applied sciences

MDPI

Article

Nutritional Profiling and Cytotoxicity Assessment of Protein Rich Ingredients Used as Dietary Supplements

Bianca-Maria Tihăuan [1,2], Ioana-Cristina Marinaș [1,*], Marian Adascălului [2], Alina Dobre [2], Grațiela Grădișteanu Pîrcălăbioru [1], Mădălina Axinie [3], Laura Mihaela Ștefan [4] and Denisa Eglantina Duță [2]

1 Research Institute of the University of Bucharest—ICUB, 91-95 Spl. Independentei, 50567 Bucharest, Romania
2 National Institute of Research & Development for Food Bioresources—IBA Bucharest, 5 Băneasa Ancuța, 020323 Bucharest, Romania
3 Department of Science and Engineering of Oxide Materials and Nanomaterials, Faculty of Applied Chemistry and Materials Science, University Politehnica of Bucharest, 011061 Bucharest, Romania
4 National Institute of Research and Development for Biological Sciences, 296 Splaiul Independenței, 060031 Bucharest, Romania
* Correspondence: ioana.cristina.marinas@gmail.com

Featured Application: Nutritional Profiling for subsequent dietary supplements/nutraceuticals development and formulation as well as by-products valorification.

Abstract: In recent years, the scientific community has made significant progress in understanding nutrition, leading consumers to shift their preferences away from animal-based protein products and towards natural, plant-based protein sources. This study aimed to determine the nutritional value, in vitro cytotoxicity and antioxidant activity for different sources of high protein content products (pea, yeast, almond, spirulina and *Pleurotus* spp.) with potential usage as raw materials for dietary supplements, especially since these products do not benefit from stricter regulation requirements regarding their actual health benefits. The characterization of raw materials consisted in evaluation of their nutritional profile (by addressing moisture content, crude protein content, extractable fat, ash, carbohydrates) and microbial contamination (TAMC, TYMC, *Enterobacteriaceae* and β-glucuronidase positive *Escherichia coli)*, total content of free amino acids, soluble proteins, phenols and flavonoids, as well as antioxidant activity through chemical assays. We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) release to evaluate the potential cytotoxicity of selected raw materials. Results obtained indicate high percentages of proteins for the pea powder (77.96%) and *Spirulina* powder (64.79%), *Pleurotus* spp. flour had strong antioxidant activity, while the highest contamination values were registered for *Pleurotus* spp. powder (4.6×10^5 CFU/g or 5.66 log CFU/g). Cytotoxicity results demonstrate that tested ingredients have an impact on the metabolic activity of cells, affecting cellular integrity and provoking leakage of DNA at several concentrations. While plant-based protein supplementation may appear to be a promising solution to balance our busy lives, there are several advantages and disadvantages associated with them, including issues related to their absorption rate, bioavailability, cytotoxicity and actual nutritional benefits.

Keywords: high protein ingredients; cytotoxicity; antioxidant activity; nutritional value; dietary supplements

Citation: Tihăuan, B.-M.; Marinaș, I.-C.; Adascălului, M.; Dobre, A.; Pîrcălăbioru, G.G.; Axinie, M.; Ștefan, L.M.; Duță, D.E. Nutritional Profiling and Cytotoxicity Assessment of Protein Rich Ingredients Used as Dietary Supplements. *Appl. Sci.* **2023**, *13*, 6829. https://doi.org/10.3390/app13116829

Academic Editor: Monica Gallo

Received: 15 April 2023
Revised: 26 May 2023
Accepted: 28 May 2023
Published: 5 June 2023

1. Introduction

In the past, protein supplements and dietary supplements, in general, were mostly associated with bodybuilders and individuals who engaged in heavy exercise. However, in recent years, there has been a significant shift in consumer behavior, with a large segment of the general population seeking out protein supplements for meal replacement, weight loss and purported health benefits [1].

This shift can be attributed to several factors, including a growing interest in healthy living and wellness, as well as an increased awareness of the importance of proper nutrition for overall health and well-being. Additionally, advancements in nutritional science have led to a better understanding of the role that proteins play in the body and the potential benefits of protein supplementation for various health goals. Overall, this shift towards protein supplementation and dietary supplementation, in general, is indicative of a larger trend towards a greater focus on nutrition and wellness in modern society [2].

Therefore, proteins, being essential macronutrients, play a crucial role in the growth and maintenance of the human body as they provide energy, amino acids, and possess various physiochemical and sensory properties, making them useful functional ingredients for promoting health [3]. Despite their importance, protein deficiency remains a major concern. Furthermore, increased protein intake has been associated with several health benefits due to its fundamental role in all body cells and maintenance of the immune system. Notably, higher protein consumption has been linked to increased muscle mass and endurance [4–7].The origin of protein can have a significant impact on the health benefits it provides. Proteins from animal sources, such as meat, dairy and eggs tend to be high in saturated fat and cholesterol, which can increase the risk of heart disease and other chronic health conditions when consumed in excess [4,5]. On the other hand, proteins from plant sources, such as legumes, nuts, seeds, and whole grains tend to be lower in saturated fat and cholesterol, and higher in fiber, vitamins, minerals, and antioxidants, which have been linked to a reduced risk of chronic diseases [6,7]. Additionally, plant-based proteins can provide several health benefits that animal-based proteins may not. For example, the use of protein isolates (hydrolysates) from peas have been shown to lower blood pressure and reduce the risk of kidney disease [8,9], the body metabolizing this protein similar to whey-based ones [10]. Almond protein powders are also metabolized similar to well-known whey-based proteins [11], being as well associated with beneficial effects against cardiometabolic diseases [12,13]. Spirulina powder's beneficial effects are identified in particular reference to obesity, hypertension and cardiovascular diseases [14,15]. Additionally, *Pleurotus* spp. protein powders have been associated with beneficial aids in various chronic diseases [16–18], as well as yeast protein powders promoting muscle protein synthesis, rapid recovery and biodiversity of gut-microbiota [19,20]. In summary, the origin of protein is an important consideration for optimizing health benefits. Consuming a variety of plant-based proteins can provide a range of health benefits, while minimizing the consumption of animal-based proteins may reduce the risk of chronic diseases associated with a high intake of saturated fat and cholesterol.

In addition, it is well known that compounds with antioxidant activity contribute to the cells' amelioration and/or protection of oxidative stress [21]. Synthetic antioxidants have been widely used in different industries being known for their health hazards such as liver damage and carcinogenesis [22]. Natural antioxidant peptides and proteins are an alternative in terms of safety and availability [23]. In the case of antioxidant peptides, the potential to reduce the risk of aging, inflammation and cardiovascular disease have been demonstrated, thus improving human health [24,25].

Up to date, numerous antioxidant peptides from various animal, plant and microbial proteins have been identified [26–29] with different mechanisms of action: inhibition of lipid oxidation, scavengers of free radicals and/or chelate pro oxidative metals [25,28,30,31]. Various studies have shown that the antioxidant properties of proteins are related to their composition, sequence, structure and hydrophobicity [11,32–34].

Keeping this in mind, we can highlight several advantages of consuming proteins of plant origin, such as lower saturated fat and cholesterol content, higher values of fiber, vitamins, minerals, and antioxidants compared to animal-based proteins, which are associated with a reduced risk of chronic diseases [35], environmental sustainability [36], cost-effectiveness, versatility and suitability for various dietary restrictions [37–40]. Overall, consuming proteins of plant origin can be a beneficial choice for both personal health and the health of the planet.

Although dietary supplementation seems like a viable solution that can balance our busy and demanding life and enhance or help maintain normal physiological functions of the human body, varying pros and cons have been associated with them regarding the absorption rate, bioavailability, cytotoxicity and actual nutritional benefits. Especially since according to the 2020 Agri-Food Fraud Network (FFN) report based on Regulation (EU) No. 1169/2011 on the food information to consumers, the majority of non-compliances regarded mislabeling, support documentation and product dilution/addition [41].

Therefore, our study analyzed the nutritional profile, physico-chemical properties, antioxidant activity and cytotoxicity degree of five high protein ingredients represented by: pea powder, almond powder, yeast powder, spirulina powder and *Pleurotus* spp. flour. These ingredients are frequently used in various diets, workout regimes or as meal replacements; as we know, the supplements are not as restricted by legislation, therefore mistakes and misconceptions are more likely to occur. Therefore, our study focuses on analyzing three key domains capable of outlining the safety consumption limits of ingredients such as these.

2. Materials and Methods

2.1. Reagents and Standards

Bovine albumin serum, Bradford regent, ninhydrin, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant potential (FRAP), 2,4,6-tripyridyl-s-triazine (TPTZ), gallic acid and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). Folin–Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical grade.

2.2. Raw Materials

The raw materials/ingredients tested were procured from Romanian manufacturers. Pea powder from SC Paradisul Verde SRL, Brasov, Romania, spirulina powder from SC Fytovital SRL, Oradea, Romania, yeast powder from SC SANOVITA SRL, Romania, almond powder from SC PRONAT SRL, Romania and *Pleurotus* spp. flour was obtained by IBA, Bucharest, from fresh *Pleurotus* through drying at 50 °C and grinding.

2.3. Assessment of the Nutritional Profile of Dry Raw Materials

2.3.1. The Moisture Content

The moisture content was achieved through a gravimetric method that involved drying the samples at 150 °C until a constant mass was reached, method described by ISO 6673:2003 [42]. Approximately 5 g of the sample were weighed into a vial, which was dried to a constant mass in an oven (Memmert UF 110, Memmert, Schwabach, Germany). The analysis was performed in triplicate. The moisture content (M, %) was determined using the following formula:

$$\mathrm{M}\ (\%) = \frac{w - d}{d} \times 100, \quad (1)$$

where w—wet weight, d—dry weight

2.3.2. Crude Protein Content

Total protein content was determined according to ISO 20483:2013 [43]. The protein content was analysed by the Kjeldahl method with a FOSS Kjeltec 2300 analyzer (FOSS Group, Hillerød, Denmark) after acid hydrolysis in an auto-digester (Behrotest InKjel, 450 P, Germany). According to the classical Kjeldahl method, samples were digested using concentrated sulfuric acid. The ammonium sulfate salt and alkali generated ammonia, which was trapped in boric acid via steam distillation. Titration was performed using a hydrochloric acid 0.2 N solution.

2.3.3. The Total Extractable Fat Content

Total fat content was measured using the Soxhlet method described in ISO 6492:1999 [44]. Briefly, the samples were transferred into the extraction cartridge. About 100 mL of petroleum ether was added to the flask attached to the Soxhlet apparatus (SoxtecTM 2050, Foss Analytical, Hilleroed, Denmark). The extraction lasted about 6 h. The total amount of lipids was determined by a gravimetric method as follows: the solvent of the extract was distilled, the residue was dried in an oven at 105 °C for 2 h and the flasks were weighed until constant mass. The fat content (FC) was calculated as follows:

$$\text{FC } (\%) = \frac{m2 - m1}{m} \times \frac{100}{100 - M(\%)} \times 100, \quad (2)$$

where $m1$—empty flask weight, $m2$—flask with fat weight, m—sample weight, $M(\%)$—moisture content

2.3.4. Determination of Ash

The ash content was determined according to ISO 2171:2010 [45] with slight modifications. The total ash content is based on the evaluation of the residue amount obtained after sample calcination at 650 ± 25 °C and the ashes were dried to constant mass at 105 °C.

2.3.5. The Total Carbohydrates Content (CC) and Energy Value

The equation used to calculate the total carbohydrate content was [46]: total carbohydrates (% FW) = 100 − moisture (%) − protein content (% FW) − crude fat (% FW) − ash (% FW). The results show total carbohydrates as g/100 g FW. The energy value was calculated using the following factors: protein 4 kcal/g, fat 9 kcal/g and carbohydrates 4 kcal/g, according to the following formula:

$$\text{EV (kcal)} = 4 \times \text{PC } (\%) + 9 \times \text{FC } (\%) + 4 \times \text{CC } (\%) \quad (3)$$

2.3.6. Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The functional groups within extracts were evaluated by Fourier transform infrared spectroscopy (FTIR, Agilent Cary 630 FTR spectrophotometer, Agilent, CA, USA) equipped with an ATR device. The spectra were recorded from 4000–650 cm^{-1} with an average of 400 scans and a resolution of 4 cm^{-1} resolution, at 22 °C.

2.4. Assessment of the Physico-Chemical Properties for the Extracts Obtained from Raw Materials

To determine the content of soluble proteins, free amino acids, phenolics and flavonoids, an aqueous extraction corresponding to a concentration of 10 mg dry material/mL distillated water was performed. The extracts were vortexed for 15 min and centrifuged at 8000 rpm for 15 min at 10 °C using a centrifuge (Thermo Scientific, Waltham, MA, USA). All assays were performed in triplicate. The results were expressed as mean ± SD.

2.4.1. Determination of Total Soluble Protein

The protein concentration from the pea powder, almond powder, yeast powder, spirulina powder and *Pleurotus* spp. flour was determined by the Bradford assay [47]. The calibration curve was made using bovine albumin serum (BSA) for concentrations that ranged between 1.4–0.1 mg/mL (R^2 = 0.9928). The extracts obtained in distillated water were used for spectrophotometric determination of protein at λ = 595 nm, using FlexStation 3 UV-Vis (Molecular Devices, San Jose, CA, SUA) spectrophotometer.

2.4.2. Determination of Total Free Amino Acids

Total free amino acids were quantified after protein precipitation with trichloroacetic acid (TCA). The samples were centrifuged (15 min, 10,000 rpm, Thermo Scientific, Waltham, MA, USA) and 10 µL of the supernatant was inserted into 300 cL of Cd-ninhydrin reagent. The samples were kept for 10 minutes at 84 °C and the absorbance was read at λ = 507 nm.

The calibration curve (glycine—R^2 = 0.9940) was measured for concentrations that ranged between 10–0.625 mg/mL. The Cd-ninhydrin reagent was prepared as follows: 1 mL of 1 g/mL $CdCl_2$ was added to a solution containing 0.8 g ninhydrin dissolved in 90 mL absolute ethanol: glacial acetic acid 8:1 (*v*:*v*). The solution formed was diluted with distilled water in a volume ratio of 1:1.5 [20].

2.4.3. Total Phenolic Content

Total phenolic content was measured by the Folin–Ciocalteu assay [48]. Over 50 µL of sample was transferred along with 50 µL Folin–Ciocalteu reagent, 0.45 mL H_2O and 0.50 mL of 7% Na_2CO_3. The samples/standard solutions were incubated in the dark for 60 min. The samples were centrifuged (5 min, 10,000 rpm, Thermo Scientific, Waltham, MA, USA) and the absorbance was read at 765 nm with FlexStation 3 UV-Vis (Molecular Devices, GA, USA) Spectrophotometer. The calibration curve was measured using gallic acid in the same condition with samples (R^2 = 0.9955). The linear domain was between 250–12.5 µg/mL. Total phenolic content was rendered as mg gallic acid equivalent (GAE)/g sample.

2.4.4. Total Flavonoid Content

The flavonoid content was determined through the $AlCl_3$ assay [49]. Briefly, over 100 µL sample/standard solution was added along with 100 µL sodium acetate 10%, 12 µL $AlCI_3$ 2.5% and 680 µL ethanol 70%. The sample/standard solutions were incubated at dark for 45 min. After that, the samples were subjected to centrifugation (5 min, 10,000 rpm). The supernatant absorbance was recorded at λ = 430 nm. A standard curve was created with quercetin (R^2 = 0.9935). The linear domain was between 200–12.5 µg/mL. Total flavonoid content was rendered as mg quercetin equivalent (QE)/g sample.

2.5. Antioxidant Activity

The DPPH assay was performed according to the method described by Madhu (2013) [50] with some minor changes. The linearity range of Trolox standard through this method was between 0 and 100 mM (R^2 = 0.9943). The absorbance was determined using a FlexStation 3 UV–VIS spectrophotometer (Molecular Devices Company, Sunnyvale, CA, USA) at λ = 515 nm.

FRAP assay. 285 µL of the FRAP reagent, prepared according to Benzie and Strain (1999) [51] were added over the 15 µL of sample. The reaction mixture was incubated at 37 °C for 30 min in the dark. The samples were centrifuged (Thermo Scientific, Waltham, MA, USA) and the absorbance was recorded at 593 nm using FlexStation 3 UV–VIS spectrophotometer (Molecular Devices Company, Sunnyvale, CA, USA). Trolox (50 to 250 µM) was used for the calibration curve (R^2 = 0.997).

2.6. Evaluation of the Microbiological Status

The vegetal high protein ingredients were tested for bacterial and fungal contamination. The monitoring of microbiological status and the quality assessment of yeast, pea, almond, spirulina and *Pleurotus* spp. powder were performed using national and international food microbiology standards. The purpose was to assess the microbiological status of these powders because the bacterial and fungal content are an important parameter for quality assurance and safe consumption.

Each sample was prepared according to specific standard requirements. An amount of 10 g of each sample was dispersed in 90 mL of either peptone water or buffered peptone water and homogenized using a Stomacher SEWARD 400 (Seward, NY, USA). Incubation steps were performed using Panasonic and Memmert incubators; the water activity was determined using an Aquaspector AQS-2-TC instrument (Nagy GmbH, Gäufelden, Germany), and additional required equipment such as Dilumat Start instrument (BioMérieux, Marcy-l'Étoile, France) and Laminar flow Faster vs.—4 (Faster SRL, Cornaredo, Italy) cabinet were used for performing microbiological assays.

2.6.1. Determination of the Total Aerobic Microbial Count (TAMC) I

Determination of contamination with both mesophilic and psychrotrophic microorganisms was performed according to standard ISO 4833-1:2014 [52] by plating 0.1 mL of each sample on Plate Count Agar (PCA—Oxoid, UK) using a Drigalski spatula, followed by incubation at 30 °C for three days. After the incubation period, all the colonies from the plates were numbered. Interpretation of results was performed using the following Formula (4):

$$CFU = (\sum C)/((n_1 + 0.1n_2) \times d) \quad (4)$$

where: CFU = average no. of colony forming units from two serial dilutions; ΣC = sum of colonies counted in all retained plates; n_1 = number of plates retained at first dilution; n_2 = number of plates retained at the second dilution; d = dilution from which the first counts were made.

2.6.2. Determination of the Total Yeast and Mold Count (TYMC)

Determination of TYMC was performed according to standard ISO 21527-2:2009 [53] by dispersing 0.1 mL of samples inoculum using a Drigalski spatula on the surface of Dichloran Glicerol Agar (DG 18) followed by incubation at 25 °C for seven days. Various molds and yeasts grow on this medium, DG-18 (Oxoid, UK) being specifically recommended for selective isolation of xerophilic molds from food samples. After the incubation period, the colonies were counted and analyzed according to Formula (4).

2.6.3. Determination of *Enterobacteriaceae*

The presence of *Enterobacteriaceae*, especially in food grade products, is in general considered an indicator of inadequate factory hygiene, prominently conditioned by the incipient microbial load of raw materials. According to the ISO 21528:2 [54] method, 1 mL of each sample was inoculated in liquefied Violet Red Bile Glucose (VRBG) media (Oxoid, UK), followed by a second layer of the same media after the solidification of the first layer. The plates were incubated at 37 °C for 24 ± 2 h. For the typical colonies (red–pink colonies), with or without a precipitation zone, biochemical tests for oxidase and glucose fermentation were performed for confirmation.

2.6.4. Determination of β-Glucuronidase Positive *Escherichia coli*

The presence of *E. coli* in food grade products is an indicator of contamination with organisms of fecal origin, thus corroborating this analysis with results from Section 2.4.3 indicates significant issues and concerns regarding compliance with hazard analysis and critical control points regulations applied to food grade products. The method described in ISO 16649-2:2007 [45] is based on the enumeration of *E. coli* using colony count technique. According to standard requirements, molten selective culture medium containing X-β-D-glucuronide, named Tryptone Bile X-glucuronide Agar (Oxoid, UK) was used for determination of β-glucuronidase positive *Escherichia coli*. Samples were incubated at 44 °C for 24 h in order to allow the selective growth of *E. coli*. On this selective chromogenic media, presence of blue colonies indicates detection of *E. coli.* The number of colony forming units (CFU) of β-glucuronidase-positive *E. coli* per gram (g) of sample was calculated using Formula (4).

2.7. Cytotoxicity Assessment by MTT and LDH

Evaluation of cytotoxicity requires sensitive, quantitative, reliable and automated methods for the precise determination of cell viability and death, therefore, we selected the MTT and LDH assays for this task.

The mouse fibroblasts L929 cell line (ECACC—European Collection of Authenticated Cell Cultures) was selected for cytotoxicity assessment of pea powder, almond powder, yeast powder, spirulina powder and *Pleurotus* spp. flour. All samples were analyzed from a starting concentration of 500 mg/mL and serially diluted in order to obtain the following concentrations: 250 mg/mL, 62.5 mg/mL, 7.813 mg/mL, and 0.651 mg/mL.

Cultivation of L929 cells was performed according to ECACC specifications in DMEM (Dulbecco's Modified Eagle Medium, Sigma-Aldrich, St. Louis, MO, USA) media supplemented with 10% FBS (Fetal Bovine Serum—Sigma-Aldrich) and 1% Pen/Strep (penicillin/streptomycin solution, 50 µg/mL—Sigma-Aldrich) for 24 h at 37 °C, at 95% humidity with 5% CO_2. After 24 h cells were washed using PBS (Phosphate Buffered Solution—Sigma-Aldrich), harvested using trypsin (Sigma-Aldrich) and counted using Trypan Blue (Sigma-Aldrich) and a hemocytometer. The seeding density for the MTT and LDH assays was optimized at 5×10^4.

Using the MTT we measured the cellular metabolic activity, which is an indicator of cell viability, proliferation and cytotoxicity. This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells [55]. The mechanism of function relies on viable cells containing NAD(P)H-dependent oxidoreductase enzymes that reduce the MTT to formazan. Moreover, the insoluble formazan crystals formed are dissolved using a cell grade solvent and the resulting colored solution is quantified by measuring its absorbance at 500–600 nm, depending on various kits specifications. This non-radioactive, colorimetric assay system using MTT was first described by Mosmann T. (1983) [56] and improved in subsequent years by several other investigators. Since disturbances in cellular metabolism cannot fully confirm the actual toxicity of a certain compound, elucidation of the process associated with reduction of cellular population is important, therefore we selected LDH assay for quantification of plasma membrane damage. Upon cellular damage, NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. Subsequently, the catalyst transfers H/H+ from NADH/H+ to the tetrazolium salt INT which is reduced to formazan. An increase in the amount of dead or plasma membrane-damaged cells results in an increase in LDH enzyme activity in the culture supernatant [56].

Cells seeded at 5×10^4 density in a clear 96 well cell culture plate were treated with pea powder, almond powder, yeast powder, spirulina powder and *Pleurotus* spp. flour (samples concentrations: 500 mg/mL, 250 mg/mL, 62.5 mg/mL, 7.813 mg/mL, and 0.651 mg/mL) and incubated for 24 h at 37 °C, 95% humidity with 5% CO_2. After 24 h of exposure to tested compounds, cells were incubated for 4 h with MTT reagent (Roche) at 37 °C, 95% humidity with 5% CO_2. After incubation, cells were treated with MTT solvent (Roche) for 15 min at room temperature. Absorbance was measured using a spectrophotometric microplate reader (ELISA reader) at OD = 570 nm. For the LDH assay we used the LDH Cytotoxicity Detection Kit (Roche). Cells exposed to tested compounds were treated with LDH reagent mixture for 15 min at 37 °C. Afterwards, the LDH activity was measured using a spectrophotometric microplate reader (ELISA reader) at 492 nm with a 600 nm wavelength reference.

2.8. Statistical Analysis

Statistical evaluations were performed using GraphPad Prism 9 (San Diego, CA, USA). Biological data were analyzed using the two-way ANOVA with Dunnett's multiple comparison test. For physico-chemical assessments, data were expressed as means ± SD determined by triplicate analysis. The statistical analysis was conducted using a one-way ANOVA with Tukey test. The level of significance was set to $p < 0.05$.

3. Results

3.1. Assessment of the Nutritional Profile and Physico-Chemical Properties

Determining key physico-chemical properties is an important step in analyzing any kind of dietary supplement.

Determination of total proteins for our samples put out high percentages of protein for the pea powder (77.96 ± 0.70%) and Spirulina powder (64.79 ± 2.20%). Determination of total extractable fat revealed that almond powder has the highest lipid content out of the tested samples (6.91 ± 0.10%). Results for all tested samples are presented in Table 1.

Table 1. Nutritional profile for the raw materials with high protein content.

Samples	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	Carbohydrates (%)	Energy Values (kcal)
Pea powder	7.43 ± 0.06 ^{b}	77.96 ± 0.70 ^{a}	0.29 ± 0.03 ^{d}	3.77 ± 0.19 ^{e}	10.55 ± 0.73 ^{e}	356.65 ± 0.56 ^{c}
Yeast powder	4.43 ± 0.18 ^{d}	46.23 ± 1.44 ^{d}	0.30 ± 0.03 ^{d}	5.80 ± 0.30 ^{c}	43.24 ± 1.27 ^{b}	360.58 ± 1.26 ^{b}
Spirulina powder	7.31 ± 0.35 ^{b}	64.79 ± 2.20 ^{b}	1.10 ± 0.05 ^{bc}	7.54 ± 0.26 ^{a}	19.26 ± 2.35 ^{d}	346.10 ± 2.22 ^{e}
Almond powder	6.04 ± 0.10 ^{c}	52.11 ± 1.55 ^{c}	6.91 ± 0.10 ^{a}	6.60 ± 0.07 ^{b}	28.34 ± 1.77 ^{c}	383.99 ± 0.23 ^{a}
Pleurotus spp. flour	8.20 ± 0.15 ^{a}	16.75 ± 0.26 ^{e}	1.36 ± 0.24 ^{b}	5.66 ± 0.20 ^{cd}	68.03 ± 0.70 ^{a}	351.36 ± 0.97 ^{d}

a–e: different superscript letters in the same column indicate significantly different values for $p < 0.05$ by Tukey's test; same superscript letters in the same column indicate not significantly different values for $p > 0.05$ by Tukey's test.

Figure 1 shows the average spectra of the five high protein ingredients analyzed. Similar bands can be observed for all five ingredients but of different intensity, which indicates the same components but of different concentrations. The FTIR spectra was divided into two distinct parts. The first one, between 750 cm^{-1} and 1900 cm^{-1} is associated with stretching vibration of C=C, C=O, C-C, C-N and C-O (Figure 1, light blue zone). The range between 2750 cm^{-1} and 3700 cm^{-1} (Figure 1, light pink zone), corresponds to the vibration of bands that contain hydrogen atoms, such as C-H, O-H and N-H. The assignment of representative bands (Table 2) was performed by analyzing lipids and protein samples and taking into account the literature data [57–59].

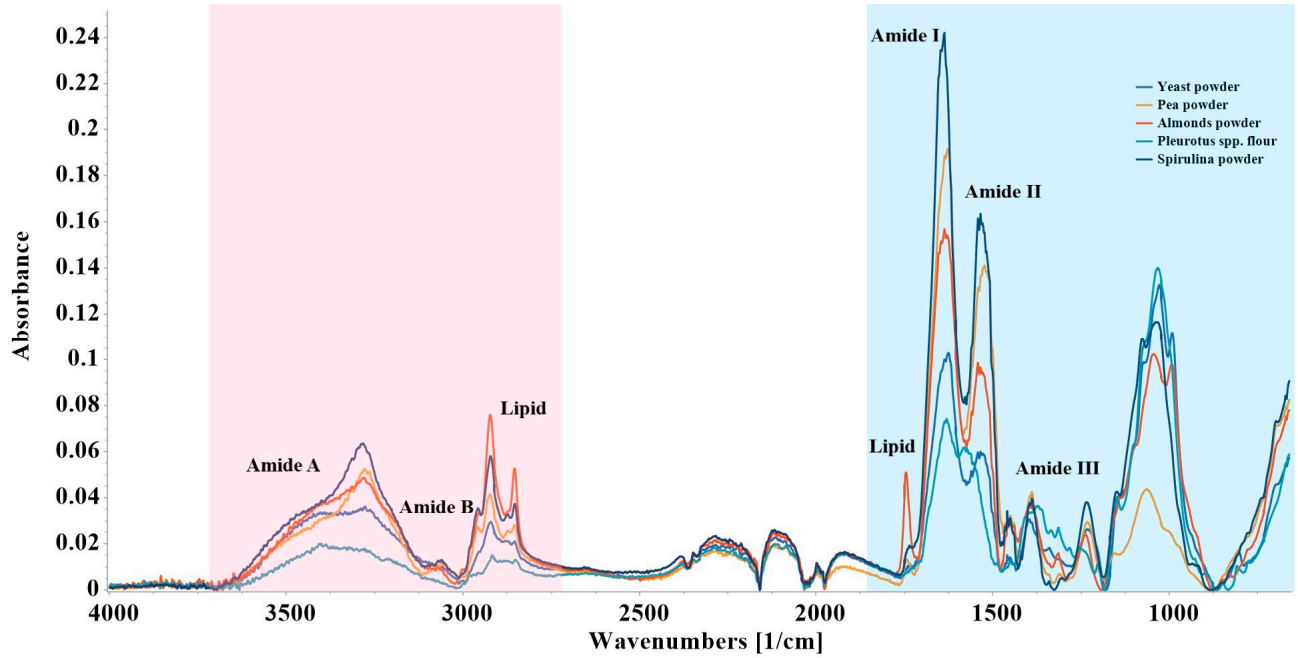

Figure 1. ATR-FTIR spectra of yeast powder (orange), pea powder (blue), almond powder (green), *Pleurotus* spp. flour (light blue), spirulina powder (red).

In order to facilitate the comparison of the five analyzed FTIR spectra, they were all normalized to the maximum. The peptide group, the structural repeat unit of proteins, shows characteristic bands named amide A, B, I, II, III. The two prominent features, namely, the amide I (1600–1650 cm^{-1}) and amide II (approximately at 1530 cm^{-1}) bands are the two major bands of the protein infrared spectrum. The amide I band is mainly associated with the C=O stretching vibration with minor contribution from the out-of-phase C-N stretching vibration, the CCN deformation and is directly related to the backbone conformation. Amide II results from the N-H bending vibration and from the C-N stretching vibration

with smaller contributions from C=O in plane bend and the C-C and N-C stretching vibrations. Like for the amide I vibration, the amide II vibration of proteins is affected by side chain vibration. Amide III (1200–1400 cm^{-1}) is a very complex band resulting from a mixture of several coordinated displacements, such as the in-phase contribution from the N-H bending and the C-N stretching vibration. The bands in the region of 1740 cm^{-1} and 2830–3010 cm^{-1} are associated with the lipids from the sample composition. The peak intensity from 1744 cm^{-1} for the almond powder sample, compared to the other samples, is in correlation with the results obtained for the total fats analysis, which reveals that almond powder has the highest lipid content out of the tested samples (6.91%) (Table 1).

Table 2. Wavenumbers assignment to the characteristic bands.

Sample	Amide A (cm^{-1})	Amide B (cm^{-1})	Amide I (cm^{-1})	Amide II (cm^{-1})	Amide III (cm^{-1})	Lipid (cm^{-1})
Yeast powder	3277	3010–3060	1625	1525	1180–1430	1733 2835–2990
Pleurotus spp. flour	3207	3020–3230	1631	1571	1185–1430	1710 2800–2945
Pea powder	3265	3010–3115	1626	1522	1185–1425	1740 2830–3000
Almond powder	3279	3020–3100	1636	1533	1200–1475	1744 2800–3020
Spirulina powder	3281	3010–3112	1638	1533	1185–1480	1734 2830–3000

The N-H stretching vibration gives rise to the amide A band between 3130 cm^{-1} and 3400 cm^{-1}. Its frequency depends on the strength of the hydrogen bond. The amide A band is usually part of a Fermi resonance doublet with the second component absorbing weakly between 3010 cm^{-1} and 3100 cm^{-1} (amide B).

The protein content obtained by the Bradford assay was comparatively lower than that obtained using the Kjeldahl method for all the samples (Tables 1 and 3), the main cause being that only the total nitrogen content was measured through Kjeldahl, whereas other biomolecules containing nitrogen, such as amino acids, nucleic acids and other nitrogenous compounds [60] or insoluble proteins may have been present. Among the products tested, the highest soluble protein content was obtained for almond powder while yeast powder had the lowest content, probably due to its nucleic acid content.

Table 3. Evaluation of total soluble proteins, free amino acids, phenol and flavonoid content and their antioxidant activity.

Sample	Total Soluble Proteins (mg/g)	Total Free Amino Acids (mg/g)	Total Phenols (mg GAE/g)	Total Flavonoids (mg QE/g)	DPPH (mM TEAC/g)	FRAP (mM TEAC/g)
Yeast powder	2.91 ± 0.16^{d}	13.00 ± 0.24^{b}	0.88 ± 0.04^{a}	0.60 ± 0.12^{e}	2.16 ± 0.69^{d}	0.82 ± 0.04^{d}
Pleurotus spp. flour	6.15 ± 0.55^{cd}	42.25 ± 1.61^{a}	6.51 ± 0.09^{b}	4.68 ± 0.1^{a}	5.48 ± 0.29^{c}	13.15 ± 1.19^{a}
Pea powder	12.73 ± 0.51^{b}	6.31 ± 0.05^{cd}	1.25 ± 0.02^{c}	0.98 ± 0.07^{d}	1.04 ± 0.17^{e}	0.52 ± 0.04^{d}
Almonds powder	95.25 ± 4.69^{a}	8.04 ± 0.5^{c}	3.90 ± 0.15^{d}	3.26 ± 0.21^{b}	6.56 ± 0.37^{b}	8.11 ± 0.63^{b}
Spirulina powder	6.23 ± 0.86^{c}	6.55 ± 0.43^{cd}	5.83 ± 0.43^{d}	2.68 ± 0.1^{c}	10.2 ± 0.13^{a}	7.52 ± 0.47^{bc}

a–e: different superscript letters in the same column indicate significantly different values for $p < 0.05$ by Tukey's test; same superscript letters in the same column indicate not significantly different values for $p > 0.05$ by Tukey's test.

In this study, the antioxidant activity was measured through two independents assays: FRAP and DPPH. Table 3 shows that the *Pleurotus* spp. flour had strong antioxidant activity through the FRAP method and was correlated with the capacity of total free amino acids (chelation metals) and in a low manner with the phenol content and flavonoids. For almond powder, where a higher content of soluble proteins was obtained, the antioxidant activity was low through both methods. For spirulina powder, the antioxidant activity could be given by c-phycocyanins [61].

3.2. Evaluation of the Microbiological Status

The results obtained from the microbiological analysis of the five products reveal the lack of contamination with pathogenic bacteria, in this case, *Escherichia coli*. The total number of aerobic bacteria, expressed in cfu/g, recorded the highest values in the case of *Pleurotus* spp. powder (4.6×10^5 CFU/g or 5.66 ± 0.1 log CFU/g), and in the case of spirulina and pea powder, the degree of contamination was lower, 10^3 cfu/g (Table 4). *Pleurotus* spp. powder has a high degree of contamination with TAMC, TYMC and *Enterobacteriaceae*, so the sample presents a microbiological risk regarding the quality of the finished products that may include this powder.

Table 4. Results for microbiological and stability indicators.

Samples	Total Aerobic Count CFU/g	*E. coli* CFU/g	*Enterobacteriaceae* CFU/g	Yeast and Molds CFU/g	Water Activity Value
Yeast powder	<10	<10	<10	<10	0.355 ± 0.1
Pea powder	7.0×10^3 3.85 log $\pm$ 0.07	<10	<10	<10	0.408 ± 0.17
Almond powder	<10	<10	<10	<10	0.424 ± 0.20
Spirulina powder	3.1×10^3 3.49 log $\pm$ 0.31	<10	<10	<10	0.363 ± 0.18
Pleurotus powder	4.6×10^5 5.66 log $\pm$ 0.1	<10	5.2×10^3 3.72 log $\pm$ 0.27	2.7×10^3 3.43 log $\pm$ 0.18	0.550 ± 0.17

Almond and yeast powder do not show microbiological contamination, thus presenting a satisfactory quality. Water activity is a critical parameter when it comes to food safety. It is a crucial factor in the microbiological control of food products. Water activity (aw) is defined as the equilibrium state achieved when a hygroscopic material is placed in a sealed container, and a balance is established between the material and the air above it. The relative humidity, which occurs at a constant air temperature, corresponds to the value of water activity multiplied by 100 (aw = relative humidity (%)/100). The majority of food items have a water activity level above 0.95, providing enough moisture to support the growth of bacteria, yeasts and mold.

The samples tested showed a low value of water activity, but the samples with aw value close to 0.600 presented microbial growth; in this case, *Pleurotus* spp. powder was the most contaminated sample tested (aw was 0.550).

3.3. Cytotoxicity Assessment by MTT and LDH

We selected colorimetric assays for cytotoxicity assessment. The MTT assay is a very popular and widely used colorimetric assay for the evaluation of cytotoxicity [62] as it provides beneficial aspects such as rapidity, reliability and significant knowledge regarding the metabolic activity of cells. The LDH assay provides information about cellular damage (lysis of cell's membranes) after cell treatment, and complements the MTT assay in drawing conclusions about the potential mechanism of action.

Our samples were analyzed from a starting concentration of 500 mg/mL, as the scientific literature indicates [63–68]. The results obtained show that this concentration provokes

a significant cell viability reduction in the case of *Pleurotus* spp. flour (up to 77% viability loss), followed by yeast powder (up to 69% viability loss). The subsequent concentrations (250 mg/mL, 62.5 mg/mL, 7.813 mg/mL and 0.651 mg/mL) tend to follow the same pattern (as seen in Figure 2A). Pea powder and almond powder present similar results, with viability reduction rates of up to 30%. Spirulina powder has great biocompatibility results (except for a concentration of 500 mg/mL—which is higher than the recommended concentration for most similar supplements). In the case of yeast powder, concentrations lower that 7 mg/mL have beneficial effects and act as nutritive substrates for cells.

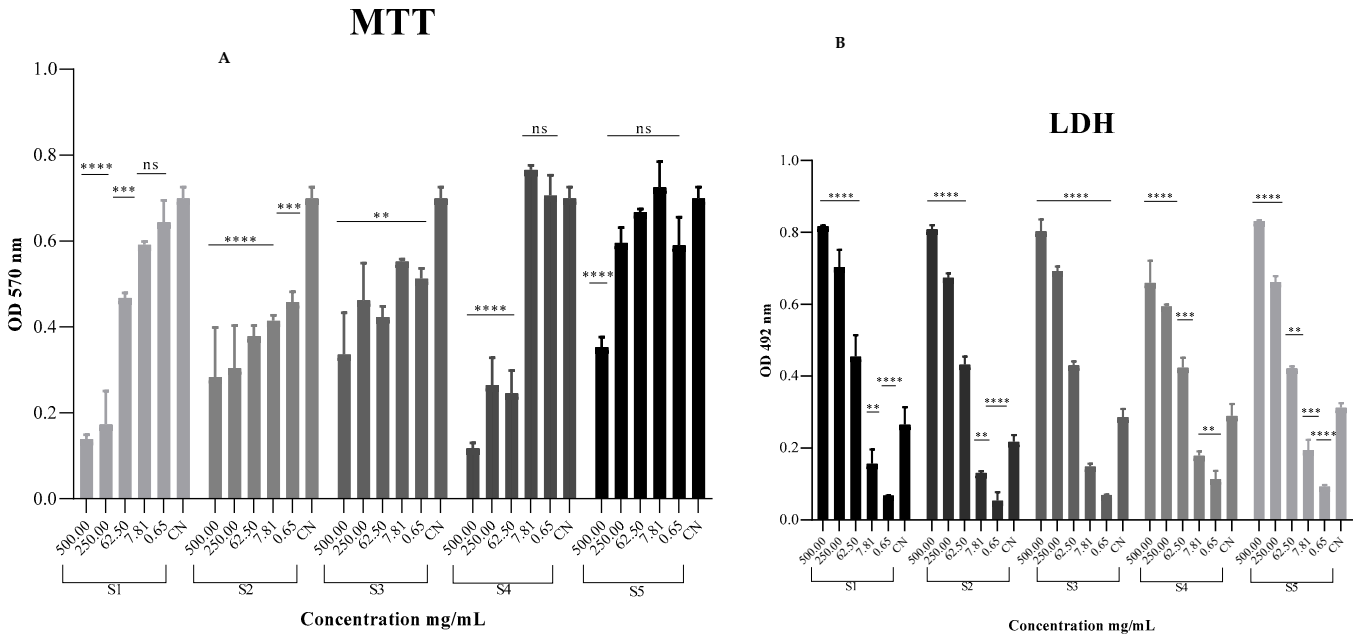

Figure 2. (**A**) Assessment of cytotoxicity using MTT; (**B**). Assessment of cytotoxicity using LDH assay. S1— *Pleurotus* spp. flour; S2—pea powder; S3—almond powder; S4—yeast powder; S5—spirulina powder; CN = Control—untreated NCTC cells with culture media; ****, ***, ** = p value < 0.05; ns = statistically non-significant.

Results obtained by the LDH assay (Figure 2B) conferee great evidence regarding cellular membrane integrity. All samples analyzed showcased membrane damage over 7.81 mg/mL. This result demonstrates that tested ingredients have an impact on the metabolic activity of cells—as MTT assay results indicate, and affect the cells integrity, provoking leakage of DNA.

4. Discussion

The human body does not store amino acids in the same way that it does fatty acids or carbohydrates. As a result, it is essential to ensure that the daily intake of amino acids needed for protein synthesis and other specific metabolic functions is sufficient. The concentration of amino acids in the blood remains relatively stable, which means that inadequate dietary protein intake can increase muscle protein breakdown, while excessive protein intake can lead to the breakdown of proteins for energy [69].

In the United States, the population's average recommended dietary allowance for protein is 0.8 grams of protein per kilogram of body weight per day [49]. In the European Union, based on nitrogen balance data, the European Food Safety Authority (EFSA) has established an average requirement of 0.66 grams of protein per kilogram of body weight per day for healthy adults, regardless of gender. Therefore, the recommendation for daily protein intake has been set at 0.83 grams of protein per kilogram of body weight per day [70], which corresponds to around 10–12% of total energy intake (E%). However, these recommendations may vary at the national level. For example, the Spanish recommen-

dation is 0.93–1.2 g of protein per kilogram of body weight per day [71] and the Finnish recommendation is 1.1–1.3 grams of protein per kilogram of body weight per day [72].

The protein content of raw materials is strongly influenced by the environment (pedoclimatic conditions, soil chemistry, pollution), variety and processing grade, which justifies the variability between the studies published so far [17,73–76]. Results obtained by our study indicate a high percentage of protein for the pea powder (77.96 ± 0.70%) and Spirulina powder (64.79 ± 2.20%); out of the tested ingredients, these are the most balanced in terms of fat and carbohydrate percentages, and their correlation with energy values. *Pleurotus* spp. has a low fat content, which is in accordance with the literature data. The lipid fraction of mushrooms generally comprises representative compounds from all lipid classes (free fatty acids, mono-, di- and triglycerides, sterols, sterol esters and phospholipids). Pea and yeast powders had a low fat content but with a high energy value due to a high protein content in the case of pea powder, and carbohydrates for yeast powder.

According to Soenen et al. (2012) [77], it was found that the relatively high protein intake highlights the success of the so-called "low-carbohydrate" diet, which is usually high in protein. Reduced carbohydrate intake had no effect on decreasing body weight and fat mass during energy restriction, while increasing daily absolute protein promoted body weight loss while reducing fat mass during the weight loss phase. Even though the highest energy value was obtained for almond powder due to the higher fat content and yeast powder for the high carbohydrate content, the preferred raw materials are pea and spirulina powder due to the total protein content.

According to Friedman et. al., 2004 [78], it was shown that the ninhydrin test for the quantification of amino groups in protein structure was more difficult than the analysis of free amino acids. The method of quantifying amino acids by Cd-ninhydrin has proven to be more effective in highlighting α-amino acids. In this context, a higher content of free amino acids for *Pleurotus* spp. flour was shown, while the soluble protein content was 6.15 mg/g. The administration of nutritional sources with a higher content of free amino acids are more indicated due to the absorption rate and assimilation efficiency higher than proteins [79].

Flavonoids are phenolic compounds with strong antioxidant activity by scavenging free radicals based on ET (electron transfer) mechanisms (DPPH) or by chelating transition metals (FRAP). Results obtained show that *Pleurotus* spp. flour had strong antioxidant activity through the FRAP method and was correlated with the capacity of total free amino acids (chelation metals) and in a low manner with phenol content and flavonoids. Almond powder has a high content of soluble proteins but with low antioxidant activity, an effect demonstrated by both antioxidant methods. For the spirulina powder, the antioxidant effect may be due to c-phycocyanins according to Zhou et. al., 2005 [61]. These compounds are known as the main light-harvesting protein pigment with nutritional and therapeutical properties.

Mesophilic aerobic bacteria and fungi are two of the most common types of microorganisms responsible for food spoilage. To prevent the contamination of finished food products with spoilage microorganisms, it is important to maintain a high level of hygiene throughout the entire food processing and handling chain. Preventing the contamination of food products with spoilage microorganisms requires a combination of good hygiene practices, effective sanitation protocols and ongoing monitoring and quality control. Results obtained for the TAMC and TYMC assessments indicate <10 CFU/g values for the yeast and almond powders. Pea, spirulina and *Pleurotus* spp. powders had $> 3 \times 10^3$ microbial contamination levels. If for pea and spirulina powders the microbial load is considered acceptable according to specific standards [52,53], for *Pleurotus* spp. powder, TAMC value of 4.6×10^5 CFU/g − 5.66 log ± 0.1 CFU/g and TYMC of 2.7×10^3 − 3.43 log ± 0.18 CFU/g are exceeding standard limits and cannot be considered suitable for integration for consumption or for composition of other products. As for determinations regarding β-glucuronidase positive *Escherichia coli*, neither of the samples tested positive, results being in accordance with standardized requirements [80].

Another category of microorganisms that are present in food in poor hygiene cases are those of the family *Enterobacteriaceae*. Bacteria from the *Enterobacteriaceae* family are commonly found in either dry or humid environments, are sensitive to heat treatment and sanitation and are desirable to be monitored in food environments. The presence of *Enterobacteriaceae* in post-hygienic processes may draw attention to the inefficiency of hygiene procedures. Our results indicate values of <10 CFU/g for yeast, pea, almond and spirulina powders. According to standardized requirements [54], these results comply. *Pleurotus* spp. powder showed a contamination of $5.2 \times 10^3 - 3.72 \log \pm 0.27$ CFU/g that exceeds standard limitations. These results indicate a high microbial contamination risk for the *Pleurotus* spp. powder. In order to exploit its beneficial effects, decontamination pre-treatments are necessary.

The assessment of toxicity of any given substance that is intended to be exposed to living organisms relies on in vitro evaluation of the dose–response. The dose–response relationship, or exposure–response relationship describes the magnitude of the response of an organism as a function of exposure (or doses) to a stimulus or stressor (usually a chemical) after a certain exposure time.

Results obtained demonstrate that tested ingredients have an impact on the metabolic activity of cells—as MTT assay results indicate, and affect the cells' integrity, provoking leakage of DNA. All in all, slow or delayed metabolic cellular activity indicates loss of balance and survival cues from the microenvironment. Such modifications do not support proper development and healthy homeostasis of mature tissues [81]. Here, we struggle with a debacle, as for some ingredients the recommended dosage for optimal effects is cytotoxic. Therefore, it should be reduced and supplemented with other products that help build the desired effect.

According to the EU Food Safety outlines, foodstuffs of animal and plant origin may present a microbiological risk. Microbiological criteria regarding the acceptability of foodstuffs and their manufacturing processes are obligated to comply with Good Hygiene and Manufacturing Practices (GHP, GMP) and the Hazard Analysis Critical Control Point (HACCP) principles that contribute to achieving food safety. Microbiological testing alone cannot guarantee the safety of a foodstuff tested, but these criteria provide objectives and reference points to assist food manufacturers and competent authorities in their activities to manage and monitor the safety of foodstuffs, respectively. Moreover, Commission Regulation (EC) EN No 2073/2005 on microbiological criteria for foods lays down food safety criteria for relevant foodborne bacteria, their toxins, and metabolites. Besides the high protein content that supports certain dietary needs (e.g., peas), the high-energy yield and notable antioxidant activity, increased levels of contamination in products tested through this study can pose several risks to human health (e.g., allergies and improper interactions with other drugs), besides false claims and regulatory issues.

5. Conclusions

Dietary supplementation does not benefit from stricter regulations regarding dosage (as pharmaceuticals do), health claims, contaminants or fraudulent practices, therefore consumers should be advised. At this moment, it falls into their responsibility to better examine what they consume and to genuinely evaluate safety. As our study presented, beyond the desirable physico-chemical and antioxidant properties, the wary microbiological status and cytotoxicity degree of such products are important parameters to consider for safe consumption. As for future perspectives, further studies are envisioned. We seek to demonstrate and confirm, on suitable final formulations, the potential beneficial effects of obtained products in order to safely place them into the current market.

Our view is that the regulatory body responsible for overseeing the placement of dietary supplements on the Romanian market should encourage manufacturers to contribute more to the research community by conducting additional testing on their products. This would be beneficial for both the manufacturers and the research community, as it could

help improve existing regulations and promote the placement of safer dietary supplements in the therapeutic market.

Author Contributions: Conceptualization, B.-M.T. and I.-C.M.; data curation, B.-M.T., I.-C.M. and L.M.Ș.; formal analysis, I.-C.M.; funding acquisition, D.E.D.; methodology, B.-M.T., I.-C.M., M.A. (Marian Adascălului), A.D., G.G.P. and M.A. (Mădălina Axinie); project administration, D.E.D.; resources, M.A. (Marian Adascălului), M.A. (Mădălina Axinie), L.M.Ș. and D.E.D.; writing—original draft, B.-M.T., I.-C.M., M.A. (Marian Adascălului), A.D., G.G.P. and M.A. (Mădălina Axinie); writing—review and editing, B.-M.T., I.-C.M., L.M.Ș. and D.E.D. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a grant of the Ministry of Research and Innovation through Program 1—Development of the National R&D System, Subprogram 1.2—Institutional Performance—Projects for Excellence Financing in RDI, project no. 26 PFE/17.10.2018. Also, this study was achieved through Core Programme (PN 19 02 01 01) and Program 1–Development of the National R&D System, Subprogram 1.2-Institutional Performance-Projects for Excellence Financing in RDI, project no. 17 PFE/2021 with the support of the Ministry of Research, Innovation and Digitization.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study is available at links associated with each reference and within manuscript.

Acknowledgments: We acknowledge the support of ICUB—Earth Environment and Life Science Department.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

References

1. Bartels, C.L.; Miller, S.J. Dietary Supplements Marketed for Weight Loss. *Nutr. Clin. Pract.* **2003**, *18*, 156–169. [CrossRef] [PubMed]
2. Samal, J.R.K.; Samal, I.R. Protein Supplements: Pros and Cons. *J. Diet. Suppl.* **2017**, *15*, 365–371. [CrossRef]
3. Europe Dietary Supplements Market (2021–2027): Market Forecast by Types, Function Type, Form, End User, Country and Competitive Landscape. Available online: https://www.researchandmarkets.com/reports/5360076/europe-dietary-supplements-market-2021-2027 (accessed on 26 April 2023).
4. Micha, R.; Michas, G.; Mozaffarian, D. Unprocessed Red and Processed Meats and Risk of Coronary Artery Disease and Type 2 Diabetes – An Updated Review of the Evidence. *Curr. Atheroscler. Rep.* **2012**, *14*, 515–524. [CrossRef] [PubMed]
5. Schwingshackl, L.; Hoffmann, G. Dietary fatty acids in the secondary prevention of coronary heart disease: A systematic review, meta-analysis and meta-regression. *BMJ Open* **2014**, *4*, e004487. [CrossRef] [PubMed]
6. Pan, A.; Chen, M.; Chowdhury, R.; Wu, J.H.; Sun, Q.; Campos, H.; Mozaffarian, D.; Hu, F.B. α-Linolenic acid and risk of cardiovascular disease: A systematic review and meta-analysis. *Am. J. Clin. Nutr.* **2012**, *96*, 1262–1273. [CrossRef]
7. Yokoyama, Y.; Nishimura, K.; Barnard, N.D.; Takegami, M.; Watanabe, M.; Sekikawa, A.; Okamura, T.; Miyamoto, Y. Vegetarian Diets and Blood Pressure: A Meta-analysis. *JAMA Intern. Med.* **2014**, *174*, 577–587. [CrossRef]
8. Shanthakumar, P.; Klepacka, J.; Bains, A.; Chawla, P.; Dhull, S.B.; Najda, A. The Current Situation of Pea Protein and Its Application in the Food Industry. *Molecules* **2022**, *27*, 5354. [CrossRef]
9. Ge, J.; Sun, C.; Corke, H.; Gul, K.; Gan, R.; Fang, Y. The health benefits, functional properties, modifications, and applications of pea (*Pisum sativum* L.) protein: Current status, challenges, and perspectives. *Compr. Rev. Food Sci. Food Saf.* **2020**, *19*, 1835–1876. [CrossRef]
10. Salles, J.; Guillet, C.; Le Bacquer, O.; Malnero-Fernandez, C.; Giraudet, C.; Patrac, V.; Berry, A.; Denis, P.; Pouyet, C.; Gueugneau, M.; et al. Pea Proteins Have Anabolic Effects Comparable to Milk Proteins on Whole Body Protein Retention and Muscle Protein Metabolism in Old Rats. *Nutrients* **2021**, *13*, 4234. [CrossRef]
11. Maykish, A.; Nishisaka, M.M.; Talbott, C.K.; Reaves, S.K.; Kristo, A.S.; Sikalidis, A.K. Comparison of Whey Versus Almond Protein Powder on Nitrogen Balance in Female College Students; The California Almond Protein Powder Project (CAlmond-P^3). *Int. J. Environ. Res. Public Health* **2021**, *18*, 11939. [CrossRef]
12. Becerra-Tomás, N.; Paz-Graniel, I.; Kendall, C.W.C.; Kahleova, H.; Rahelić, D.; Sievenpiper, J.L.; Salas-Salvadó, J. Nut consumption and incidence of cardiovascular diseases and cardiovascular disease mortality: A meta-analysis of prospective cohort studies. *Nutr. Rev.* **2019**, *77*, 691–709. [CrossRef]

13. Barreca, D.; Nabavi, S.M.; Sureda, A.; Rasekhian, M.; Raciti, R.; Silva, A.S.; Annunziata, G.; Arnone, A.; Tenore, G.C.; Süntar, I.; et al. Almonds (*Prunus dulcis* Mill. D. A. Webb): A Source of Nutrients and Health-Promoting Compounds. *Nutrients* **2020**, *12*, 672. [CrossRef] [PubMed]
14. Alfadhly, N.K.Z.; Alhelfi, N.; Altemimi, A.B.; Verma, D.K.; Cacciola, F.; Narayanankutty, A.; Rohn, S.; Alfadhly, N.K.Z.; Alhelfi, N.; Altemimi, A.B.; et al. Trends and Technological Advancements in the Possible Food Applications of Spirulina and Their Health Benefits: A Review. *Molecules* **2022**, *27*, 5584. [CrossRef] [PubMed]
15. Gogna, S.; Kaur, J.; Sharma, K.; Prasad, R.; Singh, J.; Bhadariya, V.; Kumar, P.; Jarial, S. Spirulina—An Edible Cyanobacterium with Potential Therapeutic Health Benefits and Toxicological Consequences. *J. Am. Nutr. Assoc.* **2022**, 1–14. [CrossRef] [PubMed]
16. Kumar, K. Nutraceutical Potential and Processing Aspects of Oyster Mushrooms (*Pleurotus* Species). *Curr. Nutr. Food Sci.* **2020**, *16*, 3–14. [CrossRef]
17. Lavelli, V.; Proserpio, C.; Gallotti, F.; Laureati, M.; Pagliarini, E. Circular reuse of bio-resources: The role of *Pleurotus* spp. in the development of functional foods. *Food Funct.* **2018**, *9*, 1353–1372. [CrossRef]
18. Raman, J.; Jang, K.Y.; Oh, Y.L.; Oh, M.; Im, J.H.; Lakshmanan, H.; Sabaratnam, V. Cultivation and Nutritional Value of Prominent *Pleurotus* spp.: An Overview. *Mycobiology* **2020**, *49*, 1–14. [CrossRef]
19. Jach, M.E.; Serefko, A.; Ziaja, M.; Kieliszek, M. Yeast Protein as an Easily Accessible Food Source. *Metabolites* **2022**, *12*, 63. [CrossRef]
20. Liao, Y.; Zhou, X.; Peng, Z.; Li, D.; Meng, Z.; Xu, S.; Yang, X.; Liu, L.; Yang, W. Muscle aging amelioration by yeast protein supplementation was associated with gut microbiota. *J. Funct. Foods* **2022**, *89*, 104948. [CrossRef]
21. Rizzello, C.G.; Tagliazucchi, D.; Babini, E.; Rutella, G.S.; Saa, D.L.T.; Gianotti, A. Bioactive peptides from vegetable food matrices: Research trends and novel biotechnologies for synthesis and recovery. *J. Funct. Foods* **2016**, *27*, 549–569. [CrossRef]
22. Agrawal, H.; Joshi, R.; Gupta, M. Isolation, purification and characterization of antioxidative peptide of pearl millet (*Pennisetum glaucum*) protein hydrolysate. *Food Chem.* **2016**, *204*, 365–372. [CrossRef] [PubMed]
23. Sarmadi, B.H.; Ismail, A. Antioxidative peptides from food proteins: A review. *Peptides* **2010**, *31*, 1949–1956. [CrossRef] [PubMed]
24. Zarei, M.; Ebrahimpour, A.; Abdul-Hamid, A.; Anwar, F.; Abu Bakar, F.; Philip, R.; Saari, N. Identification and characterization of papain-generated antioxidant peptides from palm kernel cake proteins. *Food Res. Int.* **2014**, *62*, 726–734. [CrossRef]
25. Lu, X.; Zhang, L.; Sun, Q.; Song, G.; Huang, J. Extraction, identification and structure-activity relationship of antioxidant peptides from sesame (*Sesamum indicum* L.) protein hydrolysate. *Food Res. Int.* **2018**, *116*, 707–716. [CrossRef] [PubMed]
26. Coda, R.; Rizzello, C.G.; Pinto, D.; Gobbetti, M. Selected Lactic Acid Bacteria Synthesize Antioxidant Peptides during Sourdough Fermentation of Cereal Flours. *Appl. Environ. Microbiol.* **2012**, *78*, 1087–1096. [CrossRef] [PubMed]
27. Maestri, E.; Marmiroli, M.; Marmiroli, N. Bioactive peptides in plant-derived foodstuffs. *J. Proteom.* **2016**, *147*, 140–155. [CrossRef] [PubMed]
28. Elias, R.J.; Kellerby, S.S.; Decker, E.A. Antioxidant Activity of Proteins and Peptides. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 430–441. [CrossRef]
29. Wen, C.; Zhang, J.; Zhang, H.; Duan, Y.; Ma, H. Plant protein-derived antioxidant peptides: Isolation, identification, mechanism of action and application in food systems: A review. *Trends Food Sci. Technol.* **2020**, *105*, 308–322. [CrossRef]
30. Qian, Z.-J.; Jung, W.-K.; Kim, S.-K. Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, Rana catesbeiana Shaw. *Bioresour. Technol.* **2008**, *99*, 1690–1698. [CrossRef]
31. Rajapakse, N.; Mendis, E.; Jung, W.-K.; Je, J.-Y.; Kim, S.-K. Purification of a radical scavenging peptide from fermented mussel sauce and its antioxidant properties. *Food Res. Int.* **2005**, *38*, 175–182. [CrossRef]
32. Nwachukwu, I.D.; Aluko, R.E. Structural and functional properties of food protein-derived antioxidant peptides. *J. Food Biochem.* **2019**, *43*, e12761. [CrossRef] [PubMed]
33. Du, Z.; Comer, J.; Li, Y. Bioinformatics approaches to discovering food-derived bioactive peptides: Reviews and perspectives. *TrAC Trends Anal. Chem.* **2023**, *162*, 117051. [CrossRef]
34. Zhu, Y.; Lao, F.; Pan, X.; Wu, J. Food Protein-Derived Antioxidant Peptides: Molecular Mechanism, Stability and Bioavailability. *Biomolecules* **2022**, *12*, 1622. [CrossRef]
35. Satija, A.; Bhupathiraju, S.N.; Spiegelman, D.; Chiuve, S.E.; Manson, J.E.; Willett, W.; Rexrode, K.M.; Rimm, E.B.; Hu, F.B. Healthful and Unhealthful Plant-Based Diets and the Risk of Coronary Heart Disease in U.S. Adults. *J. Am. Coll. Cardiol.* **2017**, *70*, 411–422. [CrossRef] [PubMed]
36. Poore, J.; Nemecek, T. Reducing food's environmental impacts through producers and consumers. *Science* **2018**, *360*, 987–992. [CrossRef] [PubMed]
37. Mistry, K.; Sardar, S.D.; Alim, H.; Patel, N.; Thakur, M.; Jabbarova, D.; Ali, A. Plant Based Proteins: Sustainable Alternatives. *Plant Sci. Today* **2022**, *9*, 820–828. [CrossRef]
38. Langyan, S.; Yadava, P.; Khan, F.N.; Dar, Z.A.; Singh, R.; Kumar, A. Sustaining Protein Nutrition through Plant-Based Foods. *Front. Nutr.* **2022**, *8*, 1237. [CrossRef]
39. Alcorta, A.; Porta, A.; Tárrega, A.; Alvarez, M.D.; Vaquero, M.P. Foods for Plant-Based Diets: Challenges and Innovations. *Foods* **2021**, *10*, 293. [CrossRef]
40. Hertzler, S.R.; Lieblein-Boff, J.C.; Weiler, M.; Allgeier, C. Plant Proteins: Assessing Their Nutritional Quality and Effects on Health and Physical Function. *Nutrients* **2020**, *12*, 3704. [CrossRef]

41. The EU Agri-Food Fraud Network and the Administrative Assistance and Cooperation System 2020 Annual Report Health and Food Safety. Available online: https://food.ec.europa.eu/safety/eu-agri-food-fraud-network_en (accessed on 14 April 2023).
42. *DIN ISO 6673*; Green Coffee—Determination of Loss in Mass at 105 °C (ISO 6673:2003). Available online: https://www.en-standard.eu/din-iso-6673-green-coffee-determination-of-loss-in-mass-at-105-c-iso-6673-2003/ (accessed on 14 April 2023).
43. *ISO 20483:2013*; Cereals and Pulses—Determination of the Nitrogen Content and Calculation of the Crude Protein Content—Kjeldahl Method. Available online: https://www.iso.org/standard/59162.html (accessed on 31 May 2023).
44. *ISO 6492*; Animal Feeding Stuffs—Determination of Fat Content. 1999. Available online: https://www.iso.org/standard/12865.html (accessed on 14 April 2023).
45. *BS EN ISO 2171:2010*; Cereals, Pulses and By-Products. Determination of Ash Yield by Incineration. 2010. Available online: https://landingpage.bsigroup.com/LandingPage/Standard?UPI=000000000030213066 (accessed on 14 April 2023).
46. Mattila, P.; Mäkinen, S.; Eurola, M.; Jalava, T.; Pihlava, J.-M.; Hellström, J.; Pihlanto, A. Nutritional Value of Commercial Protein-Rich Plant Products. *Plant Foods Hum. Nutr.* **2018**, *73*, 108–115. [CrossRef]
47. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
48. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152–178. [CrossRef]
49. Woisky, R.G.; Salatino, A. Analysis of propolis: Some parameters and procedures for chemical quality control. *J. Apic. Res.* **1998**, *37*, 99–105. [CrossRef]
50. Madhu, G.; Bose, V.C.; Aiswaryaraj, A.; Maniammal, K.; Biju, V. Defect dependent antioxidant activity of nanostructured nickel oxide synthesized through a novel chemical method. *Colloids Surfaces: A Physicochem. Eng. Asp.* **2013**, *429*, 44–50. [CrossRef]
51. Benzie, I.F.F.; Strain, J.J. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* **1999**, *299*, 15–27. [CrossRef] [PubMed]
52. European Standards. *UNE EN ISO 4833-1:2014*; Microbiology of the Food Chain—Horizontal Method for the Enumeration of Microorganisms—Part 1: Colony Count at 30 Degrees C by the Pour Plate Technique (ISO 4833-1:2013). Available online: https://www.en-standard.eu/une-en-iso-4833-1-2014-microbiology-of-the-food-chain-horizontal-method-for-the-enumeration-of-microorganisms-part-1-colony-count-at-30-degrees-c-by-the-pour-plate-technique-iso-4833-1-2013/ (accessed on 14 April 2023).
53. *EVS-ISO 21527-2:2009*; Microbiology of Food and Animal Feeding Stuffs — Horizontal Method for the Enumeration of Yeasts and Moulds — Part 2: Colony Count Technique in Products with Water Activity Less Than or Equal to 0,95 (ISO 21527-2:2008). Available online: https://www.evs.ee/en/evs-iso-21527-2-2009 (accessed on 31 May 2023).
54. *ISO 21528-2:2017*; Microbiology of the Food Chain—Horizontal Method for the Detection and Enumeration of Enterobacteriaceae—Part 2: Colony-Count Technique. International Organization for Standardization: Geneva, Switzerland, 2017.
55. Tihăuan, B.M.; Berca, L.M.; Adascalului, M.; Sanmartin, A.M.; Nica, S.; Cimponeriu, D.; Duță, D. Experimental in vitro cytotoxicity evaluation of plant bioactive compounds and phytoagents: A review. *Rom. Biotechnol. Lett.* **2020**, *25*, 1832–1842.
56. TMosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63. [CrossRef]
57. Naumann, A.; Heine, G.; Rauber, R. Efficient discrimination of oat and pea roots by cluster analysis of Fourier transform infrared (FTIR) spectra. *Field Crop. Res.* **2010**, *119*, 78–84. [CrossRef]
58. Sharma, S.; Raj, N. FTIR Spectroscopic Characterisation of Almond Varieties (*Prunus dulcis*) from Himachal Pradesh (India). *Int. J. Curr. Microbiol. Appl. Sci.* **2018**, *7*, 887–898. [CrossRef]
59. Parmar, R.; Kumar, D. Study of chemical composition in wild edible mushroom *Pleurotus cornucopiae* (Paulet) from Himachal Pradesh, India by using Fourier transforms infrared spectrometry (FTIR), Gas chromatography-mass spectrometry (GCMS) and X-ray fluorescence (XRF). *Biol. Forum* **2015**, *7*, 1057–1066.
60. Mæhre, H.K.; Dalheim, L.; Edvinsen, G.K.; Elvevoll, E.O.; Jensen, I.-J. Protein Determination—Method Matters. *Foods* **2018**, *7*, 5. [CrossRef] [PubMed]
61. Zhou, Z.-P.; Liu, L.-N.; Chen, X.-L.; Wang, J.-X.; Chen, M.; Zhang, Y.-Z.; Zhou, B.-C. Factors that effect antioxidant activity of c-phycocyanins from *Spirulina platensis*. *J. Food Biochem.* **2005**, *29*, 313–322. [CrossRef]
62. Präbst, K.; Engelhardt, H.; Ringgeler, S.; Hübner, H. Basic colorimetric proliferation assays: MTT, WST, and resazurin. In *Methods in Molecular Biology*; Humana Press Inc.: Totowa, NJ, USA, 2017; Volume 1601.
63. Babault, N.; Païzis, C.; Deley, G.; Guérin-Deremaux, L.; Saniez, M.-H.; Lefranc-Millot, C.; Allaert, F.A. Pea proteins oral supplementation promotes muscle thickness gains during resistance training: A double-blind, randomized, Placebo-controlled clinical trial vs. Whey protein. *J. Int. Soc. Sports Nutr.* **2015**, *12*, 3–9. [CrossRef] [PubMed]
64. Guasch-Ferré, M.; Liu, X.; Malik, V.S.; Sun, Q.; Willett, W.C.; Manson, J.E.; Rexrode, K.M.; Li, Y.; Hu, F.B.; Bhupathiraju, S.N. Nut Consumption and Risk of Cardiovascular Disease. *J. Am. Coll. Cardiol.* **2017**, *70*, 2519–2532. [CrossRef] [PubMed]
65. Proserpio, C.; Lavelli, V.; Laureati, M.; Pagliarini, E. Effect of *Pleurotus ostreatus* powder addition in vegetable soup on ß-glucan content, sensory perception, and acceptability. *Food Sci. Nutr.* **2019**, *7*, 730–737. [CrossRef]

66. Takahashi, T.; Yu, F.; Zhu, S.-J.; Moriya, J.; Sumino, H.; Morimoto, S.; Yamaguchi, N.; Kanda, T. Beneficial Effect of Brewers' Yeast Extract on Daily Activity in a Murine Model of Chronic Fatigue Syndrome. *Evid. Based Complement. Altern. Med.* **2006**, *3*, 109–115. [CrossRef]
67. Torres-Duran, P.V.; Ferreira-Hermosillo, A.; Juarez-Oropeza, M.A. Antihyperlipemic and antihypertensive effects of Spirulina maxima in an open sample of mexican population: A preliminary report. *Lipids Health Dis.* **2007**, *6*, 33. [CrossRef]
68. Borges, A.; Ferreira, C.; Simões, M.; Wang, M.; Tao, L.; Xu, H.; Alexandri, M.; Papapostolou, H.; Vlysidis, A.; Gardeli, C.; et al. Antibacterial Activity and Mode of Action of Ferulic and Gallic Acids Against Pathogenic Bacteria. *Microb. Drug Resist.* **2013**, *19*, 256–265. [CrossRef]
69. Kårlund, A.; Gómez-Gallego, C.; Turpeinen, A.M.; Palo-Oja, O.-M.; El-Nezami, H.; Kolehmainen, M. Protein Supplements and Their Relation with Nutrition, Microbiota Composition and Health: Is More Protein Always Better for Sportspeople? *Nutrients* **2019**, *11*, 829. [CrossRef]
70. Agostoni, C.; Bresson, J.L.; Fairweather Tait, S.; Flynn, A.; Golly, I.; Korhonen, H.; Lagiou, P.; Løvik, M.; Marchelli, R.; Martin, A.; et al. Scientific Opinion on Dietary Reference Values for protein. *EFSA J.* **2012**, *10*, 2557. [CrossRef]
71. Sánchez-Muniz, F.J.; Bastida, S.L. *En: Libro Blanco de la Nutrición en España*; Fundación Española de la Nutrición (FEN): Madrid, Spain, 2013; pp. 113–124.
72. Fogelholm, M.; Hakala, P.; Kara, R. *Terveyttä Ruoasta-Suomalaiset Ravitsemussuositukset 2014 (Finnish Nutrition Recommendations 2014)*; State Nutrition Advisory Board: Helsinki, Finland, 2014.
73. PYen, P.P.; Pratap-Singh, A. Vacuum microwave dehydration decreases volatile concentration and soluble protein content of pea (*Pisum sativum* L.) protein. *J. Sci. Food Agric.* **2020**, *101*, 167–178. [CrossRef]
74. Wang, X.-X.; Zhao, Z.-H.; Chang, T.-S.; Liu, J.-G. Yeast screening from avermectins wastewater and investigation on the ability of its fermentation. *Bioprocess Biosyst. Eng.* **2011**, *34*, 1127–1132. [CrossRef] [PubMed]
75. Jin, S.-E.; Lee, S.J.; Kim, Y.; Park, C.-Y. Spirulina powder as a feed supplement to enhance abalone growth. *Aquac. Rep.* **2020**, *17*, 100318. [CrossRef]
76. Ahrens, S.; Venkatachalam, M.; Mistry, A.M.; Lapsley, K.; Sathe, S.K. Almond (*Prunus dulcis* L.) Protein Quality. *Plant Foods Hum. Nutr.* **2005**, *60*, 123–128. [CrossRef] [PubMed]
77. Soenen, S.; Bonomi, A.G.; Lemmens, S.G.; Scholte, J.; Thijssen, M.A.; van Berkum, F.; Westerterp-Plantenga, M.S. Relatively high-protein or 'low-carb' energy-restricted diets for body weight loss and body weight maintenance? *Physiol. Behav.* **2012**, *107*, 374–380. [CrossRef] [PubMed]
78. Friedman, M. Applications of the Ninhydrin Reaction for Analysis of Amino Acids, Peptides, and Proteins to Agricultural and Biomedical Sciences. *J. Agric. Food Chem.* **2004**, *52*, 385–406. [CrossRef]
79. Rønnestad, I.; Conceição, L.E.C.; Aragão, C.; Dinis, M.T. Free Amino Acids Are Absorbed Faster and Assimilated More Efficiently than Protein in Postlarval Senegal Sole (*Solea senegalensis*). *J. Nutr.* **2000**, *130*, 2809–2812. [CrossRef]
80. Microbiologia Alimentelor Şi Nutreţurilor. Metodă Orizontală Pentru Enumerarea Escherichia Coli Pozitive La Beta-Glucuronidază. Partea 2: Tehnica de Enumerare a Coloniilor La 44 Grade C Folosind 5-Bromo-4-Cloro-3-Indolil Beta-D-Glucuronat. Available online: https://magazin.asro.ro/ro/standard/142768 (accessed on 14 April 2023).
81. Mason, E.F.; Rathmell, J.C. Cell Metabolism: An Essential Link between Cell Growth and Apoptosis. *Biochim. Biophys. Acta Mol. Cell Res.* **2011**, *1813*, 645–654. [CrossRef]