

Article

Biochemical and Antioxidant Characterization of Pigment-Deficient *Chlorella vulgaris* Flours and the Impact of Fermentation: Comparative Insights from Green, Honey, and White Variants

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Abstract

This study investigated the biochemical composition and antioxidant potential of flours from pigment-deficient *Chlorella vulgaris* variants (honey and white) and wild-type (green) and the impact of lactic acid bacteria–yeast co-culture fermentation. The three variants were characterized for composition, total polyphenol (TPC) and flavonoid (TFC) contents, antioxidant capacity (DPPH, FRAP, and ORAC assays), and reactive oxygen species production in HT-29 intestinal cells. All extracts were noncytotoxic up to 100 µg/mL. Among all variants, the green showed the highest native TPC, TFC, and overall antioxidant activity. TPC and TFC were similar between honey and white, while FRAP was higher in honey and ORAC was higher in white. Biomasses were subsequently fermented for 24 h using *Lactiplantibacillus plantarum* CR L1 or *Levilactobacillus brevis* L204 with either *Saccharomyces cerevisiae* TRE Y100 or *Kluyveromyces marxianus* MK Y55. Fermentation resulted in significant pH reduction and increases in titratable acidity and lactic acid production, particularly in co-cultures involving *K. marxianus*. However, the effects on antioxidant properties were strongly matrix-dependent, with significant increases in TPC and antioxidant activity observed only in the white variant. Overall, pigmentation and microbial pairing emerged as key determinants of metabolic outcomes. These findings highlight the potential of co-culture fermentation to enhance the bioactive profile of pigment-deficient *C. vulgaris*, supporting their application in functional foods.

Keywords: *Chlorella vulgaris*; pigment-deficient strains; polyphenols; antioxidants; alternative protein; fermentation



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1. Introduction

The research pursuit of innovative biotechnological strategies to enhance the nutritional and functional value of food products has intensified in recent years. Among emerging resources, *Chlorella vulgaris*, a unicellular microalga, stands out for its exceptional protein content, essential amino acids, polyunsaturated fatty acids, vitamins, minerals, and a diverse array of antioxidant compounds, including carotenoids, chlorophyll, and polyphenols [1–3]. Despite its nutritional richness, the conventional green variant of *C.*

vulgaris is often limited in food applications due to its intense green color, grassy taste, and sea-like aroma, which can negatively impact consumer acceptance [4].

To address these sensory challenges, pigment-deficient variants such as “white” and “honey” *C. vulgaris* have been developed through selective breeding. These variants retain the nutritional benefits of the species while exhibiting milder color and flavor profiles, making them more suitable for incorporation into a wider range of foods. The honey variant, characterized by a substantial reduction in chlorophyll and the presence of yellow carotenoids like lutein, and the white variant, almost devoid of visible pigments, both demonstrate increased protein content and altered lipid profiles compared to the wild-type [5,6].

While the green variant’s bioactive potential, including antioxidant, anti-inflammatory, and immunomodulatory effects, is well-documented [3,7,8], research on the bio-functional properties of pigment-deficient ones remains limited. Recent studies indicate that incorporating honey and white *C. vulgaris* into food products can enhance nutritional profiles and antioxidant capacity without compromising sensory quality [9–11]. However, these pigment-deficient variants generally exhibit lower intrinsic antioxidant activity than the green one, likely due to reduced pigment content.

Fermentation has emerged as a promising and sustainable approach to further improve the functional and sensory attributes of microalgal biomass, including *C. vulgaris*. Lactic acid fermentation has been shown to significantly reduce off-flavors and undesirable volatile compounds, such as aldehydes, ketones, pyrazines, and terpenes. At the same time, fermentation enhances the production of esters and other pleasant aroma compounds, thereby improving the overall sensory profile of *C. vulgaris*-based foods [12]. Multiple strains of lactic acid bacteria (LAB) have demonstrated the ability to grow robustly on *C. vulgaris* biomass, effectively modulating its volatile profile and making it more appealing for food applications [12]. Beyond sensory improvements, fermentation also enhances the nutritional and functional properties of *C. vulgaris*. The process promotes the breakdown of the rigid microalgal cell wall, increasing the release and bioaccessibility of intracellular nutrients and bioactive compounds such as polyphenols, peptides, and antioxidants [13]. Recent studies have shown that the fermentation of *C. vulgaris* with LAB and yeasts can significantly increase its total polyphenol content and antioxidant activity. Moreover, the inclusion of *C. vulgaris* in fermentation substrates can stimulate microbial growth and viability, further supporting the development of functional foods with enhanced health benefits [14–16].

This study aimed to systematically compare the biochemical composition and antioxidant potential of three pigment variant flours of *C. vulgaris* (white, honey, and green) and to evaluate the impact of fermentation on their antioxidant properties. By elucidating the interplay between pigmentation, fermentation, and bioactive compound availability, this work sought to inform the targeted use of *C. vulgaris* flour as a sustainable ingredient in functional foods and nutraceuticals.

2. Materials and Methods

2.1. Chemicals, Microalgal Biomass and Bioactive Compound Extraction

All chemicals and reference standards used in this study were of analytical or HPLC grade. Folin–Ciocalteu reagent, gallic acid, catechin hydrate, sodium carbonate, sodium acetate, potassium chloride, sodium hydroxide, phosphate-buffered saline (PBS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), acetic acid, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), sodium nitrite (NaNO_2), aluminum chloride (AlCl_3), and fluorescein sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethanol

and methanol were supplied by VWR (Radnor, PA, USA), whereas hydrochloric acid was purchased from Merck (Readington, NJ, USA). Analytical standards of all analytes (HPLC purity ≥ 99.5), including gallic acid, protocatechuic acid, vanillic acid, rutin, vitexin, trans-coumaric acid, trans-ferulic acid, and quercetin—as well as HPLC-grade methanol, gradient-grade acetonitrile, and ACS-grade phosphoric acid—were acquired from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Double-deionized water (18.2 M Ω /cm, 20 °C) was produced using a Simplicity 185 purification system (Millipore SAS, Molsheim, France).

Pure flours of the three variants of *C. vulgaris*, green (smooth, Lot N° L2450029), honey (Lot N° L23HC152), and white (Lot N° L23WC066), free from additives or preservatives, were supplied by BioSyntex S.r.l., Bologna, Italy and were used for biochemical characterization and fermentation trials. The nutritional composition of the *Chlorella* powder biomasses, as provided by the supplier, is reported in Table 1.

Table 1. Nutritional composition of white, honey and green *C. vulgaris* based on supplier-provided specifications (BioSyntex S.r.l., Bologna, Italy).

Parameters	White <i>C. vulgaris</i>	Honey <i>C. vulgaris</i>	Green <i>C. vulgaris</i>	Methods
Macronutrients (g/100 g)				
Protein	35–50	30–35	25–40	Nitrogen determination
Lipids	6–12	6–13	6–9	Soxhlet
Total Carbohydrates	<35	<45	<36	Calculus
Dietary fiber	5–25	10–20	>15	Enzymatic–gravimetric
Ash	4–7	4–7	3.5–7	Burning at 525 °C
Moisture	2–6	1–4	3–5	Drying at 105 °C
Pigments (mg/100 g)				
Total Carotenoids	-	80–120	130–250	HPLC-DAD
Fatty Acid Profile (% of Total Lipids)				
Saturated fatty acids	25–35	30–40	30–40	Calculus
Monounsaturated fatty acids	18–25	30–40	15–20	Calculus
Polyunsaturated fatty acids	45–55	25–35	45–55	Calculus
Omega-3 fatty acids	1–5	5–10	5–10	Calculus
Omega-6 fatty acids	40–50	20–30	20–30	Calculus
Palmitic acid (C 16:0)	25–30	20–30	20–25	Extraction/GC-FID
Oleic acid (C 18:1w9c)	15–20	30–40	-	Extraction/GC-FID
Linoleic acid (C 18:2w6cc)	40–50	20–30	25–35	Extraction/GC-FID
Alpha-linolenic acid (C 18:3w3c)	1–5	5–10	5–10	Extraction/GC-FID
Minerals/Trace Elements (mg/100 g)				
Potassium	500–1000	400–600	500–1000	FAAS
Phosphorous	800–1500	1000–2000	1000–2000	UV-VIS
Calcium	200–400	200–300	400–600	Spectrophotometry
Sodium	50–100	150–300	250–450	FAAS
Magnesium	100–200	100–200	80–150	FAAS
Iron	5–15	2–4	7–15	ICP
Zinc	10–20	20–30	40–60	ICP/MS
Vitamins (mg/100 g)				
Vitamin A Retinol	<0.05	<0.02	0.3–0.5	HPLC/UV-VIS
Vitamin B1 Thiamin	0.5–1.0	0.1–0.3	0.1–0.2	LC/FLD
Vitamin B2 Riboflavin	1.0–2.0	0.9–1.3	0.5–1.0	LC/FLD
Vitamin B3 Niacin	20–40	7.0–10	20–30	LC/FLD
Vitamin B6 Pyridoxal phosphate	0.8–1.5	0.5–1.0	1.0–1.5	LC/FLD
Vitamin B12	0.03–0.08	0.02–0.03	0.01–0.02	LC/DAD
Vitamin E Tocopherol	1.0–2.0	7.0–10	0.5–1.0	HPLC
Vitamin C Ascorbic Acid	50–100	5–15	3–5	HPLC/UV-VIS

For extraction, 250 mg of flour was dissolved in 5 mL of 80% ethanol, gently stirred overnight in the dark, and centrifuged at $2800 \times g$ for 25 min at 4 °C using a Jouan CR 3i centrifuge (Jouan SA, St. Herblain, France). The supernatants were collected and preserved at −20 °C until further analysis. Each extraction was conducted in triplicate to ensure accuracy and reproducibility.

2.2. Determination of Total Polyphenol Content and Antioxidant Activity

Total polyphenol content (TPC) and antioxidant activity were determined on all raw and fermented samples.

TPC was measured using the Folin–Ciocalteu colorimetric method, as described by [16]. A volume of 100 µL of each microalgal extract was mixed with 500 µL of a 1:10 diluted Folin–Ciocalteu reagent and incubated at room temperature in the dark for 5 min. Next, 400 µL of 0.7 M sodium carbonate (Na_2CO_3) was added to the mixture and incubated for an additional 2 h under the same conditions. After this reaction period, the absorbance of each sample was read at 760 nm using a FLUOstar Omega (BMG LABTECH, Ortenberg, Germany) microplate reader. TPC was then calculated from a gallic acid standard curve (0–200 µg/mL) and expressed as milligrams of gallic acid equivalent (mg GAE) per gram of dry weight (dw).

Antioxidant capacity was evaluated using three complementary assays, including the 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC), based on the procedure described by [17]. For DPPH, an aliquot of 25 µL of each suitably diluted extract was mixed with 975 µL of a 60 µM ethanolic DPPH solution. The reaction mixtures were kept in the dark at room temperature for 30 min. Following incubation, absorbance was recorded at 517 nm using a FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) to evaluate the degree of DPPH radical reduction. The results were expressed as micrograms of Trolox equivalent (TE)/grams dried weight (dw).

For FRAP, a volume of 33 µL of each algal extract was added to 967 µL of the FRAP reagent, freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl, and 20 mM ferric chloride hexahydrate ($\text{FeCl}_3 \times 6\text{H}_2\text{O}$) in a 10:1:1 (*v/v/v*) ratio, and the absorbance was measured at 593 nm after 6 and 30 min of incubation in the dark at room temperature using a FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany). Antioxidant power was quantified using a calibration curve obtained from serial dilutions of ferrous sulfate ($\text{FeSO}_4 \times 7\text{H}_2\text{O}$), and results were expressed as micromoles of Fe^{2+} equivalent per gram of dry weight ($\mu\text{mol Fe}^{2+}/\text{g dw}$).

For ORAC, an aliquot of 800 µL of 40 nM fluorescein solution (prepared in 75 mM phosphate buffer, pH 7.4), which served as the fluorescent probe, was mixed with 100 µL of appropriately diluted algal extract or Trolox standard (50 µM). To initiate the reaction, 100 µL of freshly prepared 400 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) solution was added as the peroxy radical generator. The decline in fluorescence intensity was recorded every minute for 90 min at 37 °C at an excitation wavelength of 485 nm and emission at 520 nm using the FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany). Antioxidant capacity was calculated by measuring the area under the fluorescence decay curve (AUC), and results were expressed as micromoles of Trolox equivalent per gram of dry weight ($\mu\text{mol TE/g dw}$).

2.3. Total Flavonoid Content and Phenolic Compound Profiling

Total flavonoid content (TFC), phenolic compound profiling and cell-based antioxidant activity were performed only on the raw *Chlorella* samples.

Flavonoids were quantified using the aluminum chloride colorimetric method, as described by [17]. A volume of 100 µL of each sample extract was mixed with 400 µL of distilled water, followed by the addition of 30 µL of 5% sodium nitrite (NaNO₂). After 5 min of incubation at room temperature, 30 µL of 10% aluminum chloride (AlCl₃) solution was added, and the reaction proceeded for 6 min. Next, 200 µL of 1 M sodium hydroxide (NaOH) was added to neutralize the reaction, and an additional 240 µL of deionized water was added to reach a final volume of 1 mL. The absorbance of the resulting solution was measured at 430 nm using a FLUOstar Omega microplate reader (BMG LABTECH, Ortenberg, Germany) after 30 min. The total flavonoid concentration was expressed as milligrams of catechin equivalents (mg CE/g dw), calculated using a standard curve prepared from catechin solutions ranging from 0 to 800 µg/mL.

For phenolic profiling, compounds were extracted from the three *C. vulgaris* variants—green, honey, and white—using 80% methanol (*v/v*) at room temperature. The extraction was performed for 2 h under continuous agitation using a Unimax 2010 horizontal shaker (Heidolph Instruments GmbH, Schwabach, Germany). Following extraction, the mixtures were filtered through Munktell No. 390 qualitative filter paper (Munktell & Filtrak GmbH, Bärenstein, Germany), and the clarified extracts were collected into sealed 20 mL polyethylene vials for storage. Prior to chromatographic analysis, the samples were further filtered using a 0.22 µm PVDF syringe filter (Q-Max, 25 mm; Frisette ApS, Knebel, Denmark).

Individual stock solutions were prepared at a concentration of 1.0 mg/mL in acetonitrile and stored at 4 °C protected from light. Working standard solutions were freshly prepared by serial dilution of stock solutions with a mobile phase to obtain calibration levels. The HPLC-DAD method was validated in terms of limits of detection (LOD), quantification (LOQ), and linearity (Table 2). LOD and LOQ were determined at S/N ratios of 3 and 10, respectively. Linearity was evaluated using 4-point calibration curves constructed by plotting peak area versus analyte concentration.

Table 2. Validation parameters of HPLC-DAD analysis.

Analytes	LOD (ng/mL)	LOQ (ng/mL)	Concentration Range and Linearity
Gallic acid	238.9	796.4	400–1600 ng/mL; R ² = 0.995
Protocatechuic acid	138.7	462.3	400–1600 ng/mL; R ² = 0.997
Vanillic acid	157.6	525.2	400–1600 ng/mL; R ² = 0.995
Rutin	340.3	1134.4	400–1600 ng/mL; R ² = 0.993
Trans-p-Coumaric acid	207.3	691.1	400–1600 ng/mL; R ² = 0.995
Vitexin	30.0	100.0	500–2000 ng/mL; R ² = 0.999
Trans-Ferulic acid	545.5	1818.3	400–1600 ng/mL; R ² = 0.996
Quercetin	299.5	998.5	400–1600 ng/mL; R ² = 0.997

High-performance liquid chromatography with diode array detection (HPLC-DAD) was carried out using an Agilent 1260 Infinity system (Agilent Technologies GmbH, Waldbronn, Germany), comprising a quaternary solvent pump (G1311B), degasser, autosampler (G1329B), column compartment (G1316A), and DAD detector (G1315C). Separation was achieved using a Purosphere[®] RP-C18 analytical column (250 mm × 4 mm, 5 µm; Merck KGaA, Darmstadt, Germany). The mobile phase consisted of solvent A (acetonitrile) and solvent B (0.1% phosphoric acid in deionized water), delivered according to the following gradient profile: 0–1 min, isocratic at 20% A and 80% B; 1–5 min, linear increase to 25% A; 5–15 min, increased to 30% A; and 15–25 min, ramped to 40% A. A 3 min post-run was included for re-equilibration. The flow rate was maintained at 1 mL/min with an injection volume of 10 µL. The column temperature was set at 30 °C, and the autosampler tray was maintained at 4 °C. Detection wavelengths were optimized for specific

compounds: 265 nm for vanillic acid, rutin, and protocatechuic acid; 320 nm for gallic acid, vitexin, trans-p-coumaric acid, and trans-ferulic acid; and 372 nm for and quercetin. Data acquisition and analysis were performed using Agilent OpenLab ChemStation software (version C.01.06) for LC 3D systems.

2.4. Cellular Viability and Intracellular ROS Production

For cell culture maintenance and cell viability, human colorectal adenocarcinoma cells (HT-29; DSMZ n. ACC 299, Braunschweig-Süd, Germany) were cultured in Dulbecco's Modified Eagle Medium/nutrient mixture F-12 supplemented with 10% FBS and 1% penicillin–streptomycin and maintained at 37 °C in a humidified atmosphere containing 5% CO₂.

To evaluate potential cytotoxicity, HT-29 cells were seeded into 96-well plates at a density of 2×10^4 cells per well and incubated for 24 h. Cells were then treated with various concentrations, 25, 50 and 100 µg/mL of *Chlorella* extracts, for an additional 24 h. Cell viability was determined using the MTT assay [18]. Briefly, 100 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (0.5 mg/mL in PBS) was added to each well and incubated for 1 h at 37 °C in 5% CO₂. Upon incubation, the medium was removed, and the resulting formazan crystals were solubilized with 100 µL of 10% DMSO/90% isopropanol. The amount of the dye released from the cells was quantified by reading the absorbance at 540 nm. The experiment was performed with six duplicates and results were expressed as a percentage of viable cells relative to the untreated cells (control).

Intracellular reactive oxygen species (ROS) levels were determined using the DCFH-DA dye method to evaluate the effect of the extracts on basal cellular redox status after 24 h exposure [19,20]. HT-29 cells were seeded in black 96-well plates at a density of 2×10^4 cells per well and treated with different concentrations (25, 50 and 100 µg/mL) of microalgae extracts for 24 h. Cells were then rinsed with PBS and incubated with 15 µM DCFH-DA for 30 min at 37 °C in the dark. ROS production was detected by measuring the fluorescence at 485 nm excitation and 535 nm emission using a FLUOstar Omega microplate reader. The experiment was performed with six replicates, and the results were expressed as the percentage of ROS levels compared to untreated cells.

2.5. Microorganisms, Culture Preparation and Fermentation Setup

For the fermentation of *Chlorella* flours, four microbial strains with Qualified Pre-emption of Safety (QPS) status were used. The two lactic acid bacteria (LAB) were *Lactiplantibacillus plantarum* CR L1, isolated from cricket powder fermentation [21], and *Levilactobacillus brevis* TEN L204, isolated from mealworm powder fermentation. The two yeasts were *Kluyveromyces marxianus* MK Y55, isolated from fermented milk [22], and *Saccharomyces cerevisiae* TRE Y100, isolated from brewer's spent grains. All strains belong to the Center for Fermented Food and Beverages Innovation—CIBAF culture collection, Florence, Italy. The species were selected for their technological properties and capacity to improve nutritional quality [23,24], and the single strain (lactic acid bacteria and yeasts) properties were previously evaluated in white *Chlorella vulgaris* powder fermentations (Supplementary Table S1). Strains were selected on the basis of acidification, carbohydrate consumption, and metabolite production. Lactic acid bacteria were routinely propagated for 24 h at 30 °C in MRS (Thermo Fisher Scientific Inc., Waltham, MA, USA) liquid medium before being used as inoculum. Yeasts were cultured for 24 h at 30 °C in YPD medium (10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of dextrose). Microorganism enumeration was performed by the plate count method after an appropriate dilution in MRS agar medium and MYPG agar a medium (5 g/L malt extract, 3 g/L yeast extract, 5 g/L

meat extract, 10 g/L glucose, and 20 g/L agar, bromocresol green 0.022) for LAB and yeasts, respectively, using the pour plate method. LAB colonies were counted after incubation for 48–72 h at 30 °C under anaerobic conditions, and yeast colonies were counted after incubation for 48 h at 30 °C under aerobic conditions.

2.6. Fermentation Procedures and Analytical Determinations

Flours of *Chlorella* (green, honey, and white) were suspended in sterile bidistilled water (1:2, *w/v*) to obtain homogeneous suspensions. The initial pH of each suspension was measured prior to inoculation. Microorganisms were grown for 24 h and counted by a counting chamber to obtain an initial cell density in the doughs of about 7.7 log CFU/mL for each LAB species and about 6.7 log CFU/mL for each yeast species. Fermentations were carried out at 30 °C for 24 h under static conditions. To evaluate the effect of different microbial co-cultures on the fermentation process, four combinations of lactic acid bacteria and yeast strains were applied to the three *Chlorella* variants (Table 3).

Table 3. Microbial combinations used for the fermentation of *Chlorella vulgaris* variants.

Combination	Lactic Acid Bacteria	Yeast
C1	<i>L. plantarum</i> CR L1	<i>K. marxianus</i> MK Y55
C2	<i>L. plantarum</i> CR L1	<i>S. cerevisiae</i> TRE Y100
C3	<i>L. brevis</i> TEN L204	<i>K. marxianus</i> MK Y55
C4	<i>L. brevis</i> TEN L204	<i>S. cerevisiae</i> TRE Y100

Fermentation progress was monitored over 24 h through physicochemical and microbiological analyses. The pH was measured using a pH meter (Metrohm Italiana Srl, Varese, Italy), and total titratable acidity (TTA) was determined by weighing 10 g of dough samples and homogenizing them with 90 mL of distilled water for 3 min; the TTA was then expressed as the amount (mL) of 0.1 N NaOH to achieve a pH of 8.5 [21]. Residual carbohydrates (glucose and fructose), lactic and acetic acids, glycerol, and ethanol were quantified by high-performance liquid chromatography (HPLC) analysis (Varian Inc, Palo Alto, CA, USA), according to [25]. Separation was obtained with a Rezex ROA organic acid H+ column (300 × 7.8 mm; Phenomenex, Castel Maggiore, Bologna, Italy), connected to a refractive index detector (Knauer K-2301, GmbH, Berlin, Germany) and UV detector ($\lambda = 210$). Elution was performed at 65 °C with 0.013 N H₂SO₄ eluent at a flow rate of 0.6 mL/min. Data were collected and analyzed by using the Galaxie software (version 1.9.302.530, Varian Inc., Palo Alto, CA, USA). The fermentation quotient (FQ), defined as the molar ratio between lactic and acetic acid, was calculated to assess the metabolic balance of the lactic fermentation. At the end of fermentation (24 h), the samples were dried in a static oven at 60 °C for 24 h and stored at −20 °C until further biochemical and antioxidant analyses.

2.7. Statistical Analysis

All data were expressed as mean ± standard deviation (SD) from at least three independent experiments (*n* = 3). Statistical analyses were performed using SPSS software (version 30.0; IBM Corp., Armonk, NY, USA). Differences between groups were assessed by one-way analysis of variance (ANOVA, for multiple groups) followed by Tukey's Test or Student's *t*-test (for comparisons between two groups). A *p*-value of less than 0.05 was considered statistically significant. The reproducibility of repeated experiments was evaluated by calculating the coefficient of variation (CV%), and the resulting values for all parameters are provided in the Supplementary Material Tables S2–S6.

3. Results and Discussion

3.1. Comprehensive Biochemical Characterization of Three *C. vulgaris* Variants

Table 1 reports the macronutrient composition, pigment content, fatty acid profile, mineral/trace element concentration, and vitamin levels of the green, honey and white *C. vulgaris* variants used in the present study, based on data provided by the supplier.

The white *C. vulgaris* exhibited the highest protein content, followed by honey and green. This trend was in accordance with recent findings that pigment-deficient variants, such as white and honey, redirect metabolic resources from pigment synthesis toward protein accumulation, resulting in elevated protein levels compared to the green one [6,11]. Carbohydrate content was greater in the honey and green variants. Dietary fiber was most abundant in green *C. vulgaris*, consistent with its robust cell wall structure. Ash and moisture contents were comparable across all variants, indicating similar mineral and water retention capacities [11,26].

Pigment analysis confirmed the absence of carotenoids in white *C. vulgaris*, consistent with the loss of pigment biosynthetic pathways in this variant [6]. In contrast, green and honey variants retained substantial carotenoid levels (130–250 mg/100 g and 80–120 mg/100 g, respectively), with green *C. vulgaris* also displaying high chlorophyll content. These results are in line with previous reports that the green variant is rich in chlorophylls and carotenoids such as lutein and β -carotene, while the honey accumulates lutein as the primary pigment [6,11].

Fatty acid profiling revealed notable differences among the variants. Saturated fatty acids (SFAs) accounted for 25–40% of total lipids, with honey and green *Chlorella* showing slightly higher proportions. Monounsaturated fatty acids (MUFAs) were most abundant in honey, while polyunsaturated fatty acids (PUFAs) dominated in white and green variants. Notably, honey and green *C. vulgaris* exhibited higher omega-3 PUFA levels, whereas white *C. vulgaris* was richer in omega-6 PUFAs, primarily linoleic acid [11,27]. These findings are consistent with comprehensive metabolomic studies showing that *C. vulgaris* is a valuable source of omega-6 and omega-3 fatty acids, with C16–C18 fatty acids comprising over 90% of the total lipid fraction [6,27].

All three *C. vulgaris* variants were rich in macrominerals, particularly potassium (400–1000 mg/100 g) and phosphorus (800–2000 mg/100 g).

Vitamin composition varied significantly among the variants. Vitamin A was detected only in green *C. vulgaris*, correlating with its high carotenoid content. Honey *C. vulgaris* contained the highest vitamin E, consistent with its elevated MUFA content, which enhances tocopherol stability [11]. White *C. vulgaris* exhibited the greatest vitamin C, suggesting enhanced antioxidant capacity in pigment-deficient strains. White *C. vulgaris* showed generally elevated levels of B-complex vitamins, with particularly high concentrations of B1 (thiamine), B2 (riboflavin), and B12 (cobalamin), an essential vitamin involved in red blood cell formation, neurological function, and DNA synthesis [27,28].

3.2. Polyphenol Content and Antioxidant Activity in *Chlorella vulgaris*

Polyphenols are particularly notable for their strong radical scavenging and metal-chelating abilities, contributing significantly to cellular protection against oxidative stress, and supporting their potential application in health-promoting formulations [29].

Figure 1 reports the TPC of white, honey, and green *C. vulgaris*.

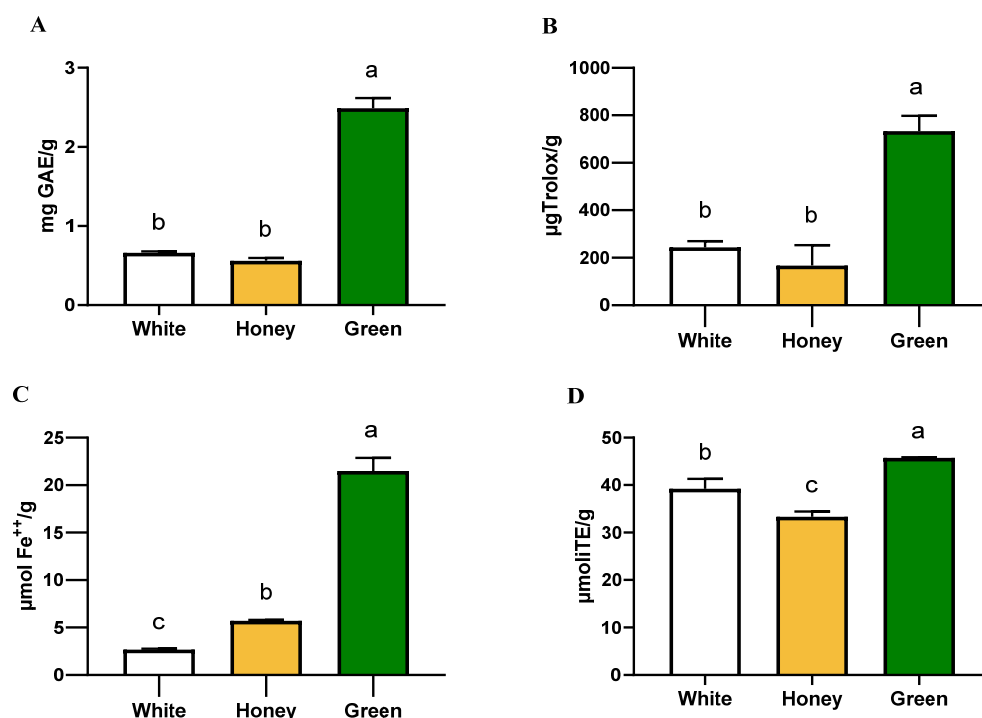


Figure 1. Total polyphenol content and antioxidant activities of the raw flour of green, honey and white *Chlorella*. (A) Total polyphenol content, expressed as milligrams of gallic acid equivalents per grams of dry weight (mg GAE/g dw); (B) DPPH, expressed as µg Trolox equivalent (TE)/grams of dry weight; (C) FRAP value, expressed as µmol Fe²⁺/ grams of dry weight; (D) ORAC, expressed as µmol Trolox equivalent (TE)/grams of dry weight. Results are expressed as mean ± SD ($n = 3$). Different letters indicate significant differences according to one-way ANOVA with Tukey's multiple-range test, $p < 0.05$.

Significant differences were observed among the variants, with green *C. vulgaris* exhibiting the highest TPC, while honey and white did not differ significantly from each other. Notably, total polyphenol content in the green strain (Figure 1A) was approximately four times greater than in the honey and white variants. This trend aligns with evidence that pigment-rich microalgae accumulate more phenolic compounds as photoprotective molecules [30,31]. These differences may be linked to the genetic background of the variants, as honey and white strains are chlorophyll-deficient mutants derived from a green parent strain through mutagenesis, resulting in major alterations in chlorophyll and carotenoid composition. Such pigment-related metabolic changes may redirect carbon allocation among biosynthetic pathways, leading to reduced phenolic accumulation, even under identical cultivation conditions [6,11]. Similar patterns have been reported in chlorophyll-deficient plant systems, including tea mutants with lower polyphenol levels than their green counterparts [32–34].

To investigate the relationship between phytochemical composition and antioxidant potential among the *C. vulgaris* variants, we evaluated antioxidant activity through three complementary *in vitro* assays such as DPPH radical scavenging, FRAP, and ORAC. These measurements provide an integrated view of both electron transfer-based and hydrogen atom transfer-based antioxidant mechanisms [35]. The DPPH assay primarily measures free radical scavenging through electron- and hydrogen-donating capacity, FRAP assesses ferric ion-reducing power as an indicator of electron transfer potential, and ORAC quantifies peroxyl radical scavenging through a hydrogen atom transfer mechanism, thereby reflecting chain-breaking antioxidant activity that is considered physiologically relevant, as the peroxyl radicals generated in this assay closely resemble those formed in biological systems during oxidative stress [35].

Green *C. vulgaris* showed the highest DPPH scavenging activity (Figure 1B), FRAP (Figure 1C), and ORAC (Figure 1D) capacity. This reflects the TPC pattern, reinforcing the strong correlation between phenolic content and antioxidant potential [30,31,36]. DPPH increased 3-fold over the white variant, and a 4-fold increase was observed over the honey one. These findings differ from those reported by Giura et al. [10], where vegetable purees enriched with 3% white *Chlorella* exhibited higher DPPH antioxidant activity than those containing green (smooth) or honey *Chlorella*. Such discrepancies may be attributed to differences in substrate matrix, as incorporation into food systems can alter the bioaccessibility and interactions of antioxidant compounds compared with analyses conducted on microalgal biomass itself. In addition, strain-specific differences in pigmentation and metabolic composition, as well as variations in processing conditions, may further influence antioxidant outcomes across studies [6,37]. The higher antioxidant capacity observed in the green variant in the present study is consistent with findings from Maurício et al. [11], who reported stronger radical scavenging activity in lipid extracts from green compared with chlorophyll-deficient variants.

The FRAP assay showed the following trend: green > honey > white, with green *C. vulgaris* showing an eightfold higher reducing power than the white variant and nearly fourfold higher than the honey variant. The moderate antioxidant activity of the honey variant may be related to residual carotenoids such as lutein [38] and traces of phenolic compounds [5]. These findings contrast with those of Giura et al. [10], who reported no significant differences in FRAP values between chlorophyll-deficient variants, and observed that the white *Chlorella* variant displayed higher FRAP values than both the green one in vegetable puree formulations designed for dysphagia diets.

ORAC results further confirmed that green *C. vulgaris* possesses the highest peroxyl radical scavenging ability, while the white and honey variants showed moderate and low activities, respectively. These findings suggest that the pigment-rich variant has more effective antioxidant defense systems, as ORAC reflects both hydrophilic and lipophilic antioxidant contributions. These results highlight that antioxidant performance among *C. vulgaris* variants is strongly matrix- and variant-dependent. Importantly, this study provides a direct comparative evaluation of multiple color variants under the same cultivation and analytical conditions, allowing clearer attribution of antioxidant differences to intrinsic compositional traits rather than external processing factors [6,30,31,39].

3.3. Flavonoid Content and Phenolic Profiles of *Chlorella vulgaris* Variants

The total flavonoid content (TFC; Figure 2) displayed pronounced pigment-dependent differences among the *C. vulgaris* variants.

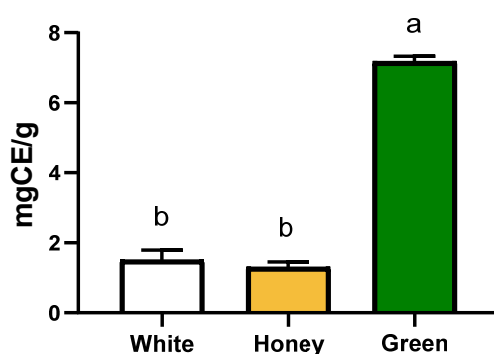


Figure 2. Total flavonoid content of the raw flour of green, honey and white *Chlorella*, expressed as milligrams of catechin equivalent per grams of dry weight (mg CE/g dw). Results are expressed as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences according to one-way ANOVA with Tukey's multiple-range test, $p < 0.05$.

The green variant consistently exhibited the highest TFC, while the honey and white variants did not differ in terms of flavonoid content, mirroring the trend observed for total polyphenols and supporting the close relationship between pigmentation and flavonoid biosynthesis [30,40]. The reduced TFC in pigment-deficient variants is consistent with previous reports suggesting that alterations in photosynthetic metabolism limit secondary metabolite accumulation [6].

The HPLC analysis showed that the most commonly reported phenolics in *C. vulgaris*, including gallic acid, protocatechuic acid, vanillic acid, rutin, vitexin, and trans-ferulic acid [41–44] were below the detection limit (Table 4), which may reflect differences in variant genetics, extraction protocols, and analytical conditions across studies, as well as the predominance of bound or glycosylated phenolics that are poorly extracted under mild conditions [15,27]. Trans-coumaric acid and quercetin were found exclusively in the green variant, further supporting the link between pigmentation and secondary phenolic metabolism [15].

Table 4. Quantification of selected phenolic compounds by HPLC-DAD in three *Chlorella vulgaris* variants (white, honey, and green) expressed as micrograms per gram of dry weight ($\mu\text{g/g dw}$). Results are expressed as mean $\pm$ SD ($n = 3$).

Compounds	RT [min]	Concentration [$\mu\text{g/g dw}$]		
		White <i>C. vulgaris</i>	Honey <i>C. vulgaris</i>	Green <i>C. vulgaris</i>
Gallic acid	2.79	$\leq\text{LOD}$	$\leq\text{LOD}$	$\leq\text{LOD}$
Protocatechuic acid	4.34	$\leq\text{LOD}$	$\leq\text{LOD}$	$\leq\text{LOD}$
Vanillic acid	7.91	$\leq\text{LOD}$	$\leq\text{LOD}$	$\leq\text{LOD}$
Rutin	12.39	$\leq\text{LOD}$	$\leq\text{LOD}$	$\leq\text{LOD}$
Trans-coumaric acid	11.08	$\leq\text{LOD}$	$\leq\text{LOD}$	1.68 ± 0.01
Vitexin	12.25	$\leq\text{LOD}$	$\leq\text{LOD}$	$\leq\text{LOD}$
Trans-ferulic acid	12.61	$\leq\text{LOD}$	$\leq\text{LOD}$	$\leq\text{LOD}$
Quercetin	18.7	$\leq\text{LOD}$	$\leq\text{LOD}$	1.25 ± 0.01

LOD = limit of detection.

3.4. Biological Activities of *Chlorella vulgaris* Extracts in HT-29 Colon Cells

To relate chemical antioxidant properties to biological relevance, the effects of raw *C. vulgaris* extracts were evaluated in HT-29 human colon cells by assessing cell viability and intracellular reactive oxygen species (ROS) modulation.

After 24 h of treatment, none of the *C. vulgaris* extracts (25, 50, or 100 $\mu\text{g/mL}$) exhibited cytotoxicity, as cell viability remained near 100% compared to the untreated control (Figure 3A), confirming the safety of the extracts at the tested concentrations and agreeing with previous studies reporting no toxicity of *C. vulgaris* extracts in epithelial and other human cell models [45]. Regarding intracellular ROS, no clear dose-dependent trend was observed across the variants (Figure 3B), which may reflect differences in cellular uptake, intercellular metabolism or saturation of antioxidant mechanisms at lower concentrations [46–48].

However, at the highest concentration (100 $\mu\text{g/mL}$), the green variant significantly reduced basal ROS level ($\sim 22\%$), followed by the white ($\sim 13\%$) and honey ($\sim 11\%$) variants, mirroring the ORAC pattern and suggesting that hydrogen atom transfer-active compounds contribute to intracellular antioxidant defense. Although similar ROS reduction effects of *C. vulgaris* have been reported in other cell systems [8,49], discrepancies among studies may arise from differences in variant characteristics, extract preparation, and experimental conditions, which strongly influence bioactive compound availability. The absence of cytotoxicity, together with the lack of basal ROS increase, supports the potential of these extracts as safe functional ingredients. Comparison of multiple *C. vulgaris* variants under

identical experimental conditions demonstrates that pigmentation-related compositional differences translate into distinct intracellular antioxidant responses, providing new insight into variant-dependent bioactivity.

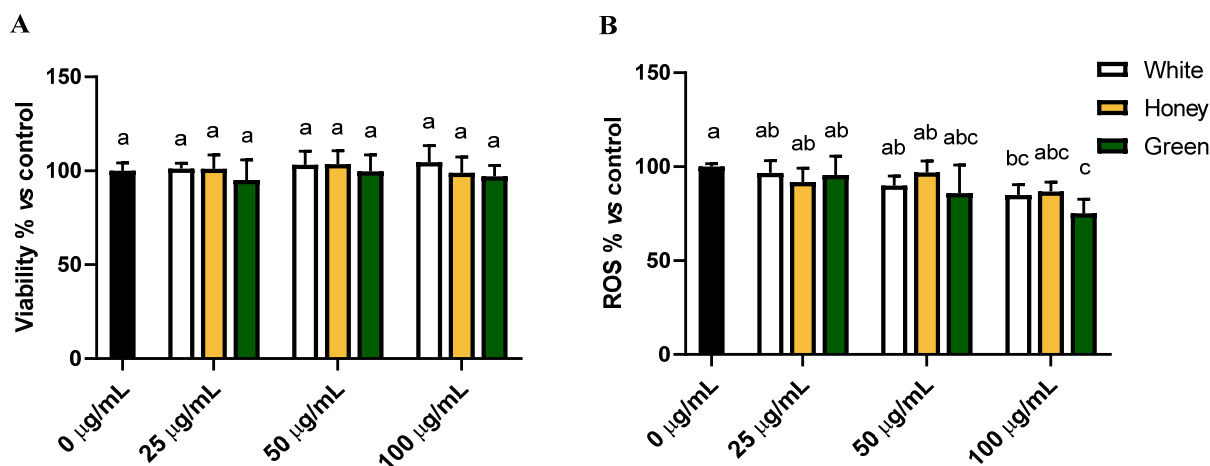


Figure 3. Effects of different concentrations, 25, 50 and 100 µg/mL of white, honey and green *Chlorella vulgaris* extracts on HT-29 cell viability (A) and intracellular reactive oxygen species (ROS) production (B). Results are expressed as mean ± SD ($n = 6$). Different letters indicate significant differences according to one-way ANOVA with Tukey's multiple-range test, $p < 0.05$.

3.5. Fermentation Performance and Bioactive Responses of *Chlorella vulgaris* Variants

3.5.1. Fermentation Dynamics and Acidification Behavior in *Chlorella vulgaris*

The determination of pH, titratable acidity, microbial growth, residual sugars and metabolite production during the fermentation of *C. vulgaris* in LAB–yeast co-cultures is essential for monitoring fermentation progress and product quality, as these parameters reflect acidification dynamics, microbial adaptation, and substrate conversion [12,50–52].

Co-culture fermentations of the three *C. vulgaris* variants (green, honey, and white) resulted in a marked decrease in pH and a corresponding increase in total titratable acidity (TTA) after 24 h, confirming active acidogenic metabolism (Figure 4).

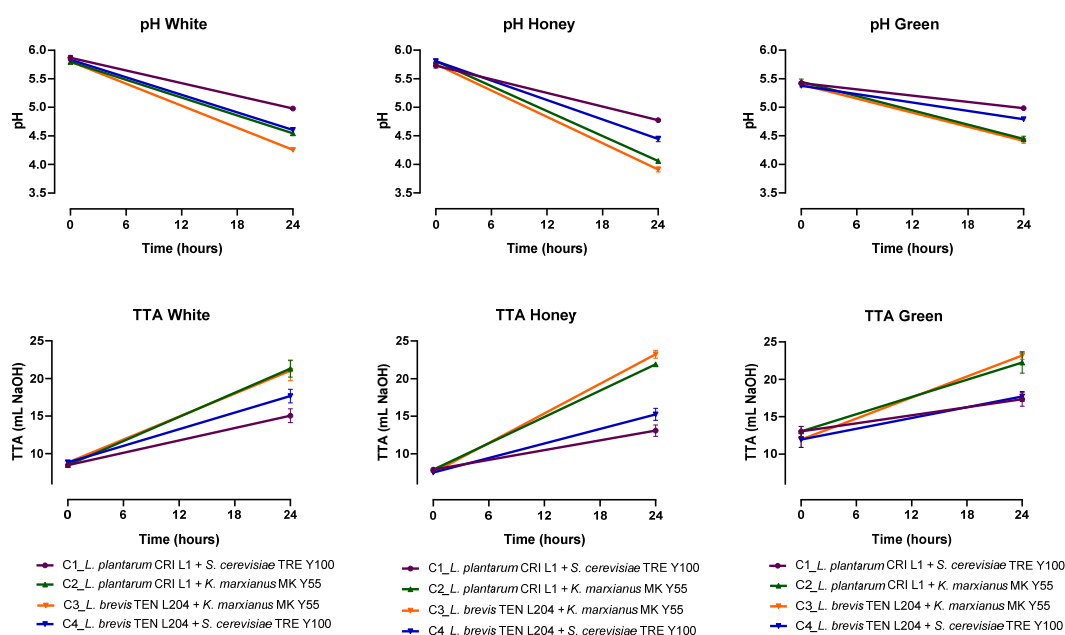


Figure 4. Changes in pH and titratable acidity (TTA) monitored over the 24 h fermentation of green, honey, and white *Chlorella vulgaris* flours with different combinations of LAB and yeast.

However, the extent of acidification varied among studies and may depend on differences in microbial consortia, substrate, and fermentation conditions, such as sugar availability and buffering capacity [53] (Adams, 2014). In the present study, co-cultures containing *K. marxianus* induced stronger acidification than those containing *S. cerevisiae*, likely reflecting strain-specific metabolic activity and synergistic interactions with LAB that enhance sugar catabolism and organic acid production [12,54,55]. Among the microalgal matrices, green *C. vulgaris* showed a less pronounced pH decline, likely due to its higher pigment and mineral content, which could confer greater buffering capacity, whereas white and honey variants showed stronger acidification, consistent with their higher fermentable sugar availability.

Both LAB and yeast populations increased substantially during fermentation (Figure 5), indicating good adaptation to the microalgal matrices. LAB populations typically reached 10^9 – 10^{10} CFU/mL, while yeast counts stabilized around 10^7 – 10^8 CFU/mL after 24 h.

C1 *L. plantarum* CR L1 + *S. cerevisiae* TREY 100

C2 *L. plantarum* CR L1 + *K. marxianus* MK Y55

C3 *L. brevis* TEN L204 + *K. marxianus* MK Y55

C4 *L. brevis* TEN L204 + *S. cerevisiae* TRE Y100

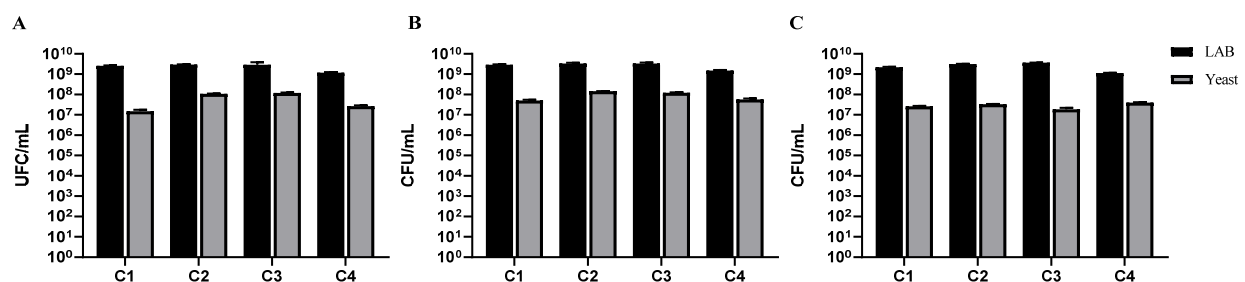


Figure 5. Microbial growth of lactic acid bacteria (*Lactiplantibacillus plantarum* and *Levilactobacillus brevis*) and yeasts (*Saccharomyces cerevisiae* and *Kluyveromyces marxianus*) in co-culture combinations C1–C4 (see legend) after 24 h of fermentation of white (A), honey (B), and green (C) *Chlorella vulgaris* flours.

LAB densities were consistently higher in co-cultures with *K. marxianus* compared to those with *S. cerevisiae*, suggesting positive microbial interactions potentially driven by oxygen scavenging or metabolite exchange [56–58]. The higher sugar content of honey *C. vulgaris* promoted yeast proliferation, highlighting a strong substrate–strain interaction [12,14,54].

Differences among variants were further reflected in metabolite profiles (Tables 5–7). Green *C. vulgaris* showed rapid sugar depletion and high lactic acid production, indicating high substrate accessibility, whereas honey *C. vulgaris* exhibited the strongest acidogenic orientation, likely due to its higher initial sugar content. In contrast, the white variant displayed more variable lactic acid production and lower fermentation quotient values, suggesting a more balanced fermentation metabolism, potentially influenced by pigmentation-related differences in cellular composition and redox balance. [55,59].

Variation in fermentation behavior among variants likely reflects differences in microalgal biochemical composition (e.g., sugar availability, pigmentation, and buffering capacity) together with strain-specific metabolic and enzymatic properties of the fermenting microorganisms, which collectively shape fermentation outcomes [57,60].

Table 5. Chemical composition of raw and fermented green *Chlorella* powder obtained using different combinations of LAB and yeast. Values are expressed as mean $\pm$ standard deviation. Values of the same row with different letters are significantly different (one-way ANOVA and Tukey's Test $p < 0.05$).

	<i>L. plantarum</i> CRI L1 <i>S. cerevisiae</i> TRE Y100	<i>L. plantarum</i> CRI L1 <i>K. marxianus</i> MK Y55	<i>L. brevis</i> TEN L204 <i>S. cerevisiae</i> TRE Y100	<i>L. brevis</i> TEN L204 <i>K. marxianus</i> MK Y55	<i>Chlorella</i> Green Powder
Glucose (g/Kg)	0.39 $\pm$ 0.02 b	<0.01	0.26 $\pm$ 0.02 b	0.39 $\pm$ 0.02 b	6.24 $\pm$ 0.52 a
Fructose (g/Kg)	<0.01	<0.01	0.07 $\pm$ 0.01 b	<0.01	9.12 $\pm$ 0.92 a
Glu+ Fru (g/Kg)	0.39 $\pm$ 0.02 b	<0.01	0.33 $\pm$ 0.02 b	0.39 $\pm$ 0.05 b	15.36 $\pm$ 0.80 a
Lactic acid (g/Kg)	11.60 $\pm$ 1.30 b	19.96 $\pm$ 1.02 a	15.32 $\pm$ 2.36 ab	21.86 $\pm$ 2.46 a	2.67 $\pm$ 0.96 c
Glycerol (g/Kg)	1.12 $\pm$ 0.09 b	1.44 $\pm$ 0.10 b	2.63 $\pm$ 0.18 a	1.70 $\pm$ 0.13 ab	1.05 $\pm$ 0.45 b
Acetic acid (g/Kg)	1.46 $\pm$ 0.06 b	1.66 $\pm$ 0.19 b	3.06 $\pm$ 0.35 a	2.75 $\pm$ 0.12 a	1.01 $\pm$ 0.30 c
Ethanol (%)	0.97 $\pm$ 0.09 b	0.32 $\pm$ 0.03 b	1.68 $\pm$ 0.14 a	0.38 $\pm$ 0.03 b	<0.01
Fermentation quotient FQ	5.29 $\pm$ 0.65 b	8.03 $\pm$ 0.46 a	3.34 $\pm$ 0.19 b	5.30 $\pm$ 0.65 b	<0.01

Table 6. Chemical composition of raw and fermented honey *Chlorella* powder obtained using different combinations of LAB and yeast. Values are expressed as mean $\pm$ standard deviation. Values of the same row with different letters are significantly different (one-way ANOVA and Tukey's Test $p < 0.05$).

	<i>L. plantarum</i> CRI L1 <i>S. cerevisiae</i> TRE Y100	<i>L. plantarum</i> CRI L1 <i>K. marxianus</i> MK Y55	<i>L. brevis</i> TEN L204 <i>S. cerevisiae</i> TRE Y100	<i>L. brevis</i> TEN L204 <i>K. marxianus</i> MK Y55	<i>Chlorella</i> Honey Powder
Glucose (g/Kg)	<0.01	1.97 $\pm$ 0.14 b	<0.01	1.32 $\pm$ 0.07 c	13.19 $\pm$ 0.17 a
Fructose (g/Kg)	<0.01	3.89 $\pm$ 0.49 b	<0.01	1.23 $\pm$ 0.12 c	13.41 $\pm$ 0.38 a
Glu+ Fru (g/Kg)	<0.01	5.86 $\pm$ 0.32 b	<0.01	2.55 $\pm$ 0.34 c	26.60 $\pm$ 0.55 a
Lactic acid (g/Kg)	10.05 $\pm$ 0.51 b	17.55 $\pm$ 2.70 a	6.74 $\pm$ 0.83 bc	17.27 $\pm$ 1.94 a	1.33 $\pm$ 0.07 c
Glycerol (g/Kg)	0.95 $\pm$ 0.07 a	nd	0.70 $\pm$ 0.05 a	2.32 $\pm$ 0.18 a	0.08 $\pm$ 0.01 b
Acetic acid (g/Kg)	0.48 $\pm$ 0.06 b	0.66 $\pm$ 0.08 b	1.16 $\pm$ 0.13 a	1.30 $\pm$ 0.06 a	0.15 $\pm$ 0.02 c
Ethanol (%)	1.00 $\pm$ 0.08 a	0.36 $\pm$ 0.08 a	1.26 $\pm$ 0.20 a	0.34 $\pm$ 0.03 b	<0.01
Fermentation quotient FQ	14.06 $\pm$ 0.81 a	17.60 $\pm$ 1.02 a	3.87 $\pm$ 0.62 c	8.86 $\pm$ 1.09 b	<0.01

nd = not detected.

Table 7. Chemical composition of raw and fermented white *Chlorella* powder obtained using different combinations of LAB and yeast. Values are expressed as mean $\pm$ standard deviation. Values of the same row with different letters are significantly different (one-way ANOVA and Tukey's Test $p < 0.05$).

	<i>L. plantarum</i> CRI L1 <i>S. cerevisiae</i> TRE Y100	<i>L. plantarum</i> CRI L1 <i>K. marxianus</i> MK Y55	<i>L. brevis</i> TEN L204 <i>S. cerevisiae</i> TRE Y100	<i>L. brevis</i> TEN L204 <i>K. marxianus</i> MK Y55	<i>Chlorella</i> White Powder
Glucose (g/Kg)	0.30 $\pm$ 0.02 b	<0.01	<0.01	<0.01	7.51 $\pm$ 0.54 a
Fructose (g/Kg)	<0.01	<0.01	<0.01	<0.01	7.44 $\pm$ 0.93 a
Glu+ Fru (g/Kg)	0.30 $\pm$ 0.02 b	<0.01	<0.01	<0.01	14.95 $\pm$ 0.63 a
Lactic acid (g/Kg)	14.37 $\pm$ 1.61 a	9.32 $\pm$ 0.48 b	3.43 $\pm$ 0.42 c	12.10 $\pm$ 0.62 ab	0.04 $\pm$ 0.01 d
Glycerol (g/Kg)	0.55 $\pm$ 0.04	0.84 $\pm$ 0.06	0.68 $\pm$ 0.05	0.64 $\pm$ 0.04	0.63 $\pm$ 0.04
Acetic acid (g/Kg)	1.14 $\pm$ 0.05 bc	1.69 $\pm$ 0.20 ab	1.15 $\pm$ 0.13 bc	1.80 $\pm$ 0.21 a	1.02 $\pm$ 0.12 c
Ethanol (%)	0.16 $\pm$ 0.01 b	0.55 $\pm$ 0.05 a	0.50 $\pm$ 0.08 a	0.19 $\pm$ 0.02 b	<0.01
Fermentation quotient FQ	8.40 $\pm$ 1.04 a	3.67 $\pm$ 0.21 bc	2.00 $\pm$ 0.32 c	4.49 $\pm$ 0.26 b	<0.01

3.5.2. Polyphenol Content and Antioxidant Activity in Fermented *Chlorella vulgaris*

In the green variant, TPC remained unchanged after fermentation (Figure 6A), with values ranging between 2.49 and 2.65 mg GAE/g. DPPH radical scavenging activity did not differ significantly from the control in the *L. brevis* + *S. cerevisiae* and *L. plantarum* + *S. cerevisiae* treatments, while *K. marxianus* co-cultures showed reduced values, with the lowest activity in the *L. plantarum* + *K. marxianus* combination (Figure 6B). FRAP (Figure 6C) and ORAC (Figure 6D) values did not show significant changes.

C1 *L. plantarum* CR L1 + *S. cerevisiae* TREY 100

C2 *L. plantarum* CR L1 + *K. marxianus* MK Y55

C3 *L. brevis* TEN L204 + *K. marxianus* MK Y55

C4 *L. brevis* TEN L204 + *S. cerevisiae* TRE Y100

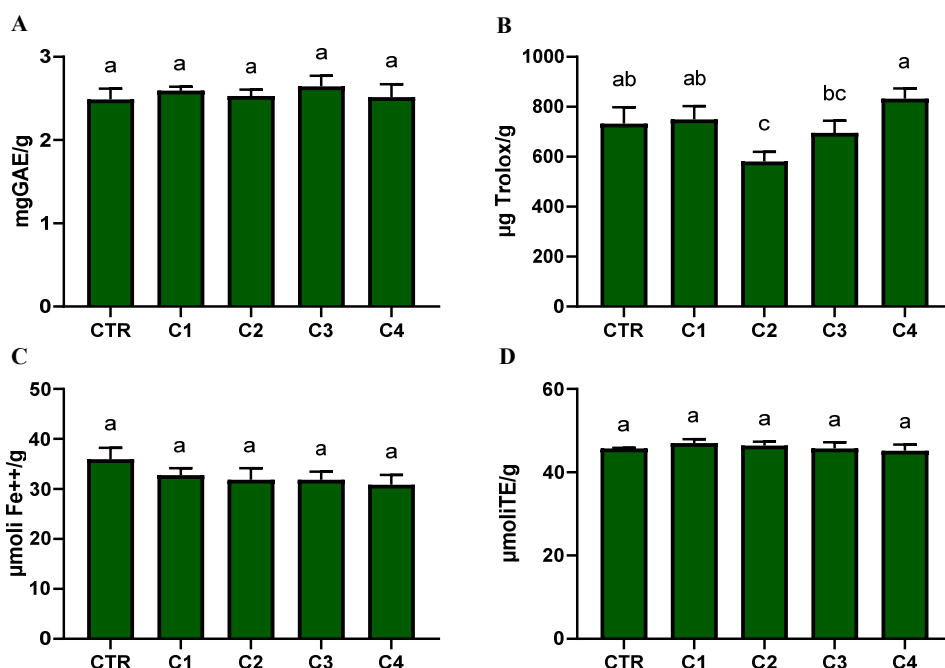


Figure 6. Bioactive content and antioxidant properties of fermented green *Chlorella* flour obtained using different combinations of LAB and yeast, compared with the control (CTR). (A) Total polyphenol content; (B) DPPH scavenging activity; (C) FRAP assay; (D) ORAC. Results are expressed as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences according to one-way ANOVA with Tukey's multiple-range test, $p < 0.05$.

The stability of TPC, FRAP and ORAC values suggests that fermentation induced qualitative rather than quantitative changes in the antioxidant pool. Microbial metabolism may have modified phenolic structures, increasing the availability of reactive hydroxyl groups or generating smaller molecules with stronger radical scavenging activity [56]. Accordingly, the DPPH assay, which is more sensitive to specific fast-reacting antioxidants [35], revealed differences among co-cultures, likely related to strain-dependent metabolic activity. This selective response is consistent with previous reports showing that fermentation may alter the qualitative profile of antioxidant compounds without changing total phenolic content and antioxidant property [16,61,62]. In the honey variant, fermentation induced only minor, non-significant changes in TPC and most antioxidant indices (Figure 7).

The only significant difference was a decrease in ORAC values in the *L. brevis* + *S. cerevisiae* treatment compared to the control and *L. plantarum* co-fermented samples. This suggests that the honey matrix, possibly due to its higher sugar content or different phenolic composition, is less responsive to fermentation in terms of antioxidant enhancement. Such matrix effects are well-documented, with some substrates showing limited or even negative changes in antioxidant indices after fermentation, depending on the initial composition and microbial metabolism [14,16]. Fermentation effects were most pronounced in the white *C. vulgaris*, where increases in TPC and antioxidant capacity were observed (Figure 8).

C1 *L. plantarum* CR L1 + *S. cerevisiae* TREY 100
 C2 *L. plantarum* CR L1 + *K. marxianus* MK Y55
 C3 *L. brevis* TEN L204 + *K. marxianus* MK Y55
 C4 *L. brevis* TEN L204 + *S. cerevisiae* TRE Y100

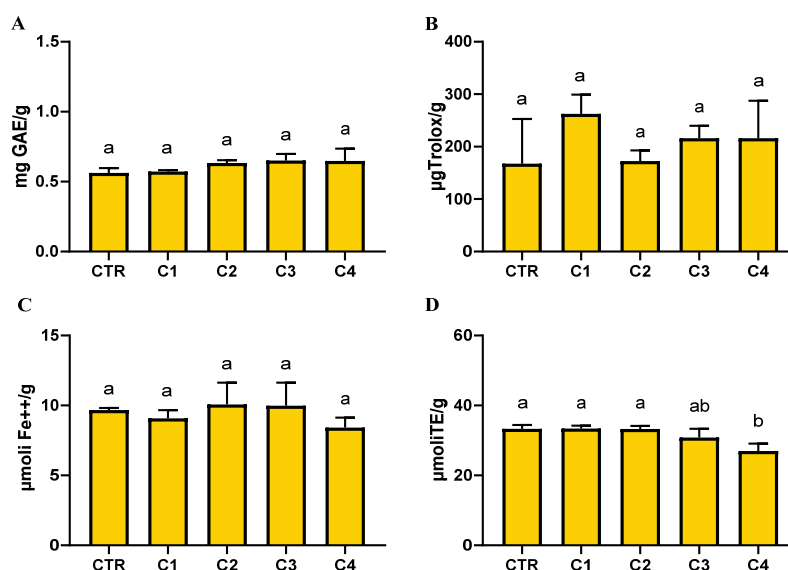


Figure 7. Bioactive content and antioxidant properties of fermented honey *Chlorella* flour obtained using different combinations of LAB and yeast, compared with the control (CTR). (A) Total polyphenol content; (B) DPPH scavenging activity; (C) FRAP assay; (D) ORAC. Results are expressed as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences according to one-way ANOVA with Tukey's multiple-range test, $p < 0.05$.

C1 *L. plantarum* CR L1 + *S. cerevisiae* TREY 100
 C2 *L. plantarum* CR L1 + *K. marxianus* MK Y55
 C3 *L. brevis* TEN L204 + *K. marxianus* MK Y55
 C4 *L. brevis* TEN L204 + *S. cerevisiae* TRE Y100

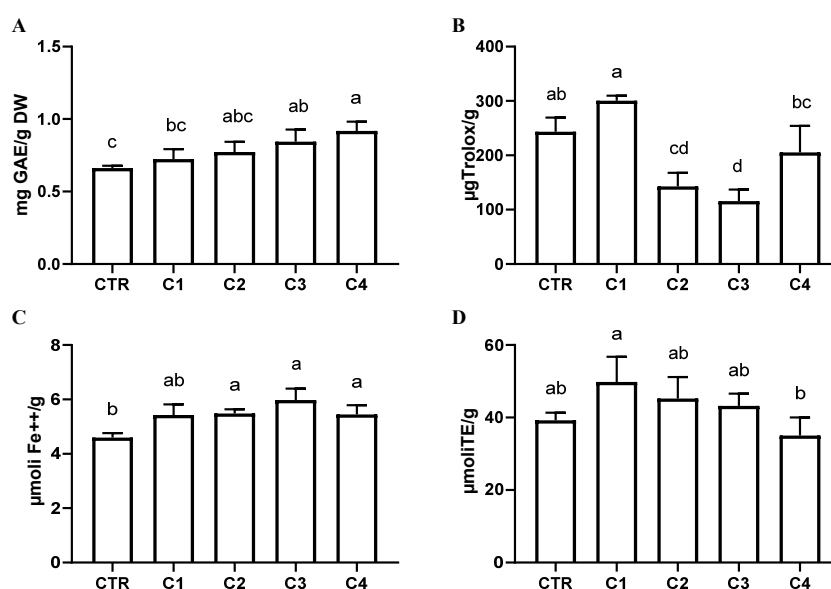


Figure 8. Bioactive content and antioxidant properties of fermented white *Chlorella* flour obtained using different combinations of LAB and yeast, compared with the control (CTR). (A) Total polyphenol content; (B) DPPH scavenging activity; (C) FRAP assay; (D) ORAC. Results are expressed as mean $\pm$ SD ($n = 3$). Different letters indicate significant differences according to one-way ANOVA with Tukey's multiple-range test, $p < 0.05$.

Specifically, TPC was highest in *L. brevis* L204 + *S. cerevisiae* TRE Y100 (0.92 mg GAE/g) and *L. brevis* L204 + *K. marxianus* (0.84 mg GAE/g), while the lowest value was found in the control (Figure 8A). Interestingly, DPPH activity decreased in *L. plantarum* CR L1 + *K. marxianus* MK Y55 and *L. brevis* L204 + *K. marxianus* MK Y55 compared to the control, but no significant differences were observed among *L. plantarum* CR L1 + *S. cerevisiae* TRE Y100, *L. brevis* L204 + *S. cerevisiae* TRE Y100, and the control (Figure 8B). FRAP values increased following fermentation in all LAB and yeast co-cultures except *L. plantarum* CR L1 + *S. cerevisiae* TRE Y100, which did not differ from the control (Figure 8C). ORAC values varied, with the highest in *L. plantarum* CR L1 + *S. cerevisiae* TRE Y100 and the lowest in *L. brevis* L204 + *S. cerevisiae* TRE Y100 (Figure 8D). These results underscore the strain- and matrix-specific nature of fermentation effects. The increase in TPC and antioxidant indices in white *C. vulgaris* is in line with studies showing that fermentation can enhance the release of bound phenolics and generate new antioxidant metabolites, especially when the substrate is initially low in extractable polyphenols [16,52,61]. The observed decrease in DPPH activity in some treatments may reflect the degradation or transformation of specific phenolics with high radical scavenging capacity, a phenomenon also reported in other plant-based fermentations [63,64]. The selective changes in antioxidant activity observed in the white *C. vulgaris* variant following fermentation may result from the combined effects of structural and compositional characteristics. Compared with the green and honey variants, the white variant may possess a less resistant or modified cell wall [65,66], facilitating microbial enzymatic penetration during fermentation. In addition, a relatively higher protein content [6] may promote the formation of antioxidant peptides, while a PUFA-rich polar lipid fraction combined with reduced intrinsic pigmentation may represent a potential antioxidant reservoir [11] that becomes more bioaccessible during fermentation. Consequently, LAB and yeast metabolism may more effectively alter the release or biotransformation of antioxidant compounds in the white variant, leading to strain-dependent changes in antioxidant activity. In contrast, pigment-rich green and honey variants, characterized by more robust cellular matrices, may undergo less extensive biotransformation [67] under the same fermentation conditions, resulting in more limited changes in antioxidant capacity. These results suggest that pigment-related metabolic traits and microbial interactions jointly influence the functional outcomes of microalgal fermentation, providing a framework for developing targeted fermentation strategies to modulate antioxidant functionality in strain-specific *C. vulgaris* biomass.

4. Conclusions

The biochemical characterization of *Chlorella vulgaris* (green, honey, and white) revealed distinct nutritional and functional profiles influenced by pigmentation. Green *C. vulgaris* exhibited a high pigment and polyunsaturated fatty acid content, conferring strong native antioxidant activity, while the white variant, pigment-deficient but protein-rich, displayed the lowest baseline polyphenol and antioxidant levels. The honey variant showed intermediate characteristics. In raw biomasses, differences in total polyphenol content, flavonoids, and antioxidant activities reflected the compositional diversity among variants. The co-culture fermentation of *C. vulgaris* with lactic acid bacteria (LAB) and yeasts significantly improved its biochemical and functional properties, depending on microbial pairing and matrix type. Combinations involving *K. marxianus* with *L. plantarum* or *L. brevis* yielded the highest lactic acid concentrations, the strongest pH reduction, and greater titratable acidity, indicating efficient sugar-to-acid conversion. In contrast, *S. cerevisiae*-based fermentations produced higher ethanol and glycerol levels, reflecting a more heterofermentative metabolism. Fermentation notably enhanced TPC and antioxidant capacity in white *C.*

vulgaris through microbial enzymatic hydrolysis and phenolic transformation, whereas green and honey variants showed limited changes due to pre-existing antioxidant richness.

These findings demonstrate that pigmentation strongly influences the biochemical composition and antioxidant profile of *C. vulgaris* variants. The green flours exhibited the highest native polyphenol content and antioxidant capacity; however, LAB–yeast co-culture fermentation can selectively enhance the bioactive profile of pigment-deficient *C. vulgaris*. Fermentation notably increased total polyphenols and antioxidant capacity in the white variant. Overall, these findings indicate that co-culture fermentation can be used as a targeted strategy to enhance the functional potential of pigment-deficient *C. vulgaris* flours, supporting their application as ingredients for functional food.

Future research should focus on optimizing process parameters such as inoculum ratio, temperature and time, and applying metabolomic and proteomic analyses to elucidate microbial interactions and metabolite pathways. Remaining research gaps include understanding the molecular basis of LAB–yeast synergy and matrix–strain specificity. Further investigation into bioavailability and product stability is also needed. Collectively, this study highlights microbial co-fermentation as a promising tool to valorize *C. vulgaris* into next-generation bioactive and functional bioproducts for sustainable food and biotechnology applications.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/foods15050955/s1>. Table S1. pH, Total Titratable Acidity (TTA), microbial count, and chemical composition of fermented and raw white *Chlorella* powder obtained using single LAB or yeast strains. Values were expressed as mean $\pm$ standard deviation. CFU: Colony Forming Units; Lp: *Lactiplantibacillus*; Ll: *Levilactobacillus*; Lc: *Lacticaseibacillus*; L: *Leuconostoc*; Lt: *Latilactobacillus*; Lm: *Limosilactobacillus*. Table S2. Coefficient of variation (CV%) for data presented in Figure 1, Figure 2 and Table 3. Table S3. Coefficient of variation (CV%) for data presented in Figure 3. Table S4. Coefficient of variation (CV%) for data presented in Figure 5. Table S5. Coefficient of variation (CV%) for data presented in Figures 6–8. Table S6. Coefficient of variation (CV%) for data reported in main Tables 5–7.

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