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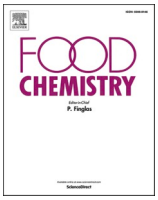

Effect of ultrasounds and ball milling on the bioaccessibility of carotenoids from the microalga *Dunaliella bardawil*

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ABSTRACT

This study evaluated the bioaccessibility of carotenoids from wild-type and phytoene-enriched *Dunaliella bardawil* biomass treated by ultrasounds and ball-milling. Fresh, freeze-dried, and alginate-encapsulated matrices were studied. The impact of yogurt addition was also evaluated on freeze-dried samples. Carotenoid bioaccessibility varied significantly depending on the matrix, treatment, and carotenoid isomer. As an example, ball-milling at 30 Hz was the treatment leading to the highest bioaccessibility in fresh samples ($p < 0.05$), whereas this treatment at 5 Hz was optimal for freeze-dried matrices, with no significant differences with the control in the wild-type matrix. The addition of yogurt enhanced carotenoid bioaccessibility ($p < 0.05$) in both freeze-dried matrices. Xanthophylls showed slightly higher bioaccessibility than carotenes, and *cis*-isomers outperformed their all-*trans* counterparts. (15*Z*)-Phytoene and (all-*E*)-lutein showed the greatest bioaccessibility in most treatments. These results can help improve the bioavailability of carotenoids from *D. bardawil* through optimized pretreatment strategies.

1. Introduction

Microalgae have attracted increasing interest as a sustainable resource due to their versatile and environmentally friendly properties. Key features include their high nutritional value and health benefits, rapid growth with high productivity, and minimal land requirement for cultivation (Mostafa & Hashem, 2024). In addition, microalgae also play a role in carbon dioxide sequestration and wastewater treatment. The diversity of species and the possibilities for genetic modification enhance their applicability. However, limitations include high production costs, challenges in scaling up and rigid cell walls that some species have and hinder digestion. Preservation and transport of live microalgae also represent technical challenges for long-term viability (Ma & Hu, 2024; Mapelli-Brahm et al., 2023). Within the broad spectrum of microalgae, the genus *Dunaliella* stands out for its ability to produce bioactive compounds of commercial interest, such as β-carotene, lutein and zeaxanthin (Mapelli-Brahm et al., 2023). Carotenoids are essential pigments for photosynthesis and photoprotection in plants and microalgae that continue eliciting increasing interest for health and beauty promotion through the diet (Baskar et al., 2024; Meléndez-Martínez et al., 2021). β-Carotene is a precursor of vitamin A, which is key for maintaining healthy vision, skin, and immune function. Moreover, lutein and zeaxanthin are important for eye health, helping protect against age-related macular degeneration, one of the leading causes of blindness (Britton, 2020).

Previous studies on *Dunaliella* have mainly focused on carotenoid production or extraction yield, with limited attention to how green processing technologies influence carotenoid bioaccessibility and bioaccessible content (Barbosa et al., 2023; Morón-Ortiz, Mapelli-Brahm, León-Vaz, Benitez-González, et al., 2024).

Bioaccessibility is considered an indicator of potential bioavailability and can be defined as the fraction of a compound released from the food matrix during digestion, making it potentially available for absorption in the gastrointestinal tract (Brodkorb et al., 2019). In this regard, carotenoids, as lipophilic molecules, need to be incorporated into mixed micelles to be effectively absorbed (Yao et al., 2022).

The bioaccessibility and bioavailability of bioactive compounds, such as carotenoids, are influenced by multiple factors summarized in the SLAMENGHI acronym. These include the Species or chemical form of the compound, its Linkage within the food matrix, Amount, Matrix

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type, Effectors of absorption (e.g., dietary fat), Nutritional status, Genetic background, Host-related factors like digestive health, and Interactions with other compounds (de Pee & West, 1996). Thus, processing and preparation methods that affect the matrix can also have a significant impact on bioaccessibility. For example, cooking foods can increase the bioaccessibility and bioavailability of certain food components, for instance by reducing hardness and breaking down cell membranes and walls and making them more accessible (Benítez-González et al., 2024; Thakur et al., 2020).

In the context of growing sustainability concerns, green technologies such as ultrasounds and ball-milling are of particular interest. They can improve bioaccessibility of food components by modifying matrix structure, through cavitation in the case of ultrasounds, and shear, friction, collision, and impact in ball-milling (Bangar et al., 2023; Rodríguez-Rodríguez et al., 2025).

To our knowledge, no previous study has simultaneously compared the effects of ultrasound- and ball-milling-assisted treatments in carotenoid bioaccessibility across multiple *D. bardawil* matrices. This study fills that gap by assessing both technologies in fresh, freeze-dried, and encapsulated biomass, including phytoene-enriched variants. Additionally, the effect of yogurt addition as a lipid source was evaluated in the freeze-dried matrix.

2. Methodology

2.1. Reagents

Tert-butyl methyl ether (HPLC-grade) was purchased from Honeywell (Seelze, Germany); ethyl acetate, and hydrochloric acid from VWR Chemicals (Leuven, Belgium); diethyl ether, and sodium chloride from PanReac AppliChem (Darmstadt, Germany); potassium chloride, sodium bicarbonate, and magnesium chloride 6-hydrate, from Panreac Quimica SA (Barcelona, Spain); potassium dihydrogen phosphate from Merck (Darmstadt, Germany); ammonium carbonate, calcium chloride dihydrate, α-amilase (≥ 5 units/mg solid), pepsin (≥ 2.500 units/mg protein), pancreatin (8 × USP specifications), and bile salts from Sigma-Aldrich (St. Louis, MO, USA); sodium hydroxide from J.T.Baker (Deventer, Netherlands).

2.2. Samples

Dunaliella bardawil (UTEX 2538) was acquired from the UTEX Culture Collection of Microalgae (University of Texas, Austin). The microalgae culture was grown under the conditions previously reported (Morón-Ortiz, Mapelli-Brahm, León-Vaz, Benitez-González, et al., 2024). Once the cultures reached the desired cell concentration, the biomass was harvested through centrifugation and stored either by freezing at −80 °C, lyophilizing in a Telstar freeze dryer (Terrassa, Spain), or encapsulating in alginate beads.

2.3. Phytoene enrichment

To induce phytoene accumulation, *D. bardawil* cultures were treated with the pyridazinone herbicide norflurazon, following the protocol amended from León et al. (2005). In brief, optimal growth standard cultures (0.5 L) were used to inoculate fresh Johnson's medium with 2 M NaCl (4 L) containing 10 μg/mL of norflurazon (previously optimized concentration). The cultures were grown for 8–10 days under regular conditions. The resulting microalgal biomass is then referred to as phytoene-enriched *D. bardawil*.

2.4. Microalgae encapsulation

The cultures of *D. bardawil* (standard and phytoene-rich) were harvested by centrifugation and resuspended in Johnson's medium with 0.5 M NaCl (Johnson et al., 1968). The cultures were mixed thoroughly with equal volumes of sterile alginate solution (6 %, *w/v*) from *Macocystis pyrifera*, and formed into 3 mm diameter beads by dropping the mixture into 0.2 M $CaCl_2$ solution at 4 °C. The process was carried out in a sterile environment within a laminar flow cabin.

2.5. Dry weight determination

The dry weight was calculated by filtering 30 mL of culture through pre-weighted glass-fiber filters (GF/F Whatman), washing with ammonium formate (0.5 M), and drying the filter at 100 °C for 24 h. The dry weight was determined by weighing the filters and subtracting the initial weight.

2.6. Experimental design

Each matrix was divided into five groups: a control group and four experimental groups subjected to different treatments by either ball-milling or ultrasounds. The ball-mill (MM400, Retsch, Germany) was applied for 5 min at two different frequencies, 5 Hz and 30 Hz. Ultrasound treatment (QSonica Ultrasonic Q500, Newtown, USA) was applied for 2 min, at 20 kHz, and at two different amplitudes, 30 % and 70 %. The ultrasound amplitudes (30 % and 70 %) and ball-milling frequencies (5 Hz and 30 Hz) were selected based on preliminary trials and our previous study on carotenoid extraction from *D. bardawil* (Morón-Ortiz, Mapelli-Brahm, León-Vaz, Benitez-González, et al., 2024). The maximum frequency available in the Retsch MM400 ball mill is 30 Hz, which was therefore used as the high-intensity condition, whereas 5 Hz was chosen as a lower setting since 3 Hz produced negligible cell disruption. Similarly, 70 % was selected as the highest amplitude achievable with the ultrasonic probe under the experimental configuration used, while 30 % corresponded to a moderate intensity level. For the application of ultrasound, a saline solution (120 mmol/L of NaCl, 6 mmol/L of $CaCl_2$, and 5 mmol/L of KCl) was added to the samples before the treatment to enable effective ultrasound propagation (Fernandes et al., 2021). Fresh or encapsulated samples (2.5 g) and freeze-dried samples (~ 0.5 g, equivalent to 2.5 g of fresh weight based on a moisture content of 80.7 %) were weighed and mixed with 10 mL of the saline solution. After ultrasound treatment, samples were centrifuged and supernatant removed before the *in vitro* digestion.

2.7. Extraction of carotenoids from microalgae

To evaluate the carotenoid content in the initial samples (before digestion), diethyl ether was used as an extraction solvent. Approximately 0.1 g of each matrix was weighed. The different treatments (ball-mill (5 Hz and 30 Hz) and ultrasound (30 % and 70 %)) were also applied, and 2 mL of solvent was added (either before the ultrasound treatment or after the ball-mill application), centrifuged (5 min, 3220 ×*g*, 4 °C) and the supernatant was extracted until the sample did not show colour. The samples were concentrated in a rotary evaporator (Eppendorf Concentrator plus™, Eppendorf, Hamburg, Germany) under its Vacuum-High Vapor mode at 30 °C for HPLC analysis.

2.8. In vitro digestion

To evaluate the bioaccessibility of carotenoids and the carotenoid bioaccesible content (CBC), the samples were subjected to an *in vitro* digestion process comprising oral, gastric, and intestinal phases, following the standardized INFOGEST protocol (Brodkorb et al., 2019). The effect of fat on the bioaccessibility was also evaluated by adding 2 g of yogurt (10 % fat) to 0.5 g of the freeze-dried sample before the *in vitro* digestion. Bioaccessibility was calculated as the percentage of carotenoid content that remained in the micellar aqueous fraction after centrifugation in relation to the respective initial content in the food matrices. The CBC was calculated by determining the absolute micellarized carotenoid level per serving size: 500 mg for lyophilized samples,

2600 mg for fresh samples (80.7 % moisture), and 9400 mg for encapsulated samples (94.68 % moisture). These doses match the 500 mg of dry biomass commonly found in algal supplements.

CBC (μg of micellarized carotenoids/ product serving) was then obtained as follows:

$$CBC_i\left(\frac{\mu g}{\text{serving}}\right) = \frac{Bioaccessiblity_i\,(\%)}{100} \times Initial\ content_i\left(\frac{\mu g}{\text{serving}}\right)$$

where *i* denotes each carotenoid.

Thus, CBC is an estimation of the amount of carotenoid that is potentially absorbable from a product serving.

2.8.1. Oral phase

For the oral phase, 2.5 g of encapsulated sample, 2.5 g of fresh sample, or 0.5 g of freeze-dried sample with or without 2 g of 10 % fat yogurt was mixed with 2.5 mL of salivary solution fluid containing 75 U/mL of α-amylase and $CaCl_2$ (1.5 mM). The samples were incubated for 2 min, at 37 °C, and at 150 rpm in a shaking incubator (Innova 40, New Brunswick Scientific). Once the incubation time was finished, the samples were quickly placed on ice to deactivate the enzymes.

2.8.2. Gastric phase

For the gastric phase, 5 mL of gastric solution fluid was added to the sample, adjusting pH at 3 with 6 M HCl. The gastric solution contained 2000 U/mL of pepsin, and $CaCl_2$ (0.15 mM). The samples were incubated for 2 h, at 37 °C, and at 150 rpm in the shaking incubator, and the samples were immersed in ice once the incubation time was complete.

2.8.3. Intestinal phase

Finally, for the intestinal phase, 10 mL of the intestinal solution was added, and the pH was adjusted to 7 with 1 M of NaOH. The intestinal solution contained 10 mM of bile salts, 100 U/mL of pancreatin, and $CaCl_2$ (0.6 mM). The samples were incubated again for 2 h, at 37 °C, and at 150 rpm in the shaking incubator, and the samples were immersed in ice for enzyme deactivation once the incubation time was complete.

2.8.4. Filtration

The samples were centrifuged (20 min, 3220 ×*g*, 4 °C) and 10 mL of the supernatant was filtered through a 0.2 μm filter (Captiva Econofltr Nylon 25 mm, Agilent Technologies).

2.9. Extraction of carotenoids from mixed samples

For carotenoid extraction, 5 mL of diethyl ether was added to the filtered sample, followed by homogenization using an Ultra-turrax (IKA® T25 digital, Staufen, Germany). Samples were centrifuged (5 min, 3220 ×*g*, 4 °C), and the colored upper phase was collected. The extraction was repeated until the residue appeared colorless. The combined extracts were then concentrated in a rotary evaporator (Eppendorf® Concentrator Plus, Germany; VC mode, 30 °C) to prevent carotenoid degradation prior to subsequent HPLC analysis.

2.10. Chromatographic analysis

The quantification of carotenoids was conducted using an ultrahigh-performance liquid chromatography (UHPLC) instrument, specifically the 1260 Infinity II Prime LC System (Agilent Technologies, Santa Clara, CA, USA). This system was equipped with a diode array detector and a C_{30} column (3 μm, 4.6 mm × 150 mm, YMC America, Inc., Allentown, PA). The extracts were dissolved in roughly 500 μL of ethyl acetate, and 10 μL was injected into the system for analysis. The mobile phase consisted of a mixture of methanol, *tert*-butyl methyl ether, and water, which was delivered at a flow rate of 1 mL/min through a linear gradient, as described in a study by Stinco et al. (2019). Carotenoid content was determined using calibration curves (Stinco et al., 2019). The total carotenoid content was calculated by summing individual carotenoids.

2.11. Statistical analysis

All experiments were performed with four replicates, and the results are presented as the mean ± standard deviation (SD). Statistical analysis was performed using InfoStat, version 2020 (InfoStat Group, Faculty of Agricultural Sciences, National University of Córdoba, Córdoba, Argentina). The analysis of variance (ANOVA) was conducted, and Tukey's post-hoc test was used to evaluate differences between groups. Statistical significance was assumed for *p*-values less than 0.05.

3. Results

3.1. Carotenoid profile

The main carotenoids found in fresh and freeze-dried wild-type *D. bardawil* were (all-*E*)-lutein, (all-*E*)-β-carotene, (all-*E*)-α-carotene, (9*Z*)-β-carotene, (all-*E*)-antheraxanthin, and (all-*E*)-zeaxanthin (Supplementary Fig. 1A and Supplementary Fig. 2A). In the encapsulated matrix, only (all-*E*)-lutein and (all-*E*)-β-carotene were found (Supplementary Fig. 3A). (All-*E*)-Lutein was the main carotenoid, followed by (all-*E*)-β-carotene, in all of the wild-type samples evaluated.

Regarding the phytoene-enriched *D. bardawil*, (15*Z*)-phytoene and (all-*E*)-phytoene were also found in the fresh and freeze-dried samples (Supplementary Fig. 1B–C and Supplementary Fig. 2B–C). (15*Z*)-Phytoene was also detected in the phytoene-enriched encapsulated matrix; however, (all-*E*)-phytoene was not found in the encapsulated sample (Supplementary Fig. 3B–C).

In all cases, (all-*E*)-lutein was the predominant carotenoid. In the wild-type fresh matrix, its content ranged from 405.37 μg/g (USD 30 %) to 1379.11 μg/g (control), while in the freeze-dried form it varied between 306.81 μg/g (control) and 411.26 μg/g (USD 70 %). The encapsulated wild-type samples showed much lower levels, from 8.68 μg/g (control) to 32.41 μg/g (USD 70 %). In the phytoene-enriched fresh matrix, values ranged from 1198.60 μg/g (BM 5 Hz) to 1932.41 μg/g (USD 70 %), and in the freeze-dried form from 1101.77 μg/g (control) to 1423.87 μg/g (BM 30 Hz). Finally, the encapsulated phytoene-enriched samples exhibited the lowest concentrations, ranging from 0.22 μg/g (BM 30 Hz) to 0.86 μg/g (USD 30 %). According to Supplementary Table 1, the total carotenoid content (TCC) varied notably across samples and treatments. In wild-type fresh *D. bardawil*, TCC ranged from 495.82 μg/g (USD 30 %) to 2058.21 μg/g (control). In wild-type freeze-dried *D. bardawil*, TCC values increased from 454.51 μg/g (control) up to 607.80 μg/g (USD 70 %). In wild-type encapsulated *D. bardawil*, TCC values were considerably lower, ranging from 9.69 μg/g (control) to 35.56 μg/g (USD 70 %).

Regarding phytoene-enriched matrices, in phytoene-enriched fresh *D. bardawil*, TCC reached the highest levels overall, from 2438.77 μg/g (ball-mill 5 Hz) to 4072.80 μg/g (USD 70 %). In phytoene-enriched freeze-dried *D. bardawil*, TCC ranged from 2542.45 μg/g (USD 70 %) to 3534.18 μg/g (control). In phytoene-enriched encapsulated *D. bardawil*, TCC was markedly lower, between 0.32 μg/g (control and ball-mill 30 Hz) and 1.20 μg/g (USD 30 %).

These results suggest that the effect of ultrasound on carotenoid content is highly matrix dependent. In both encapsulated samples, ultrasound clearly increased carotenoid release (Supplementary Table 1), probably by breaking the alginate matrix. However, in other matrices, this treatment caused a decrease in carotenoid content, which could be attributed to degradation of the compound under high-energy conditions. Further research is therefore needed to clarify the balance between compound release and degradation after ultrasound application, depending on the matrix characteristics and carotenoid profile.

3.2. Bioaccessibility of carotenoids in the fresh wild-type and phytoene-enriched *D. bardawil*

The bioaccessibility of total carotenoids in fresh wild-type *D. bardawil* ranged from 3.16 % (ball-milled sample at 5 Hz) to 11.53 % (ball-milled sample at 30 Hz), depending on the treatment applied (Fig. 1A and Supplementary Table 2A). In this strain, (all-*E*)-lutein was the carotenoid with the highest bioaccessibility, ranging from 4.20 % (ball-milled sample at 5 Hz) to 15.05 % (ball-milled sample at 30 Hz). The other carotenoids showed bioaccessibilities ranging from 0.13 % to 6.69 %, depending on the treatment.

On the other hand, fresh phytoene-enriched *D. bardawil* exhibited slightly higher bioaccessibilities of total carotenoids compared to the fresh wild-type, with values ranging from 4.94 % (sample treated by ultrasound (USD) at 30 % amplitude) to 14.70 % (ball-milled sample at 30 Hz) (Fig. 2A and Supplementary Table 2B). In the fresh phytoene-enriched *D. bardawil*, (all-*E*)-lutein exhibited a high bioaccessibility, from 3.78 % (USD 30 %) to 13.68 % (ball-milled sample at 5 Hz). However, the highest values were observed for (15*Z*)-phytoene (from 9.80 % (control sample) to 22.29 % (ball-milled sample at 30 Hz). In addition, (all-*E*)-phytoene also showed high bioaccessibility percentage values ranging from 6.26 % (control sample) to 14.75 % (ball-milled sample at 30 Hz). The remaining carotenoids in this matrix displayed bioaccessibility values ranging from 0.28 % to 13.05 %, highlighting (9*Z*)-β-carotene (2.28 %–13.05 %) and (all-*E*)-β-carotene (1.61 %–12.00 %).

CBC was also evaluated and expressed as μg of micellarized carotenoids per product serving (2600 mg fresh, 500 mg lyophilized, 9400 mg encapsulated). In fresh wild-type *D. bardawil*, (all-*E*)-lutein was the most abundant carotenoid in the micellar fraction, with levels ranging from 94.14 to 516.71 μg/product serving (2600 mg) (USD 30 % and ball-mill 30 Hz, respectively) (Fig. 2B and Supplementary Table 2A). The other carotenoids, i.e., (all-*E*)-α-carotene (3.28–5.29 μg/product serving (2600 mg)), (all-*E*)-β-carotene (1.32–14.05 μg/product serving), (9*Z*)-β-carotene (1.02–3.37 μg/product serving), (all-*E*)-antheraxanthin (0.22–0.81 μg/product serving), and (all-*E*)-zeaxanthin (0.06–0.19 μg/product serving), showed significantly lower CBC (Fig. 1B and Supplementary Table 2A).

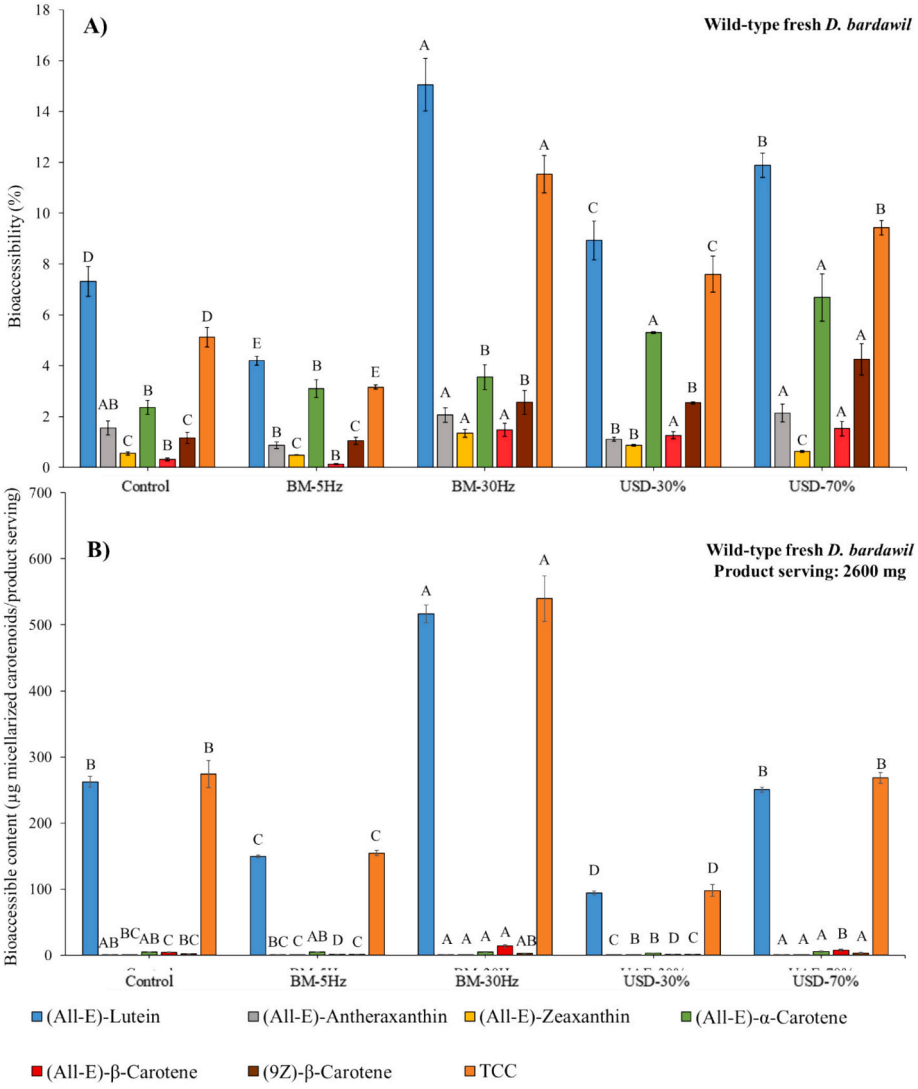

Fig. 1. Carotenoid bioaccessibility (%) (A) and CBC (μg/product serving) (B) in wild-type fresh *D. bardawil*. A product serving corresponds to 2600 mg of fresh biomass (80.7 % moisture).

General note for Figs. 1–6: Values are expressed as mean ± standard deviation ($n = 3$). Different capital letters indicate statistically significant differences among treatments for the same carotenoid ($p < 0.05$). Asterisks (*) denote significant differences ($p < 0.05$) between control and yogurt with 10 % fat; ns: not significant. BM-5 Hz: ball-milling at 5 Hz; BM-30 Hz: ball-milling at 30 Hz; USD-30 %: ultrasound at 30 % amplitude; USD-70 %: ultrasound at 70 % amplitude; CBC: carotenoid bioaccessible content; TCC: total carotenoid content.

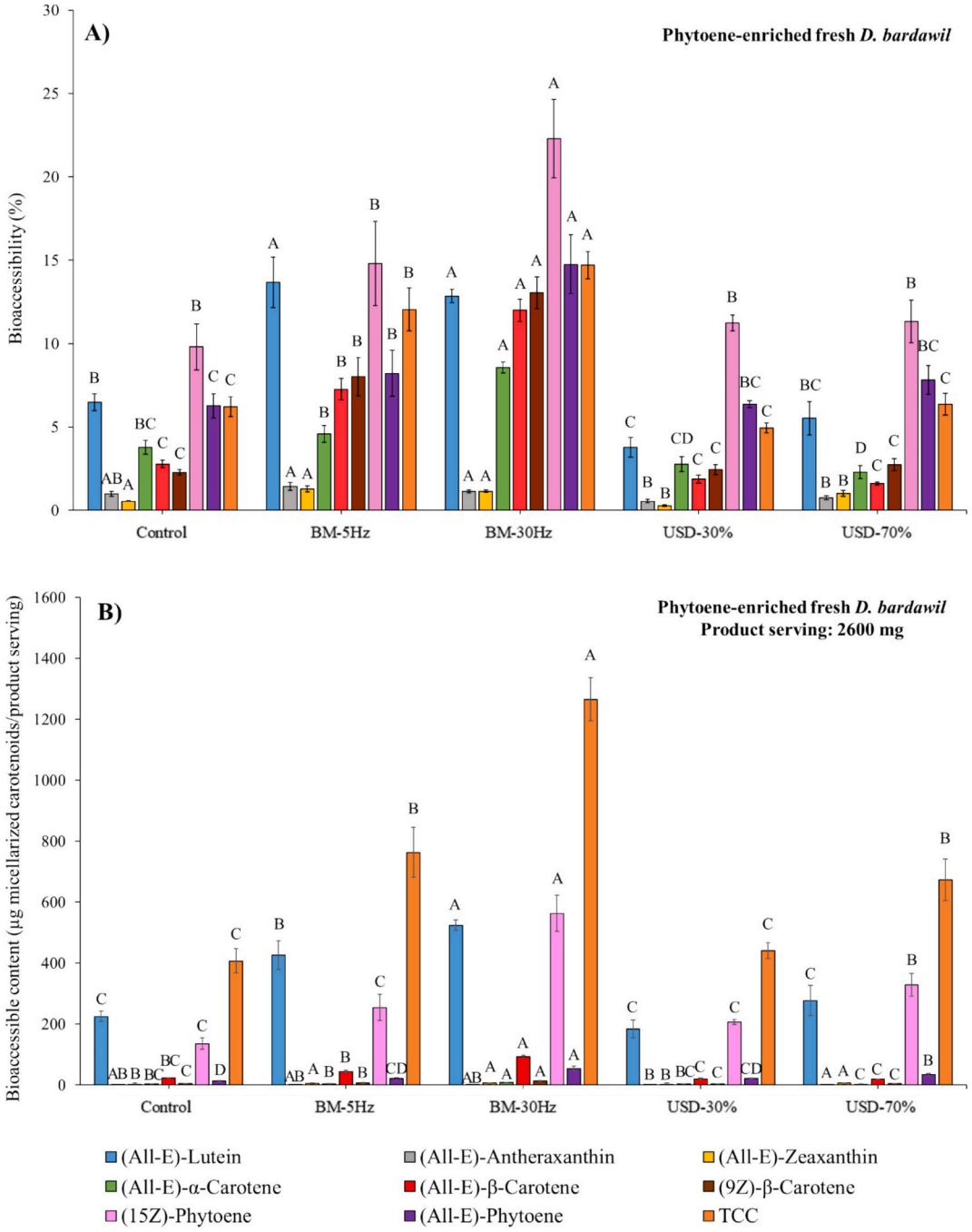

Fig. 2. Carotenoid bioaccessibility (%) (A) and CBC (µg/product serving) (B) in phytoene-enriched fresh *D. bardawil*. A product serving corresponds to 2600 mg of fresh biomass (80.7 % moisture). For more information see note in Fig. 1.

In the phytoene-enriched matrix, (all-*E*)-lutein (183.49–524.33 µg/portion (2600 mg) and (15*Z*)-phytoene (135.60–563.10 µg/product serving) were the most abundant carotenoids in the micellar fraction (Fig. 2B and Supplementary Table 2B). The rest of the carotenoids, including (all-*E*)-β-carotene (19.55–93.20 µg/product serving (2600 mg)), (all-*E*)-phytoene (12.82–54.81 µg/product serving), (9*Z*)-β-carotene (3.31–13.89 µg/product serving), (all-*E*)-α-carotene (2.74–8.69 µg/product serving), (all-*E*)-zeaxanthin (1.43–6.64 µg/product serving), and (all-*E*)-antheraxanthin (0.31–0.88 µg/product serving), showed significantly lower CBC (Fig. 2B and Supplementary Table 2B).

Ball-milling at 30 Hz yielded the highest total carotenoid bioaccessibility and CBC in both wild-type and phytoene-enriched *D. bardawil*. Regarding the phytoene-enriched *D. bardawil,* compared to the untreated sample (control), the ball-mill treatment at 30 Hz enhanced total carotenoid bioaccessibility by 2.25 times and CBC by 1.97 times. This treatment also proved to be optimal for all the individual carotenoid bioaccessibility and CBC in this matrix. Regarding wild-type matrix, compared to control, the ball-mill treatment at 30 Hz enhanced total carotenoid bioaccessibility by 2.36 times and CBC by 3.11 times.

3.3. Bioaccessibility of carotenoids in the freeze-dried wild-type and phytoene-enriched D. bardawil

In freeze-dried wild-type *D. bardawil*, the bioaccessibility of total carotenoids varied significantly, ranging from 1.21 % (USD 70 %) to

27.54 % (ball-milled 5 Hz), depending on the treatment applied (Fig. 3A and Supplementary Table 3A). In contrast, freeze-dried phytoene-enriched *D. bardawil* showed slightly lower bioaccessibility, with values between 0.33 % (USD 70 %) and 11.77 % (control) (Fig. 4A and Supplementary Table 3B).

Among the individual carotenoids in the wild-type matrix, (all-*E*)-lutein showed the highest bioaccessibility, ranging from 1.63 % (USD 70 %) to 39.13 % (ball-milled 5 Hz), depending on the treatment. The rest of the carotenoids exhibited lower bioaccessibility, with values between 0.00 % and 9.12 %, depending on the treatment.

In the phytoene-enriched matrix, (15*Z*)-phytoene (from 11.07 % (control) to 25.27 % (ball-milled 5 Hz)) and (all-*E*)-phytoene (from 6.95 % (control) to 16.39 % (ball-milled 5 Hz)) showed the highest bioaccessibility under control and ball-milling conditions. The remaining carotenoids, including (9*Z*)-β-carotene (from 3.45 % (control) to 6.20 % (ball-milled 5 Hz)) and (all-*E*)-α-carotene (from 1.71 % (control) to 5.76 % (ball-milled 5 Hz)), (all-*E*)-lutein (from 2.35 % (control) to 5.13 % (ball-milled 5 Hz)), and (all-*E*)-antheraxanthin (from 0.12 % (control) to 0.30 % (ball-milled 5 Hz)) displayed lower bioaccessibility values. However, this trend was not observed in samples subjected to USD. In

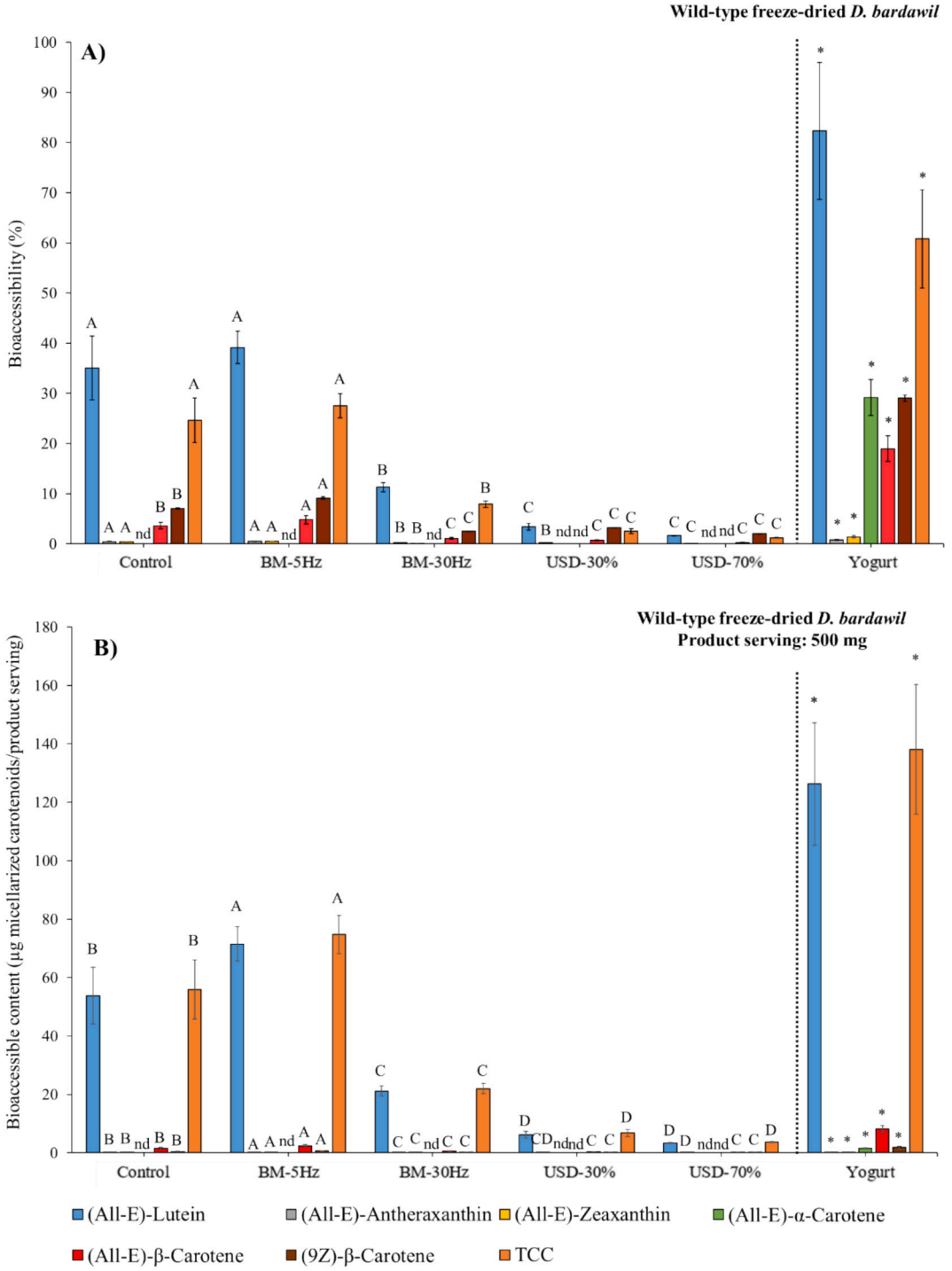

Fig. 3. Carotenoid bioaccessibility (%) (A) and CBC (µg/product serving) (B) in wild-type freeze-dried *D. bardawil*. A product serving corresponds to 500 mg of freeze-dried biomass. For more information see note in Fig. 1.

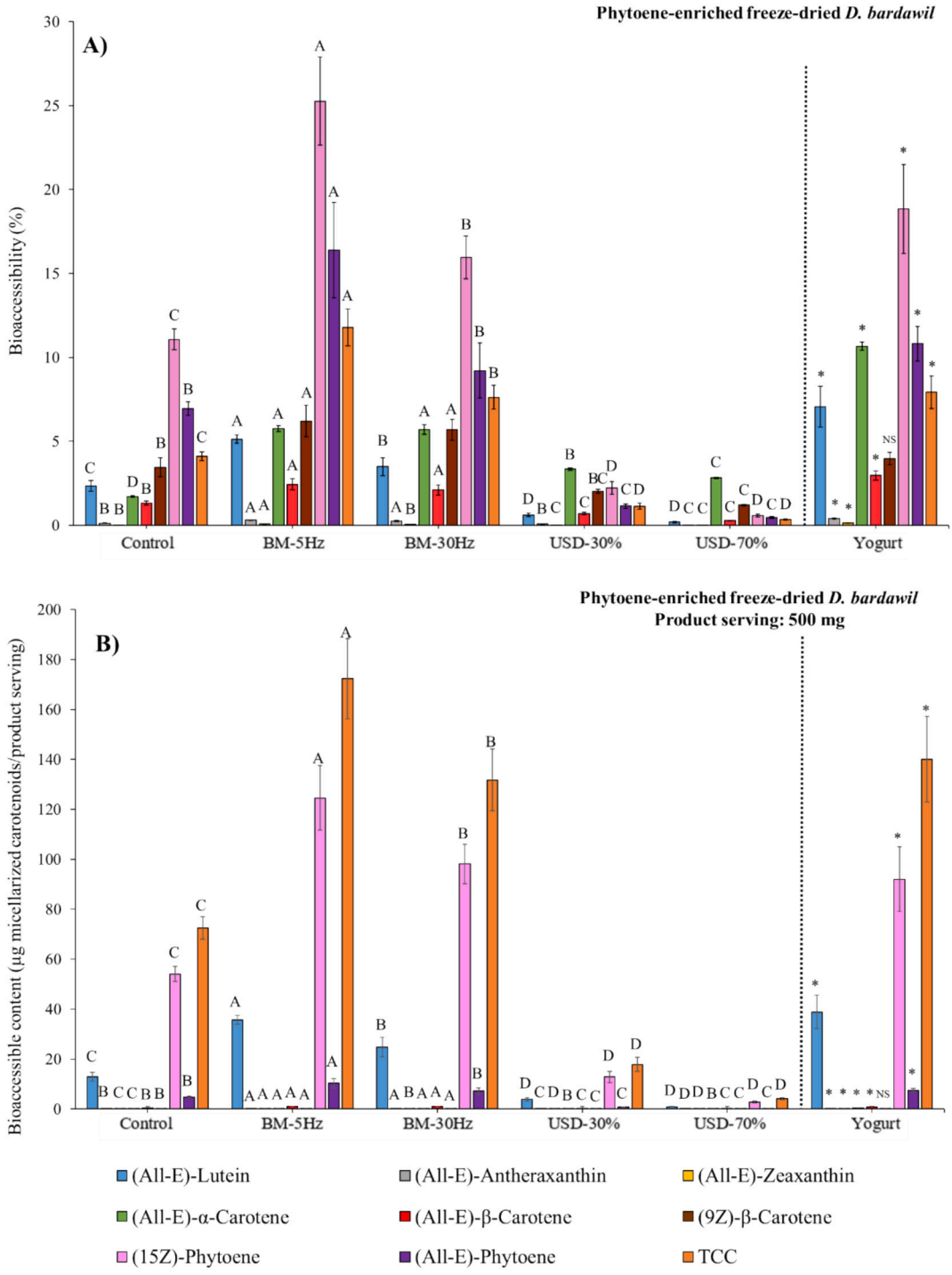

Fig. 4. Carotenoid bioaccessibility (%) (A) and CBC (µg/product serving) (B) in phytoene-enriched freeze-dried *D. bardawil*. A product serving corresponds to 500 mg of freeze-dried biomass. For more information see note in Fig. 1.

both USD treatments (30 % and 70 %), (all-*E*)-α-carotene exhibited the highest bioaccessibility (2.83 % in USD 70 %, and 3.35 % in USD 30 %), followed by (9*Z*)-β-carotene (1.21 % in USD 70 %, and 2.02 % in USD 30 %). The bioaccessibility of the remaining carotenoids were: (15*Z*)-phytoene (0.58 % in USD 70 %, and 2.23 % in USD 30 %); (all-*E*)-phytoene (0.46 % in USD 70 %, and 1.14 % in USD 30 %); (all-*E*)-β-carotene (0.27 % in USD 70 %, and 0.70 % in USD 30 %); (all-*E*)-lutein (0.19 % in USD 70 %, and 0.61 % in USD 30 %) and finally (all-*E*)-antheraxanthin (0.02 % in USD 70 %, and 0.09 % in USD 30 %).

Regarding the CBC, in the freeze-dried wild-type *D. bardawil*, (all-*E*)-lutein was notably the most abundant carotenoid in the micellar fraction, with concentrations ranging from 3.36 (USD 70 %) to 71.52 µg/product serving (500 mg) (ball-milled 5 Hz) (Fig. 3B and Supplementary Table 3A). Other carotenoids, such as (all-*E*)-β-carotene (0.15–2.45 µg/product serving), (9*Z*)-β-carotene (0.18–0.69 µg/product serving), (all-*E*)-antheraxanthin (0.02–0.09 µg/product serving), and (all-*E*)-zeaxanthin (0.00–0.03 µg/product serving), were detected in the micellar fraction in significantly lower amounts. Notably, (all-*E*)-α-carotene was not detected in freeze-dried wild-type samples treated with ultrasound or ball-mill (Fig. 3B and Supplementary Table 3A).

In the freeze-dried phytoene-enriched matrix, (15*Z*)-phytoene (from 2.86 (USD 70 %) to 124.58 µg/product serving (500 mg) (ball-milled 5

Hz)) was the most abundant carotenoid in the micellar fraction followed by (all-*E*)-lutein (from 0.94 (USD 70 %) to 35.69 μg/product serving (ball-milled 5 Hz)) and (all-*E*)-phytoene (from 0.25 (USD 70 %) to 10.30 μg/product serving (ball-milled 5 Hz)). Other carotenoids, including (all-*E*)-β-carotene (0.05–1.06 μg/product serving (500 mg)), (9*Z*)-β-carotene (0.03–0.30 μg/product serving), (all-*E*)-α-carotene (0.06–0.16 μg/product serving), (all-*E*)-zeaxanthin (0.00–0.19 μg/product serving), and (all-*E*)-antheraxanthin (0.00–0.04 μg/product serving), were present in lower concentrations (Fig. 4B and Supplementary Table 3B).

Among the evaluated treatments, ball-milling at 5 Hz consistently led to the highest total and individual carotenoid bioaccessibility and CBC in both freeze-dried wild-type and phytoene-enriched *D. bardawil*. These differences were statistically significant in most cases ($p < 0.05$), except for the bioaccessibility in the wild-type matrix, where no significant difference was observed compared to the control. Although all individual carotenoids and the TCC showed numerically higher values under this treatment, the increases in TCC and the bioaccessibility of xanthophylls ((all-*E*)-lutein, (all-*E*)-zeaxanthin, (all-*E*)-antheraxanthin) were not statistically significant when compared to the control. However, TCC was significantly higher than in other treatment conditions. The application of the ball-mill at 5 Hz led to an increase of 1.12-fold and 1.36-fold in the bioaccessibility and CBC of total carotenoids, respectively, in the freeze-dried wild-type *D. bardawil,* compared to the control (untreated sample). For the phytoene-enriched matrix, these increases were 2.87-fold and 2.38-fold, respectively.

3.4. Bioaccessibility of carotenoids in freeze-dried wild-type and phytoene-enriched D. bardawil as affected by the addition of yogurt

A yogurt containing 10 % fat was added at a proportion of 4:1 in weight relative to the algal biomass (that is, yogurt fat was added to a proportion of 0.4:1). This significantly ($p < 0.05$) enhanced the bioaccessibility and CBC of total carotenoid and each individual carotenoid in freeze-dried wild-type *D. bardawil* compared to the samples without yogurt, i.e., the untreated sample (control) and all the samples treated with ultrasound and ball-mill. In particular, considering the wild-type total carotenoids, the bioaccessibility and CBC increased by 2.47-fold compared to untreated samples (Fig. 3A and Supplementary Table 3A). Among individual carotenoids, (all-*E*)-lutein showed the highest bioaccessibility (82.34 %), followed by (all-*E*)-α-carotene (29.16 %), (9*Z*)-β-carotene (29.05 %), (all-*E*)-β-carotene (18.97 %), (all-*E*)-zeaxanthin (1.40 %), and (all-*E*)-antheraxanthin (0.80 %). Similarly, (all-*E*)-lutein had the highest CBC (126.31 μg/product serving (500 mg)), with other carotenoids following in this order: (all-*E*)-β-carotene (8.16 μg/product serving), (9*Z*)-β-carotene (1.95 μg/product serving), (all-*E*)-α-carotene (1.53 μg/product serving), (all-*E*)-antheraxanthin (0.11 μg/product serving), and (all-*E*)-zeaxanthin (0.07 μg/product serving). Thus, the CBC of lutein in freeze-dried wild-type *D. bardawil* with yogurt was between 15.48 and 1684.13 times higher than that of the rest of the carotenoids in this sample (Fig. 3B and Supplementary Table 3A).

Regarding the phytoene-enriched matrix, the addition of yogurt led to a 1.93-fold increase in both total carotenoid bioaccessibility and CBC compared to control (Fig. 4 and Supplementary Table 3B). However, the phytoene-enriched sample treated by ball-mill at 5 Hz presented a total carotenoid bioaccessibility and CBC higher than that of the untreated phytoene-enriched sample with yogurt. The bioaccessibility of carotenoids in the phytoene-enriched sample with yogurt followed the following order: (15*Z*)-phytoene exhibited the highest bioaccessibility (18.85 %), followed by (all-*E*)-phytoene (10.82 %), (all-*E*)-α-carotene (10.67 %), (all-*E*)-lutein (7.05 %), (9*Z*)-β-carotene (3.98 %), (all-*E*)-β-carotene (2.97 %), (all-*E*)-antheraxanthin (0.40 %), and (all-*E*)-zeaxanthin (0.14 %) (Fig. 4A and Supplementary Table 3B). The CBC was highest for (15*Z*)-phytoene (92.03 μg/product serving (500 mg)), followed by (all-*E*)-lutein (38.86 μg/product serving), (all-*E*)-phytoene (7.52 μg/product serving), (all-*E*)-β-carotene (0.84 μg/product serving), (all-*E*)-α-carotene (0.39 μg/product serving), (all-*E*)-zeaxanthin (0.28 μg/product serving), (9*Z*)-β-carotene (0.13 μg/product serving), and (all-*E*)-antheraxanthin (0.06 μg/product serving) (Fig. 4B and Supplementary Table 3B).

3.5. Bioaccessibility of carotenoids in encapsulated wild-type and phytoene-enriched D. bardawil

In the encapsulated wild-type *D. bardawil*, total carotenoid bioaccessibility ranged from 6.85 % (control) to 19.51 % (ball-milled 5 Hz), that is a 2.8-fold difference, depending on the treatment applied (Fig. 5A and Supplementary Table 4A). The encapsulated phytoene-enriched *D. bardawil* exhibited higher bioaccessibility, with values between 8.54 % (ball-milled 5 Hz) and 73.71 % (USD 70 %), that is an 8.6-fold difference (Fig. 6A and Supplementary Table 4B).

In the wild-type matrix, (all-*E*)-lutein showed bioaccessibility values ranging from 7.02 % (control) to 20.02 % (ball-milled 5 Hz), depending on the treatment, while for (all-*E*)-β-carotene, the bioaccessibility ranged from 5.41 % (control) to 15.42 % (ball-milled 5 Hz) (Fig. 5A and Supplementary Table 4A). In the phytoene-enriched matrix, (15*Z*)-phytoene exhibited the highest bioaccessibility, with values between 15.40 % (ball-milled 30 Hz) and 90.37 % (USD at 70 % amplitude), while the other carotenoids detected in the matrix, i.e. (all-*E*)-lutein and (all-*E*)-β-carotene, showed lower bioaccessibility, ranging from 6.42 % (ball-milled sample at 30 Hz) to 78.40 % (USD 70 %) and 5.88 % (ball-milled 30 Hz) to 29.38 % (USD 70 %), respectively (Fig. 6A and Supplementary Table 4B).

Regarding CBC, in the encapsulated wild-type *D. bardawil*, (all-*E*)-lutein was the most abundant carotenoid in the micellar fraction, with concentrations ranging from 5.73 (control) to 34.24 μg/product serving (9400 mg) (USD 70 %), followed by (all-*E*)-β-carotene (from 0.52 (control) 2.64 μg/product serving (USD 70 %)) (Fig. 5B and Supplementary Table 4A). (All-*E*)-Lutein showed a CBC, on average between all treatments, 11.27 times higher than that of (all-*E*)-β-carotene in the wild-type matrix. In the phytoene-enriched matrix, (all-*E*)-lutein also predominated in the micellar fraction, with concentrations between 0.16 (ball-milled 5 Hz) and 5.07 μg/product serving (9400 mg) (USD 70 %), followed by far by (15*Z*)-phytoene (from 0.10 (control and ball-milled) to 1.43 μg/product serving (USD 70 %)) and (all-*E*)-β-carotene (from 0.03 (control and ball-milled) to 0.37 μg/product serving (USD 70 %)) (Fig. 6B and Supplementary Table 4B). Very interestingly, in this phytoene-enriched matrix, (all-*E*)-lutein had a CBC approximately 18.17 times higher than that of (all-*E*)-β-carotene, and 3.41 times higher than that of (15*Z*)-phytoene.

The most effective treatment varied depending on the matrix. In the wild-type encapsulated matrix, the highest total and individual carotenoid bioaccessibility was achieved with ball-mill treatment at 5 Hz and ultrasound at 30 %, increasing total carotenoid bioaccessibility by 2.85-fold and 2.76-fold compared to the untreated sample, respectively. However, the highest CBC for total carotenoids and (all-*E*)-lutein occurred with ultrasound at 70 %, with a 5.94-fold increase for total carotenoids compared to untreated samples. No significant differences were found between ultrasound treatments at 70 % and 30 % for (all-*E*)-β-carotene, both of which were the most effective treatments (Fig. 5B and Supplementary Table 4A).

Concerning the encapsulated phytoene-enriched matrix, ultrasound at 70 % amplitude significantly resulted in the highest bioaccessibility for total and individual carotenoids, increasing total carotenoid bioaccessibility by 5.42-fold compared to untreated sample (control) (Fig. 6A and Supplementary Table 4B). Similarly, ultrasound at 70 % and 30 % were the most effective treatments for total CBC increasing by 16.61-fold and 14.64-fold, respectively, compared to control (Fig. 6B and Supplementary Table 4B). In particular, ultrasound at 70 % and 30 % were significantly the most effective treatments for enhancing the CBC of (all-*E*)-lutein and (15*Z*)-phytoene. However, for (all-*E*)-

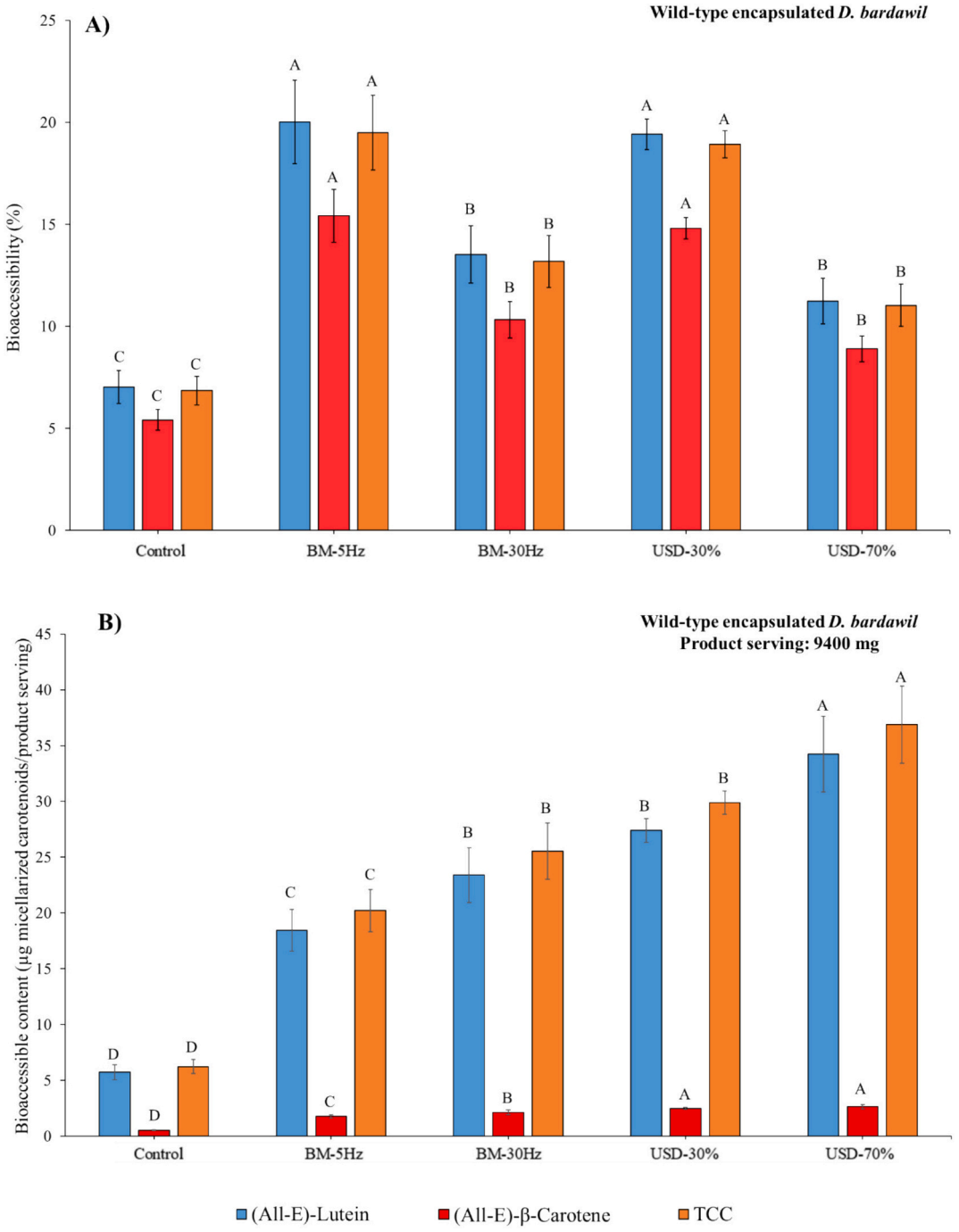

Fig. 5. Carotenoid bioaccessibility (%) (A) and CBC (µg/product serving) (B) in wild-type encapsulated *D. bardawil*. A product serving corresponds to 9400 mg of fresh biomass (94.68 % moisture). For more information see note in Fig. 1.

β-carotene, ultrasound at 70 % resulted in a significantly higher CBC compared to ultrasound at 30 %.

4. Discussion

The observed differences between treatments can be explained by the physicochemical characteristics across the matrices and the individual carotenoids. In fresh samples, ball-milling physically disrupts the cell wall and thylakoid membranes, promoting the release of carotenoids that are structurally associated with these compartments (Demarco et al., 2022). Ultrasound also relies on mechanical effects derived from acoustic cavitation, generating microstreaming and shear forces that can enhance carotenoid dispersion and mass transfer. However, its effectiveness depends on the structural rigidity and composition of the matrix; in more compact or densely organized cell structures, disruption may be limited, whereas in more porous or pre-emulsified environments ultrasound can facilitate carotenoid diffusion (Chemat et al., 2020). Moreover, carotenoid polarity and localization also influence results: xanthophylls (e.g., lutein) are more polar and located near the membrane surface, which facilitates micellization, whereas carotenes (e.g., β-carotene) are highly hydrophobic and, in some cases they can crystallize at high concentrations, reducing their transfer (Chacón-Ordóñez et al., 2019). In the case of the colorless carotenoid phytoene, its relatively low degree of conjugation makes it adopt less rigid conformations

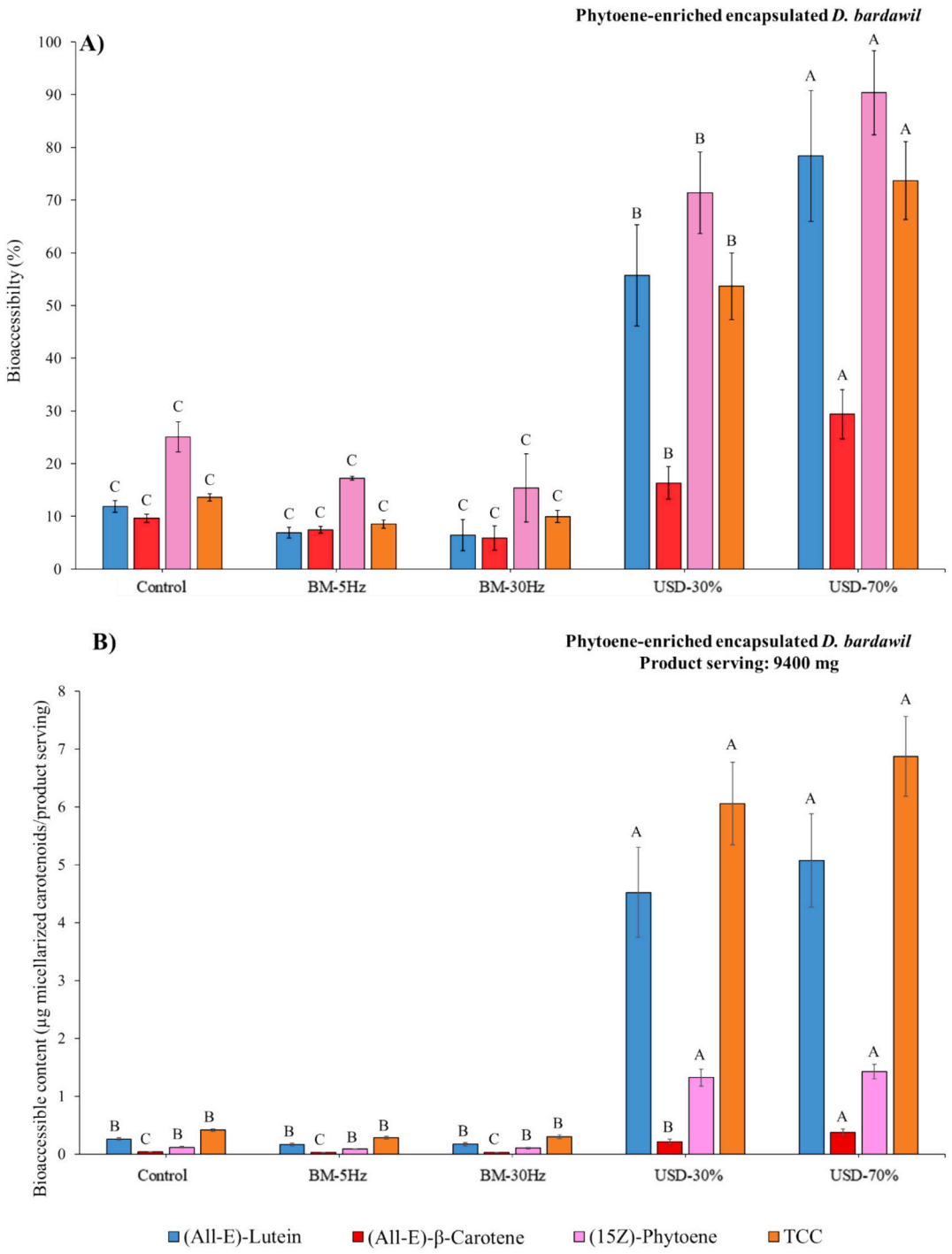

Fig. 6. Carotenoid bioaccessibility (%) (A) and CBC (µg/product serving) (B) in phytoene-enriched encapsulated *D. bardawil*. A product serving corresponds to 9400 mg of fresh biomass (94.68 % moisture). For more information see note in Fig. 1.

and be less susceptible to oxidative degradation and aggregation. These facts may favor its incorporation into mixed micelles compared to highly conjugated colored carotenoids (Mapelli-Brahm et al., 2017).

In this study, the effects of various pretreatments based on ball-milling and ultrasounds on the carotenoid bioaccessibility and CBC were evaluated in wild-type and phytoene-rich *Dunaliella bardawil.* The algal biomass was studied in three different forms, namely fresh, freeze-dried, and encapsulated. A widely used consensus INFOGEST protocol to simulate static *in vitro* digestion was applied (Brodkorb et al., 2019). Additionally, yogurt was added to the freeze-dried samples to assess the impact of fat addition (at a proportion of 40 % in weight relative to the matrices) on carotenoid bioaccessibility.

4.1. Effect of microalgae D. bardawil form on carotenoid bioaccessibility and CBC

The total carotenoid bioaccessibility in the wild-type microalgae without any treatment (control) ranged from 5.12 % to 24.62 %, depending on the sample (fresh, freeze-dried, and encapsulated), while in the case of the phytoene-rich microalga, the bioaccessibility range was from 4.10 % to 13.60 %. The wild-type fresh sample obtained a total carotenoid bioaccessibility of 5.12 %, slightly lower to that of phytoene-

enriched fresh microalgae, which was 6.22 %. Contrastingly, in freeze-dried samples, the wild-type variant showed a markedly higher bioaccessibility (24.62 %) relative to that of the phytoene-enriched freeze-dried *D. bardawil* (4.10 %).

The increased bioaccessibility in freeze-dried wild-type matrix may result from structural changes induced by freeze-drying that enhance carotenoid release. Freeze-drying reduces water content and oxidative degradation while generating a porous structure that facilitates carotenoids incorporation into mixed micelles (Grace et al., 2022). In addition, carotenoid stability in the freeze-dried sample could also play a role. In a study, the bioaccessibility of freeze-dried and raw pulp of carrot, tomato, and red pepper were evaluated, showing that freeze-drying had a strong effect on the enhancement of the gastrointestinal stability of carotenoid in the three samples, especially after simulated intestinal phase, compared to raw samples (Bilušić et al., 2019).

Zhang et al. (2018) also evaluated the effect of dying on lutein, α-carotene, and β-carotene bioaccessibility in carrots, sweet potatoes, yellow bell peppers, and broccoli florets. They reported a higher bioaccessibility compared to fresh samples, with increases from 1.33 to 3.56 times, depending on the drying temperature and the specific carotenoid.

However, this trend was not observed in the samples treated with norflurazon during cultivation (phytoene-enriched samples). The bioaccessibility of total carotenoids in the phytoene-enriched freeze-dried microalgae was 1.52 times lower than that of the phytoene-enriched fresh matrix. A similar phenomenon has been reported in spinach, where the carotenoid bioaccessibility of the fresh sample (80.06 %) was significantly higher than that of the freeze-dried sample (72.10 %), resulting in a 1.11-fold decrease. This reduction was attributed to the release of divalent minerals during processing, which subsequently interfered with lipid micellarization (Hayes et al., 2022). In this case, the effect of freeze-drying on carotenoid release and micellarization likely depended on the carotenoid profile. The phytoene-enriched microalgae accumulated large amounts of phytoene, undetected in the wild-type, with structure markedly different from most dietary carotenoids (Meléndez-Martínez et al., 2019a). Interestingly, in two recent studies with the microalgae *D. bardawil* and *Chlorella sorokiniana,* it has been observed that the ultrasound-assisted extraction of phytoene in freeze-dried phytoene-rich matrices was considerably lower than it may have been expected (Morón-Ortiz, Mapelli-Brahm, León-Vaz, Benitez-González, et al., 2024; Morón-Ortiz, Mapelli-brahm, León-Vaz, Benítez-González, et al., 2024). Additionally, norflurazon treatment, which led to the accumulation of phytoene, may also have affected other microalgal components that affected carotenoid release and micellarization, especially in freeze-dried material.

CBC is a crucial parameter for understanding the absolute amount of carotenoid incorporated into micelles that can be potentially available for intestinal absorption. In the control sample of wild-type *D. bardawil,* the total CBC of the freeze-dried sample was 4.81 times higher compared to the fresh matrix; however, the actual CBC of the freeze-dried sample was 4.90 times lower (Supplementary Tables 2 and 3). Specifically, the CBC in the fresh sample was 274.07 μg/product serving (2600 mg), while in the freeze-dried sample it was 55.94 μg/product serving (500 mg). This could be attributed to a substantial loss of carotenoids as a result of the freeze-drying process.

The effect of the encapsulation on carotenoids' bioaccessibility and CBC was also evaluated. In the control encapsulated matrices, the bioaccessibility of the wild-type *D. bardawil* (6.85 %) was lower than that of the phytoene-enriched encapsulated sample (13.60 %). However, despite this higher bioaccessibility, the CBC in the control phytoene-enriched encapsulated matrix (0.41 μg/product serving (9400 mg)) was substantially lower than in the wild-type encapsulated sample (6.24 μg/product serving). This difference is explained by the markedly lower total carotenoid content of the phytoene-enriched matrix (3.01 μg/product serving) compared to the wild-type matrix (91.09 μg/product serving).

Encapsulation did not enhance bioaccessibility vs. freeze-dried samples but showed improvement over fresh microalgae. In contrast, the phytoene-enriched encapsulated sample exhibited the highest bioaccessibility among all phytoene-enriched samples, being 2.19 and 3.32 times higher than fresh and freeze-dried matrices, respectively. However, this increase did not translate into a higher CBC (per product serving). In fact, it was 993.20 times and 176.83 times lower than that of the phytoene-enriched fresh and freeze-dried *D. bardawil*, respectively. At this point, it is important to note that the carotenoid content in the encapsulated samples was considerably lower (91.09 μg/product serving for wild-type and 3.01 μg/product serving for phytoene-enriched; product serving: 9400 mg).

The encapsulation process was carried out using alginate, which is known for its capacity to protect carotenoids from oxidation and degradation. This enhances their stability prior to digestion. However, its gel-like matrix may entrap lipophilic compounds, limiting their intestinal release and micelle formation (key for carotenoid absorption) (Gherasim et al., 2024).

In any case, the effect of alginate-containing particles in the bioaccessibility of carotenoids deserves further investigation. For instance, a recent study by Sorasitthiyanukarn et al. (2022) examined the effect of chitosan oligosaccharide/alginate nanoparticle encapsulation on the bioaccessibility of standard astaxanthin solution in acetone. The results showed that encapsulation with alginate significantly enhanced bioaccessibility, making it approximately five times higher than that of the free form, with values of 61.5 % and 12.4 %, respectively. Another study by Zipei Zhang et al. (2016) investigated the bioaccessibility of β-carotene in different systems: free lipid droplets, hydrogel beads formed with 0.5 % alginate, and hydrogel beads formed with 1 % alginate. The results indicated that β-carotene in free lipid droplets exhibited the highest bioaccessibility. The encapsulation with 0.5 % alginate significantly reduced bioaccessibility compared to free droplets. Increasing the alginate concentration to 1 % led to an even greater reduction in bioaccessibility compared to 0.5 % alginate. All these results underline the fact that both bioaccessibility and carotenoid content must be considered collectively to obtain a realistic perception of the nutritional potential of the processed matrices.

4.2. *Effect of ball-milling and USD on carotenoid bioaccessibility and CBC in D. bardawil*

Carotenoids are often found within complex food matrices and structures and their release and bioaccessibility can be enhanced by different processing techniques. Among these, mechanical cell disruption methods, such as ball-milling and USD, have been widely employed to enhance the release and bioaccessibility of bioactive compounds, including carotenoids. Ball-milling is a mechanical force-based technique, while ultrasound applies energy transfer through waves. Both effectively disrupt cell walls, which can in turn improve the release and bioaccessibility of bioactive compounds depending on the conditions.

The effects of ball-milling at two different frequencies (5 and 30 Hz) and USD at two different amplitudes (30 % and 70 %) were evaluated across all samples. In most cases, the application of at least one of these treatments significantly enhanced both bioaccessibility and CBC.

4.2.1. *Wild-type and phytoene-enriched fresh D. bardawil*

In wild-type fresh *D. bardawil*, the most effective treatment for enhancing both total carotenoid bioaccessibility and total CBC was ball-milling at 30 Hz, showing a significant improvement compared to other treatments. The total carotenoid bioaccessibility and CBC increased 2.25 and 1.97 times, respectively, compared to untreated sample (control). This treatment was also the most effective in increasing the bioaccessibility of (all-*E*)-lutein and (all-*E*)-zeaxanthin. For the remaining carotenoids ((all-*E*)-antheraxanthin, (all-*E*)-α-carotene, (all-*E*)-β-carotene, and (9*Z*)-β-carotene), the highest bioaccessibility was with USD at 70 %, although some differences were not statistically significant

(Fig. 1A and Supplementary Table 2A).

Regarding the CBC, ball-milling at 30 Hz yielded the highest values for all carotenoids, although in some cases no significant differences were observed among treatments (Fig. 1B and Supplementary Table 2A).

Similarly, in phytoene-enriched fresh *D. bardawil*, ball-milling at 30 Hz was also the most effective treatment for enhancing both bioaccessibility and CBC of total and individual carotenoids, except for (all-*E*)-lutein, (all-*E*)-zeaxanthin and (all-*E*)-antheraxanthin for which ball-milling at 5 Hz was the best treatment in terms of bioaccessibility, but without statistically significant difference with the ball-milling treatment at 30 Hz (Fig. 2A and Supplementary Table 2B). The total carotenoid bioaccessibility and CBC increased by 2.36 and 3.11 times higher, respectively, with the application of the ball-mill (30 Hz) compared to untreated sample (control).

Several studies show that bead milling facilitates cell rupture and enhance the accessibility and bioavailability of key compounds in microalga, such as lipid and fatty acids, pigments and minerals (Demarco et al., 2022). A study by Wild et al. (2018) evaluated ball-milling (0.5 mm bead diameter, 1:9 dilution in distilled water, 50 mL/min pump speed, 3200 rpm, two milling runs) on dry *Arthrospira*, *Chlorella*, *Nannochloropsis*, and *Phaeodactylum* samples, reporting improvements in protein bioaccessibility ranging from 1.05 to 1.46 times, depending on the matrix, compared to non-cell-disrupted samples. Similarly, Teuling et al. (2019) investigated the effect of ball-milling (0.5 mm bead diameter, 20 L/h pump speed, 14 m/s milling speed, three milling runs) on the bioaccessibility of *Nannochloropsis gaditana* paste. They reported a substantial increase in the apparent digestibility coefficient of proteins (from 62 % to 78 %) and lipids (from 50 % to 82 %) compared to untreated samples.

4.2.2. Wild-type and phytoene-enriched freeze-dried D. bardawil

The ball-milling at 5 Hz resulted in the highest numerical value for both bioaccessibility and CBC of total and carotenoids in wild-type freeze-dried sample. While significant differences were observed in CBC compared to the control, no significant differences were observed in bioaccessibility (Fig. 3 and Supplementary Table 3). The application of this treatment allowed for a total carotenoid bioaccessibility and CBC 1.12 and 1.34 times higher, respectively, compared to untreated sample (control).

In phytoene-enriched freeze-dried *D. bardawil*, ball-milling at 5 Hz was also the most effective treatment for total and individual carotenoid bioaccessibility and CBC, although differences with 30 Hz were not statistically significant for some individual carotenoids (Fig. 4 and Supplementary Table 3B). The application of the ball-mill (5 Hz) resulted in a total carotenoid bioaccessibility and CBC 2.87 and 2.38 times higher, respectively, compared to untreated sample (control).

4.2.3. Wild-type and phytoene-enriched encapsulated D. bardawil

In the encapsulated matrix, ball-milling at 5 Hz and USD at 30 % were the most effective treatments for enhancing total and individual ((all-*E*)-lutein and (all-*E*)-β-carotene) carotenoid bioaccessibility (Fig. 5A and Supplementary Table 4A). The application of the ball-mill (5 Hz) and USD (30 %) resulted in a total carotenoid bioaccessibility 2.85 and 2.76 times higher, respectively, compared to untreated control sample. However, the CBC was highest with USD at 70 %, with no significant differences compared to USD 30 % in the case of (all-*E*)-β-carotene (Fig. 5B and Supplementary Table 4A). The application of USD (70 %) allowed for a total CBC 5.91 times higher compared to untreated control sample.

For phytoene-enriched encapsulated *D. bardawil*, USD at 70 % was the most effective treatment for total and individual carotenoid bioaccessibility and CBC, although differences in CBC were not statistically significant. USD (70 %) increased total carotenoid bioaccessibility by 5.42 times and CBC by 18.25 times versus control.

Ultrasound treatment has been shown to enhance carotenoid bioaccessibility in other microalgae. For instance, Gille et al. (2016) assessed the application of ultrasound in fresh *Chlorella vulgaris*. The baseline bioaccessibility (without ultrasound) was 7 % for lutein and negligible for β-carotene. However, applying ultrasound for 15 min at 20 kHz and 5 cycles/min significantly improved these values, increasing lutein bioaccessibility up to 18 % (2.57-fold increase) and β-carotene up to 12.5 %.

Beyond microalgae, USD has also been shown to enhance carotenoid bioaccessibility in various food matrices. For instance, in tomato juice, ultrasound treatment (25 kHz, 400 W, 50 μm amplitude, 20 min, time intervals of 10 s) resulted in a 1.76-fold increase in total lycopene bioaccessibility (W. Zhang et al., 2019). A similar effect was observed in fresh broccoli florets, where ultrasound application (24 kHz, 400 W, 100 mm amplitude, 120 min, 25 °C) led to a 1.61-fold increase in total carotenoid bioaccessibility compared to untreated samples (Z. Zhang et al., 2021).

4.3. Effect of yogurt addition on carotenoid bioaccessibility in the freeze-dried D. bardawil

Yogurt was added to the freeze-dried wild-type and phytoene-enriched *D. bardawil* samples prior to the *in vitro* bioaccessibility assay, in order to evaluate the effect of fat on carotenoid bioaccessibility. Dietary lipids play a crucial role in stimulating bile secretion and chylomicron synthesis, facilitating micellarization and intestinal absorption of carotenoids. However, increasing evidence suggests a saturation point beyond which increased fat intake no longer improves carotenoid solubilization. A systematic review analyzed the effect of dietary fats on the bioaccessibility and bioavailability of carotenoids. The findings indicated that the addition of fat, regardless of some structural differences among carotenoids, generally enhances carotenoid absorption by promoting micelle formation and facilitating cellular uptake (Yao et al., 2022).

Corrêa-Filho et al. (2022) studied the incorporation of encapsulated tomato pomace extract in inulin (10 and 20 % concentration) into natural liquid yogurt to evaluate the influence of yogurt fat on bioaccessibility, showing a significantly higher lycopene bioaccessibility. They observed an approximately 1.4–1.6 times higher (depending on inulin concentration) compared to the encapsulated extracts without yogurt addition. Only a few studies have investigated the impact of fat on carotenoid bioaccessibility from microalgae. One such example is the work by Tudor et al. (2021), where the effect of adding 5 % coconut oil on the bioaccessibility of extracts containing zeaxanthin and β-carotene from *Arthrospira platensis*, and lutein from *Chlorella pyrenoidosa* was evaluated. While no significant improvement was observed for lutein and β-carotene, the bioaccessibility of zeaxanthin increased significantly, reaching 1.15 times higher than in samples without oil addition.

In the present study, the addition of fat significantly enhanced total carotenoid bioaccessibility and CBC in the wild-type and phytoene-enriched freeze-dried *D. bardawil* (Figs. 3 and 4, and Supplementary Table 3). In the wild-type sample the total carotenoid bioaccessibility when yogurt was added was 2.47 times higher than in the untreated control sample and in the phytoene-enriched sample the enhancement was 1.93 times higher than in the untreated control sample. When comparing carotenoids, carotene bioaccessibility increased 4.67-fold and that of xanthophylls 2.57-fold compared to control samples. However, in the phytoene-enriched matrix, carotene was enhanced by 1.69-fold, while xanthophylls showed a greater improvement, with a 3.48-fold increase. Regarding individual carotenoids, it is worth highlighting (all-*E*)-α-carotene, which was not detected in the micellar fraction of any sample. However, upon yogurt addition, although its CBC remained low (Fig. 3A and 4A, and Supplementary Table 3), its bioaccessibility (Fig. 3B and 4B, and Supplementary Table 3) increased considerably, reaching levels comparable to those of other carotenoids in the matrix.

Several studies have reported an improvement in carotenoid bioaccessibility following the addition of dietary fats. For instance, Lyu

et al. (2021) found that adding 2 % corn oil to peeled *Cucurbita maxima* powder enhanced the bioaccessibility of total carotenoids by approximately 1.9 to 2.1 times, depending on the particle size of the sample, compared to those samples without fat addition.

Similarly, (Mashurabad et al., 2017) investigated the effect of different concentrations of olive oil (1, 2.5, 5, and 10 %) on carotenoid bioaccessibility from various food matrices, including cooked carrot (*Daucus carota*), tender spinach leaves (*Spinacia oleracea*), cooked drumstick leaves (*Moringa oleifera*), and fully ripened papaya pulp (*Carica papaya*). The findings demonstrated that from 1 % fat addition onwards, bioaccessibility significantly improved, with 2.5 % appearing to be the optimal concentration. At this level, carotenoid bioaccessibility increased by 2.26 to 5.96 times, depending on the matrix and carotenoid, compared to samples without oil.

These results provide relevant insights for the formulation of functional foods and nutraceuticals enriched in carotenoids. The enhanced bioaccessibility observed after mechanical treatments and lipid addition suggests that incorporating moderate cell-disruption steps (e.g., mild ball-milling or ultrasound) or combining carotenoid-rich microalgae with fat-containing carriers (such as dairy or oil-based matrices) can significantly improve carotenoid delivery. This knowledge may guide the design of food products or supplements with higher nutritional efficiency.

4.4. Effect of the type of carotenoid on the bioaccessibility

The bioaccessibility of carotenoids depends on several factors, including the species of carotenoids. Thus, structural properties of carotenoids significantly influence their bioaccessibility, with notable differences between carotenes and xanthophylls. Carotenes such as β-carotene in carrots and lycopene in tomato tend to form compact crystalline aggregates that limit their bioaccessibility, as these aggregates must dissolve before being incorporated into mixed micelles. Furthermore, due to their strong lipophilicity, carotenes are localized in the core of lipid droplets, which makes their transfer into mixed micelles even more difficult. On the other hand, xanthophylls are more bioaccessible because they are less hydrophobic, as they contain oxygen-containing functional groups (e.g., hydroxyl, methoxy, carbonyl, and epoxide). These polar groups enhance solubility in the digestive environment and facilitate incorporation into mixed micelles. Unlike carotenes, xanthophylls tend to localize at the surface of lipid droplets, making their transfer to mixed micelles more efficient (Chacón-Ordóñez et al., 2019).

In the present study, xanthophylls showed higher bioaccessibility than carotenes on average. In all matrices and treatments (excluding yogurt), the mean bioaccessibility of (all-*E*) xanthophylls ((all-*E*)-lutein, (all-*E*)-antheraxanthin, and (all-*E*)-zeaxanthin) was 6.38 %, whereas that of (all-*E*) carotenes ((all-*E*)-α-carotene, (all-*E*)-β-carotene, and (all-*E*)-phytoene) was 5.33 %.

In the wild-type matrix, (all-*E*)-lutein had the highest bioaccessibility among all the carotenoids analyzed. For instance, in a study using hydrodynamic cavitation (1.5 kW pump, 586.43 W, 1.17 W/cm^3) to disrupt freeze-dried *Chlamydomonas reinhardtii*, lutein exhibited approximately 1.44 times higher bioaccessibility than β-carotene (Akepach et al., 2022).

In phytoene-enriched matrices, (15*Z*)-phytoene exhibited the highest bioaccessibility, even higher than that of (all-*E*)-lutein. Although phytoene is a carotene, its structural and physicochemical properties appear to enhance its micellization. Its acyclic structure, lower number of conjugated double bonds compared to other common carotenoids, and specific geometric configuration contribute to its flexibility and reduced aggregation tendency. Since crystallization can hinder carotenoid release from the food matrix, the lower propensity of phytoene to form aggregates and crystals may play a crucial role in its increased bioaccessibility (Mapelli-Brahm et al., 2017; Meléndez-Martínez et al., 2019b).

Differences in bioaccessibility between carotenoid isomers were also observed. Thus, the average bioaccessibility across all samples, excluding the sample with yogurt, for *trans* carotenoids (lutein, antheraxanthin, zeaxanthin, α-carotene, β-carotene, and phytoene) was 6.46 %, while that of *cis* carotenoids ((9*Z*)-β-carotene and (15*Z*)-phytoene) reached 13.64 %.

The higher bioaccessibility of *cis* carotenoids compared to *trans* forms can be attributed to structural and physicochemical differences between them. The *cis* carotenoids present a more flexible molecular structure with less compact packing, which facilitates their solubilization in mixed micelles, improving their bioaccessibility. In contrast, *trans* carotenoids adopt a more linear and rigid configuration, making them less soluble in micellar environments (Priyadarshani, 2017).

do Nascimento et al. (2021) found both *cis* and *trans* β-carotene isomers in the microalga *Scenedesmus obliquus* (whole dried biomass, wet ultrasonicated biomass, and isolated carotenoid extract). The bioaccessibility of (all-*E*)-β-carotene ranged from 3.24 to 5.70 %, while (9*Z*)-β-carotene was significantly higher (19.71–21.90 %), being 3.8–6 times greater.

In the present study, the *cis* isomer of β-carotene also showed a higher bioaccessibility than its *trans* counterpart. The average bioaccessibility of (all-*E*)-β-carotene in fresh and freeze-dried samples among all treatments (excluding yogurt treatments), where both carotenoids were present, was 2.38 %. In contrast, the bioaccessibility of (9*Z*)-β-carotene reached 4.13 %, representing a 1.74-fold increase compared to its *trans* form. Moreover, another study by W. Zhang et al. (2019) evaluated the bioaccessibility of carotenoids in tomato juice treated with high-pressure homogenization (homogenized twice, 15 min, 4 °C, 500 bar) and ultrasound (25 kHz, 20 min, 50 μm, 800 W, with a 10 s time interval) using an *in vitro* model. The results showed that (all-*E*)-lycopene had significantly lower bioaccessibility than its geometric isomers, including (5*Z*)-lycopene, (9*Z*)-lycopene, and (13*Z*)-lycopene. The bioaccessibility of the *cis* isomers was 1.95 times higher than that of the *trans* form in high-pressure homogenization and 1.63 times higher in ultrasound treatment.

From an industrial perspective, these results suggest that mechanical pretreatments such as ultrasound and ball-milling can be integrated into processing pipelines for microalgal biomass. Ultrasound systems can be scaled using continuous-flow reactors and modular transducer configurations, enabling controlled cavitation and efficient treatment of larger product volumes (Cauduro et al., 2025). Similarly, ball-milling can be implemented at pilot and industrial scale using planetary or stirred-media mills, which are already employed for micronization and dispersion in the food and pharmaceutical industries (Reynes et al., 2023). Together, these characteristics support the practical incorporation of *D. bardawil* biomass into carotenoid-based formulations.

5. Conclusions

This study highlights the potential of ball-milling and ultrasound to enhance carotenoid bioaccessibility from wild-type and phytoene-rich *Dunaliella bardawil* in fresh, freeze-dried and encapsulated forms. The effect of fat addition to freeze-dried samples on carotenoids bioaccessibility was also evaluated. The results showed that the bioaccessibility and CBC of carotenoids varied significantly depending on the matrix and the treatment applied. Encapsulation resulted in a much lower CBC compared to both fresh and freeze-dried forms in both the wild-type and phytoene-enriched *D. bardawil*.

Among the treatments evaluated, ball-milling at 30 Hz was the most effective in increasing bioaccessibility and CBC in the fresh samples (wild type and phytoene enriched), while at 5 Hz was optimal for freeze-dried samples (wild-type and phytoene-enriched). In the encapsulated wild-type samples, both ball-milling at 5 Hz and ultrasound at 30 % amplitude significantly improved bioaccessibility. In the case of the phytoene-enriched encapsulated matrix, ultrasound at 70 % produced the highest bioaccessibility. However, in both encapsulated matrices,

the most effective treatment for improving the total CBC was ultrasound at 70 %. The addition of yogurt (10 % fat; yogurt:matrix ratio of 4:1 *w*/w; fat:matrix ratio of 0.4:1 *w*/w) significantly improved the bioaccessibility of carotenoids in both freeze-dried matrices, confirming the crucial role of lipids in carotenoid micellarization.

Overall, xanthophylls showed higher bioaccessibility than carotenes, probably due to their relatively higher polarity. However, in phytoene-enriched matrices, (15*Z*)-phytoene exhibited the highest bioaccessibility, even higher than that of (all-*E*)-lutein. On the other hand, *cis*-isomers of carotenoids showed higher bioaccessibility than their *trans* counterparts, probably due to their less compact and more flexible molecular structure, which facilitates their solubilization in micelles.

Based on the results, it can be concluded that sustainable processing techniques like ball-milling and ultrasound improve *D. bardawil*'s nutritional value by enhancing carotenoid bioaccessibility. Enhancing carotenoid bioaccessibility from *D. bardawil* supports the development of health-promoting products from Blue Economy renewable and eco-friendly sources. Future work should assess in vivo bioavailability, cost-effectiveness, and, eventually, scalability.

CRediT authorship contribution statement

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

Declaration of competing interest

The authors 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.2025.147332.

Data availability

No data was used for the research described in the article.

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