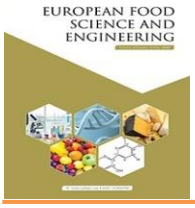

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Impact of *Chlorella vulgaris* biomass substitution on *in vitro* bioaccessibility of cookies

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ABSTRACT

This study aimed to produce low-fat cookies (C) by substituting *Chlorella vulgaris* biomass (0.5% CB1, 1.0% CB2, and 1.5% CB3) and investigating the bioavailability of minerals, total phenolic content, and antioxidant capacities of the cookies. *Chlorella* sp. microalgae is recognized for its high phenolic content, antioxidant capacity, and as a source of essential minerals. Extractable and hydrolyzable fractions were prepared to determine the total phenolic content and antioxidant capacity. The total phenolic content of CB samples ranged from 200.82 to 274.07 mg GAE/g, with bioaccessibility values from 32.31 to 47.26 mg GAE/g. The CUPRAC method provided the highest antioxidant capacity values (116.57-154.38 µmol TE/g), while the ABTS method showed the highest bioaccessibility values (6.76-9.21 µmol TE/g). Mineral content analysis (Na, Mg, P, K, Ca, Mn, Fe, Cu, Zn, and Se) revealed significant enhancements in the CB samples compared to controls, showing an approximate 2-fold increase in mineral bioaccessibility. Despite extensive research on microalgae-fortified foods, there is a notable gap in knowledge regarding their "in vitro bioaccessibility." This study aims to pioneer the exploration of bioaccessibility and highlight the positive impact of algae-based food consumption on human health.

1. Introduction

Functional foods refer to foods and/or food ingredients that offer health benefits beyond their nutritional value, reducing the risk of chronic and other diseases. Microalgae have gained considerable attention in the past two decades due to their potential as a source of protein, fatty acids, and other biologically active functional ingredients that have significant therapeutic applications, including protection against diabetes and obesity (Khan et al., 2018). *Chlorella* sp. and *Spirulina (Arthrospira)* sp. are the most cultivated microalgae for food applications worldwide. *Chlorella* biomass provides high-quality proteins thanks to their basic amino acid profile, as well as provitamin A, *β*-carotene, vitamin E, B1, B2, B3, B6, B12, and minerals. Incorporating microalgae into food products can bring about significant physicochemical changes and nutritional improvements (Bito et al., 2020).

In recent years, several studies have explored the use of microalgal biomass in the production of innovative and healthy food products such as pasta, biscuits, vegetarian mayonnaises, and gelled desserts (Ferreira et al., 2021; Udayan et al., 2021). While the bioactive properties of microalgae biomass and its extracts have been extensively studied and demonstrated, only a limited number of studies have examined the bioactivity of microalgae-based foods and how they respond to various processing techniques. As a result, there is a knowledge gap regarding the impact of food processing conditions on the digestibility, bioavailability, and bioactive properties of microalgae functional ingredients in different food matrices.

Cookies are a popular baked food product consumed worldwide, but they typically contain high levels of sugar and fat and low water content. Cookie doughs are typically made with a high amount of shortening, which is not ideal for a healthy diet. Therefore, fat-reduced or fat-replaced cookies are more acceptable to health-conscious consumers. However, replacing fat in cookies can result in cookies that are harder, and less brittle compared to their full-fat counterparts. Studies have shown that it is challenging to produce low-fat cookies without affecting their structural, visual, color, and sensorial properties. To address this, healthy ingredients such as proteins, fibers, antioxidants, vitamins, and minerals can be added to the cookie production process to create a healthier end-product. Previous studies have used microalgae biomass, such as *Chlorella vulgaris*, *Isochrysis galbana*, *Dunaliella salina*, and *Spirulina platensis*, as a coloring agent and functional food ingredient in cookie production (Batista et al., 2017, 2019; Gouveia et al., 2007; Kadam & Prabhasankar, 2010; Shahbazizadeh et al., 2015).

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The term "bioaccessibility" is often used to quantify the amount of nutrients that are released in the gastrointestinal (GI) tract and is frequently utilized as a gauge of absorption rather than the quantity ingested. It is essential to evaluate the bioaccessibility of food constituents, as only those compounds capable of withstanding the rigorous conditions of the gastrointestinal tract and effectively crossing the intestinal epithelium can be bioavailable for utilization by the human body. Although microalgae are known as superfoods because of their protein content, amino acid, and mineral profiles, there are very few studies that focus on the bioaccessibility of microalgae-rich foods (Hossain et al., 2017; Uribe-Wandurraga et al., 2020). Therefore, in order to fill the gap for microalgae-enhanced foods, the aim of this study is to determine the bioavailability of minerals, total phenolic content, and antioxidant capacity in reduced-fat cookies enhanced with *Chlorella vulgaris*.

2. Materials and Methods

2.1. Preparation of microalgal biomass

Chlorella vulgaris (UTEX 26) was grown in modified Bold's medium as described by Powell et al. (2009) and maintained at pH 6.8. The concentrations of nutrients in the medium (mg L^{-1} of reverse osmosis water) were: 75 KH_2PO_4, 50 K_2HPO_4, 75 $NaNO_3$, 25 $MgSO_4$, 7 H_2O, 12.5 $CaCl_2$, 12.5 NaCl, 60 $NaHCO_3$, 25 EDTA-sodium salt, 2.5 $FeSO_4$, 7 H_2O and 0.5 mL of trace mineral solution. The trace mineral solution consisted of (mg per 100 mL of reverse osmosis water): 1250 boric acid, 882 $ZnSO_4$, 70 MoO_3, 50 $Co(NO_3)_2$, 140 $MnCl_2$, 160 $CuSO_4$, 5 H_2O. The prepared medium was sterilized in an autoclave at 120 °C for 15 min and cooled to 22 °C prior to use. *C. vulgaris* was pre-cultured for 7 days and then cultivated in batch mode for 15 days in photobioreactor (PBR) at 25 °C with 12/12 lightening period at 3200 lux. The biomass was harvested when the growth of microalgae achieved a stationary phase, centrifuged at 6000 x*g* and then freeze dried (Teknosem, TRS2/2V, Turkey). The freeze-dried biomass consisted of 5.83±0.08% moisture, 9.85±0.02% ash, 53.75±0.09% protein, 14.09±0.45% lipid, and 16.48±0.07% carbohydrate.

2.2. Dough formulation and cookie preparation

Table 1 referenced the ingredients and specific *C. vulgaris* biomass concentration for cookies. The wheat flour used was supplied by Toru Un Inc. (Türkiye) and had 13.0% moisture, 9.80% protein, 0.65% ash, and 24.0% wet gluten. All other ingredients (powdered sugar, brown sugar, sodium bicarbonate, salt, skimmed milk powder, shortening, ammonium bicarbonate, and high fructose corn syrup) were procured from the local market (Bursa, Türkiye). The dry ingredients (excluding flour and ammonium bicarbonate) were combined and mixed well. This dry mixture and shortening were placed in the bowl of the mixer (Kitchen Aid, 5KSM150PSEAC model, USA) and mixed for 3 min in total, stopping every minute to scrape down the sides of the bowl, to obtain a cream. In a separate bowl, a liquid mixture was prepared with water, high fructose corn syrup (HFCS), and ammonium bicarbonate, which was then added to the cream and mixed for 1 min, stopping every 15 seconds. Flour or a mixture of flour and biomass was added to this mixture and mixed for 30 seconds, stopping every 10 seconds, to obtain cookie dough. The dough was then rolled out to a thickness of 10 mm, cut into 40 mm diameter and 10 mm height circle disks, and baked in an oven at 205±2 ºC for 11 min. The cookies were cooled and stored in polyethylene bags under dark conditions at room temperature.

2.3. *In vitro* digestion

In vitro digestion analysis of cookies were carried out with the method according to Brodkorb et al., (2019). For this purpose, 1 g of cookie was mixed with 5 mL of distilled water, 3.5 mL of simulated salivary fluids (SSF), 0.5 mL of α-amylase, 25 µL of 0.3 M $CaCl_2$, and 975 µL of water, and held in a water bath at 37 ºC for 2 min. Then, the mixed solution was transferred into a gastric medium containing 7.5 mL of simulated gastric fluids (SGF), 1.6 mL of pepsin solution, 5 µL of 0.3 M $CaCl_2$, 0.2 mL of 1 M HCl, and 0.556 mL of distilled water. The pH of this medium was arranged to 3 and samples were kept in a shaking water bath at 100 rpm for 2 h at 37 ºC. At the end of this period, 10 mL of solution was inserted into the intestinal medium consisting of 5.5 mL simulated intestinal fluids (SIF), 2.5 mL pancreatin solution, 1.25 mL bile solution, 20 µL 0.3 M $CaCl_2$, 0.075 mL 1 M NaOH, and 0.655 mL distilled water. The pH of this medium was arranged to 7 and the mixture was held in a shaking water bath at 100 rpm for 2 h at 37 ºC. For *in vitro* digestion of cookies, samples were centrifuged at 9 000 rpm for 30 min at 4 ºC. The supernatant was taken and stored at -18 ºC. *In vitro* digestion of samples was determined with the TPC, antioxidant capacity and mineral profile analyses as mentioned above.

Table 1. Cookie ingredients (g/100 g of cookie dough).

Ingredients	C	CB1	CB2	CB3
Flour	50.42	49.91	48.90	47.39
Sucrose (fine granulated)	16.13	16.13	16.13	16.13
Brown sugar	5.04	5.04	5.04	5.04
Skimmed milk powder	0.50	0.50	0.50	0.50
Salt	0.63	0.63	0.63	0.63
Sodium bicarbonate	0.50	0.50	0.50	0.50
Shortening	13.41	13.41	13.41	13.41
High fructose corn syrup	0.76	0.76	0.76	0.76
Water	12.60	12.60	12.60	12.60
***Chlorella vulgaris* biomass**	0.00	0.50	1.51	3.02

C: Control, CB1, 2, and 3: 0.5, 1, and 1.5% *Chlorella* biomass added samples, respectively.

2.4. Proximate analysis

The protein, lipid, ash, and moisture content of cookies were determined according to the AACC (1990) and AOAC (1990) methods. Carbohydrate values were calculated using Atwater general factor system according to FAO (2003). All proximate analyses were repeated, at least in triplicate, and were performed after cookie preparation.

Mineral composition

A sample of 500 mg was incinerated at high pressure in a microwave oven (Muffle P Selecta Mod.367PE) for 24 h at 550 °C, and ash was gravimetrically quantified. The residue of incineration was extracted with HCl (hydrochloric acid) (50% v/v) and HNO_3 (nitric acid) (50% v/v) and made up to an appropriate volume with distilled water. Minerals were measured using standard solutions for calibration purposes. The multi-mineral determination was performed by using an inductively coupled plasma optical emission spectrometer (700 Series ICP-OES; Agilent Technologies, Santa Clara, United States), with an axial viewing and a charge-coupled device detector. Results were given as mg/kg sample.

Total phenolic content and antioxidant capacity

The extraction of extractable and hydrolysable fractions, utilized for assessing the total phenolic content (TPC) and antioxidant capacity, was performed following the protocol of Vitali et al. (2009) with slight modifications. For this purpose, 2 g of each sample was mixed with 20 mL of HCl/methanol/water (1:80:10, v/v/v) and shaken for 2 hours at room temperature on an orbital shaker (Mipro/MLS3535; 250 rpm at 20°C). Afterward, the extracts were subjected to centrifugation at 3500 g for 10 min (Hettich/Universal 320R). The supernatant was employed for evaluating the extractable fractions of the TPC and antioxidant capacity of the cookies.

Following the extractable fraction, the residue was subjected to an additional treatment by adding 20 mL of methanol:H_2SO_4 (10:1, v/v) and incubated at 85 °C for 20 h. The resultant mixture was subjected to centrifugation at 3500 g for 10 min at 4 °C (Hettich/Universal 320R). The supernatant obtained after centrifugation was utilized as the hydrolysable fraction.

In vitro enzymatic digestion extraction, which imitates the gastrointestinal conditions, was employed to obtain the bioaccessible fractions of the cookies as per the method described by Bouayed et al. (2012).

Antioxidant capacity of samples was determined by cupric ion-reducing antioxidant capacity (CUPRAC) assay, free radical scavenging assay (2,2-diphenyl-1-picrylhydrazyl, DPPH) (Brand-Williams et al., 1995) and radical cation decolorization assay (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid), ABTS) (Apak et al., 2008). The calibration curve of the Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was prepared and the results were given as µmol trolox equivalent/g dry weight.

2.5. Statistical analysis

The results of the analyses were presented as mean ± standard deviation. All analyses were conducted at least in triplicate per duplicated cookie sample. The statistical differences between the cookie samples were evaluated by analysis of variance (ANOVA) and mean differences were determined using Duncan's multiple comparison at a significance level of 5% with the SPSS program (IBM Corp., USA).

3. Results and Discussion

3.1. Proximate analysis

The freeze-dried biomass of *Chlorella* sp. consists of 5.83% (±0.08) moisture, 9.85% (±0.02) ash, 53.75% (±0.09) protein, 14.09% (±0.45) lipid, and 16.48% carbohydrate. Table 2 represents the chemical composition of cookies with and without microalgal biomass addition. Owing to the fact that the protein rich microalgal biomass, there was a significant protein content increase from 5.55% to 7.08%. As expected, moisture and ash contents also showed a statistically significant increasing trend ($P< 0.05$). These findings were in consistence with previous studies (Batista et al., 2017; Fadila & Widyaningrum, 2023).

The fat content results of our study indicated a statistically significant but relatively modest increase in lipid content in cookies upon the addition of *Chlorella*. The lipid composition of *Chlorella* biomass exhibited considerable variability, dependent on the species, strain, and growth conditions. This variability was observed to range from 1-12 % of the biomass on a dry basis. However, it has been postulated that the incorporation of a specific quantity of *Chlorella* can enhance the total lipid content of food products containing *Chlorella* (Batista et al., 2017).

3.2. *In vitro* analysis

Fortification of cookies offers an alternative convenient strategy for delivering the nutritional and functional compounds. Bioaccessibility of a food component, is the fraction that release from food matrix into the digestive system. The results of the extractable and hydrolysable phenolic content, antioxidant capacities, in terms of ABTS, CUPRAC and DPPH as well as in vitro bioaccessibility are given in Table 3a-c.

Phenolic compounds are classified as phenols, flavonoids, phenolic acids and their derivatives, which are natural antioxidants.

Table 2. Chemical composition of cookies.

Parameters	Cookies			
	C	CB1	CB2	CB3
Moisture	6.80±0.16^{c}	5.86±0.28^{b}	5.63±0.84^{ab}	5.11±0.58^{a}
Ash	0.47±0.01^{d}	0.49±0.01^{c}	0.53±0.01^{b}	0.55±0.01^{a}
Protein	5.55±0.05^{d}	5.70±0.02^{c}	6.18±0.02^{b}	7.08±0.03^{a}
Fat	12.35±0.01^{d}	12.51±0.01^{c}	12.81±0.06^{b}	13.37±0.11^{a}
Carbohydrate	74.83±0.23	75.45±0.30	74.85±0.72	73.89±0.77

C: Control, CB1, 2, and 3: 0.5, 1, and 1.5% *Chlorella* biomass added samples, respectively. Values are means ± standard deviation. $^{a-d}$ Means within the same row with different letters are different ($P<0.05$).

Table 3. Total phenolic content, antioxidant capacity and *in vitro* bioaccessibility of a) indicates *Chlorella* biomass, b) indicates undigested cookie samples incorporated with *Chlorella* biomass, c) indicates cookie samples incorporated with *Chlorella* biomass after in vitro digestion protocol.

	Chlorella biomass		Total phenolic content (mg GAE/g)	Antioxidant capacity (µmol TE/g) ABTS	CUPRAC	DPPH
a)	**Extractable**		21.64±0.12	5.52±0.61	9.11± 0.19	17.22±0.12
	Hydrolyzable		401.13±12.25	156.37±1.07	388.95±1.36	232.15±3.27
	Total		422.67±12.37	161.89±1.68	398.06±1.55	249.37±3.39
	In vitro	**Oral**	20.00±0.38	9.76±0.32	12.39±0.59	ND
		Gastric	41.28±1.77	4.51±0.33	14.34±0.03	ND
		Intestinal	54.20±0.38	17.22±0.91	13.05±0.40	10.86±0.79

	Undigested samples		Total phenolic content (mg GAE/g)	Antioxidant capacity (µmol TE/g) ABTS	CUPRAC	DPPH
b)	**Extractable**	**C**	9.85±0.21^{c}	0.63±0.07^{b}	2.47±0.66^{b}	7.33±1.00^{b}
		CB1	10.59±0.18^{bc}	0.71±0.12^{a}	3.06±0.11^{ab}	7.67±0.07^{b}
		CB2	11.04±0.10^{ab}	0.84±0.05^{a}	3.82±0.28^{a}	8.06±0.09^{ab}
		CB3	12.02±0.68^{a}	1.00±0.18^{a}	4.19±0.41^{a}	8.61±0.16^{a}
	Hydrolyzable	**C**	187.57±1.23^{d}	59.70±5.04^{b}	114.10±1.85^{b}	89.59±5.23^{c}
		CB1	190.23±0.74^{c}	72.17±1.19^{a}	126.05±4.97^{ab}	100.52±0.22^{b}
		CB2	206.19±2.65^{b}	74.84±2.56^{a}	132.85±3.31^{ab}	110.98±2.73^{a}
		CB3	262.05±5.87^{a}	77.52±1.26^{a}	150.19±3.97^{a}	113.87±4.54^{a}
	Total	**C**	197.42±1.44^{d}	60.33±5.67^{b}	116.57±2.51^{b}	96.92±6.23^{c}
		CB1	200.82±0.92^{c}	72.88±1.31^{a}	129.11±5.08^{ab}	108.19±0.29^{bc}
		CB2	217.23±3.65^{b}	75.68±2.61^{a}	136.67±3.59^{ab}	119.04±2.82^{ab}
		CB3	274.07±6.55^{a}	78.52±1.44^{a}	154.38±4.38^{a}	122.48±4.70^{a}

	Digested samples		Total phenolic content (mg GAE/g)	Antioxidant capacity (µmol TE/g) ABTS	CUPRAC	DPPH
c)	**Oral**	**C**	23.13±0.56	7.57±0.13^{c}	1.29±0.18^{d}	ND
		CB1	28.51±1.72	8.20±0.16^{b}	3.30±0.15^{c}	ND
		CB2	30.53±0.27	8.36±0.05^{b}	4.77±0.20^{b}	ND
		CB3	30.92±2.28	9.38±0.09^{a}	6.17±0.56^{a}	ND
	Gastric	**C**	19.28±0.97^{c}	3.98±0.36^{b}	2.65±0.02^{b}	ND
		CB1	25.59±0.81^{b}	4.05±0.06^{b}	2.68±0.07^{b}	ND
		CB2	43.65±2.66^{a}	4.30±0.03^{b}	3.12±0.15^{ab}	ND
		CB3	44.54±1.6^{a}	5.74±0.80^{a}	3.75±0.58^{a}	ND
	Intestinal	**C**	26.93±0.19^{d}	6.48±0.09^{b}	3.12±0.15^{b}	3.14±0.06^{b}
		CB1	32.31±1.72^{c}	6.76±1.24^{b}	3.75±0.58^{b}	3.78±0.23^{a}
		CB2	39.65±0.81^{b}	8.95±0.06^{a}	6.33±0.15^{a}	3.85±0.01^{a}
		CB3	47.26±2.55^{a}	9.21±0.02^{a}	7.17±0.02^{a}	3.98±0.06^{a}

C: Control, CB1, 2, and 3: 0.5, 1, and 1.5% *Chlorella* biomass added samples, respectively. Values are means ± standard deviation. $^{a-d}$ Means within the same row with different letters are different (P<0.05).

Compared to other microalgae, such as *Spirulina sp.*, *Chlorella sp*. have lower contents (Batista et al., 2017). On the other hand, in comparison to other microalgae, green microalgae Chlorella exhibit antioxidant capacity and activity due to their high content of chlorophylls (a and b) (Wang & Wink, 2016) and vitamin E (Chini Zittelli et al., 2006), which includes compounds with antioxidant capacity has been observed in several microalgae, including tocopherols and tocotrienols (Traber & Atkinson, 2007). As reported by Lanfer-Marquez et al., (2005), chlorophylls are capable of inhibiting the DPPH radical. A study conducted by Siriwardhana et al. (2003) also demonstrated a high correlation between DPPH radical scavenging activities and total polyphenolic content. TPC of C samples varied from 9.85±0.21 to 12.02±0.68 mg GAE/g. Cookies containing 3% *Chlorella* biomass (CB3) had a significantly (P<0.05) higher extractable and hydrolysable TPC than then all samples, 12.02±0.68 mg GAE/g and 262.05±5.87 mg GAE/g, respectively. Extractable (free) and hydrolysable (bond) antioxidant capacity, which can be seen in Table 3b, were determined by the ABTS, CUPRAC and DPPH assays. For all assays, compared to control cookies, in connection with the aforementioned correlation between antioxidant and phenolic substances, an increase (P<0.05) in antioxidant capacity values was observed, when increasing biomass concentration. In terms of ABTS, replacement of flour with the increasing amount of *Chlorella* biomass did not show a significant difference (P>0.05). On the other hand, in contrast to Lanfer-Marquez et al. (2005), *Chlorella* biomass presence did not inhibit the DPPH scavenging activities. Total DPPH values of C samples were obtained as 96.92±6.23 µmol TE/g, while CB3 samples were increased to 122.48±4.70 µmol TE/g.

Similar findings were obtained for in vitro bioaccessibility. As the presence of *Chlorella* biomass increased, TPC, ABTS, CUPRAC, and DPPH intestinal bioaccessibility values also significantly increased (P<0.05). Nevertheless, due to a significant reduction of antioxidant capacity of cookies was noticed after gastric and oral digestions, the determination of the DPPH of oral and gastric bioaccessibility could not be accomplished. The intestinal bioaccessibility of total phenolic content was found to be increased from 26.93±0.19 mg GAE/g to 47.26±2.55 mg GAE/g respectively.

Table 4. Mineral content and *in vitro* mineral bioaccessibility of cookie samples.

		Na	Mg	P	K	Ca	Fe	Cu	Zn	Se
U	**C**	3932.10±45.40^{c}	172.70±30.82^{c}	1305.22±7.07^{d}	879.60±12.73^{d}	213.80±8.38^{c}	9.16±0.23^{d}	0.90±0.01^{b}	8.00±0.28^{c}	ND
	CB1	4325.90±36.63^{b}	212.40±30.39^{bc}	1406.04±8.54^{c}	992.50±16.97^{c}	263.60±4.24^{b}	10.74±0.34^{c}	1.04±0.06^{a}	8.54±0.20^{bc}	0.18±0.01
	CB2	4422.10±31.25^{b}	271.80±14.14^{ab}	1724.71±33.94^{b}	1053.70±12.43^{b}	268.40±11.31^{b}	12.98±0.68^{b}	1.05±0.04^{a}	8.83±0.11^{ab}	0.29±0.03
	CB3	4857.20±38.18^{a}	333.20±46.67^{a}	2118.68±25.46^{a}	1145.90±13.64^{a}	304.00±5.66^{a}	14.56±0.79^{a}	1.09±0.03^{a}	9.17±0.24^{a}	0.43±0.04
O	**C**	54.25±5.66^{d}	1.57±0.14^{d}	35.23±1.41^{c}	97.85±4.24^{c}	20.27±1.52^{c}	0.87±0.21^{c}	0.02±0.01^{c}	0.52±0.17^{c}	ND
	CB1	100.28±7.07^{c}	10.49±1.41^{c}	50.45±7.07^{c}	118.98±11.31^{b}	35.15±7.15^{b}	1.29±0.12^{c}	0.15±0.03^{b}	0.65±0.07^{bc}	ND
	CB2	337.79±9.90^{b}	28.96±4.24^{b}	93.68±7.07^{b}	121.24±1.41^{b}	40.35±3.25^{b}	2.03±0.14^{b}	0.20±0.04^{b}	0.99±0.12^{ab}	ND
	CB3	559.66±26.87^{a}	60.13±5.66^{a}	182.67±14.14^{a}	151.72±7.07^{a}	60.29±6.85^{a}	3.26±0.36^{a}	0.34±0.06^{a}	1.32±0.14^{a}	0.02±0.01
G	**C**	806.08±11.31^{d}	35.02±2.80^{b}	83.15±4.24^{d}	208.00±11.31^{b}	101.16±7.07^{c}	2.07±0.10^{c}	0.04±0.01^{b}	1.24±0.14^{b}	ND
	CB1	917.84±24.04^{c}	38.73±2.83^{b}	135.04±7.07^{c}	228.19±9.25^{b}	122.50±2.83^{b}	3.03±0.14^{c}	0.08±0.01^{b}	1.73±0.28^{b}	ND
	CB2	1084.15±62.23^{b}	57.54±9.90^{a}	204.92±5.66^{b}	232.14±2.82^{b}	123.15±4.24^{b}	5.89±0.42^{b}	0.13±0.04^{b}	2.99±0.42^{a}	ND
	CB3	1361.70±28.28^{a}	70.98±5.66^{a}	295.80±6.56^{a}	324.95±14.14^{a}	186.94±8.48^{a}	7.59±0.56^{a}	0.38±0.11^{a}	3.24±0.06^{a}	ND
I	**C**	1000.39±21.21^{d}	96.58±0.71^{d}	466.31±8.49^{d}	346.54±8.49^{d}	64.92±5.66^{b}	0.25±0.07^{c}	0.18±0.03^{b}	2.22±0.03^{d}	ND
	CB1	2112.70±16.97^{c}	132.40±2.83^{c}	667.10±9.89^{c}	431.93±14.14^{c}	96.56±4.24^{a}	1.91±0.01^{b}	0.21±0.01^{b}	3.53±0.04^{c}	0.05±0.01^{b}
	CB2	2442.61±24.04^{b}	137.80±9.90^{b}	720.39±14.14^{b}	582.29±7.07^{b}	99.44±2.83^{a}	2.06±0.08^{b}	0.29±0.03^{a}	4.56±0.08^{b}	0.06±0.01^{b}
	CB3	2747.04±28.28^{a}	169.34±5.66^{a}	781.72±21.21^{a}	678.62±11.31^{a}	106.45±4.24^{a}	4.79±0.28^{a}	3.43±0.11^{a}	0.35±0.01^{a}	5.38±0.11^{a}

C: Control, CB1, 2, and 3: 0.5, 1, and 1.5% *Chlorella* biomass added samples, respectively. U: undigested, O: oral phase, G: gastric phase, I: intestinal phase. Values are means ± standard deviation. $^{a-d}$ Means within the same column with different letters are different ($P<0.05$).

The antioxidant capacity of the samples was determined to be in the range of 6.48±0.09 µmol TE/g to 9.21±0.02 µmol TE/g for ABTS, 3.12±0.15 µmol TE/g to 7.17±0.02 µmol TE/g for CUPRAC, and 3.24±0.06 µmol TE/g to 3.98±0.06 µmol TE/g for DPPH, respectively. The results demonstrated that the quantity of bioactive nutrients absorbed from the intestine is which is defined as bioaccessibility was positively affected by the presence of *Chlorella* biomass as a flour substitute.

Previous studies demonstrated the potential for creating new foods especially snacks enriched with microalgal biomass by providing natural bioactive compounds derived from microalgae.

The development and in vitro bioaccessibility of cookies enriched with 1.5% or 2.0% *Chlorella* or *Arthrospira* with added functional minerals was also investigated (Uribe-Wandurraga et al., 2020). The results demonstrated that these cookies facilitated greater accessibility for the absorption of minerals such as calcium, iron, potassium, magnesium, phosphorus, selenium, and zinc in the human body. The mineral content and in vitro bioaccessibility of mineral of the cookies within the scope of this study were presented in Table 4. The commercially available *Chlorella* biomass was known as rich in phosphorus (P), sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), and iron (Fe); other mineral contents included manganese (Mn), zinc (Zn), selenium (Se), and copper (Cu) (Bito et al., 2020). All the cookie samples substitute with *Chlorella* biomass was screened and determined in terms of these minerals. As anticipated, which given in Table 4, the incorporation of *Chlorella* resulted in a notable enhancement in the mineral content of all samples ($P<0.05$). *Chlorella* is a very well-known microalgae for its Se content. Se is a vital trace mineral that plays a crucial role in human health. It is a component of seleno-proteins, including thioredoxin reductase and glutathione peroxidases, which assist in the protection of cells from oxidative damage. As illustrated in Table 4, Se was not detected in control cookie samples.

In accordance with the European Parliament and Council regulation no. 1924/2006 on nutrition and health claims for foods, cookies enriched with 1.5% or 2% of *Chlorella* or *Spirulina* are considered "high in selenium" (Tokuşoglu & Unal, 2003). This designation is based on the fact that a daily selenium intake requires plasma concentrations of 55 µg for both men and women. Although the addition of microalgae resulted in elevated levels of phosphorus, potassium, calcium, iron, magnesium, and zinc in the cookies, none of these minerals reached the levels necessary to substantiate specific health claims. The Se content of *Chlorella* biomass added (CB) samples was determined to be 0.18±0.01, 0.29±0.03, and 0.43±0.04 mg/kg, respectively. Additionally, the bioaccessibility of selenium was found to be significantly increased ($P<0.05$) in the CB3 samples, with a bioaccessibility of 0.13±0.01 mg/kg.

The Recommended Dietary Allowances (RDA) stipulate that the recommended daily intake of calcium (Ca) for an adult male is 1.000 mg, for an adult female it is 800 mg, and for children aged 4 to 8 years it is 800 mg. When the results were examined, Ca content of cookies were increased 213.80±8.38 to 304.00±5.66 mg/kg ($P<0.05$). *In vitro* Ca intestinal bioaccessibility was also recorded with an increase from 64.92±5.66 to 106.45±4.24 mg/kg.

Calcium can inhibit iron absorption when fed as inorganic calcium compounds. As is well documented, iron (Fe) is the most studied mineral both for in vivo and in vitro conditions. Fe bioaccessibility, like the other minerals, were found to be increased with significant differences ($P<0.05$). In a previous study, a decreased sodium bioaccessibility was observed in microalgae-amended cookies. This was attributed to competition with other monovalent competing ions, such as potassium (Kulkarni et al., 2007; Uribe-Wandurraga et al., 2020). However, the presented study demonstrated that both Na and K contents and bioaccessibility exhibited statistically significant increases in response to an increase in the substitution of *Chlorella* biomass.

Consequently, according to Table 4, it can be posited that the intestinal bioaccessibility values of all minerals were found to be within the range of 30% to 50%. The addition of *Chlorella* biomass to the cookies resulted in a 2-fold increase in mineral bioaccessibility.

4. Conclusions

The incorporation of microalgae as an ingredient and also as a flour substitute to enhance the functionality in terms of total phenolic, antioxidant capacity and mineral content of cookies was still a promising alternative. Cookies enriched with 1.5 or 2% of *Chlorella* or *Spirulina* are classified as "high in selenium" foods. The incorporation of *Chlorella* biomass in cookie formulations permitted greater accessibility of the aforementioned total phenolic and antioxidant capacity. The objective of functional cookie production is not merely to augment the quantity of phenolic compounds and antioxidant capacity; it is also to enhance their bioaccessibility by modifying the nutritional profile. The mineral content is a further reason for the popularity of functional products. The study demonstrated that the bioaccessibility of minerals also increased with the increase in chlorella content. The value of this increase was 5 mg/kg with the addition of 1.5% *Chlorella*, with the value of Se, in particular, exhibiting a notable trend. The presented study has shown that the bioaccessibility of total phenolics and antioxidants ranges from about 5% to 20%, while for minerals it is between 30-50%. Nevertheless, despite the extensive research and studies conducted to date, there remains a significant knowledge gap in the area of "*in vitro* bioaccessibility" of microalgae-added foods. This study is expected to be a pioneering investigation into the expansion of bioaccessibility and the elucidation of the positive impact of algae-based food consumption on human health promotion.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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