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Ongoing Research on Microgreens

Nutritional Properties, Shelf-Life, Sustainable Production, Innovative Growing and Processing Approaches

Edited by
Vito Michele Paradiso and Massimiliano Renna

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About the Editors

Vito Michele Paradiso is currently an Associate Professor of Food Science and Technology for the Department of Biological and Environmental Sciences and Technologies of the University of Salento. From 2005 to 2009 he obtained a research grant for the research program "Evaluation of the hydrolytic and oxidative of refined edible oils by innovative analytical parameters", at the University of Bari. In 2011, he obtained the PhD in Microbiology, Safety and Chemistry of Food. From 2010 to 2020 he was a researcher in food science and technology for the Department of Soil, Plant and Food Science of the University of Bari. He teaches courses such as "Food Packaging" for the Bachelor's degree in Food Science and Technology, "Food chemistry" for the Master's degree in Food Science and Technology, and "Food Technology" for the Master's degree in Science of Nutrition for Human Health, all at the University of Bari, as well as Enology I and Enology II for the Bachelor's degree of Viticulture and Enology at the University of Salento. He was a tutor for several experimental theses for the Master's degree in Food Science and Technology, three PhD theses in Soil and Food Science and several research grants.

He is responsible for funded research project under competitive calls. His research activity regards:

- innovative and sustainable technologies for winemaking;
- chemistry of lipid oxidation in bulk oils and dispersed systems;
- volatile compounds in foods;
- reduction of the environmental impact of food processes and food analysis;
- food packaging and shelf life;
- agri-food waste exploitation;
- agricultural biodiversity;
- tailored nutrition and improvement of food nutritional value.

He is author of more than 180 papers dating back to 2006, of which 111 papers are indexed in Scopus, as well as a number of contributions and oral presentations given at national and international conferences. He is a member of the Italian Society of Food Science and Technology and of the Euro Fed Lipid. He serves as a member of the Editorial Board for the MDPI journal *Foods*.

Massimiliano Renna is a researcher at the CNR—ISPA (Institute of Sciences of Food Production, National Research Council, Italy). In 2013, he received his PhD in Mediterranean Agronomy from the University of Bari Aldo Moro, Italy. From 2015 to 2018 he was a researcher for the Department of Agricultural and Environmental Science, University of Bari Aldo Moro, Italy. He is an advisory board member of the Academy of Mediterranean Biodiversity Sciences and a member of the Italian Society of Horticulture. His research interests include a broad range of aspects regarding vegetable products. Particular attention is given to vegetable landraces and wild edible plants. He is the author of three books and more than 80 scientific papers published in national and international journals, and proceedings presented at national and international conferences.

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Editorial

Ongoing Research on Microgreens: Nutritional Properties, Shelf-Life, Sustainable Production, Innovative Growing and Processing Approaches

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Abstract: Microgreens garner immense potential for improving the nutritional value of the human diet, considering their high content of healthy compounds. On the other hand, they are gaining more and more interest not only for their nutritional value but also for their interesting organoleptic traits and commercial potential. The purpose of this Special Issue is to publish high-quality research papers with the aim to cover the state-of-the-art, recent progress and perspectives related to production, post-harvest, characterization, and potential of microgreens. A broad range of aspects such as cultivation, post-harvest techniques and packaging, analytical methods, nutritional value, bioaccessibily and prospects are covered. All contributions are of significant relevance and could stimulate further research in this area.

Keywords: bioaccessibility; bioactive componds; Brassicaceae; carotenoids; hydroponic cultivation; mineral elements; polyamine; quality; nitrate; wild edible species

"Microgreens" is a marketing term used to describe young and tender edible seedlings harvested when the cotyledonary leaves have fully developed and the first true leaves emerge. This category of vegetables presents different traits as compared to the already known sprouts and the common baby leaf vegetables [1,2].

Microgreens are gaining increasing interest as potential functional foods, due to their relevant contents of micronutrients and bioactive compounds [3–7]. They are gaining popularity also due to their varying and attractive colors, textures, and flavors [8]. The wide number of species and cultivars that can be grown as microgreens and the possibility to control their growing conditions even in micro-scale production underlie their promising potential for tailored nutrition [9], as well as to address particularly demanding categories of consumers, such as vegans or raw-foodists. At the same time, microgreens can be grown in a very simple way, even in very small spaces, being suitable for urban agriculture, as well as a component of space life support systems [10]. Nevertheless, several research themes still need to be explored, throughout the chain. A representative, though non-exhaustive, list of current research themes could include:

- sustainable cultivation and growing substrates from renewable sources;
- microgreens production in indoor, urban or space growing systems;
- nutritional characterization and effects of genotypes (i.e., biodiversity exploitation) and of growing conditions (e.g., nutrition, natural and artificial lighting systems, use of selected wavelengths);
- nutritional tailoring to address specific needs (e.g., nutritional integration for children, pregnant women, elderly people; chronic diseases management; prevention; hidden hunger issues);

- packaging and shelf-life, with specific focus on safety, nutritional content, environmental and sustainability issues;
- nutrient bioaccessibility and bioavailability;
- specific analytical methods for either characterization or quality control.

The purpose of this Special Issue was to collect contributes on some of these relevant themes and publish high-quality research papers with the aim to cover the state-of-the-art, recent progress and perspectives related to production, post-harvest, characterization, and potential of microgreens.

In the first article, entitled "Evaluation of the Bioaccessibility of Antioxidant Bioactive Compounds and Minerals of Four Genotypes of *Brassicaceae* Microgreens" by Beatriz de la Fuente, Gabriel López-García, Vicent Máñez, Amparo Alegría, Reyes Barberá and Antonio Cilla [11], the contents of minerals and antioxidant bioactive compounds were evaluated in broccoli (*Brassica oleracea* L. var. *italica* Plenck), green curly kale (*Brassica oleracea* var. *sabellica* L.), red mustard (*Brassica juncea* (L.) Czern.) and radish (*Raphanus sativus* L.) hydroponic microgreens. Authors evaluated the content of potassium, calcium, magnesium, iron, zinc, ascorbic acid, total soluble polyphenols, total carotenoids, total anthocyanins, total isothiocyanates and total antioxidant capacity. For the first time, the bioaccessibility of these compounds was also evaluated on microgreens by using a simulated gastrointestinal digestion process. The authors found that all four genotypes of microgreens provided relevant amounts of ascorbic acid and carotenoids, while mineral content was comparable to those reported in the literature for microgreens hydroponically grown. Moreover, the article reported that the greatest contributors to the antioxidant capacity after the simulated digestion were polyphenols and isothiocyanates, while macroelements showed high bioaccessibility values, reaching 90% in the case of calcium. Overall, the authors suggested that the four genotypes of Brassicaceae microgreens can be considered a good source of antioxidant bioactive compounds, although radish and mustard presented the highest bioaccessibility not only for these compounds but also for minerals.

The second contribution regards the "Antioxidant and Mineral Composition of Three Wild Leafy Species: A Comparison Between Microgreens and Baby Greens" by Anna Lenzi, Alessandro Orlandini, Roberta Bulgari, Antonio Ferrante and Piero Bruschi [2]. In this study, the authors compared three wild leafy species (*Sanguisorba minor* Scop., *Sinapis arvensis* L., and *Taraxacum officinale* Weber ex F. H. Wigg.), harvested at the microgreen and baby green stages, in order to evaluate yield and content of chlorophylls, carotenoids, anthocyanins, phenolic index, nitrate and mineral elements. The authors also calculated the potential contribution to human mineral intake, showing that both micro- and baby greens could positively contribute to the dietary intake of macro- and microelements as well as non-nutrient bioactive compounds, having a contribution comparable to, or even larger than that of vegetable crop species. On the other hand, the authors concluded that, although wild edible plants may play an important role in human nutrition, the observed high amounts of nitrate and traces of some metals potentially detrimental for health, suggest the need for caution in the use of wild species to produce microgreens and baby leaves.

The third paper illustrates the "Setup of an Extraction Method for the Analysis of Carotenoids in Microgreens" by Vito Michele Paradiso, Maria Castellino, Massimiliano Renna, Pietro Santamaria and Francesco Caponio [12]. In this research, a specific extraction procedure for the analysis of carotenoids in microgreens was developed, starting from the remark that the analysis of carotenoids is inherently difficult, and that extraction is the most critical step. Authors evaluated several aspects, such as the solvent composition, extraction time, solvent/sample ratio, and repeated extractions. The results enabled the authors to develop an effective protocol for the extraction and analysis of carotenoids from microgreens that allows the recovery of 97.2%, limits of quantitation of 5.2 $\mu g\ g^{-1}$ for lutein and 15.9 $\mu g\ g^{-1}$ for β-carotene, as well as intra-day mean repeatability of 5.7% and inter-day mean repeatability of 4.7%. The authors concluded that the developed protocol proved to be more efficient in the extraction of carotenoids from the delicate tissues of microgreens, even compared to another method from the literature. Therefore, the proposed analytical method could allow improvement in

the obtainment of nutritional data on microgreens, which are claiming increasing attention for their functional potential and suitability for tailored nutrition.

The fourth article concerns the "Accumulation of Agmatine, Spermidine, and Spermine in Sprouts and Microgreens of Alfalfa, Fenugreek, Lentil, and Daikon Radish" by Irena Kralj Cigić, Sašo Rupnik, Tjaša Rijavec, Nataša Poklar Ulrih and Blaž Cigić [13]. This study was conducted firstly to determine the polyamine content in seeds, sprouts, and microgreens of three legumes (lentil, fenugreek, alfalfa) and daikon radish. The authors also evaluated whether microgreens are nutritionally superior to sprouts in terms of polyamine content. Furthermore, an evaluation of the enzymatic potential of sprouts to degrade undesirable biogenic amines was carried out. The authors observed that, in general, sprouting led to the accumulation of the total polyamine content. Alfalfa microgreens showed the highest levels of agmatine, fenugreek sprouts showed the highest content of putrescine and cadaverine, in lentil microgreens the highest content of spermidine was found, while fenugreek microgreens showed the highest content of spermine. Moreover, while a large increase in cadaverine content was observed in all three legume sprouts, the nutritionally beneficial polyamines (agmatine, spermidine, and spermine) were accumulated in microgreens, together with a lower cadaverine content. The authors also observed that daikon radish sprouts, in contrast to other ones, exhibited a nutritionally better profile of polyamines than the microgreens. Another interesting result of this study regards the enzymatic potential of fenugreek sprouts, since the authors found that homogenized fenugreek sprouts was effective in degrading exogenous putrescine, cadaverine, and tyramine at pH values above 5.

In the fifth paper, Manjula D. Ghoora and Nagarajan Srividya evaluated the "Effect of Packaging and Coating Technique on Postharvest Quality and Shelf Life of radish (*Raphanus sativus* L.) and roselle (*Hibiscus sabdariffa* L.) Microgreens" [14]. The authors studied the efficacy of two types of macro-perforated packaging (polyethylene terephthalate clamshell containers—PET-CS, and low-density polyethylene self-seal bags—LDPE-SSB) on the postharvest quality and shelf life of microgreens stored at 5 °C. Moreover, for the first time, spray- and dip-coating techniques were compared to study the effect of *Aloe vera* gel (AG) as an eco-friendly treatment on the postharvest quality and shelf life of radish and roselle microgreens. Physiological loss in weight, respiration rate, electrolyte leakage, color analysis, ascorbic acid content, microbial count, overall acceptability, and marketability were evaluated. Overall, the authors indicated that although macro-perforated PET-CS was found to be a comparatively better packaging than LDPE-SSB for postharvest quality maintenance during the storage of radish and roselle microgreens, LDPE-SSB could be used as an economical alternative in short distance markets and for sturdier microgreens. AG-coated microgreens had significantly lesser deteriorative postharvest changes and higher ascorbic acid content than the uncoated control, while AG spray coating maintained better overall acceptability and postharvest quality than AG dipping coating. Therefore, the authors concluded that AG spray coating could be suggested as an eco-friendly ergonomic pre-harvest treatment along with PET–CS for the enhancement of postharvest quality and shelf life in radish and roselle microgreens, with a high potential to be extended to other microgreens.

The sixth contribution is "Yield and Quality Characteristics of *Brassica* Microgreens as Affected by the NH_4:NO_3 Molar Ratio and Strength of the Nutrient Solution" by Onofrio Davide Palmitessa, Massimiliano Renna, Pasquale Crupi, Angelo Lovece, Filomena Corbo and Pietro Santamaria [15]. In this study, three *Brassica* genotypes (broccoli raab, broccoli and cauliflower) were fertigated using a nutrient solution with three different strength or with three different NH_4:NO_3 molar ratios percent (5:95, 15:85, and 25:75), starting from a Hoagland-like nutrient solution. Microgreen yields and content of inorganic ions, dietary fiber, proteins, α-tocopherol, and β-carotene were evaluated. The authors found that all three *Brassica* genotypes can be considered suitable for microgreen production, although micro cauliflower showed the highest yield, as well as a higher content of some mineral elements and α-tocopherol compared to other genotypes, while micro broccoli raab showed the fastest growth rate. Overall, the authors observed that the use of a Hoagland-like nutrient solution at half strength allowed them to obtain both high yield and desirable seedling height. On the other hand, the authors

highlighted the possibility of producing microgreens of broccoli raab, broccoli and cauliflower by changing the NH_4:NO_3 molar ratio in the nutrient solution without negatively affecting yield, growing parameters and an important commercial characteristic such as the nitrate content, although the highest β-carotene content was found by using a nutrient solution with a NH_4:NO_3 molar ratio of 25:75.

In conclusion, the papers of this Special Issue cover a broad range of aspects and represent some of the recent research results regarding the topic of microgreens, which are gaining more and more interest not only for their nutritional value but also for their interesting organoleptic traits and commercial point of view. We think that this Special Issue may stimulate further research in this area.

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Article

Evaluation of the Bioaccessibility of Antioxidant Bioactive Compounds and Minerals of Four Genotypes of *Brassicaceae* Microgreens

Beatriz de la Fuente [1], Gabriel López-García [1], Vicent Máñez [2], Amparo Alegría [1], Reyes Barberá [1] and Antonio Cilla [1,*]

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Abstract: Microgreens constitute an emerging class of fresh, healthy foods due to their nutritional composition. In this study the content of minerals and antioxidant bioactive compounds, and for the first time bioaccessibility, were evaluated in broccoli (*Brassica oleracea* L. var. *italica* Plenck), green curly kale (*Brassica oleracea* var. *sabellica* L.), red mustard (*Brassica juncea* (L.) Czern.) and radish (*Raphanus sativus* L.) hydroponic microgreens. Macro- (K, Ca, Mg) and oligo-elements (Fe, Zn), ascorbic acid, total soluble polyphenols, total carotenoids, total anthocyanins, total isothiocyanates and total antioxidant capacity (Trolox Equivalent Antioxidant Capacity and Oxygen Radical Absorbance Capacity) were determined before and after the standardized simulated gastrointestinal digestion process. All microgreens provided relevant amounts of vitamin C (31–56 mg/100 g fresh weight) and total carotenoids (162–224 mg β-carotene/100 g dry weight). Mineral content was comparable to that normally found in hydroponic microgreens and the low potassium levels observed would allow their dietetic recommendation for patients with impaired kidney function. Both total soluble polyphenols and total isothiocyanates were the greatest contributors to the total antioxidant capacity after digestion (43–70% and 31–63% bioaccessibility, respectively) while macroelements showed an important bioaccessibility (34–90%). In general, radish and mustard presented the highest bioaccessibility of bioactive compounds and minerals. Overall, the four hydroponic *Brassicaceae* microgreens present a wide array of antioxidant bioactive compounds.

Keywords: microgreens; *Brassicaceae*; bioaccessibility; minerals; bioactive compounds; antioxidants

1. Introduction

Microgreens are a new class of small, fresh, edible vegetables considered as a good nutritional source because of their high mineral and bioactive compound content. The meaning of microgreen refers to immature greens harvested at soil level between the first and third week after sowing, when the cotyledon is fully developed and the first true leaves have emerged [1–3], being different from both baby leaf (cut greens for salads) [4] and sprouts (germinated seeds with entire roots) [5]. Microgreens can be produced from many vegetables, herbaceous plants, aromatic herbs, grains and wild species [6–8], and possess distinctive organoleptic properties, such as color, shape, texture and taste [2,8,9].

These new and young vegetables are a versatile, nutritive and sustainable crop from cultivation to consumption. They can be adapted to different agronomic practices to obtain a final product which is of high organoleptic and nutritional quality [1]. Likewise, growing conditions (soil, compost, hydroponic) directly affect the plant growth and the levels of phytonutrients and minerals [5,10].

In this sense, soilless or hydroponic is based on the use of nutrient solution rather than soil for crop production, reducing fertilizer and water resources as well as the use of pesticides [11]. According to Weber [10], a higher amount of minerals was obtained in broccoli microgreens compared to the mature vegetable using about 200 times less water, 94% less time and without applying fertilizer, pesticides or energy-demanding transport. Besides the possibility of saving natural resources and chemicals, the production and consumption of microgreens have additional advantages, turning these products into a new, healthy, and environmentally-friendly vegetable option. For instance, the containerized production in an industrial, local or home scale implies that the final consumer can harvest them just at the moment of being used, and their consumption only without roots generates much less waste than adult vegetables [1,10,12].

In addition, microgreens have been considered as healthy foods because of their general higher levels of phytochemicals with respect to their mature counterparts [2,6,7,12]. In this context, a recent review has defined microgreens as a new food for the 21st century attributing them a potential role as anti-inflammatory, anti-carcinogenic, anti-obesogenic and anti-atherosclerotic [5]. In contrast to the great amount of nutrients expected to obtain health benefits, Renna et al. [13] developed chicory and lettuce microgreens with a reduced potassium content to be consumed by chronic kidney disease patients. Also, microgreens have been proposed as ideal food for people with a vegetable-based diet such as vegans or vegetarians, and even for space crew members due to their limited access to food diversity [14].

It is known that *Brassica* vegetables, at the mature stage, contain beneficial nutrients for human health [15], and available data reveal that their intake reduces the risk of chronic diseases [16]. Probably this is the reason why among the different species used to obtain microgreens, the *Brassicaceae* family is one of the most widely grown to date [2]. Nevertheless, information in the literature about *Brassicaceae* microgreens is limited regarding the concentrations of the antioxidant bioactive compounds and minerals that were examined in this work. There are some studies on this subject in broccoli [7,10,17–20], kale [19–21], mustard [6,8,9,20,22–24] and radish [6,8,20,24–26] microgreens. However, the health-related effects of bioactive compounds of a food depend not only on their content and the amount consumed, but also on their bioavailability. Although in vivo assays are the gold standard for this purpose, these studies are expensive, lengthy, and have some ethical concerns. In turn, in vitro digestion allows one to estimate the bioaccessibility (the total amount of a food compound in soluble form and released from the solid food matrix that is available for absorption) [27], a prerequisite of bioavailability.

The aim of the present study was to evaluate the content and for the first time the bioaccessibility of the main antioxidant bioactive compounds (ascorbic acid, total carotenoids, total isothiocyanates, total anthocyanins, total soluble polyphenols), total antioxidant capacity, as well as macro- (K, Ca, Mg) and oligoelements (Fe, Zn) provided by the four studied hydroponic *Brassicaceae* microgreens: broccoli, kale, mustard and radish.

2. Materials and Methods

2.1. Plant Material and Sample Preparation

Four microgreen species belonging to the *Brassicaceae* family were evaluated in this study: broccoli (*Brassica oleracea* L. var. *italica* Plenck), green curly kale (*Brassica oleracea* var. *sabellica* L.), red mustard (*Brassica juncea* (L.) Czern.) and radish (*Raphanus sativus* L.). Mustard and radish cultivar seeds were purchased from CN Seeds Ltd. (Cambridgeshire, UK) and kale and broccoli from Rocalba S.A. (Huesca, Spain) and Intersemillas S.A. (Valencia, Spain), respectively.

Microgreens were produced by the Agronomic Innovation Center (CIAM) of Grupo Alimentario Citrus Company (Valencia, Spain) at the end of August 2017. A hydroponic system was created by placing substrates of pine tree fibers (12 cm × 12 cm × 0.4 cm) on plastic trays. Two seeding densities were selected: 3.8 seeds cm^{-2} for broccoli and kale and 2.8 seeds cm^{-2} for mustard and

radish. The sown substrates were moistened with water and introduced into a growth chamber at 18 °C and 90% relative humidity (RH) until the germination of the seeds. Then, they were moved into an unheated greenhouse where no artificial light treatment was applied. The incidence of natural light at this time of year provided a daily average of 18 °C and 61% RH. The following nutritive solution expressed as mmol/L for each component was applied daily: NO_3^- (5.3), $H_2PO_4^{-2}$ (1.5), SO_4^{-2} (4.4), HCO_3^- (0.5), Cl^- (5.3), K^+ (1.5), Ca^{+2} (6.3), Mg^{+2} (1.3) and Na^+ (3.1). An average fertigation value of 20.4 l m^{-2} per day from June to September 2017 was recorded. No phytosanitary treatment was used.

Nine days after seeding for radish and 7 days for broccoli, kale and mustard, the microgreens were transported in plastic trays (58 cm × 39 cm) from CIAM to the University of Valencia (UV). They were fertigated just before being moved in order to maintain good humidity conditions during the 30 min period of transportation. In our laboratory at the UV, a total of 40 trays were received (8 for kale, 12 for mustard and 10 for broccoli and radish). For each microgreen, approximately 400 g were harvested as close as possible to the root using sterilized scissors. Next, a pool was made to homogenize each microgreen sample, and then they were randomly divided into several replicates. Fresh microgreens were used immediately for ascorbic acid analysis and the rest of the collected samples were weighted inside aluminum containers before freezing at −80 °C. Frozen microgreens were lyophilized for 48 h (Sentry 2.0 Virtis SP Scientific, Philadelphia, PA, USA) and maintained in a desiccator until constant weight to obtain dry weight (DW) percentage (4.76 ± 1.43, 4.71 ± 1.49, 4.25 ± 1.36 and 4.91 ± 1.55 for broccoli, kale, mustard and radish, respectively) in accordance with the range 3.9–8.1% described in previous studies on these *Brassicaceae* microgreens [6–8,19]. Next, samples were ground into a fine powder in a grinder (Super Junior "S" Moulinex, Alençon, France) and stored at −20 °C for subsequent experiments.

2.2. Reagents

2.2.1. In Vitro Gastrointestinal Digestion

Pepsin (porcine, 975 units per mg protein), pancreatin (porcine, activity equivalent to 8 × USP specifications), bile extract (porcine), ammonium carbonate ($(NH_4)_2CO_3$), sodium bicarbonate ($NaHCO_3$) and calcium chloride dihydrate ($CaCl_2(H_2O)_2$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium chloride (KCl), sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$) and potassium dihydrogen phosphate (KH_2PO_4) were supplied by Merck (Darmstadt, Germany). Culture-grade water was obtained from B. Braun (Melsungen AG, Germany). Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF), Simulated Intestinal Fluid (SIF) and enzymatic activity assays were prepared according to Minekus et al. (2014). A water bath with orbital shaking (Stuart SBS30, Staffordshire, UK) and centrifuge (Eppendorf 5810, Hamburg, Germany) were used to simulate the gastrointestinal digestion process.

2.2.2. Bioactive Compounds and Antioxidant Capacity

Glacial acetic acid, metaphosphoric acid, formic acid and L (+) ascorbic acid (≥99%) were supplied by Panreac Química (Barcelona, Spain). Sodium salt dihydrate 2,6-dichlorophenolindophenol (DCFI), Tris(hydroxymethyl)aminomethane, potassium phosphate monobasic (Na_2HPO_4), potassium phosphate dibasic (K_2HPO_4), sodium phosphate dibasic (Na_2HPO_4), potassium chloride (KCl) and sodium acetate ($C_2H_3NaO_2$) were obtained from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3), potassium persulfate ($K_2S_2O_8$), Folin-Ciocalteu reagent, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid and sulforaphane standard (≥90%) were purchased by Sigma Chemical Co. (St. Louis, MO, USA). Ethanol (96%), methanol and n-hexane (96%) were provided from Scharlau (Barcelona, Spain). Sodium fluorescein was obtained from Fluka Chemie AG (Bunds, Switzerland) and 1,2-benzenedithiol (BDT)

(96%) from Acros organics (BVBA Thermo Scientific, Geel, Belgium). Water was purified by a Milli-Q system (Milford, MA, USA).

2.2.3. Minerals

Titrisol concentrated standards (1000 mg) of macro and oligoelements (Ca, Mg, K, Fe and Zn) and nitric acid (HNO_3) (65%) were purchased by Merck (Darmstadt, Germany) while hydrochloric acid (37%) was obtained from Scharlau (Barcelona, Spain).

2.3. Methodology for In Vitro Gastrointestinal Digestion

Freeze-dried samples were rehydrated to their original moisture contents in order to be as close as possible to the edible fresh microgreen [28], and in vitro gastrointestinal digestion based on the standardized method INFOGEST [29] was applied. Because of the absence of starch in the samples, the salivary step was carried out without α-amylase enzyme. Briefly, 5 g of rehydrated microgreen or culture-grade water (blank of digestion), 3.5 mL of SSF, 25 µL of 0.3 M $CaCl_2$ and culture-grade water to a final volume of 10 mL were mixed by mechanical shaking at 95 opm and 37 °C for 2 min. Immediately afterwards, to simulate the gastric phase, 7.5 mL of SGF, 1.6 mL of pepsin solution (25,000 U/mL) and 5 µL of 0.3M $CaCl_2$ were added to the gastric solution obtained and agitated for 1 min. The pH was adjusted at 7.0 ± 0.1 with 6M NaOH and culture-grade water was added up to a final volume of 40 mL. The intestinal mixture was incubated again at 95 opm and 37 °C for 2 h and after this period the digested samples were cooled in an ice bath and centrifuged at 3100 *g* and 4 °C for 90 min to obtain the bioaccessible fraction (BF). The values of the blank of digestion obtained in each assay were subtracted from the values of the digested microgreen samples to remove possible interferences caused by digestive enzymes or simulated fluids, in order to avoid overestimation of results. The results of bioaccessibility were calculated as the ratio between the concentration of each bioactive compound in the BF and the initial concentration in microgreens. The results were expressed as percentage of bioaccessibility according the next Equation (1):

$$\text{Bioaccessibility (\%)} = (\text{content in BF/initial content}) \times 100 \quad (1)$$

2.4. Analysis of Bioactive Compounds

2.4.1. Ascorbic Acid

Total ascorbic acid (AA) was determined by the AOAC Official Method 967.21 [30] and the procedure applied by Xiao et al. [8] was used to obtain extracts from fresh samples. Just harvested microgreens (6 g) and ice-cold 5% (*w*/*v*) metaphosphoric acid (20 mL) were homogenized in a Polytron (PT 2000 AFORA S.A. Kinematica, Switzerland) at 15,000 rpm for 1 min, centrifuged at 3000 *g* for 20 min and 4 °C, and filtered through Whatman nº 4 filter paper. BF samples were directly used for the titrimetric method. Both kind of samples were mixed 1:1 (*v*/*v*) with acetic acid—Metaphosphoric acid and the amount of acid ascorbic was measured using 2,6-DCFI. Concentration of AA was calculated by using L (+)-ascorbic acid standard solution (1 mg/mL). The results were expressed as mg AA/100 g fresh weight (FW).

2.4.2. Total Carotenoids

Total carotenoids were extracted as described by Sims and Gamon [31]. Quantification for extracts and BF was determined spectrophotometrically according to Sotelo et al. [32]. Dry microgreen powder (10 mg) was ground in 30 mL cold 80/20 (*v*/*v*) acetone/Tris buffer solution (pH 7.4) and mixed overnight in darkness at room temperature. Afterwards, samples were centrifuged at 3100 *g* for 10 min and supernatants were diluted 1/6 (*v*/*v*) in acetone/Tris buffer solution before measuring absorbance at 470,

537, 647 and 663 nm. Carotenoid content was obtained by following the next Equation (2) and the results were expressed as mg of β-carotene/100 g DW.

$$\text{Carotenoids} = (A_{470} - (17.1 \times (Chl_a + Chl_b) - 9.479 \times \text{Anthocyanin}))/119.26 \quad (2)$$

where,

Anthocyanin = 0.8173 A_{663} − 0.00697 A_{647} − 0.002228 A_{663}
Chla = 0.013773 A_{663} − 0.000897 A_{537} − 0.003046 A_{647}
Chlb = 0.024054 A_{647} − 0.004305 A_{537} − 0.005507 A_{663}

2.4.3. Total Isothiocyanates

The extraction of total isothiocyanates was performed as described by Torres-Contreras et al. [33]. Freeze-dried samples (100 mg) and water (5 mL) were mixed and centrifuged at 12,000 *g* for 8 min. The supernatant was diluted 1/5 (*v*/*v*) in water and 100 µL was used for cyclocondensation reactions [34]. BF samples were directly used for cyclocondensation reactions. Umber tubes were used and the order for the mixture was the following: 900 µL of 100 mM potassium phosphate buffer solution (pH 8.5), 900 µL of methanol, 100 µL of the isothiocyanate extract dilution and 100 µL of 80 mM 1,2-BDT in methanol to initiate the reaction. The tubes were heated at 65 °C for 1 h and cooled at room temperature before measuring absorbance at 365 nm. A standard curve of DL-sulforaphane in the range of 25–800 mg/L was subjected to the same analysis conditions and the results were expressed as mg of sulforaphane/100 g of DW.

2.4.4. Total Anthocyanins

Anthocyanin pigments were extracted according to Hanlon and Barnes [25] with some modifications and total anthocyanin content was determined by the pH differential method [35]. Anthocynins from lyophilized microgreens (400 mg) were extracted with a 0.1% (*w*/*w*) acetic acid aqueous solution (4 mL) in a sonicator for 10 min. After centrifugation at 3100 *g* for 10 min at 4 °C and filtering through a Whatman n° 4 filter paper, the extract of microgreens or BF were diluted 1/5 (*v*/*v*) in two different buffer solutions (0.025 M potassium chloride pH 1 and 0.4 M sodium acetate pH 4.5). Absorbance of diluted samples in both buffers was measured at 520 and 700 nm. The anthocyanin concentration was calculated according to the following Equation (3), and the final results were expressed as mg of cyanidin-3-glucoside/100 g DW.

$$\text{Anthocyanin pigment} = A \times MW \times DF \times 10^3/\varepsilon \times l \quad (3)$$

where

A = (A520 − A700) pH1 − (A520 − A700) pH 4.5
MW (molecular weight for cyanidin-3-glucoside) = 449.2 g/mol
DF (dilution factor) = 5
10^3 = factor for conversion from g to mg
ε = 26,900 molar extinction coefficient
l = path length in cm

2.4.5. Total Soluble Polyphenols

The total soluble polyphenols content was analyzed by the Folin-Ciocalteu method with some modifications [36], and extraction was carried out according to the method described by Xiao et al. [26]. Briefly, 100 mg of lyophilized microgreen sample was mixed with 10 mL of 80% methanol and sonicated for 30 s. Then, a hexane wash procedure was applied three times (4 mL of hexane was added, sonicated again for 30 s, centrifuged at 6650 *g* for 5 min at 4 °C and the hexane phase was discarded). The washed

methanolic extract was filtered using Whatman nº 4 filter paper and an aliquot of 100 µL of sample extract, BF or standard was mixed with 3 mL of 2% (*w*/*v*) sodium carbonate aqueous solution and 150 µL of 50% (*v*/*v*) Folin-Ciocalteu reagent. The mixture was incubated at room temperature in darkness for 1 h, and the absorbance at 765 nm was measured on a spectrophotometer (Perkin Elmer lambda 2 UV-VIS, Überlingen, Germany). Quantification was achieved using a gallic acid external standard calibration curve in the range of 0–1000 mg/L. The results were expressed as gallic acid equivalent (GAE)/100 g DW.

2.5. Determination of Antioxidant Capacity

Lyophilized microgreens were previously subjected to the same methanolic extraction process described above for total soluble polyphenols and BF were directly used.

2.5.1. Trolox Equivalent Antioxidant Capacity Assay (TEAC)

TEAC assay measures the reduction of the radical cation ABTS by antioxidant compounds, and the spectrophotometric method proposed by Cilla et al. [36] was used. The $ABTS^+$ radical cation stock solution was generated by chemical reaction with 7 mM ABTS and 140 mM $K_2S_2O_8$ overnight in darkness at room temperature. Next, it was diluted in ethanol until an absorbance of 0.700 ± 0.020 at 734 nm and 30 °C to obtain the $ABTS^+$ working solution. The optimal dilution of the samples to obtain a percentage of absorbance inhibition of approximately 50% was 1/3 (*v*/*v*) in ethanol. At the same time, Trolox standard solutions were prepared in a range of 0 to 300 µM. The absorbance of 2 mL of $ABTS^+$ working solution was considered the initial point of reaction (A_0). Then, diluted samples or Trolox standards (100 µL) were added immediately and the absorbance were measured after 3 min (A_f). All readings were carried out in a thermostatized UV–vis spectrophotometer. The percentages of absorbance inhibition were obtained from the following Equation (4):

$$1 - (A_f/A_0) \times 100 \quad (4)$$

and were compared to Trolox standard curve to express the results as µM Trolox equivalents/100 g DW.

2.5.2. Oxygen Radical Absorbance Capacity Assay (ORAC)

The ORAC assay measures the capacity of the antioxidant compounds to scavenge peroxyl radicals; the fluorimetric method described by Cilla et al. [36] was used. The reaction was carried out in a Multilabel Plate Counter VICTOR^3 1420 (PerkinElmer, Turku, Finland) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm at 37 °C. The optimization of the assay parameters was required. Sodium fluorescein and freshly prepared AAPH solution were used at a final concentration of 0.015 and 120 mg/mL respectively. Samples were diluted 1/250 (*v*/*v*) and 20 µM Trolox was used as antioxidant standard. All of them were prepared with phosphate buffer (75 mM, pH 7.4). The final reaction consisted of 80 µL of fluorescein, 40 µL of AAPH and 80 µL of diluted sample, Trolox standard or phosphate buffer (blank) and the fluorescence was recorded every 5 min over 70 min (until the fluorescence in the assay was less than 5% of the initial value). The results were calculated considering the differences of areas under the fluorescence decay curve (AUC) between the blank and the sample over time, and were expressed as µM Trolox Equivalents/100 g DW.

2.6. Analysis of Minerals

The main macroelements (K, Ca, Mg) and oligoelements (Fe, Zn) were evaluated according to Cilla et al. [37,38]. Briefly, 1 g of each lyophilized microgreen was ashed in a muffle furnace (Heraeus, Eurotherm, Germany) at 450 °C for 48 h (the temperature being slowly increased at a rate of 50 °C/h). In the case of BF, 10 g were heated until complete evaporation before being introduced into the furnace. Next, 1 mL of concentrated nitric acid was added to the white ashes and heated on a hot plate to dryness. Immediately after, samples were dissolved in 3 mL of concentrated HCl and allowed to

flux for 3.5 h. Then, the digest was filtered through Whatman nº 4 filter paper and the filtrate was diluted with ultrapure water to a final volume dependent on the total concentration estimated for each element, in both lyophilized microgreens and BF samples. Titrisol standard solutions of K, Ca, Mg, Fe and Zn were prepared in ultrapure water containing the same % of HCl used to dissolve ashes. Lanthanum oxide (La_2O_3) and cesium chloride (CsCl) at 0.1% (*p*/*v*) were added to samples and standards to eliminate possible chemical interferences of phosphate on calcium and to avoid potassium ionization, respectively.

Mineral concentrations were determined by flame atomic spectrometry (Thermo Scientific ICE 3000, UK) and the quantification of minerals was calculated from their standard calibration curves (mg/L): K (0.25–2.5), Ca (0.125–5.0), Mg (0.125–1.0), Fe (0.0625–5.0) and Zn (0.0625–2.5). The results were expressed as mg of each element/100 g FW. In addition, a dried hay powder (Certifed Reference Material BCR-129) was used to confirm the accuracy of the method. It was prepared and analyzed using the same procedure as that followed for the microgreen samples. The certified and experimental values were (mg/L) 640 ± 10 and 609 ± 4 for calcium, 145 ± 4 and 115 ± 1 for magnesium, 3380 ± 80 and 2850 ± 18 for potassium, 11.4 ± 0.0 and 13.4 ± 0.1 for iron and 3.2 ± 0.17 and 4.01 ± 0.10 for zinc, respectively. The coefficient of variation with regard to the precision for all minerals was in the range of 0.62–2.42%.

2.7. Statistical Analysis

All analyses were carried out in triplicate in at least two independent experiments, and data were expressed as mean ± standard deviation. Experimental data were subjected to one-way analysis of variance (ANOVA) to determine significant differences among samples composition. Tukey's multiple range test, at a significance level of $p < 0.05$, was used. All analyses were performed with the software Statgraphics Plus 5.1 (Statpoint Technologies Inc., Warrenton, VA, USA).

3. Results and Discussion

3.1. Content and Bioaccessibility of Antioxidant Bioactive Compounds in Microgreens

The results of antioxidant bioactive compounds content in fresh microgreens and their bioaccessible fraction, as well as the bioaccessibility, are shown in Table 1. The concentration range of ascorbic acid in fresh microgreens was from 31 to 56 mg/100 g FW, which would provide between 38 and 70% of the recommended daily intake for vitamin C, justifying the inclusion of the nutritional claim "high vitamin C content" according to the Regulation (EU) 1924/2006, Annex II [39]. Kale microgreen contained the highest concentration, followed by broccoli, radish and mustard. The results of ascorbic acid content are within the ranges described in recent published data for microgreens of kale (28–66 mg/100 g FW), mustard (19–44 mg/100 g FW) and radish (25–68 mg/100 g FW) and lower than those found in broccoli (89 mg/100 g FW) [6,20,24]. Considering the National Nutrient Database for Standard Reference (USDA, 2018) [40] and data in the literature for adult plants (see Table 2), the ascorbic acid concentration in microgreen samples was higher for radish and lower for kale and mustard, while broccoli was within the range described (Table 2). On the other hand, the results obtained in the BF were 0.6–1.2 mg AA/100 g FW. The lowest content of ascorbic acid in BF was observed in the broccoli microgreen, while there were no statistically significant differences in kale, mustard and radish. These very low values seem to indicate a high loss of ascorbic acid, possibly due to instability at intestinal pH and oxidation in presence of oxygen. Although there are no bioaccessibility (BA) data for microgreens in the literature, similar vitamin C losses, i.e., greater than 95%, have been reported in pomegranate juice and in broccoli inflorescences after in vitro gastrointestinal digestion [41,42].

Table 1. Antioxidant bioactive compounds content in broccoli, kale, mustard and radish microgreens before and after simulated gastrointestinal digestion.

Microgreen	Total Content mg/100 g	Bioaccessible Fraction mg/100 g	Bioaccessibility (%)
	Ascorbic Acid [1]		
Broccoli	50.99 ± 1.91 ^{b}	0.56 ± 0.09 ^{b}	1.10 ± 0.17 ^{d}
Kale	56.14 ± 1.04 ^{a}	1.05 ± 0.09 ^{a}	1.87 ± 0.17 ^{c}
Mustard	30.67 ± 1.02 ^{d}	1.14 ± 0.10 ^{a}	3.73 ± 0.32 ^{a}
Radish	45.43 ± 1.15 ^{c}	1.19 ± 0.09 ^{a}	2.61 ± 0.21 ^{b}
	Total carotenoids (β-carotene) [2]		
Broccoli	221.80 ± 13.36 ^{a}	0.18 ± 0.02 ^{b}	0.08 ± 0.01 ^{c}
Kale	217.54 ± 18.74 ^{a}	0.12 ± 0.02 ^{c}	0.06 ± 0.01 ^{d}
Mustard	224.27 ± 9.35 ^{a}	0.25 ± 0.02 ^{a}	0.11 ± 0.01 ^{b}
Radish	162.29 ± 5.50 ^{b}	0.23 ± 0.03 ^{a}	0.14 ± 0.02 ^{ab}
	Total isothiocyanates (sulphoraphane) [2]		
Broccoli	633.11 ± 10.69 ^{b}	204.51 ± 47.94 ^{b}	32.30 ± 7.57 ^{b}
Kale	608.23 ± 35.63 ^{b}	207.18 ± 10.33 ^{b}	34.06 ± 1.70 ^{b}
Mustard	801.07 ± 51.16 ^{a}	248.90 ± 25.75 ^{b}	31.07 ± 3.21 ^{b}
Radish	809.62 ± 27.83 ^{a}	512.99 ± 33.97 ^{a}	63.36 ± 4.20 ^{a}
	Total anthocyanins (cyanidin-3-glucose) [2]		
Broccoli	12.66 ± 1.53 ^{b}	ND	-
Kale	1.39 ± 0.43 ^{d}	ND	-
Mustard	36.40 ± 0.46 ^{a}	ND	-
Radish	5.57 ± 0.86 ^{c}	ND	-
	Total soluble polyphenols (GAE) [2]		
Broccoli	2037.38 ± 103.10 ^{b}	1427.98 ± 175.00 ^{a}	70.09 ± 8.59 ^{a}
Kale	2415.95 ± 109.34 ^{a}	1447.72 ± 140.10 ^{a}	59.92 ± 5.80 ^{a}
Mustard	1889.76 ± 64.81 ^{bc}	820.57 ± 31.00 ^{b}	43.42 ± 1.64 ^{b}
Radish	2111.19 ± 132.79 ^{b}	1434.82 ± 62.34 ^{a}	67.96 ± 2.95 ^{a}

[1] Data presented in fresh weight (FW). [2] Data presented in dry weight (DW). Data are expressed as the mean ± SD ($n = 3$). Different lowercase letters in the same column for each bioactive compound indicate significant differences ($p < 0.05$). ND: not detected. GAE: Gallic acid equivalents.

Regarding to total carotenoids content, the concentration ranged from 162 to 224 mg β-carotene/100 g DW. Radish microgreen showed the lowest value before the digestion process, and no statistically significant differences ($p > 0.05$) were found between broccoli, kale and mustard. For radish, lower (46–66 mg/100 g DW) and similar contents (85–200 mg/100 g DW) have been described [6,8,20]. For broccoli microgreens, a lower concentration of total carotenoids (118–209 mg/100 g DW) was reported in several studies, regardless of the growing system applied: hydroponic [17] or peat substrate [6,7]. In the case of kale, the value obtained was higher than the range described by Xiao et al. [20] (141–197 mg/100 g). As for mustard microgreens, different amounts of total carotenoids content were found (27–270 mg/100 g DW) [6,8,20], which was in agreement with our data. The developmental stage at harvest, light intensity during the growth period, or genotypic differences between species were suggested as important factors for the final carotenoid content in microgreens [6]. Overall, microgreens have been considered as good sources of β-carotene [8]. In addition, the four *Brassicaceae* microgreens analyzed showed extremely high total carotenoid concentrations compared to their mature counterparts (Table 2), and also in accordance with the 260-fold more β-carotene determined in cabbage microgreen versus the adult plant [5]. Very low contents of carotenoids in BF were observed, and therefore, minor BA were obtained (<0.15%). The same results were reported by Courraud et al. [58] in fresh spinach using HPLC. However, some studies reported a BA from 1 to 20% in broccoli and kale vegetables analyzed by HPLC [47,59,60]. The reason for the low BA of carotenoids in microgreens could be due to differences in the digestion method conditions and to the chemical structure adopted by these compounds into the plant matrix, since it has been hypothesized that carotenoids in crystalloid form would not be transferred to the micellar aqueous phase as they do in cabbage (*Brassicaceae* family) [60].

Table 2. Antioxidant bioactive compounds and mineral elements content in mature counterparts of the microgreens evaluated in this study.

	Broccoli	Kale	Mustard	Radish	References
Antioxidant bioactive compounds					
Ascorbic acid (mg/100 g FW)	13–110	70–93	70	15–39	[33,43–45]
Total carotenoids (mg β-carotene/100 g DW)	2–28	27	0.17–0.21	43	[45–48]
Total isothiocyanates (mg sulphoraphane/100 g DW)	5–2307	NA	NA	189–368	[25,33,49]
Total anthocyanins (mg cyaniding-3-glucoside/100 g DW)	NA	NA	34–67	ND-189	[25,46,50]
Total soluble polyphenols (mg GAE/100 g DW) *	167–3606	967–3010	300–1702	0.2–13,890	[25,33,43–46,51–55]
Total antioxidant capacity (μM Trolox Eq/100 g DW)					
ORAC *	4785–15,887	28,698–36,030	NA	15,021–76,638	[51]
TEAC *	26,200	36,200	NA	NA	[54]
Mineral content (mg/100 g FW)					
K	310–599	165–348	384	233–495	[21,40,43,56,57]
Ca	27–88	169–254	115	25–752	[21,40,43,56,57]
Mg	17–40	33–98	32	10–57	[21,40,43,56,57]
Fe	0.34–0.73	0.34–1.6	1.64	0.34–3.8	[21,40,43,56]
Zn	0.41–0.85	0.39–0.61	0.25	0.28–0.39	[21,40,43,56]

ND: Not detected. NA: Not available. FW: fresh weight. DW: dry weight. GAE: Gallic acid equivalents. * calculated in dry base from values of water content from the specifics references or from USDA and ORAC Database (2018) when necessary.

The total isothiocyanate concentration in microgreens ranged from 608 to 810 mg sulphoraphane/100 g DW (Table 1). Mustard and radish showed higher values than broccoli and kale. There is no data in the literature about isothiocyanates present in microgreens. However, Hanlon and Burnes [25] reported a range from 970 to 3762 mg/100 g DW in 8 varieties of 7-day-old radish sprouts. Regarding values of isothiocyanates in adult plants, the literature is also limited and variable, from 2–4 times less content in radish taproots versus radish microgreens to a wide difference of concentrations in broccoli florets (Table 2). Both the content in the BF ranging from 205 to 513 mg/100 g DW and the BA (31–63%) were double in radish compared to the rest of the samples. In general, the results of BA of total isothiocyanates in microgreens were similar to those described in mature cruciferous vegetables such as radish and mustard (43–72%) using the same spectrophotometric methodology [53]. The reduction of the total content of isothiocyanates during the digestion process could be due to the chemical transformations caused by the action of gastric pH, obtaining new non-detectable compounds (phenethylamines) for the analytical conditions [53].

Total anthocyanin content in microgreen samples were from 1.4 to 36.4 mg cyanidin-3-glucoside/100 g DW, following this increasing order: kale, radish, broccoli and mustard (Table 1). The scarce data in the literature about anthocyanin content are quite variable, from very few to hundred μg per g FW, often depending on the colour [7]. In this sense, 30 different anthocyanins responsible for the coloration of five *Brassica* microgreens with red to purple seed-leaves have been identified [9]. Regarding mustard, very different results have been previously reported. Two varieties of 19-day-old red mustard leaves grown by natural irradiance presented concentrations of 30 and 67 mg/100 g DW [46]. In contrast, values of 760 mg/100 g DW [23] and 1480 mg/100 g DW [22] have been described in mustard microgreens grown before applying LEDs and short-term red lighting. The anthocyanin concentration in broccoli was equal to one variety (13 mg/100 g DW), but much lower than the other one (208 mg/100 g DW) analyzed by Paradiso et al. [7]. In addition, for 7-day-old radish microgreens, anthocyanin content varied from not detected to 29 mg/100 g DW [25]. Differences in the concentration of anthocyanins have also been observed in adult stage of radish and in two varieties of red mature mustard (Table 2). No data in the literature were found for broccoli and kale adult vegetables. For all the studied microgreens, no anthocyanins were detected in the corresponding BF (Table 1). In this context, Pérez-Vicente et al. [41] suggested that anthocyanins could be metabolized into colorless substances, oxidized, or degraded, giving rise to other chemical compounds which are not detectable by the spectrophotometric differential pH method. Likewise, a complete degradation or non-detection of anthocyanin pigments in some golden apple varieties after in vitro gastrointestinal digestion and applying the pH differential method have been described [61].

Total soluble polyphenol content in the microgreens varied from 1890 to 2416 mg GAE/100 g DW, with the highest value for kale and the lowest for mustard (Table 1). Two non-hydroponic varieties of broccoli microgreens showed 1092 and 1163 mg GAE/100 g DW [7], while mustard microgreens total polyphenols ranged from 536 to 2800 mg GAE/100 g DW [22,23,46]. A recent study of 13 microgreen species concluded that the polyphenol composition profiles were significantly different across species [6]. In general, the total soluble polyphenols determined in the microgreens of the present study were within the range of their corresponding adult stage (Table 2). The identification of 164 polyphenols in five *Brassica* microgreens revealed more complex profiles and a greater variability in the content of polyphenols in microgreens compared to mature plants [9,62]. As for the content of soluble polyphenols in the BF (821–1448 mg/100 g DW) the lowest amount was observed in mustard while there were no statistically significant differences between broccoli, kale and radish. The decrease in BA, showing values from 43% to 70%, could be due to the slightly alkaline conditions reached after intestinal phase, together with possible interactions with digestive enzymes. No data are available in literature about BA in microgreens; nevertheless, our results were comparable to those obtained by Puangkam et al. [53] using the Folin Ciocaltou method for conventional vegetables of the *Brassicaceae* family including radish and mustard. Lower values of BA were determined by HPLC in broccoli flavonoids (11%) and for total polyphenols in raw kale (15%) or in kale subjected to different culinary

techniques (7%) [42,55,60]. The determination of total soluble polyphenols by the Folin-Ciocalteu assay may present some interferences and limitations, but it offers a rapid chemical index. In addition, spectrophotometric methods have been regarded as useful screening techniques for comparison among samples providing an idea of the antioxidant capacity in the matrix [63]. The measurement of the phenolic profile, as well as that of other antioxidant bioactive compounds found in these microgreens through chromatographic analysis, could be interesting for future research.

The results of total antioxidant capacity determined by TEAC and ORAC methods in microgreens, their bioaccessible fractions and the percentage retained in the BF are shown in Table 3. The antiradical activity of fresh microgreens ranged from 422 to 493 and from 7579 to 9783 μM Trolox Eq/100 g DW for TEAC and ORAC assays, respectively. In general, broccoli showed a slight lower antioxidant capacity compared to the rest of microgreens. In contrast, the results of antioxidant capacity determined by DPPH method in six genotypes of microgreens showed the highest activity levels for broccoli microgreens [7]. The comparison of antioxidant activity is limited due to the different existing methods. According to the ORAC Database [51], higher values (from 2 to 8-fold) were found for mature broccoli, kale and radish compared to the microgreen samples we analyzed, and no data was available for mustard (Table 2). The antioxidant capacity in the BF varied from 78 to 138 (TEAC) and from 3646 to 7453 (ORAC) μM Trolox Eq/100 g DW. For TEAC method the highest value in BF was observed in radish, and the highest percentage retained in the BF resulted in both radish and mustard, while for the ORAC method, mustard and kale showed higher antioxidant capacities than broccoli and radish in BF and the highest antioxidant percentages retained in the BF. Different results of antioxidant capacity were observed in cruciferous vegetables (radish and mustard) subjected to a simulated gastrointestinal digestion using DPPH and FRAP methods with percentage retained in the BF of 59–69% and 12–28%, respectively [53]. These differences could be related to the compounds formed after digestion process, which are susceptible to various reactions with substrates and free radicals according to each antioxidant method, depending on the matrix. The decrease in the antioxidant capacity observed with both methods after gastrointestinal digestion, is attributable to the reduction in bioactive antioxidant compounds (ascorbic acid, total soluble polyphenols, total anthocyanines, total carotenoids and total isothiocyanates) previously discussed (Table 1). The decrease was more pronounced in the case of TEAC method showing percentage of antioxidant capacity retained in the BF between 19–28% values versus 48–82% observed with ORAC method.

Table 3. Total antioxidant capacity before and after simulated gastrointestinal digestion in microgreens.

Microgreen	Total Content μM Trolox Eq/100 g	Bioaccessible Fraction μM Trolox Eq/100 g	Antioxidant Capacity Retained in BF (%)
	TEAC [1]		
Broccoli	421.81 ± 19.35 ^{b}	78.39 ± 9.05 ^{c}	18.58 ± 2.15 ^{b}
Kale	493.21 ± 25.10 ^{a}	98.69 ± 11.26 ^{b}	20.01 ± 2.28 ^{b}
Mustard	447.98 ± 11.55 ^{b}	110.81 ± 18.57 ^{b}	24.73 ± 4.15 ^{a}
Radish	488.65 ± 19.20 ^{a}	137.70 ± 11.30 ^{a}	28.18 ± 2.31 ^{a}
	ORAC [1]		
Broccoli	7578.89 ± 815.87 ^{c}	3645.50 ± 281.21 ^{b}	48.10 ± 3.71 ^{c}
Kale	9782.57 ± 822.34 ^{a}	7391.52 ± 1162.12 ^{a}	75.56 ± 11.88 ^{a}
Mustard	9090.15 ± 907.25 ^{ab}	7452.51 ± 701.65 ^{a}	81.98 ± 7.72 ^{a}
Radish	9690.38 ± 935.81 ^{a}	5258.94 ± 721.69 ^{b}	54.27 ± 7.45 ^{b}

[1] Data presented in dry weight (DW). BF: bioaccesible fraction. Data are expressed as the mean ± SD (n = 3). Different lowercase letters in the same column in each antioxidant capacity assay indicate significant differences ($p < 0.05$).

3.2. Content and Bioaccessibility of Mineral Elements in Microgreens

The total content of mineral elements in the microgreens before and after gastrointestinal digestion and their BA are reported in Table 4. For all fresh microgreens (mg/100 g FW), the most abundant element was K (86–102), followed by Ca (31–40), Mg (11–13), Fe (0.30–0.39) and Zn (0.15–0.16).

In general, the same order was observed in different studies about macro- and micro- mineral content for the same microgreen species here evaluated [6,7,10,19,21]. Among the 30 varieties of *Brassicaceae* microgreens grown in peat moss substrate evaluated by Xiao et al. [19] and expressed in mg/100 g FW, the range of K (176–365), Ca (41–88), Mg (28–60), Fe (0.47–0.72) and Zn (0.29–0.43) content in broccoli, kale, mustard and radish was higher than those found in this study. Similarly, also for broccoli microgreens grown on a mixture of peat [7] and compost [10] macro- and oligoelements were also higher (mg/100 g FW) (K: 249–422, Ca: 59–202, Mg: 21–40, Fe: 0.59–1.2, Zn: 0.30–0.73). However, when broccoli microgreens were obtained through two different hydroponic growing systems, similar results (mg/100 g FW) were found in K (79–101), lower in Ca (29–32) and higher in the rest of the elements analyzed (Mg: 33–36, Fe: 0.48–0.61, Zn: 0.47–0.53) [9]. As for kale, three cultivars grown in soilless media and harvested at five different development stages generally showed lower K, Ca, Mg, Fe and Zn content at the microgreen stage than at the baby leaf one, and fresh microgreens also showed lower concentrations of Ca and Mg than adults [21]. In general, the concentration of all macro- and oligo-elements measured in microgreen samples were lower than those found in mature plants (Table 2). In particular, K content was more than 30% lower than the average K content found in the adult counterparts. Furthermore, Renna et al. [13] demonstrated that in hydroponically grown microgreens K can easily be modulated by controlling the element concentration in the nutrient solution. Thus, microgreens produced with these specific conditions could be labeled with the nutritional claim of "reduced potassium" (Regulation 1924/2006) [39], and could be recommended for patients with impaired kidney function [13].

The highest BA for the three macroelements analyzed was found in mustard microgreens. In contrast, broccoli microgreens showed lower BA values for Ca and Mg. Although Fe and Zn could not be detected in the BF, a decrease in the amount of macroelements occurred after digestion process, high BA (34–61% for Ca, 59–73% for Mg and 80–90% for K) was observed. This fact could be probably ascribed to the low content of ascorbic acid and high content in total soluble polyphenols in the BF (substances that promote and inhibit BA of minerals, respectively) of broccoli, in contrast to mustard. There are no data in the literature about BA of mineral elements in microgreens. However, the values of Ca BA in conventional vegetables of *Brassicaceae* have been described in two different studies. Lucarini et al. [64] obtained 27–40% BA in cooked broccoli and kale and Kamchan et al. [65] showed 33–39% BA in two kinds of kale. These values are slightly lower than those in our study.

Table 4. Mineral content before and after simulated gastrointestinal digestion in microgreens.

Microgreen	Total Content mg/100 g	Bioaccessible Fraction mg/100 g	Bioaccessibility (%)
		Potassium [1]	
Broccoli	86.21 ± 3.23 ^{d}	71.81 ± 2.63 ^{b}	83.30 ± 3.06 ^{ab}
Kale	100.97 ± 2.02 ^{b}	88.96 ± 2.30 ^{a}	88.30 ± 2.28 ^{ab}
Mustard	101.71 ± 1.10 ^{ab}	91.82 ± 2.07 ^{a}	90.27 ± 9.26 ^{a}
Radish	95.04 ± 4.65 ^{c}	76.15 ± 0.12 ^{b}	80.13 ± 0.11 ^{b}
		Calcium [1]	
Broccoli	37.38 ± 2.07 ^{b}	12.67 ± 0.12 ^{c}	33.91 ± 0.15 ^{b}
Kale	40.38 ± 0.60 ^{ab}	22.48 ± 0.18 ^{a}	55.67 ± 0.17 ^{a}
Mustard	32.20 ± 2.09 ^{c}	19.8 ± 5.78 ^{ab}	61.48 ± 17.94 ^{a}
Radish	31.02 ± 1.07 ^{c}	14.84 ± 0.15 ^{bc}	47.85 ± 0.18 ^{ab}
		Magnesium [1]	
Broccoli	11.95 ± 0.35 ^{b}	7.03 ± 0.56 ^{c}	58.83 ± 4.66 ^{b}
Kale	11.21 ± 0.15 ^{c}	7.87 ± 0.26b ^{c}	70.26 ± 2.30 ^{a}
Mustard	12.87 ± 0.19 ^{a}	9.36 ± 0.69 ^{a}	73.41 ± 6.36 ^{a}
Radish	11.21 ± 0.17 ^{c}	8.12 ± 0.42 ^{b}	72.42 ± 3.79 ^{ab}
		Iron [1]	
Broccoli	0.39 ± 0.03 ^{a}	ND	-
Kale	0.39 ± 0.01 ^{a}	ND	-
Mustard	0.32 ± 0.02 ^{bc}	ND	-
Radish	0.30 ± 0.02 ^{c}	ND	-

Table 4. *Cont.*

Microgreen	Total Content mg/100 g	Bioaccessible Fraction mg/100 g	Bioaccessibility (%)
		Zinc [1]	
Broccoli	0.15 ± 0.04 [a]	ND	-
Kale	0.16 ± 0.04 [a]	ND	-
Mustard	0.15 ± 0.03 [a]	ND	-
Radish	0.15 ± 0.02 [a]	ND	-

[1] Data presented in fresh weight (FW). Data are expressed as the mean ± SD (n = 3). Different lowercase letters in the same column for each mineral compound indicate significant differences ($p < 0.05$). ND: not detected.

4. Conclusions

In general, the four hydroponic *Brassicaceae* microgreens produced in this study could be considered as good sources of minerals and antioxidant phytochemicals in a balanced human diet. In particular, they contain relevant amounts of vitamin C, higher levels of total carotenoids than adult plants, mineral and antioxidant bioactive compound contents comparable to other hydroponic microgreens, and low K, making them suitable for patients with impaired kidney disease.

In this study, bioaccessibility data for antioxidant bioactive compounds, total antioxidant capacity, and mineral elements in microgreens are provided for the first time. Radish and mustard showed the highest BF and BA values for antioxidant parameters, while broccoli and mustard provided the lowest and highest values for minerals, respectively. Despite the expected decrease in different compounds after the in vitro digestion process, the bioaccessible fractions of microgreens still contained remarkable total antioxidant capacities and bioactive compounds with potential beneficial local effects in the gastrointestinal tract. For future studies, determining the bioaccessibility of the antioxidant phytochemicals in more microgreen species, as well as their potential bioactivity in pre-clinical and human intervention studies, ought to be addressed.

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Antioxidant and Mineral Composition of Three Wild Leafy Species: A Comparison Between Microgreens and Baby Greens

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Abstract: Wild plants may play an important role in human nutrition and health and, among them, many are the leafy species. We hypothesized that the wild greens could be profitably grown as microgreens and baby greens, specialty products whose market is increasing. We compared three wild leafy species (*Sanguisorba minor* Scop., *Sinapis arvensis* L., and *Taraxacum officinale* Weber ex F. H. Wigg.) harvested at the microgreen and baby green stages. Seedlings were grown hydroponically in a half-strength Hoagland nutrient solution under controlled climatic conditions. At harvest, the yield was assessed, and chlorophylls, carotenoids, anthocyanins, phenolic index, nitrate, and mineral elements were measured in the two types of product. The potential contribution to human mineral intake was calculated, and the possible risk due to the presence of metals potentially detrimental for health was estimated. Results showed that micro/baby greens of the studied wild plants achieved competitive yields and could contribute to the dietary intake of macroelements, microelements, and non-nutrient bioactive compounds. On the other hand, the wild greens showed high amounts of nitrate and traces of some metals potentially detrimental for health, suggesting the need for caution in the use of wild species for producing microgreens and baby leaves.

Keywords: wild plants; vegetable specialty products; immature greens; nitrate; minerals; dietary value; health risk

1. Introduction

Wild foods include leaves, flowers, fruits, and seeds gathered from spontaneous plants. In Europe, their consumption, often considered as an emergency practice associated with food shortage periods, has been almost completely neglected in the last decades. Nowadays wild food plants are gaining renewed attention for their nutritional value and their use is promoted by health-oriented people in the healthy lifestyle framework, with special reference to wild-green centered cuisines [1]. The leafy plants, also known as wild greens, have been traditionally consumed as salad, soup or vegetable dishes and have represented an important part of the daily diet in the Mediterranean countries, especially during the early spring and in the autumn [1]. Wild greens are known to be a good source of protein and fat, vitamins, sugars, and minerals [2–4]. A wide variety of phytochemicals with antioxidant effects have been also reported in many of these species [5]. Moreover, some studies demonstrated that wild plants often contain molecules showing antimicrobial potential [6] and other biological-pharmacological activities [7]. For this reason, some wild greens have recently attracted considerable attention as

a source of functional foods or fortified food additive powders. On the other hand, most of them grow in anthropogenically disturbed sites such as farmlands (weeds), places of human habitation (ruderals), borders of paths and roads, etc., in soils often rich in nitrate or contaminated by metallic trace elements [8] whose detrimental effects on human health are known [9–11]. Considering that, due to the efficiency in root-to-shoot translocation paths, the synanthropic plants can accumulate high levels of pollutants in the aerial parts [12,13], their use as food may also entail health risks.

More than 600 wild species are used in traditional rural Italian cuisine and, among them, approximately 200 are the leafy plants [14]. We hypothesized that these wild greens could be profitably grown as specialty crops like microgreens or baby greens, with the dual advantage of widening the range of these products and, at the same time, promoting the wild species.

Microgreens are tender immature greens harvested within 10–20 days from seedling emergence and about 5 cm in height, when cotyledons are fully expanded, and the first pair of true leaves are more or less developed. Recently, microgreens have been gaining more and more popularity as a novel culinary ingredient used to enhance salads and other dishes in color, taste or texture [15], and their price may exceed $100 per kg [16]. Also, baby greens (otherwise known as baby leaves) are harvested and consumed in immature plant size, but they are older and larger than microgreens (about 10 cm in height) [16]. Baby greens are widely requested as a base component of mixed salads, especially for the ready-to-eat ones, whose consumption is constantly growing [17]. Considering both fruits and vegetables, the market for fresh-cut products in Europe has shown a double-digit growth since they began to be commercialized in the early 1980s [18]. In the United States, ready-to-eat salad mixes went through a five-fold increase in supermarket sales over a period of 20 years [19].

As reviewed by different authors [20–22], several studies have recently shown that plants at the microgreen stage are particularly rich in antioxidants and other health-promoting compounds, which is a reason why microgreens have started to be appreciated also as functional food. However, literature on the chemical composition of microgreens [23–27], as well as of baby greens [17,28], is by far focused on cultivated species, while very few studies have been carried out on wild edible plants [29–31]. Furthermore, the concentration of minerals and organic bioactive compounds of micro/baby greens has often been compared with that of the mature counterparts [30,32–35], while to our knowledge only one study is available about the differences in the mineral composition between microgreens and baby greens of the same species [36].

Based on this background, the aim of the present study was to evaluate three wild leafy species (*Sanguisorba minor* Scop., *Sinapis arvensis* L., and *Taraxacum officinale* Weber ex F. H. Wigg.) as possible candidates as microgreens and baby greens. Plants were grown hydroponically until they reached the microgreen or baby leaf stage, and yield, some antioxidants, nitrate, and mineral content were analyzed. The possible contribution of the different products to human mineral requirements was calculated and the health risk due to the ingestion of heavy metals possibly resulting from their consumption was also estimated.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

Seeds of *S. minor* (small burnet), *S. arvensis* (wild mustard), and *T. officinale* (common dandelion) were used as starting material. *S. minor* and *S. arvensis* seeds were provided by "B & T World Seeds" (Aigues-vives, France), while seeds of *T. officinale* were harvested in late April from wild plants growing in uncultivated land in the peri-urban area of Lucca (Tuscany Region, Italy). Prior to use, seeds were surfaced-sterilized in 2.2% hypochlorite for 15 min and then rinsed under tap water for 2 min. Besides this, 1000-seed weight and germination percentage were determined (Table 1). Seeds were sown in polystyrene cell trays (27.0 × 53.5 cm^2, 392 cells) filled with vermiculite (Asfaltex S.A., Sant Cugat del Vallés, Barcelona, Spain). Seed amount was calculated based on 1000-seed weight and germination percentage in order to obtain about eight plants per cell. After sowing, trays were

kept in the dark at 20 °C for 48 h and then moved in a growth chamber at 25 ± 2 °C (day) and 17 ± 2 °C (night) with a photoperiod of 16 h under fluorescent lighting units OSRAM L36W/77 (36 WATT, 120 cm in length, 26 mm in diameter, four per tray). Trays were placed in polyethylene tanks containing 5 L of half-strength Hoagland's nutrient solution prepared with distilled water (macroelements expressed in mM and microelements in μM: N 7.5, P 0.5, K 3.0, Ca 2.5, Mg 1.0, Fe 25.0, B 23.1, Mn 4.6, Zn 0.39, Cu 0.16, Mo 0.06; pH: 5.56; CE: 1.12 mS/cm) and arranged in a randomized block design with three replicates (1 replicate = 1 tank). The volume of the nutrient solution consumed by the crops was reintegrated at least once a week.

Table 1. One thousand-seed weight and germination rate of *Sanguisorba minor* Scop., *Sinapis arvensis* L., and *Taraxacum officinale* Weber ex F. H. Wigg. seeds.

Species	1000-Seed Weight [1] g	Germination [2] (%)
S. minor	7.02 ± 0.30	75.5 ± 3.4
S. arvensis	2.48 ± 0.13	61.0 ± 3.7
T. officinale	0.62 ± 0.03	72.0 ± 2.9

[1] Means of eight samples of 100 seeds each × 10 ± SD. [2] Means ± SD of four samples of 50 seeds each, kept in the dark at 20 °C for 21 days.

2.2. Harvesting and Yield Assessment

At the microgreen stage (first true leaf, green and swollen cotyledons), which was reached 14 days after sowing in *S. arvensis* and 16 days after sowing in both *S. minor* and *T. officinale*, half of the plants were harvested by cutting them with scissors just above the surface of the growing medium. The remaining plants were thinned to one plant per cell and leaves were harvested by cutting them with scissors after plants had reached the baby leaf stage (5–6 true leaves), 35 days after sowing in *S. arvensis* and *T. officinale*, and 43 days after sowing in *S. minor*. Microgreens and baby greens were weighed to determine yield, which was expressed in kg FW/m^2.

2.3. Analysis

Harvested microgreens and baby greens were analyzed for the following chemical parameters: chlorophylls, carotenoids, phenols, anthocyanins, nitrate and mineral composition (Ca, Mg, P, Fe, Cu, Zn, Mn, Cr, Se, Mo, Co, Al, Ni, As, Cd, Pb).

2.3.1. Total Chlorophyll and Carotenoids

Chlorophylls and carotenoids were extracted from fresh tissues (about 200 mg) using methanol 99.9% as solvent. Samples were kept in a dark room at 4 °C for 24 h. Quantitative chlorophyll determinations were carried out immediately after extraction. Absorbance readings were measured at 665.2 and 652.4 nm for chlorophyll *a* (Chl *a*) and *b* (Chl *b*), respectively, and 470 nm for total carotenoids. Chlorophyll and carotenoid concentrations were calculated by Lichtenthaler's formula [37].

2.3.2. Phenolic Index and Anthocyanins Concentration

Samples of frozen tissue (30–50 mg) were ground in pre-chilled mortar and extracted into methanolic HCl (1%). After that, they were incubated overnight at 4 °C, in the dark. Phenols were spectrophotometrically determined by measuring directly the methanolic extract absorbance at 320 nm (phenolic index), slightly modifying the procedures reported in Ferrante et al. [38]. The phenolic index was expressed as ABS_{320nm}/g FW [38]. For anthocyanins determination, the concentration of cyanidin-3-glucoside equivalents was determined spectrophotometrically at 535 nm [39]. The same methanolic extract was used for both determinations.

2.3.3. Nitrate

Nitrate content was measured with the salicylsulphuric acid method [40]. 10 mg of oven-dried samples (80 °C for 48 h) were suspended in 10 mL of distilled water and left in agitation for 2 h. After that, 20 μL of sample were added to 80 μL of 5% salicylic acid in sulphuric acid and to 3 mL of NaOH 1.5 N. Samples were cooled at room temperature and the spectrophotometer readings were performed at 410 nm. Nitrate content was calculated referring to a KNO_3 standard calibration curve. Data were expressed on a fresh weight (FW) basis considering the fresh weight/dry weight ratio.

2.3.4. Mineral Composition

For assessing the mineral composition, oven-dried samples (80 °C for 48 h) were ground and digested with nitric acid, and elements were measured using inductively coupled plasma mass spectroscopy (ICP-MS). Data were expressed on an FW basis considering the fresh weight/dry weight ratio.

2.4. Contribution to Mineral Dietary Intake and Health Risk Assessment

The estimated dietary intake (EDI, mg/day) of mineral elements possibly resulting from the consumption of micro/baby greens of the studied species was calculated by the following formula:

$$EDI = C_{metal} \times (SP/1000) \quad (1)$$

where,

$$C_{metal} = \text{the element concentration (mg/kg FW) in the produce} \quad (2)$$

$$SP = \text{a supposed portion of 20 g of micro/baby greens} \quad (3)$$

For evaluating the contribution of microgreens and baby greens to human mineral requirements, EDI was expressed as percentage (EDI%) of the recommended dietary intake (RDI, mg/day) (for Ca, P, Mg, Fe, Cu, Zn, Mo, and Se) or adequate intake (AI, mg/day) (for Mn and Cr) as defined by Italian Society of Human Nutrition (SINU), considering RDI and AI values referred to an adult male [41].

In order to assess the possible health risk due to the intake of metals related to micro/baby greens consumption, the health risk index (HRI) was calculated for Fe, Cu, Zn, Mn, Cr, Se, Mo, Co, Ni, As, and Cd according to the following formula:

$$HRI = EDI_{Bw}/RfD \quad (4)$$

where,

$$EDI_{Bw} = \text{EDI (as defined above) per kg of body weight (BW)} \quad (5)$$

$$RfD \text{ (mg/kg BW/day)} = \text{oral reference dose} \quad (6)$$

which is an estimate of the daily exposure of humans to heavy metals having no hazardous effect during the lifetime according to US-EPA [42]

As BW an average body weight for an adult was considered and assumed to be 55.9 kg as in previous studies [43]. Since RfD is not available for Al and Pb, the possible health risk was evaluated on the basis of Al tolerable weekly intake (TWI; mg/kg BW/week) according to EFSA [44], and of Pb *Codex Alimentarius* maximum level (ML; mg/kg FW) (maximum concentration of a contaminant in a food commodity recommended by the FAO/WHO *Codex Alimentarius* Commission to be legally permitted in that commodity) [45].

2.5. Statistical Analysis

Yield and composition data were subjected to a two-way ANOVA (3 species × 2 stages of harvest) according to a randomized block experimental design with three replicates, by using CoStat Statistics

Software. Significant differences among means were determined by using Duncan's Test at $p < 0.05$. A principal component analysis (PCA) was also performed on composition data by using the software STATISTICA for Windows. Before performing PCA, all values of considered variables were replaced by standardized values, which were computed as follows:

$$\text{Standardized value} = (\text{raw value} - \text{mean})/\text{Std. deviation} \quad (7)$$

3. Results

3.1. Yield

Considering the average of the two stages of harvest, the most and the least productive species were *S. arvensis* (2.41 kg FW/m^2) and *S. minor* (0.39 kg FW/m^2), respectively. An intermediate yield was obtained in *T. officinale* (1.83 kg FW/m^2). On average, the three species resulted in higher yield when they were harvested at the baby leaf stage (2.11 kg FW/m^2) rather than as microgreens (0.99 kg FW/m^2). A significant interaction species × stage of the harvest was observed ($F = 24.66$; $p < 0.001$), revealing that *S. minor* gave higher yield as microgreens than as baby greens, while the contrary occurred in *T. officinale* (Figure 1). In *S. arvensis*, harvesting at different stages resulted in comparable yields.

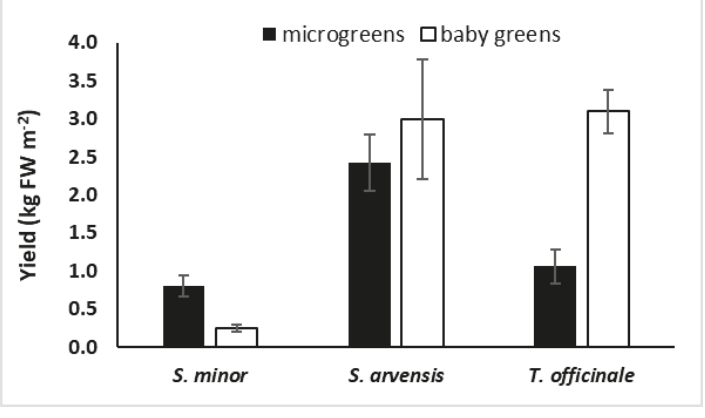

Figure 1. Yield of *S. minor*, *S. arvensis*, and *T. officinale* microgreens and baby greens grown in a hydroponic system. Data are means ± SD ($n = 3$).

3.2. Chlorophylls, Carotenoids, Phenols, Anthocyanins and Nitrate Content

Statistical analysis showed that chlorophylls concentration, considering both the total amount and the single chlorophyll types (Chl *a* and Chl *b*), as well as the phenol values (expressed as phenolic index), were not significantly different among the species and the stages of harvest (Table 2). For carotenoids, higher concentration was found in baby greens than in microgreens, while no differences were observed among the species (Table 2). On the contrary, the species, as well as the stages of the harvest, showed significant differences in anthocyanin concentration. Among the species, the highest anthocyanin amount was found in *S. minor* (0.19 mg/g FW); between microgreens and baby greens, the latter showed higher values (Table 2). However, the significant interaction species × stage of harvest highlighted that such difference did not occur in *T. officinale* (Figure 2A). The species did not differ in nitrate concentration, whose values, on average, ranged from 5205 mg/kg FW (*S. minor*) to 6833 mg/kg FW (*S. arvensis*) (Table 2). The comparison between the stages of harvest revealed a significantly higher nitrate concentration in baby greens than in microgreens. A significant interaction species × stage of harvest was found for nitrate content. Specifically, *T. officinale* microgreens showed much lower nitrate

values than baby greens, while for *S. minor* and *S. arvensis* nitrate concentration was similar in the two product types (Figure 2B).

Table 2. Chlorophylls (Chl *a*, Chl *b* and total), carotenoids, phenols, anthocyanins, and nitrate concentrations of *S. minor*, *S. arvensis*, and *T. officinale* grown in a hydroponic system and harvested at microgreen or baby green stage.

Treatments	Chl *a* mg/g FW	Chl *b* mg/g FW	Chl *a*+*b* mg/g FW	Carotenoids mg/g FW	Anthocyanins [1] mg/g FW	Phenolic Index $ABS_{320\ nm}$/g FW	Nitrate mg/kg FW
Species							
S. minor	0.84 ± 0.58	0.66 ± 0.58	1.41 ± 1.17	0.16 ± 0.07	0.19 ± 0.05 [a]	11.95 ± 2.85	5205 ± 2023
S. arvensis	1.00 ± 0.40	0.55 ± 0.35	1.55 ± 0.68	0.18 ± 0.08	0.13 ± 0.03 [b]	10.98 ± 2.51	6833 ± 1626
T. officinale	0.90 ± 0.38	0.65 ± 0.31	1.55 ± 0.64	0.11 ± 0.04	0.13 ± 0.02 [b]	10.78 ± 1.91	6368 ± 4100
Stage of harvest							
Microgreens	0.76 ± 0.38	0.50 ± 0.50	1.26 ± 0.85	0.11 ± 0.05 [b]	0.13 ± 0.02 [b]	10.30 ± 2.74	4962 ± 2231 [b]
Baby greens	1.07 ± 0.46	0.74 ± 0.26	1.75 ± 0.74	0.20 ± 0.05 [a]	0.17 ± 0.05 [a]	12.17 ± 1.54	7308 ± 2774 [a]
Significance							
Species	ns	ns	ns	ns	***	ns	ns
Stage of harvest	ns	ns	ns	**	**	ns	*
Species x stage of harvest	ns	ns	ns	ns	*	ns	*

[1] Cyanidin-3-glucoside equivalent. Means (± SD) in columns not sharing the same letters are significantly different according to Duncan's Test ($p \leq 0.05$). ns = not significant; asterisk(s) = significant at 0.05 (*), 0.005 (**) or 0.001(***) level of significance.

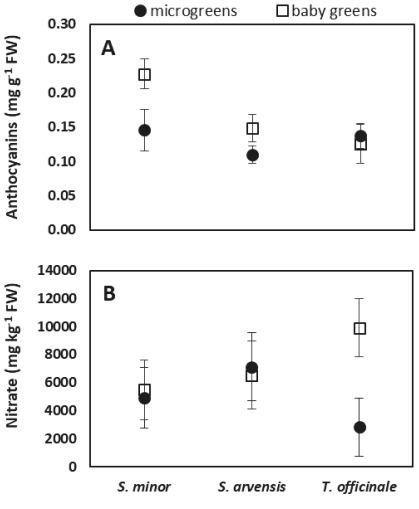

Figure 2. Interaction species × stage of harvest for anthocyanins (**A**) and nitrate concentration (**B**) of *S. minor*, *S. arvensis*, and *T. officinale* grown in a hydroponic system and harvested at microgreen or baby green stage. Data are means ± SD ($n = 3$).

3.3. Mineral Content

Significant differences in element concentration between the species were observed for Ca, Mg, P, Cu, Zn, Mn, Se, Mo, Cd, and Pb (Tables 3 and 4). *S. minor* was richer in Mg, P, Zn, Mn, Mo, and Pb than *S. arvensis* and *T. officinale*. The latter ones did not differ for these elements with the exception of Zn and Mn, which were higher in *S. arvensis* than in *T. officinale*. *S. arvensis* showed the highest concentration in Ca, but the lowest amount in Cu and Se, and *T. officinale* was richer in Cd. No significant differences between the species were noticed for the content in Fe, Cr, Co, Al, Ni, and As. As the average of the three species, baby greens were found to contain higher amounts of Ca, Mg, P, Mn, Mo, and Cd than microgreens, which, conversely, showed higher concentrations in Co, Al, and Pb (Tables 3 and 4). The interaction between species and stage of the harvest was significant for Ca, Mg, Fe, Cu, Zn, Mn,

Mo, Co, Al, Cd, and Pb (Tables 3 and 4). *S. arvensis* was particularly reached in Ca and *S. minor* in Mg, Zn, Mn and Mo at the baby green stage (Figure 3A,B,E,F and Figure 4A) On the contrary, the high accumulation of Pb in *S. minor* occurred only in microgreens (Figure 4E). *S. minor* showed a higher concentration of Fe, Cu, Co and Al when harvested at the baby greens stage, while for *S. arvensis* and *T. officinale* microgreens were richer in these elements than baby greens (Figure 3C,D and Figure 4B,C). For Cd, the difference between microgreens and baby greens was observed in *S. minor* and *T. officinale* (Figure 4D).

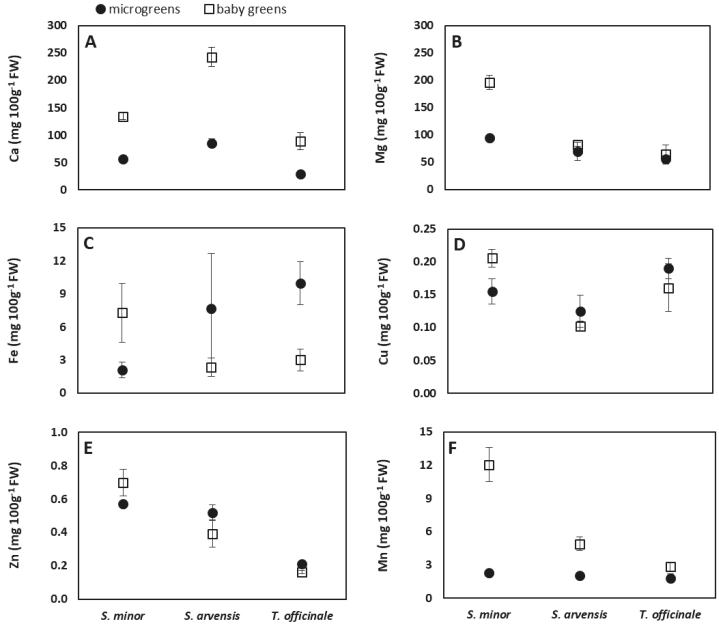

Figure 3. Interaction species × stage of harvest for Ca (**A**), Mg (**B**), Fe (**C**), Cu (**D**), Zn (**E**), and Mn (**F**) concentration in *S. minor*, *S. arvensis*, and *T. officinale* grown in a hydroponic system and harvested at microgreen or baby green stage. Data are means ± SD (n = 3).

Table 3. Calcium, Mg, P, Fe, Cu, Zn, and Mn concentration in *S. minor*, *S. arvensis*, and *T. officinale* grown in a hydroponic system and harvested at microgreen or baby green stage.

Treatments	Ca	Mg	P	Fe	Cu	Zn	Mn
	mg/100 g FW						
Species							
S. minor	95.59 ± 42.59 [b]	145.58 ± 55.71 [a]	108.11 ± 13.76 [a]	4.70 ± 3.32	0.18 ± 0.03 [a]	0.63 ± 0.09 [a]	7.16 ± 5.45 [a]
S. arvensis	163.95 ± 87.09 [a]	75.99 ± 13.21 [b]	54.08 ± 11.56 [b]	5.03 ± 4.31	0.11 ± 0.02 [b]	0.46 ± 0.09 [b]	3.48 ± 1.61 [b]
T. officinale	59.00 ± 34.22 [c]	59.79 ± 12.78 [b]	61.56 ± 20.07 [b]	6.48 ± 4.04	0.18 ± 0.03 [a]	0.19 ± 0.03 [c]	2.34 ± 0.67 [c]
Stage of harvest							
Microgreens	57.05 ± 24.60 [b]	73.39 ± 19.71 [b]	63.59 ± 25.16 [b]	6.59 ± 4.40	0.16 ± 0.03	0.43 ± 0.17	2.05 ± 0.30 [b]
Baby greens	155.31 ± 69.48 [a]	114.18 ± 62.79 [a]	85.57 ± 28.89 [a]	4.22 ± 2.74	0.16 ± 0.05	0.42 ± 0.24	6.61 ± 4.27 [a]
Significance							
Species	***	***	***	ns	***	***	***
Stage of harvest	***	***	**	ns	ns	ns	***
Species x stage of harvest	***	***	ns	**	**	**	***

Means (± SD) in columns not sharing the same letters are significantly different according to Duncan's Test ($p \leq 0.05$). ns = not significant; asterisk(s) = significant at 0.05 (*). 0.005 (**) or 0.001(***) level of significance.

Table 4. Chromium, Se, Mo, Co, Al, Ni, As, Cd, and Pb concentration in *S. minor*, *S. arvensis*, and *T. officinale* grown in a hydroponic system and harvested at microgreen or baby green stage.

Treatments	Cr	Se	Mo	Co	Al	Ni	As	Cd	Pb
	μg/100 g FW								
Species									
S. minor	256.4 ± 281.7	25.3 ± 4.2 [a]	36.3 ± 20.5 [a]	7.3 ± 4.7	2284.8 ± 2058.7	138.5 ± 130.2	2.4 ± 0.7	0.5 ± 0.2 [b]	17.9 ± 15.3 [a]
S. arvensis	59.3 ±5 1.1	10.0 ± 3.2 [b]	20.3 ± 3.2 [b]	6.6 ± 5.8	3664.8 ± 3903.1	32.3 ± 28.1	1.0 ± 0.3	0.4 ± 0.1 [b]	1.8 ± 0.6 [b]
T. officinale	267.3 ± 275.9	22.3 ± 4.1 [a]	25.2 ± 8.5	12.2 ± 7.6	4331.3 ± 3546.0	139.8 ± 146.8	4.1 ± 5.7	0.6 ± 0.2 [a]	4.6 ± 1.9 [b]
Stage of harvest									
Microgreens	192.3 ± 243.0	18.1 ± 5.8	19.3 ± 4.2 [b]	11.1 ± 7.3 [a]	4729.6 ± 3878.4 [a]	107.9 ± 125.2	1.3 ± 0.5	0.4 ± 0.1 [b]	12.2 ± 14.8 [a]
Baby greens	196.4 ± 245.6	20.4 ± 9.5	35.2 ± 16.0 [a]	6.3 ± 4.3 [b]	2124.3 ± 1674.4 [b]	99.2 ± 120.5	3.6 ± 4.6	0.6 ± 0.2 [a]	4.0 ± 1.9 [b]
Significance									
Species	ns	***	***	ns	ns	ns	ns	**	***
Stage of harvest	ns	ns	***	*	*	ns	ns	***	**
Species x stage of harvest	ns	ns	***	**	*	ns	ns	*	**

Means (± SD) in columns not sharing the same letters are significantly different according to Duncan's Test ($p \leq 0.05$). ns = not significant; asterisk(s) = significant at 0.05 (*). 0.005 (**) or 0.001(***) level of significance.

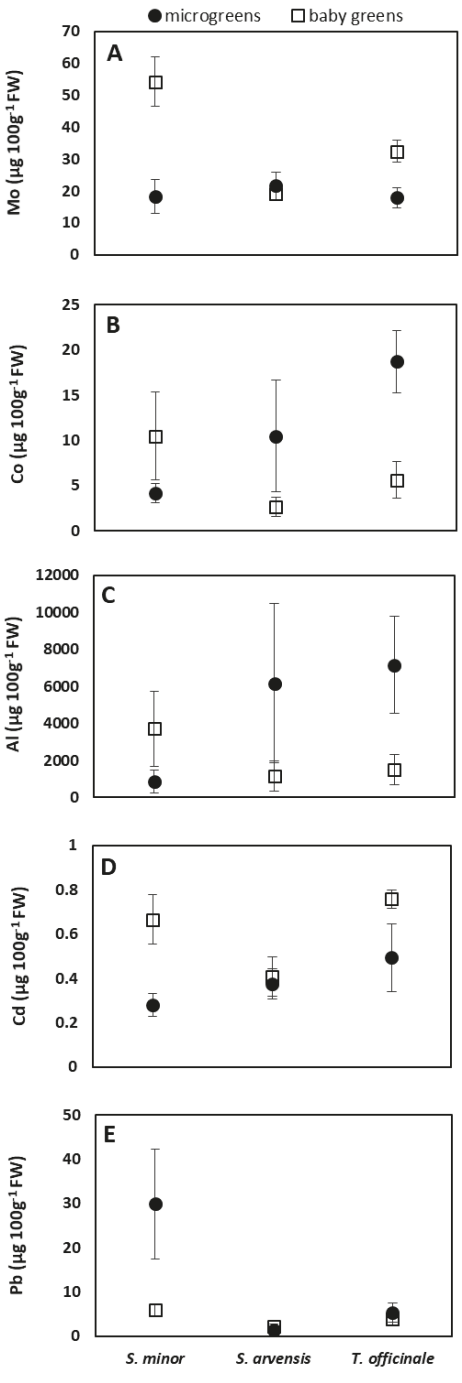

Figure 4. Interaction species × stage of harvest for Mo (**A**), Co (**B**), Al (**C**), Cd (**D**), and Pb (**E**) concentration in *S. minor*, *S. arvensis*, and *T. officinale* grown in a hydroponic system and harvested at microgreen or baby green stage. Data are means ± SD (n = 3).

3.4. Principal Component Analysis (PCA)

A PCA was carried out in order to investigate whether there were factors grouping correlated variables together and to identify clusters across species and stages of harvest. Two principal components (PCs) explaining a cumulative variance of 61.0% were identified based on a screen plot of eigenvalues (Figure 5). PC 1, which explained 35.1% of the total variance, was positively correlated with anthocyanins, Mg, Mn, Mo, and P, while PC 2 (25.9% of the total variance) was negatively correlated to carotenoids and Ca and positively to Fe, Cu, Co and Al. The loading plot reported in Figure 6A illustrates the relationships between the parameters considered in this study. Parameters located close to each other had a strong co-variance. Moreover, parameters far from the origin contributed more to the PCs than parameters close to it. In the rightmost part of Figure 6A, two clusters (the first with anthocyanins, Mo and Mg, and the second with P, Mn and Zn) suggested a strong co-variance between these variables, as well as a strong contribution to PC 1. The most important variables contributing to PC 2 were Ca and carotenoids and, on the opposite side, Al, Co, Fe and Cu. The relationship existing between the analyzed samples are shown in the score plot (Figure 6B). PC 1 and PC 2 discriminated species and stages of harvest in five groups. *S. minor* baby greens were positioned in the right half of the plot (the positive side of PC 1): they were characterized by the highest levels of anthocyanins, Mg, Mn, Mo, P, and Zn. *T. officinale* microgreens were included in the upper left quadrant (the positive side of PC 2): they were characterized by high Fe, Co and Al concentrations and low nitrate content. *S. arvensis* samples harvested at the baby leaf stage were included in the lower-left quadrant (the negative side of PC 2): they were characterized by high carotenoids and Ca content. Differently, *S. arvensis* microgreens were characterized by low anthocyanins and relatively high nitrate and Al contents. Finally, *S. minor* microgreens and *T. officinale* baby greens were closely clustered at the center of the scatterplot (Figure 6B).

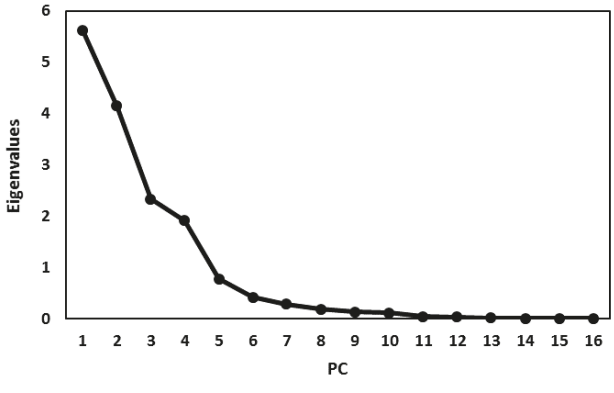

Figure 5. Screen plot of eigenvalues in PCA analysis.

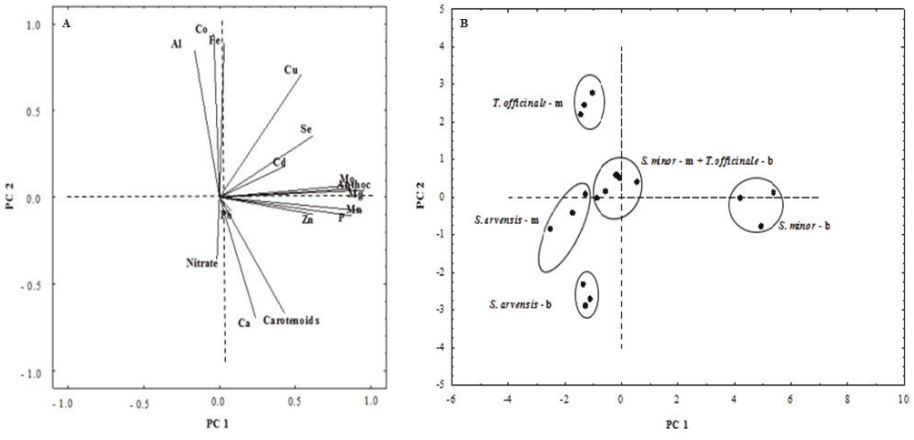

Figure 6. Loading plot (**A**) and scores (**B**) for each component (PC 1 and PC 2). Anthoc = anthocyanins; m and b correspond to microgreen and baby green stages, respectively.

3.5. Contribution to Mineral Dietary Intake and Health Risk Assessment

The potential contribution of the analyzed microgreens and baby greens to human mineral requirements was very different for the different elements (Table 5). With reference to a portion of 20 g of microgreens/baby greens, the EDI% ranged from very low values, even lower than 1% (Ca from *T. officinale* microgreens and Zn from *S. arvensis* and *T. officinale* regardless of the stage of harvest) to values higher than 100% in the case of Cr (*T. officinale* microgreens and *S. minor* baby greens), revealing a potential intake so far over the AI of this element. Values of EDI% over 10% were detected for Mg (*S. minor* baby greens), Fe (*S. arvensis* and *T. officinale* microgreens, *S. minor* baby greens), Mn (all the species, both the stages), Se (*S. minor* baby greens) and Mo (*S. minor* and *T. officinale* baby greens). Considering the average of the three species, the EDI% values from microgreens showed the following ascending order for the different elements: Zn (0.79%), Ca (1.14%), P (1.82%), Cu (3.48%), Mg (6.11%), Se (6.58%), Mo (8.56%), Fe (13.18%), Mn (15.16%) and Cr (109.90%). Similarly, the order of EDI% from baby greens was Zn (0.76%), P (2.45%), Ca (3.10%), Cu (3.46%), Se (7.40%), Fe (8.44%), Mg (9.52%), Mo (15.67%), Mn (48.95%) and Cr (112.21%).

Regarding the assessment of the health risk related to detrimental metals present in the micro/baby greens, all the EDI_{BW} values (Fe, Cu, Zn, Mn, Cr, Se, Mo, Co, Ni, As, Cd), calculated with reference to a portion of 20 g, were smaller than the corresponding RFDs (US-EPA IRIS, 2013), and the HRIs were far below 1 (Table 6). For Al, for any species and stage of the harvest, weekly consumption of 20 g of product per day would bring to an element intake far below the TWI (1 mg/kg body weight/week) recommended by EFSA (2008) (data not shown). For Pb, the ML recommended by the FAO/WHO *Codex Alimentarius* Commission to be legally permitted in leafy vegetables (30 μg/100 g) was exceeded in *S. minor* microgreens (Figure 4E).

Table 5. Estimated dietary intake expressed as percentage (EDI%) of the recommended dietary intake (RDI) or adequate intake (AI) resulting from the consumption (20 g per day) of microgreens or baby greens of *S. minor*, *S. arvensis*, and *T. officinale*.

Mineral	RDI/*AI* [1] mg/day	Microgreens			Baby Greens		
		S. minor	*S. arvensis*	*T. officinale*	*S. minor*	*S. arvensis*	*T. officinale*
Ca	**1000**	1.14	1.7	0.58	2.69	4.85	1.78
Mg	**240**	7.94	5.79	4.61	16.32	6.88	5.35
P	**700**	2.76	1.28	1.41	3.42	1.81	2.11
Fe	**10**	4.23	15.39	19.91	14.56	4.73	6.02
Cu	**0.9**	3.44	2.78	4.22	4.56	2.26	3.57
Zn	**11**	1.04	0.95	0.39	1.27	0.71	0.30
Mn	*2.7*	16.82	15.28	13.39	89.32	36.30	21.23
Cr	*0.035*	42.27	54.46	232.98	250.78	13.33	72.51
Se	**0.055**	8.16	4.24	7.35	10.26	3.05	8.88
Mo	**0.045**	8.15	9.60	7.94	24.09	8.48	14.43

[1] RDI (bold) and AI (italic) according to SINU (2014).

Table 6. Estimated daily intake per kg of body weight (EDI_{BW}, mg/kg body weight/day) and health risk index (HRI) resulting from the consumption (20 g per day) of microgreens or baby greens of *S. minor*, *S. arvensis*, and *T. officinale*.

Metal		Microgreens			Baby Greens		
		S. minor	*S. arvensis*	*T. officinale*	*S. minor*	*S. arvensis*	*T. officinale*
Fe (RfD = 0.7)	**EDI_{BW}**	0.007565	0.027538	0.035613	0.026045	0.008454	0.010773
	HRI	0.010808	0.039339	0.050876	0.037207	0.012077	0.015391
Cu (RfD = 0.01)	**EDI_{BW}**	0.000553	0.000447	0.00068	0.000735	0.000363	0.000575
	HRI	0.013835	0.011178	0.016994	0.018383	0.009082	0.014373
Zn (RfD = 0.3)	**EDI_{BW}**	0.002037	0.001861	0.000764	0.002502	0.001398	0.000581
	HRI	0.006791	0.006203	0.002548	0.008341	0.00466	0.001936
Mn (RfD = 0.14)	**EDI_{BW}**	0.008124	0.007378	0.006466	0.043143	0.017535	0.010253
	HRI	0.058028	0.0527	0.046187	0.308164	0.125247	0.073234
Cr (RfD = 0.003)	**EDI_{BW}**	0.000265	0.000341	0.001459	0.00157	0.000083	0.000454
	HRI	0.088223	0.113652	0.486246	0.523397	0.027814	0.151333
Se (RfD = 0.005)	**EDI_{BW}**	0.00008	0.000042	0.000072	0.000101	0.00003	0.000087
	HRI	0.016061	0.00835	0.014472	0.020196	0.006007	0.017484
Mo (RfD = 0.005)	**EDI_{BW}**	0.000007	0.000008	0.000006	0.000019	0.000007	0.000012
	HRI	0.001313	0.001546	0.001279	0.003879	0.001364	0.002323
Co (RfD = 0.0003)	**EDI_{BW}**	0.000015	0.000037	0.000067	0.000037	0.00001	0.00002
	HRI	0.049521	0.124754	0.223107	0.12494	0.031681	0.067146
Ni (RfD = 0.02)	**EDI_{BW}**	0.000196	0.000192	0.00077	0.000795	0.00004	0.00023
	HRI	0.009811	0.009592	0.038497	0.039736	0.001977	0.011521
As (RfD = 0.0003)	**EDI_{BW}**	0.000006	0.000003	0.000006	0.000011	0.000005	0.000024
	HRI	0.020747	0.009076	0.018335	0.035316	0.01538	0.07893
Cd (RfD = 0.001)	**EDI_{BW}**	0.000001	0.000001	0.000002	0.000002	0.000001	0.000003
	HRI	0.001003	0.001342	0.001767	0.002385	0.001467	0.002715

RfD = oral reference dose (mg/kg/body weight/day) according to USEPA (2013).

4. Discussion

The fresh biomass of *S. minor*, *S. arvensis*, and *T. officinale* microgreens (Figure 1) ranged from 0.8 kg/m^2 (*S. minor*) to 2.4 kg/m^2 (*S. arvensis*) and was consistent with that reported by Bulgari et al. [46], Paradiso et al. [47], and Renna et al. [48] for microgreens of vegetable crop species. Kyriacou et al. [27] found that the microgreens of 10 different species produced over 3 kg FW/m^2, but these authors

adopted a longer growth period, harvesting the microgreens at the second leaf stage. At the baby green stage, *S. arvensis* and *T. officinale* yield (about 3 kg FW/m^2) was higher than that of cultivated species [28,49,50]. The fresh biomass of *S. minor* baby leaves was only 0.2 kg FW/m^2. In this case, the increase in plant fresh weight from microgreens to baby leaves did not compensate for the lower plant density, suggesting that a later stage of harvest (i.e., more than 5–6 leaves) would have been more proper for *S. minor*.

Wild edible plants contain important amounts of non-nutrient compounds beneficial for health, such as carotenoids and phenolic compounds [51]. Healthy effects of these bioactive molecules are often associated with antioxidant activity, leading to the reduction in cardiovascular disease risk factors, the decrease of the incidence of cancer, and protection against a wide range of chronic diseases [52]. Besides the health benefits, carotenoids and anthocyanins influence the organoleptic quality of plant products (taste, aroma) and their visual appearance [27,53]. Together with chlorophylls, they are the main pigments contributing to leaf color, which is particularly important for leafy vegetables since it strongly conditions the evaluation by the consumer and, especially in produce like microgreens and baby leaves, should be uniform and intense [38,54].

Considering the microgreen stage, the three studied wild species showed usually higher or, sometimes, comparable chlorophyll, carotenoids and anthocyanin concentrations than those of most vegetable crop species analyzed in previous studies [25,27,46,47,55,56]. Nevertheless, under LED illumination some microgreens of Brassicaceae family showed even higher carotenoid amounts [57], and particularly high contents of total anthocyanins were measured by Samuolienė et al. [26] in the microgreens of 10 vegetable species. As reviewed by Saini et al. [17] and Di Gioia et al. [58], many studies have shown that baby greens are a good source of antioxidants. To our knowledge, no comparison between baby greens and microgreens of the same species has been carried out on this aspect yet. Among the species we analyzed, *S. minor* showed the highest anthocyanin amounts, and baby greens were richer in these compounds, as well as in carotenoids than microgreens (Table 2 and Figure 2). Considering that, in general, these phytochemicals increase during leaf development and reach the maximum level in mature leaves [59] this result is probably ascribable to the different stage of the harvest of the two products. For the same reason, the lower content of carotenoids found in *S. minor* and *T. officinale* micro/baby greens in comparison with values reported in the literature for adult plants of these species [51] is reasonable.

Microgreens and baby greens of vegetable crops show very variable nitrate contents [27,28]. Such variability is due to the different accumulation ability of the different genotypes, but it is also strongly influenced by agronomic and environmental factors [9]. When microgreens were compared to adult plants of the same species grown in the same conditions, lower nitrate content was observed in microgreens [34]. Accordingly, in our study, the more mature stage (baby greens) of *T. officinale* contained more nitrate than the microgreen counterpart. Conversely, compared to nitrate content measured in *T. officinale* adult leaves collected in the wild [60], we found much higher values, probably due to higher nitrogen availability in the nutrient solution than in the uncultivated soil. In *S. minor* and *S. arvensis*, no differences were found between microgreens and baby leaves (Figure 2).

Concerns about nitrate accumulation in vegetables are mainly related to the fact that nitrate ingestion is thought to be a risk factor for stomach cancer [9]. That has brought the EU Commission to establish maximum nitrate levels allowed for the commercialization of some vegetables (spinach, lettuce, and rocket) ranging from 2000 to 7000 mg/kg FW (Regulation No 1258/2011). On the other hand, the association between the estimated intake of nitrate in the diet and stomach cancer has been recently rejected on the basis of the review of the epidemiological literature [10]. Moreover, different authors have reported that a diet high in nitrate is beneficial to humans for cardiovascular and cerebrovascular health [61,62], in particular in older adults [63]. In our study, nitrate concentration was over 2000 mg/kg FW in all the analyzed samples, and in *S. arvensis* microgreens and *T. officinale* baby greens exceed 7000 mg/kg FW. If, on one hand, that can be considered a limitation for these products, on the other

hand, it makes them possible candidates to provide dietary nitrate supplementation for some categories of people like the elderly.

Data available in the literature demonstrate that wild edible plants may be an excellent source of macro and microelements for humans. Wild greens usually contribute to the dietary intake of minerals more than wild fruits, and for Ca, Mg, Fe, and Mn, the provided amounts may even reach half of the recommended daily requirement [4]. In *S. arvensis*, *S. minor* and *T. officinale* micro/baby greens, analyzed in this study, these elements showed concentrations sometimes higher and sometimes lower than those reported in the literature for adult counterparts [4,64,65]. In previous studies, microgreens were found to contain lower Ca amount than adults in amaranth [30] and kale [36], while the contrary was found in lettuce [34], and broccoli grown on compost [66]. Among the three analyzed species, *S. arvensis* showed higher Ca concentrations than *S. minor* and *T. officinale*, and, at the baby green stage, exceeded 200 mg/100 g FW (Figure 3A), which is considered a good Ca content [4]. In all the three species, baby greens were richer in Ca than microgreens (Table 3 and Figure 3A), confirming the results of Waterland et al. [36] in kale. These authors found that kale baby greens contained also higher amounts of Mg and Fe than microgreens of the same species. In our study, baby greens were richer in Mg than microgreens only in *S. minor* (Figure 3B). This species, on average, showed much more Mg than *S. arvensis* and *T. officinale* (Table 3). That is not surprising, considering that among wild edible greens, *S. minor* is considered one of the richest Mg sources [4]. Furthermore, *S. minor* needed eight days more than *S. arvensis* and *T. officinale* to reach the baby leaf stage and the different growth period could have affected the mineral composition [24]. In comparison with microgreens [24,27,47] and baby greens [28,67] of many vegetable crop species, the wild greens grown in our study showed medium to low content as microgreens and medium to high content as baby greens for Ca and contained medium to high amounts of Mg at both stages of harvest. For Fe, according to what was observed by Waterland et al. [36] in kale, *S. minor* baby greens showed higher concentration than microgreens, while the opposite occurred in *T. officinale* (Figure 3C). It is interesting to notice that, considering the reviewed literature on wild greens [2,4,68], vegetable microgreens [24,30,34,46,47], and vegetable baby greens [67] of different species, *T. officinale* microgreens exceeded the Fe amount of any of them. Some differences among species were observed in P, Cu, Zn, and Mn concentrations, and for P and Mn also between stages of harvest (Table 3 and Figure 3D–F). For all the three species and both the stages, values were comparable (P and Zn) or higher (Mn and Cu) than those measured by other authors in vegetable microgreens [24,47] or baby greens [67]. Waterland at al. [36], noticed higher Zn amounts in kale baby leaves in comparison with the microgreen counterparts. Contrasting this, we did not observe differences in Zn concentration between the two stages of harvest.

According to the Regulation (EU) No. 1169/2011 on the provision of food information to consumers, foods can be considered significant sources of mineral elements if they contain, per 100 g, at least 15% of the reference values reported in the Annex XIII, and corresponding to (in mg): 120.0 (Ca), 56.3 (Mg), 105.0 (P), 2.10 (Fe), 0.15 (Cu), 1.50 (Zn), 0.30 (Mn), 0.0060 (Cr), 0.0083 (Se), and 0.0075 (Mo). The comparison between these amounts and data shown in Table 3, Table 4 and Figure 3, Figure 4 would indicate that micro/baby greens of the wild species analyzed in our study should be good sources of several minerals in the human diet. Nevertheless, for evaluating their contribution it cannot be disregarded that specialty produce, especially microgreens, are normally consumed in small amounts. Therefore, in order to avoid overestimations, in our study EDI% was calculated for a portion of 20 g (Table 5), which was considered quite a reasonable amount for the comparison between microgreens and baby greens. As reference values, RDI or AI as defined in the Materials and Methods section were considered. The largest contributions were observed for Cr, Mn, Mo, Mg, and Fe. For the latter, particularly noticeable was the EDI% of *T. officinale* microgreens (almost 20%). Intermediate EDI% values were noticed for Se and Cu, and the lowest for Zn, Ca, and P. Zinc and P data are consistent with the fact that leafy vegetables, either wild or cultivated, do not stand out by their P and Zn concentrations, and thus they are not generally recognized as good sources of these elements [4].

Minor elements (Cr, Se, Mo, Co, Al, Ni, As, Cd, and Pb) have been rarely measured in micro/baby greens. Molybdenum concentration in lettuce microgreens [34] was comparable to the values found in the microgreens of the wild species considered in our study but lower than those of *S. minor* and *T. officinale* harvested at the baby stage (Figure 4A). For Se, the wild greens, independently from the stage of harvest, showed higher amounts than those measured in lettuce microgreens [34], but *S. minor* and *T. officinale* were richer in this element than *S. arvensis* (Table 4). Xiao et al. [24] investigated Cd and Pb content of 30 vegetable microgreens of the Brassicaceae family, finding that these elements were under the limit of detection. Also, Paradiso et al. [47] observed that Pb was under the detection limit in some genotypes of microgreens belonging to Brassicaceae or Asteraceae, while in the same samples Cd concentration was about 10 times over the values observed in our study (Figure 4D). The species considered in our study resulted to contain Pb, and, in *S. minor* microgreens, the amount of this metal exceeded the ML of 30 μg/100 g FW recommended by the FAO/WHO *Codex Alimentarius* Commission for leafy vegetables [45]. Other heavy metals detected in the wild greens were Cr, Co, Al, Ni, and As. That was not surprising since ruderal species, like *S. minor*, *S. arvensis* and *T. officinale*, are well-known for their capability to accumulate contaminants, especially in leaves [8,12,13]. For example, Giacomino et al. [69] and Stark et al. [70] found potentially hazardous levels of Pb and As, respectively, in some samples of spontaneously growing *T. officinale*, and *S. arvensis* stood out among different wild species for Cd and Cr accumulation in both contaminated and not-contaminated soils [12,64]. In our study, microgreens and baby greens were grown in a controlled environment and hydroponically, using a nutrient solution prepared with distilled water, therefore it can be supposed that the detected trace elements derived from the mineral fertilizers used to prepare the nutrient solution [71,72] and from vermiculite used as growing medium [73]. However, since HRI values <1 are assumed to be safe in terms of population exposure to metals [43], HRI calculated for Fe, Cu, Mn, Cr, Se, Mo, Co, Ni, As and Cd considering a portion of 20 g, being far below 1 (Table 6), excluded health risks due to the consumption of micro/baby greens in relation to these elements. Health risks were excluded also for Al, whose ingestion was calculated on a weekly basis according to EFSA recommendation [44]. Even considering portions of 100 g, which are quite improbable for these products, HRI values would still be below 1 in most cases. Only *S. minor* baby greens and *T. officinale* microgreens would show HRI >1 for Mn and Cr, and for Co and Cr, respectively, if EDI_{Bw} was reported to 100 g product.

5. Conclusions

The results of this study showed that *S. minor*, *S. arvensis*, and *T. officinale* would be interesting species for producing specialty crops like microgreens and baby greens. Actually, not only did they achieve competitive yield, but also demonstrated that their contribution to the dietary intake of macroelements, microelements, and non-nutrient bioactive compounds would be comparable, or even larger, than that of vegetable crop species. Among the species, *S. minor* showed the highest amounts of Mg, P, Zn, Mn, and Mo, and *T. officinale* microgreens stood out by Fe content. Between microgreens and baby greens, the latter were often richer in minerals and antioxidants. On the other hand, the wild greens showed high amounts of nitrate, which could be a limitation for commercialization, and the presence of some metals potentially detrimental for human health. Although micro/baby greens are normally consumed in small portions, and the calculated HRI values were far below 1, such a finding suggests caution. Therefore, the aspect of the accumulation ability of wild ruderal species should always be considered prior to introducing them in cultivation, and, in this case, strict control of possible sources of chemical contamination (water, salts used to prepare the nutrient solution, and substrates) would be necessary.

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Setup of an Extraction Method for the Analysis of Carotenoids in Microgreens

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Abstract: Microgreens are gaining increasing interest as a potential functional food due to their relevant contents of micronutrients and bioactive compounds, including carotenoids. Nevertheless, the analysis of carotenoids is inherently difficult, due to their thermal and chemical susceptibility, as well as to their varying polarity. From this point of view, extraction is the most critical step, compared to chromatographic separation and detection. Thus, the reliability of data on carotenoids should be guaranteed by a constant focus on analytical issues, with appropriate adaptations to each sample matrix. In this research, a specific extraction procedure for the analysis of carotenoids in microgreens was developed. Solvent composition, extraction time, solvent/sample ratio, and repeated extractions were evaluated. The obtained protocol showed recovery of 97.2%, limits of quantitation of 5.2 $\mu g \cdot g^{-1}$ for lutein and 15.9 $\mu g \cdot g^{-1}$ for β-carotene, as well as intra-day mean repeatability of 5.7% and inter-day mean repeatability of 4.7%.

Keywords: microgreens; carotenoids; bioactive compounds; antioxidants; extraction; lettuce; linen

1. Introduction

Traditionally used for garnishing gourmet dishes, microgreens have been reconsidered over the last years as basic ingredients in several types of dishes [1], as well as for their potential in enhancing human diets due to relevant contents in micronutrients and phytochemicals [2–5]. In this regard, several reviews have been published in the last years [6–9].

Carotenoids are among the phytochemicals present in microgreens in considerable amounts that can be significantly affected by various endogenous and exogenous factors [10]. Carotenoids are one of the major classes of phytochemicals, and their importance in diet is not only related to their role as vitamin A precursors, but also to their antioxidant anti-tumor activities and their role in gene function regulation, gap-junction communication, and hormone and immune modulation [11,12]. Moreover, they cannot be synthesized by animals and need to be consumed through diet [11]. In this context, vegetable sources of carotenoids are obtaining a great interest [13,14]. As a matter of fact, several papers have evaluated the carotenoid contents in microgreens, reporting results varying in a wide range. To this end, irrespective of absolute concentrations, green leaves show quite a constant qualitative carotenoid pattern, referred to as a chloroplast carotenoid pattern, with lutein (about 45%), β-carotene (25–30%), violaxanthin (10%), and neoxanthin (10%) as the most represented carotenoids. Lactucaxanthin is another major carotenoid in lettuce [13,15,16]. In particular, lutein, the most represented xanthophyll, has been determined in microgreens of different genotypes and grown under different conditions in

amounts ranging from 13 to 191 mg·kg^{-1} on fresh weight [10,17–21]. In most cases, these contents are quite higher than those observed in common fruits and vegetables [22]. Exceptionally higher amounts (from 105.7 to 503.5 mg·kg^{-1}, with a mean content of 291.6 mg·kg^{-1} of lutein) were reported by Brazaitytė et al. [23] in three *Brassicaceae* microgreens grown under different lighting conditions. Regarding β-carotene, the most abundant carotene, the ranges observed in literature are even wider: from 0.11 to 121 mg·kg^{-1} [10,17–21,23]. Also in this case, outstanding results have been reported in another study, in which contents up to 8592.2 mg·kg^{-1} on a dry weight basis (corresponding to 451.9 mg·kg^{-1} on fresh weight) were reported for several species of microgreens grown under controlled conditions [24]. The wide range of carotenoids content in vegetables can be explained by genetic variability (intra- and inter-species biodiversity), as well as by different growing conditions. Nevertheless, analytical issues, particularly in carotenoid extraction, should not be disregarded [25,26]. In fact, carotenoids are easily degradable by diverse factors, and show varying affinity towards extraction solvents, due to their wide range of polarity [27]. As an example, xanthophylls, being oxygenated molecules, can be extracted with polar solvents such as alcohols, acetone, and acetone/water mixtures, while carotenes are more easily extracted by non-polar solvents [25]. Therefore, a possible underestimation of certain carotenoids could have occurred depending on the extraction solvent adopted, such as in some studies wherein 80% aqueous acetone was used as an extraction solvent. Moreover, some undervalued phenomena (degradation, isomerization) could also occur when the extraction procedure involves overnight extractions or some analytical steps, such as saponification, intended for different carotenoid patterns (e.g., carotenoid esters of fruit) or detection methods (e.g., direct spectrophotometry without chromatographic separation) [26,28].

With all the above remarks as a starting point, the aim of the present study was to set up an optimized extraction of carotenoids focused on microgreens as a specific food matrix. The general goal was to critically evaluate the effects of solvent polarity, extraction time, solvent/sample ratio, and repeated extractions.

2. Materials and Methods

2.1. Materials and Reagents

Microgreens of *Lactuca sativa* L. Group *crispa* (cultivar 'Bionda da taglio') and *Linum usitatissimum* L. were used. Seeds were purchased from Riccardo Larosa Company (Andria, Italy). The selected species was one of those characterized in our previous papers [2,3], showing intermediate levels of carotenoids compared to other genotypes.

Acetone (>99.5%), ethanol (96%), methyl *tert*-butyl ether (MTBE) for HPLC (≥99.8%), ammonium acetate, β-Carotene (≥93%), and trans-β-apo-8'-carotenal (≥96%) were purchased from Sigma-Aldrich, Milan, Italy. Methanol for HPLC (99.9%) was purchased from Honeywell (Monza, Italy). Lutein was provided by Extrasynthese (Genay Cedex, France). Butylated hydroxytoluene (BHT) was purchased from Fluka (Honeywell, Bucharest, Romania).

2.2. Microgreens Production and Storage

Microgreens were grown according to Paradiso et al. [2]. Batch samples were obtained by pooling microgreens harvested from at least three growing trays, lyophilized and stored at −20 °C until analyzed.

2.3. Sample Pre-Treatments

Lyophilization was chosen as the best dehydration method for both storage and sample pre-treatment, since it does not cause thermal degradation of carotenoids [25]. No other physical pre-treatment was used to facilitate the release of carotenoids, since microgreens are characterized by tender tissues, with very a low fiber content [2].

2.4. Protection against Degradation

BHT (0.1%) was added to the extraction solvent to prevent carotenoid oxidation. Extraction was carried out in dim light and the extraction vessels were covered with aluminum foil in order to protect carotenoids from photodegradation and isomerization during extraction [25,27,29].

2.5. Optimization of the Carotenoids' Extraction

Lyophilized samples were weighted (0.05 g) in test tubes covered with aluminum foil and added with the extraction solvent and *trans*-β-apo-8'-carotenal (40 mL, 1 g·mL^{-1}) as internal standard. Cold acetone, either pure or in mixture with water, was used as extraction solvent [29]. After centrifugation (3000 *g*, 5 min) the acetone layer was collected, and the pellet was submitted to further extraction where provided. The extraction procedure was set up through the subsequent steps:

i. Evaluation of the solvent polarity mixing acetone with varying amounts of water (acetone 70%, 80%, 90%, 100%);
ii. Evaluation of different extraction times (30 s, 10 min, 1, 5, 24 h);
iii. Evaluation of the solvent/sample ratio (4, 5, 6, 12 mL of solvent per 0.05 g of sample);
iv. Evaluation of repeated extractions.

Saponification is another critical step during carotenoid extraction. This procedure is aimed to remove chlorophylls, in case they could interfere during HPLC separation, and to improve extraction and separation of esterified xanthophylls [25]. Yet, saponification has important side effects (carotenoid degradation and loss, isomerization and formation of artefacts, especially involving more polar carotenoids such as lutein, violaxanthin, and neoxanthin [30]), and is often considered unnecessary in leafy vegetables, in which carotenoids are not esterified [16], a fortiori when HPLC analysis obtains appropriate separation of chlorophylls [13,16,25]. Therefore, due to the abundance of evidence in the literature, saponification was avoided.

2.6. HPLC Analysis of Carotenoids

The extracts were filtered using a 0,45 μm nylon filter and immediately analyzed by HPLC-DAD (Agilent Technologies, 1260 Infinity, USA), in accordance with the procedures reported by Rasmussen et al. [31]. Chromatography was carried out on a C_{30} column (3 μm, 150 × 4.6 mm, YMC, Japan). The mobile phase consisted of two components: eluent A, methanol:MTBE:water (95:3:2, by volume, with 1.5% ammonium acetate in water) and eluent B, methanol:MTBE:water (8:90:2, by volume, with 1.0% ammonium acetate in water). The flow rate was 0.4 mL·min^{-1}, the injection volume was 25 μL, and all carotenoids were monitored at 445 nm. The gradient procedure (10 °C), was as follows: Start at 100% solvent A; a 22 min linear gradient to 45% solvent A and 55% solvent B; an 11 min linear gradient to 5% solvent A and 95% solvent B; a 4 min hold at 5% solvent A and 95% solvent B; a 2 min linear gradient back to 100% solvent A; a 28 min hold at 100% solvent A.

Carotenoid identification was carried out by means of analytical standards (β-carotene and lutein), comparison with retention times in literature, and UV spectra examination. Carotenoid quantification was performed using calibration curves of lutein for xanthophylls (in the range 0.1–10 μg·mL^{-1}), β-carotene for carotenes (in the range 0.5–10 μg·mL^{-1}), trans-β-apo-8'-carotenal (in the range 0.5–6 μg·mL^{-1}) for the recovery evaluation. The linearity of calibration curves, expressed as adjusted R^2, was 0.999.

2.7. Method Validity

Recovery of the optimized method was evaluated according to the following formula [32], applied to the internal standard:

$$R'_A = Q_A(yield)/Q_A(orig)$$

where QA(*orig*) is the known original and QA(*yield*) is the recovered quantity of the analyte A.

Intra-day repeatability was evaluated repeating a series of six extractions in the same day, while inter-day repeatability was evaluated repeating a series of three extractions in three consecutive days.

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated on the basis of the signal-to-noise (S/N) ratio in HPLC analysis, with LOD = 3 × S/N and LOQ = 10 × S/N, and were reported as $\mu g \cdot g^{-1}$ of sample (dry weight), considering the percent recovery [33]. An experimental limit of quantitation (ELOQ), the minimum quantified amount extracted by an exhaust real sample matrix, was also reported.

The method validity was also checked by comparison with another method applied in literature for microgreens by Kyriacou et al. [24].

2.8. Statistical Analysis

All the extractions were carried out at least in duplicate. One-way (to evaluate the effect of solvent/sample ratio and repeated extractions) and two-way (to evaluate the effect of solvent and extraction time) analysis of variance (ANOVA), followed by honestly significant difference (HSD) Tukey's test for multiple comparisons, were carried out using Origin Pro 2019 (OriginLab, Northampton, Massachusetts, USA).

3. Results and Discussion

3.1. Optimization of the Carotenoids' Extraction

3.1.1. Effect of Solvent Polarity and Extraction Time

Considering the differences in polarity of the carotenoids existing in foods and their consequent differing affinity towards polar and non-polar solvents [29], the choice of the extraction solvent should consider the type of food matrix and its typical carotenoid pattern. Acetone and hexane are the most commonly used solvents for carotenoid extraction from food matrices [25,29]. Literature regarding carotenoids in vegetables and microgreens mainly reports methods using either acetone mixed with water [10,19,23,34], or hexane [24] and hexane/toluene [18] combined with saponification. Regarding 80% acetone, its high polarity should be taken under examination for the possible underestimation of non-polar carotenes, as pointed out in the introduction. On the contrary, hexane is mostly indicated for the extraction of non-polar carotenes and esterified xanthophylls [25]. Yet, green leafy vegetables, including microgreens, present the typical chloroplast carotenoid pattern [16], for which polar solvents are generally used. Therefore, we chose acetone for these reasons, as well as for its tunable polarity by mixing with water, and pure cold acetone or acetone mixed with varying amounts of water (10–30%) were evaluated. Different extraction times were also evaluated, ranging from 30 s to 24 h, considering that some extraction protocols provide overnight contact [22,29].

The results are reported in Figure 1, while the results of Tukey's test for multiple comparisons are reported in the Supplementary Table S1. Regarding polar xanthophylls (a–e), the use of acetone:water mixtures provided high extraction yields in one hour of sample –solvent contact. The response to solvent polarity was, as expected, related to the xanthophyll polarity: Xanthophylls with epoxide moieties (violaxanthin, neoxanthin, luteoxanthin) were extracted in higher amounts by 70% acetone. Lutein and lactucaxanthin, having diol structure, gave similar results with 70% and 80% acetone. Extraction times longer than 1 h with acetone:water mixtures caused xanthophyll losses, probably due to oxidative enzymes activation and to isomerization phenomena, such as epoxide-furanoid rearrangement (isomerization of 5,6-epoxy- to 5,8-epoxycarotenoids), as pointed out by the marked decrease of violaxanthin and corresponding increase of luteoxanthin, its corresponding furanoid [25,27,28]. Acetone with 10% water showed an intermediate behavior between acetone:water mixtures and pure acetone. Extraction carried out with pure acetone gave the highest extracted amounts of xanthophylls after 24 h of contact. The extracted amounts were in almost all cases higher than those obtained with the acetone:water mixture, with the exception of luteoxanthin, which was presumably formed de novo,

as stated above, due to epoxide rearrangement. Regarding carotenes (f–h), the performances of the solvent were quite different. Due to their non-polar nature, carotenes were poorly extracted by acetone 70% and 80% mixtures. On the contrary, relevant amounts were extracted with acetone 90% and, above all, with pure acetone. The highest extracted amounts were obtained after 24 h extraction. These results suggest the possibility that some low β-carotene contents reported in literature for microgreens could derive from an underestimation of carotenes due to the polarity of the adopted solvent (acetone 80%).

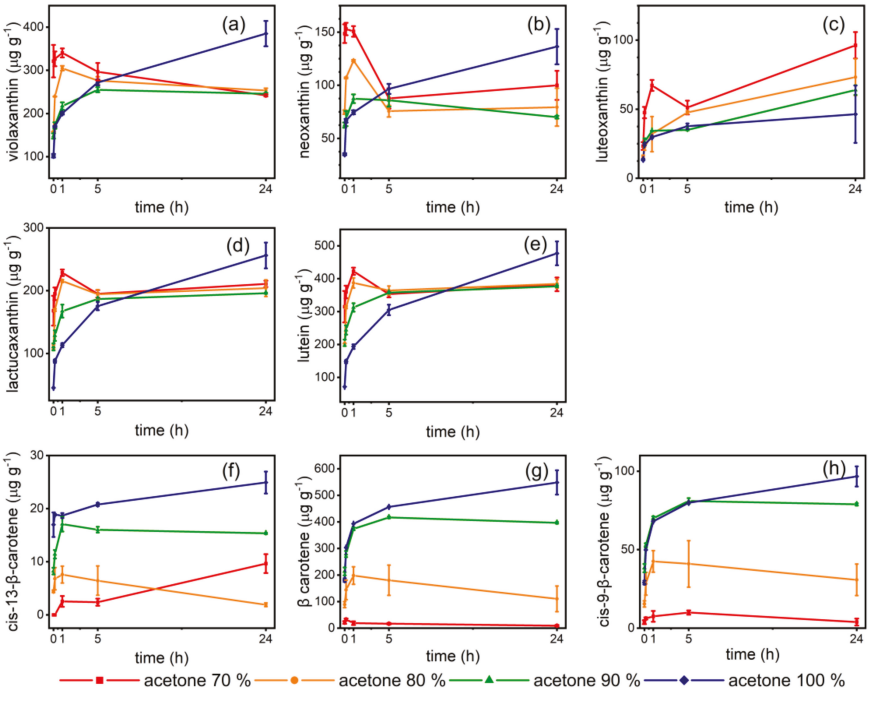

Figure 1. Carotenoids extracted from lettuce microgreens with different acetone:water mixtures and varying extraction times. (**a**–**e**), xanthophylls; (**f**–**h**), carotenes. Error bars indicate standard deviation. Results of ANOVA and post-hoc Tukey's test are reported in Supplementary Table S1.

3.1.2. Effect of Solvent/Sample Ratio

The effect of solvent/sample ratio was also evaluated. Different volumes of cold acetone (4, 5, 6, and 12 mL) were tested on the same amount of sample (0.05 g).

Figure 2 reports the results for three carotenoids (violaxanthin, lutein, and β-carotene), the most abundant and characterized by decreasing polarity. For all three molecules, the extracted amounts after 24 h extraction were comparable but with significant differences. The highest amounts were extracted using 5 mL of solvent for 0.05 g of sample. Higher volumes of solvent determined poorer extraction. This could be due to the fact that, during agitation, mechanical friction could have facilitated the extraction of carotenoids, while this effect would have been reduced by using higher volumes of solvent.

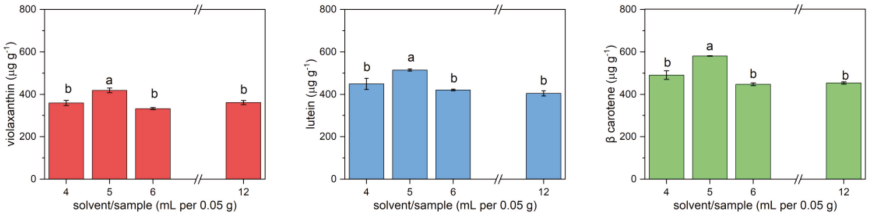

Figure 2. Carotenoids extracted from lettuce microgreens with different solvent/sample ratios. Error bars indicate standard deviation. Different letters mean a significant difference at $p < 0.05$.

3.1.3. Repeated Extractions

Since a single extraction step did not provide sufficient recovery, as pointed out by recovery data on the internal standard (data not shown), series of repeated extraction were evaluated. Repeated extraction is very common in carotenoid analysis [29]. This allowed to opt for shorter extraction times and to avoid possible analyte degradation during long-term analysis, observed by simulated extractions with a standard solution of β-carotene instead of microgreen sample (with a degradation higher than 18%; data not shown). For the repeated extractions testing, extracts of each step were injected separately to evaluate the contribution of each step to overall extraction.

Extraction steps of one hour were carried out, considering that the extraction time (Figure 1) could extract large amounts of carotenes and significant amounts of xanthophylls. Two extraction steps with 5 mL of cold acetone, followed by a step with 5 mL of acetone 70% to extract residue xanthophylls were tested (Figure 3). The last step was effective for polar carotenoids. A slight improvement of extracted amounts was observed after a slight hydration of the sample before analysis [29], while longer extraction times caused a decrease in extracted amounts.

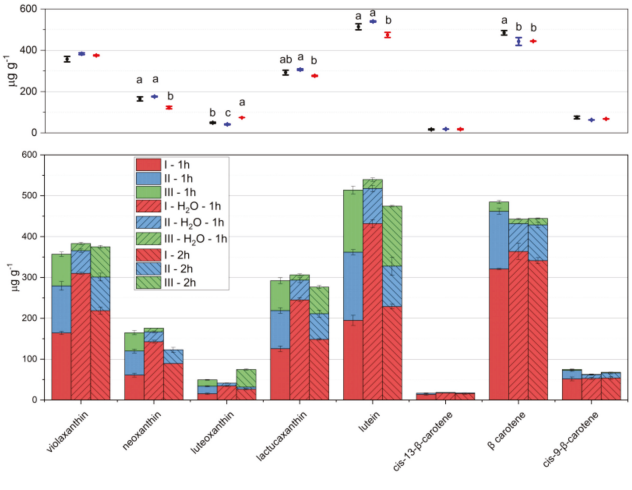

Figure 3. Carotenoids extracted from lettuce microgreens with three repeated extraction steps (I–III in stacked bars), with 1-hour extractions (left bars); initial sample rehydration and 1-hour extractions (central bars); 2-hour extractions (right bars). For steps I and II, 5 mL of pure acetone were used; for step III 5 mL of acetone 70% were used. Means and standard deviations of each step are reported in the stacked bar plot of lower panel; means and standard deviations of overall extraction are reported in the scatter plot of upper panel. Different letters mean a significant difference at $p < 0.05$.

Extraction times higher than 2 h per step were not considered in order to keep reasonable analysis duration. Therefore, the volumes of extracting solvents (10 mL of acetone and 5 mL of acetone 70%) were split into four aliquots (4, 3, and 3 mL of acetone and 5 mL of acetone 70%) and four 1-hour extraction steps were performed.

The results are reported in Figure 4, in comparison with those obtained with the same amount of solvents divided into three extraction steps. This change determined relevant increases in the extracted amounts of both carotenes and xanthophylls. Therefore, this procedure was adopted and considered for the evaluation of recovery and repeatability. Further extractions with 5 mL aliquots of solvents (either a fourth pure acetone step, before acetone 70% step, or a fifth step with acetone 70%) provided limited increases in extracted carotenoids, in the ranges 0–1.5% and 0–3.7%, respectively. Though a third extraction could provide a more efficient procedure, we believe that environmental issues should not be disregarded even in analytical chemistry; therefore, this increase in solvent volumes could be avoided without relevant analyte losses [35,36]. Figure 5 reports the flowchart of the analytical protocol.

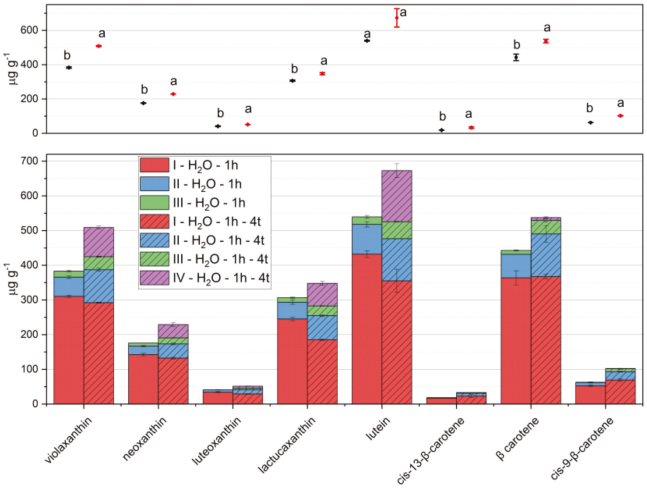

Figure 4. Carotenoids extracted from lettuce microgreens with three repeated 1-hour extraction steps (I–III in left stacked bars, steps I and II with 5 mL of pure acetone, step III with 5 mL of acetone 70%); with four repeated 1-hour extraction steps (I–IV in right stacked bars, step I with 4 mL of pure acetone, steps II and III with 3 mL of pure acetone, step III with 5 mL of acetone 70%). Means and standard deviations of each step are reported in the stacked bar plot of lower panel; means and standard deviations of overall extraction are reported in the scatter plot of upper panel. Different letters mean a significant difference at $p < 0.05$.

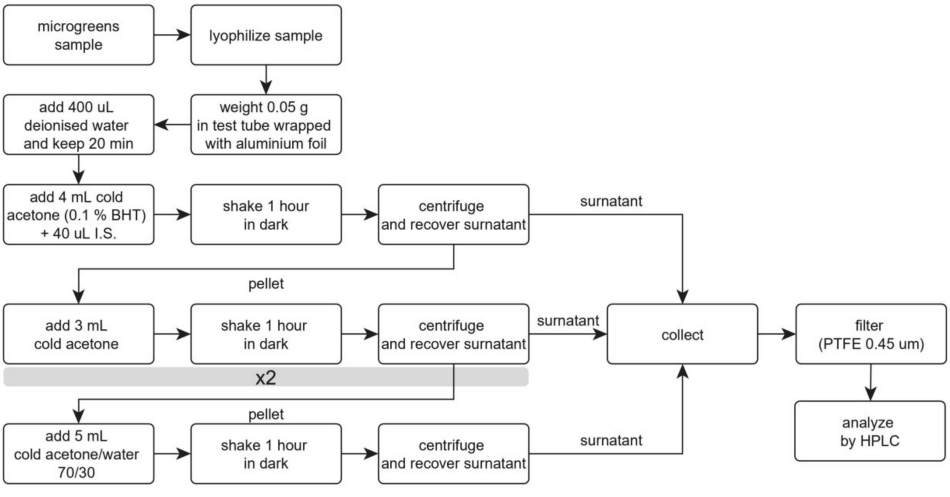

Figure 5. Flowchart of the extraction protocol for the analysis of carotenoids in microgreens.

A typical chromatographic separation is reported in Figure 6.

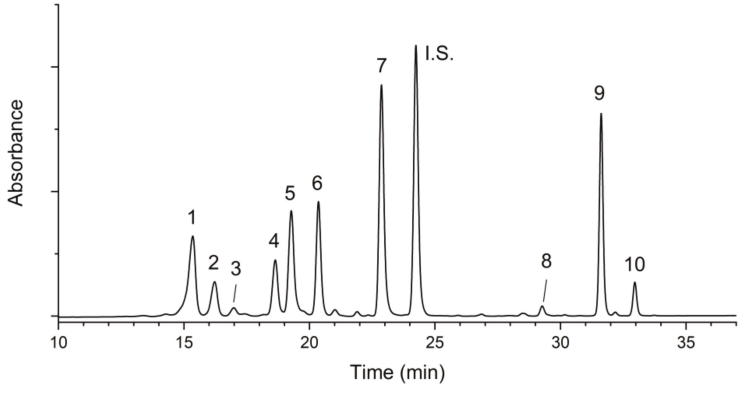

Figure 6. Typical chromatogram of carotenoids extracted from a microgreen sample of *Lactuca sativa* L. Group *crispa* (cultivar 'Bionda da taglio'). For chromatographic and detection conditions, please see Section 2.8 in the text. 1, violaxanthin; 2, neoxanthin; 3, lutheoxanhin; 4, lactucaxanthin; 5, chlorophyll b; 6, lutein; 7, chlorophyll a; I.S., internal standard; 8, cis-13-β- carotene; 9, β-carotene; 10, cis-9-β-carotene.

3.1.4. Method Validity

The recovery of the internal standard is reported in Figure 7. The adopted extraction procedure allowed to recover 97.2% of the internal standard. Further extraction steps allowed further recoveries of 1.1% (acetone 100%) and 0.5% (acetone 70%).

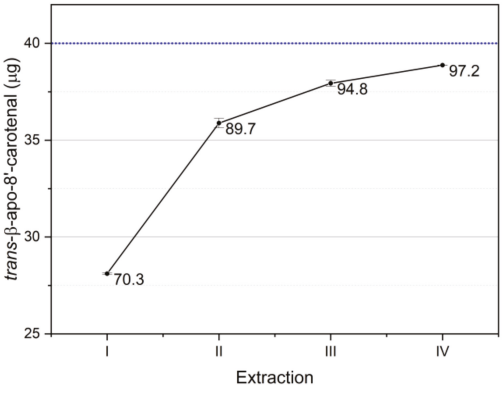

Figure 7. Recovery of the internal standard added to the sample before extractions. Blue reference line indicates the spiked amount. Data labels indicate the cumulative percent recovery after each extraction step of the tested extraction protocol. Error bars indicate standard deviation.

The figures of merit of method validation are reported in Table 1.

Table 1. Figure of merit of the optimized method (intra-day repeatability, n = 6; inter-day repeatability n = 3 × 3; limits are expressed as μg g^{-1} of sample on dry weight basis).

	Internal Standard	Violaxanthin	Lutein	β-Carotene
Recovery	97.2%			
Linearity (Adjusted R^2)	0.999		0.999	0.999
Limit of detection (LOD)			1.6 μg g^{-1}	11.3 μg g^{-1}
Limit of quantitation (LOQ)			5.2 μg g^{-1}	15.9 μg g^{-1}
Experimental limit of quantitation (ELOQ)			8.4 ± 0.5 μg g^{-1}	9.1 ± 1.1 μg g^{-1}
Intra-day repeatability (C.V.%)		4.4%	5.7%	6.9%
Inter-day repeatability (C.V.%)		4.1%	4.8%	5.3%

Limits of detection (LOD) and quantitation (LOQ), calculated on the basis of S/N ratio, were quite below 10 μg g^{-1} for lutein, while the indices slightly exceed this threshold for β-carotene. Experimental limit of quantitation determined as the lowest real measure obtained by submitting to extraction an exhausted sample matrix substantially complied. Violaxanthin, lutein, and β-carotene were considered to be the most abundant and showed decreasing polarity. The C.V.% of repeatability tests ranged 4.1–5.3% (5.7% mean value) for inter-day repeatability and 4.4–6.9% (4.7% mean value) for inter-day repeatability and was considered satisfactory.

As a last step, the method developed was compared with another method from literature applied to carotenoids [24], involving a saponification step followed by hexane extraction. Besides lettuce microgreens, characterized by very tender tissues, microgreens of linen (*Linum usitatissimum* L.), characterized by tough tissues, were also submitted to carotenoid extraction. The results are reported in Figure 8.

As can be observed, the method developed in the present study provided higher amounts of extracted carotenoids compared to the method which involves saponification and hexane extraction. The largest differences could be observed for the more polar xanthophylls, probably underestimated when extraction was carried out with hexane, compared to carotenes, for which the differences between the two methods were less marked. Moreover, differences between the methods were more pronounced for lettuce microgreens compared to linen microgreens. This is because saponification probably caused

a certain degradation of carotenoids when applied to more tender tissues. Therefore, the method developed appeared to be suitable to be applied to microgreens.

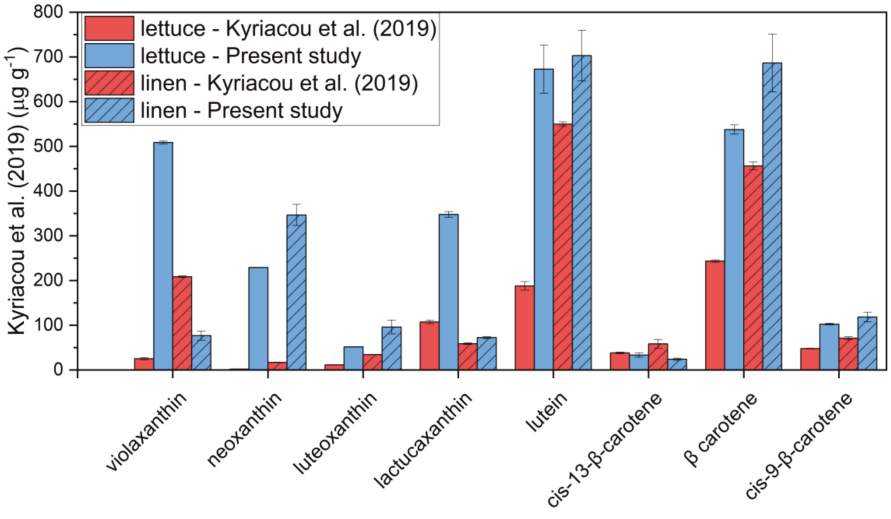

Figure 8. Carotenoids extracted from lettuce and linen microgreens with the method developed in the present study and with a method in literature [24]. Error bars indicate standard deviation.

In conclusion, an effective protocol for the extraction and analysis of carotenoids from microgreens was setup in the present research. The protocol was developed considering several variables (i.e., solvent polarity, extraction time, solvent/sample ratio, repeated extractions) and was optimized on this matrix according to its typical carotenoid pattern, characterized by a wide range of polarity, and possible degradation/isomerization phenomena that could occur. Good recovery, mean repeatability, and limits of detection and quantitation characterized this method, which proved to be more in the extraction of carotenoids from the delicate tissues of microgreens, even compared to another method from literature. The critical development of a reliable analytical method can allow for affordable nutritional data on such an emerging food, which is claiming increasing attention for its functional potential and for its suitability for tailored nutrition.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/4/459/s1, Table S1: Carotenoids extracted from lettuce microgreens with different water:acetone mixtures and varying extraction time. Results of Tukey's post-hoc test for multiple comparisons. Extracted amounts are expressed in mg g^{-1}.

Author Contributions: Conceptualization, V.M.P.; Data curation, M.C. and M.R.; Formal analysis, M.C.; Funding acquisition, P.S. and F.C.; Investigation, M.C.; Methodology, V.M.P., M.R., and F.C.; Project administration, V.M.P.; Supervision, V.M.P., P.S. and F.C.; Writing—Original draft, V.M.P. and M.C.; Writing—Review and editing, M.R., P.S., and F.C. All authors have read and agreed to the published version of the manuscript.

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foods

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Article

Accumulation of Agmatine, Spermidine, and Spermine in Sprouts and Microgreens of Alfalfa, Fenugreek, Lentil, and Daikon Radish

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Abstract: Sprouts and microgreens are a rich source of various bioactive compounds. Seeds of lentil, fenugreek, alfalfa, and daikon radish seeds were germinated and the contents of the polyamines agmatine (AGM), putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM) in ungerminated seeds, sprouts, and microgreens were determined. In general, sprouting led to the accumulation of the total polyamine content. The highest levels of AGM (5392 mg/kg) were found in alfalfa microgreens, PUT (1079 mg/kg) and CAD (3563 mg/kg) in fenugreek sprouts, SPD (579 mg/kg) in lentil microgreens, and SPM (922 mg/kg) in fenugreek microgreens. A large increase in CAD content was observed in all three legume sprouts. Conversely, the nutritionally beneficial polyamines AGM, SPD, and SPM were accumulated in microgreens, while their contents of CAD were significantly lower. In contrast, daikon radish sprouts exhibited a nutritionally better profile of polyamines than the microgreens. Freezing and thawing of legume sprouts resulted in significant degradation of CAD, PUT, and AGM by endogenous diamine oxidases. The enzymatic potential of fenugreek sprouts can be used to degrade exogenous PUT, CAD, and tyramine at pH values above 5.

Keywords: polyamines; biogenic amines; germination; medicago sativa; trigonella foenum-graecum; lens culinaris; raphanus sativus; diamine oxidase

1. Introduction

Sprouts and microgreens are popular and trendy foods [1]. The wide variety, available even in a relatively small surface area, offers the opportunity to practice urban gardening under space-limited conditions in houses and apartments. The popularity of sprouts and microgreens is related to their high aesthetic potential and intense taste. The sensory and nutritional properties depend on the content of secondary metabolites [2]. The results of various studies have shown that the content of many bioactive compounds increases significantly during sprouting and in the microgreens [3]. High contents of polyphenols, anthocyanins, and other redox-active compounds, i.e., vitamins, glucosinolates, and minerals, have been found [4–7]. Modulation of the light regime [8] and the composition of the growth solution for microgreens have shown a high potential for biofortification with minerals [9] and secondary metabolites [10].

Polyamines, such as agmatine (AGM), putrescine (PUT), cadaverine (CAD), spermine (SPM), and spermidine (SPD) are secondary metabolites with two or more amino groups that are closely related to plant growth and development, stabilization of cellular structures, and stress resistance [11]. Increased endogenous synthesis as well as the exogenous application of polyamines, improve seed germination and growth [12]. The results of several studies show that germination leads to a change in the content

and profile of polyamines. In the soybean, germination leads to an accumulation of all analyzed polyamines. Maximum values were determined after 48 h, followed by slightly lower values after 96 h of germination. These are still three-fold higher than in ungerminated seeds [13]. The content of all analyzed polyamines increased during germination in lupin sprouts whereas, in fenugreek, only PUT and CAD accumulated, while SPM and SPD remained constant [14]. There are some other reports of changes in polyamine content during germination of legume seeds, where a larger increase of PUT and CAD, compared to SPM and SPD, was observed when dry weight is assumed [15,16]. Accumulation of all polyamines was observed in germinated corn [17] and a large increase in agmatine content in radish [16] and flaxseed sprouts [18]. Reports related to the polyamine transformation in microgreens are rare. In lettuce, a gradual decrease in free SPM and SPD was observed from the microgreens stage (2 weeks) to commercial maturity (10 weeks) [19].

The polyamines, which accumulate in the germinating seeds, not only have intracellular functions but can also serve as a substrate for diamine oxidases. The enzymatic oxidation of predominantly PUT and CAD produces H_2O_2, which is involved in cell wall differentiation and programmed cell death and has direct antimicrobial activity when tissue integrity is broken [20,21]. Copper amine oxidases (CuAO) are diamine oxidases with copper ion in the active site and are expressed at high levels in legumes [22]. They are localized either in the apoplasts, in the intercellular spaces, or loosely bound to the cell walls [23,24]. Diamine oxidases are expressed in various tissues of germinated seeds of the *Leguminosae* family. In soybean sprouts, the enzyme is predominantly expressed in the hypocotyl and root system. The activity in bean sprouts has been found mainly in the cotyledons [25], and, in fava beans, in all parts except the cotyledons [26]. The higher enzyme activities were found to be correlated with higher contents of CAD or PUT [27] in the hypocotyl and root of chickpeas. Enzymes that catalyze the oxidative deamination of biogenic amines can be used as dietary supplements. Diamine oxidases of animal origin, incorporated in capsules, can be consumed in the intestinal tract for more efficient oxidation of undesirable dietary biogenic amines. Such treatment effectively reduces the severity of migraine episodes [28]. On the other hand, excessive oxidation of polyamines in the digestive tract is problematic, as the H_2O_2 generated is toxic to the intestinal cells. A dietary supplement with a combination of white pea diamine oxidase with catalase, which catalyzes the decomposition of H_2O_2 generated by diamine oxidase, resulting in reduced toxicity [29]. The direct oxidation of biogenic amines in the food matrix, prior to ingestion, could be a viable alternative to the use of amine oxidases as dietary supplements.

From the published results, it can be concluded that the content of polyamines generally increases during sprouting. From a nutritional point of view, there is no simple answer to whether this is beneficial or not. Large contents of PUT and CAD that accumulate as a result of endogenous synthesis in plants or by microbial decarboxylation of amino acids [30] are certainly not desirable. These foul-smelling compounds are slightly toxic to the intestinal cells [31] and, mainly interfere with the enzymatic oxidation of tyramine (TYR) and histamine (HIS) in the digestive tract [32], which increases their negative effects. Dietary intake of AGM, SPM, and SPD may be desirable. SPM and SPD, in particular, appear to have cardioprotective and neuroprotective effects [33]. AGM, which can cross the blood–brain barrier, can be consumed in large quantities without adverse health effects and can relieve the symptoms of central nervous system disorders, including major depression [34]. Endogenous synthesis of polyamines in mammals decreases with age [35], and dietary intake of polyamines, particularly SPD, is directly related to lower mortality, as has been found in a prospective population-based study [36]. However, dietary polyamines are a double-edged sword, as they can potentiate the growth of certain cancers, most probably due to the stabilizing of DNA [37]. Dietary intake of polyamines is therefore generally desirable, but should also be controlled because of the possible adverse effects. The content of SPM, SPD [38], and AGM [39] in the diet is becoming an important issue. Different seeds and meats are quantitatively the main sources of SPM and SPD, while certain types of fermented foods are rich in AGM (Table 1).

Table 1. Food sources and health effects of dietary polyamines.

	Agmatine (AGM)	Putrescine (PUT)	Cadaverine (CAD)	Spermidine (SPD)	Spermine (SPM)
Biosynthesis in humans	Putative From arginine (arginine decarboxylase)	Yes From ornithine/agmatine (ornithine decarboxylase/ agmatinase)	No	Yes From putrescine and S-adenosylmethioninamine (spermidine synthase)	Yes From spermidine and S-adenosylmethioninamine (spermine synthase)
Main dietary sources	Fermented food, various sprouts [16,39]	Fermented foods, Citrus fruits and vegetables, legumes [38,40,41]	Fermented foods, legume sprouts [16,42]	Legumes, brassica vegetables, mushrooms, cheese [38,40]	Liver, meat, legumes, brassica vegetables, cheese [38,40]
Oral intake **Positive health effects** **Animal/human studies**	• Large number of studies showing an antidepressant effect in rodents (0.0001–80 mg/kg) [34,42] • Antidepressant effect in humans (2–3 g/day) [43] • Pain relief in humans related to radiculopathy (0.75–2 g/day) [44]	• Improved intestinal immune function in piglets (10 mg/kg day) [45] • Longer survival of mice on high daily polyamine diet (15 mg PUT/kg BW) [46]	/	• Longer life span of humans with higher dietary intake of spermidine (5.7 years) [36] • Statistically significant increase of life span in mice (0.3 mM in water) [47] • Prevention of arterial aging in mice (3 mM in drinking water) [48] • Longer survival of mice on high daily polyamine diet (35 mg SPD/kg BW) [46] • Daily intake of 29 mg/kg promotes liver regeneration in rats [49]	• Statistically significant increase in life span in mice (3 mM in water) [47] • Antidiabetic effect on mice at 10 mg/kg daily intake [50] • Daily supplement of 80 mg/kg increases the antioxidant capacity rat spleen and liver under oxidative stress [51] • Longer survival of mice on high daily polyamine diet (12 mg SPM/kg BW) [46] • Daily intake of 10 mg/kg promotes liver regeneration in rats [49] • Daily supplement of 80 mg/kg in piglet diet alleviate inflammatory response and enhance the immune function [52]
Oral intake **Toxicity and negative health effects** **Animal/human studies**	• No adverse effect of 5 years daily agmatine intake of 1.5 g/day for humans [53] and 21 days of 2 g/day for humans [44]	• Non-observed adverse effect of daily putrescine or cadaverine intake (180 mg/kg for rats) [54] • Formation of nitrosamine [32] • Potentiation of histamine and tyramine toxicity [32]		• Non-observed adverse effect of daily spermidine intake (60 mg/kg for murines) [55], (83 mg/kg for rats) [54] • Promotion of growth of existing tumors in mice [56]	• Non-observed adverse effect of daily spermine intake (19 mg/kg for rats) [54]

The objectives of the present study are (I) to determine the polyamine content in seeds, sprouts and microgreens of three legumes and one cruciferous plant, (II) to evaluate whether microgreens are nutritionally superior to sprouts in terms of polyamine content, and (III) to evaluate the enzymatic potential of sprouts to degrade undesirable biogenic amines.

2. Materials and Methods

2.1. Materials

Acetonitrile (gradient HPLC grade) was obtained from Fischer Scientific (Hampton, NH, USA). Ultrapure water was obtained with a Milli-Q water system (Millipore Merck, Darmstadt, Germany). Acetone (≥99.8%), n-hexane (≥95%), HCl (37%) were obtained from Honeywell (Charlotte, NC, USA), NaOH (p.a.), NH_3 (25%), acetic acid (glacial), $NaH_2PO_4 \times 2H_2O$ (p.a.) and $NaHCO_3$ (p.a.) from Merck (Darmstadt, Germany). Dansyl chloride (≥99%) and amines were obtained from Sigma-Aldrich (St. Louis, MO, USA): 1,7-diaminoheptane (98%), agmatine sulfate (≥97%), phenethylamine (99%), histamine (≥97%), cadaverine (≥96.5%), putrescine (≥98.5%), spermidine (≥98%), spermine (≥97%), tyramine (≥98.5%) and tryptamine (TRP) (≥98%).

Fenugreek (*Trigonella foenum-graecum*), lentil (*Lens esculentum*), alfalfa (*Medicago sativa*), and daikon radish (*Raphanus sativus*) seeds designated for sprouting were supplied by Amarant (Kresnice, Slovenia).

2.2. Seed Sprouting

The seeds of each species were rinsed, then soaked in tap water (23 °C, pH 7.5, 450 μS/cm) at room temperature for 6 h. The soaked seeds were germinated in Schnitzer (Offenburg, Germany) sprouting trays (diameter 18.5 cm, depth 4 cm) with a built-in drainage system. Four trays, each containing different seeds, were organized in a vertical tower to retain moisture. Every 8 h, the tower was dismantled, and the germinated seeds were rinsed in separate trays with tap water (approx. 500 cm^3 per tray) to prevent microbial spoilage. After the water had been drained off, the tower was reassembled. The germinated seeds were incubated at 23 ± 1.5 °C for 4 days. The seeds of all four species were germinated three times (independent experiments).

2.3. Growing Microgreens

The seeds were soaked, as described in Section 2.2, and spread on a fully hydrated Urbanscape rockwool slab 12 × 12 × 2 cm (Knauf insulation, Škofja Loka, Slovenia) with half-strength Hoagland nutrient solution [57]. The hydrated slabs were placed separately in plastic trays and filed up to a height of 1 cm with half-strength Hoagland nutrient solution. The germinated seeds were incubated for 10 days at 23 ± 1.5 °C (relative humidity 60%) under 16 h/8 h (light/dark cycle) photoperiod and a photon flux density of 36 μmol $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps MASTER TL D 58W/840 (Philips, Amsterdam, The Netherlands). The germinating seeds and later the seedlings were moistened once a day by spraying with distilled water. The loss of solution in the trays was compensated by a daily addition of distilled water. Microgreens of all four species were grown four times (independent experiments).

2.4. Sample Preparation

2.4.1. Extraction Procedure

All sprouts and microgreens were homogenized fresh, unless otherwise indicated. Approximately 1.5 g (known mass) of sprouts or microgreens (upper two-thirds of the seedling height) were weighed into 50 mL polypropylene centrifuge tubes, filled with 15 mL of 0.4 M HCl containing 10 mg/mL of 1,7-diaminoheptane (IS) and immediately homogenized (30 s homogenization/30 s resting time-repeated 3 times) with the T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany) at 13,500 rpm. The ungerminated seeds were homogenized in the same way as sprouts and microgreens.

The homogenized samples were left at room temperature for 5 min and then centrifuged at 4000× *g* for 5 min. Aliquots of the partially cleared homogenates were transferred to 2 mL centrifuge tubes and further centrifuged at 15,000× *g* for another 5 min. The supernatant was transferred to new centrifuge tubes and used for derivatisation, which was performed within 2 h after homogenization. The polyamines extracted in 0.4 M HCl can also be stored at −20 °C for one week, as this storage had no influence on the determined polyamine content.

The moisture content of sprouts and microgreens was determined by oven drying the samples at 105 °C to constant weight (≈6 h).

2.4.2. Freezing and Thawing

Liquid nitrogen was poured over the sprouts to induce immediate freezing. Frozen sprouts were immediately transferred into polypropylene bags and stored at −20 °C. One week storage at −20 °C of frozen sprouts did not result in lower polyamine content if they were immediately transferred to 0.4 M HCl containing 10 mg/L IS and homogenized as explained in Section 2.4.1. To assess the influence of thawing on the polyamine content, frozen sprouts were evenly spread on a glass Petri dish and homogenized in 0.4 M HCl containing 10 mg/L of IS after 5, 20, 60, and 180 min of thawing at room temperature.

2.4.3. Fenugreek Sprouts as a Source of Amine Oxidases

Fresh fenugreek sprouts (5 g) were homogenized (30 s homogenization/30 s resting time-repeated twice) with the T-25 Ultra-Turrax (Ika-Labortechnik, Staufen, Germany) at 13,500 rpm in 25 mL of MQ water. 4 mL of fresh homogenates were transferred into 80 mL glass beakers containing a mixture of 100 mM buffer with suitable pH (6 mL) and 10 mL of a mixture of biogenic amines. Buffers (100 mM) with pH 4 and 5 were prepared previously from acetic acid with the addition of NaOH. Buffers (100 mM) with pH 6, 7, and 8 were prepared from sodium dihydrogen phosphate with the addition of NaOH. The mixture of polyamines (100 mg/L of CAD, HIS, PHE, PUT, TRP, and TYR) was previously adjusted to pH 7 by the addition of HCl solution. The concentration of individual polyamines in the reaction mixtures was 50 mg/L, 30 mM for buffer, and 33 g/L for fenugreek sprouts (assuming that the densities of all solutions/sprouts are approximately 1 g/mL). The reaction mixtures were incubated at 25 °C on a magnetic stirrer at a stirring speed of 250 min^{-1}.

At specified time intervals (2, 5, 12, 25, 60, and 120 min), 750 µL of the reaction mixtures were transferred into 2 mL centrifuge tubes containing 750 µL of IS (20 mg/L) in 0.8 M HCl, thoroughly mixed to stop the reaction, centrifuged and proceed as described in Section 2.4.1.

As the pKa values of all amino groups (except the imidazole group of HIS) are above 9, the neutral solution of polyamines had no significant influence on the pH value of the reaction mixtures. The pH value of the reaction mixtures was also checked at the end of the incubation period (120 min), and we found that it did not differ from the initial pH value by more than for ±0.1. Buffers at the concentration used in the reaction mixture did not seem to affect the derivatization yield of the biogenic amines.

2.5. Preparation of Standard Solutions and Derivatization

2.5.1. Internal Standard

1,7-diaminoheptane (IS) was used as an internal standard in amine standard solutions and at various levels of sample preparation to control all steps of sample manipulation from homogenization, derivatization, and injection into HPLC. Stock IS solution with a concentration of 1.0 g/L was prepared by weighing 10 mg of IS and dissolving it in 10 mL of 0.4 M HCl or 0.8 M HCl.

2.5.2. Amine Standards and Calibration Solutions

Standard solutions of individual amine (AGM sulfate, TRP, PEA, PUT, CAD, HIS, TYR, SPD, and SPM) were prepared with a concentration of 1.0 g/L. Then, 10 mg of solid amines (AGM sulphate,

TRP, HIS, TYR, and SPM) were dissolved in 10 mL of 0.4 M HCl solution containing 10 mg/L of IS. Afterwards, 10 µL of liquid amines (CAD, PUT, SPD, PEA) were pipetted and dissolved in different amounts of 0.4 M HCl with IS, according to their density (ρ(CAD) 0.873 g/mL, ρ(PUT) 0.877 g/mL, ρ(SPD) 0.925 g/mL, ρ(PEA) 0.962 g/mL). Mixed calibration standard solutions containing all 9 amine compounds were prepared in the concentration range of 0.3–45.0 mg/L, with a 0.4 M HCl solution containing 10 mg/L of IS.

2.5.3. Derivatization Procedure with Dansyl Chloride (DNS–Cl)

The solution of DNS–Cl with a concentration of 10 g/L was prepared in acetone. The derivatization was performed in a 1.5 mL centrifuge tube, as previously described [58]. Then, 250 µL of the calibration solution or sample was pipetted, and then 50 µL of 2 M NaOH, 75 µL of the saturated solution of $NaHCO_3$, and 500 µL of DNS–Cl solution were added, each addition followed by vortexing. The derivatization was carried out in a heating block at 40 °C for 60 min. After incubation, 25 µL of a 25% aqueous NH_3 solution was added to the solution and left at room temperature for 30 min. Afterwards, 350 µL of acetone was added, the solution was mixed again, and filtered through a 0.45-µm nylon filter before HPLC analysis.

2.6. HPLC Analyses

The HPLC determinations were performed with UV–vis and fluorescence detectors. All chromatograms were recorded using both detectors (Figure 1). Due to better sensitivity and selectivity of the dansylated amines obtained by a fluorescence detector, the signals for the latter were used for peak-area integration and further evaluation. The only exception was histamine, where spectrophotometric signals were employed because the fluorescence yield of its dansylated derivative was low. Across all samples, the ratio of peak areas in the chromatograms was constant by the respective detectors for the individual amine derivative, which corroborated the supposition that the integrated peak actually reflected the content of the amine analyzed. All peak areas were normalized to those of IS. The matrix showed only a small influence on the derivatization yield of IS. The median derivatization yield of IS in the complex matrix was 86% (upper quartile 91% and lower quartile 80%).

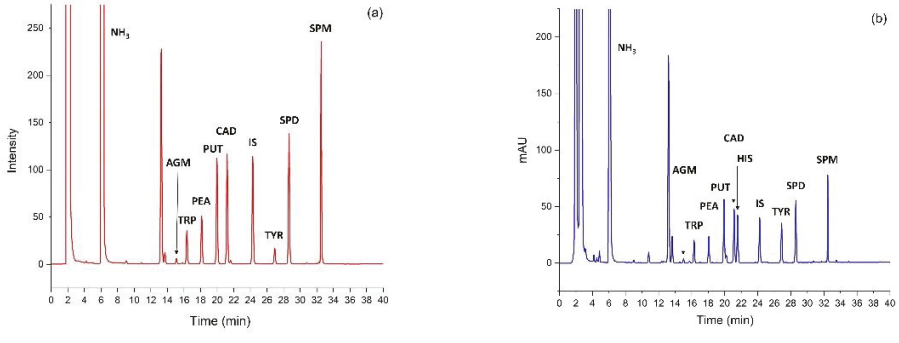

Figure 1. Chromatograms of the standard solution (11 mg/L) of the dansylated biogenic amines agmatine (AGM), tryptamine (TRP), phenethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), 1,7-diaminoheptane (IS), tyramine (TYR), spermidine (SPD), and spermine (SPM). (**a**) fluorescence detector (350/520 nm) and (**b**) UV–vis (254 nm).

Instrumentation: Agilent HPLC system 1100 (Palo Alto, CA, USA), equipped with a degasser, a quaternary pump, an autosampler, a UV–vis and a fluorescent detector was used. The wavelength of the UV–vis detector was 254 nm, the excitation wavelength of the fluorescence detector was 350 nm, and the emission wavelength 520 nm. A Kinetex XB-C18 (5 µm, 100 Å, 150 × 4.6 mm) column with a

guard column of the same particle size was used (Phenomenex, Torrence, CA, USA). The flow rate of the mobile phase was 0.7 mL/min. The separation was performed with a gradient of two eluents. Eluent A was MQ water and eluent B was acetonitrile. The initial composition of the mobile phase was 40% B, which changed linearly from 0 to 25 min to 80% B. At 25 to 30 min, a second linear gradient was used to change the mobile phase from 80% B to 100%, where it remained constant until 35 min. Then the composition changed linearly within 5 min to the initial 40% B. The column was then equilibrated for 2 min.

2.7. Statistical Analysis

A non-parametric Mann–Whitney test [59,60] based on the data ranking was used for the statistical analysis. The differences in the content of a particular polyamine in ungerminated seeds, sprouts and microgreens were significant at the $p < 0.05$ level.

3. Results and Discussion

3.1. Polyamine Content in Sprouts and Microgreens of Lentil, Fenugreek, Alfalfa, and Daikon Radish

Polyamine contents were determined in different stages of plant growth for four different species—lentil, fenugreek, alfalfa, and daikon radish. Sprouting and microgreen formation resulted in a large transformation of polyamines in all species analyzed. In general, a large accumulation of polyamines was observed. In each species, the content of at least one of the polyamines increased by two orders of magnitude compared to that in ungerminated seeds. The changes in polyamine content that occurred during growth from seed to sprout, and, finally, to microgreen, were specific to each species, so the results are presented separately. A comparison of the polyamine content in the different species and growth stages was carried out in terms of dry weight (DW) values.

3.1.1. Lentil

The contents of AGM (38 mg/kg), PUT (50 mg/kg), CAD (2 mg/kg), SPD (101 mg/kg), and SPM (35 mg/kg) and their relative proportions in lentil seeds were in similar ranges to those previously observed [16]. Sprouting only led to an increase in the contents of PUT (238 mg/kg) and CAD (742 mg/kg), as in previous germination experiments with lentil [16]. A different scenario was observed in soybeans [13], in which the content of all polyamines in sprouts increased. Since both PUT and CAD are nutritionally unfavorable, the nutritional value of the sprout in terms of polyamine composition was lower than that of ungerminated seeds. As the polyamine content was reported on a DW basis, it was still several times lower than that found in certain fermented foods of plant and animal origin [41] and was therefore highly unlikely to pose a health risk. The separate analysis of the polyamines in the epicotyl (part of the seedling above the cotyledons) and in the hypocotyl (part of the seedling below the cotyledons) of sprouts showed that the polyamine content in the epicotyl was an order of magnitude lower. In addition, a large difference in the spatial distribution of CAD in chickpea seedlings was found previously [27].

It was found that the content of CAD (47 mg/kg) in microgreens was one order of magnitude lower than in sprouts (Figure 2a), which is consistent with the spatial distribution in sprouts. CAD was the only polyamine with a lower content in lentil microgreens than in sprouts, while values four times higher were found for SPD (579 mg/kg) and three times higher for SPM (88 mg/kg). Levels above 500 mg/kg SPD are extremely high compared to that in other foods. A similar concentration range was only found for some mature cheddar cheeses [61], mushrooms [62], and germinated flaxseeds [18]. The estimated average daily dietary intake of SPD for the USA population [63] is achieved by the consumption of 100 g lentil microgreens (on a fresh weight basis (FW)). The higher contents of SPD and SPM and lower content of CAD indicate that lentil microgreens are nutritionally superior to sprouts in terms of polyamine composition.

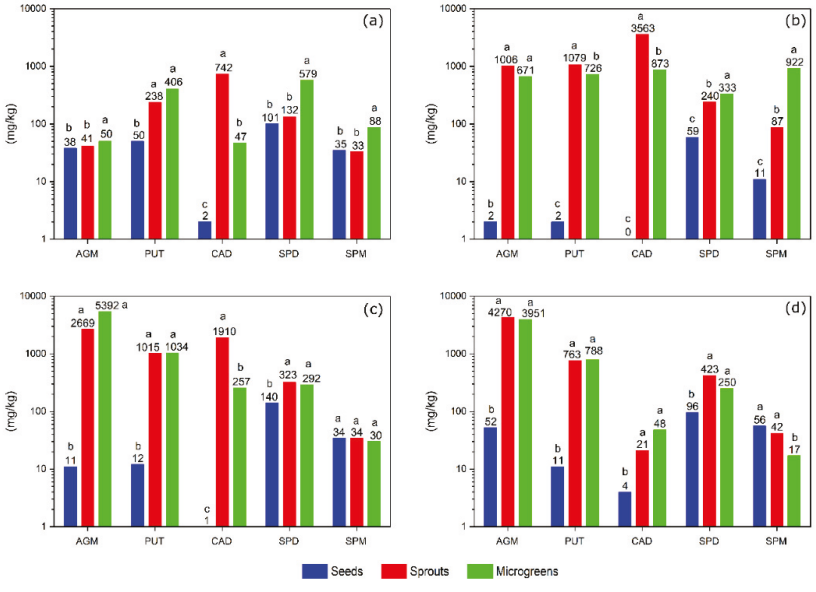

Figure 2. Content of the polyamines agmatine (AGM), putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM) in seeds, sprouts and microgreens of (**a**) lentil, (**b**) fenugreek, (**c**) alfalfa, and (**d**) daikon radish. The data are presented on a logarithmic scale and expressed on a dry weight basis. When the content of a given polyamine in seeds, sprouts, and microgreens differs significantly, it is labeled with different letters.

3.1.2. Fenugreek

Fenugreek is a traditional medicinal plant found in many cultures around the world [64]. Its culinary uses include the use of seeds as a spice and fresh leaves or sprouts and microgreens, which have a mild but slightly bitter taste. The absolute contents of AGM (2 mg/kg), PUT (2 mg/kg), SPD (59 mg/kg), and SPM (11 mg/kg) were lower in ungreminated seeds (Figure 2b, Figure A3) than in lentil seeds (Figure 2a). They were in a similar range as in the literature [14], except for SPD, in which a higher amount was determined in current work. Sprouting led to a large increase in practically all polyamines. CAD was below the limit of detection in ungerminated seeds and accumulated to more than 3000 mg/kg DW during sprouting. Such high levels are unusual in food samples and are twice as high as the maximal levels found in some acid-cured cheeses [65] or fresh scallops [62]. Higher DW based values were only found in some fermented soybean sauces [66]. AGM (1006 mg/kg) and PUT (1079 mg/kg) levels were above 1000 mg/kg DW but PUT levels were still in the range of foods such as sauerkraut [67], aged cheeses and even freshly squeezed citrus juices, which are among the richest sources of polyamines in unfermented foods [40]. Little information has been published on the AGM content of foods, but levels above 1000 mg/kg DW are undoubtedly high, as higher levels are found only in some fermented soybean products [39]. Despite initially lower contents in seeds, SPM (240 mg/kg) and SPD (87 mg/kg) in fenugreek sprouts have accumulated to twice the levels found in lentil sprouts. The accumulation of SPM and SPD during germination has not been observed before [68], and the increases of PUT and CAD contents were much less pronounced. The reason for the observed differences could be either their biological origin or the growth regime or methodology. In the previous study, the samples were processed by freeze-drying, which may have influenced the profile of the polyamine content, as shown in Section 3.2. Due to the extremely high content of CAD

(3563 mg/kg), fenugreek sprouts cannot be considered as a healthy food, despite the high amounts of the nutritionally beneficial polyamines AGM, SPD, and SPM.

The composition of the polyamines in fenugreek microgreens has been improved in terms of nutritional value, as well as in lentils. The four-fold lower content of CAD (873 mg/kg) and an order of magnitude higher content of SPM (922 mg/kg) in microgreens were the most pronounced changes compared to sprouts. None of the foods listed in the published databases [38,40,62] showed similarly high levels of SPM on a DW basis as we found in fenugreek microgreens. The estimated average daily dietary intake of SPM for the USA population [63] is exceeded by the consumption of 90 g of fenugreek microgreens (based on FW).

3.1.3. Alfalfa

Alfalfa, fenugreek, and lentil all belong to the legume family. The contents of AGM (11 mg/kg), PUT (12 mg/kg), CAD (1 mg/kg), SPD (140 mg/kg), and SPM (34 mg/kg) in ungerminated alfalfa seeds were lower than those for lentil, but higher than for fenugreek. Similar to lentil sprouting, alfalfa sprouting did not result in a higher content of SPM (34 mg/kg), while the content of SPD (323 mg/kg) doubled compared to ungerminated seeds. Sprouting resulted in two orders of magnitude greater contents (Figure 2c) of AGM (2669 mg/kg) and PUT (1015 mg/kg), and the accumulation of CAD (1910 mg/kg) resulted in higher levels than that for lentil sprouts and lower levels than for fenugreek. Polyamines PUT and CAD present in sprout samples could be formed as a result of microbial spoilage [16] or endogenous synthesis by plants. Insufficient hygienic standards could impair microbial spoilage. However, even with 11 log colony-forming units/g and five orders of magnitude higher microbial load after the end of the 12-day storage period of four sprout varieties, only a minor increase in CAD and PUT content was observed [69]. In order to test whether some polyamines were outside the plant cells, due to microbial spoilage, the sprouts were rinsed with extraction buffer (30 s), and only about 10% polyamines were found in such extracts. The ratios of the polyamine contents reflected those in sprouts, strongly suggesting that polyamines in the "rinsing solution" originated from ruptured cells (0.4 M HCl).

The contents of PUT (1034 mg/kg), SPD (292 mg/kg), and SPM (30 mg/kg) in microgreens remained in similar ranges to those in sprouts. The content of CAD (257 mg/kg) was lower by an order of magnitude, as was also observed in lentil and fenugreek microgreens. Alfalfa sprouts, and especially, microgreens (Figure 2c), stood out as extremely rich sources of AGM (5392 mg/kg). Such high contents were found only in some samples of fermented soybean pastes [70]. As noted above, there are relatively few data on the content of AGM in foods [39]. This is rather surprising, considering the many health benefits of AGM supplements and the fact that long-term intake of relatively high doses of AGM should be safe [53]. Dietary AGM can be absorbed in the small intestine and pass the blood–brain barrier [71], where it can interfere with some important central nervous system disorders. The majority of the experiments were conducted in a rodent model with ingested daily doses of 10 mg/kg or higher [34]. Human trials are rare; daily doses of about 10 mg/kg [44] have been shown to be effective in relieving pain in lumbar disc-associated radiculopathy. An intake of more than 1 kg of alfalfa microgreens (FW) would be necessary to reach an equivalent level, which is unrealistic. However, some rodent experiments [42] have shown that oral administration of 0.1 mg/kg of AGM alone produces the antidepressant effects. If similar results are confirmed in human studies, this would put a different perspective on alfalfa sprouts as a source of dietary AGM.

3.1.4. Daikon Radish

The absolute content of AGM (52 mg/kg), PUT (11 mg/kg), CAD (4 mg/kg), SPD (96 mg/kg), and SPM (56 mg/kg) in ungerminated seeds of daikon radish was similar to that in lentil seeds. The germination of this cruciferous vegetable (Figure 2d) resulted in some differences from the analyzed legumes. There was only a slight increase in the content of CAD (21 mg/kg), and the values in sprouts were one or two orders of magnitude lower than in legume sprouts. On the other hand,

the contents of AGM (4270 mg/kg) and SPD (423 mg/kg) in daikon sprouts were the highest of all four plants analyzed. In combination with the relatively low content of CAD, this indicates that daikon radish sprouts are an excellent source of nutritionally beneficial polyamines. Previous reports on the change in polyamine content during sprouting [16,72], were consistent with the current results and showed a large accumulation of AGM. In the same studies, it was found that SPD content in sprouts decreased on an FW basis, but, assuming 10% DW, the recalculated values were consistent with the observations of our study. An increase in SPD content was also observed in [73], but the reported levels are an order of magnitude lower in both ungerminated seeds and sprouts.

In contrast to legumes, in which microgreens were nutritionally better than sprouts, due to a lower content of CAD and a higher content of AGM, SPD or SPM, daikon radish microgreens were nutritionally inferior to sprouts. Contents of the nutritionally beneficial polyamines AGM (3951 mg/kg), SPD (250 mg/kg), and SPM (17 mg/kg) were lower in microgreens than in sprouts. The content of SPM in microgreens was even lower than in ungerminated seeds.

3.2. The Effect of Thawing on The Determined Polyamine Contents in Frozen Sprouts

The general protocol for the extraction of biogenic amines, described in materials and methods (Section 2.4.1), explicitly requires immediate extraction of freshly harvested microgreens and sprouts. The reason for this rigor is that the freeze/thaw cycle initiates extensive transformations of polyamines. Consequently, the polyamine contents determined depend strongly on the time delay of sampling after the start of thawing (Figure 3, Figure A1). Freezing (in liquid nitrogen) and storage for a few hours at −20 °C was in itself not problematic, but thawing at room temperature was. The contents of the majority of the polyamines analyzed already decreased on a time scale of minutes. In the case of fenugreek (Figure 3), approximately 30% lower contents of PUT and CAD were found in frozen sprouts (−20 °C) that were left at room temperature for only 5 min before homogenization in 0.4 M HCl. During longer thawing times, considerable amounts of SPD and AGM were also degraded. Similar results were observed for lentil and alfalfa sprouts, in which CAD and PUT were most susceptible to degradation (Figure A1a,b). Daikon radish sprouts were much less affected by freeze/thawing (Figure A1c).

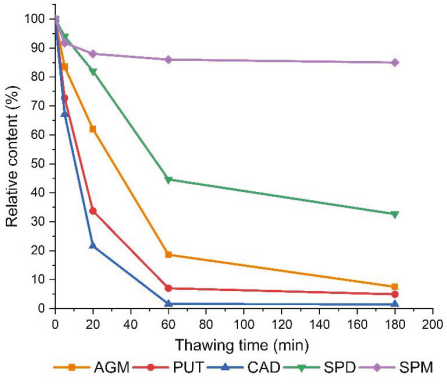

Figure 3. Time-dependent changes in the content of polyamines (agmatine (AGM), putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM)) during thawing of the frozen fenugreek sprouts.

Experimental artifacts, resulting from sample preparation, are not unusual and are often related to the enzymatic conversion of metabolites. A typical example is the oxidation of ascorbic acid to dehydroascorbic acid, catalyzed by ascorbate oxidase, which can lead to a distorted ratio of both forms of vitamin C when samples are homogenized under conditions where the enzyme remains active [74].

As shown in Figure 3, the polyamines are also highly susceptible to degradation, which can lead to experimental artifacts if the samples are not handled properly. Indeed, there is little overall consistency in the sample preparation protocols for the analysis of polyamines in sprouts. Fresh sprouts could be ground or homogenized in a grinder before extraction in acid [13,15,16], stored in a frozen state before extraction [75], lyophilized before extraction [68], or directly homogenized in acid [14]. Any protocol that disrupts tissue integrity prior to extraction could result in a significant transformation of polyamines if the criteria for enzyme activity are met.

It has been observed that freezing and thawing of soybean sprouts [76] resulted in significant accumulation of γ-aminobutyric acid (GABA), which is formed in two enzymatic steps from PUT, including the action of amine oxidases as the first step. Recently it was shown that GABA accumulation in thawed sprouts is most probably the result of ruptured cellular structures rather than the higher enzyme activity itself [77]. In neither of these two studies, where such enzymatic activity could be observed with certainty, was the change in polyamine profiles assessed. Since the transformation of polyamines in legume sprouts was much more pronounced than in radish, the most likely candidates are the diamine oxidases with copper ion in the active site, termed copper amine oxidases (CuAOs). CuAOs are expressed in high levels in legumes [22] and are localized in apoplasts, intercellular spaces, or loosely bound to the cell walls [23,24]. The freezing and thawing of plant tissue is detrimental to its structural integrity and leads to rapid changes in the content of some secondary metabolites, often resulting in lower sensory quality and nutritional value [78]. However, this enzymatic potential can also be used to increase the content of desired bioactive constituents [77].

3.3. Degradation of Exogeneous Biogenic Amines by Homogenized Sprouts

Copper amine oxidases (CuAO) from the legume family have, in general, optimal activities at neutral or slightly alkaline pH in general and relatively broad substrate specificity, as they catalyze the oxidation of diamines and polyamines, with PUT and CAD as optimal substrates [79–82]. A similar substrate specificity could be derived from the degradation pattern of polyamines observed in thawed fenugreek (Figure 3), lentil, and alfalfa seeds (Figure A1a,b). According to some older studies [83], the substrate specificity of CuAO could be even broader, including monoamines. The monoamines TYR, PEA, and TRP are often present in fermented foods together with PUT, CAD, and HIS. The content of PEA and TRP in foods is generally low, while HIS and TYR may be present in relatively high concentrations and may have adverse health effects associated with a local immune response (HIS) or increased blood pressure and migraine (TYR). PUT and CAD are less toxic to intestinal cells than TYR and HIS, and only some foods with the highest content [31] could potentially have an adverse effect. Of the sprouts included in the study, even those with the highest CAD/PUT content do not pose a direct risk if consumed in the hydrated form. PUT and CAD are nutritionally problematic, mainly due to their interference with the activity of intestinal amine oxidases with broad substrate specificity, since, at high concentrations of these diamines, less HIS and TYR is oxidized [32]. Even low dietary doses of biogenic amines are problematic for persons taking monoamine oxidase inhibitors as part of their antidepressant therapy [84].

Optionally, biogenic amines can be degraded in the food matrix before consumption. Accordingly, it was investigated as to whether homogenized sprouts could be used as a source of the enzyme for the oxidation of biogenic amines in complex mixtures. Homogenized fenugreek sprouts (less than 0.5% DW in suspension) efficiently oxidized PUT, CAD, and even TYR, when all analyzed biogenic amines were present in the mixture at concentrations of 50 mg/L and in the pH range of 6 to 8. TYR was even more efficiently oxidized than PUT and CAD (Figure 4). After 5 min of incubation at pH 6, 53% of TYR, 32% of PUT, and 22% of CAD were oxidized (Figure 4a–c). At the end of the incubation period (2 h), only traces of these 3 polyamines were observed. At pH 5, the activity was much lower so that after 2 h, the values observed were closely similar to those at pH 6 after 5 min. At pH 4, the enzyme was not active against any of the substrates.

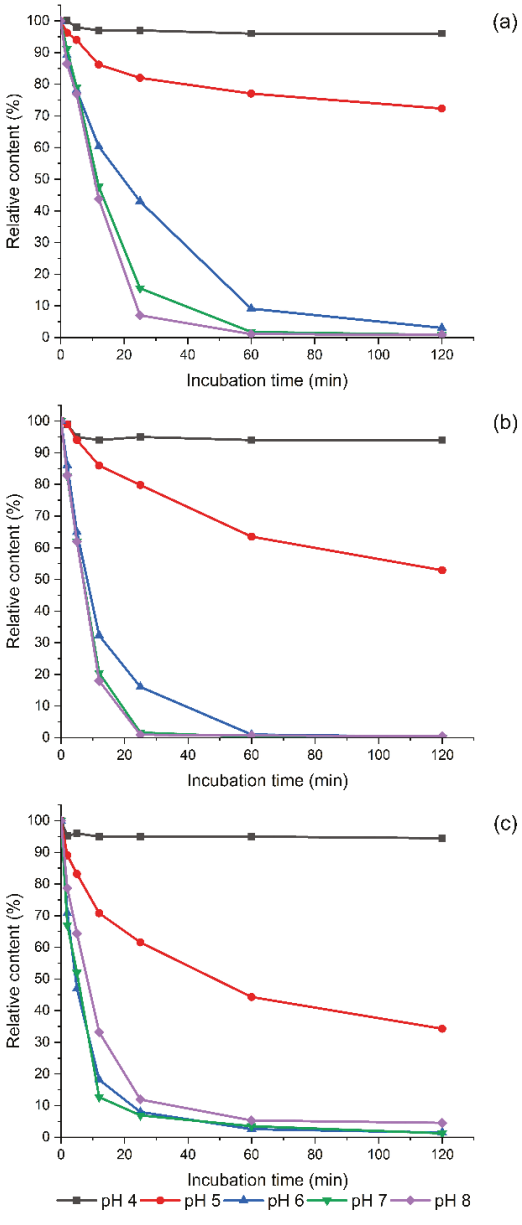

Figure 4. Time-dependent degradation of (**a**) putrescine, (**b**) cadaverine, and (**c**) tyramine by homogenized fenugreek sprouts at different pH values.

Three other substrates were much less susceptible to oxidation (Figure A2). Under optimal conditions, only 40% of TRP was oxidized after 2 h of incubation, while homogenized sprouts had practically no effect on the stability of HIS and PEA. The substrate specificity of the amine oxidases in fenugreek appears to differ from that of the white pea, for which HIS is a better substrate than TYR [29].

Fenugreek sprouts could, potentially, be used to reduce PUT, CAD, and TYR loads of fermented high protein food products that stabilize the pH of the matrix above 5. Some examples are sausages and cheeses or, when more acidic fermented vegetables and pulses are added to the matrix, with pH above 5. On the other hand, the content of biogenic amines PUT and CAD in legume sprouts could be reduced simply by freezing and thawing for several dozen minutes. Under these conditions, considerable amounts of PUT and CAD are degraded, while the content of nutritionally beneficial SPD is only slightly reduced (Figure 3).

4. Conclusions

Germination of lentil, fenugreek, alfalfa, and daikon radish led to the accumulation of their total polyamines. A large increase in CAD content was observed in all three legume sprouts. In the microgreens of these legumes, the CAD content was substantially reduced, whereas here, compared to the sprouts, more AGM, SPD, or SPM accumulate. These are considered to be nutritionally beneficial. In daikon radish sprouts, AGM is the major polyamine, while the formation of microgreens led to a reduction in the content of the nutritionally beneficial polyamines. This behavior was reversed with respect to the changes in the content in legumes.

Tissue damage of legume sprouts led to considerable degradation of polyamines, especially PUT, CAD, and AGM. Experimental artifacts or modulation of the polyamine composition of sprouts are among the possible implications. Homogenized fenugreek sprouts can be used for the degradation of exogenous PUT, CAD, and TYR at pH-values above 5.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

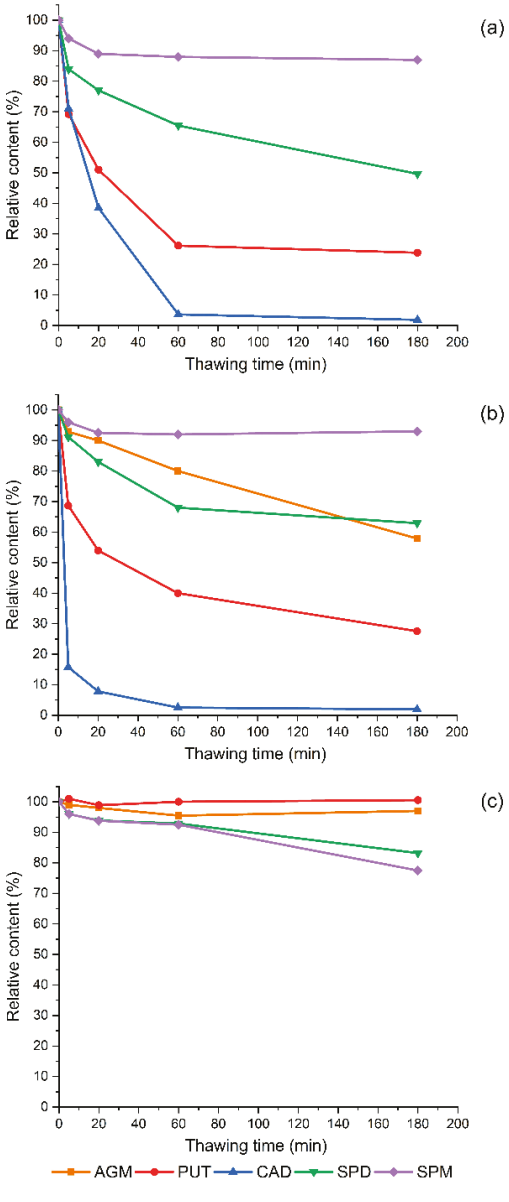

Figure A1. Time-dependent change in the content of polyamines (agmatine (AGM), putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM)) during thawing of the frozen sprouts of (**a**) lentil, (**b**) alfalfa, and (**c**) daikon radish.

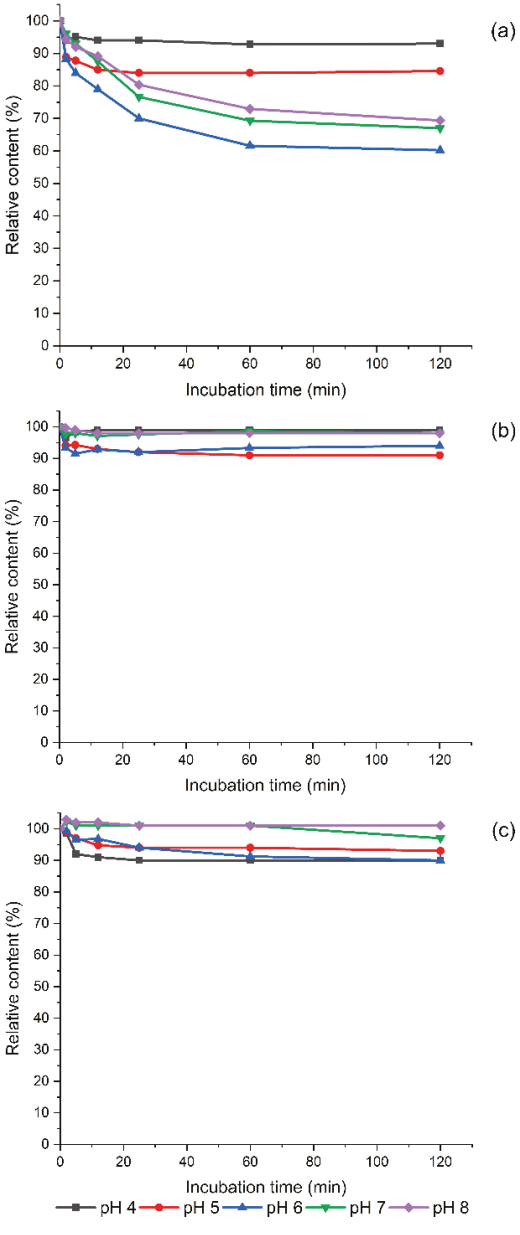

Figure A2. Time-dependent degradation of (**a**) tryptamine (TRP), (**b**) phenethylamine (PHE), and (**c**) histamine (HIS) by homogenized fenugreek sprouts at different pH values.

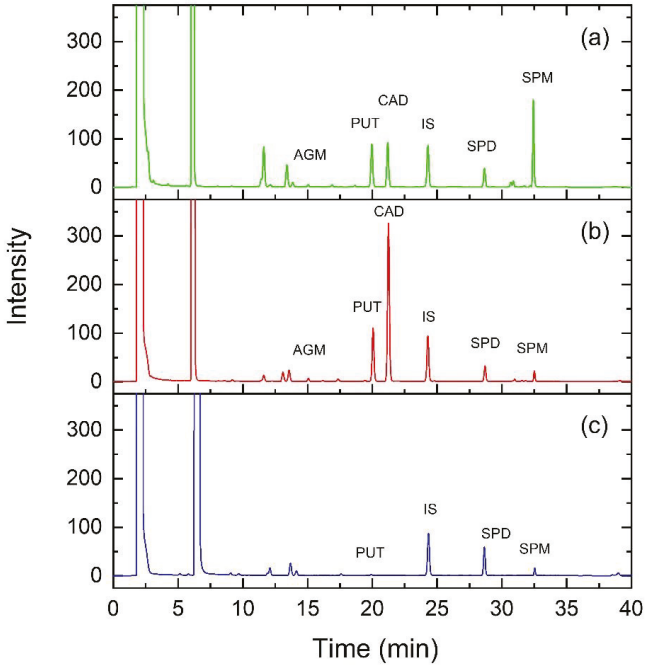

Figure A3. Chromatograms of dansylated 1,7-diaminoheptane (IS) and the polyamines agmatine (AGM), putrescine (PUT), cadaverine (CAD), spermidine (SPD), and spermine (SPM) extracted from (**a**) microgreens, (**b**) sprouts, and (**c**) ungerminated seeds of fenugreek—fluorescence detector (350/520 nm).

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Effect of Packaging and Coating Technique on Postharvest Quality and Shelf Life of *Raphanus sativus* L. and *Hibiscus sabdariffa* L. Microgreens

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Abstract: Microgreens are highly respiring produce characterized by a relatively short shelf-life. In this study, the efficacy of two types of macro-perforated packaging, PET clamshell (PET–CS) and LDPE self-seal bag (LDPE–SSB), was assessed on the postharvest quality and shelf life of radish (RaS) and roselle (HbS) microgreens stored at 5 °C. Pre-harvest spray treatment (AGSC) was compared with postharvest dip coating (AGDC) using *Aloe vera* gel (AG) for the first time in microgreens for postharvest quality improvement. PET–CS had a lower physiological loss in weight (PLW), respiration rate (RR), electrolyte leakage (EL), microbial counts (MCs), and higher overall acceptability (OA) than LDPE–SSB. AG-coated microgreens had significantly ($p \leq 0.05$) lesser deteriorative postharvest changes and higher ascorbic acid content than uncoated control. AGSC maintained better OA and postharvest quality than AGDC, especially at the end of the study period in terms of reducing EL, retaining greenness ($-a^*$), and chroma value in HbS microgreens. In RaS microgreens, AGSC helped to maintain lower PLW, MC, and higher ascorbic acid levels. AGSC could be suggested as an eco-friendly ergonomic pre-harvest treatment along with PET–CS for enhancement of postharvest quality and shelf life in RaS and HbS microgreens, with a tremendous potential to be extended to other microgreens.

Keywords: LDPE bag; PET clamshell; *Aloe vera*; edible coating; pre-harvest spray; dip-coating; postharvest quality; microgreens

1. Introduction

Microgreens are high-value crops considered the latest innovation in the vegetable sector [1]. Their supply and demand are highly influenced by emerging gastronomic trends and consumer familiarisation with the sensory attributes [2]. However, industrial production and marketing are limited since this highly respiring produce has a very short shelf life at ambient temperature [3,4]. Microgreens are known to respire during the germination process, metabolising stored carbohydrates in the cotyledonary leaves [5]. Once the carbohydrate sources are depleted, degradation of the microgreens occurs. Thus, modification of the plant metabolic activity and extending their shelf life by even a few days could be advantageous.

The main objectives of any postharvest technology are quality optimisation and loss reduction in fresh produce. Modification of the package atmosphere is one of the important methods in extending the shelf life. Passive modified atmosphere packaging (MAP) with relatively high oxygen transmission rate (OTR) films or perforated packaging is suggested to favour postharvest performance in microgreens [2]. High OTR polyethylene bags were preferred for the storage of radish microgreens over laser microperforated polypropylene packaging [6]. In another study, better postharvest quality

of "Tah Tasai" Chinese cabbage microgreens was maintained in polyethylene bags compared to polypropylene packaging [3]. Sunflower microgreens have been reported to have a better shelf life and nutritional quality when packed in polystyrene trays compared to LDPE bags [7]. However, at the commercial front, microgreens are mostly packaged in plastic clamshell containers. To the best of our knowledge, there are no scientific reports comparing the efficacy of such commercial packaging with polyethylene packaging on the postharvest quality and shelf life of microgreens. Therefore, such comparison warrants further studies.

Macro-perforated packaging, commonly preferred in commercial set-ups, is known to provide additional gaseous diffusion and is beneficial in reducing off-flavour of fresh produce [8,9]. In addition, our earlier observations have shown that it minimises surface condensation on the packaging used for highly respiring produce, such as microgreens. However, it is often accompanied by higher weight loss, and the content is potentially exposed to outside contaminants. These limitations could be addressed using natural polymeric coating materials as primary packaging on the surface of fresh produce.

Edible coatings represent new packaging strategies in the postharvest management of fresh produce. They are reported to create a micro-modified atmosphere around the produce by acting as a gas and water vapour barrier [10]. This helps in retarding food deterioration and enhancing its quality. Edible coatings are made up of natural polymers such as carbohydrates, proteins, and lipids. Edible coating applications have previously been reported to improve postharvest quality of fresh-cut produce such as celery sticks [11], and minimally processed lettuce [12,13]. However, to date, there are no published reports on the use of edible coating on microgreens.

In the last decade, there has been increased interest in using *Aloe vera* gel, as an edible coating on fruits and vegetables, due to its film-forming, antimicrobial, biodegradable and biochemical properties [14]. Benítez et al. [15] reported *Aloe vera* gel to be a better coating compared to chitosan and alginate coatings to extend the postharvest quality and shelf life of kiwi slices. *Aloe vera* gel (50%) was reported to reduce enzymatic browning in fresh-cut lotus roots and conserve the overall quality when stored at 5 °C [16].

The successful application of the edible coating on foods is dependent on several factors, including the method of application and its cost [17]. Dip coating technique is one of the age-old methods used commercially to coat fresh, whole, and minimally processed fruits and vegetables [18]. In earlier studies conducted in our laboratory, *Aloe vera* gel dip-coating gave promising results by reducing weight loss, minimising changes in the physicochemical parameters, reducing decay and extending the shelf life of papaya [19], figs [20] and litchi fruits [21]. A similar but less pronounced effect was observed in fenugreek and sunflower microgreens (unpublished data) using Aloe gel dip-coating. Dip-coating was found to be a little harsh on delicate and tender microgreens. There are also other drawbacks of dip-coating, such as the requirement of a large quantity of dip solution and quality deterioration of dip solution. Powder-coating was successfully used in our lab instead of dip coating for carrot shreds [22] and radish shreds [23]. However, this is not suitable for microgreens.

The spray-coating technique, which has recently attracted considerable industrial interest [24], was hence considered as an alternative technique. Chitosan postharvest dip-coating alone or combined with preharvest chitosan spray has been reported to enhance fruit quality and lower decay incidence in table grapes [25]. Recently, pre-harvest $CaCl_2$ spray has been used to delay senescence in broccoli microgreens [26,27]. In another study, preharvest calcium spray displayed better overall quality and longer shelf life in broccoli microgreens than postharvest dip treatment [28]. However, to the best of our knowledge, there are no published scientific studies evaluating the efficacy of preharvest spray treatment using a bio-based coating such as *Aloe vera* gel on fresh-cut leafy produce or microgreens.

In the present study, radish and roselle microgreens belonging to the Brassicaceae and Malvaceae families, respectively, with different leaf morphologies, were selected. Radish microgreens are characterised by succulent cotyledonary leaves, while roselle have broad, thin, and flat leaves. Based on our nutritional evaluation studies among ten microgreens, these two microgreens were also found to be among the nutrient-rich ones [29], hence the need to optimise their postharvest quality.

In the first phase of this study, the postharvest quality of these microgreens was assessed in two macro-perforated packaging, PET clamshell containers, and LDPE self-seal bags, commonly used for packaging fresh-cut produce, salad mixes and microgreens at the commercial and household levels. In the second phase of the study, spray- and dip-coating techniques were compared to study the effect of *Aloe vera* gel as an eco-friendly treatment on the postharvest quality and shelf life of radish and roselle microgreens.

2. Materials and Methods

2.1. Plant Material Cultivation

Good quality seeds (germination rate > 90%) of radish (RaS; *Raphanus sativus* L.) and roselle (HbS; *Hibiscus sabdariffa* L.) were purchased from government-approved outlets of seed corporations (Anantapur, India). Seeds were broadcast in plastic trays (L: 24 × W: 17 × D: 4 cm) containing cocopeat in triplicates. The seeded trays were germinated in darkness at a relative humidity of 95% ± 5%. After two to three days of germination, they were exposed to sunlight (photoperiod 11.5 h; light intensity 2500–4400 lux) with an average air temperature of 25 ± 5 °C and relative humidity of 65% ± 10%. Seven-day old RaS and HbS microgreens were harvested by cutting the stem ends with sharp and sterile scissors. Microgreens were inspected prior to storage, and plants with defects or discoloured leaves were discarded.

2.2. Experimental Design

The study was conducted in two phases—Phase I determined the effect of packaging, and Phase II determined the effect of edible coating techniques on the postharvest quality and shelf life of RaS and HbS microgreens in the packaging which maintained better postharvest quality. The summary of the experimental design is represented in Figure 1.

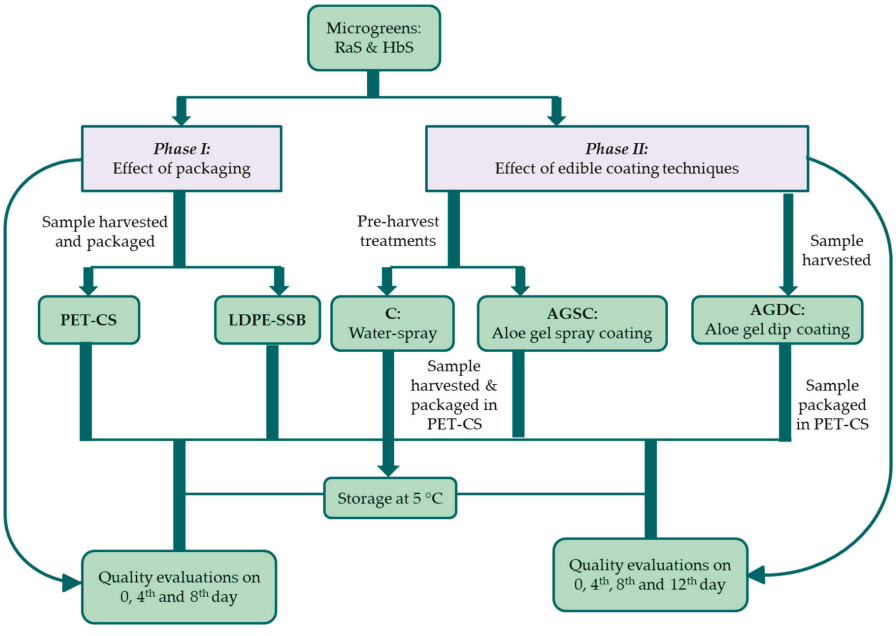

Figure 1. Experimental design and sample coding. RaS: radish microgreens; HbS: roselle microgreens; PET–CS: PET clamshell container; LDPE–SSB: LDPE self-seal bag.

2.2.1. Postharvest Packaging—Phase I

Fifteen grams of freshly harvested microgreens were packaged in clear macro-perforated (4 perforations of 3 mm diameter) polyethylene terephthalate clamshell containers (PET–CS) with hinged lid (dimension: 12.5 × 10 × 3.5 cm; thickness: 0.2 mm) or low-density polyethylene self-seal bags (LDPE–SSB) (dimension: 12.5 × 12.5 cm; thickness: 0.14 mm). The number of perforations was optimised in earlier experiments in our laboratory in order to minimise condensation on the inner package surface and, at the same time, retain the fresh weight of the produce (unpublished data). Samples were stored at 5 °C for 8 days. Quality evaluations were performed on 0, 4 and 8 days of storage, except for physiological loss in weight (PLW), which was measured every two days. Three replicates of each packaging were prepared for every analysis. A total of 84 packaged samples were obtained in Phase I (two microgreens × two packaging × seven parameters × three replicates).

2.2.2. Edible Coating Techniques and Application—Phase II

Medium-sized and freshly harvested *Aloe vera* leaves were used to extract the gel according to a previously standardised protocol [20], and suitable dilutions were prepared for application on RaS and HbS microgreens. Two common techniques viz. spray coating and dip coating, were adopted in this study. The uncoated control (C) comprised of microgreens sprayed with water, prior to harvest. Preliminary trials were conducted to optimise the concentration of *Aloe vera* gel for application (unpublished data). The edible coating comprised of Aloe gel in an amount ranging from about 25 to 50 wt %, with the Aloe gel dip-coating (AGDC) having double the concentration of Aloe gel spray coating (AGSC). Prior to the harvest of microgreens, three trays were randomly selected for the spraying of Aloe gel. The AGSC was applied as a fine mist in the early hours of the morning as multilayers, with intermittent drying periods between the coating application. Harvesting was done upon complete drying of the Aloe gel coating on the surface of microgreens, and they were packaged in PET–CS. In the AGDC treatment, the microgreens were harvested from 3 random trays and dipped in Aloe gel and fan-dried for 5–10 min without allowing wilting to take place. Quality evaluations were performed on 0, 4, 8 and 12 days of storage, except for PLW, which was measured every two days. Three replicates of each treatment were selected for quality evaluations on every sampling day. The final number of packaged samples in Phase II was 126 (two microgreens × three treatments × seven parameters × three replicates).

2.3. Quality Evaluations

2.3.1. Physiological Loss in Weight

The physiological loss in weight (PLW) was determined by accurately weighing the bagged samples at the beginning of storage and during storage at regular intervals (every two days). Results were expressed as a percentage of weight loss relative to the initial fresh weight of the microgreens [6].

2.3.2. Respiration Rate

Respiration rates of the microgreens were determined in a closed system every 4 days during the storage period. Preliminary trials were conducted to determine the optimal incubation time for the studied microgreens. Gas samples were taken from the microgreens container every 15 min for a period of 1 h and evaluated until the CO_2 level reaches a steady point. Thirty minutes was found to be the time when equilibrium was reached. Hence, 30 min was selected as the incubation time. In the case of LDPE–SSB, the macro-perforated package was placed inside a rigid container of a known volume containing ambient air as the initial atmosphere and incubated for 30 mins at 5 °C. This was carried out to minimise handling of the greens and ensure an air-tight atmosphere for gas sampling. In PET–CS containers, the macro-perforations were sealed during the incubation period. Gas composition (O_2 and CO_2%) in the headspace of the packaged sample was measured using a needle connected to the CO_2/O_2 gas analyser (PBI Dansensor, Checkmate II, Ringsted Denmark). The needle was inserted through a septum (silicone sealant) placed on the rigid container (in the case

of LDPE–SSB package) and directly through the septum of the PET–CS containers. The change in the concentration of CO_2 evolved during the incubation period was used in the calculation of respiration rate using the following Equation (1) [30]:

$$Respiration\ rate\left(uL\ CO_2\ g^{-1}h^{-1}\right) = \frac{(\%\ CO_2)_{Final} - \ (\%\ CO_2)_{initial}}{Sample\ weight\ \times\ incubation\ time\ \times 100} \times headspace\ volume \quad (1)$$

where (% CO_2) $_{final}$ is the CO_2 concentration after 30 min; (% CO_2) $_{initial}$ is the CO_2 concentration at the beginning of the incubation period; headspace volume is the volume of the container minus the volume occupied by the microgreens, expressed in µL; sample weight is the weight of microgreens on the evaluation day in g; incubation time is expressed in h.

2.3.3. Electrolyte Leakage

Tissue electrolyte leakage was measured following the procedure given by Xiao et al. [31]. Samples (5 g) were submerged in 150 mL deionized water at 20 °C and shaken for 30 min. The electrolyte of the solution was measured using a conductivity meter (ELICO CM-180, India). Total electrolytes were obtained after freezing the samples at −20 °C for 24 h and subsequent thawing. Tissue electrolyte leakage was expressed as a percentage of the total electrolyte.

2.3.4. Instrumental Colour

The instrumental colour of samples was measured with a Konica Minolta colour reader CR-10 (Minolta Co. Ltd., Osaka, Japan), equipped with an 8 mm aperture and calibrated with a white tile before the measurement was performed. The instrumental colour was measured in the form of CIELAB colour coordinates. The colour coordinate L^*, which denotes lightness, was measured in both microgreens. To trace the degradation of chlorophyll in the microgreens, a^* (−) corresponding to greenness was recorded. The coordinate b^* (+) denoting yellowness was measured in RaS as leaf yellowing was observed. In the case of HbS microgreens, since browning and not yellowing was a problem, chroma value, which denotes the overall chromacity, was calculated using the formula $(a^2 + b^2)^{1/2}$. Leaves were plucked and placed in a 3-inch petri plate until filled with the sample. The probe of the colour reader was placed onto the adaxial surface of the leaves in the dish, and the reflectance spectra were measured by the instrument directly at three different locations and the mean was calculated.

2.3.5. Ascorbic Acid

The extraction and estimation of free, dehydro- and total ascorbic acid were performed according to the method given by Kampfenkel et al. [32], and the DHA/FAA ratio was computed and expressed. The concentration of ascorbic acid was calculated based on values obtained from the L-ascorbic acid standard curve (100–500 µg/mL). Results were expressed as mg/100 g fresh weight.

2.3.6. Microbial Enumeration

To assess the microbial quality of microgreens, total aerobic mesophilic bacterial count (APC), and total yeast and mold count (YMC) were determined. Aseptically weighed sample (1 g) was homogenised in a sterilised diluent (0.1% peptone water). The extract was centrifuged, filtered under sterile conditions, and volume was made up to 10 mL. The filtrate was serially diluted (10^{-1} to 10^{-5}), and 100 µL of the appropriate dilution was spread on the agar plate using a spiral plater. The APC was determined by plating samples on the plate count agar, while YMC was determined by culturing on the potato dextrose agar. The incubation time was 24 and 48 h for APC and YMC, respectively. Microbial colonies were counted using a digital colony counter (Scan100 Interscience, St Nom, France), and results were reported as log CFU/g of sample.

2.3.7. Overall Acceptability and Marketability

Microgreens were evaluated for overall acceptability by a group of 25 female panel members (selected from the authors' department). The panel members were familiarised with the samples and scoring system, but not specifically trained as they were to reflect consumer acceptability. Samples were coded and presented to the panelists immediately after opening the containers, in a randomised manner. The panelists were asked to rate the samples based on their degree of liking, using a 9-point hedonic scale.

End of shelf life was determined based on marketability score derived from the percentage loss of saleability (Equations (2)–(6); Table 1). The latter was a composite value calculated as a sum of 40% of the degree of wilting, 40% of the degree of yellowing/browning and 20% loss of overall acceptability. The degree of wilting/discoloration was determined by counting the number of wilted/discoloured leaves and expressed as a percentage of the total number of leaves in the package. The parameters were determined on duplicate samples. The overall acceptability (OA), as determined by the sensory panel on a 9-point hedonic scale, was first converted to a percentage. Hundred minus the % OA was the percent loss of acceptability. The equations used to derive the loss of overall saleability are given below:

$$Degree\ of\ wilting\ (\%) = \frac{Number\ of\ leaves\ wilted}{Total\ number\ of\ leaves\ in\ package} \quad (2)$$

$$Degree\ of\ discolouration\ (\%) = \frac{Number\ of\ discoloured\ leaves}{Total\ number\ of\ leaves\ in\ package} \quad (3)$$

$$Overall\ acceptability\ (\%) = \lfloor \frac{OA\ score}{9} \rfloor \times 100 \quad (4)$$

$$Loss\ of\ overall\ acceptability\ (\%) = 100 - \%\ OA \quad (5)$$

$$Loss\ of\ overall\ saleability\ (\%) = 40\%\ wilting + 40\%\ discolouration + 20\%\ loss\ of\ OA \quad (6)$$

Marketability scores were assigned as follows:

Table 1. Percentage loss of saleability and marketability score for shelf life quality assessment.

Loss of Saleability (%)	Marketability Score
0 < 10	5
10 < 19	4
20 < 29	3
30 < 39	2
>40	1

A loss of saleability of 20–29%, corresponding to a marketability score of ≤3, denoted loss of marketable shelf life of the produce.

2.3.8. Scanning Electron Microscopy

The surface morphologies of coated (AGSC and AGDC) and uncoated (C) HbS microgreens were observed using an environmental scanning electron microscope (ESEM/VP–SEM–JEOL IT-300, Tokyo, Japan) operated in high vacuum mode. Samples were mounted on an aluminium stub using a double-sided adhesive carbon tape, sputter-coated with a thin layer of platinum and observed.

2.3.9. Statistical Analysis

Three replications per treatment were employed, and results were expressed as means along with their standard deviation. All statistical analyses were performed using SPSS software (IBM SPSS Statistics 25, New York, NY, USA). Data obtained for the seven parameters (PLW, RR, EL, colour, ascorbic acid, microbial quality, and sensory acceptability) across the storage period were subjected to analysis

of variance (ANOVA) using the generalised linear model. This was followed by a posthoc Tukey HSD test at $p \leq 0.05$ to determine significantly different groups. Graphical representations were performed using OriginPro® 2020 Graphing and Analysis software (OriginLab, Northampton, MA, USA).

3. Results

3.1. Effect of Packaging on Postharvest Quality and Shelf Life of Radish (RaS) and Roselle (HbS) Microgreens

3.1.1. Physiological Loss in Weight (PLW)

Significantly lower PLW ($p < 0.05$) was recorded in samples (Figure 2) stored in PET–CS than LDPE–SSB throughout storage. At the end of 8-day storage, a PLW of 6.8% and 8.1% in PET–CS stored samples, and 10.2% and 10.9% in LDPE–SSB stored samples were recorded in RaS and HbS microgreens, respectively.

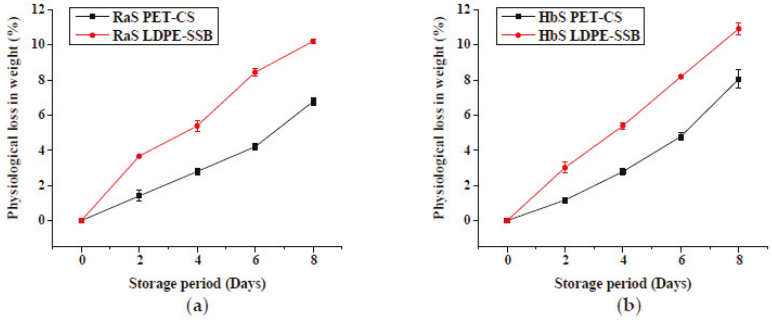

Figure 2. Effect of packaging on the physiological loss in weight in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C. PET–CS: PET clamshell containers; LDPE–SSB: LDPE self-seal bags; data are means ± SD ($n = 3$).

3.1.2. Respiration Rate (RR)

The effect of packaging on the RR is represented in Figure 3. Respiration rates were significantly ($p \leq 0.05$) affected by the type of packaging during storage, and PET–CS stored samples, maintaining lower RR throughout storage. A comparatively lower initial RR was obtained in RaS microgreens (55.2 μL CO_2 g^{-1} h^{-1}) than HbS microgreens (77.9 μL CO_2 g^{-1} h^{-1}). In both microgreens, a significant decrease ($p \leq 0.05$) was noted in RR from days 0 to 4 of storage, and it remained relatively unchanged from the 4th day till the end of storage. Significantly lower RR was recorded in PET–CS (RaS: 41.5 μL CO_2 g^{-1} h^{-1}; HbS: 45.8 μL CO_2 g^{-1} h^{-1}) compared to LDPE–SSB (RaS: 47.3 μL CO_2 g^{-1} h^{-1}; HbS: 51.3 μL CO_2 g^{-1} h^{-1}) at the end of storage.

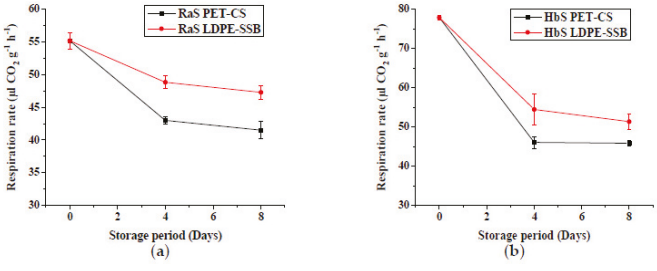

Figure 3. Effect of packaging on the respiration rate (μL CO_2 g^{-1} h^{-1}) in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C. PET–CS: PET clamshell containers; LDPE–SSB: LDPE self-seal bags; data are means ± SD ($n = 3$).

3.1.3. Electrolyte Leakage (EL)

The initial values of EL of RaS and HbS microgreens were 2.4% and 3.1%, respectively (Figure 4). The EL remained relatively constant for up to 4 days. The values subsequently increased to 9% and 11% in RaS microgreens, and 14.8% and 16.3% in HbS microgreens, stored in PET–CS and LDPE–SSB packaging, respectively, on the 8th day of storage. PET–CS stored samples had lower EL compared to LDPE–SSB stored samples. However, the difference between the two packaging was significant ($p \leq 0.05$) only in RaS microgreens at the end of storage.

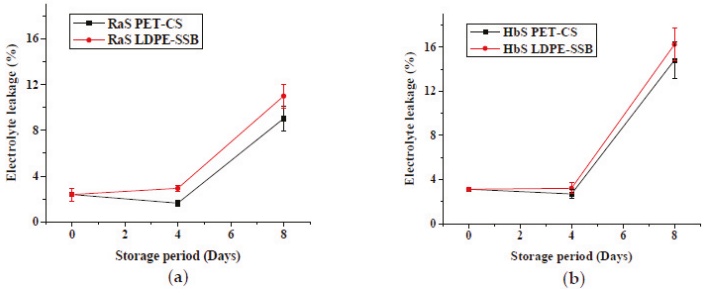

Figure 4. Effect of packaging on the electrolyte leakage (%) in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C. PET–CS: PET clamshell containers; LDPE–SSB: LDPE self-seal bags; data are means ± SD (n = 3).

3.1.4. Instrumental Colour

Instrumental colour was recorded to quantify the colour change in microgreens. Yellowing was the major discolouration seen in radish microgreens. Figure 5a represents the lightness (L^*), degree of greenness ($-a^*$), and yellowness (b^*) of RaS microgreens during storage. At harvest, RaS microgreens had an average L^* of 44.1, $-a^*$ of 8.5 and b^* of 19.7. A significant increase in the L^* coordinate was observed during storage with PET–CS having significantly lower L^* value on the 8th day. No significant change was observed in $-a^*$ coordinate in PET–CS across storage, while on the 8th day, significantly lower $-a^*$ value was recorded in LDPE–SSB. Yellowing was observed in both types of packaging. At the end of the storage period, PET–CS RaS recorded significantly lower (32.6) b^* values compared to LDPE–SSB samples (35.7). In HbS microgreens (Figure 5b), there was a significant reduction ($p \leq 0.05$) in the L^* value from 44.4 on day 0 to 35.9 in PET–CS, and 34.2 in LDPE–SSB on the 8th day. The $-a^*$ coordinate had a reduction from 10.8 on day 0 to 6.4 in PET–CS and 5.8 in LDPE–SSB after the 8th day of storage. Chroma value was calculated in HbS microgreens to trace the discolouration, as instead of yellowing, browning was observed. The chroma value decreased from 27.9 to 16.8 in both packaging across the storage. No significant difference was noted between the two packaging on the degree of discoloration of HbS microgreens.

3.1.5. Microbial Quality

Changes in the aerobic plate count (APC) and yeast and mold count (YMC) in the two microgreens (Ras and HbS) during storage are given in Figures 6 and 7, respectively. RaS microgreens had an initial APC and YMC of 5.6 and 4.9 log CFU/g, respectively. In the case of the HbS sample, an initial APC of 6 log CFU/g and YMC of 4.6 log CFU/g were recorded. The APC increased by 0.7 and 0.43 log in LDPE–SSB, while PET–CS sample showed a lower increase of 0.6 and 0.38 log at the end of storage in RaS and HbS microgreens, respectively. A lower increase in YMC was observed in PET–CS RaS (0.47 log) and HbS (0.67 Log), compared to LDPE–SSB RaS (0.55 log) and HbS (0.85 log) microgreens.

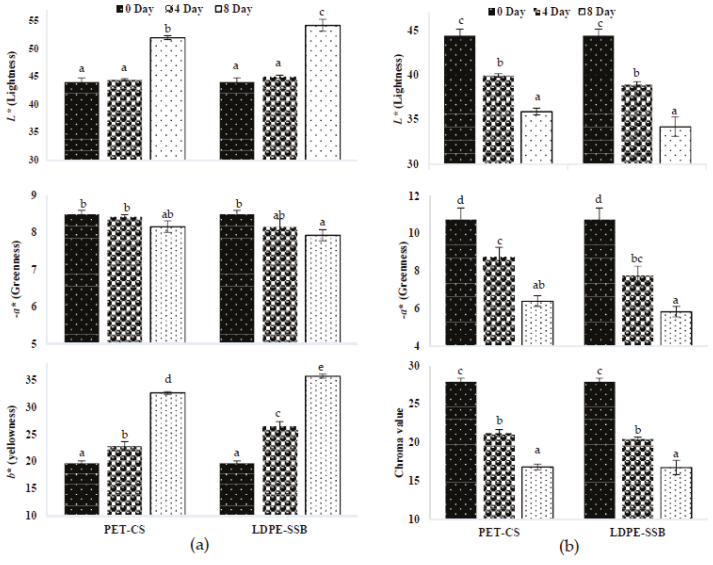

Figure 5. Effect of packaging on the colour coordinates, L^* (lightness), $-a^*$ (greenness) and b^* (yellowness) of (**a**) radish microgreens (RaS) and (**b**) roselle (HbS) microgreens during storage at 5 °C. PET–CS: PET clamshell containers; LDPE–SSB: LDPE self-seal bags; Different alphabets within the graph indicate a significant difference between packaging across storage at $p \leq 0.05$; data are means ± SD ($n = 3$).

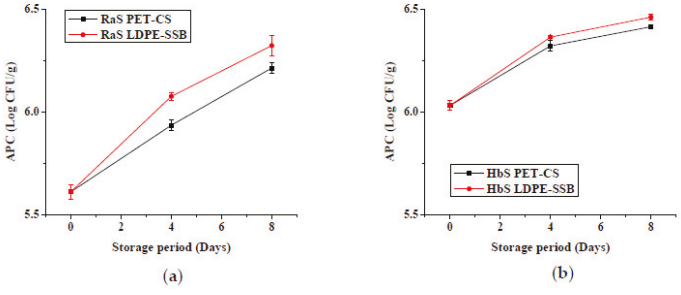

Figure 6. Effect of packaging on the aerobic plate count (APC; log CFU/g) in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C. PET–CS: PET clamshell containers; LDPE–SSB: LDPE self-seal bags; data are means ± SD ($n = 3$).

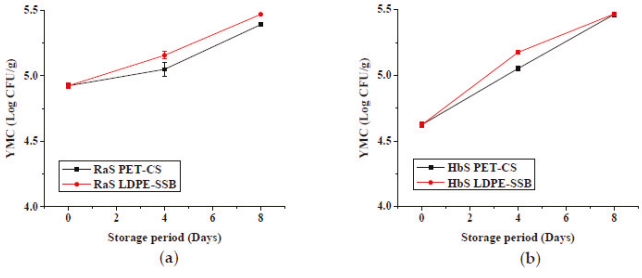

Figure 7. Effect of packaging on the yeast and mold count (YMC; log CFU/g) in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C. PET–CS: PET clamshell containers; LDPE–SSB: LDPE self-seal bags; data are means ± SD ($n = 3$).

3.1.6. Ascorbic Acid

The effect of packaging on the total (TAA), free (FAA), and dehydro-ascorbic acid (DHA) content along with the DHA/FAA ratio is depicted in Table 2. At harvest, HbS microgreens had higher TAA and FAA, and comparatively lower DHA/FAA ratio compared to RaS microgreens. As the storage period increased, there was a substantial reduction in FAA and TAA content in all the samples, with a concomitant increase in DHA. At the end of the storage period, no significant differences in AA levels were found with respect to the effect of packaging in RaS microgreens. However, roselle microgreens stored in the PET–CS package had significantly ($p \leq 0.05$) lower DHA and DHA/FAA ratio compared to LDPE–SSB.

Table 2. Effect of packaging on the free (FAA), dehydro (DHA) and total ascorbic acid (TAA) contents of radish and roselle microgreens across storage.

Packaging	Storage Period (Days)	Ascorbic Acid (mg/100 g)			
		FAA	DHA	TAA	DHA/FAA Ratio
		Radish Microgreens			
PET–CS	0	67.83 ± 0.69 ^{d}	6.15 ± 0.79 ^{a}	73.98 ± 3.82 ^{d}	0.091 ^{a}
	4	55.08 ± 1.03 ^{c}	10.85 ± 1.19 ^{b}	65.93 ± 2.54 ^{c}	0.197 ^{b}
	8	38.29 ± 0.68 ^{a}	16.30 ± 0.78 ^{cd}	54.59 ± 2.46 ^{a}	0.426 ^{d}
LDPE–SSB	0	67.83 ± 0.69 ^{d}	6.15 ± 0.79 ^{a}	73.98 ± 3.82 ^{d}	0.091 ^{a}
	4	49.96 ± 2.26 ^{b}	14.08 ± 2.61 ^{c}	64.04 ± 2.51 ^{bc}	0.284 ^{c}
	8	38.40 ± 0.76 ^{a}	18.98 ± 0.88 ^{d}	57.38 ± 1.92 ^{ab}	0.495 ^{d}
		Roselle Microgreens			
PET–CS	0	98.71 ± 0.44 ^{d}	7.92 ± 0.51 ^{a}	106.62 ± 1.77 ^{d}	0.080 ^{a}
	4	52.58 ± 1.52 ^{c}	15.14 ± 1.75 ^{b}	70.01 ± 3.02 ^{c}	0.289 ^{b}
	8	28.66 ± 0.84 ^{b}	20.58 ± 2.04 ^{c}	49.24 ± 2.21 ^{a}	0.718 ^{c}
LDPE–SSB	0	98.71 ± 0.44 ^{d}	7.92 ± 0.51 ^{a}	106.62 ± 1.77 ^{d}	0.080 ^{a}
	4	48.92 ± 3.18 ^{c}	18.80 ± 3.67 ^{bc}	62.00 ± 0.99 ^{b}	0.390 ^{b}
	8	24.22 ± 1.78 ^{a}	25.51 ± 1.91 ^{d}	49.73 ± 2.00 ^{a}	1.061 ^{d}

PET–CS: PET clamshell containers; LDPE–SSB: LDPE self-seal bags. Different alphabets within the same column for each microgreen indicate significant difference at $p \leq 0.05$ according to the Tukey HSD test; data are means ± SD ($n = 4$).

3.1.7. Overall Acceptability and Marketability

The effect of packaging on the overall acceptability (OA) and marketability scores (MS) of RaS and HbS microgreens across storage is shown in Table 3. The detailed computation leading to the marketability scores is given in Table S1. At harvest, the OA for RaS and HbS microgreens were 8.3 and 8.4, respectively, and both had the highest MS of 5. A gradual reduction was observed in the OA during storage in both samples but remained within acceptable levels (>6.5). Overall, microgreens packed in PET–CS showed slightly higher consumer acceptability compared to LDPE-packaged ones in both the microgreens. However, this difference was not significant. On the 8th day, the highest OA was recorded in RaS PET–CS. HbS samples stored in PET–CS also had greater OA than LDPE–SSB stored samples. Marketability was not affected greatly by the type of packaging. All samples, except HbS LDPE–SSB, had an MS of 3 on the 8th day. Digital photographs depicting the effect of packaging on RaS and HbS microgreens are presented in Figure S1.

Table 3. Effect of packaging on the overall acceptability and marketability score of radish (RaS) and roselle (HbS) microgreens during storage at 5 °C.

Packaging	Storage Period (Days)	Radish Microgreens		Roselle Microgreens	
		OA	MS	OA	MS
PET–CS	0	8.3 ± 0.5 ^{c}	5	8.4 ± 0.5 ^{c}	5
	4	7.8 ± 0.4 ^{b}	4	7.6 ± 0.6 ^{b}	4
	8	7.0 ± 0.4 ^{a}	3	6.6 ± 0.7 ^{a}	3
LDPE–SSB	0	8.3 ± 0.5 ^{c}	5	8.4 ± 0.5 ^{c}	5
	4	7.5 ± 0.5 ^{b}	4	7.4 ± 0.5 ^{b}	4
	8	6.7 ± 0.7 ^{a}	3	6.2 ± 0.7 ^{a}	2

PET–CS: PET clamshell containers; LDPE–SSB: LDPE self-seal bags; OA: overall acceptability; MS: marketability score. Different alphabets within the same column indicate significant difference at $p \leq 0.05$ according to the Tukey HSD test; data are means ± SD (n = 2).

3.2. Comparative Effect of Edible Coating Techniques on Postharvest Quality and Shelf Life of Radish (RaS) and Roselle (HbS) Microgreens

3.2.1. Physiological Loss in Weight

The effect of the edible coating technique on the PLW is shown in Figure 8. A significantly ($p \leq 0.05$) lower PLW was noted in all the coated samples in both RaS and HbS microgreens compared to the respective controls throughout storage. No significant difference was observed between spray- and dip-coated (AGDP) microgreens, except on the 12th day. The values in coated samples were lower than uncoated samples with RaS C microgreens recording a PLW of 10.5%, while HbS C had a relatively higher PLW of 15.5%. Least PLW of 4.7% and 8.3% were obtained in spray-coated Aloe gel (AGSC) RaS and HbS microgreens, respectively. These values were lower than that of the PLW recorded in AGDP RaS (6.3%) and HbS (9.4%) microgreens at the end of the storage period.

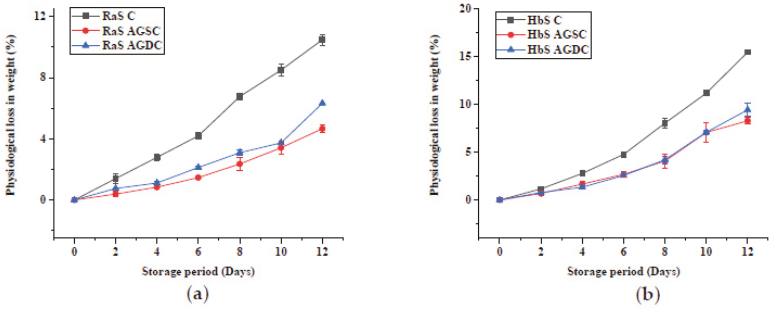

Figure 8. Effect of edible coating technique on the physiological loss in weight (%) in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C; C—control, AGSC—Aloe gel spray-coated, AGDC—Aloe gel dip-coated; data are means ± SD (n = 3).

3.2.2. Respiration Rate

The effect of the edible coating technique on the RR of microgreens is represented in Figure 9. In both RaS and HbS microgreens, significantly lower initial RR was recorded in AGSC (RaS: 27.4 μL CO_2 g^{-1} h^{-1}; HbS: 32 μL CO_2 g^{-1} h^{-1}) and AGDC (RaS: 27.1 μL CO_2 g^{-1} h^{-1}; HbS: 31.3 μL CO_2 g^{-1} h^{-1}) microgreens compared to C (RaS: 55.2 μL CO_2 g^{-1} h^{-1}; HbS: 77.9 μL CO_2 g^{-1} h^{-1}). The RR of C samples showed a pronounced decrease during storage up to the 4th day and remained relatively constant till the end of storage with values of 42.5 μL CO_2 g^{-1} h^{-1} in RaS and 47.6 μL CO_2 g^{-1} h^{-1} in HbS microgreens on the 12th day. No significant differences in RR were observed between the two edible coating techniques during the storage of RaS microgreens. RaS AGDC

maintained slightly lower values compared to AGSC for up to 8 days. However, the initial RR of the coated RaS microgreens remained relatively unchanged up to the 12th day of storage in AGSC (27.4 μL CO_2 g^{-1} h^{-1}) and AGDC (29.2 μL CO_2 g^{-1} h^{-1}) samples. With respect to HbS microgreens, a relatively lower initial RR was obtained in the AGDC sample compared to the AGSC sample on days 0 and 4 of storage; however, this difference was not significant. A gradual increase was noted from the 4th day in the AGDC sample, ending with a significantly higher ($p \leq 0.05$) RR of 36.4 μL CO_2 g^{-1} h^{-1}compared to AGSC (30.7 μL CO_2 g^{-1} h^{-1}) sample on the 12th day of storage. These values were significantly lower ($p \leq 0.05$) than HbS C.

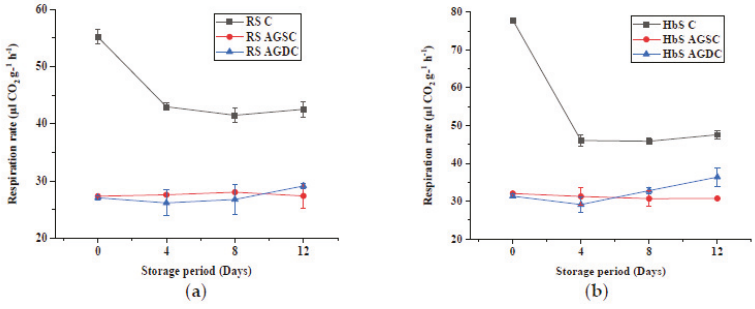

Figure 9. Effect of edible coating technique on the respiration rate (μL CO_2 g^{-1} h^{-1}) in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C. C—control uncoated, AGSC—Aloe gel spray-coated, AGDC—Aloe gel dip-coated; data are means ± SD (n = 3).

3.2.3. Electrolyte Leakage

Figure 10 shows the effect of *Aloe vera* gel spray and dip coating on the electrolyte leakage (EL) of RaS and HbS microgreens. Negligible EL was noted in the case of RaS microgreens from 0 to 4 days of storage. The presence of edible coating reduced the initial EL (0.2% in both AGSC and AGDC RaS) compared to RaS C (0.6%). In HbS microgreens too, AGDC and AGSC samples recorded lower EL of 2% and 1.98%, respectively, compared to the HbS C sample (3.1%). All samples had an initial reduction in the EL, followed by different degrees of increase after the 4th day. On the 12th day of storage, the least increase in EL was observed in RaS and HbS AGSC microgreens (4.1% both) compared to the respective controls (RaS C—14% and HbS C—15.3%) and the AGDC samples. HbS AGDC microgreens had a sharp increase after the 4th day, resulting in an EL of 9.9% at the end of storage. The less pronounced increase was noted in AGDC RaS microgreens with a final value of 6.1%. With respect to EL, the spray-coating technique showed better results compared to dip-coating in the studied microgreens.

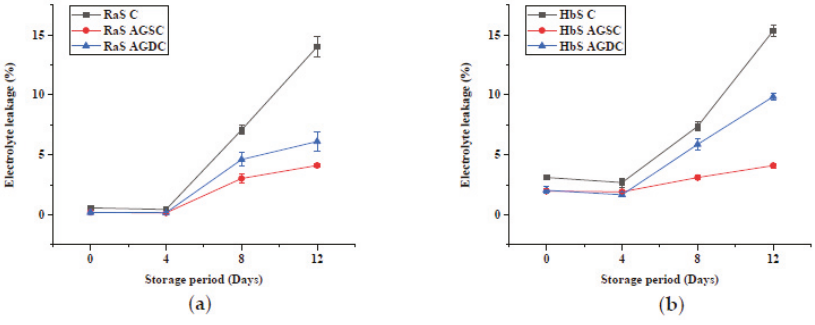

Figure 10. Effect of the edible coating technique on the electrolyte leakage (%) in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C; C—control uncoated, AGSC—Aloe gel spray-coated, AGDC—Aloe gel dip-coated; data are means ± SD (n = 3).

3.2.4. Instrumental Colour

The effect of the edible coating technique on the instrumental colour of RaS and HbS microgreens during storage is represented in Figure 11a,b, respectively. In RaS microgreens, L^* coordinate in C had a pronounced increase during storage (44.4 to 54.7). However, a very slight increase occurred in the L^* coordinate of the Aloe gel-coated sample (AGSC: 39.8 to 42.6 and AGDC: 40.2 to 42.8) during the storage period. Aloe gel-coated RaS microgreens had minimal change in the $-a^*$ coordinate during storage (AGSC: 8.6 to 7.7; AGDC: 8.5 to 7.6), while the control sample had a significant reduction ($p \leq 0.05$) from 8.5 to 5.3 at the end of the 12th day. With respect to the b^* coordinate, the highest increase was noted in the C sample (19.7 to 42.1). A significantly lower degree of increase was noted in AGSC (17.3 to 23.4) and AGDC (18.5 to 24.7) samples from 0 to 12th day in terms of b^*. No significant difference was noted between the coating techniques on the colour coordinates with respect to RaS microgreens. In the case of HbS microgreens, a significant reduction was observed in L^* coordinate in C sample (44.4 to 32.6) at the end of storage. AGSC maintained significantly higher ($p \leq 0.05$) L^* value (39.3) than C and AGDC (33.8). AGSC maintained the highest $-a^*$ value of 8.2 at the end of the storage, with significantly lower ($p \leq 0.05$) values in AGDC (6.5) and C (5.9). The chroma values followed the same trend, with AGSC (23.6 to 19.8) having a significantly lower reduction ($p \leq 0.05$) compared to AGDC (23.4 to 15.9) and C (27.9 to 13.5) on the 12th day of storage.

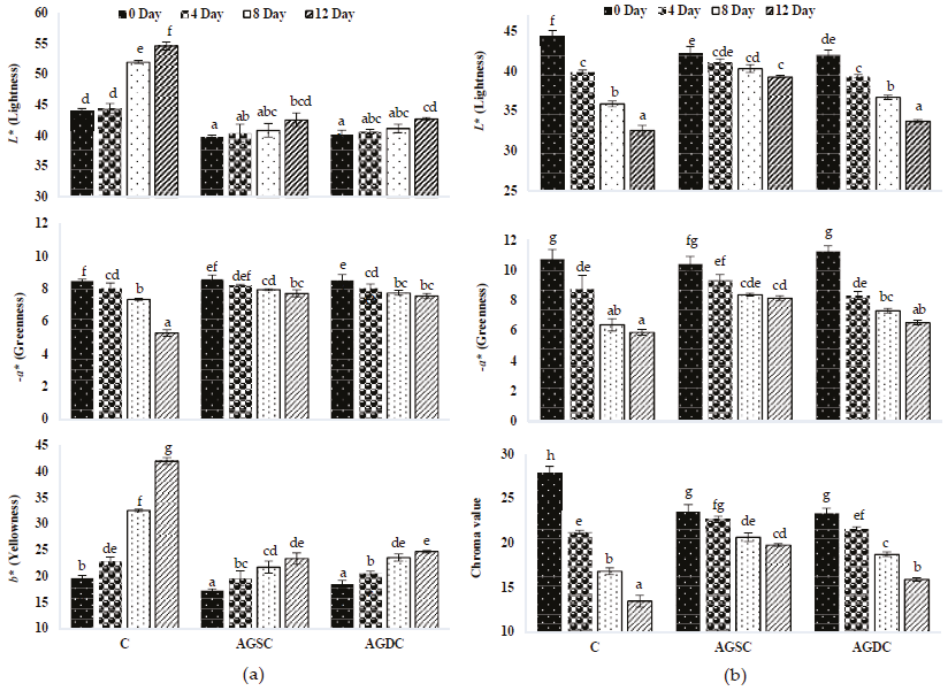

Figure 11. Effect of edible coating technique on the colour coordinates, L^* (lightness) $-a^*$ (greenness) and b^* (yellowness) of (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C; C—control uncoated, AGSC—Aloe gel spray-coated, AGDC—Aloe gel dip-coated; Different alphabets within the graph indicate a significant difference between packaging across storage at $p \leq 0.05$; data are means ± SD ($n = 3$).

3.2.5. Microbial Quality

The effect of the edible coating technique on the microbial quality (APC and YMC) of RaS and HbS microgreens is represented in Figures 12 and 13, respectively. Significantly lower ($p \leq 0.05$) initial APC was recorded in both Aloe gel-coated RaS (AGSC: 5.2 log CFU/g; AGDC: 5.5 log CFU/g) and HbS (AGSC: 5.4 log CFU/g; AGDC: 5.5 log CFU/g) microgreens compared to control (RaS C: 5.6 log CFU/g; HbS C: 6.0 log CFU/g). In both microgreens, AGSC samples maintained significantly lower ($p \leq 0.05$) APC than control throughout the storage period. In RaS microgreens, significantly lower APC was observed in AGSC compared to AGDC treatments on 0 and 12th day of storage. In the case of HbS, AGSC samples maintained significantly lower ($p \leq 0.05$) APC than AGDC throughout storage. The YMC followed a similar trend to the APC. At the end of storage, the RaS C sample had a pronounced increase ($p \leq 0.05$) in the YMC of 1.4 log. In both microgreens, AGSC maintained relatively lower YMC than AGDC throughout storage, but this change was not significant in RaS. In HbS microgreens, significantly lower ($p \leq 0.05$) YMC was recorded in AGSC on the 12th day of storage compared to AGDC, with values of 4.5 and 5.1 log CFU/g, respectively. Both the treatments, however, recorded a larger and significantly lower YMC than the control uncoated samples.

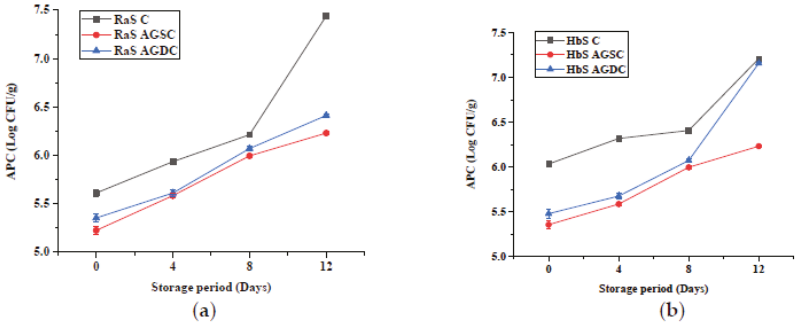

Figure 12. Effect of edible coating technique on the aerobic plate count (APC; log CFU/g) in (**a**) radish microgreens and (**b**) roselle microgreens during storage at 5 °C; C—control uncoated, AGSC—Aloe gel spray-coated, AGDC—Aloe gel dip-coated; data are means ± SD (n = 3).

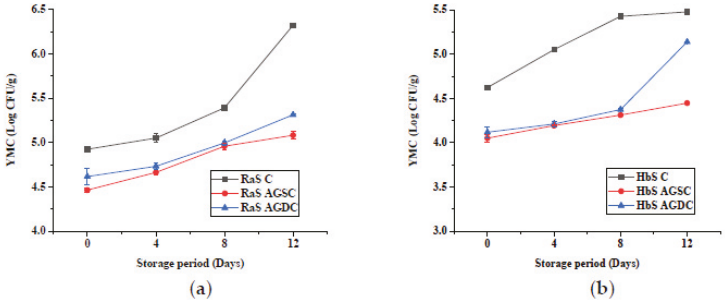

Figure 13. Effect of edible coating technique on yeast and mold count (YMC; log CFU/g) in (**a**) radish microgreens (RaS) and (**b**) roselle microgreens (HbS) during storage at 5 °C; C—control uncoated, AGSC—Aloe gel spray-coated, AGDC—Aloe gel dip-coated; data are means ± SD (n = 3).

3.2.6. Ascorbic Acid

Table 4 shows the effect of the edible coating technique on the TAA, FAA, and DHA contents along with the DHA/FAA ratio across storage. The results show significantly higher ($p \leq 0.05$) initial contents of TAA and FAA in AGSC and AGDC samples than the C in both RaS and HbS microgreens. A gradual

reduction in the TAA and FAA contents occurred in all samples, accompanied by an increase in the DHA/FAA ratio. Among the edible coating techniques, the highest content of TAA was recorded in AGSC samples of both microgreens throughout storage. However, the differences between AGSC and AGDC were not significant. Interestingly, the DHA/FAA ratio showed a significant difference ($p \leq 0.05$) between RaS AGSC and RaS AGDC on the 12th day of storage. In both microgreens, AGSC and AGDC DHA/FAA ratios were significantly lower ($p \leq 0.05$) than the controls. Maximum TAA loss was noted in HbS C microgreens (63%), followed by RaS C microgreens (42%) at the end of 12-day storage.

Table 4. Effect of edible coating technique on the free (FAA), dehydro (DHA) and total ascorbic acid (TAA) contents of radish and roselle microgreens across storage.

Treatments	Storage Period (Days)	Ascorbic Acid (mg/100 g)			
		FAA	DHA	TAA	DHA/FAA Ratio
		Radish Microgreens			
RaS C	0	78.56 ± 0.37 ^{g}	6.63 ± 0.43 ^{a}	85.19 ± 1.08 ^{d}	0.084 ^{ab}
	4	56.40 ± 1.13 ^{d}	12.54 ± 1.30 ^{bc}	68.94 ± 1.68 ^{c}	0.223 ^{abc}
	8	42.22 ± 1.34 ^{b}	17.16 ± 1.54 ^{de}	59.38 ± 2.92 ^{b}	0.408 ^{de}
	12	26.41 ± 2.41 ^{a}	23.32 ± 2.78 ^{f}	49.74 ± 1.97 ^{a}	0.899 ^{f}
RaS AGSC	0	99.88 ± 0.51 ^{j}	8.16 ± 0.59 ^{a}	108.04 ± 4.52 ^{f}	0.082 ^{a}
	4	83.45 ± 1.30 ^{h}	9.68 ± 1.50 ^{ab}	93.13 ± 3.55 ^{e}	0.116 ^{abc}
	8	70.97 ± 2.02 ^{f}	12.79 ± 1.75 ^{bc}	83.76 ± 2.91 ^{d}	0.181 ^{abc}
	12	56.25 ± 2.19 ^{d}	14.55 ± 2.53 ^{cd}	70.81 ± 3.36 ^{c}	0.261 ^{cd}
RaS AGDC	0	94.07 ± 0.73 ^{i}	8.31 ± 0.84 ^{a}	102.38 ± 1.47 ^{f}	0.088 ^{ab}
	4	72.90 ± 0.90 ^{f}	12.38 ± 1.04 ^{bc}	85.29 ± 3.24 ^{d}	0.170 ^{abc}
	8	63.49 ± 0.78 ^{e}	15.34 ± 0.90 ^{cd}	78.84 ± 1.88 ^{d}	0.242 ^{bc}
	12	47.07 ± 0.78 ^{c}	20.12 ± 0.90 ^{ef}	67.18 ± 0.98 ^{c}	0.428 ^{e}
		Roselle Microgreens			
HbS C	0	107.67 ± 3.60 ^{h}	7.40 ± 1.26 ^{a}	115.07 ± 2.68 ^{g}	0.069 ^{a}
	4	50.21 ± 1.14 ^{c}	15.00 ± 3.19 ^{c}	65.19 ± 5.09 ^{bc}	0.357 ^{cd}
	8	31.97 ± 0.51 ^{b}	16.29 ± 0.59 ^{cd}	48.26 ±1.12 ^{a}	0.510 ^{d}
	12	23.45 ± 2.44 ^{a}	19.66 ± 2.82 ^{de}	43.11 ± 3.06 ^{a}	0.858 ^{e}
HbS AGSC	0	127.43 ± 0.84 ^{j}	6.93 ± 0.97 ^{a}	134.36 ± 6.81 ^{h}	0.054 ^{a}
	4	83.63 ± 0.57 ^{g}	9.74 ± 0.57 ^{ab}	93.37 ± 4.01 ^{f}	0.116 ^{b}
	8	67.41 ± 1.83 ^{e}	14.69 ± 1.58 ^{bcd}	82.10 ± 3.24 ^{def}	0.219 ^{abc}
	12	52.76 ± 2.02 ^{c}	20.27 ± 1.75 ^{de}	73.03 ± 7.49 ^{bcd}	0.386 ^{cd}
HbS AGDC	0	119.99 ± 1.76 ^{i}	6.59 ± 1.53 ^{a}	126.58 ± 2.67 ^{h}	0.055 ^{a}
	4	75.48 ± 1.40 ^{f}	10.57 ± 1.22 ^{abc}	86.05 ± 1.79 ^{df}	0.140 ^{ab}
	8	58.61 ± 1.19 ^{d}	19.33 ± 1.03 ^{de}	77.93 ± 1.81 ^{cde}	0.330 ^{bcd}
	12	49.19 ± 1.65 ^{c}	23.84 ± 1.43 ^{e}	62.30 ± 2.60 ^{b}	0.486 ^{d}

C—uncoated control; AGSC—Aloe gel spray-coated; AGDP—Aloe gel dip-coated; different alphabets within the same column indicate significant difference at $p \leq 0.05$, according to Tukey HSD test; data are means ± SD ($n = 4$).

3.2.7. Overall Acceptability and Marketability

The effect of the edible coating technique on the OA and MS of RaS and HbS microgreens during storage is given in Table 5. The detailed computation leading to the marketability scores is given in Table S2. Comparatively higher initial OA was obtained in Aloe gel-coated (AGSC and AGDC) samples compared to control in both microgreens. With increasing storage period, a reduction in the OA occurred in all samples, with C samples rapidly losing their marketability (score of 3) on the 8th day of storage. RaS and HbS AGSC microgreens maintained the highest OA till the end of storage. In RaS microgreens, AGDC maintained equally good marketability, with a slightly lower OA score. However, in the case of HbS microgreens, the AGDC sample lost its marketability by the 12th day of storage. Digital photographs depicting the effect of edible coating on RaS and HbS microgreens are presented in Figure S2.

Table 5. Effect of edible coating techniques on the overall acceptability and marketability of radish and roselle microgreens during storage at 5 °C.

Edible Coating Technique	Storage Period (Days)	Radish Microgreens		Roselle Microgreens	
		OA	MS	OA	MS
C	0	8.3 ± 0.5 ^{f}	5	8.4 ± 0.5 ^{f}	5
	4	7.8 ± 0.4 ^{de}	4	7.6 ± 0.6 ^{cde}	4
	8	7.0 ± 0.4 ^{b}	3	6.6 ± 0.7 ^{b}	3
	12	6.4 ± 0.6 ^{a}	2	5.8 ± 0.5 ^{a}	1
AGSC	0	8.4 ± 0.5 ^{f}	5	8.5 ± 0.5 ^{f}	5
	4	8.1 ± 0.2 ^{ef}	5	8.1 ± 0.4 ^{ef}	5
	8	7.9 ± 0.5 ^{cde}	5	7.9 ± 0.5 ^{de}	5
	12	7.5 ± 0.6 ^{cd}	4	7.4 ± 0.6 ^{cd}	4
AGDC	0	8.4 ± 0.5 ^{f}	5	8.5 ± 0.5 ^{f}	5
	4	7.9 ± 0.3 ^{def}	5	8.0 ± 0.4 ^{ef}	5
	8	7.5 ± 0.6 ^{cd}	4	7.3 ± 0.5 ^{c}	4
	12	7.3 ± 0.5 ^{bc}	4	6.7 ± 0.5 ^{b}	3

OA—overall acceptability; MS—marketability score; C—uncoated control; AGSC—Aloe gel spray-coated; AGDP—Aloe gel dip-coated; different alphabets within the same column indicate significant difference at $p \leq 0.05$ according to Tukey HSD test; data are means ± SD ($n = 3$).

3.2.8. Scanning Electron Microscope (SEM) Image Analysis

The SEM images showing the surface morphology of uncoated control and Aloe gel-coated HbS microgreens with spray and dip techniques are presented in Figure 14. Stomatal apertures, guard cells and epidermal cells were distinctly visible on the surface of uncoated control. The SEM image of AGSC revealed the presence of a well-distributed thin coating forming a semi-permeable barrier showing indistinct stomata and epidermal cells. In the case of AGDC, the surface was covered with a relatively thicker and non-uniform coating showing very few stomatal apertures and no distinct epidermal cells visible.

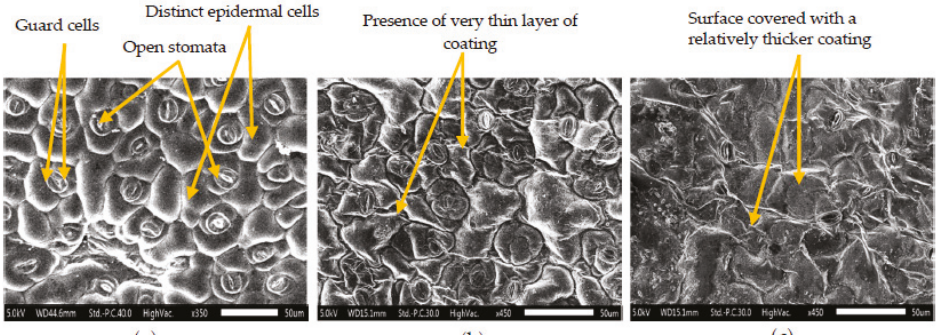

Figure 14. SEM images of roselle microgreens (**a**) uncoated, (**b**) Aloe gel spray-coated, and (**c**) Aloe gel dip-coated.

4. Discussion

Physiological loss in weight is a natural process of catabolism brought about by enzymes, and accelerated by mechanical injuries like cutting and slicing, in fresh horticultural produce [33]. The decrease in weight may be attributed to respiration and other senescence-related metabolic processes during storage [34]. In a study carried out in macro-perforated BOPP packaging, a weight loss of 60% was reported in traditional leafy vegetables stored for 4 days at 10 °C [35]. The much lower weight loss in the present study may be attributed to the lower storage temperature (5 °C). The better

performance of PET-CS could be attributed to the lower water vapour permeability of the packaging material compared to LDPE [36]. Macro-perforated packaging, commonly preferred in commercial set-ups, is beneficial in reducing off-flavour of fresh produce [8], as it prevents anaerobic atmosphere, even under temperature abuse situations [37]. However, it leads to higher fresh weight losses and requires other strategies to minimize the PLW. Considerable reduction in the PLW was obtained with Aloe gel-based edible coating in the present study. The spray coating technique was found to be as good or even better than the dip-coating in minimising losses in fresh weight. This could possibly be due to uniformity of the coating and immediate packaging of the microgreens after harvest in the case of AGSC compared to the time delay in allowing the coating to dry in the case of AGDC. Significant reduction in weight loss was also reported in Aloe gel-treated fresh-cut lotus root slices with weight losses of ~6% in 25% AG and ~ 4% in 50% AG compared to ~10% in control on the 8th day of storage at 5 °C [16].

Based on the classification of vegetables given by Kader and Salveit [38], according to their relative respiration rates and degree of perishability, the studied microgreens can be categorized as fresh produce with very high respiration rates (>30 μL CO_2 g^{-1} h^{-1}) when stored at 5 °C. Such high respiration rates limit their shelf life [3], and their storage requires packaging with good O_2 permeability to prevent anaerobic conditions and off-odour development [39]. Thus, perforated packaging material, generally used for salad crops, is expected to enhance the post-harvest performance of microgreens [2]. The initial respiration rates of the studied microgreens were found to be comparable to that of young leaves of parsley (~ 50 μL CO_2 g^{-1} h^{-1}) [40] and asparagus (96 μL CO_2 g^{-1} h^{-1}) [38] when stored at 5 °C. Very high respiration rates (99–111 μL CO_2 g^{-1} h^{-1}) were also reported in arugula, radish and red cabbage microgreens when stored at 4 °C [5]. The decrease in the respiration rate during storage may be attributed to the depletion of carbohydrate reserves, which function as substrates for the respiration process [41]. The lower RR obtained PET-CS could be due to the lower O_2 permeability compared to LDPE-SSB packaging [42]. In addition, the RR was also species-specific as they undergo senescence due to differences in the surface area to volume ratio and their surface characteristics (e.g., cuticle thickness, stomata, lenticels) [38]. Interestingly, the treatment of microgreens with *Aloe vera* gel coating considerably reduced the RR throughout storage in both samples with AGDC having a slightly lower respiration rate than AGSC initially. This was evidenced by the SEM images, which showed partial covering of the stomatal apertures in the case of AGSC, while AGDC images showed the presence of unclear stomata only in certain regions of the surface, indicating a thicker coating. However, as storage progressed, AGDC samples showed a comparatively higher RR than AGSC at the end of storage. This could be due to relatively thicker coating in AGDC samples leading to excess inhibition of O_2 and more production of CO_2 as a result of anaerobic respiration [42]. Similar results were obtained by Nasrin et al., who observed a significant reduction in the respiration rate of Aloe gel-coated strawberries compared to uncoated ones [43].

Electrolyte leakage is a common index of senescence that reflects the deterioration of membranes caused by physiological stress or mechanical injury [44]. Electrolyte leakage has been linked to the postharvest quality and shelf life of microgreens [3,4,31]. Comparable EL was reported in previous studies in broccoli microgreens [27,28]. Our observation with respect to the effect of packaging on EL is in line with the results of Xiao et al. [31], who also found no significant effect of packaging on the EL of radish microgreens. However, the comparatively lower EL obtained in PET–CS could be due to the nature of packaging. PET–CS being a thicker and sturdier packaging than LDPE–SSB could have protected against mechanical injuries during handling, leading to lesser injured plant tissues, hence lower EL. The lower EL in Aloe gel treated microgreens (AGSC and AGDC) compared to the control could be due to improved cell integrity, as shown in an earlier study on Aloe gel-coated bell pepper [45]. A significant reduction in the EL was also observed in Aloe-treated lotus root slices compared to uncoated ones [16]. In another study, chitosan treated fruit had a more structured cell arrangement than the uncoated cell, which was characterized by cellular plasmolysis, loss of turgor pressure as senescence progresses [46]. Between the edible coating treatments, AGSC of both samples

had lower EL than AGDC. In an earlier study, postharvest dip treatment, in general, was found to have accelerated tissue senescence and quality deterioration due to mechanical damage, which can incur during spinning and drying after the dip [28].

The colour of microgreens is an important factor affecting their visual appearance. While RaS microgreens had an increase in lightness, corresponding to the increase in yellowing, HbS samples had a decrease in lightness with storage, which could be due to browning of the leaves. Both microgreens had a reduction in greenness during storage when stored in PET–CS and LDPE–SSB. The type of packaging did not have any significant effect on the colour coordinates of the microgreens. However, during senescence, the pattern of change in colour can differ among different vegetables [47]. RaS microgreens exhibited yellowing of leaves during storage, as indicated by an increase in the b^* coordinate. A similar trend was observed by Supapvanich et al. [48] in sweet leaf bush during 8 days of storage. This loss of greenness could be attributed to the breakdown of chlorophyll molecules by the chlorophyll-degrading enzymes such as chlorophyll oxidase, chlorophyll peroxidase, and chlorophyllase, revealing the pre-existing yellow carotenoid pigments [49]. HbS microgreens, on the other hand, showed evidence of browning during the storage period, corresponding with the loss of overall chromaticity of HbS microgreens. Earlier studies have reported changes in colour coordinates correlating with the incidence of browning [50,51]. Browning is generally considered to be caused by a range of endogenous phenolic compounds containing an *o*-dihydroxy group that gets oxidised to the corresponding *o*-quinone by oxidising enzymes in the presence of oxygen, leading to the formation of brown pigments (melanin) [52]. Roselle microgreens indeed had the highest content of total phenolics among ten microgreens analysed [53]. However, the application of edible coating (AGSC and AGDC) helped to reduce discolouration and retained the greenness of microgreens. The gas-barrier function of edible coating could retard loss of colour components and enzymatic oxidation, protecting fresh produce from discoloration and texture softening during storage [35]. In an earlier study on minimally processed vegetables, the application of chitosan coating exhibited higher chroma values throughout storage compared to uncoated samples [22]. In the case of HbS microgreens, the overall chromaticity was better maintained in AGSC samples, as evidenced by a lower incidence of browning compared to HbS AGDC samples, observed during the latter part of storage. A similar reduction in the degree of browning was also observed in fresh-cut lotus root slices coated with 50% Aloe vera gel [16]. This could be attributed to the higher EL recorded in the AGDC HbS samples. EL is an indicator of senescence due to physical damage/wounding. This is expected to have occurred during the dip-coating process, leading to an increased rate of biochemical reactions responsible for changes in colour (browning) [54]. It is also important to note that HbS microgreens have softer and thinner cotyledonary leaves compared to RaS microgreens, making it comparatively more susceptible to mechanical injuries than the latter.

Microgreens are generally characterized by high moisture and nutrient contents, which create an environment conducive for the growth of microorganisms. The initial APC and YMC were comparable to those obtained in buckwheat microgreens [4], broccoli and chicory microgreens [55]. Between the two packaging, the significantly lower microbial count in PET–CS could be attributed to the sturdiness of the packaging, which could have reduced chances of mechanical injuries, hence limiting the proliferation of the microorganisms. This packaging also helped better retention of fresh weight. Weight loss is characterized by an increase in intercellular spaces within the plant tissue, which can facilitate the entry of microorganisms into cells [46]. In the phase II study, the increase in microbial load in the control uncoated samples during storage corresponded with an increase in weight loss and electrolyte leakage of the microgreens. Leakage of juices and sugars from damaged plant tissues has been reported to favour microbial growth [56]. *Aloe vera* gel coating significantly reduced the APC and YMC in both microgreens during storage. *Aloe vera* gel is known to contain several compounds such as saponins, tannins, flavonoids, and terpenoids, which are responsible for its antimicrobial activity [57]. The results obtained were comparable with the effects of *Aloe vera* gel coating in sweet cherry [58] and apple slices [59], which showed a reduction in the mesophilic aerobic bacteria and yeast and mold counts during storage. With respect to the edible coating techniques, the higher microbial load

recorded in AGDC compared to AGSC samples at the end of storage could be attributed to the higher EL, RR and/or contamination during the postharvest dip.

Ascorbic acid is the vitamin that is most sensitive to destruction during the storage of fresh commodities and hence can be used as a chemical indicator of shelf life quality [60]. Similar contents of total, free and dehydro-ascorbic acid were reported in microgreens in an earlier study [61]. In the current study, the macro-perforated packaging increased the availability of oxygen, which could have favoured ascorbate oxidase, the enzyme responsible for the oxidation of FAA to DHA [62]. Loss of fresh weight has been associated with rapid ascorbic acid degradation as well [63]. However, no significant difference was obtained between the two forms of packaging, which could be due to the macro-perforated nature of the packaging. The reduction of TAA and FAA and the accumulation of DHA during storage were also observed in fresh-cut spinach [64] and minimally processed lettuce [65]. A significantly higher ascorbic acid content was obtained in Aloe gel-coated samples compared to uncoated control. This could be attributed to the presence of ascorbic acid in the inner gel [66]. This could be a promising way to enhance the ascorbic acid content of the microgreens using plant-based bio-treatments like *Aloe vera* gel. Similar observations were reported earlier in litchi fruit [21], raspberry fruits [67] and tomato [68] coated with *Aloe vera* gel. At the end of storage, AG treatments exhibited lower reductions in ascorbic acid in RaS microgreens (AGSC: 34.5%; AGDC: 34.4%) and HbS microgreens (AGSC: 45.6%; AGDC: 50.8%) compared to respective controls (41.6% and 62.5%). The oxidative loss of ascorbic acid could have been reduced by the presence of the protective coating [69]. Between the two edible coating techniques, a significantly lower DHA/FAA ratio was observed in AGSC compared to AGDC at the end of storage. This could be attributed to the higher levels of oxidative stress as indicated by higher RR, and injury to the membranes during the dip-coating treatment indicated by higher EL, leading to greater activity of ascorbic acid degrading enzymes [70] in AGDC samples.

Overall acceptability of the produce directly influences their marketability. The relatively higher OA in PET–CS could be attributed to the better appearance and texture of the microgreens packaged in PET–CS compared to that in LDPE. This could be due to the sturdy nature of the PET–CS package, which conferred better protection to the microgreens during storage. Also, clamshell containers are known to provide efficiency in terms of shipping and merchandising [71]. On the other hand, LDPE–SSB stored samples could be more prone to mechanical injuries during handling due to the flexible nature of the packaging. The loss of OA during storage is related to aging processes and senescence [60], as observed by an increase in PLW, RR, and EL, leading to a reduction in marketability. HbS LDPE–SSB samples lost their marketability on the 6th day of storage, while PET–CS stored was marketable up to the 8th day of storage. The comparatively sturdier nature of RaS microgreens enabled it to better withstand the effect of handling and thus showed a marketability of 8 days in both packaging. Application of *Aloe vera* gel coating resulted in better retention of freshness and added a glossy sheen on the surface of microgreens resulting in better overall acceptability compared to the uncoated samples. It also reduced the pungent taste of RaS microgreens. HbS AGDC lost its shelf marketability by the 12th day of storage due to browning of leaves and lower consumer acceptability, while HbS AGSC was marketable beyond 12 days. In the case of RaS samples, though relatively higher OA was recorded in AGSC than AGDC, both treatments were marketable beyond 12 days. It could be hypothesised that the two techniques are equally effective in retaining the freshness of the microgreens, as indicted by a much lower degree of witling. In samples prone to browning such as HbS, a thinner coating delivered by the spray technique could be more advantageous.

5. Conclusions

In conclusion, macro-perforated PET-CS was found to be a comparatively better packaging than LDPE–SSB for postharvest quality maintenance during the storage of RaS and HbS microgreens. Though PET–CS would be commercially preferred as a rigid packaging during long-distance transportation, LDPE–SSB could also be used as an economical alternative in short distance markets and for sturdier microgreens. *Aloe vera* gel edible coating acted as a primary packaging and helped to

overcome the drawbacks of macro-perforated packaging and significantly enhanced the postharvest quality and shelf life of the studied microgreens. Aloe gel coated samples also had ~40% to 70% higher total ascorbic acid content and maintained 2- to 3-fold lower DHA/FAA ratios compared to uncoated ones. Aloe gel coating is an eco-friendly and sustainable alternative to chemical pre-treatments for shelf-life quality enhancement in the studied microgreens. The spray-coating technique performed similar to or better than the dip-coating technique. It was found to be a superior option as it helped to circumvent the drawbacks of the dip-coating technique, such as dilution of coating solution and risk of contamination. The AGSC technique also offers the advantages of uniform coating, lesser handling of microgreens and lower coating solution requirement, leading to reduced cost (approx. 10-fold less). In addition, it is also amenable to large scale setups, making it a promising preharvest treatment for enhancing the postharvest quality and shelf life of radish and roselle microgreens. The AGSC technique, along with PET–CS, has potential for applications in other microgreens and fresh-cut produce. Future work will consider the use of biodegradable packaging along with edible coating as a total sustainable packaging approach for premium produce like microgreens. In addition, edible coating formulated for nutrient enrichment of high-value microgreens is also under evaluation.

Supplementary Materials: The following are available online at http://www.mdpi.com/2304-8158/9/5/653/s1, Figure S1: Digital photographs showing effect of packaging on radish (RaS) and roselle (HbS) microgreens on 0 day and 8th day of storage, Figure S2: Digital photographs showing effect of edible coating techniques on radish (RaS) and roselle (HbS) microgreens on 0 day and 12th day of storage. Table S1: Effect of packaging on the marketability of radish and roselle microgreens during storage at 5 °C, Table S2: Effect of edible coating treatment on the marketability of radish and roselle microgreens during storage at 5 °C.

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Yield and Quality Characteristics of *Brassica* Microgreens as Affected by the NH_4:NO_3 Molar Ratio and Strength of the Nutrient Solution

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Abstract: Microgreens are gaining more and more interest, but little information is available on the effects of the chemical composition of the nutrient solution on the microgreen yield. In this study, three *Brassica* genotypes (*B. oleracea* var. *italica*, *B. oleracea* var. *botrytis*, and *Brassica rapa* L. subsp. *sylvestris* L. Janch. var. *esculenta* Hort) were fertigated with three modified strength Hoagland nutrient solutions (1/2, 1/4, and 1/8 strength) or with three modified half-strength Hoagland nutrient solutions with three different NH_4:NO_3 molar ratios (5:95, 15:85, and 25:75). Microgreen yields and content of inorganic ions, dietary fiber, proteins, α-tocopherol, and β-carotene were evaluated. Micro cauliflower showed the highest yield, as well as a higher content of mineral elements and α-tocopherol (10.4 mg 100 g^{-1} fresh weight (FW)) than other genotypes. The use of nutrient solution at half strength gave both a high yield (0.23 g cm^{-2}) and a desirable seedling height. By changing the NH_4:NO_3 molar ratio in the nutrient solution, no differences were found on yield and growing parameters, although the highest β-carotene content (6.3 mg 100 g^{-1} FW) was found by using a NH_4:NO_3 molar ratio of 25:75. The lowest nitrate content (on average 6.8 g 100 g^{-1} dry weight) was found in micro broccoli and micro broccoli raab by using a nutrient solution with NH_4:NO_3 molar ratios of 25:75 and 5:95, respectively. Micro cauliflower fertigated with a NH_4:NO_3 molar ratio of 25:75 showed the highest dry matter (9.8 g 100 g^{-1} FW) and protein content (4.2 g 100 g^{-1} FW).

Keywords: broccoli; broccoli raab; cauliflower; hydroponic; mineral elements; nitrate; vitamins

1. Introduction

Microgreens can be described as young and tender edible seedlings, produced by using seeds of different vegetable species, herbaceous plants, aromatic herbs, and wild edible plants, which are considered as 'functional foods' or 'super foods' because of their high nutritional value [1–3]. In recent years, microgreens have been increasingly used as basic ingredients in culinary preparations to obtain both sweet and savoury dishes with peculiar organoleptic traits [4]. Many species and local varieties of several botanical families, such as Brassicaceae, can be used for microgreen production [5,6]. The Brassicaceae family offers some of the most consumed vegetables worldwide and their seedlings have a generally good taste and high nutritional value. Many studies have been carried out on the nutritional propriety of different Brassicacea genotypes consumed as microgreens.

For example, in a study by Xiao et al. [7], 30 genotypes of *Brassica* were analyzed in regards to the content of elements, while Sun et al. [5] analyzed the polyphenols profile of five Brassicacea species. Other authors [8] also evaluated the bioaccessibility of mineral elements and antioxidant compounds in some Brassicaceae microgreens.

Microgreens can be also used, instead of common vegetables, to reduce the daily intake of some elements when their restriction is required for health reasons. For example, Renna et al. [9] showed that a useful reduction in potassium can occur with three genotypes of microgreens in order to propose low-potassium vegetables for subjects affected by renal failure. Recently, many studies were carried out on microgreens in regards to the effect of artificial light on carotenoid content [10,11], growth and nutritional quality [12], antioxidant properties [13], and content of bioactive compounds [14]. Nevertheless, only a few studies have been done on the effects of nutrient solution strength on the growth and nutritional quality of microgreens [5]. On the other hand, the strength and optimal electric conductibility (EC) of the nutrient solution to maximize yield and content of bioactive compounds, and reduce fertilizer waste during microgreens production, are currently not clear. Some authors [15,16] used a nutrient solution with an EC of 1.12 mS cm^{-1}, Kyriacu et al. [17] reported an EC of 0.3 mS cm^{-1} (but with organic substrate with an EC of 0.2 mS cm^{-1}), Di Gioia et al. [18] indicated an EC of 1.3 mS cm^{-1}, while an EC of 1.8 mS cm^{-1} was reported by Renna et al. [9]. In regards to the chemical composition, some authors [19,20] used a modified Hoagland nutrient solution containing 31.5, 24.2, 6.2, 30.0, 4.1, and 8 mg L^{-1} of N, K, P, Ca, Mg, and S, respectively. Di Gioia et al. [21] fertigated microgreens with nutrient solution containing 105.1, 117.4, 15.5, 92.5, 26.0, and 34.6 mg L^{-1} of N, K, P, Ca, Mg, and S, respectively, while Wieth et al. [22] used three concentrations (0, 50 and 100%) of a nutrient solution containing 214.2, 250.6, 43.7, 136.0, 26.5, and 35.0 mg L^{-1} of N, K, P, Ca, Mg, and S, respectively. The optimal nutrient solution is not clear and much work needs to be done in this area.

An important aspect of the nutritional quality of vegetable products is their nitrate (NO_3) content. Nitrate per se is relatively non-toxic, but its reaction products and metabolites, such as nitrite, nitric oxide and N-nitroso compounds have raised concerns because of their implications for adverse health effects, such as methemoglobinemia or 'blue baby syndrome' [23]. In this context, it is interesting to highlight that hydroponic cultivation systems allow a reduction in nitrate content in leafy vegetables, without negatively affecting yield and quality, due to strategies such as partially replacing nitrate-based fertilizers with ammonium-based ones [24,25].

Few studies have been carried out until now on the influence of the NH_4:NO_3 molar ratio in nutrient solutions on mineral and phytochemical content of microgreens. Some authors [17,19,20] reported a NH_4:NO_3 molar ratio of 11:89 in nutrient solutions, while Wieth et al. [22] used a nutrient solution with a NH_4:NO_3 molar ratio of 9:91. At the same time, only the NO_3 form was used in the nutrient solution by other authors [15,16,21]. Nevertheless, based on the studies carried out on mature vegetables [26–28], it is possible to hypothesize a potential reduction in nitrate content, as well as an improvement in nutraceutical value, in microgreens grown in varying NH_4:NO_3 molar ratios of the nutrient solution.

Starting from these remarks, the aims of the present study on three *Brassica* microgreens were to evaluate: (i) the effects of the nutrient solution strength on yield and quality parameters; and (ii) the physiological behaviour and some quality traits of microgreens fertigated with three different NH_4:NO_3 molar ratios.

2. Materials and Methods

2.1. Experimental Set-Up

Two experiments were conducted using a hydroponic system during the spring of 2015 in the greenhouse at the Experimental Farm 'La Noria' of the Institute of Sciences of Food Production of the Italian National Research Council (CNR), located in Mola di Bari (BA, Southern Italy). The first

experiment was carried out from 16 March to 3 April, while the second one was carried out from 22 April to 5 May.

Three different genotypes of Brassicaceae were grown for both experiments: *Brassica rapa* L. subsp. *sylvestris* L. Janch. var. *esculenta* Hort, local variety 'Cima di rapa novantina' (broccoli raab); *Brassica oleracea* L. var. *italica*, cultivar 'Broccolo natalino' (broccoli); *Brassica oleracea* L. var. *botrytis*, cultivar 'Cavolfiore violetto' (cauliflower) (Figure 1). The seeds were purchased from 'Riccardo Larosa Sementi' (Andria, Italy) and their germination, tested at a constant temperature of 20 °C, was higher than 95%.

Figure 1. Genotypes used for producing microgreens: (**A**) broccoli, cultivar 'Broccolo natalino'; (**B**) broccoli raab, local variety 'Cima di rapa novantina'; (**C**) cauliflower, cultivar 'Cavolfiore violetto'.

Microgreens were grown by using a hydroponic system with polyethylene terephthalate fiber pads (40 cm × 24 cm × 0.89 cm; Sure to Grow®; Sure to Grow, Beachwood, OH, USA) as a growing medium, which was placed on an aluminium bench (180 × 80 cm) with a slope of 0.05%. The seeds were uniformly broadcasted on the surface of the growing media using a seeding density of 4 seeds cm^{-2}. The sown fiber pads were irrigated manually using a water-nozzle and were covered with a black polyethylene film until the germination was complete.

During the first experiment, three nutrient solutions (NSs), type-like Hoagland and Arnon [29], with different strengths (1/2 strength, 1/4 strength and 1/8 strength), prepared with rain water were used (Table 1). From germination until harvest, the NS was supplied for one minute in the morning and one minute in the afternoon.

Table 1. Characteristics of the nutrient solutions (NS) used during the first experiment.

	NS Strength		
	1/2	**1/4**	**1/8**
	(mg L^{-1})		
$N\text{-}NO_3$	100	50	25
$N\text{-}NH_4$	5	2.5	1.25
K	117	58.5	29.25
P	16	8	4
Mg	24	12	6
Ca	86	43	21.5
Cl	0	0	0
S	31	15.5	7.75
pH	6.5	6.5	6.5
EC (mS cm^{-1})	1.37	0.77	0.43

For the second experiment, three half-strength NS with different ratios of NH_4:NO_3 were used (Table 2).

Table 2. Characteristics of the nutrient solutions used during the second experiment.

	Molar Ratio NH_4:NO_3 (%)		
	5:95	**15:85**	**25:75**
	(mg L^{-1})		
$N\text{-}NO_3$	100	90	80
$N\text{-}NH_4$	5	16	26
K	117	117	117
P	16	16	16
Mg	24	24	24
Ca	86	86	86
Cl	0	0	20
S	31	57	71
pH	6.3	5.9	5.8
EC (mS cm^{-1})	1.12	1.42	1.40

To prepare the nutrient solutions, fertilizers for hydroponic production were used. More specifically, the following salts were used: calcium nitrate, potassium nitrate, ammonium nitrate, potassium sulphate, magnesium sulphate, calcium chloride, and potassium dihydrogen phosphate. In order to obtain the element composition reported in Tables 1 and 2, the amount of each salt was calculated, while also considering their titre and purity.

During the second experiment, being in late spring, the temperature in the greenhouse was higher than in the first experiment, for this reason another minute of fertigation was supplied at noon. During both the first and second experiment, after epicotyl emission, NSs were distributed by a drip tape line with pressure-compensated drippers (each with a delivery rate of 0.133 L min^{-1}). An open cycle management was used; therefore, the drainage was collected but not reused. The experimental scheme used was split-plot where each plot was represented by the bench and each sub-plot was represented by a genotype.

2.2. Harvesting and Physical Analysis

Harvesting was carried out by cutting microgreens just above the growing media surface, when the first true leaves were at least 1 cm long. Within each experiment, three samples were considered for each experimental unit (genotype and treatment), and analysed as independent replicates. Each field replicate was obtained by harvesting three sub-samples within the same growing pad.

For both experiments and for each cultivar, we recorded how many days passed from sowing until: breaking seed integuments, radicle spillage, hypocotyl emission, cotyledons formation, first true leaf formation, second true leaf formation (true leaf was formed when it was at least 0.5 cm long). Immediately before the harvesting, other parameters were collected: presence of true leaves, leaf length (true leaf eventually present), shoot height and substrate coverage. To determine presence of true leaves, shoot height and leaf length, three random microgreens were selected for each sub-parcel. The substrate coverage included the distribution and microgreens overlap in the substrate. We used three different categories: 1—low; 2—good; 3—excessive. Each sub-parcel was observed at 30 cm, orthogonally from the growth plan and when possible, between the shoots to watch spare space, where we used category 1. If it was not possible to watch the growth media and there was not any overlap between the shoots, we used category 2, and category 3 was used when there was overlap between the shoots.

The harvested microgreens were weighed to determine the shoot fresh weight (FW) per unit area. The dry matter (DM) was measured in triplicate by oven-drying at 65 °C until a constant weight of the samples. The oven-dried samples were used for cation and anion content determination, while freeze-dried (ScanVac CoolSafe 55-9 Pro; LaboGene ApS, Lynge, Denmark) samples were used for chemical analysis.

2.3. Inorganic Ion Content

The content of inorganic ion was determined by ion exchange chromatography (Dionex DX120; Dionex Corporation, Sunnyvale, CA, USA) with a conductivity detector, as reported by D'Imperio et al. [17]. The content of Na^+, K^+, Mg^{2+}, and Ca^{2+} was determined in 1 g of dried sample, using an IonPac CG12A guard column and an IonPac CS12A analytical column (Dionex Corporation); the elution was performed with 18 mM of methanesulfonic acid (Thermo Scientific™ Dionex™, Waltham, MA, USA). Peaks identification and calibration were performed using the Multi Element IC Standard solution Fluka TraceCERT®, Supelco® (Merck KGaA, Darmstadt, Germany). The contents of Cl^- and NO_3^- were determined in 0.5 g of dried sample using an IonPac AG14 precolumn and an IonPac AS14 separation column (Dionex Corporation). The eluent consisted of 3.5 mmol·L^{-1} of sodium-carbonate (Thermo Scientific™ Dionex™, USA) and 1.0 mmol·L^{-1} of sodium-bicarbonate solution (Thermo Scientific™ Dionex™, USA), and 50 mL of the same eluent was used to extract the anions. Inorganic cation content determination was carried out in triplicate. Peaks identification and calibration were performed using the Multi Element IC Standard sol. IC-MAN-18 (6E) of Chem-Lab (Palin Corporation, Elderslie, UK).

2.4. Dietary Fiber Content

Dietary fiber content was determined according to AOAC methods [30] with a slight modification. First, a sample of lyophilized microgreen powder (250 mg) was boiled in 32.5 mL of H_2SO_4 0.64 N for 10 min, adding a few drops of n-octanol as antifoam agent. The resulting insoluble residue was filtered, washed with warm distilled water, and boiled in 32.5 mL of KOH 0.56 N for 10 min. After filtering and washing the sample three times with acetone RPE, it was dried at 105 ± 2 °C for 1 h. Weight loss, corresponding to the raw fiber, was determined after cooling the sample at RT in a dryer. Then, ash content was determined by weighing the obtained residue before and after a strong heat treatment (550 °C for 3 h). Finally, fiber content was expressed relative to the fresh weight (FW). Crude protein was assessed by the micro-Kjeldahl method, with a nitrogen to protein conversion factor of 6.25, according to the AOAC method 976.05 [30]. Dietary fiber content determination was carried out in triplicate. All chemicals used were supplied by Sigma-Aldrich (Milan, Italy) and were of analytical grade.

2.5. Content of α-Tocopherol and β-Carotene

For α-tocopherol and pro-vitamin A expressed as β-carotene, the extraction procedure simultaneously extracts water-soluble vitamin (WSV) and fat-soluble vitamin (FSV). During the extraction process, samples were always protected from direct exposition to light and kept on ice to minimize vitamin degradation. Briefly, 0.050 g of each sample was first extracted with 7.5 mL of 1% BHA in ethanol and 500 µL of internal standard (86.82 µM trans-β-apo-8 carotenal) were added. Samples were placed in an ultrasound bath for 15 s and 180 µL of 80% KOH were added and heated for 45 min at 70 °C. Three milliliters of water and 3 mL of hexane/toluene were added (10:8 v/v), and centrifuged at 1000× *g* for 5 min. The supernatant was recovered and the bottom solution was extracted with hexane/toluene at least two times. The phases were reunited and the solvent was evaporated under the nitrogen stream. It was recovered with 500 µL of acetonitrile/ethanol 1:1 for HPLC analysis. Separation and identification of lipophilic vitamins in microgreen extracts were carried out with a HPLC 1100 equipped with quaternary pump solvent delivery, thermostatic column compartment, and diode array detector (DAD) (Agilent Technologies, Palo Alto, CA, USA). The samples (20 µL) were injected onto a reversed stationary phase ZORBAX EC18 (Agilent Technologies) (5 µm (150 × 4.6 mm i.d.)), following an isocratic program with ethanol/acetonitrile 1:1 as mobile phase according to the method previously published by Xiao et al. [7]. Stop time was set at 30 min with a re-equilibration time of 10 min corresponding to ~20 column volume (Vc = 0.52 mL). The column temperature was not controlled, while the flow was maintained at 1.2 mL/min. Diode array detection was between 250 nm and 650 nm and absorbance was recorded at 450 nm for β-carotene and 290 nm for α-tocopherol.

Compounds identification was achieved by combining different information: positions of absorption maxima (λmax), the degree of vibration fine structure (% III/II), and retention times were compared with those from pure standards. To evaluate linearity, calibration curves with five concentration points for each compound were prepared separately. Calibration was performed by linear regression of peak-area ratios of the vitamins to the internal standard (β-apo-8'-carotenal) versus the respective standard concentration, obtaining R^2 values of 0.9992 and 0.9999 for β-carotene and α-tocopherol, respectively. Finally, vitamins were quantified as mg of β-carotene and α-tocopherol per 100 g of microgreens. The determination of α-tocopherol and β-carotene content was carried out in triplicate. All chemicals used were supplied by Sigma-Aldrich (Milan, Italy) and were of analytical grade.

2.6. Statistical Analysis

The data were analysed by a two-way analysis of variance (ANOVA), using the general linear model procedure of SAS software (SAS Version 9.1, SAS Institute, Cary, NC, USA) and applying a split-plot design with genotype (G) and nutrient solution (NS) as main factors for all measurements. All means were compared using the Student–Newman–Keuls (SNK) test at $p = 0.05$, and standard deviation (SD) was also calculated. Significance of main factors and their interaction are reported in tables. Average values of main factors are reported in tables, while average values of significant interactions G x NS are showed by using histograms.

3. Results

3.1. First Experiment

At harvest, broccoli raab showed twice the number of true leaves per seedling compared to other genotypes, while the average leaf length was about 1.28 cm, without any difference between genotypes, treatments and their interaction (Table 3). In regards to yield, broccoli raab fertigated with 1/8 strength NS showed an amount 43% lower compared with cauliflower, and 40% lower compared with broccoli raab fertigated with NS 1/2 (Figure 2). Microgreens fertigated with the 1/8 strength NS showed the lowest seedling height, which was 17% lower than those fertigated with 1/4 strength NS and 25% lower than those fertigated with 1/2 strength NS (Table 3). On the other hand, broccoli raab microgreen height was 9% lower compared with broccoli (Table 3).

Table 3. Main effects of genotypes and nutrient solution strength on number and length of true leaves, yield and seedling height of microgreens (first experiment).

	True Leaves	Leaves Length	Yield	Seedling Height
	Number Seedling^{-1}	cm	g cm^{-2}	cm
Genotype (G)				
Broccoli raab	2.00 ± 0.30 a	1.43 ± 0.24	0.20 ± 0.02 b	8.60 ± 1.60 ab
Broccoli	1.00 ± 0.10 b	1.17 ± 0.32	0.20 ± 0.05 b	9.70 ± 1.10 ab
Cauliflower	1.00 ± 0.10 b	1.23 ± 0.19	0.24 ± 0.02 a	9.10 ± 1.40 b
Nutrient solution strength (NSS)				
1/2	1.03 ± 0.50	1.37 ± 0.40	0.23 ± 0.01 a	10.30 ± 0.90 a
1/4	1.03 ± 0.50	1.33 ± 0.14	0.22 ± 0.03 a	9.30 ± 1.00 b
1/8	1.03 ± 0.50	1.13 ± 0.15	0.19 ± 0.04 b	7.70 ± 0.80 c
Significance				
G	***	NS	**	*
NSS	NS	NS	**	***
G * NSS	NS	NS	**	NS

Significance: ***, **, and * respectively for $p \leq 0.001$, $p \leq 0.01$, and $p \leq 0.05$; NS, not significant. Means values (± standard deviation) within each column and main effect followed by different letters are significantly different, according to SNK test ($p = 0.05$).

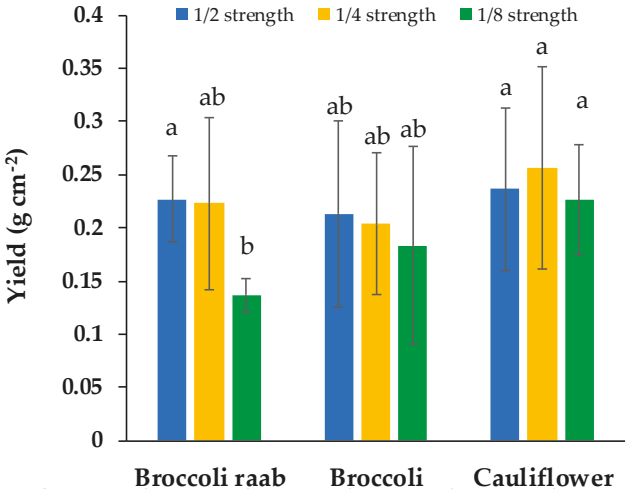

Figure 2. Yield of three genotypes of microgreens grown with three NS strengths: 1/2 strength, 1/4 strength and 1/8 strength (first experiment). Different letters indicate that mean values are significantly different, according to the SNK test ($p = 0.05$). Vertical bars represent ± standard deviation of mean values.

The average values of development stage and density were, respectively, 3.0 and 4.0, without differences between genotypes, nutrient solution strength and their interaction (Table 4). Cauliflower showed a substrate coverage 27% lower than the other genotypes, while broccoli raab showed a value of substrate uniformity 43% higher than cauliflower (Table 4).

Table 4. Main effects of genotypes and nutrient solution strength on development stage, substrate coverage, substrate uniformity and density of microgreens (first experiment).

	Development Stage [(1)]	Substrate Coverage [(2)]	Substrate Uniformity [(3)]	Density
	1-3	1-5	1-3	Microgreens cm^{-2}
Genotype (G)				
Broccoli raab	3.0 ± 0.1	2.2 ± 0.4 a	2.0 ± 0.0 a	4.1 ± 0.5
Broccoli	3.0 ± 0.1	2.0 ± 0.1 a	1.8 ± 0.4 ab	4.3 ± 1.5
Cauliflower	3.0 ± 0.1	1.6 ± 0.5 b	1.4 ± 0.5 b	3.7 ± 0.5
Nutrient solution strength (NSS)				
1/2	3.0 ± 0.1	2.1 ± 0.6	1.8 ± 0.4	3.9 ± 0.5
1/4	3.0 ± 0.2	1.9 ± 0.3	1.7 ± 0.5	4.0 ± 1.4
1/8	3.0 ± 0.1	1.8 ± 0.4	1.8 ± 0.4	4.1 ± 0.6
Significance				
G	NS	**	*	NS
NSS	NS	NS	NS	NS
G * NSS	NS	NS	NS	NS

[(1)] Development stage: 1—cotyledonary leaves; 2—true leaves (≤5 mm); 3—true leaves (>5mm). [(2)] Substrate coverage: 1—low; 2—good; 3—excessive. [(3)] Substrate uniformity: 1—not uniform in the centre; 2—uniform; 3—not uniform along the side. Significance: **, and * respectively for $p \leq 0.01$, and $p \leq 0.05$; NS, not significant. Means values (± standard deviation) within each column and main effect followed by different letters are significantly different, according to SNK test ($p = 0.05$).

3.2. Second Experiment

Even in this experiment, broccoli raab showed twice the number of true leaves compared to broccoli and cauliflower, with leaves longer than 1 cm and seedling height 7% lower compared to the

other species (Table 5). Cauliflower yield was 35% higher than broccoli raab and broccoli, beyond the chemical forms of nitrogen used (Table 5).

Table 5. Main effects of genotypes and the NH_4:NO_3 ratio on the number and length of true leaves, yield and height of microgreens (second experiment).

	True Leaves	Leaves Length	Yield	Seedling Height
	Number Seedling^{-1}	**cm**	**g cm^{-2}**	**cm**
Genotype (G)				
Broccoli raab	2.00 ± 0.10 a	1.39 ± 0.10 a	0.21 ± 0.02 b	9.09 ± 0.27 b
Broccoli	1.00 ± 0.10 b	0.40 ± 0.01 b	0.22 ± 0.03 b	9.84 ± 0.19 a
Cauliflower	1.00 ± 0.10 b	0.40 ± 0.01 b	0.29 ± 0.03 a	9.78 ± 0.48 a
NH_4:NO_3 (%) (R)				
5–95	1.33 ± 0.05	0.71 ± 0.46	0.23 ± 0.04	9.46 ± 0.65
15–85	1.33 ± 0.05	0.75 ± 0.53	0.25 ± 0.05	9.63 ± 0.35
25–75	1.33 ± 0.05	0.73 ± 0.50	0.24 ± 0.05	9.62 ± 0.47
Significance				
G	***	***	***	*
R	NS	NS	NS	NS
G * R	NS	NS	NS	NS

Significance: ***, and * respectively for $p \leq 0.001$ and $p \leq 0.05$; NS, not significant. Means values (± standard deviation) within each column and main effect followed by different letters are significantly different, according to SNK test ($p = 0.05$).

By using a NS with a NH_4:NO_3 25:75 molar ratio, microgreens showed the highest content of Cl^- and K^+. Cl^- was 75% higher in microgreens grown with a NH_4:NO_3 25:75 molar ratio than other samples, while K^+ was 6% and 19% higher in microgreens grown with a NH_4:NO_3 25:75 molar ratio than NH_4:NO_3 15:85 and 5:95 molar ratios, respectively. Between genotypes, broccoli showed a K^+ content 11% higher than other genotypes (Table 6).

Microgreens grown by using a NS with a NH_4:NO_3 15:85 molar ratio showed a SO_4^{2-} content 13% higher than the other molar ratio (Table 6). Between the genotypes, cauliflower showed a SO_4^{2-} content of 14% and 28% higher than broccoli and broccoli raab, respectively (Table 6). Ca^{2+} content was 14% higher in microgreens grown with the molar ratio NH_4:NO_3 5:95 than 25:75, while the average Mg^{2+} content was 0.3 g 100 g^{-1} DW, without differences between genotypes, NH_4:NO_3 ratios and their interaction (Table 6).

Broccoli raab had the lowest and highest nitrate content with the molar ratio NH_4:NO_3 5:95 and 15:85, respectively, while broccoli showed the lowest and highest nitrate content with NH_4:NO_3 25:75 and 15:85, respectively. No differences were found in nitrate content in cauliflower by using different NH_4:NO_3 ratios (Figure 3).

Cauliflower grown using a NS with a NH_4:NO_3 25:75 molar ratio showed the highest sodium content, which was 31% higher than the two other molar ratios used for the same genotype (Figure 4). Broccoli raab grown by using a NS with a NH_4:NO_3 5:95 molar ratio showed a sodium content 36% higher than the other molar ratios of the same genotype. The average sodium content in broccoli was 0.15 g 100^{-1} DW, without differences between NH_4:NO_3 molar ratios (Figure 4).

Table 6. Main effects of genotypes and NH_4:NO_3 ratio on dry matter and inorganic anion of microgreens (second experiment).

	Dry Matter	Cl^-	NO_3^-	SO_4^{2-}	Na^+	Mg^{2+}	K^+	Ca^{2+}
	g 100 g^{-1} FW	g 100 g^{-1} DW						
Genotype (G)								
Broccoli raab	6.29 ± 0.23	0.97 ± 0.25	8.03 ± 1.34 b	2.18 ± 0.18 c	0.16 ± 0.03 b	0.30 ± 0.03	2.23 ± 0.31 b	1.08 ± 0.11
Broccoli	5.81 ± 1.01	0.89 ± 0.31	8.29 ± 1.25 b	2.45 ± 0.36 b	0.15 ± 0.02 b	0.30 ± 0.02	2.51 ± 0.17 a	1.10 ± 0.11
Cauliflower	6.53 ± 1.41	0.91 ± 0.33	9.57 ± 0.55 a	2.80 ± 0.26 a	0.18 ± 0.03 a	0.30 ± 0.02	2.27 ± 0.29 b	1.06 ± 0.11
NH_4:NO_3 (%) (R)								
5–95	6.45 ± 0.32	0.78 ± 0.15 b	8.22 ± 1.50 b	2.36 ± 0.35 b	0.18 ± 0.02	0.32 ± 0.01	2.12 ± 0.29 c	1.16 ± 0.08 a
15–85	6.04 ± 0.94	0.74 ± 0.14 b	9.55 ± 0.59 a	2.68 ± 0.38 a	0.14 ± 0.01	0.30 ± 0.02	2.37 ± 0.21 b	1.08 ± 0.09 ab
25–75	6.09 ± 1.47	1.25 ± 0.24 a	8.13 ± 1.08 b	2.38 ± 0.34 b	0.17 ± 0.04	0.29 ± 0.03	2.52 ± 0.18 a	1.01 ± 0.11 b
Significance								
G	NS	NS	***	***	*	NS	*	NS
R	NS	**	**	**	NS	NS	***	*
G*R	**	NS	**	NS	**	NS	NS	NS

Significance: ***, **, and * respectively for $p \leq 0.001$, $p \leq 0.01$, and $p \leq 0.05$; NS, not significant. Means values (± standard deviation) within each column and main effect followed by different letters are significantly different, according to SNK test ($p = 0.05$).

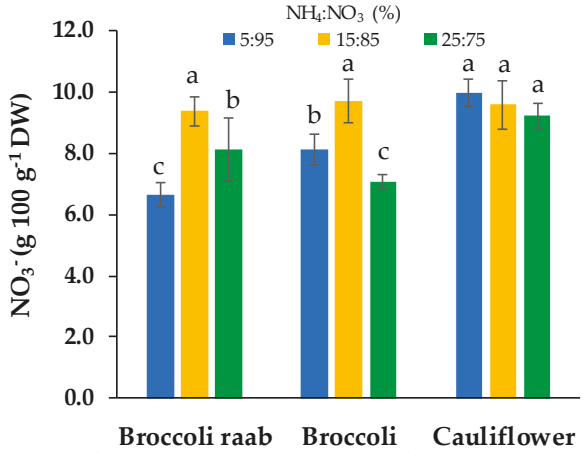

Figure 3. Nitrates (NO_3^-) content of three genotypes of microgreens grown by using a NS with three different NH_4^+:NO_3^- (%) molar ratios: 5:95, 15:85 and 25:75. Different letters indicate that mean values are significantly different, according to the SNK test (p = 0.05). Vertical bars represent ± standard deviation of mean values.

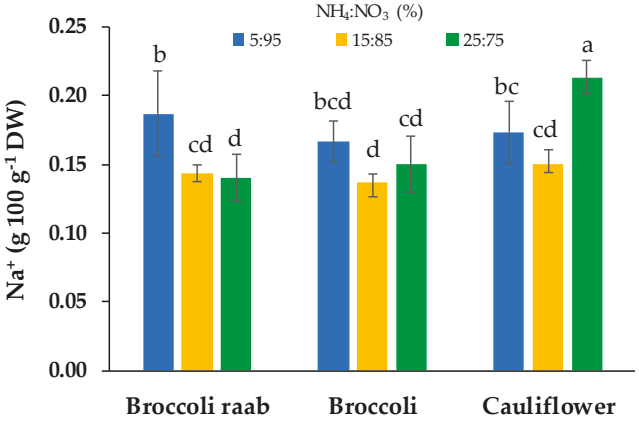

Figure 4. Sodium (Na^+) content of three genotypes of microgreens grown by using a NS with three different NH_4^+:NO_3^- (%) molar ratio: 5:95, 15:85 and 25:75. Different letters indicate that mean values are significantly different, according to the SNK test (p = 0.05). Vertical bars represent ± standard deviation of mean values.

The highest value of dry matter was obtained from cauliflower grown with a NH_4:NO_3 25:75 molar ratio that resulted in 66% higher content than the two other molar ratios of the same genotype (Figure 4). Broccoli showed a dry matter content 31% lower with a NH_4:NO_3 25:75 molar ratio compared to other molar ratios. The average content of dry matter in broccoli raab was 6.3 g 100^{-1} FW, without differences between NH_4:NO_3 molar ratios (Figure 5).

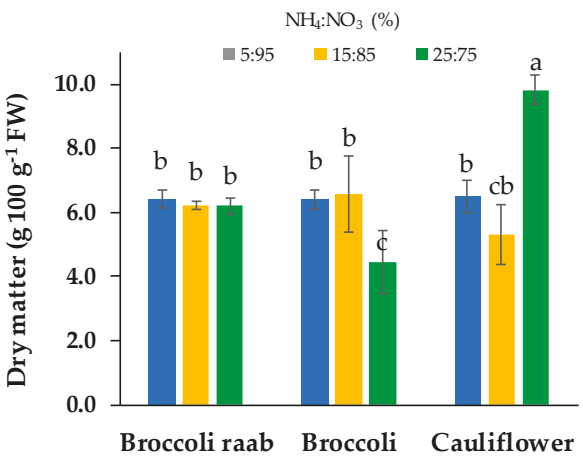

Figure 5. Dry matter content of three genotypes of microgreens grown by using a NS with three different NH_4^+:NO_3^- (%) molar ratios: 5:95, 15:85 and 25:75. Different letters indicate that mean values are significantly different, according to the SNK test (p = 0.05). Vertical bars represent ± standard deviation of mean values.

The average fiber content was 0.518 g 100 g^{-1} FW without significant differences between genotypes, NH_4:NO_3 molar ratios and their interaction (Table 7). Cauliflower showed an α-tocopherol content 194% higher than other genotypes, while broccoli raab showed a β-carotene content about 40% lower than other genotypes. The highest value of β-carotene was obtained with a NH_4:NO_3 25:75 molar ratio that resulted in 40% higher content than the two other molar ratios (Table 7).

Table 7. Effects of genotypes and NH_4:NO_3 ratio on fiber, protein, α-tocopherol and β-carotene content (second experiment).

	Fiber	Protein	α-Tocopherol	β-Carotene
	g 100 g^{-1} FW		mg 100 g^{-1} FW	
Genotype (G)				
Broccoli raab	0.355 ± 0.220	2.35 ± 0.21 b	2.02 ± 0.59 b	3.57 ± 0.95 b
Broccoli	0.517 ± 0.095	2.34 ± 1.09 b	5.08 ± 2.47 b	5.35 ± 1.54 a
Cauliflower	0.681 ± 0.259	3.12 ± 0.32 a	10.45 ± 7.71 a	6.48 ± 2.43 a
NH_4:NO_3 (%) (R)				
5–95	0.493 ± 0.073	2.31 ± 0.19	4.29 ± 3.76	4.37 ± 1.09 b
15–85	0.459 ± 0.229	2.40 ± 0.21	4.98 ± 4.64	4.60 ± 1.14 b
25–75	0.600 ± 0.337	3.01 ± 1.01	7.86 ± 7.58	6.29 ± 2.69 a
Significance				
G	NS	**	*	**
R	NS	NS	NS	**
G*R	NS	*	NS	NS

Significance: **, and * respectively for $p \leq 0.01$, and $p \leq 0.05$; NS, not significant. Means values (± standard deviation) within each column and main effect followed by different letters are significantly different, according to SNK test (p = 0.05).

As for protein content, cauliflower grown with a NH_4:NO_3 25:75 molar ratio gave the highest value, which resulted in 79% higher content than the other nutrient solutions (Figure 6).

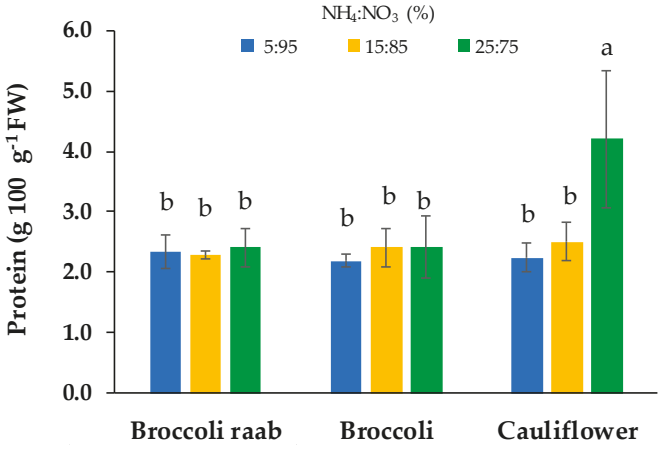

Figure 6. Protein content of three genotypes of microgreens grown by using a NS with three different NH_4^+:NO_3^- (%) molar ratios: 5:95, 15:85 and 25:75. Different letters indicate that mean values are significantly different, according to the SNK test ($p = 0.05$). Vertical bars represent ± standard deviation of mean values.

4. Discussion

In this study, we produced microgreens of some Brassicaceae genotypes by using a hydroponic system to evaluate the effects of element concentration and chemical form of nitrogen in the nutrient solution on yield and some quality traits. We conducted an exploratory experiment by using a NS type-like Hoagland and Arnon [29] but at three different strengths (1/2 strength, 1/4 strength and 1/8 strength). This, we started from the fact that some authors reported the use of a quarter-strength Hoagland nutrient solution [19,20], while other authors reported the use of a half-strength Hoagland nutrient solution [15,16] as well as three different strengths of nutrient solution [22]. Therefore, considering the short growth cycle of microgreens, we decided to also evaluate if nutrient concentration lower than half strength may satisfy seedling needs, without negatively affecting yield and other important parameters. In this context, it is important to highlight that the optimal choice of element concentration in the NS may allow one to reduce production costs and environmental impact. In the first experiment, we observed that growing parameters were not affected by NS strength (Table 4). In addition, yield was not affected by the NS strength except for broccoli raab, which showed a lower yield when 1/8 NS was used (Figure 2) and for this cultivar, the growth rate was faster than for broccoli and cauliflower (Table 4). On average, we found that seedling height significantly decreased when passing from NS at 1/2 strength to NS at 1/8 strength (Table 3). Considering that the harvesting of microgreens is usually done manually, the higher the seedling height, the easier the harvesting can be made. Therefore, for the second experiment, we decided to use a NS at 1/2 strength but with three different NH_4:NO_3 molar ratios to evaluate the effect of another aspect of fertigation on physiological behaviour and some quality traits of different Brassicaceae microgreens. The choice of NS at 1/2 strength instead of other ones was also made by considering the higher temperature and photosynthetic photon flux (PPF) forecasted for the second experiment than the first one. Effectively, the rate of nutrient uptake was related to current seedling nutrient demand, positively correlated with PPF and air temperature [31].

By changing the NH_4:NO_3 molar ratio, no differences were found on yield and growing parameters (Table 5), while significant differences were found in regards to dry matter and content of inorganic cations, proteins and β-carotene (Tables 6 and 7). For dry matter, nitrates, sodium and proteins, we observed important interactions between genotypes and the molar ratio between the chemical forms

of nitrogen. The most abundant cation in all the microgreens samples was K^+, followed by Ca^{2+}, Mg^{2+} and Na^+, while, in regards to anion content, NO_3^- was followed by SO_4^{2-} and Cl^- (Table 6). A similar mineral composition was observed in previous studies [17,32]. In regards to the differences in nitrates content (Figure 3), Santamaria [23] reported that the large variation in nitrate accumulation among plant species could be associated with genetic factors. At the same time, different genotypes may show different nitrate uptake, translocation and accumulation in the vacuoles of mesophyll cells [33]. In agreement, we observed that by using a NS with the NH_4:NO_3 molar ratio of 5:95, broccoli raab showed a nitrate content lower than other NH_4:NO_3 molar ratios, while broccoli showed the lowest nitrates content when the NS with the NH_4:NO_3 molar ratio of 25:75 was used (Figure 3). At same time, no differences in nitrates content were found by changing the NH_4:NO_3 molar ratio in cauliflower (Figure 3). These results suggest that the nitrate content in different *Brassica* microgreens can be affected by the interaction between genotypes and the NH_4:NO_3 molar ratio in the NS. This is in agreement with Dikson and Fisher [34], who observed that genotypes had a central role in anion and cation uptake by varying root zone pH. In the same way, during this study, changing the NH_4:NO_3 molar ratio and substrate/root zone pH changes influenced cation and anion (nitrates) uptake differently for each genotype.

From a commercial point of view, it could be interesting to evaluate the nitrate content in microgreens observed in our study in relation to the tolerable levels of nitrates in foodstuffs. On average, we found a content of 5051, 4816 and 6249 mg NO_3^- kg^{-1} FW, respectively for broccoli raab, broccoli and cauliflower (processed data from Table 6). It is important to note that for Brassicaceae species the European Regulation (EU) No 1258/2011 [35] reports maximum levels of nitrate only for the "rucola" group (*Eruca sativa*, *Diplotaxis* spp, *Brassica tenuifolia*, *Sisymbrium tenuifolium*). European Regulation fixed a maximum level of 7000 mg NO_3 kg^{-1} FW for "rucola" vegetables harvested from 1st of October to 31st of March (the period of our study), and a maximum level of 6000 mg NO_3 kg^{-1} FW in the other year period. Considering these maximum levels, our results suggest that by changing the NH_4:NO_3 molar ratio in the NS, it is possible to produce microgreens of broccoli raab, broccoli and cauliflower without negatively affecting an important commercial characteristic such as the nitrate content.

In regards to the nutritional quality, we found that all three genotypes of *Brassica* microgreens showed a high content of mineral elements (Table 6). This is agreement with several authors [17,32,36,37] confirming that microgreens can be considered as a good source of minerals in the human diet. Apart from the content of mineral elements, microgreens can provide higher amounts of other nutrients compared to their mature leaf counterparts [1]. To this end, we found that 100 g of mature cauliflower supplies about 2 g of fibers, 1.92 g of proteins and 0.08 mg of α-tocopherol [38]. The same serving size of mature broccoli supplies 2.6 g of fibers, 2.82 g of proteins and 0.78 mg of α-tocopherol [39], while 100 g mature broccoli raab supply 2.7 g of fibers, 3.17 g of proteins, and 1.62 mg of α-tocopherol [40]. Results of the present study show a fiber content (Table 7) much lower than mature plants independently of genotypes and the NH_4:NO_3 molar ratio. Therefore, according to Renna et al. [9], microgreens of this study can be considered as a low content fiber food for subjects with gastrointestinal disorders, such as bowel colon syndrome. Regarding protein content, microgreens showed values similar to mature *Brassica* vegetables with the exception of micro-cauliflower fertigated by using a NS with a NH_4:NO_3 molar ratio of 25:75, which showed a higher protein content than mature cauliflower. This, could be due to the fact that the NH_4:NO_3 molar ratio of 25:75 caused an increase in dry matter content compared with other treatments and proteins are one of the major constituents of the dry matter [41].

α-Tocopherol is the most common and biologically active form of vitamin E. Effectively, although the term vitamin E can refer to different types of tocopherols and tocotrienols, it should be considered the selective degradation and excretion of other vitamin E forms and the selective retention of α-tocopherol, mediated by the hepatic α-tocopherol transfer protein (α-TTP) [42]. In our study, we observed a higher α-tocopherol content, independently of the NH_4:NO_3 molar ratio, in microgreens than in the mature counterparts, especially in micro cauliflower (Table 7). α-Tocopherol represents part of the fat-soluble

antioxidant system of the cell, since it terminates the chain reaction of lipid peroxidation. Vitamin E deficiency is associated with a progressive necrosis of the nervous system and muscle. In this context, it is important to note that the recommended dietary allowance (RDA) of vitamin E (α-tocopherol) for people aged 14 years and over, including pregnant women, is 15 mg per day [42]. Therefore, 100 g of microgreens produced in this study can satisfy about 70, 34 and 13% of the RDA, respectively, for micro cauliflower, micro broccoli and micro broccoli raab.

β-Carotene is the principal pro-vitamin A carotenoid considering that its symmetrical chemical structure always provides vitamin A regardless of the metabolic process. Other forms of provitamin A are α-carotene, γ-carotene and β-cryptoxanthin. β-Carotene is the most abundant dietary carotenoid present in yellow-orange fruits and vegetables, and green leafy vegetables. In humans, it plays a potent antioxidant role known to prevent oxidative damage to biological membranes by quenching free radicals [42]. Mature cauliflower lacks β-carotene [38], while 100 g of mature broccoli and broccoli raab contain 0.36 and 1.57 mg of β-carotene, respectively [39,40]. Therefore, results of the present study show a higher β-carotene content in microgreens than the mature counterparts, especially by using a NH_4:NO_3 molar ratio of 25:75 (Table 7). In a study aimed to evaluate the nutrient composition of ten culinary microgreens, Ghoora et al. [43] found a β-carotene content ranging from 3.1 to 9.1 mg 100 g^{-1} FW. Our results are in agreement with these authors, confirming that microgreens can be considered a good source of β-carotene, although the amount can vary depending on genotype.

5. Conclusions

All three *Brassica* genotypes can be considered suitable for microgreen production, although micro cauliflower showed the highest yield, as well as a higher content of some mineral elements and α-tocopherol compared to other genotypes, while micro broccoli raab showed the fastest growth rate. The use of a nutrient solution type-like Hoagland and Arnon at half strength allowed us to obtain both high yield and desirable seedling height. By changing the NH_4:NO_3 molar ratio in the nutrient solution, no differences were found on yield and growing parameters, while the highest β-carotene content was found by using a nutrient solution with a NH_4:NO_3 molar ratio of 25:75. The lowest nitrate content was found in micro broccoli by using a nutrient solution with a NH_4:NO_3 molar ratio of 25:75 and in micro broccoli raab by using a nutrient solution with a NH_4:NO_3 molar ratio of 5:95. Micro cauliflower grown by using a nutrient solution with a NH_4:NO_3 molar ratio of 25:75 showed the highest dry matter and protein content. From a commercial point of view, we highlight the possibility of producing microgreens of broccoli raab, broccoli and cauliflower by changing the NH_4:NO_3 molar ratio in the nutrient solution without negatively affecting an important characteristic such as the nitrate content. It could be interesting to assess the optimal strength and NH_4:NO_3 molar ratio of the nutrient solution to obtain the best yield performance and quality for microgreens of other botanic families. Moreover, quality evaluation during cold storage of fresh-cut microgreens obtained by using nutrient solutions with different strengths and NH_4:NO_3 molar ratios may be a possible next goal.

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