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Bioaccessibility of carotenoids (β-carotene and lutein) from intact and disrupted microalgae (*Chlamydomonas reinhardtii)*

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ABSTRACT

Chlamydomonas reinhardtii (*C. reinhardtii*) has a potential as a novel source for food/feed because it contains several constituents including bioactive compounds. However, its multilayer cell wall (hydroxyproline-rich glycoprotein [HRPGs]) may restrict the bioaccessibility of its nutrients. Therefore, using disruption techniques such as hydrodynamic cavitation (HDC) can be useful for assessing single cell compounds. This work aims to evaluate the impact of HDC on the bioaccessibility of carotenoids (β-carotene and lutein) from *C. reinhardtii*. Our results illustrated that digestive enzymes cannot fully break down the cell walls beside HDC process generates their significant change. The intact *C. reinhardtii* (ICR) and disrupted *C. reinhardtii* (DCR) have a comparable lutein bioaccessibility, in contrast, DCR decreased the biocessibility of β-carotene. HDC decreased the biocessibility of β-carotene in the small intestine although 37% of total carotenoids from DRC were absorbed.

1. Introduction

A healthy human diet has been a worldwide concerned due to the life dynamic in great cities. Thus, food industries and scientists have been putting loads of enforces for the understanding of food ingredients (Benlloch-Tinoco et al. 2015). As a consequence, the seeking of new nutrients sources for food production including probiotic agent with a positive influence on human health has been increasing (Darwish et al., 2020). Lately, microorganisms such as microalgae has been widely studied for industrial compounds production, which can be applied for bio-products, biofuels, biochemicals, pharmaceutical, chemical, cosmetic industries, aquacultures, food, and feed (Hamed, 2016; Ting et al., 2017; Vigani et al., 2015).

New food and feed resources are worldwide concerning, and microalgae is a promising source of nutrients (Vigani et al., 2015). Microalgae contains several constituents such as proteins, amino acids, minerals, lipids, vitamins, and pigments (including carotenoids). Carotenoids are an important group of organic pigments due to their application in the food industry (Maroneze et al., 2019). Furthermore, they are beneficial for human health because of its biological activities such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic, and neuroprotective (Benlloch-Tinoco et al., 2015; Chen & Roca, 2018; Ciccone et al., 2013; Guedes et al., 2011).

Among all microalgae species studied in science *C. reinhardtii* is well characterised, which has been used as a model microalgae. However, the *C. reinhardtii* cell wall is almost entirely built up from extensin-like HRGPs, that may restrict the bioaccessibility of its nutrients (Green & Lowther, 1959; Harris, 2009; Marzol et al., 2018). The extensin (EXT) molecule also contains tyrosine residues, which enable them to behave as self-assembling units and form a three-dimensional network organized by inter- and intra-Tyr linkage intermolecules (Kieliszewski et al., 2010; Marzol et al., 2018). The chemistry of an EXT cross-linking has three types of covalent bridge such as isodityrosine (Idt), di-isodityrosine (di-Idt) or pulcherosine (Brady et al., 1998; Brady & Fry, 1997). Therefore, the assess of carotenoids (β-carotene and lutein) from *C. reinhardtii* can be increased by using cell disruption techniques,

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mainly, when considered the amount of cell disruption techniques recognised.

Cell disruption is an essential aspect of biotechnology and the downstream processes related to the manufacturing of biological products (Waghmare et al., 2019). Mechanical disruption has been more used than chemical disruption because it avoids chemical contamination and preserves cell functionality (Gogate and Pandit. 2008). Among all mechanical disruption techniques that are known, hydrodynamic cavitation (HDC) is produced by pressure variations obtained by using the geometry of the system to create velocity variations (Gogate and Pandit 2005; Waghmare et al. 2019). The processing of foods can influence the bioaccessibility of nutrients, mainly through changes in the cell wall structure and properties (Berry et al. 2008). Bioaccessibility refers to the amount of an ingested nutrient that is potentially available for absorption (Hurst, 2002; Yonekura & Nagao, 2009).

Several promising research have been conducted using microalgae-based nutrients and its application in human foods (Bleakley & Hayes, 2017). In a previous work, our team investigates the nutritional content of *C. reinhardtii* as an additional value for this species beyond its known potential in biofuels and bio-products production (Darwish et al. 2020). Gille et al. (2016) evaluated the effect of cell disruption on the bioaccessibility of carotenoids (*β*-carotene and lutein) from *C. reinhardtii* using sonication as a cell-wall disruption technique, the authors concluded sonication had no effect on bioaccessibility of carotenoids (*β*-carotene and lutein). From the best of our knowledge, accessing *C. reinhardtii* nutrients by the hydrodynamic cavitation of its cell wall to improve *in vitro* bioaccessibility of lipophilic pigments such as carotenoids (*β*-carotene and lutein) can pave the way for a more comprehensive understanding of microalgae assembly and its application. Therefore, this work aims to evaluate the impact of HDC on the bioaccessibility of carotenoids (*β*-carotene and lutein) from *C. reinhardtii*.

2. Methodology

2.1. C. reinhardtii culture preparations

A microalgae strain (*C. reinhardtii,* Wild Type CC-125 mt (+)), which was supplied from the Department of Plant Science, University of Cambridge, Cambridge, UK) was used during this study.

The Tris-Acetate-Phosphate (TAP) growth medium was prepared by combining stock solutions containing nitrogen, phosphate, and a mix of trace elements (Table S1). All stock solutions were stored at 4 °C and filled up to 1 L (all stock solution was kept no more than a month). The pH of the medium was adjusted to 7.0 with acetic acid, and it was then autoclaved at 121 °C for 20 min.

C. reinhardtii were pre-grown in 300 mL of TAP medium at 23 °C under continuous illumination (cool white fluorescent bulbs, an intensity at 70 μmole/m^2 s) for 3 days. Afterwards, 5 × 10^6 microalgae cells/mL were inoculated in 2 L of TAP medium and maintained at 23 °C under a light intensity at 70 μmole/m^2sat for 7 days, which reached the stationary growth phase (8 × 10^6 cell/mL).

2.2. Hydrodynamic cavitation disruption

The hydrodynamic cavitation device (Efficiency Technologies Limited, UK) in this study was fabricated using a motor (1.5 kW pump, Pedrollo, Italy), tubulation and cavitator version 1.4 (cavitation power 586.43 W and power density 1.17 (W/cm^3)). The liquid flow into the inlet system through the orifices leads to a rapid change of pressure. According to the Bernoulli's principle, velocity and static pressure are inversely related. Thus, when fluid velocity is increased the flow restrictive element in the reactor leads to a decrease in the static pressure, which triggers the formation of cavitating flows. Therefore, a numerous of cavities grow and collapse, consequently, this emits secondary spherical shock waves.

The disruption tests were conducted using 8 L of suspended *C. reinhardtii* cells and treated for 2 min. HDC process generates a linear variation of temperature (20–60 °C), therefore, before and after HDC process, cell suspension was maintained at 4 °C using an ice bucket to avoid overheat.

2.3. Freeze-dried C. reinhardtii biomass

2 L of fresh intact *C. reinhardtii* suspensions were harvested by centrifugation (J2-21 Centrifuges, Beckman Coulter) at 5,000×*g* for 30 min and frozen at −20 °C for 24 h. HDC-disrupted *C. reinhardtii* were immediately frozen at −20 °C for 24 h (without centrifugation step). Afterwards, both samples were freeze-dried for seven days (Freeze Dryer, Super Modulyo, Edwards, UK). The approximate 30 g of the intact *C. reinhardtii* (ICR) and disrupted *C. reinhardtii* (DCR) freeze-dried biomass were weighted and then kept at −80 °C.

2.4. Carotenoids analysis

2.4.1. Carotenoids extraction

The carotenoids (*β*-carotene and lutein) were extracted from the freeze-dried samples (ICR and DCR) using a modified Folch et al. (1957). A chloroform: methanol (1.2 mL (2:1, v:v)) was added to samples (0.1 g of freeze-dried samples), and samples were then vortexed for 1 min. A sodium chloride solution (1 mL of 0.9 g/100 mL) was added and the mixture was vortexed for 1 min and then centrifuged at 3,000×*g* for 10 min at 4 °C (Thermo Jouan CR3i multifunction). After centrifugation, sample was separated into 3 layers. The lower layer is a lipid layer, which was transferred to a clean glass vessel. The process was repeated three times where lipid layer could not more be observed. All of lipid layer collected was dried using a flow of nitrogen and kept at −80 °C for carotenoids analysis.

2.4.2. High-performance liquid chromatography (HPLC)

The carotenoids (*β*-carotene and lutein) content of freeze-dried ICR and DCR were analysed by HPLC with PDA detection using an Agilent 1100 system. The dried lipid extract was dissolved in 1 mL of acetone (containing 0.1% butylated hydroxytoluene (BHT)) and filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter membrane into amber HPLC vials. The mobile phase consisted of acetonitrile, methanol, ethyl acetate containing 0.05% triethylamine (TEA)) at a flow rate of 0.5 mL/min. At the start of the run the proportions of the solvents were 95:5:0 which changed to 60:20:20 after 25 min and these latter proportions were maintained until the end of the run. Re-equilibration took 15 min. Samples (10 μL) were injected through a guard column and separated on a Waters, Nova-Pak C18 analytical column (4 μm, 3.9 × 200 mm) with the column temperature set at 22 °C. *β*-carotene and lutein were detected at a wavelength of 454 nm. The *β* -carotene and lutein contents were determined using linear equations from standard curves.

2.5. In vitro digestion

A liquid matrix was prepared by mixing 0.5 g of sample plus 4.5 g of pure water at 200 rpm at room temperature for 5 min. The bioaccessibility process followed three steps including oral, gastric, and intestinal simulation.

During oral step, 5 mL of liquid matrix was mixed with 4 mL of SSF electrolyte stock solution and minced together at 100 rpm at 37 °C for 2 min. 0.5 mL salivary α-amylase solution of 1500 U/mL made up in Simulated Salivary Fluid (SSF) (α-amylase from human saliva Type IX-A, 1000–3000 U/mg protein, Sigma) was added followed by 25 μL of 0.3 mol/L $CaCl_2$ and 0.475 mL of water and thoroughly mixed using orbital shaker incubator (SI500, Bibby Scientific Ltd, UK) at 100 rpm at 37 °C for 2 min.

Upon the completion of oral step, samples (oral bolus) were used by adding 8.0 mL of Simulated Gastric Fluid (SGF), 1.0 mL of porcine pepsin stock solution of 25 000 U/mL made up in SGF electrolyte stock

solution (pepsin from porcine gastric mucosa 3200–4500 U/mg protein, Sigma), 5 μL of 0.3 mol/L $CaCl_2$, 0.2 mL of 1 M HCl to reach pH 3.0 and 0.075 mL of water and then, were shaken at 100 rpm at 37 °C for 2 h using orbital shaker incubator (SI500, Bibby Scientific Ltd, UK).

Finally, 20 mL of samples from gastric step (gastric chyme) were mixed with 11 mL of Simulated Intestinal Fluid (SIF), 5.0 mL of a pancreatin solution, 800 U/mL made up in SIF based on trypsin activity (pancreatin from porcine pancreas, Sigma), 2.5 mL fresh bile (160 mmol/L in fresh bile), 40 μL of 0.3 mol/L $CaCL_2$, 0.15 mL of 1 mol/L NaOH to reach pH 7.0 and 1.31 mL of water. Samples were incubated using orbital shaker incubator (SI500, Bibby Scientific Ltd, UK) at 100 rpm at 37 °C for 2 h (Minekus et al., 2014).

2.6. Phase behaviour

The phase behaviour study of ICR was conducted using pre-digested, gastric and duodenal phases. 1 mL of samples were taken into eppendorfs. Two sample groups were prepared: (1) with digestive enzyme and (2) absence of digestive enzyme. All treatments were kept at room temperature and the phase behaviour were observed.

2.7. Bioaccessibility of carotenoids (β-carotene and lutein)

2.7.1. Bioaccessibility

Bioaccessibility was determined according to method describe in section 2.5.1. After all digestion steps, total digesta and micelle fractions were obtained. The micelles fraction was separated from total digesta by the end of the simulated digestion by centrifugation at 5,000×*g* for 120 min using a Rotina 380R centrifuge. A clear supernatant containing mixed micelles (micelle fractions) was separated from the total digesta. The micelle fraction was filtrated through 0.2 μm Glass Microfiber Filter (GMF) to remove unmicellised carotenoids (modified from Mieko & Delia, 2003). The filtrates containing the micellised carotenoids were quantified by HPLC.

2.7.2. Extraction of carotenoids (β-carotene and lutein) from the total digesta and micelle fractions

5 mL of total digesta and micelle fractions were used. 5 mL of chloroform: methanol (1:1, v:v) was added into samples and vortexed for 1 min. Then, 1 mL of RO water was added into samples and vortexed for 1 min. The sample were centrifuged for 10 min at 3,000 rpm at 4 °C. The extracts were repeated until the three-layer fractions appeared. The combined chloroform fraction (lowest phase) was dried using nitrogen gas. The extracted residues were redissolved in 0.7 mL of acetone plus 0.1% butylated hydroxytoluene (BHT), and filtered through a 0.45 μm GMF filter. Samples were ready for HPLC analysis.

2.7.3. Bioaccessibility and nutrient assimilation of carotenoids (β-carotene and lutein)

The bioaccessiblity index was calculated using Eq. (1):

$$B = \left(\frac{C_m}{C_t}\right) \times 100 \quad (1)$$

where, B is the bioaccessibility of microalgae (%), C_m is the concentration of the compound present in the micelle fraction (μg/g of an initial freeze-dried microalgae), C_t the concentration of the compound present in the total digesta (μg/g of an initial freeze-dried microalgae).

N*utrient assimilation* ***(NA)*** was calculated using Eq. (2):

$$NA = B \times C_i \quad (2)$$

where, NA is a nutrient assimilated (μg/g DWB), B is the bioaccessibility of microalgae (%), and C_i is the initial concentration of the compound present in a freeze-dried microalgae (μg/g DWB).

2.8. Microscope protocols

2.8.1. Ultra-structural analysis by transmission electron microscope (TEM)

The cell morphology study of ICR samples were conducted using 1 mL of pre-digested, gastric, and duodenal phases. The presence and absence of digestive enzyme were visualized due to the cell wall's integrity using TEM.

1 mL of fresh cells culture, pre-digested, gastric, and duodenal phases of the ICR were prepared using 3% glutaradehyde in 0.1 M cacodylate buffer. Samples were transferred into an Eppendorf tube and centrifuged at 4000×*g* for 4 min. Supernatant was removed and resuspended in a 1% aqueous osmium tetroxide for 1 h. Samples were double washed for 5 min using distilled water and centrifuged at 4000×*g* for 4 min for supernatant removal. Afterwards, samples were dehydrated by washing using a series of different solutions of ethanol and propylene oxide. Firstly, samples were double washed using three different ethanol solution (50%, 70% and 90% of ethanol) and left for 15 min until ethanol evaporation. Afterwards, samples were triple washed using 98% of ethanol for 20 min. Then, samples were washed using 100% of propylene oxide for 15 min. An agar Araldite resin (in the ratio of 1:3 and 1:1, resin: propylene oxide) was prepared. 1 mL of resin (1:3) was added into samples and were kept at room temperature for 3 h. Samples were then centrifuged at 13 000×*g* for removing the supernatant and 1 mL of resin (1:1) was added.

Tubes were kept overnight in the laminar flow with the lids off. Samples were triple suspended using 1 mL of pure resin for 2.5 h, and kept in the oven at 60 °C for 48 h. A thin section of the samples was cut using an ultra-microtome (Leica EM) ad diamond knife. Finally, a selected thin section of sample was visualized under the TEM.

2.8.2. Structured illumination microscopy (SIM)

1 mL of total digesta from each digestion steps (initial, oral, gastric, and intestinal) were taken to prepare for ultrastructure visualisation under SIM and confocal. Samples were stained with Nile Red (5 μg/μL) and FM4-64FX (1 μg/μL) at room temperature for 10 min. 20 μL polyvinylpyrrolidone (PVP1A, Citifluor) and 10 μL of cell suspension was mounted on a slide using a high precision cover slip (Zeiss, Cat 1,5H, Cat. No. 474030-9010-000). Visualisation was performed 15 min after the sample preparation.

SIM imaging: Zeiss Elyra PS1 was used to visualize the stained ICR and DCR freeze-dried biomass using the hardware and settings: objective Plan-Apochromat 63x/1.4 Oil DIC M27; for Nile red and FM4-64FX band pass filter: BP 570–620 + LP 750, Laser 561 nm: 4.0%. Chloroplast: long-pass filter LP 655, Laser 642 nm: 5.0%, SIM grating period 34.0 μm. Chloroplast's visualisation was difficult because they were too bright, therefore, the imaging of the FM4-64FX was better visualized using the red channel for chloroplast visualisation. Processing was done using Zen2 Black Edition, with SIM parameters set to auto noise, sectioning at 100/83/83 and original recorded intensity values maintained (Raw Scale set on).

2.9. Statistical analysis

All data was subjected to the normality and homoscedasticity tests and as a result did not show significance. Therefore, the hypothesis Ho of normality of the data and homoscedasticity of variance were accepted. In order to identify significant differences, all the obtained data was subjected to one-way ANOVA and grouping was made according to Tukey's test. These analyses were made employing Minitab V.17 statistical package® and Microsoft excel®. A value of ($p < 0.05$) considered as statistically significant.

3. Results

A digestion process (oral, gastric, intestine) was conducted to evaluate the influence of digestive enzymes on the ICR. Therefore, a

comparison between a control condition (in an absence of digestive enzymes, CC)) and a real digestive condition (in a presence of digestive enzymes, RC) were evaluated using TEM images of ICR throughout the digestion steps (oral, gastric, intestine); and the pigment release from the total digesta of both samples (CC and RC) were analysed. Afterwards, HDC technique was applied to disrupt the microalgae cell wall. Thus, the composition of carotenoids (β-carotene and lutein) of starting freeze-dried biomass, bioaccessibility, and nutrient assimilable of the ICR and disrupted DCR were evaluated. Finally, the comparison of assessing carotenoids (β-carotene and lutein) from ICR and DCR by HDC process and its bioaccessibility was provided.

3.1. The changes on cell wall morphology and phase behaviour of ICR

3.1.1. Changing of cell morphology and the phase behaviour

The changing of cell wall morphology of *C. reinhardtii* during *in vitro* digestion was applied using two digestion conditions: First, a control condition (CC) where *in vitro* digestion was evaluated without using digestive enzymes; and second, a real digestive condition (RC) where *in vitro* digestion was evaluated using digestive enzymes (α-amylase, pepsin and pancreatin). Fig. 1 presents TEM imaging of the total digesta, which confirms that CC retained the oval shape of the cells with the cell wall (CW), cell membrane layer (CM) and the thylakoid membrane (TY) stack overshadowing most of the other organelles after the oral and stomach phases. In contrast, RC showed morphological changes, especially, during the stomach and intestinal phase. The digestion of CW and CM were continued through oral and stomach stage. The enzyme may digest a part of outlier and central triplet layer but inner most layer is still untouched even though the cell shape has changed. Moreover, at the small intestine stage, TY and LD are still intact.

Particulate/membrane structures were retained to some extent, but this qualitative data suggests that a measurable proportion of the solid material has been digested, and that the ordered structure of the thylakoid membranes was lost (Fig. 1). Furthermore, phase behaviour and phase separation of the *C. reinhardtii* were observed. During *in vitro* digestion, CC and RC presented its phase behaviour in oral digestion. However, a phase separation was observed only on CC sample during the intestinal digestion, on the other hand, RC samples was observed as a homogenous suspension due to the digestive enzyme presence. As expected, the digestive enzyme digested the cell membrane layer and the releasing of the intracellular compound into the suspension were emulsified with bile salt (Fig. 1). Thus, a colour changes from dark green to yellowish brown colour was observed, which was expected due to as the addition of bile salts in the intestinal phase (Fig. 1). Finally, CC and RC presented different pigment release because of the influence of digestive enzymes, which generated a change of cell structure and shape (Fig. 2). As expected, CC presented higher concentration of β-carotene and lutein content than RC.

3.2. Assessing carotenoids (β-carotene and lutein) from ICR and DCR

Bioactive compounds (including carotenoids) play a part important of biological activities such as bioavailability process, pro-vitamin A activity, regulation of gene transcription and antioxidant activity (Ambati et al. 2014). Because of all cited biological activities, several researchers have been searching for new sources of natural pigment, which can be applied in food industry. Among all industrial/consumption challenges such identifying, adding, and accessing food

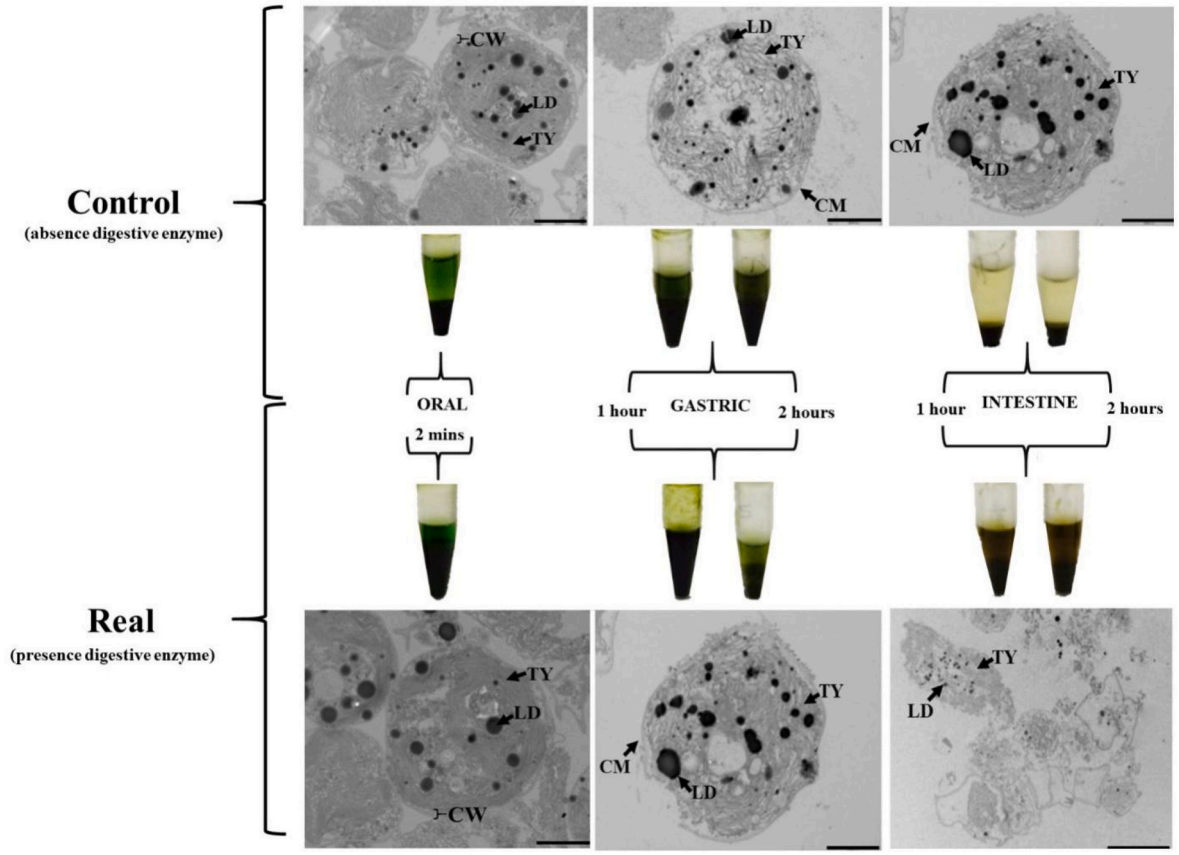

Fig. 1. TEM images of the intact *C. reinhardtii* (ICR) throughout the digestion steps (oral, gastric, intestine). A control sample represents the ICR passing through the digestion steps (oral, gastric, intestine) in an absence of digestive enzymes, and a real sample represents the ICR passing through the digestion steps (oral, gastric, intestine) with all digestive enzymes present. Both treatments were conducted with the same pH, temperature and shaking. CW: Cell Wall, CM: Cell Membrane, TY: Thylakoid Membrane, LD: Lipid Droplet. Scale bars = 2000 nm.

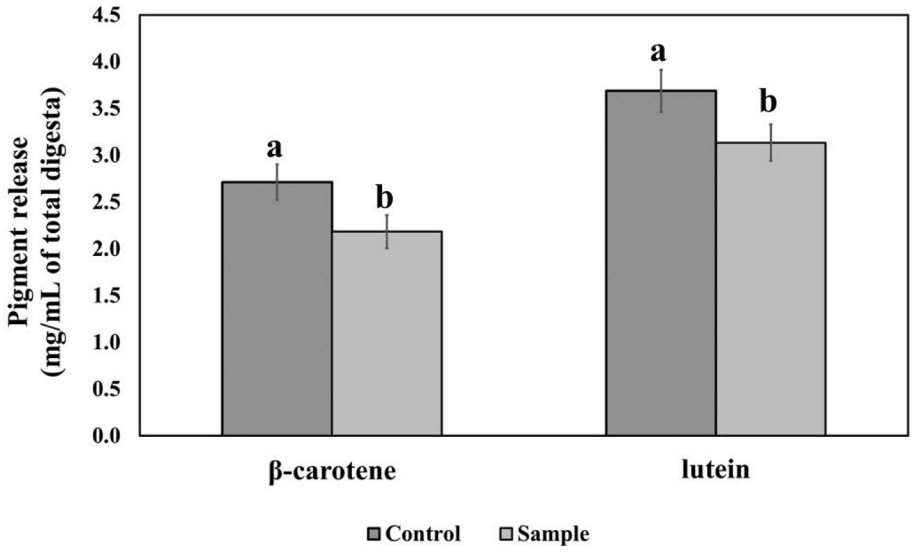

Fig. 2. The pigment release (β-carotene and lutein) from a control and a real *in vitro* digestion. A control sample represents the intact *C. reinhardtii* (ICR) passing through the digestion steps (oral, gastric, intestine) in an absence of digestive enzymes, and a real sample represents the ICR passing through the digestion steps (oral, gastric, intestine) with all digestive enzymes present. All data is present: average of concentration, Tukey test and standard deviation. The lower-case letters indicate the significant differences ($p < 0.05$). Values not sharing the same letter are significantly different according to the Tukey's test.

compounds in the food matrix, the use of pigments and the understanding of how to release all these compounds from the food matrix to its absorption is a key (Benlloch-Tinoco et al. 2015). Lately, several new sources of chlorophylls and carotenoids have been studied and microalgae is still a promising source of several nutrients. *C. reinhardtii* contains a good source of carotenoids (β–carotene and lutein) (Table 1); however, the concentration of carotenoids (β–carotene and lutein) from microalgae can change according to species/strain, medium composition, temperature, growth condition, and mainly different light regimes (intensity, photoperiod, and wavelengths) (Maroneze et al. 2019). In the studied condition, ICR and DCR contain similar β-carotene concentration; on the other hand, ICR (2.8 mg/g) and DCR (2.6 mg/g) differ its lutein concentration ($p < 0.05$) (Table 1). Therefore, HDC affected the initial concentration of lutein.

3.3. Bioaccessibility of carotenoids (β-carotene and lutein)

Results showed that ICR (43.8%) and DCR (37.4%) differ its bioaccessibility of carotenoids ($p < 0.05$). These significant differences are driven by β-carotene was less accessible in DCR (3.3%) than ICR (9.1%) ($p < 0.05$) because of the bioaccessibility of lutein in both samples were similar in ICR (34.7%) and DCR (34.1%) (Table 1). Carotenoids are also sensitive to temperature, enzymes and pH (Gille et al., 2016). Therefore, the reduction of β-carotene bioaccessible occurred because carotenoids are highly stable within their natural plant cell environment; however, once isolated, they are sensitive to situations exposing them to light, heat, and acids, which promotes *cis*-trans isomerisation. In addition, plant tissue contains lipoxygenase, which catalyses carotenoid oxidation (Ball, 1995).

Vitamin A intakes or requirements are generally expressed in terms of retinol equivalent (RE). The Recommended Dietary Allowance (RDA) for vitamin A is 1,000 RE/day for men and 800 RE/day for women. An additional 500 RE/day is recommended during the first six months of lactation and a 400 RE/day increment for the second six months (National Research Council, 1989). Therefore, fortifying 10 g of microalgae into food would provide 0.2 mg of β-carotene assimilation (Table 1). RDA for lutein has been established. Some studies have reported the benefit of lutein for age-related macular degeneration (AMD) patients (Ma et al., 2012; Weigert et al., 2011). Therefore, consuming 10 g of freeze-dried *C. reinhardtii* provide 9.6 mg of lutein assimilation (Table 1), which approaches the daily recommendation (10–20 mg/day).

3.4. Structural change of IMC and DMC during in vitro digestion

Cell walls and chloroplast are the two primary physical barriers to nutrients release, mainly carotenoids, from the food matrix (structure) during digestion (Jeffrey & Hallegraeff, 1987). Consequently, it is crucial to an understanding of both physical and chemical changes of *C. reinhardtii* during the digestive process to clarify their nutrient bioaccessibility (Table 1). Cell walls and chromoplasts are the primary physical barriers to nutrients release, mainly carotenoids, from the food matrix (structure) during digestion (Jeffery et al., 2012). Consequently, it is crucial for an understanding of both physical and chemical changes of *C. reinhardtii* during the digestive process to clarify their nutrient bioaccessibility. To provide an indication of assessing nutrients from ICR and DCR via *in vitro* digestion, a structured illumination microscopy (SIM) was used to observe the ICR and DCR cell through the *in vitro* human digestion (Fig. 3). Fig. 3A and E illustrates ICR and DCR at the initial stage. It can be observed some differences between them and also the most pro-eminent red colour on Fig. 3E than Fig. 3A. This is an indication of nutrient release from the cells by HPRGs cell broken apart. Moreover, in Fig. 3A, the shape of chloroplast cells (green colour) was easily observed, in contrast, ICR (Fig. 3E) showed an overlap, which

Table 1
The composition of carotenoids (β-carotene and Lutein) of starting freeze-dried biomass, bioaccessibility, and nutrient assimilable of the intact *C. reinhardtii* (ICR) and disrupted *C. reinhardtii* (DCR).

	Starting freeze-dried *C. reinhardtii* (mg/g)		Bioaccessibility (%)		Nutrient Assimilable (mg/g)	
	ICR	DCR	ICR	DCR	ICR	DCR
β-carotene	$0.22^{a}\pm0.01$	$0.22^{a}\pm0.00$	$9.1^{a}\pm2.8$	$3.3^{b}\pm0.2$	$0.02^{a}\pm0.01$	$0.01^{b}\pm0.00$
Lutein	$2.8^{a}\pm0.1$	$2.6^{b}\pm0.0$	$34.7^{a}\pm3.6$	$34.1^{a}\pm8.6$	$0.96^{a}\pm0.10$	$0.9^{a}\pm0.2$
Total (β-carotene + Lutein)	$3.02^{a}\pm0.5$	$2.8^{a}\pm0.0$	$43.8^{a}\pm3.3$	$37.4^{b}\pm4.4$	$0.98^{a}\pm0.10$	$0.9^{a}\pm0.2$

All data is present: average of concentration, Tukey's test and standard deviation. The lower-case letters on the same row, which is not sharing a same letter indicate significant differences ($p < 0.05$) within the data set.

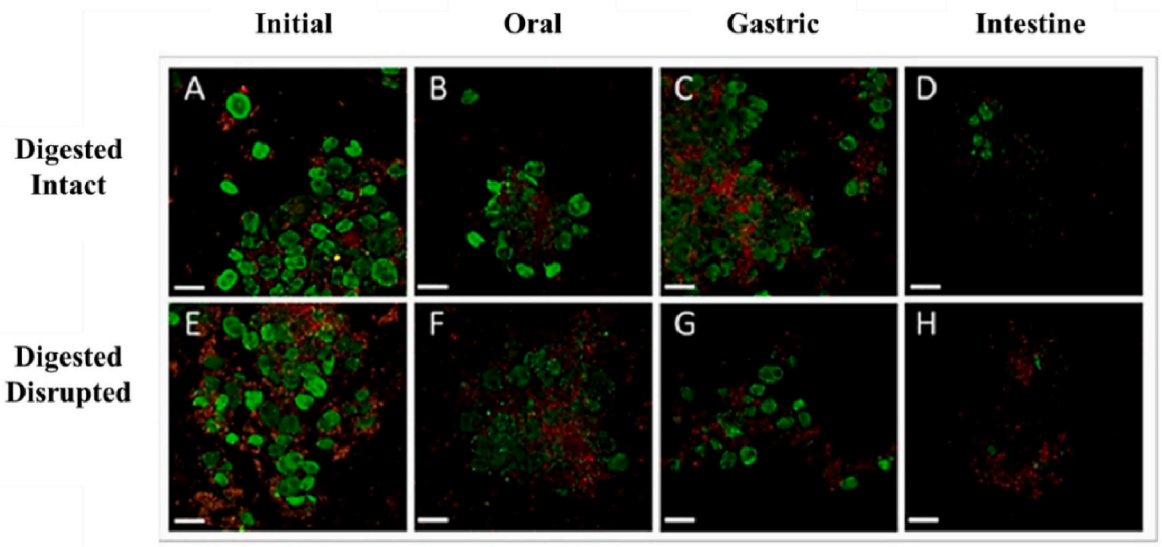

Fig. 3. Particulate material from the intact *C.reinhardtii* (ICR) and disrupted *C.reinhardtii* (DCR) at different stages during simulation of digestion. Cell membrane and lipid droplets ICR (A–D) and disrupted DCR (E–H) during each stage of the *in vitro* digestion model; initial (A&E), oral (B&F), stomach (C&G) and intestine (D&H). Images were visualized by structured illumination microscopy (SIM). *C. reinhardtii* labelled for cell membrane and lipid (red; Nile Red and FM4 - 64FX) and chloroplast (green). Scale bars = 10 μm.

represents a hard shape of some cells. From Fig. 3B–D illustrate the *in vitro* digestion of ICR. Comparing to Fig. 3A and B displays less red colour points, which may be due to the nutrient absorption in mouth. Fig. 3C showed the red colour returned, which may be occurred due to highest cell break down by gastric juice activity on stomach. Then, in Fig. 3D red colour (nutrients and HPRGs cell wall) and also green colour (chloroplast of microalgae) were less observed.

From Fig. 3E–H illustrate the *in vitro* digestion of DCR. Comparing to Fig. 3E and F displays a considerable quantity of red colour points although it is still less than Fig. 3E. Therefore, HDC increased the nutrient release and HPRG cell wall was disrupted in mouth (Fig. 3E and F). Fig. 3G showed more prominent presence of chloroplast cell (green colour) and a similar red colour points compared to Fig. 3F, which advocates to a high break down by gastric juice activity on stomach. Then, in Fig. 3H illustrates a small quantity of nutrient release and HPRG cell wall (red colour) and small quantity of microalgae's chloroplast (green colour) was observed. Therefore, 37.4% of carotenoids present in ICR were absorbed in the small intestine (Fig. 3). In conclusion, breaking down *C. reinhardtii* cell wall using HDC decreased the bioaccessibility of carotenoids during small intestine step and may facilitate its accessing for chemical industries.

This study has added a more brink to the knowledge of microalgae and hydrodynamic cavitation as a technique to assess microalgae nutrients, which can be particularly useful for food industry for a rapid accessing of microalgae compounds. Basically, intact microalgae have 10% of its β-carotene absorption during intestine step; and disrupted microalgae has a lower β-carotene absorption (3.3%) during intestine step, which may happen because β-carotene could be absorbed on the mouth mucosa or stomach mucosa. Further studies are needed. However, this information can be useful for delivering nutrient as medicine to patients through oral-gastro-intestinal systems because they may need to be encapsulated to guarantee its absorption in the intestine. In contrast, in the future, chemical companies may use our findings for assessing microalgae compounds as a pre-processing of purification. Our findings are important and the application of HDC breaks a paradigm by making easier the access of microalgae compounds; however, they are preliminary, and, as a consequence, several future works are needed. The study of bioaccessibility of other compounds from microalgae such as lipids, carbohydrates, amino acids using HDC is needed. Likewise, the quantification of compounds lost in the mouth and stomach is necessary.

4. Conclusions

The comparison of assessing carotenoids (β-carotene and lutein) from ICR and DCR by HDC process and its bioaccessibility was studied. The cell morphology using TEM and SRM did not show any the structural changes on the microalgae cell wall caused by in an absence of digestive enzymes during *in vitro* digestion. In a presence of digestive enzymes was observed significant changes on the microalgae cell wall. Digestive enzymes cannot fully break down *C. reinhardtii* cell wall. HDC generates significant changes on *C. reinhardtii* cell wall. DCR showed a lower degree of lipophilic pigmentation (β-carotene and lutein) than ICR during the *in vitro* digestion. The sum of carotenoids (β-carotene + lutein) bioaccessibility from ICR (43.8%) and DCR (37.4%) are significant different. HDC process decreased the amount of β-carotene retained in the cell structure and the biocessibility of β-carotene in the small intestine from ICR (9.1%) and DCR (3.3%) decreased. The bioaccessinility of Lutein content from ICR (34.7%) and DCR (34.1%) are similar. The HDC process together with the acid-liability of the pigment during the gastric phase may cause nutrient lost. HDC process can be beneficial for accessing carotenoids content such as β-carotene and lutein for bioacessibility and for industrial uses, which should be studied.

CRediT authorship contribution statement

Patchaniya Akepach: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Normando Ribeiro-Filho:** Conceptualization, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. **Jutarat Wattanakul:** Formal analysis, Writing – original draft, Writing – review & editing. **Randa Darwish:** Formal analysis, Writing – original draft, Writing – review & editing. **Mohamed A. Gedi:** Formal analysis, Writing – original draft, Writing – review & editing. **David A. Gray:** Funding acquisition, Supervision, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that there is no known competing financial

interest or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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