaccharides, omega-6 fatty acids, especially gamma-linolenic fatty acid, vitamins B (except B_{12}) and E, pigments and phenolic compounds [2–6]. The rich content of these compounds in Spirulina results in high antioxidant, anti-inflammatory, anti-cancer and other functional properties [6–10]. To further improve Spirulinas' bioactive properties, lactic acid fermentation can be applied, since it has been shown to reduce the antinutritional factors' activity in foods, and to improve nutrient digestibility and therapeutic properties [11,12].

Lactic acid bacteria use enzymatic hydrolysis for degradation of plant and cyanobacterial cell walls and to convert organic macromolecules to smaller compounds [11–13]. In this way, antioxidants, bioactive peptides, exopolysaccharides and vitamins are produced, which contribute to the antioxidant, immunomodulatory, antimicrobial, cholesterol-lowering and antitumor activity of the food products [14–16]. Along with the improved bioactive molecule profile and content, lactic acid fermentation also improves their bioavailability and, therefore, efficiency [17]. *Lactobacillus plantarum* is the most versatile species within the genus *Lactobacillus* and it is widely used in industrial fermentation and raw food processing due to its efficiency, many favorable properties and the gained QPS (Qualified Presumption of Safety) and GRAS (Generally Recognized as Safe) statuses [18–21]. The Spirulina fermentation studies using *L. plantarum* resulted in an increased C-phycocyanin and total phenolic content, protein bioavailability and β-carotene extraction yield. Furthermore, the fermented Spirulina biomass showed and improved antioxidant and immunostimulatory activity, neuroprotective effect and antimicrobial activity [13,22–26].

The use of 'omic' methods offers the possibility of studying the role of microorganisms and their metabolic activity in the fermentation process and, consequently, the bioactive properties of the fermented foods [27]. In this study we focused on the proteomic and metabolomic approach to explore the influence of lactic acid fermentation on the Spirulina extract composition, since the analysis of proteome enables insight into the protein activity, which is directly involved in the structural and functional information of the organism [28,29]. Metabolite analysis offers a possibility to assess the metabolic activity and consequential metabolite production, which is a result of the fermentation process. This gives us an opportunity to understand the functional, nutritional and safety aspects of the fermented product [30–33].

To understand the changes that occur in Spirulina biomass after fermentation using *L. plantarum*, in this study we characterize the Spirulina biomass extracts before and after fermentation for the first time at the proteome and molecular level.

2. Materials and methods

2.1 Preparation of Lactobacillus plantarum Inoculum

A stock culture of *Lactobacillus plantarum* (LMG 6907) was provided by the Institute of Dairy Science and Probiotics, Department of Animal Science, Biotechnical Faculty, Slovenia. The *L. plantarum* culture in 20% (v/v, 50 µL) glycerol was added to 20 mL of De Man, Rogosa, and Sharpe broth (MRS, Merck, Darmstadt, Germany). Then overnight incubation on a rotary shaker was performed (30°C, 150 rpm) until the late exponential phase was reached. Centrifugation of the overnight culture (2 mL) (14,000× g, 5 min) and double washing with the physiological solution (0.9% (w/v) NaCl) followed in order to prepare the suspension for inoculation.

2.2 Arthrospira platensis Cultivation

Arthrospira platensis (hereinafter referred to as Spirulina) fresh biomass was provided by algae farm Severino Becagli (Grosseto, Italy) in collaboration with AlgEn (Slovenia). The microalgae were cultivated under controlled conditions (pH 10.6) in 500 m^2 ponds, with constant mixing provided by a paddle wheel. Quality of the microalgae was ensured in all phases of the production with the critical control points by high-tech processes. The insects' access to the cultivating ponds was prevented by the use of nets within the greenhouses.

2.3 Spirulina Lactic Acid Fermentation

Dry Spirulina biomass (10 g) was added with physiological solution (30 mL) to get non-fermented broth (NF) which was sampled immediately after preparation. *L. plantarum* suspension (1% (v/v) inoculum) was used for the NF inoculation, and the fermentation was allowed to proceed for 24 h at 30 °C. The samples of both, non-fermented and fermented broths (FB) prepared in this manner were frozen at –20°C until further analyses.

2.4 Spirulina Extract Preparation

For the proteomic analysis, both fermented and non-fermented Spirulina broths were extracted with 70% ethanol. To obtain higher yields, two-stage extraction was carried out. Spirulina broths (8 g) were mixed in the first stage with ethanol (12 mL) and placed in a water bath (40 °C, 30 min) with constant shaking. After, 10 min centrifugation (6000 rpm) of the samples followed and the supernatant was collected. In the second stage, the remaining sediment was extracted once more in ethanol (12 mL) following the same method and both supernatants were finally combined. The extracts were then evaporated under vacuum (GeneVac HT-4 Series II, Genevac Ltd., Ipswich, UK) and lyophilized.

2.5 Spirulina Proteome Analysis

The freeze-dried microalgal biomass of Spirulina microalgae before and after fermentation was first dissolved in the RIPA lysis and extraction buffer in biological triplicates. The total protein content was first evaluated by the Bradford essay. The samples were further analyzed in triplicates using a shotgun bottom-up proteomic approach. The proteomic samples were prepared using filter-aided sample preparation (FASP) protocol followed by a trypsin digestion. The desalting of the samples was carried out by Evotips-C18 (Evosep, Denmark) following the guide of Evosep, just before the treatment by LC-MS. LC-MS analysis was performed using Evosep-One (Evosep, Denmark) liquid chromatography coupled to Q-Exactive Orbitrap (Thermo Scientific) mass spectrometer, using a nanospray source. Protein identification was performed by comparing all the gathered MS and MS/MS data with the *Arthrospira platensis* proteome databases using MaxQuant software. The statistical analysis was performed by Perseus software (version 1.6.10.43).

2.6 Spirulina pigment analysis

First, carotenoid and chlorophyll pigment extraction from Spirulina was performed. Non-fermented and fermented Spirulina samples were freeze-dried and ground to powder. Once in powder form, samples (50 mg) were added with ice-cold methanol (1 mL) and the mixture was sonicated for 20 minutes at 50 kHz. After, 5 minute centrifugation (2000 rpm) followed and supernatant was collected. Extraction procedure was repeated once more and supernatants were combined. The resulting solution was filtered through 0.22 μm syringe-tip filters (Millipore, Bedford, MA, USA) and evaporated to dryness using a solvent evaporator (EZ-2, GeneVac Ltd., Ipswich, UK). The extracts were then resuspended in methanol (100 μL) and analyzed as described below.

For pigment analysis, an ultrahigh-performance liquid chromatography equipped with diode array detector (Waters, Milford, MA, USA) and tandem mass spectrometry (Micromass, Manchester, UK), UPLC–DAD–MS/MS, was used. Separation was achieved at 50°C on a 150 mm × 4.6 mm × 3.5 μm Zorbax Eclipse XDB-C8 column (Agilent, Santa Clara, CA, USA) at a flow rate of 1.1 mL/min, using 5 μL of injection volume and a solvent system composed of methanol–tetrabutyl ammonium acetate (TBAA) buffer (28 mM, pH 6,5) (70:30, v/v) (A) and methanol (B). The gradient profile was the following: 0 min, 35% B isocratic; from 0 to 7 min, linear gradient to 50% B; from 7 to 36 min, linear gradient to 95% B; from 36 to 44 min, 95% B isocratic; from 44 to 46 min, re-equilibration to the initial conditions of 35% B; from 46 to 50 min, 35% B isocratic. The DAD spectrum was recorded between 200 and 700 nm with detection at 400, 450 and 550 nm.

2.7 Spirulina metabolite analysis

2.8 Statistical analysis

3. Results and discussion

3.1 Proteome analysis results

In Spirulina biomass 847 proteins were identified before and 490 proteins after fermentation. The results reveal a higher number of over-expressed proteins (625) before fermentation than after fermentation (268). String protein-protein interaction network revealed the over-expressed proteins to be involved in photosynthesis, bacterial secretion system and pentose phosphate pathway. On the other hand, after fermentation, under-expression of proteins involved in cellular metabolic and biosynthetic processes, and cell antioxidant activity has been determined (Figure 1). The results were processed with a high confidence minimum required interaction score (0.70).

The high decrease in the total number of proteins after fermentation in Spirulina can be explained by the proteolytic activity during fermentation and protein breakdown into bioactive peptides and amino acids. These results will further be compared to metabolite analysis results to examine the potential proteomic and metabolic pathways induced by fermentation.

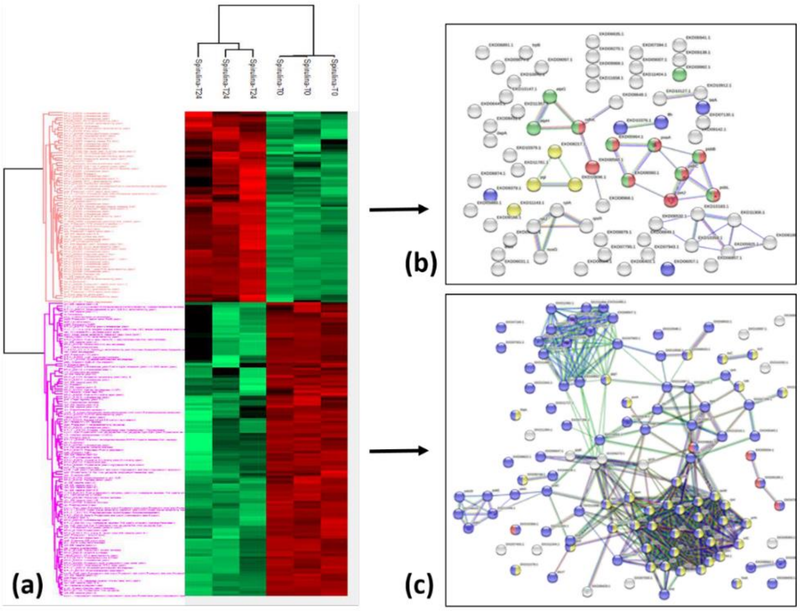


Figure 1. Protein-protein interaction network of Spirulina after fermentation. (a) Heatmap of significantly over-expressed (red) and under-expressed (green) proteins (b) Over-expressed proteins: red and/or green: photosynthesis; blue: proteins of bacterial secretion system; yellow: pentose phosphate pathway. (c) Under-expressed proteins: blue: cellular metabolic processes; yellow: cellular biosynthetic processes; red: antioxidant activity.

3.2 Carotenoid and chlorophyll composition results

The non-fermented Spirulina biomass was richer in carotenoid and chlorophyll pigment related metabolites content than the fermented Spirulina biomass. The profiles of the non-fermented and fermented Spirulina biomass show qualitative similarities, e.g., main peaks in the fermented Spirulina profile, which appear at 11.6, 16.3 and 36.9 min, were determined in the non-fermented Spirulina as well (Figures 2 & 3). Seven peaks in the non-fermented Spirulina extract profile show characteristic peak profiles of carotenoids antheraxanthin, canthaxanthin, a/b-carotene and additional four unidentified carotenoids. Furthermore, four peaks in the in the same sample, show characteristic peak profiles of chlorophyll a and additional three unidentified chlorophyll compounds (Figure 2).

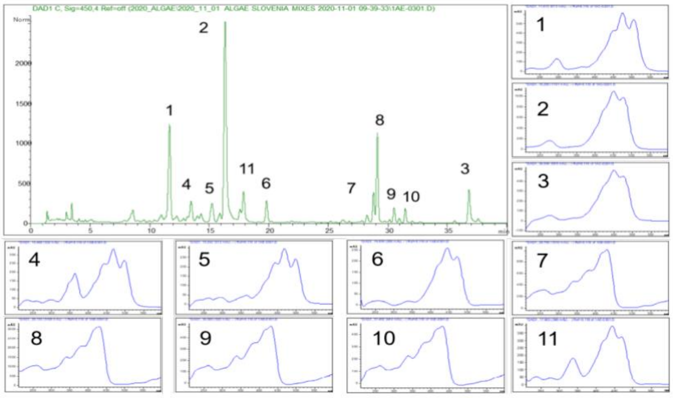


Figure 2. The diode array signal at 450 nm: pigment peak profiles in the non-fermented Spirulina extract. 1: antheraxanthin; 2: canthaxanthin; 3: a/b-carotene; 8: chlorophyll a; 4, 5, 6, 11: not identified carotenoid compounds; 7, 9, 10: not identified chlorophyll compounds.

In the fermented Spirulina extract, the three main peaks show characteristic profiles of carotenoids antheraxanthin, canthaxanthin and a/b–carotene, while the intensity of minor peaks was too weak to make any postulations. Characteristic peak profiles of chlorophyll compounds were only detected by UV profiles in the non-fermented Spirulina extract (Figure 3).

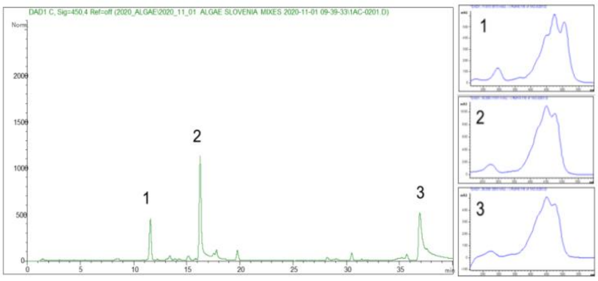


Figure 3. The diode array signal at 450 nm: pigment peak profiles in the fermented Spirulina extract. 1: antheraxanthin; 2: canthaxanthin; 3: a/b-carotene.

The loss of carotenoid and chlorophyll compounds in the fermented Spirulina biomass is consistent with the reported increase of the lactic acid content during lactic acid fermentation and lowering of the pH in the biomass [26,34,35].

3.3 Spirulina metabolite analysis results

The metabolomic analysis of the Spirulina biomass before and after fermentation showed a high increase in amino acid content after fermentation, which is consistent with previously mentioned decrease in the total number of proteins after fermentation due to proteolytic breakdown to amino acids (Table 1).

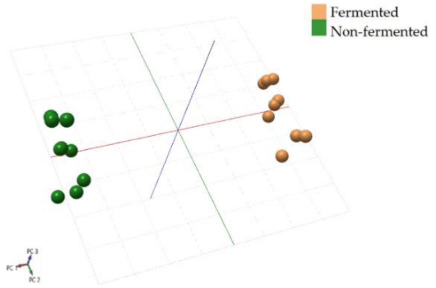
Table 1. Metabolite composition of Spirulina biomass before and after fermentation.

Metabolite	Non-fermented Spirulina (mg/L)	Fermented Spirulina (mg/L)
Amino acids		
Alanine	92.891	107.192
Beta-alanine	0.018	0.023
Gamma-aminobutyric acid	0.994	5.677
Ornithine & arginine	12.662	14.363
Asparagine	18.235	19.552
Aspartic acid	101.076	276.639
Glycine	7.526	17.161
Proline	3.817	28.613
Serine	17.888	2.270
Tyrosine	493.393	689.101
Isoleucine	27.028	48.615
Leucine	76.908	133.424
Lysine	17.608	27.068
Phenylalanine	18.891	38.402
Threonine	13.937	19.511
Valine	52.465	85.076
Sugars		
XylosE	19.089	28.510
Ribose	5.525	0.289
Alpha-rhamnose	0.667	1.436
Fructose	1.706	0.342
Galactose	0.939	0.100
Glucose	7.866	1.126
Myo-inositol	10.050	11.386
Sucrose	1.319	NF
Fucose	0.406	0.468
Acids		
Oxalic acid	6.317	NF

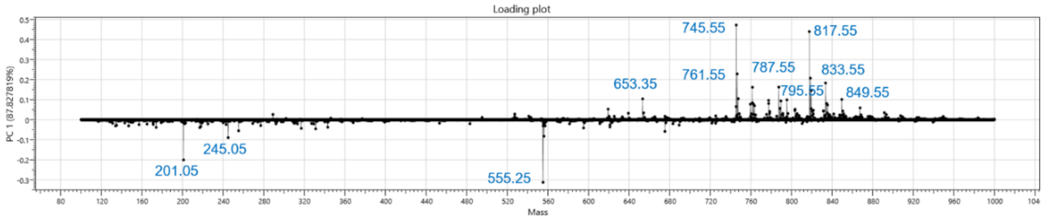
Metabolite	Non-fermented Spirulina (mg/L)	Fermented Spirulina (mg/L)
Glutaric acid	0.043	0.044
Citric acid	1.655	0.408
Succinic acid	85.808	125.686
Glyceric acid	1.545	11.634
Citramalic acid	0.249	0.316
Malic acid	1.760	0.433
Benzoic acid	8.476	8.780
Fumaric acid & maleic acid trans	0.346	0.183
Cinnamic acid	1.302	1.249
Threonic acid	0.112	0.107
Alpha-ketoglutaric acid	2.852	3.274
Alcohols		
Threitol	1.839	2.265
Meso-erythriol	0.469	0.647
Arabitol	0.128	0.122
2-pyrrolidinone	0.814	1.035
Other		
Nicotinic acid	3.555	4.013
Uracil	7.433	10.136
Pyroglutamic acid	49.050	50.068

3.3 SpiderMass analysis

The fermented and non-fermented Spirulina extracts were analyzed by SpiderMass ambient MS technology. SpiderMass provides lipid and metabolite analysis of the sample. The obtained MS spectra were submitted to a Principle Component Analysis (PCA) to assess clustering of the data. The PCA features were then analyzed by Linear Discriminant Analysis (LDA) where the fermented and non-fermented Spirulina extracts were separated in two groups based on their lipid content (Figure 4a). Examples of discriminant ions (m/z) between the two regions, which correspond to lipids, are presented as their normalized intensities (Figure 4b). The most discriminating peaks for fermented Spirulina extracts correspond to m/z 201.05, 245.05 and 555.25, and for non-fermented Spirulina extracts to m/z 653.35, 745.55, 761.55, 787.55, 795.55, 817.55, 833.55, and 849.55.



(a)



(b)

Figure 4. PCA-LDA classification model based on fermented (orange) and non-fermented (green) Spirulina extracts. (a) LDA representation of the 3-class PCA-LDA (right). (b) PC1 loading spectra indicates discrimination between the fermented (orange) and non-fermented (green) Spirulina extract.

4. Conclusions

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Supplementary Materials: The following supporting information can be downloaded at:

Author Contributions: "Conceptualization, X.X. and Y.Y.; methodology, X.X.; software, X.X.; validation, X.X., Y.Y. and Z.Z.; formal analysis, X.X.; investigation, X.X.; resources, X.X.; data curation, X.X.; writing—original draft preparation, X.X.; writing—review and editing, X.X.; visualization, X.X.; supervision, X.X.; project administration, X.X.; funding acquisition, Y.Y. All authors have read and agreed to the published version of the manuscript."

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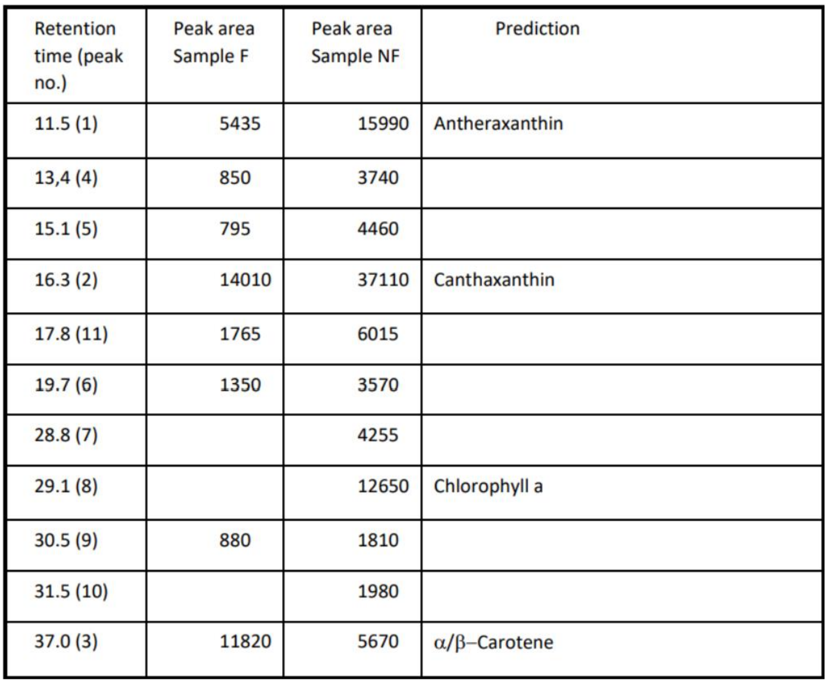
Supplementary material

Table S1. Pigment analysis results.



Retention time (peak no.)	Peak area Sample F	Peak area Sample NF	Prediction
11.5 (1)	5435	15990	Antheraxanthin
13,4 (4)	850	3740	
15.1 (5)	795	4460	
16.3 (2)	14010	37110	Canthaxanthin
17.8 (11)	1765	6015	
19.7 (6)	1350	3570	
28.8 (7)		4255	
29.1 (8)		12650	Chlorophyll a
30.5 (9)	880	1810	
31.5 (10)		1980	
37.0 (3)	11820	5670	α/β–Carotene

3.5 Scientific Paper: "Determining the Authenticity of *Spirulina* Dietary Supplements Based on Stable Isotope and Elemental Composition"

The paper entitled "Determining the Authenticity of *Spirulina* Dietary Supplements Based on Stable Isotope and Elemental Composition" by Jasmina Masten Rutar, Lidija Strojnik, Marijan Nečemer, Luana Bontempo and Nives Ogrinc is presented in this chapter. I collected and prepared commercial *Spirulina* samples for further analysis. Additionally, I was responsible for the design of the experiment, statistical analysis, data interpretation, writing the manuscript and preparing the manuscript for publication. The article was published in Foods (IF 5.2) in 2023 and discussed the potential of using stable isotope ratios of light elements and elemental composition in assessing the authenticity and geographical origin of commercially available *Spirulina* dietary supplements from the Slovenian market.

Forty-six commercial *Spirulina*-based food supplements were collected for this study in different forms (powder, capsule, tablet, flake or fresh) and declared to originate in different regions (Japan, India, Hawaii, Taiwan, Portugal, Italy, China, European Union (EU), outside the EU, and no declared origin). The elemental composition (Si, P, S, Cl, K, Ca, Ti, Mn, Fe, Zn, Br, Rb and Sr) was then analyzed using ED-XRF. Stable isotope ratio analysis of C, N and S was carried out using an elemental analyzer-isotope ratio mass spectrometer (EA-IRMS) and, for the first time, stable isotope ratio of H and O using a DELTA XP IRMS coupled with a TC/EA pyrolizer was determined. Based on the measured variables, PCA, DA and OPLS-DA were applied; *Spirulina* samples were classified according to their declared origin and composition.

Multivariate statistical analyses achieved a reliable differentiation of the Hawaiian, Italian and Portuguese samples (100%) and good discrimination of the Chinese samples. The clustering of Taiwanese and Indian samples was somewhat less successful but still notable. A wide variability in the stable isotopic ratios and elemental composition among *Spirulina* samples of different declared origins was observed due to different culturing and environmental conditions, processing methods and *Spirulina* production geographical location. Additionally, successful separation of samples containing material other than *Spirulina* was also achieved, which indicates the possibility of exposing adulterated samples using this method. Finally, combining elemental composition and stable isotopic ratio of light elements C, N, S, H, and O is a promising tool for determining *Spirulina* food supplements' authenticity and geographical origin.

This work was presented and at the 10th Jožef Stefan International Postgraduate School Students' Conference and the 12th Young Researchers' Day in Piran, Slovenia, 10th to 11th May 2018; and at the 11th Jožef Stefan International Postgraduate School Students' Conference and 13th Young Researchers' Day, 15th – 16th May 2019, in Planica, Slovenia, as an oral presentation and as a scientific conference poster presentation at the 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality in Portorož, Slovenia, 1st – 3rd April 2019, at 9th International Symposium on Recent Advances in Food Analysis from 5th to 8th November in Prague, Czech Republic, 2019, and the 2nd ISO-FOOD Symposium: ISO-FOOD From Food Source to Health, Portorož, Slovenia from the 24th – 26th April 2023.

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Article

Determining the Authenticity of *Spirulina* Dietary Supplements Based on Stable Isotope and Elemental Composition

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Abstract: While the demand for *Spirulina* dietary supplements continues to grow, product inspection in terms of authenticity and safety remains limited. This study used the stable isotope ratios of light elements (C, N, S, H, and O) and the elemental composition to characterize *Spirulina* dietary supplements available on the Slovenian market. Forty-six samples were labelled as originating from the EU (1), non-EU (6), Hawaii (2), Italy (2), Japan (1), Portugal (2), Taiwan (3), India (4), and China (16), and nine products were without a declared origin. Stable isotope ratio median values were –23.9‰ (–26.0 to –21.8‰) for δ^{13}C, 4.80‰ (1.30–8.02‰) for δ^{15}N, 11.0‰ (6.79–12.7‰) for δ^{34}S, –173‰ (– 190 to –158‰) for δ^{2}H, and 17.2‰ (15.8–18.8‰) for δ^{18}O. Multivariate statistical analyses achieved a reliable differentiation of Hawaiian, Italian, and Portuguese (100%) samples and a good separation of Chinese samples, while the separation of Indian and Taiwanese samples was less successful, but still notable. The study showed that differences in isotopic and elemental composition are indicative of sample origins, cultivation and processing methods, and environmental conditions such that, when combined, they provide a promising tool for determining the authenticity of *Spirulina* products.

Keywords: *Spirulina*; *Arthrospira* spp.; dietary supplements; stable isotope ratio; elements; authenticity; quality; geographical origin

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1. Introduction

As demand for high-quality food supplements continues to grow, combined with a greater awareness of the importance of food quality and safety, consumers are prioritizing products with declared composition and geographical origin [1–6]. There is a limited understanding of nutritional composition across microalgal species, geographical regions, and seasons, all of which can substantially affect quality and safety value of products based on microalgae, which are available on the market, mostly in the form of nutritional supplements [7]. Interest in *Spirulina* dietary supplements is also proliferating due to its accepted nutritional properties, such as high protein, mineral, vitamin, pigment, and other beneficial phytochemicals content [5,8,9]. The high proportion of adulterated algal food products discovered in the past (more than 50%), resulting partly from the lack of quality control measures, reveals the severity of the fraud, which cheats the consumer and poses a potential health risk [10,11].

Microalgal products that are subjected to variations in quality due to unstable culturing conditions are likely to be deliberately adulterated [12]. Common adulterants in microalgae products are flour and mungbean powder, which contain significantly less protein compared to *Spirulina* and *Chlorella* and have lower production costs [5]. Environmental conditions such as climate change and variability that influence algal growth may affect potential biomarkers, but also the stable isotopic composition of sulfur, carbon, and nitrogen. These parameters could be used to verify the origin of microalgae and to detect adulterants in algal products, and can be even more efficient when combined with elemental composition [13]. For example, the changes in isotopic composition of *Spirulina* products are also expected due to different production locations and cultivation techniques used for *Spirulina* production such as open or closed systems. Open systems, while being easier to operate and having lower operating costs, contribute certain negative aspects, such as water loss due to evaporation, carbon loss due to its diffusion to the atmosphere, and changing environment (light intensity, temperature, pH). Additionally, contamination of the *Spirulina* culture with external materials presents a significant problem in this cultivating system [14,15]. In contrast, closed systems are enclosed culture vessels for controlled algal biomass production. They have no direct exchange of gasses or contaminants with the environment. Instead, they provide an environment with controlled water and carbon dioxide supply, temperature, light intensity, and pH regulation, and determined aeration and gas exchange [16]. Additionally, it was found that CO_2 $\delta^{13}C$ values are lower in closed systems compared to open systems and reflect in lower $\delta^{13}C$ values in algal biomass [17].

The information concerning the distribution of the $\delta^{34}S$ values in aquatic organisms and resources is scarce. Generally, there are three potential parameters affecting sulfur isotopic composition in algae: geology, proximity to the sea, and redox chemistry, which can help to provide information about the algae origin. For instance, the $\delta^{34}S$ values of marine sulfate and vegetation near the sea are about +20‰ and continue to decrease over 100 km distance from the sea to +6‰ [18,19]. Further, the hydrogen and oxygen isotopic composition can provide additional information about the region of growing, since they differ according to latitude, altitude, and proximity to the sea [20,21]. After passing through the evaporation, condensation, and precipitation cycle, the meteoric water makes up groundwater with a systematic geographical variation in isotopic composition. Ocean water evaporation causes, through fractionation, a decrease of heavy water isotopomers concentration in the clouds, as compared to the sea. A decrease in the heavy isotope content of the precipitation also occurs due to decreasing temperatures when the equatorial ocean water vapor moves to higher latitudes and altitudes. With clouds moving further inland and gaining altitude, more evaporation, condensation, and precipitation occur, further decreasing the heavy isotope concentrations in water. Accordingly, the isotopic gradient from coastal to inland regions is reflected in the ground waters [22], and finally, the products prepared using this water [20], which, in our case, were *Spirulina* food supplements.

Furthermore, different nutrient mediums are used in *Spirulina* cultivation (Zarrouk medium, wastewater, manure, fresh, and seawater), contributing mainly to different isotopic composition of nitrogen, but also elemental compositions of the *Spirulina* culturing medium and the *Spirulina* biomass [23–27]. The elemental composition of *Spirulina* products has been shown to vary according to changes in the composition of culturing medium and deliberate enrichments of *Spirulina* products, as it has been shown that micronutrient addition in the growth medium notably improves the accumulation of macro- and micronutrients [28–30]. Additionally, the pH of the culturing medium has an important impact on mineral assimilation by *Spirulina* biomass, as higher assimilation of metal ions is stimulated at higher pH [31]. However, the cell mineral content grows only to a certain extent, after mineral concentration lowers or stagnates [28]. In addition, commercialization methods, such as processing, packaging techniques and transport, can affect the chemical composition of *Spirulina* products [32].

In Slovenia, Kejžar et al. [32] performed a preliminary characterization of algae dietary supplements from Slovenian market, which contributed towards a better overview of quality and authenticity of the products in this market niche. However, this research was restricted to only a limited number of samples, and only a part of analyzed samples were *Spirulina* products. Additionally, only carbon, nitrogen, and sulfur isotopic composition of the tested samples was determined. In the present study, however, all of the available *Spirulina* commercial food supplements from the Slovenian market and more stable isotope parameters were included to achieve a thorough overview of these products in Slovenia. Due to a lack of inspection in this market section, it is important to provide quality control and to raise awareness about quality and origin of these products among consumers. Thus, the objective of this study was to verify the quality and origin of all *Spirulina* dietary supplements sold commercially on the Slovenian market with combining carbon, nitrogen, sulfur and, for the first time, hydrogen and oxygen stable isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$, $^{2}H/^{1}H$, $^{18}O/^{16}O$), with elemental composition, to verify the type of production and country of origin. To our knowledge, this is the first time that such an approach has been used to determine the authenticity of *Spirulina* products.

2. Materials and Methods

2.1. Sample Collection and Preparation

An attempt was made to collect all available *Spirulina* products on the Slovenian market. In total, 46 samples of *Spirulina* food supplements were gathered from physical and online stores over two months in 2018. The majority of samples (44 samples) contained only *Spirulina* spp., while two were mixed samples also containing other plant material (wheat grass and barley grass) or algae (*Chlorella*, *Lithothamnium*). Of the samples containing only *Spirulina* as an active ingredient, the majority (34 samples) were labelled as pure, and ten were declared to contain excipients. The samples were labelled as originating from Hawaii ($n = 2$), Italy ($n = 2$), Japan ($n = 1$), Portugal ($n = 2$), Taiwan ($n = 3$), India ($n = 4$), European Union (EU) ($n = 1$), non-EU ($n = 6$), China ($n = 16$), or were without declared origin (NS; $n = 9$). The samples were sold either dried in tablet, capsule, or powder form, or fresh (Table 1). No additional information was available regarding production and processing.

Table 1. List of collected pure *Spirulina* or mixed supplements on the Slovenian market with product content, form, and geographical origin, as declared on the label.

Sample	Declared Origin	Product Content	Form
S1	Japan	*Spirulina*, edible scallop shell powder, edible refined processing fat	Tablets
S2	NS	*Spirulina pacifica*, $Mg(C_{18}H_{35}O_2)_2$	Capsules
S3	Outside EU	*Spirulina platensis*	Powder
S4	Hawaii	*Spirulina pacifica*, SiO_2, chicory inulin, $Mg(C_{18}H_{35}O_2)_2$	Tablets
S5	Outside EU	*Spirulina*	Powder
S6	Outside EU	*Spirulina platensis*	Powder
S7	India	*Spirulina platensis*	Tablets
S8	China	*Spirulina platensis*	Tablets
S9	Mongolia–China	Wheatgrass, Barley grass, *Spirulina*, *Chlorella*	Powder
S10	China	*Spirulina*	Powder
S11	China	*Spirulina platensis*	Powder
S12	China	*Spirulina*	Powder
S13	China	*Spirulina*	Tablets
S14	Taiwan	*Spirulina platensis*	Tablets
S15	Taiwan	*Spirulina platensis*	Powder
S16	Outside EU	*Spirulina*	Powder
S17	China	*Spirulina platensis*	Powder
S18	EU	*Spirulina*, *Chlorella*, *Lithothamnium*	Capsules
S19	India	*Spirulina platensis*, $CaCO_3$, micro-crystalline cellulose, stearic acid, CMC, SiO_2	Tablets
S20	NS	*Spirulina*, SiO_2, $Mg(C_{18}H_{35}O_2)_2$	Tablets
S21	NS	*Spirulina*	Tablets
S22	NS	*Spirulina platensis*, maltodextrine, SiO_2, $Mg(C_{18}H_{35}O_2)_2$, HPMC	Tablets
S23	China	*Spirulina*	Tablets
S24	China	*Spirulina*	Powder
S25	China	*Spirulina*	Tablets
S26	Hawaii	*Spirulina pacifica*, SiO_2	Tablets
S27	China	*Spirulina*	Powder
S28	NS	*Spirulina*	Tablets
S29	Portugal	*Spirulina platensis*, SiO_2, $Mg(C_{18}H_{35}O_2)_2$	Tablets
S30	Portugal	*Spirulina platensis*, SiO_2, $Mg(C_{18}H_{35}O_2)_2$	Tablets

Table 1. *Cont.*

Sample	Declared Origin	Product Content	Form
S31	China	*Spirulina platensis*	Powder
S32	China	*Spirulina*	Tablets
S33	China	*Spirulina*	Powder
S34	India	*Spirulina platensis*	Tablets
S35	Outside EU	*Spirulina*	Tablets
S36	Outside EU	*Spirulina*	Powder
S37	NS	*Spirulina maxima*, corn maltodextrin, $Mg(C_{18}H_{35}O_2)_2$	Tablets
S38	India	*Spirulina platensis*	Tablets
S39	NS	*Spirulina*	Tablets
S40	Taiwan	*Spirulina platensis*	Tablets
S41	China	*Spirulina platensis*	Powder
S42	NS	*Spirulina*	Capsules
S43	China	*Spirulina*	Powder
S44	Italy	*Spirulina platensis*	Flakes
S45	NS	*Spirulina*	Powder
S46	Italy	*Spirulina platensis*	Fresh

NS—Not Specified; CMC—Croscaramellose sodium; HPMC—Hydroxypropyl methyl cellulose.

Sample preparation included opening the capsules to extract the dry material, grinding the tablets to powder and, in the case of fresh samples, freeze-drying the contents followed by grinding to a powder. Once in powder form, all the samples were kept in sealed plastic containers in the dark at 4 °C.

2.2. Stable Isotope Ratio Analysis of Light Elements Using Isotope Ratio Mass Spectrometry

Measurements of the stable isotope ratios of light elements ($^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) were performed using Isotope Ratio Mass Spectrometry (IRMS) and are expressed in the δ-notation in ‰ according to Equation (1) [33]:

$$\delta^i E = \frac{R(^iE/^jE)_{sample} - R(^iE/^jE)_{standard}}{(^iE/^jE)_{standard}} \tag{1}$$

where i stands for the highest, j stands for the lowest atomic mass number of the element E (H, C, N, O, S), and R is the isotope ratio between the heavier and the lighter isotope of the element ($^2H/^1H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$) in the sample or standard. The $\delta^{13}C$ values are expressed relative to V-PDB (Vienna-Pee Dee Belemnite) standard, $\delta^{15}N$ values relative to AIR, $\delta^{34}S$ values relative to V-CDT (Vienna Cañon Diablo Troilite) standard, and the δ^2H and $\delta^{18}O$ values relative to the VSMOW (Vienna-Standard Mean Ocean Water) standard.

For the stable isotope ratio analysis of light elements C, N, and S, powdered samples (4 mg) and tungsten oxide (WO_3) (4 mg) were weighed directly into tin capsules which were then sealed and placed into the autosampler of the elemental analyzer. The samples were prepared and analyzed in triplicate. Finally, the mean values were used. The $^{13}C/^{12}C$, $^{15}N/^{14}N$ and $^{34}S/^{32}S$ values were measured simultaneously by IsoPrime 100 (IsoPrime, Cheadle Hulme, UK)—Vario PYRO Cube (Elementar, Langenselbold, Germany) (OH/CNS Pyrolyser/Elemental Analyzer) with preparation system for solid samples. The following reference materials were analyzed for quality assurance: B2155 (Protein Sercon; $\delta^{13}C$: -26.98 ± 0.13‰, $\delta^{15}N$: +5.94 ± 0.08‰, $\delta^{34}S$: +6.32 ± 0.8‰; CRP (Casein Protein; $\delta^{13}C$: -20.34 ± 0.09‰, $\delta^{15}N$: +5.62 ± 0.19‰, $\delta^{34}S$: +4.18 ± 0.74‰; USGS43 (Indian human hair powder; $\delta^{13}C$: -21.28 ± 0.10‰, $\delta^{15}N$: +8.44 ± 0.10‰, $\delta^{34}S$: +10.46 ± 0.22‰). The measurements' analytical precision was ± 0.2‰ for $\delta^{13}C$ and $\delta^{15}N$ and 0.3‰ for $\delta^{34}S$.

The δ^2H and $\delta^{18}O$ measurements were performed at the Fondazione Edmund Mach Research and Innovation Centre, Department of Food Quality and Nutrition (San Michele all' Adige, Italy). For the analysis of the H and O stable isotope ratios, the *Spirulina* powdered samples were weighed directly into silver capsules (0.20 mg) and then analyzed simultaneously using DELTA XP IRMS (Thermo Scientific, Waltham, MA, USA), coupled with a TC/EA pyrolyzer (Thermo Finnigan, Waltham, MA, USA)). Reference materials applied for normalization of the data were USGS 54 (Pinus contorta, Canadian Lodgepole pine; δ^2H_{VSMOW}: –150.40 ± 1.1‰ and $\delta^{18}O_{VSMOW}$: 17.79 ± 0.15‰) and USGS 56 (Berchemia cf. zeyheri, South African red ivory wood; δ^2H_{VSMOW}: –44.00 ± 1.8‰ and $\delta^{18}O_{VSMOW}$: 27.23 ± 0.03‰; VSMOW stands for Vienna Standard Mean Ocean Water [34]). The measurements' analytical precision was ±1‰ for δ^2H and ±0.2‰ for $\delta^{18}O$.

2.3. Macro-Elemental Composition Analysis by X-ray Fluorescence Spectrometry

Analysis of *Spirulina* samples' macro-elemental composition was performed non-destructively by Energy Dispersive X-Ray Fluorescence Spectrometry (EDXRF) to determine the following elements (13): phosphorous (P), titanium (Ti), zinc (Zn), silicon (Si), bromine (Br), sulfur (S), chlorine (Cl), manganese (Mn), rubidium (Rb), strontium (Sr), potassium (K), calcium (Ca), and iron (Fe). Powdered samples were pressed into 0.5–1.0 g pellets for analysis using a pellet die and a hydraulic press. For fluorescence excitation disc radioisotope, excitation sources Cd-109 (20 mCi, Eckert and Ziegler, Berlin, Germany) and Fe-55 (25 mCi, Eckert and Ziegler, Berlin, Germany) were used. An EDXRF spectrometer with a PX5 digital pulse processor (Amptek, Bedford, MA, USA), an XR-100 SDD detector (Amptek, Bedford, MA, USA), and a PC-based, multichannel analyzer software package (DPPMCA) were used for the emitted fluorescence radiation detection. For light element analysis (Si, P, S, and Cl), the spectrometer operating in Fe-55 mode was equipped with a vacuum chamber, and for K, Ca, Ti, Mn, Fe, Zn, Br, Rb, and Sr analysis, measurements in Cd-109 mode were performed in the air. The energy resolution of the spectrometer was 125 eV at 5.9 keV. AXIL Spectral Analysis software was used to analyze the complex X-ray spectra. For quantification, the Quantitative Analysis of Environmental Samples (QAES) software developed in our laboratory was used [35,36]. Method validation was performed using 1573a (tomato leaves) and 1547 (peach leaves) NIST standard reference materials. The EDXRF analysis estimated uncertainty budget was 11% and was incorporated in the QAES software procedure.

2.4. Statistical Analysis

XLSTAT software (Addinsoft, Long Island, NY, USA, 2019) and SIMCA-P (version 17, Sartorius Stedim Biotech, Umeå, Sweden) were used for statistical analysis. Following basic statistical methods (maximum, minimum, median, and quartiles), multivariate statistical analyses methods, including Principal Component Analysis (PCA), Discriminant Analysis (DA), and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), were applied to identify further characteristic parameters for discrimination of samples based on their elemental and isotopic composition. Internal sevenfold cross-validation was used to determine the significant components of the models and thus minimize overfitting. The OPLS-DA study evaluated performance using the explained variation (R^2X for PCA and R^2Y for OPLS-DA) and predictive ability (Q^2). The OPLS-DA model prediction performance was also evaluated via specificity (true negatives) and sensitivity (true positives), calculated as described by Fiamegos et al. [37]. The accuracy (TP/TN)/(TP + FP + FN + TN) and F1 Score (F1 Score = 2 × (Recall × Precision) / Recall + Precision) of the model were calculated as described by Strojnik et al. [38]. Candidates for discriminant markers were selected by loading plots, which allow for visualization of the relationships between the formed groups and the variables and by the variable importance in the projection (VIP) values of the OPLS-DA models, where a value higher than one was considered the threshold.

3. Results and Discussion

3.1. Isotopic Composition of Spirulina Food Supplements from the Slovenian Market

While the detailed elemental composition of *Spirulina* dietary supplements from a nutritional point of view has already been presented [9], *Spirulina* stable isotopic profiles, including hydrogen and oxygen isotopic composition, were characterized here for the first time and, together with their macro-elemental composition, were used to verify the country of origin and authenticity of the samples. In the text, the data are presented as the median value (M) and interquartile range (IR, in parentheses) of elemental or isotopic composition. Results of the elemental composition are presented in Table S1, while stable isotope ratios of light elements C, N, S, O, and H (‰) are collected in Table 2.

Table 2. Stable isotope ratios of light elements C, H, S, O, and N (‰) in *Spirulina* dietary supplements sold on the Slovenian market.

Sample Number	**^{13}C/^{12}C, ^{2}H/^{1}H, ^{34}S/^{32}S, ^{18}O/^{16}O, ^{15}N/^{14}N Isotope Ratio Expressed in δ-Notation (‰)**				
	δ^{13}C	**δ^{2}H**	**δ^{34}S**	**δ^{18}O**	**δ^{15}N**
S1	−28.0	−152	12.3	19.3	12.2
S2	−25.1	−141	7.46	21.5	8.81
S3	−20.4	−197	11.8	15.3	5.72
S4	−25.8	−141	8.78	21.6	10.8
S5	−26.1	−203	11.3	16.7	8.81
S6	−22.1	−119	−0.60	17.1	7.61
S7	−29.6	−165	−1.75	18.8	8.62
S8	−25.1	−163	13.4	18.0	1.16
S9	−26.6	−146	6.72	20.6	5.59
S10	−22.9	−177	12.8	16.9	6.44
S11	−26.1	−200	13.8	12.8	2.31
S12	−22.3	−179	12.9	16.6	−0.88
S13	−22.7	−179	12.9	16.7	−1.97
S14	−21.8	−172	11.5	18.8	6.22
S15	−21.7	−171	11.7	18.8	6.56
S16	−27.9	−164	2.67	16.8	9.84
S17	−22.2	−180	13.4	16.6	−2.40
S18	−18.1	−138	10.2	19.9	0.77
S19	−21.0	−170	11.1	20.3	4.12
S20	−23.9	−174	13.8	16.8	5.87
S21	−22.4	−173	3.53	15.8	1.72
S22	−24.9	−97.4	3.07	27.2	4.63
S23	−25.1	−158	13.8	17.6	0.95
S24	−27.1	−158	3.99	18.4	2.32
S25	−19.8	−190	13.8	15.6	3.61
S26	−24.4	−136	7.81	21.1	13.8
S27	−21.4	−199	11.1	15.2	1.74
S28	−19.6	−198	12.6	13.9	6.46
S29	−23.6	−180	7.34	15.4	3.27
S30	−23.5	−182	7.01	15.3	3.04
S31	−23.9	−169	6.55	15.7	2.59
S32	−24.6	−170	13.4	17.1	7.64
S33	−16.7	−189	10.5	16.4	−3.82
S34	−28.0	−183	11.0	17.4	8.15
S35	−20.0	−197	11.5	15.0	8.27
S36	−25.0	−180	9.39	17.2	9.47
S37	−17.4	−105	11.0	25.8	13.3
S38	−29.4	−164	0.36	18.7	2.13
S39	−24.2	−199	11.0	14.4	−4.79
S40	−30.0	−161	0.43	18.6	0.90
S41	−23.2	−195	13.6	18.5	8.73
S42	−22.3	−194	13.7	17.5	4.97
S43	−20.6	−192	10.2	17.6	5.88
S44	−28.9	−128	−0.61	22.0	−3.92
S45	−24.2	−207	10.2	12.9	−0.35
S46	−32.3	ND	0.94	ND	−5.35

ND—not determined.

The content of macro-elements (>1 g/kg) in the commercial *Spirulina* supplements was as follows: K > P > S > Si > Cl > Ca, and of micro-elements (>1 mg/kg): Fe > Mn > Sr > Zn > Ti > Br > Rb. Among the macro-elements, potassium values in *Spirulina* samples ranged from 5.83 to 26.9 g/kg with a median value of 15.2 g/kg (IR: 14.3–16.8), phosphorus values ranged from 5.06 to 14.7 g/kg (M: 10.1, IR: 10.1–12.1 g/kg), sulfur from 3.14 to 9.91 g/kg (M: 7.65, IR: 7.14–8.26), silicon from 0.68 to 21.7 g/kg (M: 5.06, IR: 1.46–14.9), chlorine from 0.09 to 5.77 g/kg (M: 2.02, IR: 0.88–2.97), and calcium from 0.46 to 63.5 g/kg (M: 1.56, IR: 0.98–2.82). Among the micro-elements, iron values in *Spirulina* supplements ranged from 0.28 to 3.48 g/kg with a median value of 0.69 g/kg (IR: 0.49–1.13), manganese from 14.7 to 195 mg/kg (M: 33.1, IR: 27.5–46.2), strontium from 4.39 to 478 mg/kg (M: 23.0, IR: 13.1–30.8), zinc from 2.30 to 52.7 mg/kg (M: 15.1, IR: 10.3–21.5), titanium from 2.58 to 65.8 mg/kg (M: 10.4, IR: 5.90–27.3), bromine from 0.47 to 17.4 mg/kg (M: 1.84, IR: 1.25–3.18), and rubidium from 0.50 to 11.9 mg/kg (M: 1.45, IR: 1.11–2.39).

Hawaiian samples (S4 and S26) showed the highest values of Cl, Fe, Zn, Br, and Rb, and the samples declared to originate outside EU had the highest values of Si (S6) and the lowest Cl (S6) and Rb (S16) values. Sample from EU (S18) had the highest values of Ca and Sr, and Italian samples (S44 and S46) had the highest K and the lowest Si, Ca, and Sr (S46) values. The lowest values of P, K, and Fe were found in sample of undeclared origin (S37), while the lowest values of Br were measured in an Indian sample (S7). Chinese samples were the highest in Ti (S23) and S (S43) and the lowest in Zn (S43). Furthermore, the lowest values of Ti and Mn were found in samples of undeclared origin (S28 and S39, respectively). Finally, Portuguese samples (S29, S30) showed the highest Mn values.

The δ^{13}C values in *Spirulina* commercial samples ranged from –32.3 to –16.7‰, with a median value of −23.9‰ (IR: –26.0 to –21.8‰). The lowest values were measured in Italian (S44, S46) and Indian (S7, S38) samples, the highest in Chinese samples (S33), and those without declared origin (NS; S37). The high δ^{13}C value of –17.4‰ in the S37 sample could be explained by the presence of corn maltodextrin excipient in the final product (Table 1), which has δ^{13}C values similar to C_4 plants (−17 to −9‰), while measured values in *Spirulina* samples are closer to those for C_3 plants (−40‰ to −20‰) [39]. The same could be assumed for the sample S33, where the presence of undeclared excipient with a higher δ^{13}C values (such as corn maltodextrin) could explain the high measured δ^{13}C value of –16.7‰. West et al. [17] found in their research that carbon values higher than −32‰ and lower than −29‰ are characteristic of crops grown in the shade or indoors, and crops with δ^{13}C values higher than −29‰ were identified as grown outdoors. However, the classification of crops grown outdoors could also include crops grown indoors when good ventilation in the indoor environment was included. Therefore, the classification of an outdoor-grown crop includes open-grown crops cultivated both outside or inside a structure. Sample separation according to δ^{13}C values is less reliable in our case, as the producers use several different synthetic or organic products to enrich the *Spirulina* growth medium. Therefore, the final products do not reflect the actual δ^{13}C isotopic composition of the environment and microalgae, but we may assume that algae grown indoor would have lower δ^{13}C values than −28‰. Our case samples were S1, S7, S34, S38, S40, S44, and S46.

The δ^{15}N values spanned a broad range of values, i.e., –5.35 to 13.8‰ (M: 4.80‰, IR: 1.30–8.02‰), where the lowest values were again measured in Italian samples (S46, S44) and the highest in Hawaiian (S4, S26), Japanese (S1), and samples without declared origin (S37). A high variability in δ^{15}N values can be attributed to using organic (manure, wastewater) and inorganic (synthetic) fertilizers. Since the nitrogen source in synthetic fertilizers is atmospheric N_2, their δ^{15}N value is around 0‰. In organic fertilizers, the δ^{15}N values are higher, since they are primarily derived from animal waste [40]. Consequently, the crops fertilized with synthetic fertilizers obtain lower δ^{15}N values than those fertilized with organic fertilizers. Additionally, a mixture of different fertilizers could be used, resulting in a relatively ambiguous nitrogen isotopic composition. Finally, variability in crop δ^{15}N value could also result from using different amounts of fertilizer [17,40]. The field and laboratory study performed on algae also indicates that higher δ^{15}N values (up to 11.1‰)

are observed in algae exposed to organic manure compared to those exposed to synthetic inorganic fertilizers [41]. Another explanation for high δ^{15}N values could be the use of a pool of NH_4^+ enriched in ^{15}N. For instance, in Delaware estuary, the δ^{15}N values of seston reached a maximum of +18‰ due to the fractionation during assimilation of NH_4^+ ions [42].

The δ^{34}S values ranged from –1.75 to 13.8‰ (M: 11.0‰, IR: 6.79–12.7‰). Here, the lowest values belonged to Indian (S7) and Italian (S44) samples, while the highest values were measured in Chinese (S11, S23, S25) and NS (S20, S42) samples. Studies on algae have shown little isotopic discrimination during the assimilation and reduction of sulfate. The isotopic composition of total sulfur in algae is depleted in ^{34}S by only 1–2‰ regarding the dissolved sulfate, which indicates that algae sulfate metabolism involves little or no isotope fractionation [43]. Therefore, δ^{34}S values in algae will reflect those of meteoric water or water used in their growth medium and geology [44,45]. It is interesting to note that in the Hawaiian Islands, δ^{34}S values of sulfates from volcanic ash and basalt-derived soils range from 6.3 to 15.4‰ [46] that are also in agreement with our data.

The lowest δ^{2}H was determined in the NS (S39, S45), Chinese (S11, S27), and sample S5 (outside EU), while the highest values were found in NS samples with undeclared origins (S22, S37). The δ^{2}H values ranged from –207 to –97.4‰ (M: –173‰; IR: –190 to –158‰). Finally, the δ^{18}O values ranged from 12.8 to 27.2‰ (M: 17.2‰; IR: 15.8–18.8‰), with examples of NS (S2, S22, S37) and Hawaiian (S4, S26) samples possessing the highest values and Chinese (S11) and certain NS (S28, S45) samples possessing the lowest.

The data also show a good linear correlation between δ^{2}H and δ^{18}O (Figure 1), with the slope ($y = 7.8x - 308.8$; $r^2 = 0.82$, $p < 0.001$) being comparable to the Global Meteoric Water Line (GMWL).

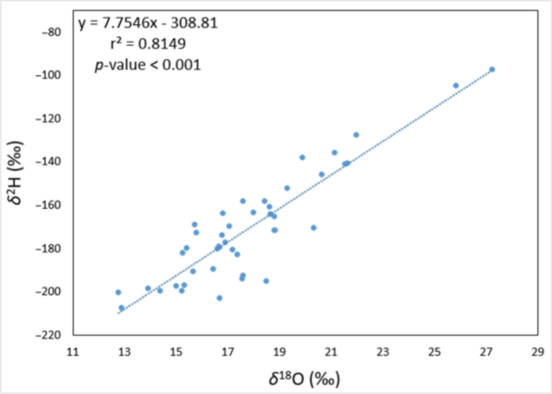


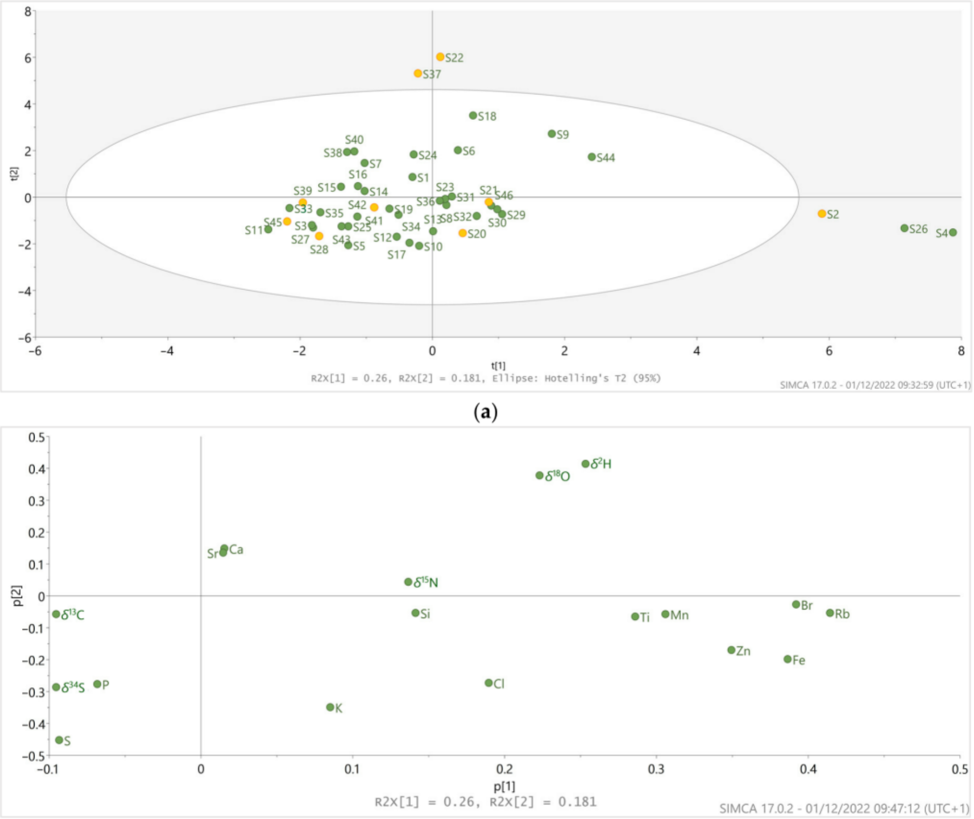
Figure 1. A plot of δ^{2}H and δ^{18}O relationship in *Spirulina* food supplements. The blue dots in the plot represent the analyzed *Spirulina* samples and the dotted line through the data points indicates a correlation of the data ($y = 7.8x - 308.8$; $r^2 = 0.82$, $p < 0.001$).

The GMWL defines the ratio of the stable isotopes in natural meteoric waters (i.e., water derived from snow, rain, and other forms of precipitation) and is typically defined by the following equation: $\delta^{2}H = 8.2 \times \delta^{18}O + 10.8$ [22]. Thus, our data indicate that hydrogen and oxygen isotopes in analyzed *Spirulina* samples originate mainly from local meteoric water and are only minimally affected by other processes such as metabolism.

3.2. Geographical Discrimination of Spirulina Samples from the Slovenian Market

3.2.1. Principal Component Analysis of All *Spirulina* Samples

Principal Component Analysis (PCA) was applied to identify trends and examine the distribution of variables in the analyzed samples. For this analysis 46 *Spirulina* commercial samples from the Slovenian market were obtained, and 18 analyzed parameters (macro-elemental and isotopic composition data) were used (Figure 2). In the PCA score plot (Figure 2a), a grouping of the samples can be observed corresponding to different elemental and isotopic compositions of the included samples. Here, three outstanding groups represented by different variables can be identified. Information about the variables that contributed most to the grouping of the samples in the PCA is provided in the PCA variables loading plot (Figure 2b).



(**b**)

Figure 2. (**a**) PCA score plot of *Spirulina* food supplements ($n = 46$) available on the Slovenian market and (**b**) PCA variables loading plot. Orange circles in figure (**a**) mark the samples of undeclared origin (NS).

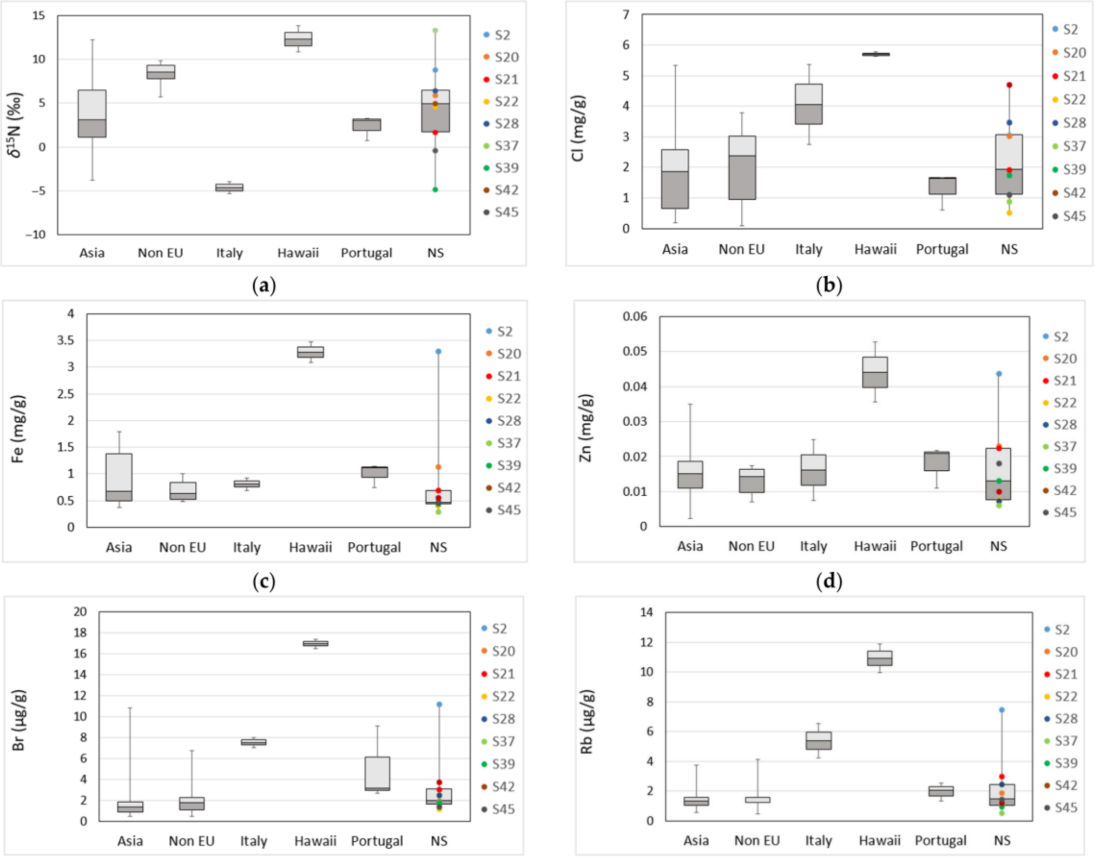
The first group of outliers (S37 and S22; Figure 2a) is characterized by having lower levels of P (S37: 6.16 and S22: 6.82 g/kg; M: 10.9 g/kg, IR: 10.1–12.1 g/kg), K (S37: 5.83 and S22: 7.40 g/kg; M: 15.2 g/kg, IR: 14.2–16.8 g/kg), Fe (S37: 0.28 and S22: 0.39 g/kg; M: 0.69 g/kg, IR: 0.49–1.13 g/kg), and S (S37: 3.88 and S22: 3.60 g/kg; M: 7.65 g/kg, IR: 7.14–8.26 g/kg). The lower content of these elements suggests a lower *Spirulina* content representing possible adulteration, since *Spirulina* typically contains high levels of P, K, Fe, and Zn [9,47,48]. Alternatively, a different growth medium could also result in different mineral compositions. For example, Michael et al. [49] showed a connection between using a poorer culturing medium (regarding elemental composition) and a lower mineral content in the final *Spirulina* product. However, given that S37 had the second highest δ^{15}N value (13.3‰) among all samples, the use of organic fertilizers in its cultivation can be suggested. As this type of cultivation medium is rich in nutrients and has a positive effect on *Spirulina* mineral uptake [25–27], it is unlikely for the growth medium to be responsible for the poor mineral content in this sample. Additionally, the highest δ^{18}O (S37: 25.8‰, S22: 27.2‰) and δ^{2}H values (S37: –105‰, S22: –97.4‰) among all samples were observed for S37 and S22 (Figure 2b). High δ^{18}O and δ^{2}H values could be attributed to the proximity of the *Spirulina* culturing site to the equatorial region and the short distance from the sea, as hydrogen and oxygen isotopic composition are strongly latitude- and altitude-dependent [20,22].

Among the samples within the elliptical field in Figure 2a, samples S18, S9, and S44 in the upper right quadrant appear to form a separate group with distinct characteristics. Here, S18 and S44 are differentiated by their high Sr values (478 and 86.2 mg/kg, respectively; M: 23.0 mg/kg, IR: 13.1–30.8 mg/kg) and low δ^{15}N values (0.77 and –3.92‰, respectively) compared to other samples (median value for δ^{15}N: 4.80‰, IR: 1.30–8.02‰). Additionally, S18 also has the highest Ca content. High Ca and Sr values in S18 can be explained by the algae *Lithothamnium* in this sample. In contrast to *Spirulina* and *Chlorella*, which also make up this product and contain moderate Ca and Sr levels, *Lithothamnium* algae contain higher values of these elements [50,51]. In S44, the higher level of Sr could be attributed to contamination during the flaking process, as this is the only sample in a flake form. The flaking process's impact on contamination with certain elements has been shown several times in previous research, where metal contamination came from the enameled parts of the flaking rollers [52,53]. As δ^{15}N values are primarily influenced by the nitrogen isotopic composition of the nitrogen source used during cultivation and internal transformations, they can indicate the type of fertilizer used, e.g., lower δ^{15}N values, as are observed in S18 and S44, point to the use of inorganic fertilizers [17,40,54].

Additionally, S9 and S44 possess lower P content (S9: 5.06 and S44: 8.56 g/kg) than most samples (M: 10.9 g/kg, IR: 10.1–12.1 g/kg). This could be explained by the samples' lower amount of algal material since this is a mixed product, containing, in addition to *Spirulina*, *Chlorella*, barley and wheat grass. While algae (in this case, *Spirulina* and *Chlorella*) are rich in phosphorus, this is not true for cereal grasses, whose content is lower [47,48]. The lower P content in S44 (and partially also S9) could be due to the drying technique used in its production. It has been shown that different drying techniques reduce the P levels in the dried material compared to the fresh one [55]. As can be observed in Figure 2b, all the samples in this group (S9, S18, and S44) have somewhat higher oxygen and hydrogen stable isotope ratios in common, which range from 19.9 to 21.9‰ (M: 17.2‰, IR: 15.8–18.8‰) and from –146 to –128‰ (M: –173‰, IR: –190 to –158‰), respectively.

Rubidium (Rb), Br, Fe, Zn, and Cl appear to play an essential role (due to their high value) in the grouping of the Hawaiian samples (S26 and S4), as well as S2, which are outlying in the lower right quadrant (Figure 2a). Here, the Rb values are as follows: S26: 9.96 mg/kg, S4: 11.9 mg/kg, and S2: 7.47 mg/kg (Rb median value (M) for *Spirulina* samples: 1.45 mg/kg, interquartile range (IR): 1.11–2.39 mg/kg). The Br values for S26 are 17.4 mg/kg, for S4 16.5 mg/kg, and for S2 11.2 mg/kg (M: 1.84 mg/kg, IR: 1.25–3.18 mg/kg). The Fe content in S26, S4, and S2 was 3.09, 3.48, and 3.29 g/kg, respectively, with a median of 0.69 g/kg (IR: 0.49–1.13 g/kg), while the Zn values in S26, S4, and S2 were 35.5, 52.7, and 43.6 mg/kg, respectively, and the median value for all samples was 15.1 mg/kg (IR: 10.3–21.5 mg/kg). The Cl content was 5.63 g/kg for S26, 5.77 g/kg for S4, and 3.07 g/kg for S2 (M: 2.02 g/kg, IR: 0.88– 2.97 g/kg). The higher content of these elements in the Hawaiian and S2 samples may be due to their deliberate addition to the growth medium, which enables *Spirulina* to uptake and accumulate these elements. *Spirulina* elemental content has been previously shown to reflect that of the culturing medium [28,30,31]. Additionally, mineral addition to the *Spirulina* culturing medium to enhance its efficiency as a nutritional source of various minerals is not uncommon [28,30,31,56]. Additionally, the high content of elements Rb, Fe, Zn, and Cl could result from manure used as a fertilizer in the growth medium, as it has been shown that adding manure in crop cultivation results in elemental composition enhancement of the plants [57,58]. A deviation in δ^{18}O and δ^{2}H has also been observed in Hawaiian samples (S4, S26) and S2, where δ^{18}O values ranged from 21.1 (S26) to 21.6‰ (S4) and δ^{2}H values from –141 (S2) to –136‰ (S26) (median values for δ^{18}O: 17.2‰

(IR: 15.8–18.8‰) and δ^2H: –173‰, (IR: –190 to –158‰)). Similarly, as previously shown in samples S22 and S37, high δ^{18}O and δ^2H values can be attributed to the proximity of the production site to the equatorial region and sea and production at low altitudes [20,22]; all parameters are true for Hawaii. However, Figure 3 shows how samples S22 and S37 cannot be placed under Hawaiian samples due to differences in elemental composition. Contrarily, these samples appear to coincide with samples from non-EU regions or Asia, as the content of elements Cl, Fe, Zn, Br, and Rb (Figure 3b–f) is similar.



(**a**) (**b**)

(**c**) (**d**)

(**e**) (**f**)

Figure 3. Box plots of selected elemental and nitrogen isotopic composition of *Spirulina* food supplements according to declared geographical origin, with specifically marked samples of undeclared origin (NS). (**a**) δ^{15}N, (**b**) Cl, (**c**) Fe, (**d**) Zn, (**e**) Br, and (**f**) Rb. The values presented under the label 'Asia' include Japanese, Indian, Chinese, and Taiwanese samples.

Another parameter, common to S4, S26, and S2 samples, is the high δ^{15}N values. Measured values are 10.8‰, 13.8‰, and 8.81‰, respectively, with a median value for all samples of 4.80‰ (IR: 1.30–8.02‰). The nitrogen isotopic composition of the samples provides information about regional agricultural practices [20]. High δ^{15}N values indicate the use of organic fertilizers, as the manure's stable isotopic composition has higher δ^{15}N values than mineral fertilizers [59]. While Zarrouk's medium (Table S2) [60] is widely used as a standard medium for *Spirulina* production, manure (chicken, cow, pig) in *Spirulina* cultivation is also a common practice, as it represents a low-cost source of nitrogen and other necessary nutrients. The use of manure in *Spirulina* production results in good cellular growth and high pigment content while reducing production costs [25–27]. Additionally, the organic manures are enriched with microflora, which induces crops to uptake micronutrients [56]. The latter can be seen in our study in the high content of specific elements in S4, S26, and S2, as mentioned earlier.

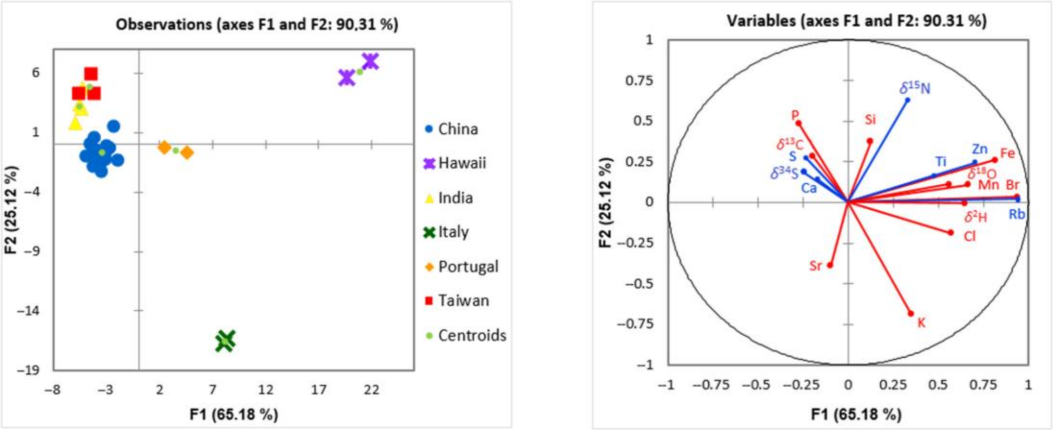
Looking at these results, we can see a close connection between the Hawaiian samples, S4 and S26, and the sample without specified country of origin, S2. Figure 3 presents box plots of δ^{15}N, Cl, Fe, Zn, Br, and Rb composition of *Spirulina* food supplements according to declared country of origin. The samples of undeclared origin (NS) are marked to compare their isotopic or elemental values with those with declared origins. Sample S2 shows the highest values of Fe, Zn, Br, and Rb among the NS samples. Fe and Zn values fall in the range of the values measured in Hawaiian samples (Figure 3c,d), indicating that it might also originate from Hawaii.

The clustering of the Italian samples (S44 and S46) using PCA analysis was unsuccessful (Figure 2a), despite having many common characteristics. One of the parameters separating them is the high Sr value in the S44, which was sold as flakes. As mentioned earlier, this observation is believed to be due to contamination arising during the flaking process. Unlike S44, S46 was obtained fresh and was subsequently lyophilized. Unlike the flaking process, lyophilization does not cause contamination and has little effect on mineral loss, except in the case of Mn [52,53,61]. Sample S44 has higher Ca content (3.45 g/kg), while the content in S46 is lower (0.46 g/kg). This finding could result from different processing, drying, and flaking techniques and undeclared excipients, as shown in previous research [55,62,63]. Additionally, the drying technique used can explain the higher level of Mn in S44 (84.9 mg/kg) than in S46 (32.9 mg/kg). As previously shown, freeze-drying can cause a decrease in the Mn content compared to oven drying [55]. The Zn content was also higher in the flaked sample (24.9 mg/kg) than in S46 (7.59 mg/kg), which is again believed to be a result of the flaking process [63] or selected drying method, as different drying techniques affect the Zn content differently [55]. Substantial variations in mineral profile have been observed between natural *Spirulina* and commercial products by Campanella et al. [62], which could result from various changing parameters introduced by the commercialization of the product, such as *Spirulina* biomass treatment, processing (washing, drying), packaging, and distribution.

3.2.2. Discriminant Analysis of Spirulina Samples

Discriminant analysis (DA) was performed using macro-elemental and isotopic composition data (Figure 4) to investigate the distribution of variables in more detail and reveal possible differences among the samples originating from China (n = 16), Hawaii (n = 2), India (n = 4), Italy (n = 2), Portugal (n = 2), and Taiwan (n = 3). The first two discriminant components (F1 and F2) account for 90.3% of the total variance. In the discriminant function score plot (Figure 4a), each cluster (centroid) is represented by a scatter plot. In the loadings plot (Figure 4b), they appear as vectors demonstrating a degree of association of the corresponding initial variable with the first two discriminant components. Red vectors indicate the most significant variables, and blue vectors represent the least significant variables for sample separation and clustering. Six groups of samples are identified in the DA score plot (Figure 4a).

A leave-one-out cross-validation (LOOCV) classified 82.8% of the samples correctly. The prediction ability was the highest for Hawaii, Italy, and Portugal (100%), and was the lowest for Taiwan (66.7%). The most critical variables for sample separation are Fe, Br, K, and P. DA analysis confirms our previous findings regarding distinct elemental and isotopic composition of Hawaiian samples. In addition, the separation of Italian and Portuguese samples was achieved using DA (Figure 4a).



(**a**) (**b**)

Figure 4. Discriminant function score plot (**a**) and discriminant loadings plot (**b**) for *Spirulina* food supplements available on the Slovenian market (China (n = 16), Hawaii (n = 2), India (n = 4), Italy (n = 2), Portugal (n = 2), and Taiwan (n = 3)). Red vectors indicate the most, and blue vectors are the least significant variables for sample separation.

Despite previously presented differences, the Italian samples have several similar characteristics which separate them from other samples in this study. For example, they possess the lowest content of Si (S44: 0.78 g/kg, S46: 0.68 g/kg; M: 5.06 g/kg, IR: 1.46–14.9 g/kg) and one of the lowest values of P (S44: 8.56 g/kg, S46: 6.64 g/kg; M: 10.9 g/kg, IR: 10.1–12.1 g/kg), the highest content of K (S44: 20.6 g/kg, S46: 26.9 g/kg; M: 15.2 g/kg, IR: 14.2–16.8 g/kg) among all samples, and a high Br content (S44: 8.00 mg/kg, S46: 7.07 mg/kg; M: 1.84 mg/kg, IR: 1.25–3.18 mg/kg). Similarly, as with samples from Hawaii, such an elemental composition could reflect the growth medium used, which appears to be specific for this production region. Moreover, S44 and S46 have the lowest δ^{15}N values (–3.92 and –5.35‰, respectively), which points to the absence of organic fertilizers and the possible influence of natural processes such as nitrification, providing them with sufficiently specific δ^{15}N composition that distinguishes these samples from others. The Italian samples also have one of the lowest δ^{34}S values (S44: –0.61‰ and S46: 0.94‰; M: 11.0‰, IR: 6.79–12.7‰), which could be a result of combined meteoric water δ^{34}S value and that of the added sulfur compounds in the *Spirulina* culturing medium. It could also indicate that *Spirulina* in S44 and S46 was cultivated in freshwater, since the δ^{34}S values in seaweed are closer to seawater values, i.e., 17 to 21‰ [64,65].

Commercial *Spirulina* samples of Portuguese-declared origin appear to possess a distinct Mn and Br composition (Figure 4a,b). The Mn values for S29 and S30 are 192 and 195 mg/kg, respectively, with a median value for samples analyzed in DA (M_{All}) of 33.3 mg/kg (IR_{All}: 28.0–38.2 mg/kg). The Br values in these samples are 2.71 mg/kg for S29 and 3.21 mg/kg for S30 (M_{All}: 1.62 mg/kg, IR_{All}: 1.04–3.21 mg/kg). Additionally, Portuguese samples have the highest Fe content, i.e., 1.14 (S29) and 1.12 g/kg (S30) (M_{All}: 0.72 g/kg, IR_{All}: 0.56–1.39 g/kg) and Si content (S29: 15.6 g/kg, S30: 15.1 g/kg; M_{All}: 7.56 g/kg, IR_{All}: 1.43–15.1 g/kg). Mn is the most important parameter for separating Portuguese and Chinese samples, together with δ^{34}S values (S29: 7.34‰, S30: 7.01‰). The Mn, Br, and Fe levels in *Spirulina* products are highly dependent on their concentration in the growth medium; therefore, adding these minerals will result in increased levels in the microalgae. Moreover, the uptake of these elements is strongly affected by growth conditions, i.e., light intensity [28,30,31,66,67]. In this respect, Portuguese *Spirulina* product production might be specific. Regarding the Mn and Br content, using rich wastewater in these elements also increases their concentration in *Spirulina* [58]. High Si content results from silicon dioxide excipient's addition to the final product, which is evident from the declared product content (Table 1). The δ^{34}S composition of Portuguese *Spirulina* samples is in agreement with local δ^{34}S results for rainwater (δ^{34}S: 7.2‰) measured in neighboring Spain [68].

Further, OPLS-DA was applied to analyze the observed differences between six countries in the DA analysis and to investigate the goodness of fit (R^2X) and prediction (Q^2) for the model. Obtained OPLS-DA resulted in five predictive and no orthogonal components (5 + 0), producing an $R^2X = 0.73$, $R^2Y = 0.68$, and $Q^2 = 0.48$. The F1 Score rate obtained by internal cross-validation was 86.2%, sensitivity was 96.2%, specificity was 57.1%, and accuracy was 87.9%. This model displayed high quality and goodness of fit and predictability ($\geq$0.93) to differentiate among different countries, with the exception of Taiwan, where we obtained 0% predictability, which also supports our DA model. Moreover, OPLS-DA analysis for pairwise comparisons among all six countries (Figure 5) was calculated, similarly as in the study performed by Potočnik et al. [69]. The separation between classes in the OPLS-DA score plots is evident. In Figure 5, separation of the most numerous class (China) from Hawaii, India, Italy, Portugal, and Taiwan is presented, while additional pairwise comparisons are presented in Supplementary Materials (Figures S1 and S2). The prominent factors influencing OPLS-DA models were δ^{18}O, Rb, Br, Fe, δ^{2}H, Mn, Zn, K, and Cl. These results can be used to delve deeper into discussion regarding distinction of *Spirulina* sample groups, more precisely, Chinese, Indian, and Taiwanese groups.

The *Spirulina* samples of Chinese origin form the strongest group, with good discrimination results. The most critical variables for separation of this group (Figure 4a) are δ^{34}S, Sr, Si, and δ^{15}N. Specifically δ^{34}S values are high in these samples with the median value (M_{Ch}) of 12.9‰, which is higher than the median of all samples (11.0‰, IR_{All}: 6.72–12.9‰) included in the DA (M_{All}). High δ^{34}S values could result from combined rainwater δ^{34}S values from the production area and the addition of sulfur compounds in the *Spirulina* growth medium. Several studies have shown that rainwater in various Chinese regions contains a wide range of δ^{34}S values (0.30‰ to 19.1‰), with higher values attributed mainly to high atmospheric pollution from coal burning in the area. Additionally, heavier δ^{34}S values were observed in the winter, when coal burning from southern China is the dominant source of pollution [70–72]. Air pollution could, therefore, also be reflected in *Spirulina* products produced in polluted areas. The Sr values are also the highest among these samples, M_{Ch} for Sr is 28.1 mg/kg (M_{All}: 26.1, IR_{All}: 15.2–31.1 mg/kg), which could be attributed to specific *Spirulina* processing techniques used [52,53]. In contrast, Chinese samples contain the lowest amount of Si (M_{Ch}: 1.71 g/kg) among the samples included in the DA (M_{All}: 7.56 g/kg, IR_{All}: 1.43–15.1 g/kg), and the lowest δ^{15}N value (M_{Ch}: 2.32‰; M_{All}: 3.04‰, IR_{All}: 0.95 to 6.44‰). Lower Si content could partially be explained by the declared pure *Spirulina* composition of Chinese samples, while all Portuguese and half of the Indian samples contain Si as a part of the silicon dioxide excipient. Additionally, higher Si content in non-Chinese samples could come from various *Spirulina* growth medium mineral additions and Si in the water used for cultivation and different processing techniques. Different food processing techniques are known to reduce the Si content in the final product [73].

Results presented in Figure 5a–d confirm the results presented by PCA and DA analyses regarding distinct composition of Hawaiian, Italian, and Portuguese samples. Additional pairwise comparisons to confirm these findings are available in Figures S1 and S2.

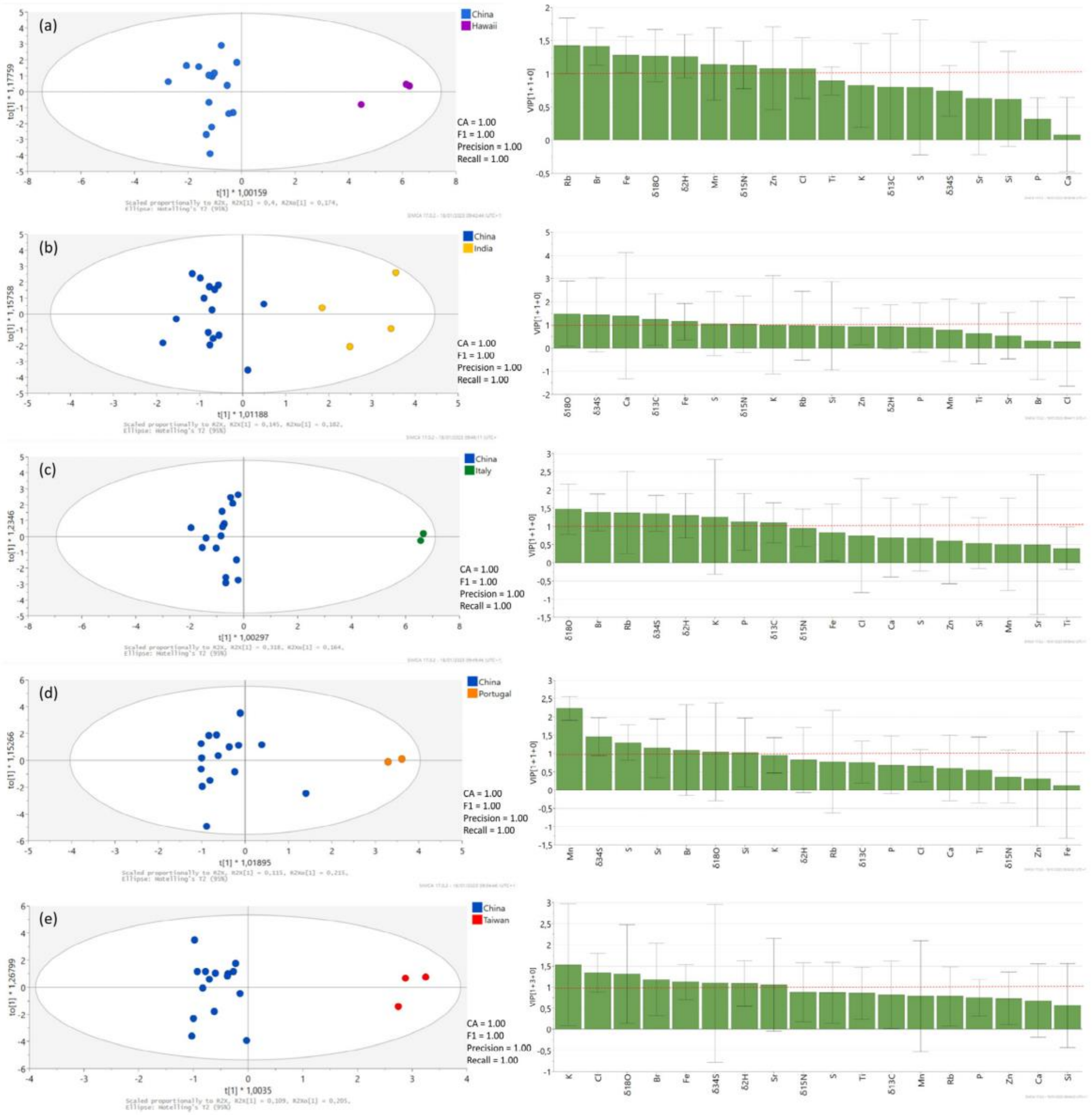


Figure 5. OPLS-DA score plots and VIP values in the pairwise comparisons between different declared countries of origin of *Spirulina* products derived from all isotopic and elemental composition data. The ellipse on the score plot represents the 95% confidence interval. Red dotted line indicates a criteria for identification of the variables, important for the developed model. Separation of the most numerous class, China, is presented, from Hawaii (**a**), India (**b**), Italy (**c**), Portugal (**d**), and Taiwan (**e**).

In addition, Figure 5b,e offer a closer look into separation of Chinese, Indian, and Taiwanese samples, where separation by DA was less successful. In Taiwanese samples, the highest δ^{13}C values were determined among samples analyzed with DA (M_{Taiw}: –21.8‰), while Chinese (M_{Ch}: –23.1‰) and Indian (M_{Ind}: –28.8‰) samples possess lower δ^{13}C values (M_{All}: –23.9‰, IR_{All}: –26.6 to –22.18‰). Differences in δ^{13}C composition could be due to the addition of different nutrients to the growth medium, excipients to the final product, and cultivation conditions (open or closed system). Closed production systems enable better control over cultivation conditions, such as loss of CO_2 to the atmosphere, temperature, and pH, and possess lower carbon dioxide δ^{13}C values [17,74]. There is a considerable difference in the δ^{34}S values among Indian, Taiwanese, and Chinese samples as well—the δ^{34}S values in Indian samples are substantially lower (5.7‰) than in Taiwanese samples (11.5‰) and Chinese samples (12.9‰). As Taiwan is an island, a higher influence of the sea than on the mainland (India, China) is expected. The δ^{34}S values in rainwater decrease while moving inland and further from the sea. Therefore, higher δ^{34}S values in rainwater and plants and algae are expected in regions closer to the sea [75]. In addition, Taiwan is a volcanic island, and therefore higher δ^{34}S values can be also expected [46,76]. Higher δ^{34}S values in Chinese samples, on the other hand, could be attributed, as mentioned previously, to high pollution levels in the area [70–72]. Separation of the Indian and Taiwanese samples from Chinese samples is also due to higher δ^{15}N values (M_{Ch}: 2.32‰, M_{Ind}: 6.13‰, M_{Taiw}: 6.22‰), which points to a specific fertilizing technique for these areas which possibly includes the use of organic fertilizers. Important parameters for distinction between Chinese, Taiwanese, and Indian samples are also δ^{18}O and δ^{2}H values, which are the highest in Taiwanese (δ^{18}O: 18.8‰, δ^{2}H: –171‰) and Indian samples (δ^{18}O: 18.7‰, δ^{2}H: –168‰), while the values in Chinese samples are lower (δ^{18}O: 16.8‰, δ^{2}H: –179‰). This could be attributed to the production of Taiwanese and Indian samples at lower latitudes, altitudes, or closer to the sea [20,22], similarly to Hawaiian samples. Another important variable that separates *Spirulina* samples from China, Taiwan, and India is the Si content, the median of which for Indian samples (M_{Ind}) is 13.8 g/kg, for Taiwanese samples (M_{Taiw}) is 7.94 g/kg, and for Chinese samples (M_{Ch}) is 1.71 g/kg (M_{All}: 7.56, IR_{All}: 1.43–15.1 g/kg), which can be explained by SiO_2's addition to the final product in the case of Indian samples. Taiwanese and Chinese *Spirulina*, on the other hand, are declared as pure. As described above, there are various parameters affecting the Si content in these products, such as addition during cultivation, varying water Si concentration, and different processing techniques [73]. Differences were also found in Zn (M_{Taiw}: 15.7 mg/kg, M_{Ch}: 15.8 mg/kg, M_{Ind}: 10.9 mg/kg), Fe (M_{Taiw}: 0.66 g/kg, M_{Ch}: 0.77 g/kg, M_{Ind}: 0.46 g/kg), and K (M_{Taiw}: 13.6 g/kg, M_{Ch}: 15.6 g/kg, M_{Ind}: 15.1 g/kg) values, which could be a result of mineral addition and use of different fertilizers in *Spirulina* growth medium [28,30,31,57,58].

According to the presented data, reliable and specific classification of samples S20, S21, S28, S39, S42, and S45 of undeclared origin (NS) is not possible. However, their elemental and isotopic compositions show similarity with Asian and declared non-EU samples (Figure 3). The findings of this study show the importance of combining both elemental and isotopic values in verifying the country of origin and authenticity of *Spirulina* samples. However, the prediction ability and assessment of authenticity should be improved in the future by including a higher number of samples, as well as verified pure *Spirulina* samples.

4. Conclusions

Interest in *Spirulina* dietary supplements is growing among consumers due to vegetarianism, increasing malnutrition, and health awareness in the population. The high demand for *Spirulina* products and the challenging culturing conditions needed for producing high-quality products make them a target for intentional adulteration and mislabeling [12,77]. This study has shown that combining stable isotope ratios of light elements (C, N, S, H and O) and elemental composition creates a promising tool for determining the authenticity of the commercial *Spirulina* dietary supplements, regarding their composition and geographical origin. Hydrogen and oxygen stable isotope ratios have been determined in *Spirulina*-based products for the first time in this study and show a correlation, as it occurs also in water, indicating that they originate mainly from local precipitation and that the influence of other parameters on their values is negligible.

A wide variability in the stable isotopic ratios and elemental composition among *Spirulina* samples of different declared origins was observed. Different statistical methods and reliable discrimination of Hawaiian, Italian, and Portuguese samples were also achieved, together with a good separation of Chinese samples. Discrimination between Taiwanese and Indian samples, however, was less successful but still notable. The parameters responsible for sample discrimination appear to be different culturing and processing techniques, environmental conditions (including pollution), and the geographical location of *Spirulina* production.

Additionally, this method shows promising results in exposing adulterated samples and samples mixed with other products and could be used in future studies for assessing product authenticity. A higher number of samples and more precise information on product culturing conditions, composition, and origin would result in more reliable results in *Spirulina* product authentication.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12030562/s1, Table S1: Elemental composition of *Spirulina* dietary supplements from the Slovenian market; Table S2: Composition of Zarrouk's medium; Figure S1 and S2: Pairwise comparisons between different declared countries of origin of *Spirulina* products.

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Table S1. Elemental composition of *Spirulina* dietary supplements from the Slovenian market.

S. No. [1]	Si (g/kg)	P (g/kg)	S (g/kg)	Cl (g/kg)	K (g/kg)	Ca (g/kg)	Ti (mg/kg)	Mn (mg/kg)	Fe (g/kg)	Zn (mg/kg)	Br (mg/kg)	Rb (mg/kg)	Sr (mg/kg)
S1	1.57	10.5	7.53	0.66	16.1	8.18	18.2	43.4	0.81	14.0	1.29	1.55	37.9
S2	7.69	11.4	7.53	3.07	14.8	1.32	56.9	159	3.29	43.6	11.2	7.47	10.0
S3	1.16	10.6	8.64	3.18	15.4	0.82	5.45	29.4	0.49	8.33	1.91	1.67	17.8
S4	13.5	10.0	7.57	5.77	17.1	2.20	46.5	128	3.48	52.7	16.5	11.9	24.3
S5	1.34	12.4	9.04	3.78	20.8	0.74	8.77	22.1	0.48	14.6	1.77	1.23	25.1
S6	21.7	12.7	6.47	0.09	11.0	1.28	9.31	54.9	0.90	13.9	0.50	4.12	10.2
S7	10.7	11.8	7.77	0.60	14.3	5.10	3.96	32.8	0.37	10.4	0.47	0.81	22.1
S8	16.0	11.5	7.53	1.03	14.7	2.04	60.6	35.0	1.39	16.0	0.91	0.91	27.3
S9	5.21	5.06	3.14	2.61	19.7	2.83	10.4	43.0	0.44	19.1	10.8	3.75	12.7
S10	2.34	14.1	9.38	2.11	18.5	3.09	35.5	36.6	1.68	18.5	1.34	1.58	34.7
S11	1.42	12.9	8.29	0.48	15.2	1.20	4.42	26.5	0.57	11.1	1.19	1.07	28.2
S12	1.63	13.9	8.42	1.77	16.8	5.24	12.3	38.2	1.38	33.0	1.39	1.48	31.8
S13	16.6	12.6	7.79	1.97	15.5	5.39	14.8	34.4	1.79	33.6	1.47	0.92	31.1
S14	7.94	12.2	7.54	0.19	13.6	1.00	9.91	34.9	0.69	16.5	0.57	2.23	7.41
S15	1.43	11.9	7.32	0.21	13.7	0.89	5.07	33.3	0.66	15.7	0.48	1.60	6.82
S16	1.59	14.7	7.50	0.52	14.3	2.78	6.08	36.3	0.65	17.5	0.91	0.50	12.7
S17	1.61	13.5	8.64	2.21	16.6	5.34	12.3	30.9	1.74	34.9	1.88	1.20	32.3
S18	2.74	12.6	6.17	0.60	8.63	63.5	43.1	47.1	0.75	11.1	9.11	2.55	478
S19	19.4	12.2	9.29	2.55	18.4	28.0	15.9	30.3	0.56	9.74	2.26	1.24	27.6
S20	16.8	12.1	8.39	3.04	16.3	2.43	47.5	29.1	1.13	23.0	2.00	1.91	32.2
S21	14.7	9.77	7.30	1.94	16.2	1.35	28.2	150	0.69	22.4	3.09	2.97	9.66
S22	1.85	6.82	3.60	0.55	7.40	1.37	11.1	21.1	0.39	7.69	1.24	1.06	8.00
S23	15.1	11.2	7.12	1.12	14.2	2.03	65.8	28.3	1.39	14.0	0.88	0.78	28.0
S24	1.40	9.27	6.05	0.87	12.5	2.45	19.0	88.3	0.77	15.4	1.04	1.16	11.2
S25	15.4	9.61	8.32	2.68	17.3	1.02	9.37	51.5	0.60	10.0	1.60	1.43	18.7
S26	15.0	10.9	7.91	5.63	17.5	2.28	42.3	185	3.09	35.5	17.4	9.96	14.1
S27	1.79	11.2	8.30	2.07	16.8	0.80	8.61	22.8	0.72	15.6	1.27	1.04	22.3
S28	4.91	10.3	8.66	3.47	17.4	0.72	2.58	26.2	0.47	7.27	2.54	2.44	15.4
S29	15.6	10.9	7.24	1.67	15.6	1.62	35.7	192	1.14	20.9	2.71	2.06	15.6
S30	15.1	10.1	6.99	1.63	15.2	1.50	34.5	195	1.12	21.7	3.21	1.33	15.2
S31	1.53	11.4	6.72	1.36	14.7	1.67	13.0	178	0.78	22.0	1.82	2.85	7.85
S32	16.1	10.2	8.15	2.62	14.2	2.54	39.4	35.9	1.39	22.9	1.86	2.30	35.5
S33	1.06	10.2	7.99	2.18	14.9	1.00	5.84	28.0	0.64	5.42	1.62	1.55	29.7
S34	16.9	10.9	8.09	4.34	15.9	0.91	7.82	27.8	0.52	11.3	4.47	0.57	31.2
S35	7.52	10.1	7.85	2.20	14.4	0.96	6.85	26.5	0.63	7.18	2.38	1.27	26.3
S36	1.97	11.4	7.10	2.58	14.9	2.17	24.7	34.5	1.01	17.0	6.74	1.23	55.0
S37	6.43	6.16	3.88	0.91	5.83	0.75	12.8	19.3	0.28	6.02	1.67	0.55	9.75
S38	7.56	10.3	8.04	0.30	9.00	4.93	2.81	24.9	0.41	13.3	0.67	1.33	26.1
S39	12.1	9.93	6.71	1.76	12.7	0.97	8.88	14.7	0.44	13.1	1.86	0.99	19.8
S40	8.49	10.2	8.06	0.34	8.84	5.52	4.39	28.3	0.42	14.7	0.85	1.09	29.3
S41	0.94	8.64	7.18	5.03	16.4	0.52	3.58	18.6	0.38	10.3	2.93	2.42	15.9
S42	1.07	10.1	7.72	4.70	15.1	0.69	8.89	29.1	0.55	9.91	3.78	1.21	23.7
S43	1.34	10.1	9.91	5.34	15.7	1.42	3.16	23.2	0.41	2.30	5.51	1.34	71.8
S44	0.78	8.56	6.63	2.75	20.6	3.45	10.4	84.9	0.69	24.9	8.00	6.55	86.2
S45	1.02	11.0	7.87	1.13	14.3	1.26	3.18	27.4	0.45	18.1	1.43	1.47	22.0
S46	0.68	6.64	7.38	5.36	26.9	0.46	5.36	32.9	0.93	7.59	7.07	4.21	4.39

[1] Sample number.

Table S2. Composition of Zarrouk's medium [1].

Constituents	Content (g/L)
K_2HPO_4	0.5
$NaNO_3$	2.5
K_2SO_4	1.0
NaCl	1.0
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.04
$FeSO_4 \cdot 7H_2O$	0.01
EDTA	0.08
$NaHCO_3$	16.8
	Content (mL)
Micronutrient solution (H_3BO_3 (2.86 g/L), $MnCl_2 \cdot 4H_2O$ (1.81 g/L), $ZnSO_4 \cdot 4H_2O$ (0.222 g/L), Na_2MoO_4 (0.0177 g/L), $CuSO_4 \cdot 5H_2O$ (0.079 g/L))	1.0

Reference:

1. Michael, A.; Kyewalyanga, M.S.; Mtolera, M.S.; Lugomela, C.V. Antioxidants Activity of the Cyanobacterium, *Arthrospira* (*Spirulina*) *Fusiformis* Cultivated in a Low-Cost Medium. *Afr. J. Food Sci.* **2018**, *12*, 188–195, doi:10.5897/AJFS2018.1688.

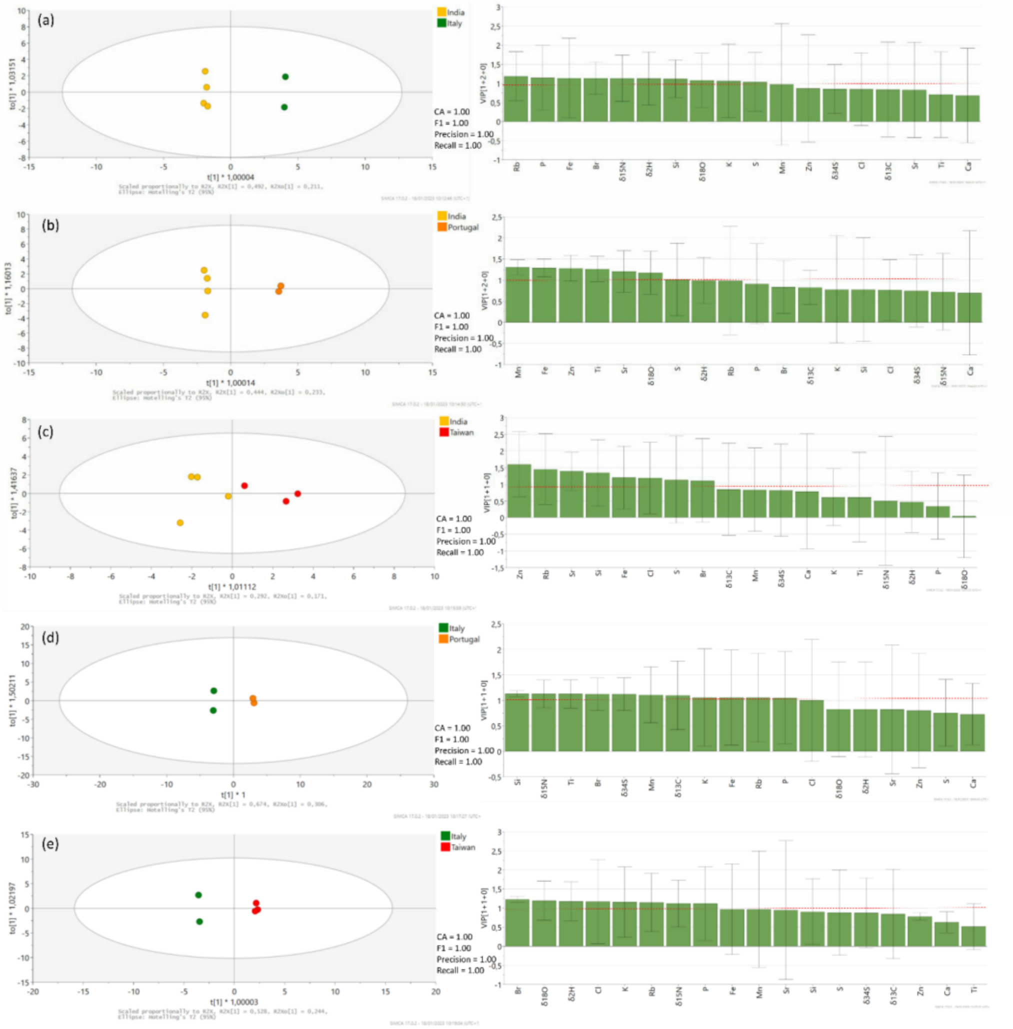

Figure S1: Pairwise comparisons between different declared countries of origin of *Spirulina* products: OPLS-DA score plots and VIP values, derived from all isotopic and elemental composition data. The ellipse on the score plot represents the 95% confidence interval. Red dotted line indicates a criteria for identification of the variables, important for the developed model. Separation of India from Italy (a), Portugal (b), Taiwan (c) and separation of Italy from Portugal (d) and Taiwan (e).

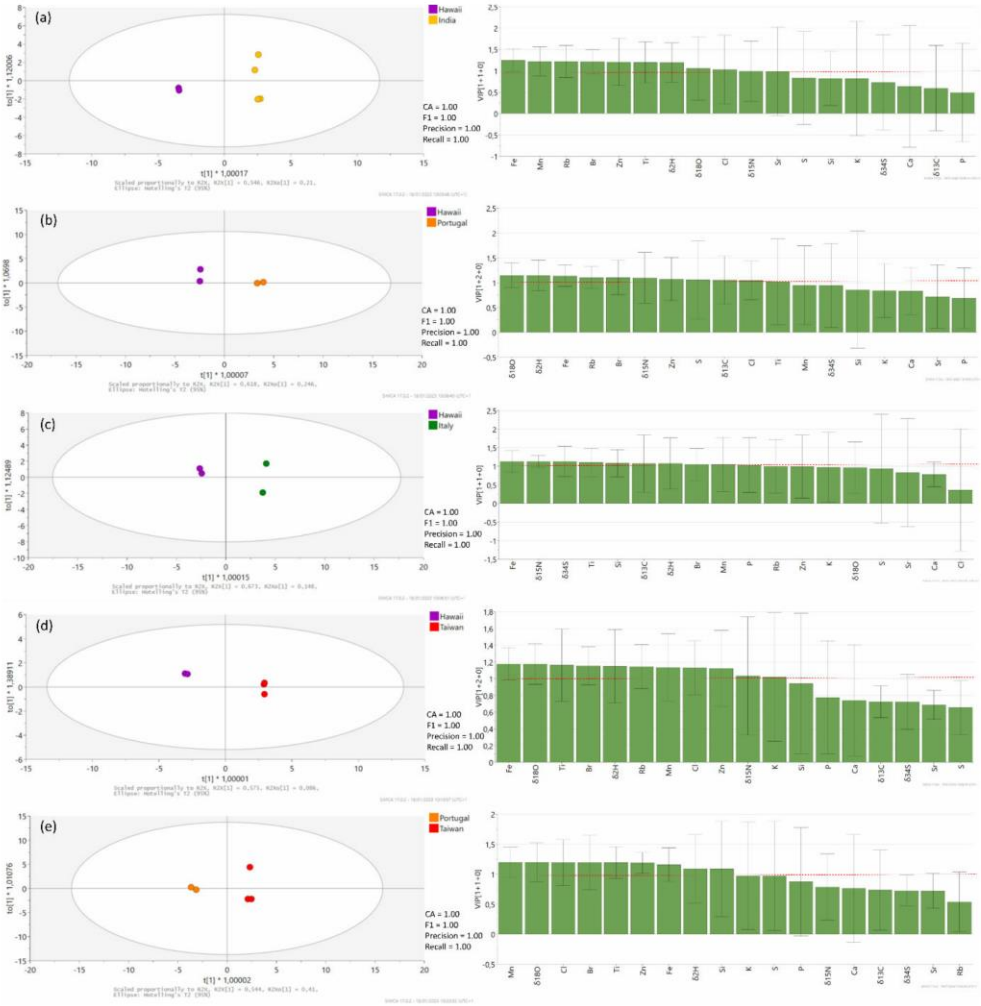

Figure S2: Pairwise comparisons between different declared countries of origin of *Spirulina* products: OPLS-DA score plots and VIP values, derived from all isotopic and elemental composition data. The ellipse on the score plot represents the 95% confidence interval. Red dotted line indicates a criteria for identification of the variables, important for the developed model. Separation of Hawaii from India (a), Portugal (b), Italy (c), Taiwan (d) and separation of Portugal and Taiwan (e).

3.6 Scientific Paper: "Characterization of Algae Dietary Supplements Using Antioxidative Potential, Elemental Composition, and Stable Isotopes Approach"

In this chapter, I present the paper entitled "Characterization of Algae Dietary Supplements Using Antioxidative Potential, Elemental Composition, and Stable Isotopes Approach" by Jan Kejžar, Marta Jagodic Hudobivnik, Marijan Nečemer, Nives Ogrinc, Jasmina Masten Rutar and Nataša Poklar Ulrih, which was published in Frontiers in Nutrition (IF 6.13) in 2021 and represented a part of the research performed in this PhD study. As a part of this study, my responsibilities were sample selection, collection and preparation for elemental and isotopic ratio content analysis. The research involved analysis of commercial algal products available on the Slovenian market and their differentiation based on their elemental and C, N and S stable isotope ratio profiles and antioxidative potential.

From several physical and web stores in Slovenia, eighteen samples of different types of algal products were obtained (Laminariales (kelps), *Spirulina* spp., *Aphanizomenon flos-aquae* (AFA) and *Chlorella* spp.) with conventional or organic production declared on the products. The samples were analyzed for their total phenolic content, radical scavenging activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, elemental composition by ED-XRF and ICP-MS and isotopic composition by EA-IRMS.

A 4.4 times higher and 2.7 times higher antioxidative potential was found in AFA compared to *Chlorella* spp. and *Spirulina* spp., respectively. High δ^{15}N values in *Spirulina* spp. (7.4‰ ± 4.4‰) samples were also found, which points to the use of organic nitrogen fertilizers. Differences in elemental composition and stable isotopic ratios of elements C, N and S among *Chlorella* spp. and *Spirulina* spp. samples are attributed to different nutrient sources used and cultivation techniques. A clear separation of *Spirulina pacifica* samples of Hawaiian declared origin was achieved due to a high content of manganese, iron, cobalt, zinc, vanadium and nickel. Furthermore, significantly higher phosphorus levels were observed for *Chlorella* spp., whereas *Spirulina* spp. had the highest calcium levels among all samples. The iodine content was the highest in kelp samples. Notably, mercury, lead, arsenic and cadmium levels were lower than the maximum allowed values and did not threaten consumer health. Principal component analysis showed that all tested AFA samples originate from the same location, which is believed to be the Klamath Lake (Oregon, USA), which is based on the characteristically high antioxidative potential, low P and high Ca, Sr and Mo content, high δ^{13}C values and relatively low δ^{15}N values. The separation between *Spirulina* spp. and *Chlorella* spp. samples was less successful using PCA. However, a lack of characteristic parameters and the limited number of samples means additional studies are required.

As a scientific conference poster presentation, part of this study was presented at the 1st ISO-FOOD International Symposium on Isotopic and Other Techniques in Food Safety and Quality, 1–3 April, Portorož, Slovenia in 2019, and in Prague, Czech Republic, at the 9th International Symposium on Recent Advances in Food Analysis, 5–8 November 2019. This study was partially presented orally at the 10th Jožef Stefan International Postgraduate School Students' Conference and the 12th Young Researchers' Day in Piran, Slovenia, 10th to 11th May 2018; and at the 11th Jožef Stefan International Postgraduate School Students' Conference and the 13th Young Researchers' Day in Planica, Slovenia, 15th to 16th May 2019.

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Characterization of Algae Dietary Supplements Using Antioxidative Potential, Elemental Composition, and Stable Isotopes Approach

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Dietary supplements based on algae, known for their nutritional value and bioactive properties, are popular products among consumers today. While commercial algal products are regarded safe by numerous studies, information about the production and origin of such products is scarce. In addition, dietary supplements are not as strictly regulated as food and medicinal drugs. We characterized different algal products (kelps: Laminariales, *Spirulina* spp., *Chlorella* spp., and *Aphanizomenon flos-aquae*), obtained on Slovenian market, based on their elemental composition (X-ray fluorescence, inductively coupled plasma–mass spectrometry), antioxidative potential [DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, total phenolic content], and stable isotope values [carbon (C), nitrogen (N), and sulfur (S); elemental analyzer isotope ratio mass spectrometry (EA-IRMS) method]. Antioxidative potential is consistent among products of the same type, with *A. flos-aquae* samples having 4.4 times higher antioxidative potential compared to *Chlorella* spp. and 2.7 times higher compared to *Spirulina* spp. Levels of toxic trace elements (arsenic, cadmium, mercury, and lead) are below the maximum allowed values and as such do not pose risk to consumers' health. Samples of *Spirulina* spp. have relatively high δ^{15}N (7.4 ‰ ± 4.4‰) values, which indicate use of organic nitrogen sources in certain samples. Likewise, different elemental composition and isotopic ratios of stable elements (C, N, and S) for the samples with *Spirulina* spp. or *Chlorella* spp. are the consequence of using different nutrient sources and algae-growing techniques. Statistical analysis (principal component analysis) has confirmed that all tested *A. flos-aquae* samples originate from the same source, supposedly Klamath Lake (Oregon, USA). Hawaiian *Spirulina pacifica* can also be differentiated from all the other samples because of its characteristically high metal content (iron, manganese, zinc, cobalt, nickel, vanadium). *Chlorella* spp. and *Spirulina* spp. require further analyses with larger number of samples, as differentiation is not possible based on results of this study.

Keywords: algae, *Spirulina*, *Chlorella*, *Aphanizomenon flos-aquae*, antioxidative potential, stable isotopes, elemental composition, toxic elements

INTRODUCTION

There are numerous algae-based dietary supplements available on the market, which indicates their widespread use among consumers. Dietary supplements are not subject to strict regulations like drugs and imported food. Therefore, continuous evaluation of efficacy, safety, and origin is required to guarantee quality of dietary supplements. Comparison between different products is also complicated because of addition of unknown compounds, which is a common practice among manufacturers. Microalgae (unicellular eukaryotes and cyanobacteria) are interesting organisms to cultivate because of their ability to synthesize bioactive compounds and accumulate minerals and high nutritional value. They are able to grow in modified mediums, including wastewater, which additionally improves economic viability of cultivating microalgae (1). Currently, most producers of microalgae-based commercial products are located in Asia or Australia and show an impressive growth. Production share of food/feed microalgae products owned by European companies is estimated to be approximately 5% of the global market (2).

Microalgae-based products (*Spirulina* spp.—*Arthrospira* spp., *Chlorella* spp., and *Aphanizomenon flos-aquae* or AFA) have the highest market share among "algal" dietary supplements. *Spirulina* spp. products, in tablet or powder form, are mostly consumed because of their nutritional profile: protein (60–70%), carbohydrates (14–19%), fat (8%), dietary fibers (3%), vitamins (<1%), and phytochemicals (3, 4). Algae products are also regarded as a significant source of major elements, such as iron (Fe), calcium (Ca), phosphorus (P), potassium (K), sodium (Na), and magnesium (Mg), and trace elements, including manganese (Mn), zinc (Zn), copper (Cu), selenium (Se), and chromium (Cr). Recommended daily amount of aforementioned algae can provide substantial amount of these minerals and even fulfill recommended dietary allowance for iron intake (4, 5). Studies on content of toxic elements and cyanotoxins are scarce (6). Studies (7–10) done on safety of microalgae do not necessarily reflect safety of algal products, as commercial cultivation practice is unknown and not subject to strict regulations. Results acquired from laboratory grown algae are potentially misleading as different growing conditions significantly impact the content of certain elements and synthesized metabolites in algae (1). Determining efficacy and safety of algal products therefore requires analysis and comparison of individual samples from different manufacturers.

Manufacturers provide little to no information regarding the origin and manufacturing practices of their algae-based dietary supplements. Nutrient composition and toxic compounds differ, depending on the location of sample production. Reasons are various environmental conditions and agrotechnical measures. Thus, to ensure quality and safety of the products used in daily nutrition, determination of product's origin is of great importance (11). Variable environmental conditions that influence microalgal growth can consequently affect the stable isotopic composition of carbon, nitrogen, and sulfur (C, N, and S). These parameters along with elemental composition could be used to verify the quality and origin of microalgal products.

The aim of our study is to differentiate commercially available algal products on Slovenian market by characterizing them based on antioxidative potential, elemental composition and stable isotope composition of C, N, and S, as they can reflect different growing conditions, sources of nutrients and origin. To our knowledge, such an approach has not yet been used in any previous study of algae-based supplementary products.

MATERIALS AND METHODS

Sample Collection

In the present study, 18 samples were obtained from several physical stores and web stores in Slovenia (2018). Dietary supplements were selected based on several types of algae [kelps: Laminariales ($n = 2$), *Spirulina* spp. ($n = 7$), *Chlorella* spp. ($n = 5$), and AFA ($n = 4$)] with different types of production—conventional and organic. The samples were intended for sale on Slovenian market and have declaration in Slovene language (**Table 1**).

Sample Preparation

Samples in tablet form were ground to fine powder, and samples in capsules were opened. All samples were subsequently stored in powdered form in plastic containers with screw caps. During analysis, all samples were stored at room temperature and kept away from direct sunlight, following the manufacturers' storage guidelines. Sample preparation step was repeated for each individual analysis.

Sample Extract Preparation for Determination of Total Phenolic Compounds and Antioxidative Potential

Five hundred milligrams of fine powder sample was added to 10 mL 80% methanol solution in a centrifuge tube. After vortex mixing it for 5 min, it was incubated in ultrasound bath for 30 min at 40°C. Following incubation, samples were centrifuged for 20 min at 9,400 rcf (at 20°C) and filtrated through filters with 0.32-µm pore width into centrifuge tubes. Ten milliliters of 80% methanol solution was added to resulting sample sediment, and the whole procedure was repeated for each sample. We added 80% methanol solution until 20-mL volume was reached. Sample extracts were prepared in duplicate. Extract of each duplicate sample was stored in four vials, containing 5 mL extract each (total 20 mL per sample duplicate) at −20°C.

Total Phenolic Content

The total phenolic content (TPC) of algal methanolic extracts was determined by Folin–Ciocalteu method (12). Twenty milliliters of Folin–Ciocalteu reagent solution was prepared by mixing MilliQ water (resistivity of 18.2 MΩ*cm (at 25°C) and total organic C value <5 ppb) in ratio 1:10. Mixture in screw cap tube was prepared by adding 0.2 mL Folin–Ciocalteu solution, 0.2 mL sufficiently diluted methanolic sample extract, 1 mL Na carbonate solution (mass concentration of 75 g/L), and 2 mL MilliQ water. After thorough vortex mixing, the mixture was incubated for 2 h in the dark at room temperature, followed by 5-min centrifugation at 2,000 RPM.

TABLE 1 | List of algae-based dietary supplement samples obtained on Slovenian market with the information on purity, origin, and suggested daily use.

Algae	Sample	Purity	Origin	Declared growing practice
Laminaria digitata and *Ascophyllum nodosum*	S1	Additives	Not specified	Conventional
Macrocystis pyrifera	S5	Additives	Not specified	Conventional
Aphanizomenon flos-aquae	S2	Additives	Klamath Lake	Conventional
A. flos-aquae	S10	Pure	Klamath Lake	Organic
A. flos-aquae	S11	Pure	Klamath Lake	Conventional
A. flos-aquae	S12	Additives	Klamath Lake	Conventional
Chlorella pyrenoidosa	S3	Pure	Not specified	Conventional
Chlorella sp.	S4	Pure	China	Conventional
Chlorella sp.	S7	Additives	Not specified	Conventional
Chlorella vulgaris	S8	Pure	Outside of EU	Organic
Chlorella sp.	S9	Pure	Outside of EU	Organic
Spirulina platensis	S6	Pure	Not specified	Conventional
Spirulina pacifica	S13	Additives	Hawaii	Conventional
Spirulina sp.	S14	Pure	Outside of EU	Organic
S. platensis	S15	Pure	Outside of EU	Organic
S. platensis	S16	Pure	Taiwan	Organic
S. pacifica	S17	Additives	Hawaii	Conventional
Spirulina maxima	S18	Additives	Italy	Conventional

Spectrophotometer was calibrated using blind sample. The sample was prepared in the same way as other samples, except that 0.2 mL of 80% methanol was added instead of 0.2-mL sample extract. All measurements were performed at wavelength of 750 nm. Sample dilution ratio was determined for each individual sample by test runs using the same procedure. Kelp samples' total phenolic compounds content was too low to be detected by our method (even after the manipulation of sample dilution ratio in final mixture). Calibration curve for TPC analysis was prepared with gallic acid in triplicate with concentrations 5, 10, 15, and 20 mg/mL. Gallic acid solutions were prepared according to the same protocol as samples, in 80% methanol solution. Results were expressed as mg gallic acid equivalent (GAE)/g solid sample mass.

DPPH Assay

The free radical–scavenging activity of algal extracts were measured by the decrease of absorbance of methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (13). A stock methanolic solution of DPPH (0.0837 µM) was prepared by mixing 3.3 mg DPPH in 100 mL of pure methanol. Absorbance of stock DPPH solution was ~1.1 at 517 nm. Final mixture was prepared by mixing 0.5 mL of sufficiently diluted sample extract and 2.5 mL of methanolic DPPH solution. Blank samples were prepared with 0.5 mL diluted sample extract and 2.5 mL pure methanol. Control sample was prepared with 0.5 mL pure methanol and 2.5 mL methanolic DPPH solution. Absorbance was measured after 30-min incubation period at room temperature in the dark.

Sample dilution ratio was determined for each individual sample by test runs using the same procedure. Antioxidative potential of kelp samples was below detection of our method and could not be determined. On the contrary, AFA samples required dilution in ratio 1:5. Calibration curve for DPPH analysis was prepared with gallic acid in triplicate with concentrations 6, 4.5, 3, 1.5 and 0.75 µg/mL. Like samples, gallic acid was prepared in 80% methanol solution.

Elemental Composition Determination Using X-Ray Fluorescence

X-ray fluorescence (XRF) analysis was performed at Jožef Stefan Institute, Ljubljana. Powdery samples were pressed into tablets. Quality assurance for element analysis was performed using standard reference materials 1573A National Institute of Standards and Technology (NIST) tomato leaves and 1547 NIST peach leaves, acquired from the NIST (Gaithersburg, MD, USA). XRF was used to determine the following elements: bromine (Br), calcium (Ca), chlorine (Cl), iron (Fe), iodine (I), potassium (K), manganese (Mn), phosphorus (P), rubidium (Rb), sulfur (S), silicon (Si), strontium (Sr), titanium (Ti), and zinc (Zn).

Sample Preparation for Inductively Coupled Plasma–Mass Spectrometry

Samples were digested using an UltraWAVE digestion system (Millestone, Italy); 0.05–0.1 g of sample was weighted directly into Teflon vial, followed by addition of 2 mL of 65% HNO_3 (Suprapur, Merck, Germany). Digestion temperature program was as followed: from room temperature to 240°C in 20 min, held on 240°C for 15 min, and then cooled to 40°C (approximately 1 h). Maximum pressure was set at 100 bar. Loading gas (N_2) was set at 25 bar and room temperature.

Digested samples were transferred into plastic vials and filled to 10-mL mark with MilliQ water. Samples were additionally diluted with 5% HNO_3 in 1:5 ratio. Because of visible residuals, the samples were filtered through 0.45-µm hydrophilic syringe filters (Millipor Millex-HV, Merck, Germany). Quality assurance for element analysis was performed using standard reference material BCR-414 (trace elements in plankton) with known elemental composition. Reagents' blanks were prepared according to the same protocol as samples.

Elemental Composition Determination Using Inductively Coupled Plasma–Mass Spectrometry

Inductively coupled plasma–mass spectrometry (ICP-MS) analysis was performed on an Agilent 8800 triple quadrupole instrument (Agilent Technologies, California, USA). Calibration curve for mercury (Hg) content determination was prepared using NIST 3133 Hg standard solution in the following concentrations: 0, 0.1, 0.5, 1, and 5 ng/mL. Elements [V, Mn, Co, Ni, Cu, Zn, As, Se, Sr, Mo, Cd, Pb and Fe] were determined using MULTI XVI (Merck, Germany) multielement standard solution for ICP-MS. Calibration curve was prepared in 5% HNO_3 by using the following concentrations of MULTI XVI: 0, 0.1, 0.5, 1, 5, 10, 50, 100, and 250 ng/mL.

Each sample was analyzed in dilutions (with 5% HNO_3) to 10 and 100 mL. Samples S1, S4, S7, and S10 were prepared in duplicate (two parallels for 10-mL dilution, two parallels for 100-mL dilution). ICP-MS was used for determination of following trace elements: As, Cd, Co, Cu, Hg, Mn, Mo, Ni, Pb, Se, Sr, V, and Zn.

Stable Isotope Ratio Analysis of Light Elements Using EA-IRMS

Stable isotope ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{34}S/^{32}S$ were expressed as δ values in ‰ according to the following equation (14):

$$\delta\,^{i}_{j}E = \frac{^{i}_{j}R_{sample} - ^{i}_{j}R_{ref}}{^{i}_{j}R_{ref}} \qquad (1)$$

where E represents element (C, N, S), R is isotope ratio between heavier "i" and lighter "j" isotopes ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) in the "sample" and reference material ("ref"). Values for C were expressed relative to V-PDB (Vienna-Pee Dee Belemnite) standard, N values relative to AIR, and S values relative to V-CDT (Vienna Cañon Diablo Troilite) standard.

Stable isotope ratios of light elements ($^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$) in algae samples were simultaneously determined by isotope ratio mass spectrometry with preparation system for solid samples IsoPrime 100–Vario PYRO Cube (OH/CNS Pyrolyser/Elemental Analyzer, Elementar Analysensysteme GmbH, Germany). Four milligrams of sample and 4 mg of tungsten oxide (WO_3) were weighted directly into tin capsules, sealed, and placed into the automatic sampler of the elemental analyzer. Each sample was measured in triplicate, and the average value was considered. Quality assurance for stable isotope ratio analysis was performed using the following reference materials: USGS-43: $\delta^{13}C = -21.28 \pm 0.10‰$, $\delta^{15}N = +8.44 \pm 0.10‰$, $\delta^{34}S = +10.46 \pm 0.22‰$; B2155 Protein Sercon: $\delta^{13}C = -26.98 \pm 0.13‰$, $\delta^{15}N = +5.94 \pm 0.08‰$, $\delta^{34}S = +6.32‰ \pm 0.8‰$; Casein Protein CRP: $\delta^{13}C = -20.34 \pm 0.09‰$, $\delta^{15}N = +5.62 \pm 0.19‰$, $\delta^{34}S = +4.18‰ \pm 0.74‰$. Measurement precision value was $\pm 0.2‰$ for $\delta^{13}C$, and $\pm$ 0.3‰ for $\delta^{15}N$ and $\delta^{34}S$.

Statistical Analysis

Statistical calculations were carried out using the XL-STAT software package (Addinsoft, Long Island, NY, USA, 2019). First, basic statistics were applied to the data. Because most of the data were not normally distributed (Shapiro-Wilk test, $p < 0.05$), the nonparametric Mann–Whitney U test was used for comparison of element content between different microalgae products. In all analyses, $p < 0.05$ was considered as statistically significant.

Further, principal component analysis (PCA) was applied. PCA is an unsupervised pattern recognition multivariate statistical tool able to analyze numerical dataset structured in an M observations/N variables table and recognize underlying patterns in the dataset. The results of such analysis are displayed as biplots, which are simultaneous representations of variables and observations in the space of selected two PCA axes (e.g., PC1/PC2). The biplots enable visualization and increase interpretability of relation and trends among observations and variables on a two-dimensional map and identify similarities and differences among observations, association with variables, and also impact and role of a particular variable in discrimination and clustering of observations.

PCA was used to enable identification of characteristic parameters that are able to discriminate samples based on antioxidative potential, stable isotope composition of light elements and elemental composition. Samples of kelps (Laminariales) were excluded from PCA, because of relatively low content of algae in dietary supplement products and significant physiological differences compared to microalgae.

RESULTS AND DISCUSSION

Total Phenolic Content and Antioxidative Potential

Average values of TPC of different dietary supplements based on algae, expressed as mg GAE/g solid sample, were 12.3 ± 1.2 for samples of *Chlorella* spp., 23.2 ± 6.4 for *Spirulina* spp., 96.4 ± 7.5 for AFA, and 1.6 ± 1.6 for kelp (**Figure 1**). AFA samples contained the highest amounts of TPC, followed by *Spirulina* spp. and *Chlorella* spp. For comparison, Al-Dhabi and Valan Arasu (15) determined TPC values of 2.4–24.4 mg GAE/g sample for *Spirulina* spp. Kelp samples had negligible TPC content, presumably due to the low algae content in the sampled product itself (13–14% according to the declaration). It should be noted that kelp samples were not homogenous (brown algal parts tabletized with filler); therefore, the measurements might be incorrect.

Average antioxidative potential values, determined by the DPPH method, for different types of algae-based dietary supplement samples (expressed as mg GAE/g solid sample) were 1.33 ± 0.08 for *Chlorella* spp., 2.17 ± 0.43 for *Spirulina* spp., and 5.90 ± 0.71 for AFA (**Figure 1**). Antioxidative potential of kelp was significantly lower in comparison to other algae samples and as such could not be measured by using our method.

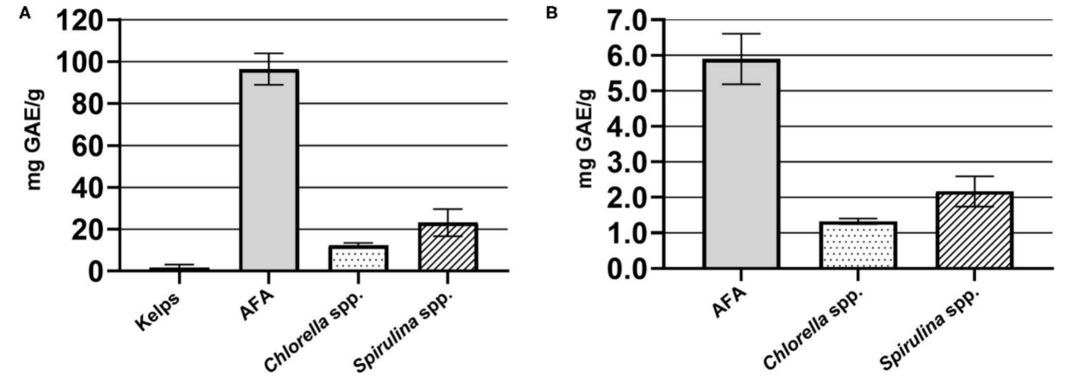

FIGURE 1 | (A) Average total phenolic compounds (Folin–Ciocalteu method); **(B)** average antioxidative potential values (DPPH method) in different types of algae-based dietary supplement samples, expressed as mg gallic acid equivalent (GAE)/g solid sample.

AFA samples showed the highest measured antioxidative potential, which was 4.4 times the value of *Chlorella* spp. samples and 2.7 times the value of *Spirulina* spp. samples. There were no significant differences among similar products from different manufacturers regarding antioxidative potential. The latter is evident from the relatively small deviations in measured values among sample groups with the same algae type (**Figure 1**). Consequently, antioxidative potential allows discrimination of samples between different algae types. It should be noted that presented results of antioxidative potential do not assess the efficacy in relation to health benefits of algal products, as our research goal is mainly characterization of different product types.

Stable Isotope Composition of Light Elements

δ^{13}C value in algae is reflected by their C source (16). Average δ^{13}C value of *Spirulina* spp. samples was −23.0‰ ± 4.0‰. Unusually high δ^{13}C value of −17.4‰ of sample S18 could be explained by declared addition of corn maltodextrin, which has characteristic δ^{13}C value of C4 plants (from −15‰ to −12‰) (17). *Chlorella* spp. had similar δ^{13}C values with an average value of −27.5‰ ± 5.7‰, with sample S3 having the lowest value of −37.1‰ (**Table 2**). The low δ^{13}C value determined in *Chlorella* spp. could be related to the growing conditions in a closed system. Closed systems are closed bioreactors with higher control over growing conditions (pH and temperature), higher photosynthetic efficiency, lower water evaporation rate, and lower CO_2 loss to the atmosphere (18). It was found that the δ^{13}C value in a closed system exhibits lower δ^{13}C value of CO_2. Because of relatively small deviation among samples of *Spirulina* spp. and *Chlorella* spp., we assume they utilize similar sources of HCO_3^- and CO_2 during cultivation, presumably ones that have shown to be the most efficient from manufacturer's point of view.

TABLE 2 | δ^{13}C, δ^{15}N and δ^{34}S values in algal dietary supplement samples.

Algae	Sample	$\delta^{13}C_{V-PDB}$ (‰)	$\delta^{15}N_{AIR}$ (‰)	$\delta^{34}S_{V-CDT}$ (‰)
Kelp	S1	−25.5	8.8	15.5
	S5	−22.1	10.3	16.8
Aphanizomenon flos-aquae	S2	−16.8	1.8	4.9
	S10[a]	−15.4	−0.5	5.1
	S11	−15.8	0.4	5.3
	S12	−14.1	−0.6	5.3
Chlorella spp.	S3	−37.1	−0.7	1.7
	S4	−25.9	6.6	−0.9
	S7	−25.7	3.4	−3.0
	S8[a]	−22.0	−3.4	1.3
	S9[a]	−27.0	11.8	−0.9
Spirulina spp.	S6	−27.7	1.2	−0.2
	S13	−25.8	10.8	8.8
	S14[a]	−26.1	8.8	11.3
	S15[a]	−22.1	7.6	−0.6
	S16[a]	−21.8	6.2	11.5
	S17	−24.4	13.8	7.8
	S18	−17.4	13.3	11.0

[a] *Declared as organic.*

Spirulina spp. samples had an average δ^{15}N value of 7.4‰ ± 4.4‰, indicating organic source of N in samples with high δ^{15}N values (>6‰), possibly due to wastewater use (19). This also applies to kelp samples. Higher δ^{15}N values also indicate usage of modified mediums for algae cultivation, as the latter can significantly improve economic viability of the project. One sample (S6) of *Spirulina platensis* differed from other samples with δ^{15}N value of 1.2‰, indicating inorganic source of N or molecular N (air) fixation. Differences in δ^{15}N values between samples can also be explained by other factors, such as (i) different climate, which is hard to control in open bioreactors, and (ii) recycling of growth medium (1). Samples of *Chlorella* spp. generally showed lower δ^{15}N values (3.5 ± 6.0‰) compared to *Spirulina* spp. samples. This is probably due to *Chlorella* manufacturers using less optimized and modified growing methods compared to *Spirulina* manufacturing. *Spirulina* is able to grow in saline environments (8), which is exploited to prevent contamination by other microorganisms when growing in "low-quality" media. As original media use mostly inorganic source of N (1), we can assume that most samples were grown using modified media. Lack of information from manufacturers makes interpretation of results rather difficult, as we do not have any insight into geographical factors that may affect fractionation.

AFA samples had similar stable isotope composition of C and N, which indicates that samples originate from the same source (all AFA products are declared to originate from Klamath Lake, OR). Small deviations in stable isotope composition of C and N in sample S2 were probably due to presence of additives in the product. Relatively high values of δ^{13}C (−15.5 ± 1.1‰) in AFA samples might indicate photosynthetic fixation of CO_2 from air as their primary source of C. Values of δ^{15}N were around zero (0.3 ± 1.1‰), indicating fixation of molecular N from air.

The δ^{34}S values in algae-based dietary supplement samples ranged from −3.0 to 16.8‰, with the lowest values observed in *Chlorella* spp. and the highest in kelps (brown algae). Variability in δ^{34}S values among the samples of same algae species (with the exception of AFA) was on average higher for C and N. This variability can be explained by different origins of samples or differences in organic load during growing conditions. However, the information regarding the distribution of the δ^{34}S values of aquatic resources and organisms is scarce. There are three potential sources of S in algae, depending on the proximity to the ocean, geology, and redox chemistry. For example, the δ^{34}S of marine sulfate and vegetation near the ocean are approximately +20‰ but decrease to +6‰ over 100 km (20, 21). In the Hawaiian Islands, δ^{34}S values of sulfates from volcanic ash and basalt-derived soils ranging from 6.3 to 15.4‰ (22) have been reported and are also in agreement with our data.

Elemental Analysis

The results of the elemental composition in microalgae supplements are presented in **Supplementary Table 1**. No statistically significant difference between different types of algae supplements was observed for Si, V, Mn, Ti, Co, Ni, Rb, Cu, Se, Mn, Fe, and Hg. A significantly higher content of Ca was observed for *Spirulina* spp. products, whereas *Chlorella* spp. displayed the highest P level. This observation agrees with the study performed by Rzymski et al. (7). AFA products exhibited high Ca and Mo concentrations that differ statistically significantly from other products. The highest concentrations of Sr, Br, and Cl were determined in kelps samples and differ significantly from other products mainly from *Chlorella* spp. Zn levels did not differ between *Spirulina* spp. and *Chlorella* spp., but they are significantly higher than those observed in AFA and kelps.

Iron Content

Average Fe content (mg Fe/g solid sample) in samples of *Chlorella* spp. was 1.00 ± 0.52, *Spirulina* spp. 1.36 ± 1.33, AFA 0.44 ± 0.05, and kelps 0.13 ± 0.01 (**Figure 2**). High deviation among *Spirulina* spp. samples is due to higher Fe content in Hawaiian *Spirulina pacifica* samples (3.29 ± 0.27). Iron content in *Spirulina* reflects the Fe content in the medium used for cultivation (23).

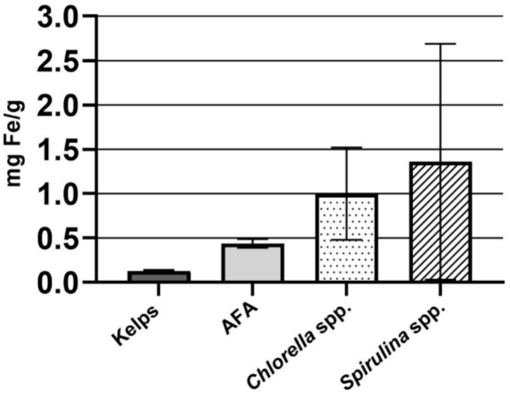

FIGURE 2 | Average iron content in algal dietary supplement samples, expressed as mg iron/g solid sample.

Iodine Content

Iodine content in kelp samples was 183 mg/kg solid sample for S1 and 221 mg/kg for S5, with 3% and 11% deviation from values declared on the product. Variability of I content in kelp-based supplements is therefore lower compared to edible seaweed (24). Other algae samples had I content below the limit of detection of XRF method.

Toxic Elements Content in Algal Dietary Supplements

Statistically significant difference in As concentration was found between *Chlorella* spp. and *Spirulina* spp. samples and AFA and kelp-based algae. Average total arsenic (As) content of samples was 0.26 ± 0.17 mg/kg for *Chlorella* spp. and 0.73 ± 0.96 mg/kg for *Spirulina* spp. samples (**Figure 3**). AFA and kelp-based samples had higher total As content, which was between 3.5 and 6.5 mg/kg solid sample. At the time of writing, European Commission (25) has not set upper tolerable level for As content in dietary supplements. It should be noted that our analysis determined only total As. For health risk assessment, determination of As species is required as the inorganic form of As is more toxic compared to the organic form.

Cd content was below maximum allowed value for dietary supplements of 1.0 mg/kg, set by European Commission (25) (**Figure 4**). Kelp sample (S5) had notable Cd content of 0.55 mg/kg solid sample and as such differs significantly from other samples. Other samples had Cd content between 0.011 and 0.064

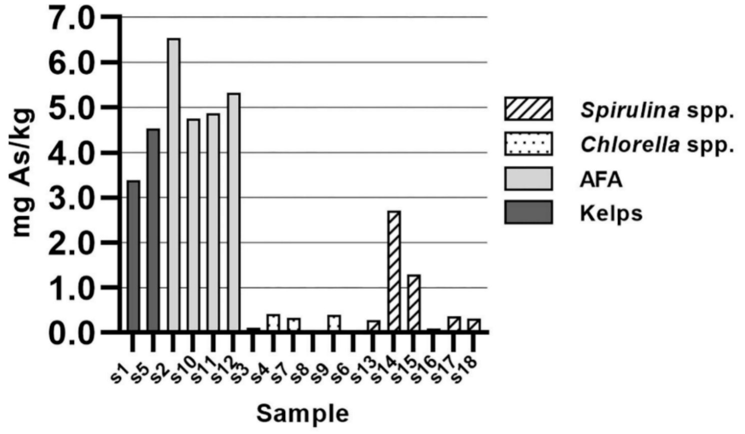

FIGURE 3 | Arsenic content in samples of algal dietary supplements, expressed as mg total arsenic/kg solid sample.

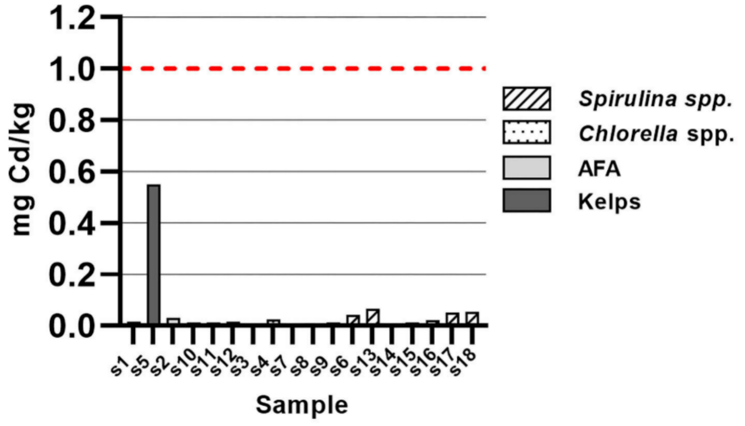

FIGURE 4 | Cadmium content in samples of algal dietary supplements, expressed as mg total cadmium/kg solid sample.

mg/kg. With respect to Cd content, none of the samples pose risk to consumers' health.

Samples of kelps (S1) and AFA (S2) exceeded maximum allowed value of Hg in dietary supplements by factor of 3.5 and 4.4, respectively (**Figure 5**). Maximum allowed value of Hg in dietary supplements is set at 0.1 mg/kg by European Commission (25). Hg content of other samples was below maximum allowed value. One sample of *Chlorella* spp. (S8) had Hg content below LOD. Despite the exceeded values of Hg in two samples, it should be noted that maximum Hg content for fish is set much higher (compared to dietary supplements) at 1.0 mg/kg fish muscle (25). By ingesting manufacturer's recommended daily dose of sample (4.02 g) with highest Hg content, we would ingest 1.76 µg of Hg. In contrast, eating 100 g of fish flesh with Hg content at limit (1.0 mg/kg) would equate to ingesting 0.1 mg Hg, which is 57 times higher than daily dose of sample (S2) with the highest Hg content.

Pb content was below maximum allowed value (3.0 mg/kg) (25) in all samples of algal dietary supplements (**Figure 6**). Kelps sample (S1) had Pb content below LOD. Pb content of samples was 0.35 ± 0.22, 0.23 ± 0.19, and 0.02 ± 0.00 mg/kg for *Spirulina* spp., *Chlorella* spp., and AFA, respectively, where *Spirulina* spp. differ significantly from AFA samples. In contrast with our

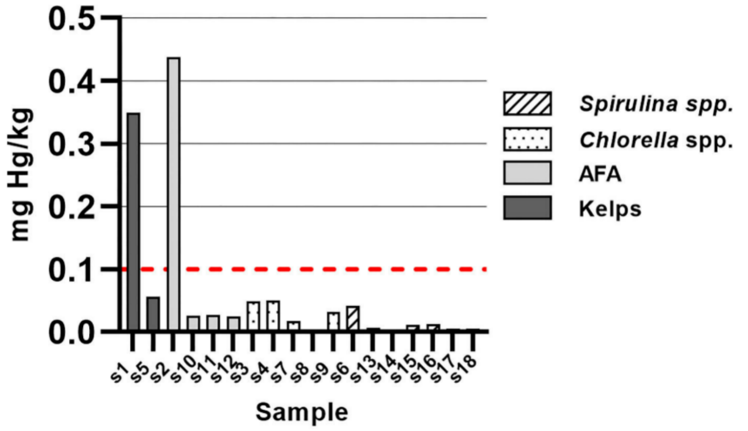

FIGURE 5 | Mercury content in samples of algal dietary supplements, expressed as mg total mercury/kg solid sample.

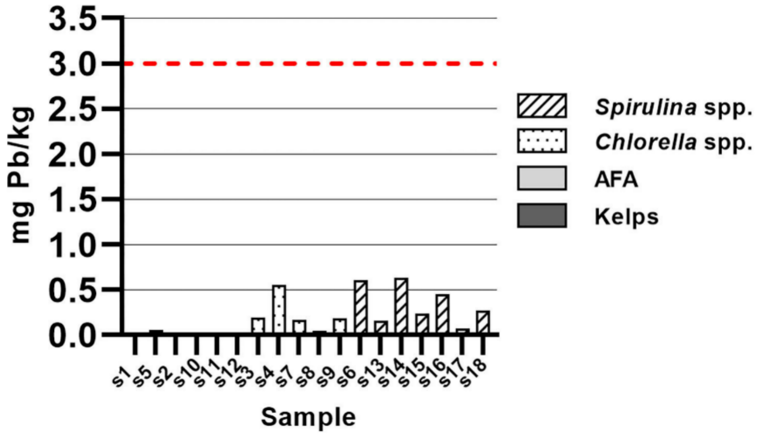

FIGURE 6 | Lead content in samples of algal dietary supplements, expressed as mg total Pb/kg solid sample.

results, Rzymski et al. (7) report of average Pb content of 2.6 ± 1.3 mg/kg for *Chlorella* spp. samples and 2.6 ± 1.9 mg/kg for *Spirulina* spp. samples, where 40% of *Chlorella* spp. and 30% of *Spirulina* spp. samples exceeded maximum allowed value.

Toxic elements such as Hg, Cd, Pb, and As were detected only in trace amounts and as such do not pose risk to consumers' health, which is in agreement with other studies (7, 10). It should be noted that algal products also require determination of algal toxins to fully evaluate their safety (6).

PCA of Microalgae Samples

AFA-based products are distinguishable from other samples (**Figure 7**). They have characteristically high antioxidative potential (TPC and DPPH); high Mo, Ca, and Sr content; low P content; relatively low δ^{15}N values; and high δ^{13}C values. Sample S2 slightly deviates from AFA group, possibly due to additives (all other AFA samples are declared as pure). Based on our PCA, we can claim that AFA samples originate from the same source, supposedly Klamath Lake, OR. Interestingly,

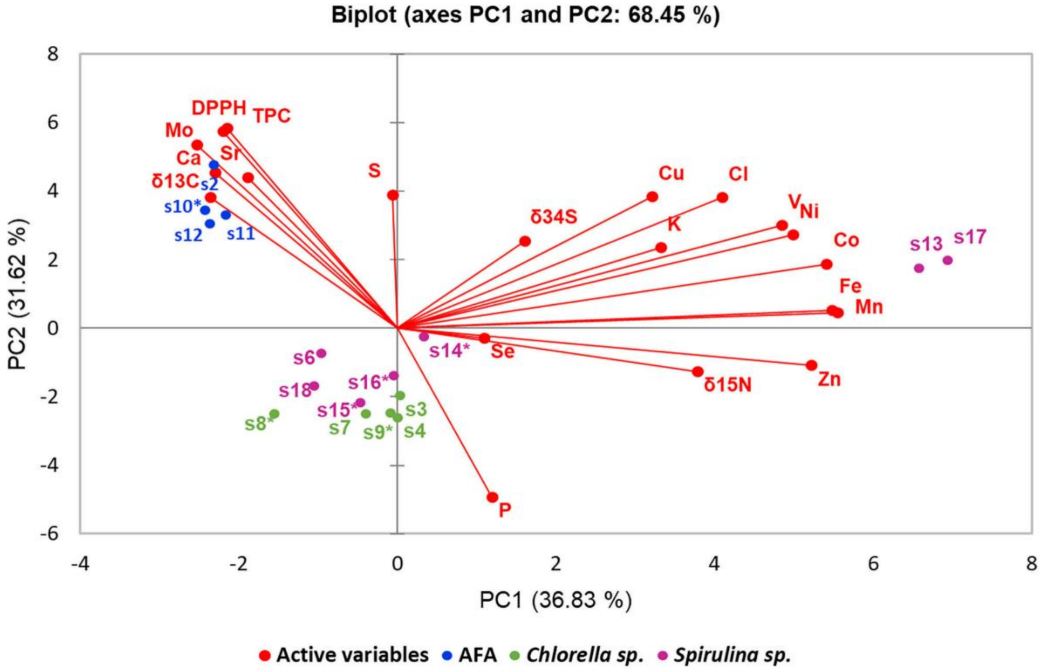

FIGURE 7 | Principal component analysis of algal dietary supplements analysis results (antioxidative potential, elemental, and stable isotope composition data). The biplot shows data of samples as dots (scores) and analysis results (loadings) as vectors. Samples marked with * are declared as organic.

sample S10 is declared as organic, whereas other samples have no such declaration. Such labeling discrepancy is unexpected, considering all our AFA samples are advertised to originate from Klamath Lake.

Samples of *Chlorella* spp. and *Spirulina* spp. cannot be reliably distinguished using PCA (with exception of Hawaiian *S. pacifica*) because of lack of characteristic parameters of respective microalgae (**Figure 7**). Organically grown *Spirulina* spp. and *Chlorella* spp. also do not exhibit any characteristic parameters, including δ^{15}N values, where high values usually indicate assimilation of organic N originating from wastewater. Two *S. pacifica.* samples (S13 and S17), originating from Hawaii, are well separated from other samples based on Fe, Mn, Zn, Co, Ni, V, K, Cl, Cu, and δ^{15}N values and δ^{34}S values, whereas the sample from Italy (S18) cannot be distinguished from other *Spirulina* spp. samples originating from non-EU countries.

Hawaiian *S. pacifica* samples S13 and S17 (**Figure 7**) significantly differ from other analyzed samples. That is largely due to significantly higher content of elements such as Co, Mn, Fe, Ni, V, and Zn compared to other samples, which is shown by their respective loadings (**Figure 7**).

By combining results from stable isotope composition, antioxidative potential, and elemental composition, we can reliably discriminate *S. pacifica* and AFA from our samples. Discrimination between *Chlorella* spp. and *Spirulina* spp. is not possible based on our results because of insufficient number of samples and scarce information provided by the manufacturers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

NO, NP, and JK: Conceptualization. JK, NP, NO, and JM: Methodology. MJ, JK, and MN: Validation. JK, MN, and MJ: Formal analysis. JK, MJ, JM, and MN: Investigation. NP, NO, and MN: Resources. JK: Writing—original draft preparation and visualization. JK, MJ, NO, NP, and MN: Writing—review and editing. NP and NO: Supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2020.618503/full#supplementary-material

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Supplementary Material

1 Supplementary Data

Supplementary Table 1. Mean concentration (Mean) ± standard deviation (SD), minimum (Min) and maximum (Max) of elements in different algae food supplement samples (mg element/kg solid sample) determined by XRF (Br, Ca, Cl, Fe, K, Mn, P, Rb, S, Si, Sr, Ti, Zn) and ICP-MS (As, Cd, Co, Cu, Hg, Mn, Mo, Ni, Pb, Se, Sr, V, Zn) methods.

	***Spirulina* spp. (n = 7)**			***Chlorella* spp. (n = 5)**			***Aphanizomenon flos-aquae* (n = 4)**			**Kelp (n = 2)**		
	Mean ± SD	**Min**	**Max**	**Mean ± SD**	**Min**	**Max**	**Mean ± SD**	**Min**	**Max**	**Mean ± SD**	**Min**	**Max**
As	0.73 ± 0.96	0.05	2.70	0.26 ± 0.17	0.04	0.41	5.38 ± 0.81	4.76	6.54	3.96 ± 0.81	3.39	4.54
Br	5.57 ± 7.80	0.50	17.40	0.93 ± 0.51	0.55	1.80	3.34 ± 1.46	1.51	4.77	81.85 ± 58.19	40.70	123.00
Ca	2551 ± 3178	737	9610	1970 ± 1396	1090	4430	9048 ± 3805	6430	14700	1178 ± 937	515	1840
Cd	0.04 ± 0.02	0.01	0.06	0.01 ± 0.01	0.00	0.02	0.02 ± 0.01	0.01	0.03	0.28 ± 0.38	0.01	0.55
Cl	2367 ± 2613	92	5770	219 ± 236	82	638	2045 ± 574	1340	2630	6420 ± 4950	2920	9920
Co	3.41 ± 5.36	0.16	13.14	0.63 ± 0.34	0.10	1.00	0.70 ± 0.21	0.40	0.90	0.05 ± 0.01	0.05	0.06
Cu	3.85 ± 3.52	0.83	9.97	3.28 ± 1.98	1.94	6.75	5.31 ± 0.63	4.51	5.99	0.32 ± 0.16	0.21	0.44
Fe	1360 ± 1334	281	3480	996 ± 524	544	1900	438 ± 49	377	491	126 ± 6	122	130
Hg	0.01 ± 0.01	0.01	0.04	0.03 ± 0.02	*	0.05	0.13 ± 0.21	0.03	0.44	0.20 ± 0.21	0.06	0.35
K	13904 ± 5003	5830	20800	9528 ± 1310	7570	11100	11800 ± 1538	10000	13700	4465 ± 3500	1990	6940
Mn^1	69.0 ± 63.1	19.3	185.0	43.7 ± 16.1	16.7	55.8	21.5 ± 3.4	17.6	25.4	8.4 ± 0.4	8.2	8.7
Mn^2	65.4 ± 59.2	15.4	169.0	40.6 ± 14.9	16.1	51.2	19.1 ± 0.6	18.6	19.7	2.6 ± 0.3	2.4	2.8
Mo	0.30 ± 0.24	0.12	0.77	0.24 ± 0.07	0.18	0.36	4.86 ± 0.77	4.11	5.83	0.09 ± 0.04	0.07	0.12
Ni	1.96 ± 1.93	0.22	5.10	0.40 ± 0.12	0.19	0.51	1.03 ± 0.35	0.61	1.47	0.33 ± 0.04	0.30	0.35
P	11137 ± 2495	6160	13600	14460 ± 1742	12800	16700	6095 ± 814	4940	6800	538 ± 575	131	944
Pb	0.35 ± 0.22	0.08	0.63	0.23 ± 0.19	0.05	0.55	0.02 ± 0.00	0.02	0.03	0.03 ± 0.03	*	0.06
Rb	4.47 ± 4.59	0.55	11.90	2.38 ± 1.21	0.87	3.82	2.26 ± 0.63	1.47	2.99	3.00 ± 3.11	0.80	5.20
S	7203 ± 1651	3880	9040	6638 ± 740	5870	7730	8683 ± 1215	7470	10100	980 ± 750	449	1510
Se	0.55 ± 0.97	0.02	2.70	0.05 ± 0.03	0.02	0.10	0.11 ± 0.02	0.08	0.12	0.01 ± 0.01	0.01	0.02
Si	10699 ± 6630	1340	21700	8962 ± 6122	2840	15600	3790 ± 3075	2120	8400	1175 ± 7	1170	1180
Sr^1	21.0 ± 17.0	7.4	56.2	15.8 ± 12.8	7.9	38.5	55.2 ± 29.2	36.1	98.7	79.1 ± 63.6	34.1	124.0
Sr^2	19.1 ± 16.7	6.1	54.3	19.5 ± 12.8	7.9	37.2	52.0 ± 29.6	32.2	96.0	80.2 ± 64.0	34.9	125.4
Ti	20.0 ± 16.8	8.8	46.5	33.8 ± 45.6	8.3	115.0	6.8 ± 1.3	5.5	8.5	5.7 ± 2.6	3.9	7.6
V	1.93 ± 2.98	0.08	7.31	0.23 ± 0.16	0.11	0.50	1.19 ± 0.74	0.44	2.21	0.47 ± 0.04	0.44	0.50
Zn^1	22.3 ± 16.1	6.0	52.7	18.5 ± 9.1	8.2	31.2	3.7 ± 1.1	2.6	5.0	1.1 ± 0.3	0.9	1.3
Zn^2	26.0 ± 18.4	10.2	60.5	24.1 ± 13.7	10.4	41.7	3.8 ± 1.1	2.8	5.3	1.4 ± 0.5	1.1	1.8

[1]Measured by XRF. [2]Measured by ICP-MS. *Below limit of detection of the method.

Chapter 3

Conclusions

This thesis offers new insight into using *Spirulina* microalgae as a new food product. This insight was achieved by determining the nutritional value of *Spirulina* dietary supplements available on the Slovenian market by analyzing the products' fatty- and amino acid and elemental profiles. Furthermore, as a part of the elemental analysis, iron speciation was conducted, which allowed for an estimation of iron bioavailability. In order to confirm the safety of these products, toxic trace elements were also analyzed.

To further improve its nutritional and therapeutic properties, lactic acid fermentation of fresh *Spirulina* biomass using *Lactobacillus plantarum* was carried out. The results of this experiment on the bioactive properties of *Spirulina* microalgae were assessed on a cellular and proteome level, where yeast *Saccharomyces cerevisiae* was used as the model organism. The ethanol *Spirulina* extracts were further assessed using proteome and metabolome profiling.

This work is important because it provides the first assessment of *Spirulina* microalgae products' geographical origin, using the stable isotope ratio of light elements C, N, S, O and H, combined with their elemental composition. Additionally, an attempt was made to verify their authenticity. For this, not only was the stable isotope ratio and elemental composition used, but also their amino- and fatty acid composition were considered. Finally, the *Spirulina* products' compliance with their declarations was evaluated for the first time.

The data obtained in this research is presented in six articles – five already published in international scientific peer-reviewed journals with one paper in preparation. The results were presented at 11 international conferences as oral and poster contributions.

The research work included in this thesis addresses the following topics which are interchangeably connected:

1. **Nutritional quality assessment of the *Spirulina* products available in the Slovenian market:** Analysis of the commercial *Spirulina* products' nutritional composition was performed in order to assess the quality of these products on the Slovenian market and to improve consumer trust in *Spirulina* products, which otherwise lack inspection control. The analyzed *Spirulina* food supplements are a good source of minerals phosphorous, selenium, potassium and calcium, and non-essential and essential amino acids when consumed within the amounts recommended by the producers. The fatty acid analysis has shown, however, that while *Spirulina* is a good source of polyunsaturated ω-6 fatty acids, the content of polyunsaturated ω-3 fatty acids is low. Also, even though *Spirulina* has been shown to contain relatively high levels of iron, its bioavailability is believed to be low, according to the performed iron speciation study. The latter has shown that iron in

Spirulina products is mainly (82–92%) present in the less bioavailable ferric (Fe^{3+}) form, which suggests that it would be necessary to reconsider the promotion of *Spirulina* as a rich dietary source of iron.

Pure *Spirulina* samples have been shown to contain higher linoleic and γ-linolenic fatty acid, amino acid, Se and P content, which suggests that choosing pure *Spirulina* products for consumption over samples containing excipients may contribute to a higher nutrient intake. Additionally, different *Spirulina* products may differ in nutrient content levels. For example, *Spirulina* products originating from Hawaii are higher in Mn, Cl, Fe, Br, Rb, Zn and Ti compared to other samples. Therefore, consumer's specific nutrient needs should be considered when choosing a specific *Spirulina* product.

Overall, the results mean that the first hypothesis "*Spirulina* (*Arthrospira* spp.) products from the Slovenian market are highly nutritional and rich in amino acids, fatty acids and essential elements" is partially confirmed: commercial *Spirulina* products from the Slovenian market are a good source of amino acids, elements and ω-6 fatty acids. However, as the analyzed samples contained only low levels of ω-3 fatty acids and the bioavailability of its iron was proven to be low, this hypothesis is also partially rejected.

2. **Safety and compliance with declaration analysis of the *Spirulina* products from the Slovenian market:** The toxic trace element analysis and analysis of the sample content compliance with declaration were performed to determine the safety and quality of *Spirulina* products from the Slovenian market. The levels of toxic trace elements, including Pb, Cd, Hg and As, in the sampled *Spirulina* products were significantly below the maximum allowable values set for dietary supplements. This finding means that the consumption of *Spirulina* products does not contribute significantly to their intake and is thus safe to consume. More concerning results were found regarding the accuracy of the information presented in the product declaration. Even though no upper safety levels were exceeded, in 86.7% of the analyzed products, inaccurate declarations were found regarding their Ca, P, Fe, Zn, K and Mn mineral content. This high proportion of products with inappropriately declared mineral values is concerning, as high content deviations from the declared values may cause excessive mineral intake in specific individuals and raise doubt among consumers about the product quality.

   According to these results, the second hypothesis "*Spirulina* products available in physical and online stores in Slovenia are safe for consumption, and their composition is consistent with the declared values" is partially confirmed regarding levels of toxic trace elements, and partially rejected due to inaccurate product declarations.

3. **Effect of fresh *Spirulina* biomass lactic acid fermentation and bioactive compound extraction solvent on its bioactivity and antioxidant activity – on a cellular, proteome and metabolome level:** Lactic acid fermentation of the *Spirulina* microalgae was performed by *L. plantarum* starter culture to study its effect on the nutritional and functional properties of this microalgae. The fermented *Spirulina* biomass has been determined to be a potential source of antioxidants, which resulted in reduced intracellular levels of reactive oxygen species, as well as reduced oxidative lipid damage. The non-protein nitrogen levels increased after fermentation, showing a higher protein bioavailability in the fermented *Spirulina* biomass than in the non-fermented biomass. The fermentation process has not altered the *Spirulina* microbiological safety, as no pathogenic

bacteria were found in the biomass after fermentation. A lower pH after fermentation also indicates a prolonged shelf life of the *Spirulina* biomass.

The antioxidant efficiency of non-fermented and fermented *Spirulina* water and ethanol extracts was determined at the proteome level, where yeast *S. cerevisiae* was used as a model organism. The analysis of protein expression alterations showed a distinct separation of the yeast treated with non-fermented and fermented *Spirulina* water and ethanol extracts. Ethanol extracts presented a higher antioxidant efficiency than water extracts when comparing fermented *Spirulina* to the non-fermented-one, thus confirming our third hypothesis "*Spirulina* biomass lactic acid fermentation and subsequent bioactive compounds' extraction in ethanol result in significantly enhanced antioxidant activity in treated model organisms (yeast cells), reflected at the cellular and molecular level". Fermentation was also crucial in lowering the cell stress response-related protein expression. The following protein and metabolite profiling of the ethanol *Spirulina* extracts resulted in significant separation of the fermented and non-fermented *Spirulina* extracts due to a decrease in protein and lipid content after fermentation and consequent increase in amino acid and lipid metabolite content. Fermentation also resulted in lowering of chlorophyll and carotenoid content in ethanol *Spirulina* extracts.

4. **Geographical origin discrimination among *Spirulina* products from the Slovenian market by combining stable isotope ratio and elemental composition analysis:** Stable isotope ratios of light elements C, N, S, and for the first time also O and H, together with elemental composition were determined in commercial *Spirulina* samples in order to assess the authenticity of their declared geographical origin. Results of the δ^2H and δ^{18}O analysis showed a correlation similar to that in water, which means that the local water is the main source of H and O isotopes in *Spirulina* and that other parameters have little impact on the δ^2H and δ^{18}O values of the final product. Also, the *Spirulina* samples of different declared origins showed significant variability in the elemental and stable isotope composition. Using various multivariate statistical analysis methods (OPLS-DA, PCA and DA), reliable discrimination of Italian, Portuguese and Hawaiian samples was achieved, including a good separation of samples from China. However, discrimination between the samples from Taiwan and India was somewhat less successful. Discrimination between the samples of different origins was achieved due to various parameters, such as the *Spirulina* production geographical location and environmental conditions of the region, culturing methods, pollution in the area, processing techniques and excipient addition in the final product. While the stable isotope ratio and elemental composition of the samples of undeclared origin show similarity with the Asian and declared non-EU samples, a specific and reliable classification of these samples was not possible. Therefore, the fourth hypothesis "The geographical origin of *Spirulina* products can be differentiated by combining the results of their stable isotope ratio and multi-elemental composition analyses" is also only partially confirmed, as for reliable differentiation of the commercial products, a reliable database consisting of isotopic and elemental composition data from authentic *Spirulina* samples would be required.
5. ***Spirulina* product authenticity assessment, regarding the product composition:** Nutrient (amino-, fatty acid and elemental analysis), as well as combined elemental and stable isotope ratio analysis, were used to determine whether the presence of other algal or plant material in the commercial *Spirulina* products alters the nutrient and isotopic composition of the final product. Separation of mixed products from those containing only *Spirulina* microalgae was

achieved using multivariate analysis. Additionally, the results of amino acid, fatty acid and elemental composition suggest that at least two of the analyzed *Spirulina* samples from the Slovenian market were adulterated, due to notable differences in their composition, compared to pure *Spirulina* samples. However, the excipients in the products cannot explain the deviation of the measured values. Similar results were obtained when the combined elemental and isotopic compositions were assessed. Here, differences in the isotopic and elemental composition of the analyzed samples resulted in the notable separation of mixed samples from other commercial *Spirulina* samples when using PCA. A good separation of the samples with mixed composition (*Spirulina* and other algal or plant material) was achieved. Also, as in the previous study, two samples declared *Spirulina* stood out, suggesting the presence of undeclared material in their composition. Using the nutritional composition and stable isotope ratio data, we can distinguish between the samples containing only *Spirulina* and samples mixed with other plant or algal material. Thus, our fifth hypothesis "The presence of adulterants in *Spirulina* products can be detected using elemental composition combined with stable isotope ratio analysis results and amino acid and fatty acid compositional data" is confirmed.

4.1 Scientific Relevance

The research presented in this doctoral dissertation has led to several original and significant scientific contributions. Firstly, it provides the first systematic quality and safety characterization of commercial *Spirulina* products available in the Slovenian market. The comprehensive analysis of *Spirulina*'s amino acid, fatty acid, and elemental composition offers valuable insights into the quality and safety of these products on the Slovenian market. It shows that even though most of the products are authentic, some still do not correspond to the typical composition of *Spirulina*, suggesting the possibility of adulteration. In this way, this study serves as a foundation for future product control and monitoring of their quality over time.

Iron speciation was performed in *Spirulina* products for the first time, showing that despite high iron content, its bioavailability for humans is low. This finding contrasts with previously published results and highlights the importance of considering the iron content and the iron species in future studies on iron bioavailability. Also, when conducting *in vivo* iron bioavailability studies in animals, the effect of the metabolic processes specific to the animal concerned should be considered, and compatibility with the human metabolic system should be evaluated.

The findings of this study further originally demonstrate that stable isotope ratio and elemental composition analysis are valuable tools for determining the authenticity of *Spirulina* products regarding their composition and geographical origin. Using the multivariate statistical analysis, the separation of the samples, which do not comply with the authentic *Spirulina* product isotopic and elemental composition, is achieved. In this way, exposing the products with adulterated composition and declared geographical origin is possible. This method could be used primarily for authentication studies of these and similar commercial products, protecting producers and consumers from potential fraud.

The first assessment of the impact of lactic acid fermentation on *Spirulina* bioactivity at the cellular, proteome and metabolome level offers a novel insight into the intracellular activity induced by fermented *Spirulina* bioactive compounds. The study shows that fermented *Spirulina* biomass is a potential source of antioxidants, whose activity is also determined at the cellular level. This finding is expressed as a reduced intracellular level of ROS, lower lipid oxidative damage and, at a proteome level, a lower expression of proteins related to the cell stress response. Furthermore, the proteomic and metabolomic analysis of fermented *Spirulina* biomass gives us new insight into the essential role of lactic acid fermentation in metabolite transformation and the role of microorganisms and their metabolic potential during fermentation. These results are the first step to understanding the processes involved in the bioactive effect of fermented *Spirulina* on the human organism.

4.2 Future Work

Combining all of the gathered data on the *Spirulina* microalgae, this thesis shows the high potential of this product to be used as an alternative food source. Nevertheless, even though the *Spirulina* products on the Slovenian market show excellent nutritional quality, specific issues have arisen, showing that improved inspection control would be required to increase safety and consumer trust in these highly nutritious products. Using the stable isotope ratio of light elements C, N, S, O and H, combined with elemental composition analysis, and the nutrient composition analysis, has proven to be efficient in *Spirulina* authenticity studies, regarding the product geographical origin discrimination, as well as the product composition. However, the authenticity assessment of the commercial *Spirulina* products should be improved, which means developing a reliable database including verified pure and authentic *Spirulina* samples from various production regions produced using different production methods. A similar approach could also be used for quality control of other dietary supplements available on the market.

Additionally, studying the extraction and purification processes of *Spirulina*'s bioactive compounds may lead to the development of novel pharmaceuticals, nutraceuticals, or functional food ingredients. Moreover, while this thesis provides a better insight into the lactic acid fermentation effect on the *Spirulina* antioxidant activity – at the cellular and molecular level and the role of the bioactive compound extraction solvent, additional studies are still required to provide an in-depth insight into fermented *Spirulina* bioactive compound activity mechanism on the subcellular level. Additionally, the therapeutic effects of fresh *Spirulina* have been reported in numerous publications, while the role of *Spirulina* biomass fermentation in this field is still scarce and has yet to be thoroughly investigated. One potential area of research could focus on elucidating the molecular mechanisms underlying the numerous health benefits of *Spirulina* consumption. Researchers could also gain insights into its antioxidant, anti-inflammatory, and immunomodulatory effects by investigating its bioactive compounds and their interactions with cellular pathways, while assessing the impact of *Spirulina* supplementation on various pathological health conditions could uncover new therapeutic avenues.

From this study, it is clear that future research on *Spirulina* holds tremendous promise for expanding our knowledge and harnessing its full potential in various industries, contributing to human health and sustainable development.

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Publications Related to the Thesis

Journal Articles

Masten Rutar, J., Vrhovšek, U., Poklar Ulrih, N., Ogrinc, N., Jamnik, P., & Ogrinc, N. (2023). Exploring the proteome and metabolome of fermented *Spirulina* biomass. Manuscript in preparation.

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Other Publications

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Biography

Jasmina Masten Rutar was born on January 31, 1989 in Šempeter pri Gorici, Slovenia. She finished her primary education in Komen, Slovenia and her secondary education in Nova Gorica, Slovenia. She began her studies at the Faculty of Medicine, University of Ljubljana, Slovenia, in 2008 and transferred to the Biotechnical Faculty, University of Ljubljana, Slovenia, in 2012, where she later finished her BSc with a thesis entitled “Active Food Packaging” and was awarded a BSc degree in Food Science and Nutrition in 2014. She continued her studies in the Food Science program of the Biotechnical Faculty at the University of Ljubljana, Slovenia, in 2014 and received her MSc degree and title Master of food science for her thesis entitled “Quality Parameters of Lettuce, Tomato and Bell Peppers from Supermarkets” in 2017. The thesis was awarded the Biotechnical Faculty Prešeren award in 2018. In 2017 she enrolled on the Ecotechnology program at the Jožef Stefan International Postgraduate School (IPS), Ljubljana, Slovenia as a PhD student under the mentorship of Prof. Dr. Nives Ogrinc. She got employed as a Young Researcher at the Department of Environmental Sciences at Jožef Stefan Institute, Ljubljana, Slovenia, under the financial support of the Slovenian Research Agency (ARRS). Her research focuses on the characterization of *Spirulina* from a chemical, safety, quality, authenticity and proteomic point of view. She specialized in amino- and fatty acid analysis using gas chromatography-mass spectrometry (GC-MS). She is also interested in biotechnological and microbiological techniques in proteomic and metabolomic research. She has gained experience in sample preparation for isotope, element and trace element analysis using microwave digestion and statistical data analysis. She has attended several workshops and training sessions during her studies, including in proteomics analytical approaches, LC-MS/MS (HF Orbitrap) analysis and data analysis at the University of Maastricht, Maastricht, Netherlands. She has presented her work at 11 international conferences. From 2017 to 2019, she was an active member and vice president of the IPS Student Council, where she played a key role in organizing the yearly IPS student conference.