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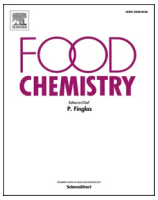

Effect of ultrasounds and ball milling on the bioaccessibility of carotenoids from the microalga *Chlorella sorokiniana*

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ABSTRACT

The bioaccessibility of *Chlorella sorokiniana* carotenoids is typically low due to the rigidity of its cell wall. This study evaluated the effect of ultrasound and ball milling (BM) on carotenoid bioaccessibility in wild-type and phytoene-enriched *C. sorokiniana* in three types of matrices: fresh, freeze-dried, and alginate-encapsulated. The *in vitro* digestion methodology followed the standardized INFOGEST protocol. Ball milling at 30 Hz significantly improved bioaccessibility in wild-type matrices, whereas milder conditions (BM-5 Hz or USD-30%) were more effective in phytoene-enriched and encapsulated samples. Freeze-drying and encapsulation also improved carotenoid bioaccessibility compared to fresh biomass. The addition of yogurt (10% fat) increased the incorporation of carotenoids into micelles, particularly the highly hydrophobic ones α- and β-carotene. Furthermore, the *cis* isomers showed greater bioaccessibility than their *trans* counterparts. These results highlight the importance of applying disruption treatments, encapsulation, or fat-addition to improve the bioaccessibility and bioavailable content of microalgal carotenoids.

1. Introduction

Carotenoids are natural lipophilic compounds widely distributed in foods, mainly fruits and vegetables, but also in cereals, eggs, dairy and fish, among others. Alternative carotenoid sources that can be produced sustainable are attaining growing attention. Examples are microalgae, seaweeds, fungi, bacteria or insects (Li et al., 2024; Meléndez-Martínez et al., 2022). They are essential for food security, sometimes as precursors of apocarotenoids, due to their important roles in processes such as photosynthesis, pollination, seed dispersal and plant development and resilience (Bufka et al., 2024; Meléndez-Martínez et al., 2021). Their biological actions range from vitamin A activity (in the case of certain carotenoids, such as α- and β-carotene), to protection against oxidation, inflammation or photodamage (especially in the case of the colorless carotenoids phytoene and phytofluene, capable of absorbing ultraviolet radiation), among others. A diet rich in carotenoids has been associated with a reduction in the risk of suffering from cardiovascular diseases, cancer, eye disorders and other conditions (Meléndez-Martínez et al., 2019). It is no wonder that carotenoids are interesting as ingredients of diverse products intended for human consumption, including functional foods, nutraceuticals, nutricosmetics or supplements, among others (Meléndez-Martínez et al., 2021). However, it is worth noting that pharmacological doses of the vitamin A precursor β-carotene were associated with an increased risk of lung cancer in subjects at high risk of developing such malignancy (smokers or workers with high exposure to asbestos), as shown in intervention studies from the 1990s (Omenn et al., 1996; The ATBC Cancer Prevention Study Group, 1994).

To fully realize the health benefits of bioactive compounds in foods, both their concentration and their bioavailability are critical. Bioaccessibility, defined as the fraction of a compound released from the food matrix during gastrointestinal digestion and rendered available for absorption (Parada & Aguilera, 2007), is often positively correlated with bioavailability; compounds efficiently released are more likely to be absorbed and exert biological effects.

To enhance this process, various physical treatments have been applied to promote matrix disruption and carotenoid release, thereby directly influencing bioaccessibility. These include thermal, high-pressure, or homogenization methods and, more recently, ball milling and high-intensity ultrasound (Jiang et al., 2024). Sonication is recognized as a sustainable, efficient, and potentially scalable technology

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(Bas, 2024; Singla & Sit, 2021). Its capacity to enhance bioaccessibility is driven by the physical effects of acoustic cavitation, with treatment efficacy depending on specific conditions and matrix type (Costa et al., 2023).

The exploitation of aquatic species for foods, ingredients, cosmetics, energy or biomaterials is a central pillar of the European Blue Economy. Microalgae offer numerous advantages, such as carbon fixation, no requirement for arable land, and easily modulated biosynthetic routes (Mapelli-Brahm et al., 2023; Razzak, 2024). Microalgae, such as those of *Chlorella* species, are rich and sustainable sources of carotenoids. However, their bioaccessibility is particularly low due to the mechanical resistance of their cell walls (Demarco et al., 2022), an effect documented in earlier studies (Becker, 1994; Lubitz, 1963) as a major limiting factor for nutrient bioavailability during digestion. In this context, Hendrickx' group investigated the role of structural barriers in limiting carotenoid bioaccessibility, showing that while certain treatments effectively promote release, others may not yield significant improvements (Colle et al., 2010; Knockaert et al., 2012; Palmero et al., 2016). Consequently, sonication is especially relevant for these species, yet studies evaluating its effect on microalgal carotenoid bioaccessibility remain limited. For instance, Gille et al. (2016) reported an up to 18% increase in the carotenoid bioaccessibility from *Chlorella vulgaris* following ultrasound treatment.

The use of ball milling as a technology to improve the bioaccessibility of microalgal lipophilic compounds has also been little explored. However, its potential to disrupt the rigid cell walls of microalgal biomass has been proposed, thus facilitating carotenoid release and improving their bioaccessibility (Demarco et al., 2022). In this sense, recent applications of ball milling to *C. sorokiniana* have shown promising results, especially in lyophilized and encapsulated matrices, where a significant improvement in the extraction of carotenoids from this matrix has been observed (Morón-Ortiz et al., 2024).

Likewise, scientific literature indicates that the presence of lipids in food is a key factor promoting the carotenoid micellization during intestinal digestion, thus increasing their bioaccessibility (Molteni et al., 2022). In this regard, several studies have shown that the addition of lipid sources, such as vegetable oils or dairy products, can significantly improve the solubility and absorption of carotenoids, depending on the type of matrix and the nature of the added lipids. For example, Xavier et al. (2014) demonstrated that the *in vitro* bioaccessibility of pure lutein is significantly improved in the presence of dietary fats, specifically with the use of whole (3.60% fat) and semi-skimmed (1.55% fat) milk and yogurt.

This study was designed to evaluate the effect of two physical cell disruption technologies, ultrasound and ball milling, on carotenoid bioaccessibility in wild and phytoene-enriched *Chlorella sorokiniana*. Three types of matrices were studied: fresh, freeze-dried, and encapsulated. Furthermore, in the freeze-dried matrix, the effect of adding yogurt as a fat source to promote carotenoid bioaccessibility was evaluated.

2. Methodology

2.1. Reagents

Methyl-*tert*-butyl ether and methanol (HPLC-grade) were supplied by Honeywell (Seelze, Germany), while ethyl acetate and hydrochloric acid were obtained from VWR Chemicals (Leuven, Belgium). Diethyl ether, sodium chloride, potassium chloride, sodium bicarbonate, and magnesium chloride hexahydrate were purchased from Panreac Química SA (Barcelona, Spain). Monopotassium phosphate was provided by Merck (Darmstadt, Germany). The following compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA): ammonium carbonate, calcium chloride dihydrate, sodium alginate (medium viscosity, from *Macrocystis pyrifera*), α-amylase (≥ 5 U/mg), pepsin (≥ 2500 U/mg protein), pancreatin (8 × USP specifications) and bile salts. Finally, sodium hydroxide was obtained from J.T.Baker (Deventer, Netherlands). Carotenoid identification was based on an in-house library previously established using authentic standards (including α-carotene, β-carotene, and lutein, among others) and validated according to Stinco et al. (2019).

2.2. Samples

The microalga *Chlorella sorokiniana* (strain 211–32) was provided by the algae collection of the Institute of Plant Biochemistry and Photosynthesis (IBVF-CSIC, Seville, Spain). The cultures were grown under photomixotrophic conditions using TAP (Tris-acetate-phosphate) liquid medium, following the specifications described in the Chlamydomonas Sourcebook (Harris, 1989). Incubation conditions were kept constant in a climate chamber at 25 °C, with continuous white light irradiation at an intensity of 100 $\mu E\, m^{-2}\, s^{-1}$ of photosynthetically active radiation (PAR), measured using a Delta OHM quantum photoradiometer equipped with a PAR sensor.

To achieve high cell density cultures, the suspensions were subcultured in TAP medium supplemented with sodium acetate and ammonium chloride, according to the procedure optimized by León-Vaz et al. (2019). Once the desired biomass concentration was reached, it was collected by centrifugation at 4400 rpm and subsequently frozen at −20 °C, lyophilized or encapsulated in alginate beads (see Section 2.3).

2.3. Microalgae phytoene enrichment

To induce phytoene accumulation, *C. sorokiniana* cultures were harvested at mid-exponential phase, resuspended in fresh TAP medium, and divided into 50 mL aliquots. Each culture was treated with increasing concentrations (0–20 μg/mL) of the herbicide norflurazon (NF), according to previously optimized protocols (Morón-Ortiz et al., 2024). Over 72 h, growth and phytoene, colored carotenoids and chlorophyll contents were monitored and compared with untreated cultures. Biomass obtained from NF-treated samples was considered phytoene-enriched and used for further analysis.

2.4. Encapsulation of microalgae

The alginate bead encapsulation technique was carried out according to the procedure described by León and Galván (1994), with slight modifications. Specifically, *C. sorokiniana* cultures were harvested at the end of the exponential phase, when they had a biomass of between 1 and 1.2 g/L dry weight (equivalent to an optical density at 660 nm of approximately 3.5AU). The biomass was resuspended in fresh buffered TAP medium and mixed homogeneously with an equal volume of a sterile 6% (*w/v*) alginate solution, prepared from medium-viscosity sodium alginate, previously sterilized by autoclaving (20 min at 120 °C).

The bead formation was carried out by dripping the mixture onto a cold 0.1 M calcium chloride solution, maintained at 4 °C. The beads obtained, approximately 3 mm in diameter, were stored refrigerated (4 °C) until use. The entire procedure was performed under sterile conditions in a laminar flow cabinet.

2.5. Determination of dry weight

The dry biomass content was determined by filtering 30 mL of culture through pre-weighed glass fibre filters (GF/F, Whatman). After filtration, the retained residues were washed with a solution of ammonium formate (0.5 M) to remove residual salt, and the filters were dried in an oven at 100 °C for 24 h. Finally, the dry weight was calculated as the difference between the initial and final weights of the dry filter, using an analytical balance.

2.6. Experimental design

Each of the *C. sorokiniana* matrices (fresh, freeze-dried and encapsulated; in their standard and phytoene-enriched versions) was divided into five experimental groups: a control (untreated) and four groups subjected to different physical processing conditions, using a ball mill or ultrasound (see Fig. 1 for a detailed schematic of the experimental workflow). The selection of these treatment conditions (time; frequency for ball milling; amplitude for ultrasound) was based on the parameters previously established for the assessment of carotenoid bioaccessibility in *Dunaliella bardawil* (Morón-Ortiz et al., 2025).

Ball milling (model MM400, Retsch, Germany) was performed in a wet state for fresh and encapsulated samples, and in a dry state for freeze-dried matrices. The treatments were carried out at two different frequencies (5 Hz and 30 Hz) for 5 min. The brief processing time was specifically chosen to ensure high reproducibility and minimize potential thermal effects on carotenoid stability.

On the other hand, sonication was carried out using a Q500 ultrasound equipment (QSonica, Newtown, USA), at a frequency of 20 kHz, for 2 min, and assessing two amplitude levels: 30% and 70%. To facilitate the propagation of ultrasonic waves in the matrices, the treatments were carried out in the presence of a saline solution composed of 120 mM NaCl, 6 mM $CaCl_2$ and 5 mM KCl (Fernandes et al., 2021). Thus, before the ultrasound treatment, 10 mL of this solution was mixed with 2.5 g of fresh or encapsulated biomass, or 0.5 g of freeze-dried sample, equivalent to 2.5 g of fresh biomass based on an estimated moisture content (80.9%). After the ultrasound treatment, the supernatant solution was carefully removed by centrifugation, and the pellet was subjected to an *in vitro* digestion protocol to evaluate carotenoid bioaccessibility. The effect of adding lipids on carotenoid bioaccessibility was evaluated by incorporating 2 g of yogurt (10% fat) into 0.5 g of freeze-dried sample before starting the gastrointestinal digestion. Yogurt was selected as a complex fat source to simulate realistic dietary conditions, providing a food matrix where the interaction between lipids and proteins reflects a physiologically relevant scenario for carotenoid micellarization.

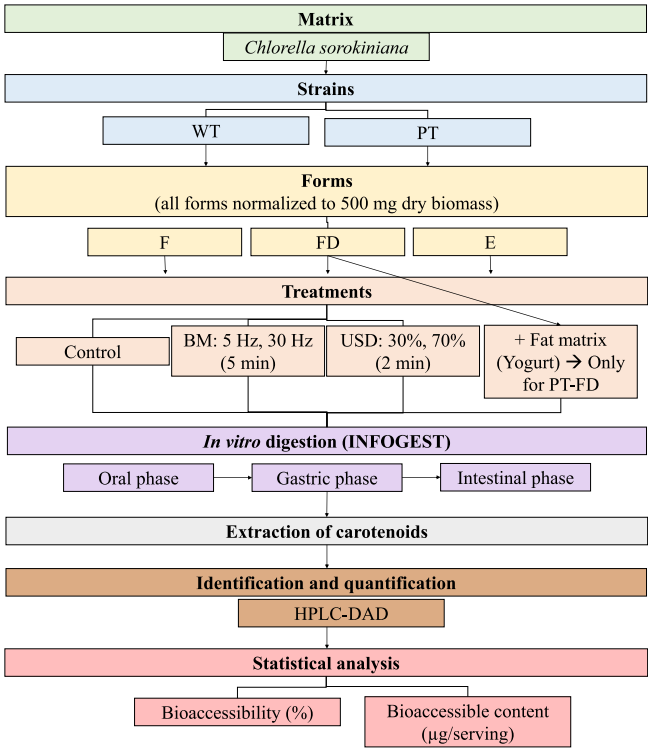

Fig. 1. Experimental design flowchart. The diagram illustrates the processing of *C. sorokiniana* biomass (WT: wild-type; PT: phytoene-enriched) through different delivery systems (F: fresh; FD: freeze-dried; E: encapsulated) and mechanical treatments (BM: ball milling; USD: ultrasound), followed by standardized *in vitro* digestion (INFOGEST 2.0) and carotenoid quantification by HPLC-DAD.

2.7. Extraction of carotenoids from microalgae

To determine the initial carotenoid content in the different matrices (prior to digestion), an extraction protocol using diethyl ether as a solvent was applied (Estévez-Santiago et al., 2016). This solvent was selected for its high affinity for carotenoids and its high volatility, which allows for efficient concentration of the extracts at low temperatures, thus minimizing the risk of carotenoid degradation or isomerization. Approximately 100 mg of each sample was weighed and subjected to the respective treatments (ball mill or ultrasound). In the case of sonicated samples, 2 mL of the extraction solvent was added before treatment, while in ball milled samples, it was added afterwards. After shaking and centrifugation (5 min, 3220 ×*g*, 4 °C), the organic phase was recovered, and the extraction was repeated until the pellet lost its visible colour. Finally, the extracts were concentrated in a rotary evaporator (Eppendorf Concentrator plus™, Hamburg, Germany) and stored at −80 °C until the chromatographic analysis.

To account for any potential changes in carotenoid stability or extractability induced by physical processing, bioaccessibility was calculated relative to the non-digested counterpart of each treated sample (*e.g.*, US-70% digested *vs.* US-70% non-digested). This approach ensures that the observed differences are strictly related to micellarization efficiency rather than alterations in the initial carotenoid yield or profile caused by ball-milling or ultrasound.

2.8. INFOGEST in vitro digestion

Gastrointestinal digestion was simulated following the standardized INFOGEST protocol (Brodkorb et al., 2019), including oral, gastric and intestinal phases.

2.8.1. Oral phase

The samples (2.5 g of fresh biomass; 2.5 g of encapsulated biomass; 0.5 g of freeze-dried biomass, *i.e.*, the dry weight equivalent) were mixed with 2.5 mL of artificial salivary fluid containing α-amylase (75 U/mL) and $CaCl_2$ (1.5 mM). The mixtures were incubated for 2 min at 37 °C in an orbital shaker (SKI 4 Shaker Incubator, ARGO LAB, Italy) at 160 rpm. At the end of incubation, they were placed in ice to stop enzyme activity.

2.8.2. Gastric phase

Subsequently, 5 mL of simulated gastric fluid was added, adjusting the pH to 3 with 6 M HCl. This phase included pepsin (2000 U/mL) and $CaCl_2$ (0.15 mM). Incubation was carried out at 37 °C for 2 h under the same stirring conditions. Enzymatic activity was then stopped by placing the samples in ice.

2.8.3. Intestinal phase

To simulate intestinal digestion, 10 mL of intestinal fluid was added, adjusting the pH to 7 with 1 M NaOH. This phase contained bile salts (10 mM), pancreatin (100 U/mL) and $CaCl_2$ (0.6 mM). The mixture was incubated for another 2 h at 37 °C and 160 rpm. As in the previous phases, after incubation the samples were cooled on ice.

2.8.4. Filtration of the micellar phase

Once digestion was complete, the samples were centrifuged for 20 min at 3220 ×*g* and 4 °C. Then, 10 mL of the micellar phase (supernatant) was collected and filtered using 0.2 μm nylon filters (Captiva Econofltr 25 mm, Agilent Technologies).

2.9. Extraction of carotenoids after digestion

To recover the carotenoids in the micellar fraction, 5 mL of diethyl ether was added to the filtrate, followed by homogenization with an Ultra-turrax (IKA® T25 digital, Staufen, Germany). The mixture was centrifuged (5 min, 3220 ×*g*, 4 °C) and the colored upper phase was collected. The process was repeated until the solvent became colorless, and the combined extracts were concentrated in an evaporator for subsequent analysis by ultra-high-performance liquid chromatography (UHPLC).

2.10. Ultra-high-performance liquid chromatography analysis

Carotenoids were identified and quantified by UHPLC using the 1260 Infinity II Prime LC system (Agilent Technologies, Germany) equipped with a diode array detector and a C_{30} column (3 µm, 4.6 × 150 mm; YMC America, Allentown, PA, USA). Colored carotenoids and phytoene were monitored at 450 nm and 286 nm, respectively. Identification of *cis*-isomers was performed by comparing chromatographic and spectral data with authentic standards available in our in-house library. Dried extracts were reconstituted in 500 µL of ethyl acetate and 10 µL was injected into the system. Quantification was performed using external calibration curves ($R^2 > 0.99$) following previously validated protocols (Stinco et al., 2019). The method's selectivity and high resolution ensure the accurate separation and quantification of carotenoids, including minor *cis*-isomers. The LOD and LOQ were below the concentrations found in most samples, ensuring the method is fit for the quantitative analysis of *C. sorokiniana* extracts. However, in highly diluted matrices such as the encapsulated beads, minor carotenoids fell below these limits due to the high moisture content of the hydrogel. The method's linearity, precision (as %CV), and accuracy were verified prior to analysis to ensure the consistency of the in-house library data for all carotenoids, including *cis*-isomers. The mobile phase consisted of a mixture of methanol, methyl-*tert*-butyl ether, and water, following a linear gradient described by (Stinco et al., 2019). Quantification was performed using calibration curves previously established by the same authors.

2.11. Calculations and statistical analysis

All experiments were performed in triplicate, representing independent biological replicates (separate microalgal cultures and independent extraction/digestion procedures). To evaluate the main effects and interactions on carotenoid bioaccessibility, a three-way analysis of variance (ANOVA) was performed using R Studio (version 4.3.3), considering matrix type, treatment, and carotenoid species as factors (Matrix type × Treatment × Carotenoid). Additional, one-way ANOVA and Tukey's post-hoc test were performed using InfoStat software, version 2020 (InfoStat Group, Faculty of Agricultural Sciences, National University of Córdoba, Argentina). In all cases, differences were considered statistically significant at $p < 0.05$. Tukey's HSD post-hoc test was specifically used to control the family-wise error rate, ensuring the robustness of the results against false-positive discoveries (Type I errors) during multiple comparisons. The total carotenoid content was calculated as the sum of the individual carotenoids.

The bioaccessibility was calculated as the percentage of carotenoids present in the micellar fraction (aqueous phase after digestion and centrifugation) relative to the initial total content of each specific sample (untreated biomass for the control or the corresponding treated sample for the rest of the samples).

Likewise, the carotenoid bioaccessible content (CBC) was calculated by quantifying the absolute amount of micellarized carotenoids, expressed as micrograms, obtained per serving size. The reference portions used for the calculations were: 500 mg for freeze-dried samples, 2620 mg for fresh samples (80.9% moisture) and 9780 mg for encapsulated samples (94.89% moisture), all equivalent to 500 mg of dry biomass. This normalization ensures that CBC values are compared across equivalent microalgal loads, regardless of the moisture or matrix content of each delivery system.

3. Results

3.1. Carotenoid profile of treated samples

Total carotenoid content (TCC) varied among the processed matrices (Supplementary Table 1), likely due to differences in moisture (80.9% for fresh; 94.9% for encapsulated), matrix composition, and the specific extraction efficiency of each system. Moreover, physical treatments could impact the TCC by potentially enhancing extractability through structural disruption or, conversely, affecting carotenoid stability. Thus, TCC values represent the carotenoid levels after physical processing (ball milling or ultrasound) and served as the baseline for bioaccessibility calculations to ensure the results strictly reflect micellarization efficiency. Furthermore, differences in carotenoid profiles were observed among the *C. sorokiniana* matrices.

In the wild-type fresh and freeze-dried *C. sorokiniana*, the main carotenoids identified were, in descending order of concentration, (all-*E*)-lutein, (all-*E*)-α-carotene, (all-*E*)-β-carotene, and (9*Z*)-β-carotene (Supplementary Table 1, Supplementary Fig. 1A and Supplementary Fig. 2A). In the encapsulated wild-type matrix, (all-*E*)-lutein was the only carotenoid quantified (Supplementary Fig. 3A). This observation is likely attributed to the lower algal biomass-to-matrix ratio in the beads, which may dilute minor carotenoids below the method's limit of quantification (LOQ). Additionally, matrix-related effects associated with the alginate hydrogel may influence extraction efficiency compared to the more accessible freeze-dried or fresh matrices.

In all phytoene-enriched matrices (fresh, freeze-dried, and encapsulated), the same colored carotenoids were detected as in the wild-type samples, with lutein being predominant and (9*Z*)-β-carotene the least abundant. Unlike the wild type, which did not contain colorless carotenoids, these matrices showed the presence of (15*Z*)-phytoene. In fresh controls, (15*Z*)-phytoene accounted for 52.0% of total carotenoids, whereas in freeze-dried controls it accounted for 46.1%. In encapsulated controls, (15*Z*)-phytoene accounted for 36.7% of total carotenoids (Supplementary Table 1, and Supplementary Figs. 1B, 2B, 1C, and 2C).

3.2. Carotenoid bioaccessibility in fresh wild-type and phytoene-enriched *C. sorokiniana*

The bioaccessibility of (all-*E*)-lutein, (all-*E*)-α-carotene, (all-*E*)-β-carotene, and (9*Z*)-β-carotene in all matrices, in addition to (15*Z*)-phytoene and (all-*E*)-phytoene in the phytoene-enriched matrices, were evaluated through the standardized INFOGEST protocol (Brodkorb et al., 2019). The total carotenoid bioaccessibility (%) and total carotenoid bioaccessible content (CBC) for all investigated matrices and treatments are summarized in Table 1.

In the fresh wild-type sample, the TCC bioaccessibility varied depending on the treatment, ranging from 0.1% (USD-70%) to 1.3% (BM-30 Hz) (Fig. 2A and Supplementary Table 4A). The carotenoid that showed the highest bioaccessibility under control conditions in this matrix was (all-*E*)-lutein (1.4%); however, under the best treatment for TCC bioaccessibility (BM-30 Hz, without significant differences with BM-5 Hz, $p > 0.05$), the highest bioaccessibility was found for (9*Z*)-β-carotene (2.1%), followed by (all-*E*)-α-carotene, (all-*E*)-β-carotene, and finally (all-*E*)-lutein (Fig. 2A and Supplementary Table 4A).

Regarding CBC, and considering 2620 mg as a product serving, the TCC varied from 1.9 µg/product serving (USD-70%) to 22.4 µg/product serving (BM-30 Hz) (Fig. 2B and Supplementary Table 4A). Under BM-30 Hz, the carotenoid that showed the highest CBC was (all-*E*)-lutein (11.6 µg/product serving), followed by the other individual carotenoids (Fig. 2B and Supplementary Table 4A).

In the phytoene-enriched fresh matrix, the TCC bioaccessibility ranged from 0.6% (USD-70%) to 1.4% (BM-5 Hz) (Fig. 3A and

Table 1
Total carotenoid bioaccessibility (%) and Carotenoid Bioaccessible Content (CBC, μg/serving) of *C. sorokiniana* delivery systems. BM5: Ball-mill 5 Hz; BM30: Ball-mill 30 Hz; CBC: Carotenoid bioaccessible content; E: Encapsulated; F: Fresh; FD: Freeze-dried; PT: Phytoene-enriched; USD 30: Ultrasound 30% amplitude; USD 70: Ultrasound 70% amplitude; WT: Wild-type. Different capital letters refer to statistical differences between treatments in each matrix. Asterisks correspond to statistical differences with yogurt addition compared to control in PT-FD.

Matrix	Form	Treatment	Total carotenoid bioaccessibility (%)		Total CBC (μg per serving)	
WT	F	Control	0.9 ± 0.1	B	3.1 ± 0.2	C
		BM5	1.2 ± 0.1	A	11.2 ± 1.2	B
		BM30	1.3 ± 0.0	A	22.4 ± 0.6	A
		USD30	1.0 ± 0.1	B	11.4 ± 1.1	B
		USD70	0.1 ± 0.0	C	1.9 ± 0.1	C
	FD	Control	7.7 ± 0.3	D	3.0 ± 0.1	D
		BM5	17.4 ± 1.3	C	7.0 ± 0.5	CD
		BM30	58.9 ± 2.6	A	78.6 ± 3.4	A
		USD30	48.8 ± 4.0	B	55.8 ± 4.6	B
		USD70	42.8 ± 1.2	B	11.9 ± 0.3	C
	E	Control	16.7 ± 1.2	D	0.6 ± 0.0	B
		BM5	56.1 ± 4.9	A	3.7 ± 0.3	B
		BM30	23.6 ± 2.8	CD	2.0 ± 0.2	B
		USD30	42.0 ± 3.6	B	2.1 ± 0.2	B
		USD70	25.6 ± 1.8	C	92.7 ± 4.0	A
PT	F	Control	1.2 ± 0.1	A	6.1 ± 0.2	C
		BM5	1.4 ± 0.1	A	7.6 ± 0.4	C
		BM30	1.1 ± 0.1	B	6.9 ± 0.4	C
		USD30	1.3 ± 0.1	A	40.6 ± 3.0	A
		USD70	0.6 ± 0.1	C	17.2 ± 1.8	B
	FD	Control	43.2 ± 3.5	B	76.7 ± 6.1	C
		BM5	56.4 ± 5.7	A	128.6 ± 13.0	AB
		BM30	29.5 ± 2.3	D	142.4 ± 11.3	AB
		USD30	39.4 ± 2.4	BC	119.5 ± 7.2	B
		USD70	32.1 ± 3.1	CD	145.2 ± 13.8	A
		Yogurt	65.3 ± 7.2	*	115.9 ± 8.6	*
	E	Control	41.4 ± 1.9	C	11.8 ± 0.5	D
		BM5	60.2 ± 3.7	A	33.0 ± 2.1	D
		BM30	6.4 ± 0.5	D	96.9 ± 7.4	C
		USD30	59.2 ± 2.4	B	130.0 ± 5.3	B
		USD70	42.3 ± 3.9	C	232.4 ± 14.4	A

Supplementary Table 4B). Although the treatment BM-5 Hz showed the highest bioaccessibility for TCC in this matrix, no significant differences were found with control and USD-30% ($p > 0.05$). Under BM-5 Hz treatment, the carotenoid that showed the highest bioaccessibility was (15*Z*)-phytoene (1.7%) (Fig. 3A and Supplementary Table 4B). While no significant differences were found between the best treatments in TCC bioaccessibility ($p > 0.05$), USD-30% significantly showed the highest TCC CBC (40.6 μg/product serving (2620 mg)) ($p < 0.05$) (Fig. 3B and Supplementary Table 4B). Under this treatment, the carotenoid with the highest CBC was (all-*E*)-lutein (25.2 μg/product serving), followed by (15*Z*)-phytoene (13.9 μg/product serving), while the remaining carotenoids exhibited CBC values more than ten-fold lower than those of lutein and (15*Z*)-phytoene (Fig. 3B and Supplementary Table 4B).

In the wild-type fresh *C. sorokiniana*, the application of BM-30 Hz led to a total carotenoid bioaccessibility improvement of 1.5-fold compared to control (BM-30 Hz: 1.3%; control: 0.9%) (Fig. 2A and Supplementary Table 4A), and a total carotenoid bioaccessible content of 7.2-fold compared to control (BM-30 Hz: 22.4 μg/product serving (2620 mg); control: 3.1 μg/product serving) (Fig. 2B and Supplementary Table 4A). Regarding the phytoene-enriched matrix, the application of BM-5 Hz enhanced the total carotenoid bioaccessibility by 1.1-fold compared to control (BM-5 Hz: 1.4%; control: 1.2%, with no significant differences between them ($p > 0.05$)) (Fig. 3A and Supplementary Table 4B). While BM-5 Hz showed the highest bioaccessibility improvement in the phytoene-enriched matrix, the CBC was significantly enhanced by USD-30% by 6.7-fold compared to control (USD-30%: 40.6 μg/product serving (2620 mg); control: 6.1 μg/product serving) ($p < 0.05$) (Fig. 3B and Supplementary Table 4B).

3.3. Carotenoid bioaccessibility in freeze-dried wild-type and phytoene-enriched *C. sorokiniana*

The bioaccessibility of individual and total carotenoids under different USD (30 and 70%) and ball-milling (5 and 30 Hz) conditions were also assessed in freeze-dried matrices.

In the wild-type matrix, the bioaccessibility of TCC varied from 7.7% (control) to 58.9% (BM-30 Hz) (Fig. 4A and Supplementary Table 5A). Under the significantly best treatment for total carotenoid bioaccessibility, BM-30 Hz, the carotenoid that showed the highest bioaccessibility in this matrix was (9*Z*)-β-carotene (75.4%), followed by (all-*E*)-lutein, (all-*E*)-α-carotene, and (all-*E*)-β-carotene (Fig. 4A and Supplementary Table 5A) ($p < 0.05$). For the remaining treatments in the freeze-dried wild-type matrix, (all-*E*)-β-carotene was also the carotenoid with the lowest bioaccessibility. BM-30 Hz was also the treatment that significantly showed the highest total carotenoid CBC in this matrix (78.6 μg/product serving (500 mg)) (Fig. 4B and Supplementary Table 5A) ($p < 0.05$), being (all-*E*)-lutein the carotenoid with the highest bioaccessible content (58.9 μg/product serving). The rest of carotenoids showed substantially lower bioaccessible content (Fig. 4B and Supplementary Table 5A). For the remaining treatments in the freeze-dried wild-type matrix, (all-*E*)-lutein was also the carotenoid with the highest bioaccessible content.

In the phytoene-enriched freeze-dried *C. sorokiniana* the ball-mill also showed a significant total carotenoid bioaccessibility enhancement ($p < 0.05$), however, with the lowest frequency of 5 Hz (56.4%) (Fig. 5A and Supplementary Table 5B). Under this treatment, (15*Z*)-phytoene showed the highest bioaccessibility (74.8%), followed by (all-*E*)-lutein, (all-*E*)-phytoene, (9*Z*)-β-carotene, (all-*E*)-β-carotene, and (all-*E*)-α-carotene (Fig. 5A and Supplementary Table 5B). For the remaining treatments in the freeze-dried phytoene-enriched matrix, (all-*E*)-β-carotene and (all-*E*)-α-carotene showed also the lowest bioaccessibility, while lutein or (15*Z*)-phytoene exhibited the highest values. Although the BM-5 Hz showed the highest bioaccessibility in the matrix, the treatment that presented the highest total carotenoid bioaccessible content was USD-70% (145.2 μg/product serving (500 mg)). The lowest CBC was found in the control group (76.7 μg/product serving) (Fig. 5B and Supplementary Table 5B). In all treatments for this sample, the highest CBC was observed for (15*Z*)-phytoene and lutein, with values clearly higher than those of the other carotenoids, while the lowest CBC corresponded to (9*Z*)-β-carotene (Fig. 5B and Supplementary Table 5B).

In the wild-type freeze-dried *C. sorokiniana*, BM-30 Hz significantly showed the highest bioaccessibility and CBC compared with the other treatments ($p < 0.05$). Specifically, the improvement in total carotenoid bioaccessibility was 7.6-fold compared to control (BM-30 Hz: 58.9%; control: 7.7%) (Fig. 4A and Supplementary Table 5A), and in the CBC by 26.3-fold (BM-30 Hz: 78.6 μg/product serving (500 mg); control: 3.0 μg/product serving) (Fig. 4B and Supplementary Table 5A). In the phytoene-enriched freeze-dried matrix, the application of BM-5 Hz enhanced bioaccessibility by 1.3-fold compared to the control (BM-5 Hz: 56.4%; control: 43.2%) (Fig. 5A and Supplementary Table 5B), and the application of USD-70% enhanced CBC by 1.9-fold compared to control (USD-70%: 145.2 μg/product serving (500 mg); control: 76.7 μg/product serving) (Fig. 5B and Supplementary Table 5B).

The addition of yogurt (10% fat, at a proportion of 4:1 in weight relative to the algal biomass (0.4:1)) was also assessed in the freeze-dried phytoene-enriched *C. sorokiniana*. The individual and total carotenoid bioaccessibility and bioaccessible content was significantly enhanced ($p < 0.05$) with the yogurt addition compared to the control group (Fig. 5A-B and Supplementary Table 5B). Specifically, the total carotenoid bioaccessibility and the total bioaccessible content were improved by 1.5 times compared to control (control: 43.2% and 76.7 μg/

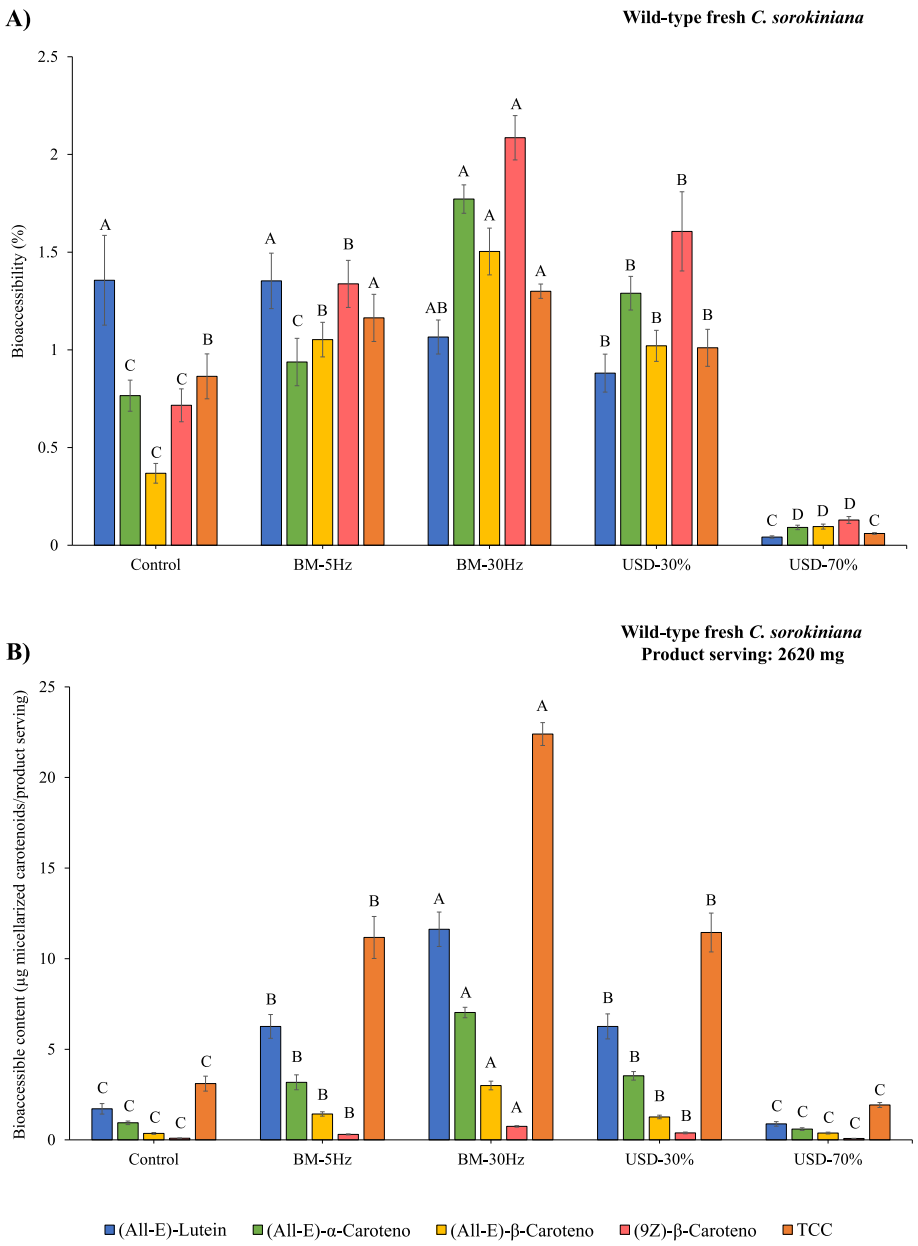

Fig. 2. Carotenoid bioaccessibility (%) (A) and bioaccessible carotenoid content (µg micellarized carotenoids/product serving) (B) in wild-type fresh *C. sorokiniana*. A product serving corresponds to 2620 mg of fresh biomass (80.9% moisture). For the same carotenoid, different capital letters indicate statistically significant differences ($p < 0.05$) between treatments. BM-5 Hz: ball-mill at 5 Hz; BM-30 Hz: ball-mill at 30 Hz; USD-30%: ultrasound with 30% amplitude; USD-70%: ultrasound with 70% amplitude; TCC: total carotenoid content. The error bars in the figures represent the standard deviation (SD).

product serving (500 mg); yogurt: 65.3% and 115.9 µg/product serving) (Fig. 5A-B and Supplementary Table 5B). The carotenoid that showed the greatest improvement in bioaccessibility with fat addition was (all-E)-α-carotene (4.2-fold), followed by (all-E)-β-carotene (1.9-fold), whereas (all-E)-lutein exhibited the lowest enhancement (1.3-fold) (Fig. 5A and Supplementary Table 5B). Among the carotenoids, the highest CBC enhancement was observed for (all-*E*)-α-carotene, followed by (all-*E*)-β-carotene, whereas (all-*E*)-lutein showed the lowest improvement (Fig. 5B and Supplementary Table 5B).

3.4. Carotenoid bioaccessibility in encapsulated wild-type and phytoene-enriched C. sorokiniana

Finally, the bioaccessibility and CBC of (all-*E*)-lutein in the wild-type encapsulated matrix, in addition to (all-*E*)-β-carotene and (15*Z*)-phytoene in the phytoene-enriched encapsulated matrix were evaluated.

In the wild-type matrix, the only carotenoid identified in the micellarized fractions was (all-*E*)-lutein. Among the treatments, BM-5 Hz most significantly enhanced (all-*E*)-lutein bioaccessibility (56.1%, $p < 0.05$), whereas the lowest value was observed under control conditions (16.7%) (Fig. 6A and Supplementary Table 6A). Although USD-70% did not show the highest bioaccessibility (25.6%), was significantly the best treatment for (all-*E*)-lutein bioaccessible content (92.7 µg/product serving (9780 mg)) ($p < 0.05$) (Fig. 6B and Supplementary Table 6A).

In the phytoene-enriched encapsulated matrix, the total carotenoid bioaccessibility varied from 6.4% (BM-30 Hz) to 60.2% (BM-5 Hz) (Fig. 7A and Supplementary Table 6B). Although BM-5 Hz was the treatment that most enhanced total carotenoid bioaccessibility, the highest total CBC was obtained with USD-70% (Fig. 7B and Supplementary Table 6B), as the wild-type matrix. The total carotenoid bioaccessible content varied from 11.8 µg/product serving (9780 mg) (control) to 232.4 µg/product serving (USD-70%) (Fig. 7B and

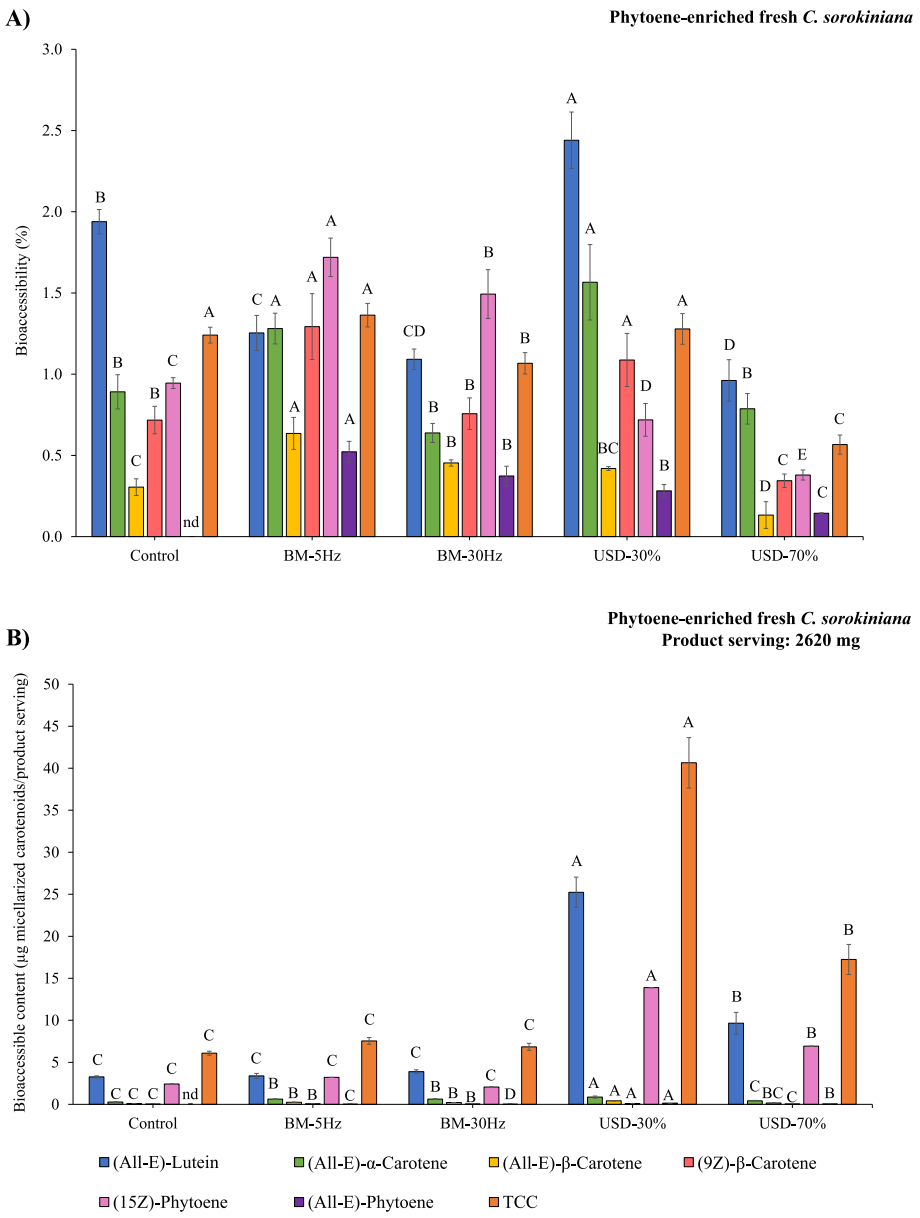

Fig. 3. Carotenoid bioaccessibility (%) (A) and bioaccessible carotenoid content (µg micellarized carotenoids/product serving) (B) in phytoene-enriched fresh *C. sorokiniana*. A product serving corresponds to 2620 mg of fresh biomass (80.9% moisture). For the same carotenoid, different capital letters indicate statistically significant differences ($p < 0.05$) between treatments. BM-5: Hz: ball-mill at 5 Hz; BM-30 Hz: ball-mill at 30 Hz; USD-30%: ultrasound with 30% amplitude; USD-70%: ultrasound with 70% amplitude; TCC: total carotenoid content. The error bars in the figures represent the standard deviation (SD).

Supplementary Table 6B). Under USD-70%, both (all-*E*)-lutein and (15*Z*)-phytoene contributed equally to the CBC (approx. 111.5 µg/serving each) (Fig. 7B and Supplementary Table 6B).

Both encapsulated matrices showed the highest carotenoid bioaccessibility with the application of BM-5 Hz (Fig. 6A and A, and Supplementary Table 6A-B), and the highest CBC with USD-70% (Fig. 6B and B, and Supplementary Table 6A-B). The application of BM-5 Hz in the wild-type matrix enhanced (all-*E*)-lutein bioaccessibility by 3.4-fold compared to control (Fig. 6A, and Supplementary Table 6A), and an improvement of 1.5-fold in TCC in the phytoene-enriched matrix, compared to control (Fig. 7A, and Supplementary Table 6B). Finally, the application of the USD-70% produced an increase of (all-*E*)-lutein bioaccessible content of 157.0-fold in the wild-type matrix compared to the control (Fig. 6B, and Supplementary Table 6A), and an increase of total carotenoid bioaccesible content of 19.7-fold in the phytoene-enriched matrix, compared to the control (Fig. 7B, and Supplementary Table 6B).

3.5. Global analysis of factors influencing bioaccessibility

To understand the combined impact of processing, matrix type, and carotenoid species, a three-way ANOVA was performed (Supplementary Table 2). The analysis revealed that Treatment was the most influential factor ($F = 34.5$, $p < 0.001$), followed by Carotenoid species ($F = 11.0$, $p < 0.001$). Matrix type also showed a significant effect ($F = 4.0$, $p = 0.046$). Notably, a significant interaction was found between Matrix type and Treatment ($p < 0.001$). This synergy indicates that the efficiency of mechanical disruption is highly dependent on the initial physical state of the biomass. Specifically, physical treatments (BM and USD) triggered a higher improvement in fresh and encapsulated matrices, where the cell walls are intact and offer high resistance. In contrast, the relative impact of these treatments was lower in freeze-dried samples, as the lyophilization process itself already induces substantial structural damage, leaving less margin for further enhancement by mechanical means (Baudelet et al., 2017; Bernaerts et al., 2026; Gille

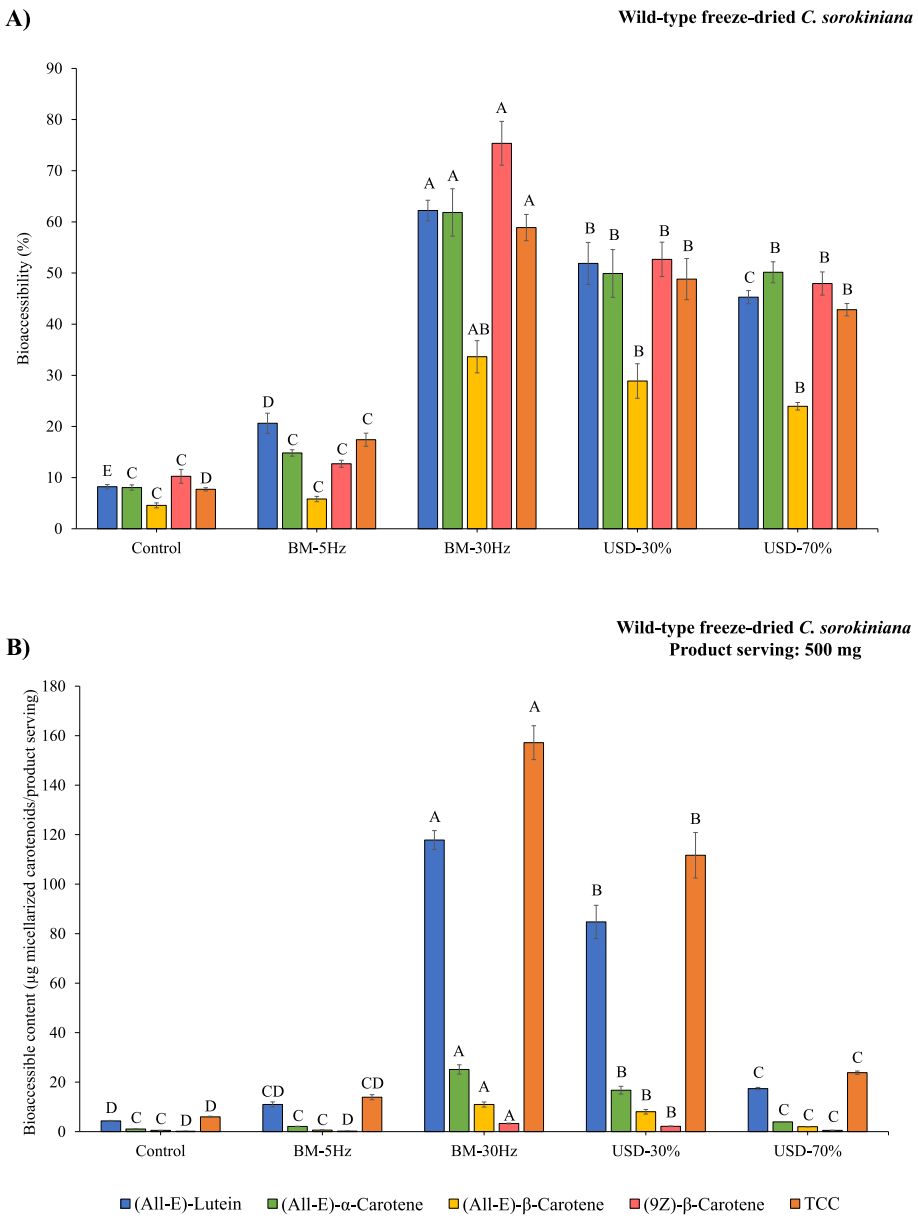

Fig. 4. Carotenoid bioaccessibility (%) (A) and bioaccessible carotenoid content (µg micellarized carotenoids/product serving) (B) in wild-type freeze-dried *C. sorokiniana*. A product serving corresponds to 500 mg of freeze-dried biomass. For the same carotenoid, different capital letters indicate statistically significant differences ($p < 0.05$) between treatments. BM-5 Hz: ball mill at 5 Hz; BM-30 Hz: ball mill at 30 Hz; USD-30%: ultrasound with 30% amplitude; USD-70%: ultrasound with 70% amplitude; TCC: total carotenoid content. The error bars in the figures represent the standard deviation (SD).

et al., 2016).

4. Discussion

4.1. Carotenoid profile

The carotenoid profiles across all matrices, including both the wild-type and the phytoene-enriched microalgae, were consistent with our previous findings (Morón-Ortiz et al., 2024). (all-*E*)-Lutein was the major pigment in wild-type samples, while phytoene-enriched matrices were characterized by a high accumulation of (15*Z*)-phytoene. In the latter, the high accumulation of this colorless carotenoid is driven by the inhibition of the enzyme phytoene desaturase by norflurazon. The enrichment strategy resulted in a phytoene content of 52.6%, 48.1%, and 36.7% of the total carotenoids in the fresh, freeze-dried, and encapsulated samples, respectively (Supplementary Fig. 1). Beyond redirecting the metabolic flux, this treatment may alter the alga's internal structure and membrane proteins (Leetanasaksakul et al., 2025), potentially explaining the variations in extractability observed between the wild-type and phytoene-enriched matrices.

As detailed in Section 3.1 and Supplementary Table 1, the TCC used as the baseline for bioaccessibility calculations represents the concentration in the matrix after the physical processing. The higher recovery observed after mechanical treatments is due to enhanced analytical extractability from cell wall disruption, rather than *de novo* biosynthesis. Regarding digestion, the high bioaccessibility of phytoene found here agrees with the results of Mapelli-Brahm et al. (2018). This is likely due to the fact that colorless carotenoids are more flexible and less crystalline than colored ones, which makes it easier for them to be incorporated into micelles during digestion, regardless of the matrix used.

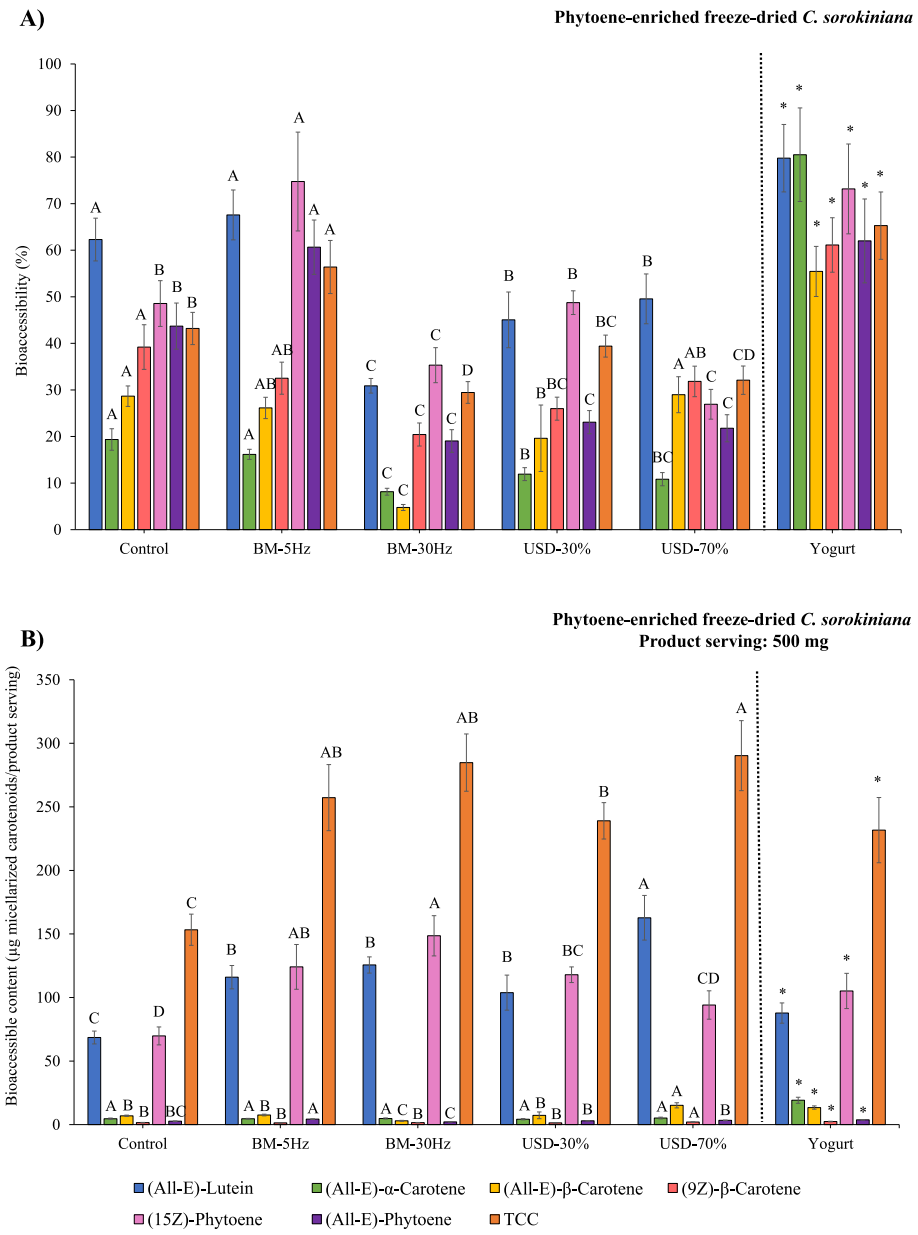

Fig. 5. Carotenoid bioaccessibility (%) (A) and bioaccessible carotenoid content (µg micellarized carotenoids/product serving) (B) in phytoene-enriched freeze-dried *C. sorokiniana*. A product serving corresponds to 500 mg of freeze-dried biomass. For the same carotenoid, different capital letters indicate statistically significant differences ($p < 0.05$) between treatments. BM-5 Hz: ball mill at 5 Hz; BM-30 Hz: ball mill at 30 Hz; USD-30%: ultrasound with 30% amplitude; USD-70%: ultrasound with 70% amplitude; TCC: total carotenoid content. Asterisks (*) indicate significant differences ($p < 0.05$) between control and yogurt with 10% fat; ns: not significant. The error bars in the figures represent the standard deviation (SD).

4.2. Effect of microalgae C. sorokiniana form and matrix type on carotenoid bioaccessibility and bioaccessible content

The carotenoid bioaccessibility and CBC of six different *C. sorokiniana* matrices (wild-type fresh, phytoene-enriched fresh, wild-type freeze-dried, phytoene-enriched freeze-dried, wild-type encapsulated, and phytoene-enriched encapsulated) were assessed (Figs. 2–7).

Significant differences in bioaccessibility were observed among the untreated matrices. Phytoene enrichment consistently improved bioaccessibility across all matrices, reaching its maximum increase in the phytoene-rich freeze-dried matrix, which exhibited a 5.6-fold higher bioaccessibility compared to its respective wild-type sample. The increased bioaccessibility observed in phytoene-enriched matrices could be associated with structural alterations in plastids and cell membranes, as suggested by recent proteomic analyses of *Chlamydomonas reinhardtii* exposed to norflurazon. In this model, phytoene desaturase inhibition leads to plastid remodeling and altered carotenoid accumulation patterns, which might potentially facilitate their release during digestion (Leetanasaksakul et al., 2025). Overall, fresh matrices showed significantly lower carotenoid bioaccessibility (<1.3%), likely due to the recalcitrance of the trilaminar outer cell wall (Baudelet et al., 2017). The higher values in freeze-dried samples suggest that these treatments may increase cell wall fragility, potentially facilitating nutrient release (Bilušić et al., 2019). Regarding encapsulated samples, the high bioaccessibility observed could be attributed to the stabilization and enhanced dispersion of carotenoids within the alginate matrix during digestion, rather than to mechanical damage inflicted to the microalgal cells during the encapsulation procedure. The encapsulation improved carotenoid bioaccessibility in all cases, both in phytoene-enriched and wild-type samples, and regardless of whether they were untreated, ball-

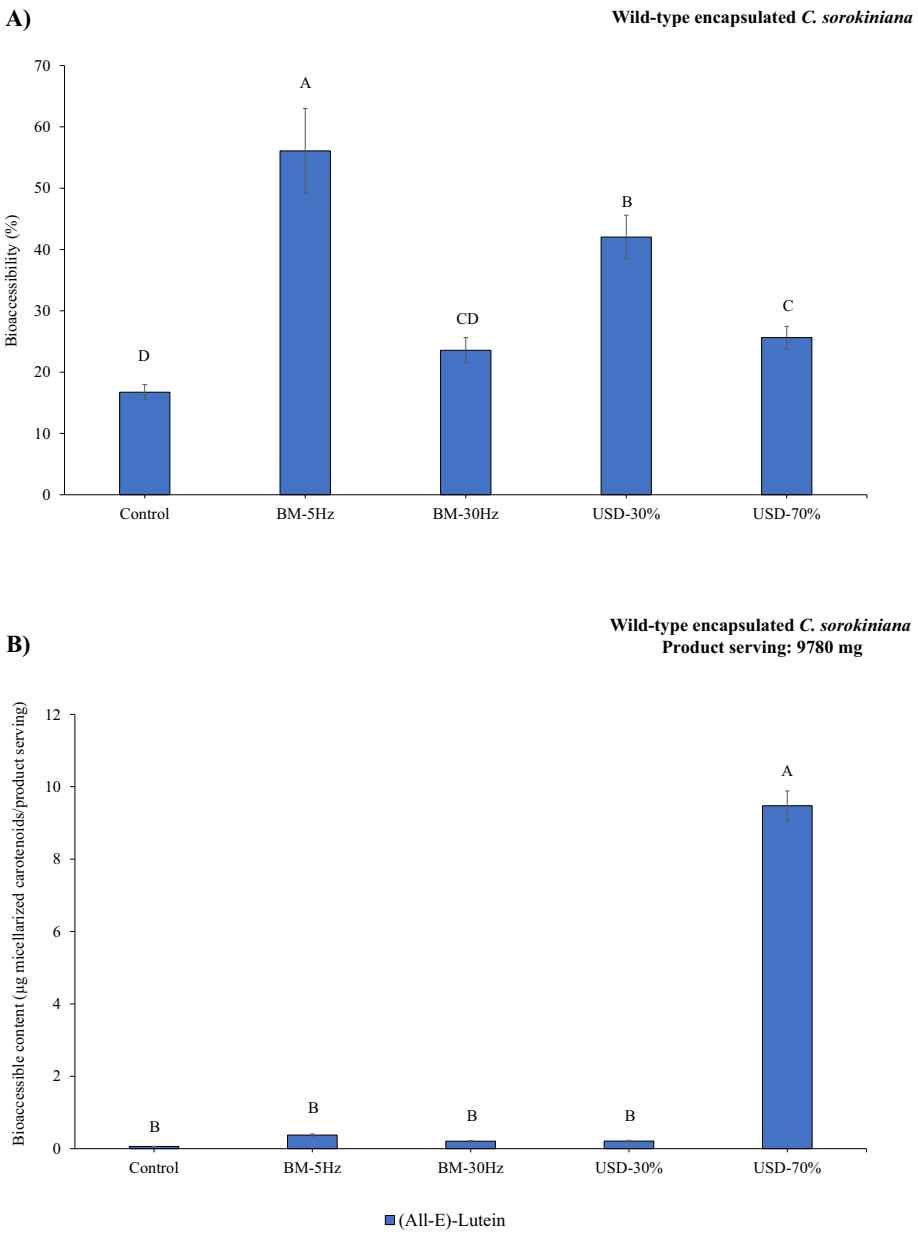

Fig. 6. Carotenoid bioaccessibility (%) (A) and bioaccessible carotenoid content (µg micellarized carotenoids/product serving) (B) in wild-type encapsulated *C. sorokiniana*. A product serving corresponds to 9780 mg of fresh biomass (94.89% moisture). For the same carotenoid, different capital letters indicate statistically significant differences ($p < 0.05$) between treatments. BM-5: Hz: ball-mill at 5 Hz; BM-30 Hz: ball-mill at 30 Hz; USD-30%: ultrasound with 30% amplitude; USD-70%: ultrasound with 70% amplitude; TCC: total carotenoid content. The error bars in the figures represent the standard deviation (SD).

milled, or ultrasound-treated. Thus, the total carotenoid bioaccessibility of wild-type encapsulated matrix under control conditions exhibited 48.2 times higher bioaccessibility than that of the wild-type fresh sample, while the total carotenoid bioaccessibility of phytoene-enriched encapsulated sample (control) was 33.4 times higher than that of the phytoene-enriched fresh sample (control). Alginate and its hydrogels have already been widely used to encapsulate various bioactive compounds, such as carotenoids. This type of encapsulation has been reported to improve the stability and solubility of carotenoids, allow for more controlled release, and consequently, enhance their bioavailability and the preservation of their beneficial properties (Milivojević et al., 2023). While encapsulation significantly increased bioaccessibility (up to 48-fold, $p < 0.05$), the CBC was lower in some encapsulated samples. Given that serving sizes were normalized on an equivalent dry biomass basis across all samples, this reduction is likely due to processing losses during bead formation rather than a dilution effect. This suggests that high bioaccessibility efficiency does not always translate to a higher nutritional dose per serving.

In terms of carotenoid bioaccessible content (CBC) per serving, differences were influenced not only by bioaccessibility percentage and concentration, but also by the serving size chosen for each matrix (2620 mg for fresh samples, 500 mg for freeze-dried samples, and 9780 mg for encapsulated samples). The carotenoid bioaccessible content (CBC) was higher in the three phytoene-enriched *C. sorokiniana* matrices (fresh, freeze-dried, and encapsulated) compared to their respective non-enriched samples. Thus, for control samples, the wild-type fresh matrix yielded 3.1 µg of bioaccessible carotenoids per serving, whereas the phytoene-enriched fresh matrix reached 6.1 µg/serving. For the freeze-dried matrices, the wild-type and phytoene-enriched samples provided 3.0 µg and 76.7 µg per serving, respectively. For the encapsulated matrices, the wild-type and phytoene-enriched samples provided 0.6 µg and 11.8 µg per serving, respectively. These results show that the

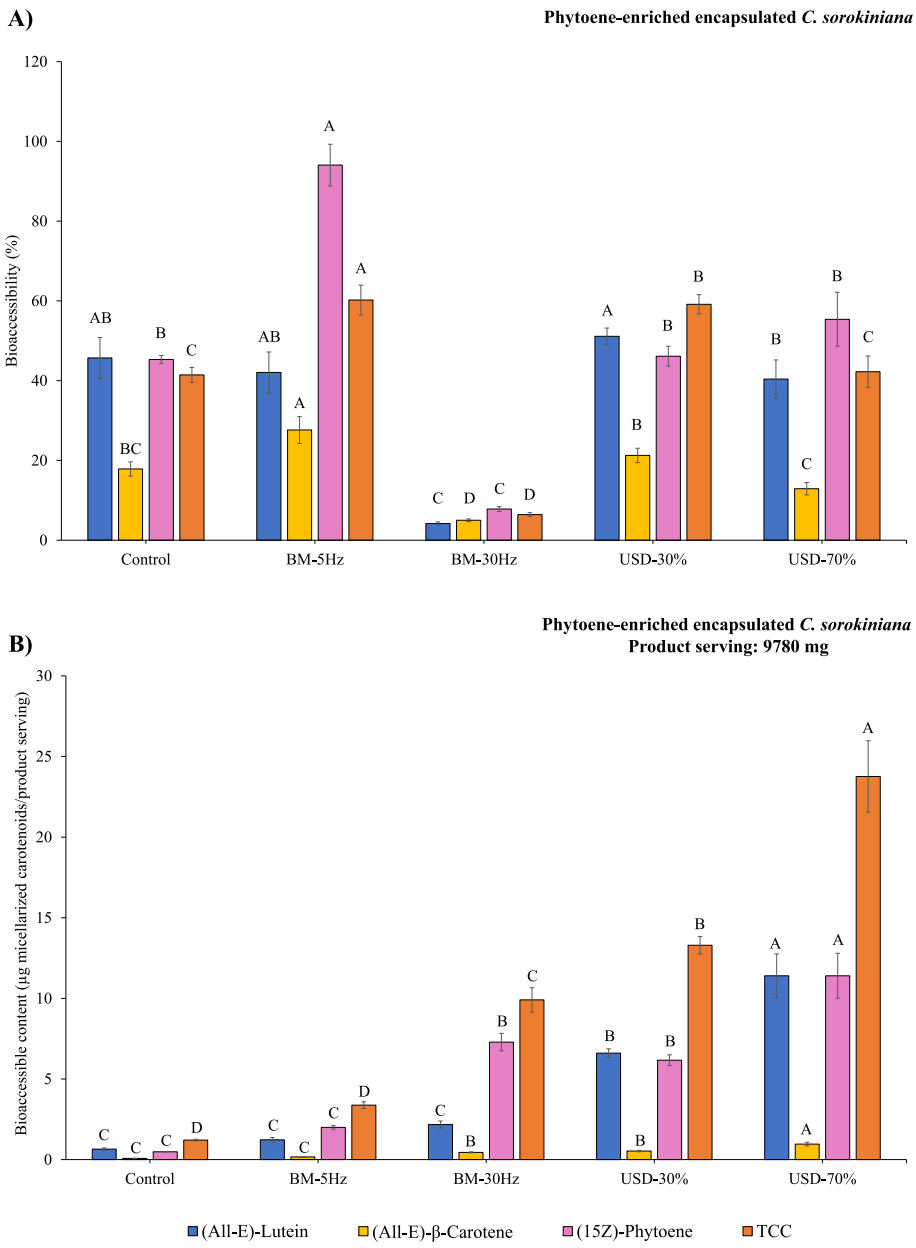

Fig. 7. Carotenoid bioaccessibility (%) (A) and bioaccessible carotenoid content (µg/product serving) (B) in phytoene-enriched encapsulated *C. sorokiniana*. A product serving corresponds to 9780 mg of fresh biomass (94.89% moisture). For the same carotenoid, different capital letters indicate statistically significant differences ($p < 0.05$) between treatments. BM-5: Hz: ball-mill at 5 Hz; BM-30 Hz: ball-mill at 30 Hz; USD-30%: ultrasound with 30% amplitude; USD-70%: ultrasound with 70% amplitude; TCC: total carotenoid content. The error bars in the figures represent the standard deviation (SD).

carotenoid bioaccessible content in *C. sorokiniana* improves with phytoene enrichment. This improvement in CBC could be related to both the enhanced bioaccessibility, and the increased carotenoid concentration observed in phytoene-enriched samples (Figs. 2, 4, and 6, and Supplementary Table 1).

4.3. Effect of ball milling and USD on carotenoid bioaccessibility and bioaccessible content in C. sorokiniana

The effect of two different disruption methods (ball-milling and ultrasound) on carotenoid bioaccessibility and bioaccessible content was assessed in the six different *C. sorokiniana* matrices. Ball-milling was assessed under two different frequencies, 5 and 30 Hz, and ultrasound under two different amplitudes 30 and 70%. The summarized results are shown in Supplementary Table 3.

4.3.1. Wild-type and phytoene-enriched fresh C. sorokiniana

The present study findings demonstrate that, although the effect of ultrasound and ball milling depended on the applied conditions, at least one of the applied treatments (ultrasound or ball milling) in both types of matrices (wild-type and phytoene-enriched) increase carotenoid bioaccessibility and CBC compared to untreated controls. In fresh wild-type matrices, high-frequency ball milling (BM-30 Hz) was the most effective treatment, significantly enhancing both bioaccessibility and CBC ($p < 0.05$). This increase in CBC (7.2-fold) reflects enhanced analytical extractability following matrix disruption rather than *de novo* biosynthesis. In fresh untreated biomass, the recalcitrant cell wall hinders full pigment recovery; however, ball-milling overcomes this structural resistance, resulting in a higher carotenoid content value used as the baseline for CBC calculations. These findings are consistent with recent structural evidence showing that both bead milling and ultrasonication effectively overcome the recalcitrant *C. vulgaris* cell wall

(Bernaerts et al., 2026; Demarco et al., 2022). During milling, the rotational motion of spheres generates collisions, friction and shearing potentially leading to cell rupture. The efficiency of the process depends on parameters such as the speed, weight, and density of the spheres, as well as the speed and type of biomass introduced (Nunes et al., 2024). For instance, Schüler et al. (2020) compared the efficiency of mechanical disruption by ball milling wet and freeze-dried *Tetraselmis sp.* biomass, using solvents such as acetone and tetrahydrofuran (THF), among others. The use of glass spheres with wet biomass favoured the carotenoid release from the matrix, with THF being the solvent that produced the highest concentrations of lutein (622 μg/g) and β-carotene (618 μg/g).

In the phytoene-enriched matrix, the highest TCC bioaccessibility was achieved with BM-5 Hz, although the differences with USD-30% and the control were not statistically significant ($p > 0.05$). However, the CBC was significantly higher with USD-30% compared to the control (7.6 times higher) ($p < 0.05$). Although USD-70% showed the lowest bioaccessibility among all treatments (2.2-fold lower than the control), its CBC was significantly high, being 2.8-fold that of the control ($p < 0.05$).

In a study optimizing carotenoid extraction from *Citrus reticulata* peel by response surface methodology, ultrasonic amplitude (10–40%), solvent-to-solid ratio, and ultrasonic time were evaluated as factors. The optimized amplitude was around 30% (32.9%), while higher amplitudes led to carotenoid degradation in the matrix (Saini et al., 2021). Our results confirm that moderate ultrasonic amplitudes (30%) are more favorable for carotenoid bioaccessibility than higher intensities (70%). This suggests that while high-energy ultrasound may facilitate cell wall rupture, it might simultaneously induce the oxidative degradation of the highly unsaturated carotenoid structure, as evidenced by the decrease in TCC observed in samples treated at 70% amplitude compared to those at 30% (Supplementary Table 1). Such degradation, along with a possible impairment in the formation of stable mixed micelles (Saini et al., 2021), are aspects that need to be studied in future investigations.

4.3.2. *Wild-type and phytoene-enriched freeze-dried C. sorokiniana*

The application of mechanical treatments to freeze-dried *C. sorokiniana* matrices had an impact on both carotenoid bioaccessibility and CBC, although the effects varied depending on the sample type (wild or phytoene-enriched) and the nature and intensity of the treatments. While CBC generally responded to the treatments, bioaccessibility showed limited or inconsistent improvements, especially in the phytoene-enriched samples.

In the freeze-dried matrices, ball mill was the most effective treatment for bioaccessibility enhancement. Ball milling was the most effective strategy for freeze-dried biomass. Wild-type cells performed better at higher frequency (30 Hz), whereas the phytoene-enriched matrix achieved better results with lower intensity (5 Hz), likely due to its plastid and membrane remodeling previously discussed. However, in contrast to the wild-type matrix, USD-70% led to the highest CBC, reaching 145.2 μg/serving product (500 mg), nearly double that of the control (76.7 μg/serving product). This finding highlights the importance of compatibility between the treatment and the matrix characteristics. In wild-type strain cells, stiffer cell walls might necessitate more vigorous ball milling for effective disruption, which enhances carotenoid release. In contrast, the phytoene-enriched matrix appears to be more sensitive to mechanical stress, so gentler milling is sufficient to improve bioaccessibility, as excessive disruption could potentially compromise carotenoid stability or micelle formation. In addition, in previous studies conducted in our laboratory, the application of the ball mill (30 Hz, 5 min) before the ultrasound-assisted extraction (UAE; 30%, 2 min, 20 kHz) of carotenoids, significantly enhanced the total carotenoid release from both freeze-dried *C. sorokiniana* matrices (wild-type and phytoene-enriched) by 1.1–2.4 times ($p < 0.05$), depending on the solvent used and the matrix, compared to those samples where only UAE was applied without the ball mill pretreatment (Morón-Ortiz et al., 2024).

4.3.3. *Wild-type and phytoene-enriched encapsulated C. sorokiniana*

Although sodium alginate encapsulation has been proposed as an effective strategy for preserving carotenoids against adverse environmental and digestive conditions, the treatments applied to these delivery systems can strongly influence their behaviour during digestion. In the wild-type encapsulated matrix, (all-*E*)-lutein was the only carotenoid detected in the micellar fraction; however, (all-*E*)-lutein, (all-*E*)-β-carotene, and (15*Z*)-phytoene were found in the phytoene-enriched encapsulated matrix. In both matrices, the best treatment for bioaccessibility enhancement was BM-5 Hz. Interestingly, moderate treatments (BM-5 Hz or USD-30%) enhanced bioaccessibility in encapsulated samples more effectively than high-intensity ones. This could suggest that that more intense mechanical treatment (BM-30 Hz or USD-70%) might compromise the structural integrity of the alginate hydrogel through polymer degradation, reducing its ability to protect carotenoids from the acidic gastric environment. Our findings align with Wardhani et al. (2021), who reported that ultrasound-degraded alginate has been reported to form fragile beads with reduced retention capacity due to the loss of matrix integrity.

Interestingly, while USD-70% did show a similar or significantly reduced bioaccessibility, depending on the matrix, compared to the control, it showed the highest CBC ($p < 0.05$) in both encapsulated matrices (wild-type and phytoene enriched). Specifically, in the wild-type matrix, USD-70% showed a 157-fold increase over the control (92.7 *vs.* 0.6 μg/serving product (9780 mg)), and in the phytoene-enriched matrix it reached 232.4 μg/serving, representing nearly 20-fold increase over the control (11.8 μg/serving product).

Taken together, the results discussed above highlight that a high bioaccessibility does not always correspond to greater nutritional value, particularly when the initial carotenoid content is low. It should be noted that while encapsulation introduces a dilution effect due to the hydrogel weight, the serving sizes were normalized to 500 mg of dry biomass for CBC calculations. This ensures that the CBC values are directly comparable between matrices, reflecting the actual amount of carotenoids released from an equivalent dose of microalgae, regardless of the moisture or matrix content of each delivery system. Therefore, alongside bioaccessibility values, it is important to consider the actual amount of carotenoids that is potentially absorbable per food serving.

Furthermore, each matrix responds differently to each treatment. These results highlight the importance of assessing not only carotenoid bioaccessibility but also bioavailable content to properly assess the nutritional potential of matrices. In addition, it appears important to adapt cell disruption treatments to the specific characteristics of each matrix.

4.4. *Effect of yogurt addition on carotenoid bioaccessibility in the phytoene-enriched freeze-dried C. sorokiniana*

In the phytoene-enriched freeze-dried matrix, where the effect of yogurt (10% fat) was evaluated, its addition significantly improved the bioaccessibility and CBC of individual and total carotenoids ($p < 0.05$). This matrix was selected to evaluate how the yogurt matrix (providing both lipids and proteins, among other constituents) influences the micellarization of colored and colorless carotenoids with varying hydrophobicity. While lipids are crucial for micelle formation, the protein fraction and the overall food matrix structure also play a significant role interact with these colloidal systems, potentially modulating the stability and efficiency of carotenoid solubilization (Xavier et al., 2014). Specifically, phytoene remains less explored than colored ones despite its growing biological interest (Mapelli-Brahm et al., 2018; Meléndez-Martínez et al., 2015, 2018). Additionally, the freeze-dried format ensured superior homogeneity and precise dosing.

It should be noted that the use of yogurt as a lipid source introduces additional components, such as dietary proteins, which may also

influence carotenoid bioaccessibility. Therefore, the observed effects cannot be exclusively attributed to lipid content and may result from combined matrix interactions, such as protein-carotenoid stabilization or the structural effect of the dairy matrix. This approach prioritizes the evaluation of bioaccessibility within a structured food environment, providing a more representative assessment of how these microalgae behave when incorporated into a fortified dairy product rather than as isolated ingredients.

The addition of yogurt notably enhanced the bioaccessibility of (all-*E*)-α-carotene and (all-*E*)-β-carotene by 4.2 and 1.9-fold, respectively ($p < 0.05$). This trend aligns with the high hydrophobicity of these carotenes, which results in a low affinity for mixed micelles, thereby restricting their bioaccessibility in the absence of a lipid carrier. As stated before, the complex dairy matrix may further stabilize carotenoids during digestion (Yao et al., 2022). These findings align with (Nagao et al., 2013), who observed that while β-carotene exhibits a lower baseline bioaccessibility than lutein within the same edible plant matrices, the latter—being a more polar carotenoid—showed a less pronounced increase in micellarization after the addition of soybean oil.

4.5. Effect of the type of carotenoid on the bioaccessibility

The bioaccessibility of carotenoids from *C. sorokiniana* matrices was influenced by their chemical nature and isomeric configuration.

(all-*E*)-Lutein consistently showed the highest bioaccessibility (peaking at 62.3% in phytoene-enriched freeze-dried samples), significantly surpassing (all-*E*)-carotenes. This is attributed to its greater polarity, which facilitates preferential micellarization compared to more hydrophobic carotenes (Bohn, 2008). Notably, (15*Z*)-phytoene reached its maximum bioaccessibility under BM-30 Hz, whereas colored carotenoids peaked at USD-70%. This divergence could be attributed to the unique features of this colorless carotenoid; the interplay between its specific molecular flexibility and its physical state within the plastids (Meléndez-Martínez et al., 2015) may account for a different susceptibility to mechanical disruptive forces compared to colored carotenoids. Consequently, these results suggest that processing treatments should be specifically tailored to the targeted carotenoid profile of the matrix.

Isomeric configuration also played a critical role, with *Z*-isomers consistently exhibiting higher bioaccessibility than their all-*E* counterparts. Specifically, (9*Z*)-β-carotene and (15*Z*)-phytoene showed up to 4-fold higher bioaccessibility than their respective all-*E* forms. This trend is consistent with studies on lycopene Yu et al. (2025) and lutein Yang et al. (2018), where *Z*-isomers demonstrated superior micellarization efficiency.

The enhanced bioaccessibility of *Z*-isomers is primarily due to their higher solubility and greater capacity for micellar incorporation during intestinal digestion (Yang et al., 2020). Furthermore, the dynamic nature of carotenoids in lipid environments suggests that isomerization and subsequent equilibrium may occur once incorporated into micelles (Meléndez-Martínez et al., 2014), potentially further influencing their final bioavailability.

5. Conclusions

This study suggests that both USD and ball milling application can be in general effective physical strategies for improving the CBC of *Chlorella sorokiniana* carotenoids, although in some samples the improvement in CBC with certain treatments was not statistically significant. The best treatment varies depending on the matrix type (fresh, freeze-dried, encapsulated) and strain (wild, phytoene-enriched), indicating that the efficacy of these methods is closely linked to the specific characteristics of the microalgal matrix. Ball milling, especially at 30 Hz, was the most effective treatment for improving total carotenoid bioaccessibility in fresh and freeze-dried wild matrices, while milder conditions (BM-5 Hz or USD-30%) showed the best results in all phytoene-enriched and encapsulated samples, albeit in the fresh phytoene-enriched matrix the improvement was not statistically significant. Furthermore, freeze-dried and encapsulated forms of *C. sorokiniana* showed significantly greater carotenoid bioaccessibility compared to fresh biomass, especially when combined with disruption techniques. The addition of yogurt (10% fat) to phytoene-enriched freeze-dried *C. sorokiniana* biomass significantly improved the micellization of carotenoids, especially the most hydrophobic ones, such as α-carotene and β-carotene. This observation highlights the potential role of the complex yogurt matrix in enhancing micellization. Although this phenomenon is likely driven primarily by the presence of lipids, the contribution of other components, such as proteins, should also be considered. Thus, the specific impact of different fat and protein levels remains to be further explored.

The chemical structure of the carotenoids influenced their bioaccessibility, with higher values observed for all-*E* xanthophylls compared to their carotenes counterparts. Isomeric configuration also had an impact, as *Z* isomers showed greater bioaccessibility than their corresponding *E* isomers.

The findings highlight the importance of assessing not only the carotenoid bioaccessibility, but also their CBC, which represents the actual, potentially absorbable amount of carotenoids per serving size.

Furthermore, each matrix needs to be studied individually to better understand how strategies such as cell disruption, encapsulation, or co-ingestion with dietary lipids could potentially enhance the nutritional value of microalgal carotenoids in functional foods. Overall, this study suggests how processing strategies might be tailored to maximize the nutritional potential of *C. sorokiniana*. By comparing different matrix forms, biomass types, and disruption conditions, our findings indicate that the efficacy of each treatment in enhancing carotenoid bioaccessibility appears to be highly dependent on matrix characteristics. However, further studies are required to determine if these matrix-dependent effects are also reflected in actual bioavailability. These findings are highly relevant for the preliminary design of functional foods enriched with microalgal carotenoids, highlighting the need for targeted matrix and compound optimization to ensure stability and bioaccessibility. Our work therefore contributes to the development of sustainable and healthy food products enriched with microalgal carotenoids.

CRediT authorship contribution statement

Ángeles Morón-Ortiz: Writing – original draft, Investigation, Formal analysis. **Paula Mapelli-Brahm:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Antonio León-Vaz:** Writing – original draft, Investigation, Formal analysis. **Rosa León:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Antonio Jesús Meléndez-Martínez:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antonio J. Melendez Martinez reports a relationship with carries out consultancy work for companies occasionally that includes: consulting or advisory. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.149627.

Data availability

The data can be found in Supplementary Material

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