Semi-dynamic in vitro digestion of honey chlorella vulgaris reveals biochemical and structural insights during gastro-intestinal transit

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Abstract

Concerns about current food systems have prompted increased exploration of sustainable alternative protein sources, such as microalgae. This study investigated honey *Chlorella vulgaris*, a chlorophyll-deficient mutant, distinguished by its consumer-friendly honey colour, milder flavour and improved texture. To facilitate the nutritional transition towards this source, a standardised *in vitro* semi-dynamic INFOGEST digestion model was employed to analyse the digestive behaviour of *C. vulgaris*, focusing on the biochemical and structural changes during *in vitro* digestion. Gastric digestion was conducted over 67.5 min with dynamic fluid addition and gastric emptying. Results indicated slow gastric digestion of *C. vulgaris* due to the initially low pepsin activity and low protein solubility. Significant protein breakdown commenced when the pH dropped to 3.5. By the end of the gastric phase, 11.8 % of the protein and 3.0 % of free amine groups were released, generating new peptides of 0.3-1 kDa. Followed by 2h static intestinal digestion, some cell structures remained intact, indicating a barrier to nutrient release. Pancreatic enzymes caused substantial protein hydrolysis, generating a higher fraction of 0.1-0.3 kDa peptides, with a notable release of essential amino acids as well as phenolic compounds.

This study highlighted that protein insolubility and the cell wall structure of *C. vulgaris* may impede enzyme effectiveness, leading to a reduced protein breakdown. Furthermore, introduction of

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processing steps may enhance bioaccessibility in microalgae-derived foods, thereby contributing to the development of nutritional and sustainable food productions.

Key Words

Chlorella vulgaris; Microalgae; Protein; INFOGEST; *in vitro* digestion

Abbreviations

GE, gastric emptying; EAAs, essential amino acids; (e)SSF, (electrolyte) simulated salivary fluid; (e)SGF, (electrolyte) simulated gastric fluid; (e)SIF, (electrolyte) simulated intestinal fluid; CLSM, confocal laser scanning microscopy; TCA, trichloroacetic acid; BCA, bicinchoninic acid; OPA, o-phthaldialdehyde; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; HPLC, high-performance liquid chromatography; AUC, area under the curve; TPC, total phenolic compounds; GEA, gallic acid equivalent; HPH, high-pressure homogenization; PEF, pulsed electric field.

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1 Introduction

Considering the environmental challenges and rising protein demand, a transition from animal-based to alternative protein sources has become imperative. Traditional livestock production adversely affects greenhouse gas emissions, deforestation, terrestrial acidification, eutrophication and freshwater withdrawals . Furthermore, the COVID-19 pandemic, coupled with the ongoing global conflicts, has strained global food security. Therefore, various alternative protein sources have been proposed for research investigation to ease environmental degradation while promoting human health.

Desirable alternative proteins should minimise environmental impact while maximising protein yield and quality to meet the growing demands. Microalgae is one of these promising sources of alternative protein. As a single cellular marine organism, microalgae excel in their fast-growing rates and high protein content. They utilise solar energy to convert CO_2 and nitrogen compounds into biomass and require minimal land use for cultivation . Several microalgae, such as *Chlorella vulgaris*, *Arthrospira platensis*, *Auxenochlorella protothecoides*, and *Dunaliella bardawill*, were authorised as food in the EU before 1997 and classified as Generally Recognized As Safe (GRAS) status by the Food and Drug Administration (FDA) . Among these, *C. vulgaris* has gained wide research attention due to its long-standing history in human diets, early commercial applications, and excellent nutritional profile . *C. vulgaris* has 51-58 % protein on a dry weight basis and offers a complete profile of essential amino acids (EAAs) . In addition to its pronounced protein profile, *Chlorella* contains various phenolic compounds, such as caffeic acid, ferulic acid, p-coumaric acid, etc. . These compounds exhibit antioxidant activity and potentially hepatoprotective activity highlighting their potential application in the development of functional foods or dietary supplements . Although *Arthrospira platensis* contains higher protein content than *C. vulgaris*, *C. vulgaris* is notably richer in various vitamins and minerals . Furthermore, *Chlorella* was reported to have better techno-functional properties (higher solubility, higher oil absorption capacity and stronger foaming capacity and

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stability than other two microalgae species, which enhance its versatility in food applications . Overall, *C. vulgaris* is widely regarded as a prospective candidate for facilitating a dietary shift.

Although *C. vulgaris* have been incorporated into various food systems, such as bakery products and beverages, challenges remain in achieving consumer acceptance. Obstacles are associated with unfavourable sensory properties of aversive green colour, fishy taste and odour . A random chemical-induced chlorophyll-deficient mutant, known as honey *C.vulgairs* due to its cream yellow colour, exhibits milder taste and odour, improved texture and higher protein content than its wild-type counterpart . Consequently, the improved properties of honey *C. vulgaris* create greater opportunities to develop market-viable products and support the transition to sustainable and nutritional protein alternatives.

To ensure a successful protein shift for consumers, it is essential to study the digestion pathway of microalgae, as it could be a new substrate introduced to the human body. Undoubtedly, *in vivo* methods, such as human and pig trials, are considered as the golden standard for digestion studies. However, these studies are often limited by cost, time and ethical concerns . Furthermore, in accordance with the principles of the 3Rs, Replacement, Reduction and Refinement, there is an emphasis on minimising animal research. Consequently, *in vitro* models have become practical alternatives for assessing nutrient quality.

Currently, the *in vitro* static INFOGEST method is the most commonly used method . Several preliminary studies have investigated the digestibility of *Chlorella* using this method and revealed a relatively low degree of digestion (42-51 %) . However, this method could only replicate the endpoint of the chemical process but was unable to capture the complexity of the dynamic chemical and physiological changes involved. Consequently, it leaves a gap in understanding the digestive behaviour of the substrate including how its structural and physical properties influence the breakdown of nutrients in the digestive tract. To address these limitations, a standardised semi-dynamic *in vitro* method was developed to study the physiological relevance and the food structure

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variation in digestion , Currently, this model has been applied to study the digestive behaviour of dairy and plants proteins , but not yet to microalgae protein. To address this gap, a honey *C. vulgaris* suspension with 4 % protein (w/w) was selected to understand the digestive behaviour of this substrate using a semi-dynamic *in vitro* digestion method. This suspension was chosen to match the approximate protein content of bovine milk, as a typical nutritional beverage, and to eliminate the effects of additives and processing methods associated with food production. This study aims to explore the digestive behaviour of microalgae protein and the release of polyphenols, thereby evaluating *C. vulgaris* as a viable substitute for traditional proteins. This research is anticipated to provide crucial information towards consumers for nutrition consideration, and for industry bodies to guide the manufacturing practice in manipulating digestive behaviour for microalgae food production.

2 Materials and methods

2.1 Materials

The spray-dried biomass of honey *C. vulgaris* was supplied by Allmicroalgae, Natural Products S.A. All chemicals, reagents, and enzymes for simulated digestion, including pepsin from porcine gastric mucosa (lot SLBV3776), pancreatin from porcine pancreas (lot SLCM8903) and bile bovine (lot SLBV1780) were analytical grade and purchased from Sigma-Aldrich (Dublin, Ireland). Milli-Q water (resistivity 18.2 MO, Purelab® Flex 1, Germany) was used to prepare all reagents and samples.

2.2 Food characterisation

2.2.1 Dry matter content

The dry matter content was analysed in duplicate by a Leco TGA701 gravimetric oven (Leco Instruments UK Ltd., Cheshire, UK). Around 0.8 g of microalgae powder placed in the crucible was

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heated to 102 ℃ and maintained for 4h to determine the moisture content. The dry matter content was determined by subtracting the weight loss percentage during heating.

2.2.2 Protein content

The protein contents of the biomass and digested sample in section 2.3 were determined in duplicate using a LECO FP628 nitrogen analyser (LECO Corporation, UK). A specific nitrogen-to-protein conversion factor (4.78) was determined for microalgae to convert the nitrogen percentage into protein content (Lourenço et al., 2004).

2.2.3 Protein solubility

Microalgae suspension (4 % protein, w/w) was prepared by overnight hydration in dH_2O. The same composition of electrolyte simulated salivary fluid (eSSF) and electrolyte simulated gastric fluid (eSGF) (without enzyme), as described in section 2.3, was added to the suspension. The solubility test was conducted within the pH range of digestion by adjusting the pH 2-7 using 1M HCl or 1M NaOH. The concentration was diluted to precisely 0.97 % w/w protein by dH_2O, the theoretical protein concentration at the end of the gastric phase. Each tube was gently stirred in an overhead rotator (Stuart rotator SB3, Stuart Equipment, Cole-Parmer, UK) for 90 min at ambient temperature before centrifuging (Eppendorf centrifuge, 5417 R) at 5,000 × *g* for 15 min. The soluble fractions (supernatants) were separated and further analysed for protein content using a Leco nitrogen analyser. The protein solubility (%) was determined by the protein content of the soluble fraction over the protein content in the initial suspension.

2.3 Semi-dynamic *in vitro* digestion

Microalgae suspensions (4 % protein, w/w) were hydrated overnight and digested following a standardised semi-dynamic *in vitro* digestion method to evaluate the physiochemical changes during gastrointestinal digestion .

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The digestive fluids were prepared following the semi-dynamic *in vitro* digestion method , and the enzymatic activities were assessed according to the INFOGEST 2.0 protocol . The pepsin and pancreatin activity were 2997 Sigma pepsin units/mg and 8.9 (TAME) U/mg solid respectively. Bile was estimated to have 2.27 mmol bile salts per g of bile. The oral phase started by mixing a 1:1 ratio with eSSF (dry weight of food:eSSF volume) for 2 min at 37 ℃.

The gastric emptying time was deliberated by the caloric profile of the food. The caloric profile (Table 1) was calculated by the average of 5 commercial protein drinks to project the practical application of protein drinks fortified with microalgae. Following the gastric emptying rate of 2 kcal/ min, the total gastric digestion time was 67.5 min, involving five gastric emptying (GE) intervals. Prior pH tests were conducted, and the optimal eSGF pH was obtained to achieve the final GE pH of 2, which was 0.097 M HCl at pH 1.40.

Table 1. Nutritional profiles of 5 commercial protein drinks and C. vulgaris suspension

Nutritional profile (250mL per serving)	Tesco Fresh Milk*	The Original Oatly Whole Oat Long Life Drink*	Alpro This is Not M*lk Whole Chilled Oat Drink*	Alpro Soya Original Long Life Dairy Free Drink*	Alpro Soya Vanilla Original Long Life Dairy Free Drink*	*C. vulgaris* suspension (4 % protein, w/w)
Fat (g)	8.8	7.0	8.8	4.8	4.5	3.8
Carbohydrates (g)	9.4	16.5	16.0	6.5	16.8	15.3
Protein (g)	8.5	1.0	1.8	8.3	7.5	10.0
Energy (kcal)	150.4	133.0	149.8	101.8	137.5	135.0

**The nutritional information was sourced from the numbers listed on the food labels.*

Gastric digestion is carried out in a v-shaped vessel with a 37 ℃ water bath circulating its exterior space. An overhead stirrer (Ingenieurbüro CAT, Germany) fitted with a 3D-printed stirrer head was employed for agitation at 18 rpm. To mimic the physiological condition of an empty gut, the digestive system was started with 10 % of the SGF and pepsin. Subsequently, pepsin (4,000 U/mL) was added by the syringe pump (KD Scientific, USA) at 0.0151 mL/min, while eSGF was added by titrator (Metrohm, UK) at a rate of 0.2868 mL/min. Aliquots of 8 mL digesta were taken from the bottom of the vessel by the approximate 3 mm diameter pipette every 13.5 min. After the digesta

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collection, 1 M NaOH was added to reach pH 7 to inhibit pepsin activity. Each gastric emptying digesta followed 2h static intestinal digestion . The SIF was added to the sample with pancreatin (200 trypsin U/ml SIF) and bovine bile (20 mM). The reaction happened with mixing by rotator and incubating (Binder BF056 Incubator, BINDER GmbH, Germany) at 37 ℃ for 2h. To stop the protease activity, 0.1 M Pefabloc® SC (5 mM final concentration) were added into the digesta, followed by snap frozen using liquid nitrogen, and stored at -20 ℃ for further analysis. Protein-free cookies digestion and water digestion were performed as blank digestion for different experimental purposes.

2.4 Particle size distribution

To evaluate the changes of microalgae cell aggregations during digestion, particle size distribution of digested microalgae samples was performed by laser diffraction in a Mastersizer 3000 with a Hydro MV accessory (Malvern Instruments, UK). The measurements were conducted with a refractive index value of 1.47 for microalgae and 1.31 for water dispersants . $D_{[3,4]}$ value was used to evaluate the volume mean diameter. Despite triplicate measurements, a consistent decreasing trend led to using only the first measurement of each sample for analysis, as it was the most representative of the digesta.

2.5 Confocal Laser Scanning Microscopy

To visualise the cell structure throughout the digestion process and to detect any cell disaggregation or debris, Confocal Laser Scanning Microscopy (CLSM) (Leica TCS SP5, Leica Microsystems CMS GmbH, Germany) was employed. Three dyes were mixed in advance with the ratio of 1:2:2 by Calcofluor White 1:20 water, 1 % Fast Green FCF (w/v) and 0.02 % Nile Red in 1.2 propanediol buffer (w/v). Aliquots of samples were mixed with the dyes in a 2:1 volume ratio. Fast Green is an anionic triphenylmethane dye which stains net positive charge protein and the arginine group, where it can be used to detect the presence of protein ; Calcofluor White is believed to react with β-D-glucopyranose polysaccharide (cellulose) where it can indicate the *Chlorella* cell wall ; Nile Red as a

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benzophenoxazone dye could interact with the hydrophobic environments where it was used to detect the fat droplets in *C. vulgaris* cell . Fast Green FCF, Nile Red and Calcofluor White were excited at 633 nm, 488 nm and 405 nm, respectively, and the emission wavelengths were 695-765 nm, 560-627 nm and 440-480 nm, respectively. Images were taken using a 20× oil immersion objective.

2.6 Bioaccessible fraction

To quantify the bioaccessible protein released from the matrix, 5 % trichloroacetic acid (TCA) was added to the digested aliquots to reach the final concentration of 3.2 % TCA and centrifuged at 10,000 × *g* at 25˚C for 30 min.

The protein released from the food matrix into the bioaccessible fraction was quantified using a Bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Ireland). Briefly, 25 µL aliquots of supernatant were mixed with 200 µL BCA working solution in the 96-well microplate and incubated for 30 min at 37 ˚C. The absorbance was measured at 562 nm, and the calibration curve was established using bovine serum albumin (0-2000 µg/mL). Cumulated protein release (%) was calculated by the bioaccessible fraction's protein content over the corresponding digesta protein content.

The free amine group in the bioaccessible protein fraction of each digesta and the total amine group from the undigested substrate were determined by the o-phthalaldehyde (OPA) spectrophotometric assay . The total amino group in the substrate was released by acid hydrolysis of 5 mg sample with 4 mL hydrolysation volume (1040 µL water, 480 µL 1 % DPP, 480 µL 0.2 M HCl and 2000 µL 37 % HCl) at 110 ˚C for 24h. Hydrolysed samples were filtered by a 0.45 µm PTFE filter (VWR International, USA) for further quantification.

The OPA reagents were prepared from 12.5 mL of 0.1 mol/L borate solution, 2.5 mL of 10 % SDS-solution, 0.5 mL of 40 g/L OPA-solution in ethanol, 0.5 mL of 200 g/L Na-MES solution, 1.25 mL 100 g/L Triton X-100 solution and titrated to 25 mL with MQ-H_2O. The standard curve was performed

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with 0-8 mmol/L L-glutamic acid solution. The sample (8 µL) and OPA reagent (232 µL) were added to the microplate and incubated for 10 min at 30 ˚C before measurement at 340 nm. The equation below calculated the bioaccessible protein of each gastric emptying point.

$$Cumulated\ degree\ of\ hydrolysis\ (\%) = i \quad \text{Equation 1}$$

C_{Fx} - free amine group concentration (mmol/L) of each GE point during food digestion

C_{Bx}- free amine group concentration (mmol/L) of each GE point during cookies blank digestion

$C_{hydrolysed}$-*free* amine group concentration (mmol/L) of the acid hydrolysed food sample

N_{Fx}- nitrogen concentration (%) of each GE point during food digestion

N_{Bx} - nitrogen concentration (%) of each GE point during cookies blank digestion

$N_{hydrolysed}$ - nitrogen concentration (%) of the acid hydrolysed food sample

2.7 Protein size distribution

Protein hydrolysis during digestion was evaluated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to examine changes in protein molecular weight. Each sample, diluted to 5 mg protein/mL, was mixed with MQ-H_2O, NuPAGE® LDS Sample Buffer and NuPAGE® Reducing Agent (Thermofisher, Waltham, MA, USA) in the ratio of 3:3.5:2.5:1. The mixture was heated at 90˚C for 5 min and cooled down on ice. Subsequently, 10 µL samples were loaded into the well of the NuPAGE® SDS-PAGE Bis-Tris gels (4–12 %). The separation was run at 150 V for 100 min with the running buffer NuPAGE® MES. Protein bands were stained by InstantBlue Coomassie Protein Stain overnight and washed with water.

2.8 Peptide molecular weight distribution

Size exclusion chromatography (SEC) was performed to estimate the peptide profile of the undigested sample, gastric and intestinal digesta to access protein hydrolysis. Digesta was diluted to

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0.25 % protein dispersion and filtered by 0.2 µm PES membrane (Agilent Technologies, Ireland). TSKgel G2000SWXL column was equipped with a Waters 2695 high-performance liquid chromatography (HPLC) with UV/Vis detector monitored by software EMPOWER®. The injection volume was 10 µL and isocratically eluted with the mobile phase (30 % v/v acetonitrile, 0.1 % trifluoroacetic acid buffer at a 0.5 mL/min flow. The elution lasted 70 min per sample, and the absorbance was detected at 214 and 220 nm. The calibration curves were drawn by the elution time of the standards: bovine serum albumin (67 kDa), carbonic anhydrase equine (29 kDa), β-lactoglobulin (18.4 kDa), bacitracin (1.4 kDa) and glycine (0.075 kDa).

To correct the permeability of the dispersion from the filter, the BCA protein assay evaluated the protein content of the permeate (soluble protein). The soluble fraction (%) was determined by the protein content in the permeate over the protein content before filtration.

2.9 Free amino acid profile

Free amino acid analysis was conducted to evaluate the hydrolysis pattern of digestive enzymes over the simulated gastro-intestinal digestion. Undigested sample, gastric digesta and intestinal digesta were each mixed with 24 % (w/v) TCA in a 1:1 ratio. After standing for 10 min, the mixture was centrifuged at 14,400 × *g* for 10 min. The supernatant was collected and diluted with 0.2 M sodium citrate buffer at pH 2.2 to adjust the concentration of amino acid residues to 250 nmol in each sample. Norleucine, at 125 nmol/mL, was used as an internal standard and added to the samples accordingly. The samples were analysed using a JEOL JLC-500/V Amino Tac amino acid analyser fitted with a JEOL Na^+ high-performance cation-exchange column (JEOL Ltd., UK).

2.10 Total phenolic compounds

The quantification of total phenolic compounds (TPC) was analysed in the supernatant of digesta centrifuged at 3,000 × g for 10 min and was performed to assess the polyphenols released from the substrate using the Folin-Ciocalteu method . Briefly, 15 µL of supernatant was mixed with 75 µL of

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Folin-Ciocalteu reagent (diluted 1:10 in water) in the 96-well plate. After 5 min incubation, 60 µL of 7.5 % Na_2CO_3 was added to each well. The plate was incubated at 45 ˚C for 30 min in the dark. Absorbance was measured at 765 nm. A standard curve was performed using gallic acid (25-600 µg/mL). Results were expressed in µg of Gallic Acid Equivalent (GEA) relative to g of sample.

2.10 Statistical analysis

Experiments were conducted and reported in independent triplicate. One set of SEC data was defined as an outliner using the z-score outlier removal method (cut-off value of 3). Statistical analysis was performed with IBM SPSS v22 (Armonk, IBM Corp, New York, USA). The progress of digestion (Undigested sample, Gastric 1-5, Intestinal 1-5 digesta) and the analytic results of the digesta (protein content, BCA and OPA assay, TPC, EAA/NEAA ratio) were defined as the independent and dependent variable respectively. The normality of the dependent variables of each factor was assessed using the Shapiro-Wilk test. The majority of the data set conformed to normal distribution, except 4 data points, which failed across the whole sample sets. Considering the small sample sizes (n=3), a robust normal distribution was assumed to perform the parametric test. The homogeneity of variance was checked by Levene's test. To access significant differences, data which had homogenous variance was performed using one-way analysis of variance (ANOVA), followed by Tukey's posy-hoc test ($p<0.05$). For the data set that violated the assumption of homogeneity of variance, the Welch test was performed, followed the Games-Howell post-hoc test ($p<0.05$).

3 Results and discussion

3.1 Food characterisation

3.1.1 Relative protein solubility

Relative protein solubility is a fundamental parameter in food science, affecting food production, storage quality, sensory profile, digestibility, etc. To investigate the physical properties of *C. vulgaris* protein during digestion, a solubility test was conducted across the pH range (pH 2-7) representative

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of the entire digestive process. *C. vulgaris* protein revealed generally poor solubility behaviour (16.2-22.9 %) with the lowest solubility (16.2 ± 0.5 %) at pH 4 (Figure 1). This is likely due to the isoelectric point near pH 4, as reported in the literature . Minimal pH dependency was observed across the tested range, consistent with findings in *C.protothecoides* . The researchers attributed the soluble fraction to a high degree of glycosylation and a high proportion of hydrophilic amino acids, while the insoluble fraction consisted of protein aggregates. In addition to aggregation, the cell wall also acts as a barrier to protein solubility. The rigid cell wall of *Chlorella* was reported to resist water penetration, thus hindering interaction between intracellular substrates and water molecules (Safi et al., 2014b). The low solubility of *C. vulgaris* protein may pose challenges for its application in food design. For instance, poor solubility could lead to turbidity and sediment in beverages, potentially compromising product appeal and stability. Moreover, it may negatively affect protein techno-functional properties and digestibility, thereby impacting its nutritional value. To address these limitations and expand its potential integration into diverse food systems, it is recommended to further explore strategies to enhance protein solubility.

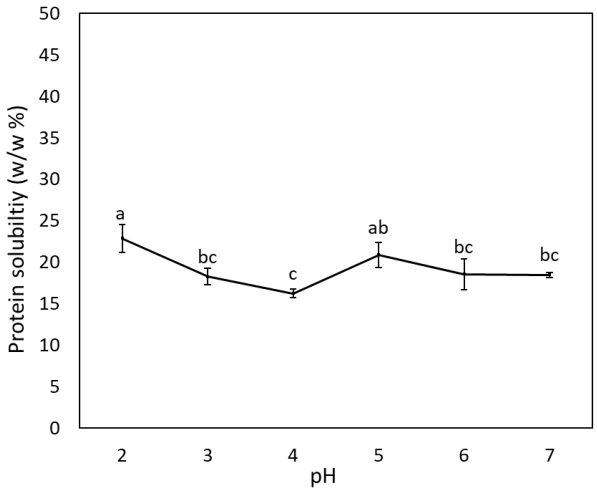

Figure 1. Relative protein solubility of C.vulgaris suspension (4 % w/w) in stimulated digestion fluid expressed as the ratio of protein concentration in the supernatant after incubation for 90 min and centrifugation (5,000 × g, 15 min) at room temperature over the protein concentration before centrifugation. Different lower case letters refer significant differences between the means of each pH sample (p<0.05).

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3.2 Semi-dynamic *in vitro* digestion

3.2.1 Protein content and microstructural changes

A semi-dynamic *in vitro* digestion was performed to investigate the physiochemical changes considering the physiologically relevant conditions, including gradual enzyme addition, progressive acidification and dynamic gastric emptying. The protein content of each digesta is presented in Figure 2, and the visual inspection and microstructural changes are depicted in Table 2. During gastric digestion, a phase separation was observed, attributed to the low solubility of the substrate. This phase separation resulted in varying protein content across different gastric digesta. Given that each gastric emptying (GE) fraction was emptied from the lower section of the gastric reaction vessel to mimic *in vivo* gastric emptying through the pylorus, this resultant inhomogeneous system would lead to variable protein concentration across different GE points. More proteins, particularly insoluble proteins, were evacuated in the earlier state of GE points, resulting in higher protein content in the digesta than the theoretical values for a homogenous system. Whereas, digesta from G3 onwards contained less protein than the expected value. Distinctive phase separation by turbidity could be visually observed in GE 4 and 5. The insoluble fraction showed a sandiness texture; above that, there was a soluble fraction with higher clarity and lighter colour. Meanwhile, it is noteworthy that the protein-free cookies used as a blank accounted for a notable portion of protein content in the digesta, especially in the intestinal phase. According to the literature, endogenous nitrogen accounted for 2.6 % and 53 % of the total nitrogen in the *in vitro* gastric and intestinal digesta, respectively . Thus, it is crucial to consider the enzyme background when performing quantitative analysis.

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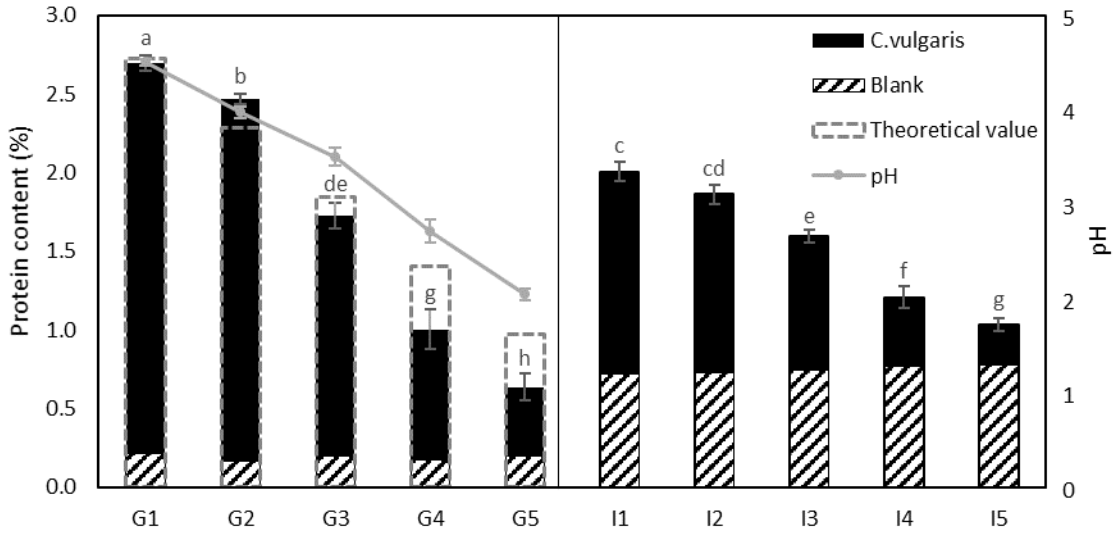

Figure 2. Protein content (%, w/w) and pH in each digesta during semi-dynamic in vitro gastrointestinal digestion of C.vulgaris suspension (4% protein w/w). The protein content, as determined by the Dumas method with a conversion factor of 4.78 is displayed on the left-Y-axis and pH is displayed on the right-Y-axis. Gastric emptying points (G1-5) correspond to the gastric emptying time of 13.5, 27.0, 40.5, 54.0, 67.5 min, respectively. I1-5 correspond to the individual 2h intestinal digestion performed on the G1-5 digesta, e.g. sample I1 has gone through 13.5min of gastric phase followed by 2h of intestinal phase. Blank refers to the protein-free cookies digestion performed under the same condition. Theoretical values are calculated based on the volume of fluid added at each time point, as described in . Different lower case letters refer significant differences between the means of each pH sample (p<0.05).

The results of CLSM and particle size distribution revealed the microstructure changes of the microalgae during digestion. Prior to digestion, a spherical shape of the *C. vulgaris* cell revealed a diameter of around 3-5 µm (Supplementary Figure S1), which is in agreement with the 2-10 µm diameter reported in the literature . Calcofluor White dyed cell wall presented a blue ring outside the cell, whereas Fast Green dyed cytoplasm protein showed a green circle (Table 2). In the gastric digestion, large aggregates, of up to 30 µm, were observed. As the digestion process advanced and protein content decreased, the degree of aggregations decreased. This trend was particularly evident in the particle size distribution results, where the $d_{[4,3]}$ of initial two GE points significantly decreased ($p<0.001$). Meanwhile, larger particle size spans were observed in the first two GE points, followed by a trend towards more uniform sizes. This trend may be attributed to the continuous stirring of the system, coupled with the action of digestive enzymes, which disrupted cell aggregation.

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Meanwhile, digestion started from pH 5.2 and dropped to 4.5, 4.0, 3.5, 2.7 and 2.0 at each GE point, respectively, as acidic gastric fluid was added gradually into the system. Pepsin would present different activities during gastric digestion at different pH. This could serve as a better imitation of *in vivo* digestion. According to the , the optimal pH for pepsin activity is pH 2. Between pH 2 and pH 3.5, pepsin activity will decrease significantly by 30 %. From pH 3.5 to pH 5.5, the enzyme activity plateaus. Above pH 6, pepsin stability will decrease rapidly, and the enzyme becomes irreversibly inactivated at pH 8. Therefore, it is anticipated that *C. vulgaris* undergoes more efficient pepsin hydrolysis in the latter phase of digestion from G3.

Despite the variations found in different gastric phases, the particle size distribution of each intestinal endpoint consistently exhibited similar bell curve and $d_{[4,3]}$ values. The CLSM confirmed that most of the cell aggregates were disassociated after intestinal digestion. More importantly, even in the final intestinal digestion, a small quantity of cell structures could be observed by the presence of protein within the cell wall. This suggested the hindering effect of the cell wall from protein digestion. *C. vulgaris* has a rigid three-layer structure cell wall composed of chitin- or chitosan-like polysaccharides, cellulose, hemicellulose, rhamnose proteins and lipids . This strong mechanical nature of the cell wall and the organelles work collectively to protect the cell against the external environment. Therefore, disrupting cell wall or disaggregating cell clusters could offer a potential solution to enhance nutrient release from microalgae cell wall.

There are several research on the effects of processing on cell wall disruption. The processing methods are generally categorised into three types: physical methods, chemical methods and enzymatic methods . Among these, physical methods obtained the most attention which included milling, high-pressure homogenization (HPH), ultrasonication, pulsed electric field (PEF) and more. A study compared different methods and reported HPH had a stronger effect on cell disruption than chemical treatment followed by ultrasonication . HPH treatment (2 × 2.7 kbar) was reported to be efficient in releasing 93 % more soluble protein from the substrate . PEF showed immediate and

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irreversible damage to the permeability of the cell wall of *C. vulgaris* . observed *C. vulgaris* after bead-milling and HPH, the cell lost its globular shape, but intact cells were observed after ultrasonication and chemical hydrolysis. explored the effects of acid and alkaline treatments on cell wall disintegration. Findings indicated that both methods were effective; however, alkaline catalysed hydrolysis showed higher protein release. The study further recommended mild chemical treatments to minimize protein and pigment degradation. Additionally, enzymatic treatments, including cellulose, pectinase, lysozyme and hemicellulase, have been proven to effectively enhance lipid release from *C.sorokinana* . Therefore, future research should explore the effectiveness of cell wall disruption methods to support microalgae-derived food products with enhanced nutrient accessibility.

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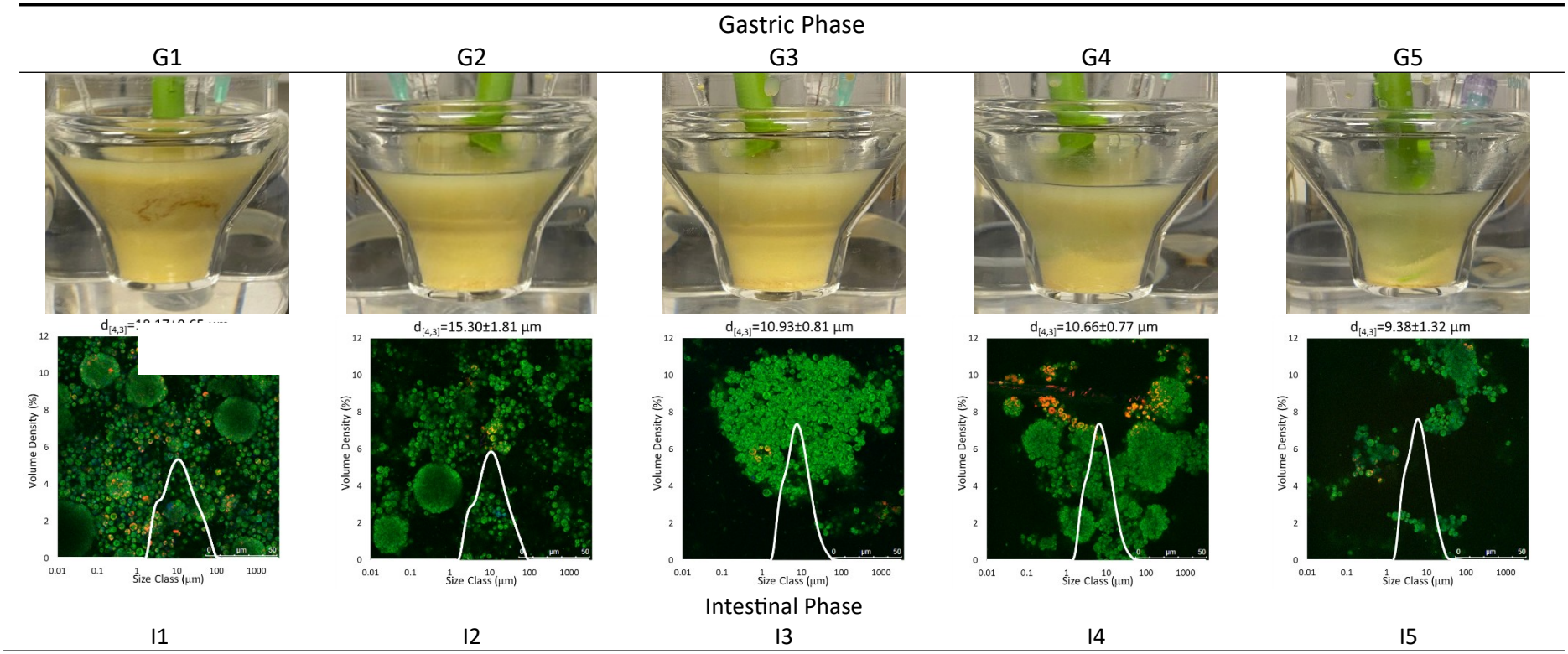
Table 2. Photos of the gastric reaction vessels, particle size distribution and representative Confocal Scanning Laser Microscopy micrographs over the development of semi-dynamic in vitro gastro-intestinal digestion of C. vulgaris suspension (4 % protein w/w). G1-5 correspond to the gastric emptying time of 13.5, 27.0, 40.5, 54.0, and 67.5 min, respectively. I1-5 correspond to the individual 2h intestinal digestion performed on each G1-5 digesta. Proteins were dyed with 1 % Fast Green FCF (w/v) shown in green; lipids were dyed with 0.02 % Nile Red shown in red; Cellulose (cell wall) was dyed with Calcofluor White 1:20 water shown in blue. The scale bar of the micrographs is 50 μm. Particle size distribution curves are shown overlapping on the microscopy picture. d[4,3] refers to the mean diameters of the particle size based on volume-weighted mean results.



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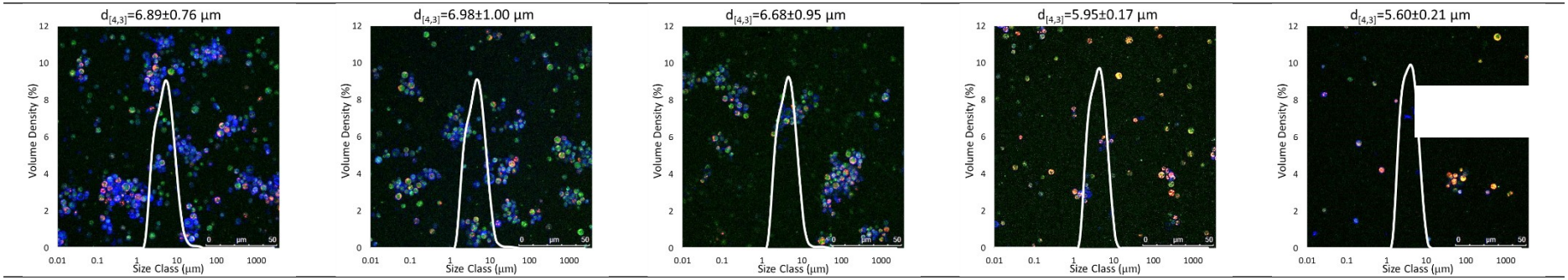

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3.2.2 Bioaccessible protein fraction

The bioaccessible protein fraction was obtained by TCA precipitation, commonly used to separate large proteins and peptides. suggested that 4 % TCA was the optimal concentration for maximising the yield of peptides without unstable large peptides and proteins. As a result, the TCA-soluble fraction would reflect the bioaccessible fraction of digesta and provide information on microalgae suspension's digestive behaviours.

In this study, the BCA assay measured protein release from the suspension, while the OPA assay measured the free amine groups release from the protein (Figure 3). In the gastric phase, G1-3 showed steadily low hydrolysis (3.9-5.2 % protein release; 1.5–1.8 % protein hydrolysis), possibly due to the low *C. vulgaris* solubility and low enzyme activity of pepsin. A significant increase ($p<0.05$) in hydrolysis was observed from G4 onwards by both the BCA and OPA methods. In the last GE point, 11.8 ± 0.9 % of the protein was released into the bioaccessible fraction, and 3.0 ± 0.5 % of amine groups were detached from the proteins. The increase in pepsin activity and higher solubility of microalgae protein collectively contributed to the increased level of proteolysis. A more significant increase in protein release than free amine groups release suggested that pepsin tends to cleave proteins in the middle rather than at the end of the chains, producing larger fragments instead of small peptides and amino acids. Driven by the higher efficiency of trypsin, chymotrypsin and carboxypeptidase in intestinal digestion, sharp increases in protein hydrolysis were observed during this phase. However, there is no significant increase ($p>0.05$) in protein release between the G5 and intestinal phases. This suggested that pancreatin had more pronounced effects on releasing proteins into small peptides and amino acids, while the overall protein release remained similar in the TCA-soluble fraction. The higher affinity to pancreatin aligned with the finding of .

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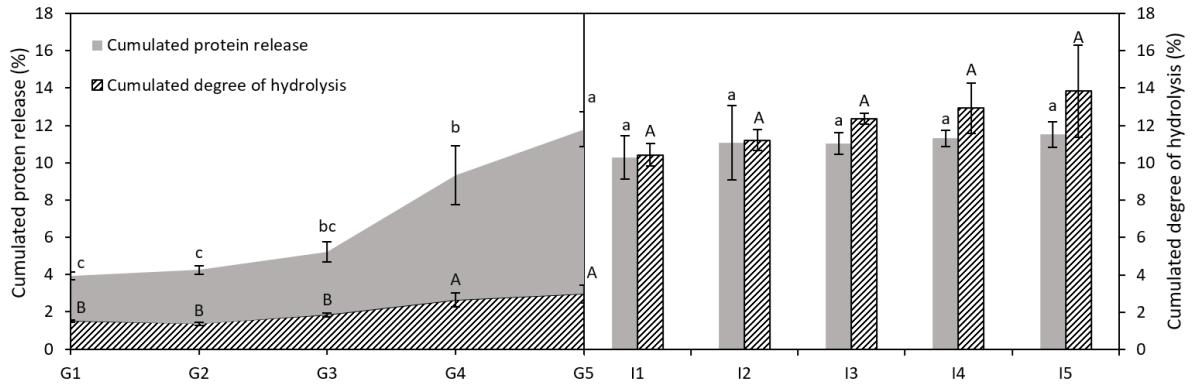

Figure 3. The proportion of protein release (%) (filled areas on the left Y-axis) and hydrolysed (striped areas on the right Y-axis) during semi-dynamic gastro-intestinal digestion of C.vulgaris suspension. Cumulated protein release (%) is calculated by the protein content in the TCA-soluble fraction of each digesta over the protein content in the digesta. Cumulated protein hydrolysis (%) is calculated by the amount of free amine group in TCA-soluble fraction over the total amine group presented in the acid-hydrolysed undigested C.vulgaris powder. Gastric emptying points (G1-5) correspond to the gastric emptying time of 13.5, 27.0, 40.5, 54.0, and 67.5 min, respectively. I1-5 correspond to the individual 2h intestinal digestion performed on the G1-5 digesta, e.g. sample I1 has gone through 13.5min of gastric phase followed by 2h of intestinal phase. Statistical analysis was performed separately for the gastric and intestinal phases. Different lowercase letters refer to significant differences between the means of each pH sample ($p<0.05$).

Currently, several research have reported the *in vitro* protein digestibility of *C. vulgaris*, ranging from 51% to 87 %, using various methods . It is important to note that the protein digestibility could vary widely due to differences in stains, cultivation conditions, harvest time, and post-harvest processing. Many of these studies lack detailed insights into the digestive behaviour, particularly regarding their physical performance and response to the decreasing gastric pH conditions that occur during human *in vivo* gastro-intestinal digestion. The semi-dynamic model used in this study simulated the changing gastric conditions in terms of pH, enzyme activity, gradual dilution and transit into the intestinal phase. Measuring free amine groups is an indirect method to access protein breakdown during gastric phase, hence it may yield lower values compared to overall gastro-intestinal digestibility. Semi-dynamic methods also accurately simulate the kinetics of food digestion, where static digestibility assays fail due to the static, unchanged digestive conditions (pH, enzyme activity, food/substrate ration etc.). Future research should focus on developing analytical methods tailored to the semi-dynamic model.

It is important to highlight that cell wall disruption processes could be applied to increase protein digestibility by increasing enzyme accessibility through loosening the cytoplasmic matrix and

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enhancing enzyme mobility within the cell. reported positive effects of bead milling on *in vitro* protein digestibility among 16 microalgae species, including *Chlorella*. A notable increase in *in vitro* protein digestibility was reported in bead-milled and high pressure homogenized *Picochlorum* sp. biomass . Results showed that both treatments could improve protein solubility and nitrogen release from the cell. In an *in vivo* fish trial, bead-milling had the most significant improvement on protein digestibility coeffients, followed by pasteurised, freeze dried, frozen-thawed on *N. gaditana*. The disrupted cell wall is hypothesised to release cellular contents and provide additional binding sites for digestive enzymes. Similar positive effects on digestibility by applying cell wall disruption technologies have also been reported in plant proteins, such as red seaweeds and legume seeds . However, cell wall disruption was not always equivalent to increased nutritional value. For examples, bead-milling was reported to reduce apparent protein digestibility of *P. tricornutum* but enhanced that of *T. chui.* in a fish trial . Another study observed a digestibility loss by electroporation and believed the over-intensive destruction led to cell molecule damage, which accounted for the lower digestibility . These findings suggest that different microalgae species might exhibit various response to cell disruption processes. In order to develop new food system with microalgae protein, the knowledge on protein solubility and digestibility will be crucial to guide manufacturer. Thus, *C. vulgaris* protein requires further study on improving nutrient release by applying different cell disruption methods.

3.2.3 Protein size distribution

To study the kinetics of protein breakdown during semi-dynamic *in vitro* digestion, the undigested sample, gastric and intestinal digesta were used to evaluate the protein breakdown in molecular weight using SDS-PAGE (Figure 4). In particular, many insoluble proteins were trapped in the well and failed to migrate into the gel. The intestinal phase revealed a less insoluble fraction than the undigested sample and gastric digesta. This result indicated that the proteolysis introduced by pancreatin positively increased *C. vulgaris* protein solubility.

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The molecular range of *C. vulgaris* was in accordance with the literature . Overall, the undigested sample showed a smearing background, indicating the presence of numerous protein types with various sizes. Band a at 50 kDa could be assigned to α-tubulin, which is the cytoskeleton of the cell and/or stress response heat-shock proteins (Hsp70); band b at 39 kDa could be assigned to ATP synthase subunit beta, which is responsible for energy metabolism; band d at 31 kDa could be assigned to biotin carboxylase and/or chlorophyll a/b binding; band e at 24 kDa could be assigned to superoxide dismutase that prevents cell damage, and/or chloroplast light-harvesting complex II; band f at 21 kDa could be assigned to peroxiredoxin, Fe-superoxide dismutase and/or photosystem I subunit chloroplast precursor; band g at 15 kDa could be assigned to Rubisco small submit that regulate photosynthesis of the cell . The pepsin lane displayed an intensive band at 38.3 kDa. Pancreatin lane displayed multiple bands, and these bands were suspected to be pancreatic α-amylase (55.4 kDa), pancreatic lipase (52 kDa), chymotrypsin (27 kDa), and trypsin (24 kDa) and their breakdown products (. However, precise protein identification requires further analysis, such as mass spectrometry.

In the gastric emptying points, the decrease in density could be observed for bands a, b, d, e, f, and g, while band c and h showed slightly stronger intensity as the digestion developed. The most noticeable difference in gastric phase was observed between G3 and G4. The increase in band c revealed the progressive addition of pepsin. Besides, visible bands at 15-50 kDa faded as gastric digestion developed, but new bands of pepsin and 12 kDa gradually appeared. It could be translated that pepsin hydrolysis breaks down protein into moderate sizes around 12 kDa. Moreover, there was a significant reduction in bands from *C. vulgaris* in size >14 kDa of intestinal digesta. This indicated that proteolysis was more intense during intestinal digestion. The differences in each subsequent intestinal digestion were minor; a gradual decrease could be observed in bands I (4-6 kDa). This suggested that the lower degree of hydrolysis during the gastric phase may result in more peptide releases in the 4-6 kDa range.

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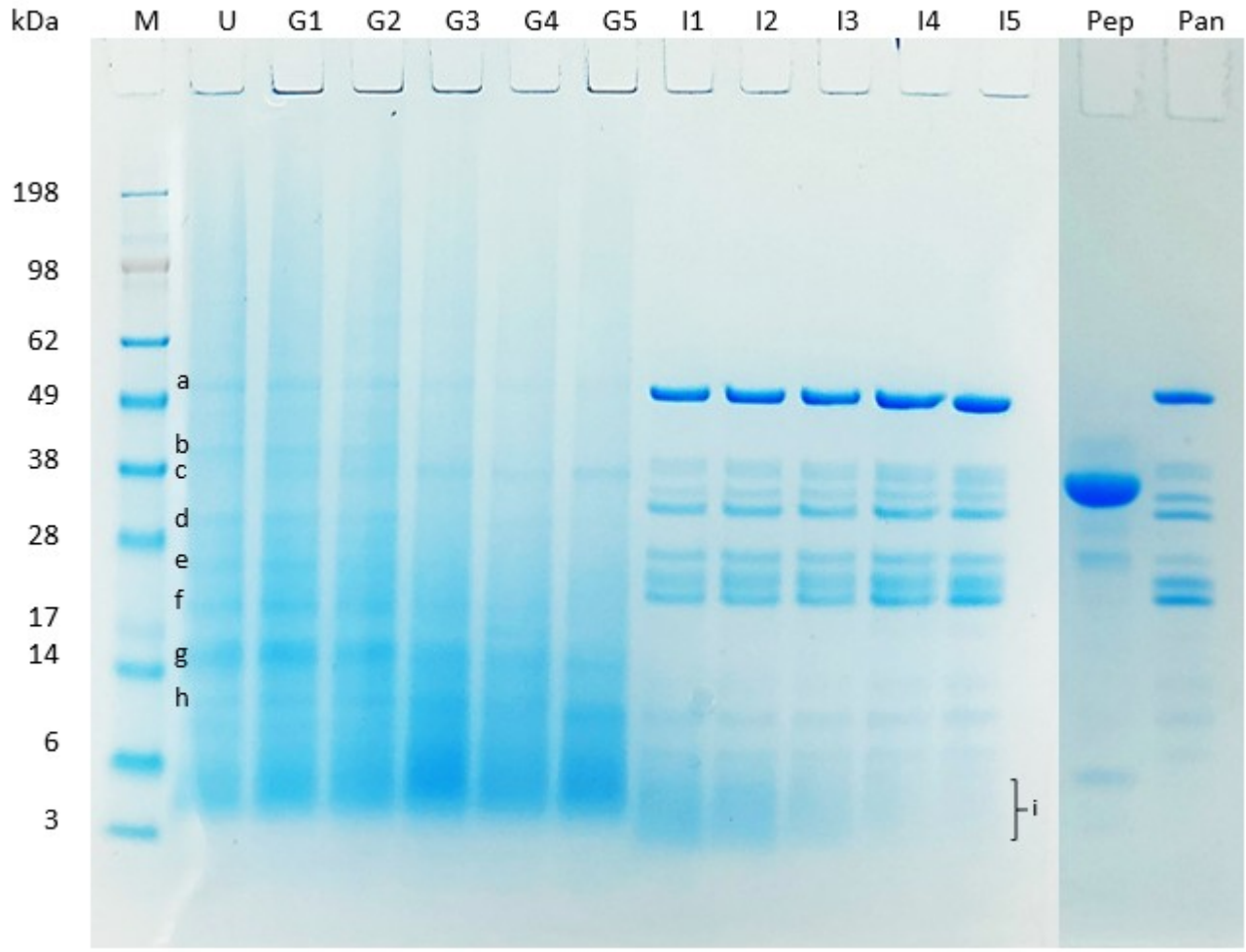

Figure 4. SDS-PAGE pattern (reducing condition) of marker (M), undigested C.vulgaris (U), digesta over semi-dynamic in vitro digestion (G1-I5), pepsin (Pep) and pancreatin (Pan). G1-5 correspond to the gastric emptying time of 13.5, 27.0, 40.5, 54.0, and 67.5 min, respectively. I1-5 correspond to the individual 2h intestinal digestion performed on the G1-5 digesta. The molecular weights of bands from the marker were shown in the left column. Bands that appeared in the samples were named in bands a-i.

3.2.4 Peptide molecular weight distribution

Before determining the peptide profile, the soluble fractions of the digesta were separated (Figure 5A). The initial undigested sample revealed 93.5 % insoluble protein. This fraction gradually decreased to 85.2 % at the finalised stage of gastric digestion, with a notable drop between G3 and G4. Following intestinal digestion, the insoluble fraction was further reduced to 65.5-70.1 %. This result is independent of pH, as the fractions were analysed at a constant pH of 7. Results implied the positive effects of digestive enzymes on increasing protein solubility. Literature supports this finding, showing a more than 40 % increase in protein solubility of extracted *Chlorella* protein after hydrolysed with pepsin, trypsin and pancreatin for 3h . The enhanced solubility was speculated by

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the increased hydrophilicity resulting from reduced molecular weight and the formation of carboxyl groups.

Size exclusion chromatography (SEC) was performed to obtain the molecular weight profile of peptides in undigested and digesta subjected to different degrees of digestion (Figure 5B). For the gastric emptying points, an increase in the area under the curve (AUC) was observed from the chromatograph (Supplementary Figure S2) in the digesta as gastric digestion progressed. Therein, 0.3-1 kDa was the most abundant fraction among gastric digesta. The ratio of peptides at > 30 kDa and 10-30 kDa decreased, while a significant increase was found in the ratio of peptides at 0.3-1 kDa. This observation elucidated that proteins higher than 10 kDa were most likely hydrolysed into 0.3-1 kDa peptide by pepsin. This pepsin effect corroborates with results observed in pea and rice protein digestion .

In intestinal digesta, the dominating peptides were sizes between 0.3-1 kDa, and this fraction revealed a negative correlation with the extent of gastrointestinal digestion. Meanwhile, an increase of peptides with molecular weight between 0.1-0.3 kDa was observed. This result is consistent with the findings in pea protein . Interestingly, a notable increase in the fraction of large proteins (>30 kDa) was observed in the advanced stages I4 (2.94 %) and I5 (3.47 %), compared to the earlier stages I1-3 (0.05-0.30 %). A comparable phenomenon was revealed in peptides from 10-30 kDa. It is presumed that the higher degree of gastrointestinal digestion enhanced the solubility of larger proteins. Therefore, although there was a decreasing fraction of proteins larger than 10 kDa during gastric digestion, the combined effects of gastric and intestinal digestion facilitated a more effective release of larger peptides into the soluble phase.

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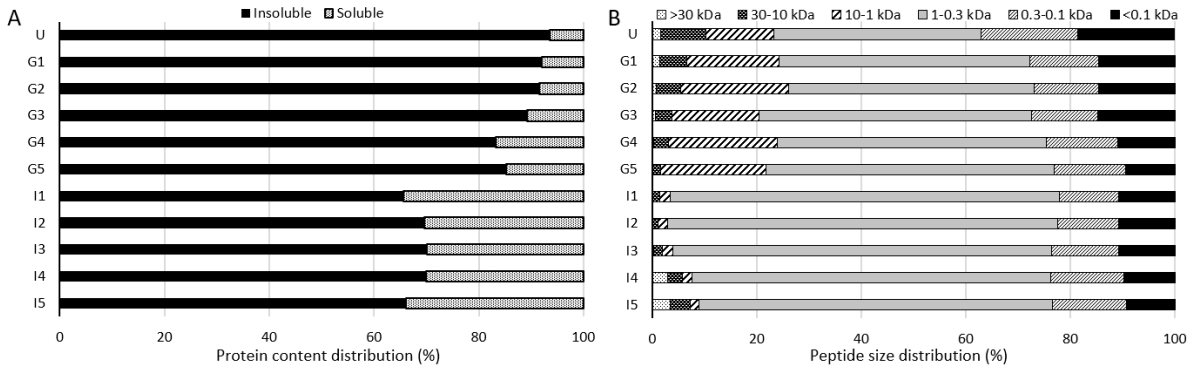

508

509 *Figure 5. The protein solubility (A) and peptide size profile (B) of undigested sample and different stages digesta during*
510 *semi-dynamic in vitro digestion. Protein solubility was calculated by the protein content in the filtered permeate over the*
511 *protein content in the according digesta. G1-5 correspond to the gastric emptying time of 13.5, 27.0, 40.5, 54.0, and 67.5*
512 *min, respectively. I1-5 correspond to the individual 2h intestinal digestion performed on the G1-5 digesta.*

513 ### 3.2.5 Free amino acid composition

514 The amino acid profiles at each stage of digestion are presented in Figure 6 and Supplementary
515 Table S1. The undigested sample exhibited a nearly complete amino acid profile, except tryptophan,
516 predominantly released in the subsequent intestinal phase. A notable increase in free amino acid
517 content was only observed from the G4 onwards. As discussed in the previous section, the minimal
518 or negligible protein hydrolysis in G1 to G3 may be attributed to the pH. Phenylalanine had the
519 highest increase at the end of the gastric digestion, followed by tyrosine and cysteine. Recent
520 research on α-lactalbumin demonstrated that pepsin exhibited different selectivity over different
521 pH . At pH 1-3, phenylalanine, leucine, tyrosine and aspartic acid were the most frequent cleavage
522 points for pepsin. However, at pH 4-5, the C-terminal of the hydrolysed peptides were found absent
523 in asparagine, serine, threonine, methionine, valine, isoleucine, or cysteine. Hence, employing
524 dynamic gastric digestion could enhance the accuracy in reflecting the pepsin selectivity occurs *in*
525 *vivo* condition.

526 During intestinal digestion, where amino acids were mainly released, the six most abundantly
527 released amino acids were phenylalanine, leucine, aspartic acid, methionine, lysine and isoleucine,
528 the majority of which were essential amino acids (EAAs). According to the literature, trypsin is highly
529 specific and shows a strong affinity for cleaving protein into peptides with arginine and lysine on the
530 C-terminal site. Meanwhile, chymotrypsin is less specific and shows a higher affinity to hydrophobic

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amino acids, such as phenylalanine, tryptophan and tyrosine . Although the cleavage sites reported in this study did not fully match those from the literature, they are generally in accordance with it, considering multiple factors that could affect enzyme specificity, such as protein folding state, substrate ratio, temperature, etc.

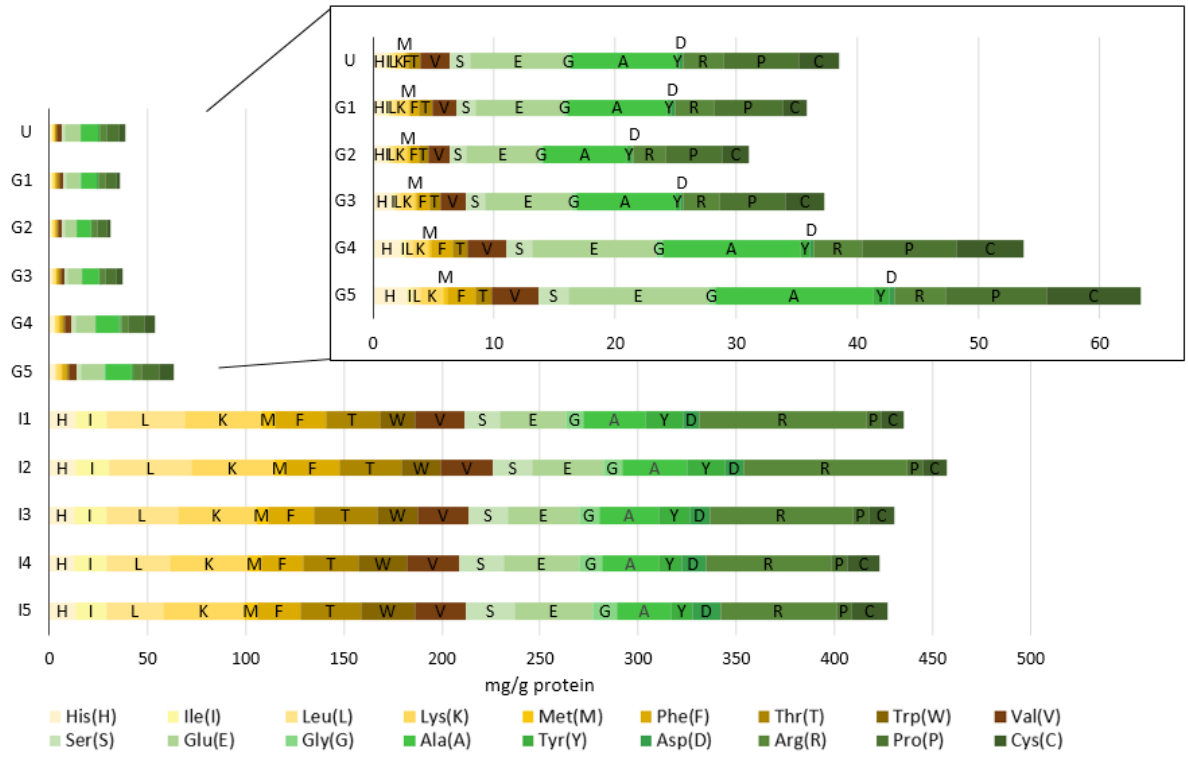

Figure 6. Free amino acid profile for the undigested (U), gastric (G1-5) and intestinal (I1-5) digesta of C.vulgaris samples by semi-dynamic in vitro digestion. Essential amino acids are shown in yellow and non-essential amino acids in green. G1-5 correspond to the gastric emptying time of 13.5, 27.0, 40.5, 54.0, and 67.5 min, respectively. I1-5 correspond to the individual 2h intestinal digestion performed on the G1-5 digesta. Each amino acid is shown in a Three-letter abbreviation.

As mentioned, EAAs are the major amino acids released from intestinal digestion. EAAs are those amino acids that cannot be synthesised by the human body and must be obtained through diet. The release of EAA benefits protein synthesis . Conversely, non-essential amino acids (NEAAs) could be synthesised endogenously. In this study, the undigested samples exhibited an EAA: NEAA ratio of 0.20. This ratio increased to 0.26 during gastric digestion and further elevated to 0.95-0.99 by the end of the intestinal digestion. However, the differences in EAA: NEAA ratios among different digesta at the same digestion stage were statistically insignificant. The literature reports different EAA/NEAA ratios for animal and plant-based proteins. Animal proteins generally had higher values, ranging

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548 from 1.7 to 2.9 . In contrast, legumes and cereal grains typically had a ratio of around 1, except for
549 chickpeas, which had a ratio of 3 . An early *in vivo* pig study suggested an optimal ratio of 1:1 for
550 nitrogen (N) retention and utilisation and emphasised the importance of EAA for N utilisation at
551 lower protein intake. They concluded that nitrogen utilisation increased with the EAA ratio, but
552 nitrogen retention declined once it exceeded 2.3. . Therefore, the free amino acid profiles along the
553 digestive pathway elucidated the potential of *C. vulgaris* as an alternative protein source.

554 ### 3.2.6 Total phenolic compounds

555 The total phenolic compounds (TPC) of *C. vulgaris* and water blank digesta were quantified using the
556 Folin-Ciocalteu method, shown in Figure 7. This method measures the reducing capacity of the
557 sample through electron transfer and correlates it with phenolic content. Prior to digestion, the
558 initial *Chlorella* sample exhibited a TPC level of 1.3 mg GAE/g DM, consistent with the literature
559 range of 0.8-3.0 mg GAE/g DW . The variability in TPC can be attributed to differences in cultivation
560 conditions and extraction methods, with higher values typically reported when using organic
561 solvents or solely aqueous phases.

562 During the initial 40.5 min (G3) of gastric digestion, the concentration of polyphenols remained
563 unchanged from the undigested sample. As gastric digestion continued, a moderate increase in TPC
564 was observed from G4 onwards. This increase is likely due to the dissociation of cell aggregates,
565 facilitating the greater release of polyphenols from the substrate. A substantial release of phenolic
566 compounds was noted by the end of intestinal digestion compared to the previous. However, no
567 significant differences were observed in the intestinal digesta among the various gastric phases.
568 Overall, a general upward trend in TPC observed with each successive phase during digestion was in
569 agreement with the finding of on the free polyphenol level of 3 microalgae species: *Spirulina*,
570 *Dunaliella* and *Isochrysis*.

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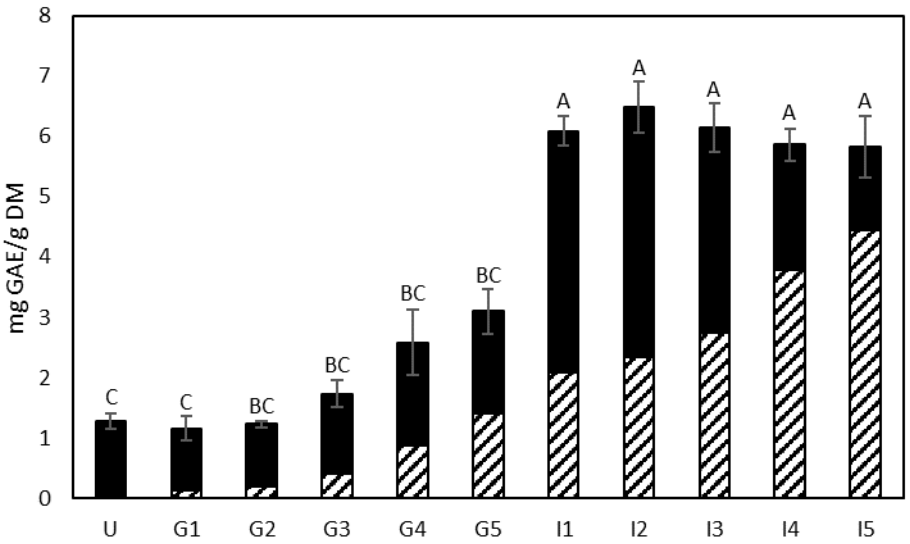

571

572 *Figure 7. Changes of total phenolic compounds (mg gallic acid equivalent (GAE)/g DM) during semi-dynamic in vitro*
573 *digestion of C.vulgaris suspension. G1-5 correspond to the gastric emptying time of 13.5, 27.0, 40.5, 54.0, and 67.5 min,*
574 *respectively. I1-5 correspond to the individual 2h intestinal digestion performed on the G1-5 digesta. Solid bars representing*
575 *the values of C. vulgaris digesta overlap with diagonal-filled bars representing the values of the water blank digesta. Bars*
576 *with different letters are statistically significantly different ($p<0.05$). The error bars refer to the standard deviation of values*
577 *from three individual C.vulgaris digestions.*

Revised and accepted in March, 2025
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Interestingly, the TPC in water blank digesta also gradually increased during gastric digestion, inherently affecting the TPC in the subsequent intestinal phase. This finding might indicate that while the overall TPC levels of microalgae intestinal digesta were similar, the net TPC released from microalgae decreased between stages I1 and I5. It is important to note that the Folin-Ciocalteu method estimates the antioxidant capacity or reducing power of the substrate instead of quantifying polyphenol directly . Based on these observations, two hypotheses were proposed. First, the high TPC level reported in the water blank might be due to enzyme autolysis. This autolysis, particularly enzyme fragmentation, is likely reflected in a loss of enzymatic activity. suggested that pepsin peptide hydrolysates fell between 6.9-12.1 amino acid residues and 5.4-3.1 amino acid residues for pancreatic enzymes. Meanwhile, they reported that the pancreatic enzyme half-life increased from 1.6 h without food to 88.4 h with the presence of food. This protection effect of food was only found in pancreatic enzymes but not in pepsin. Subsequently, these enzyme hydrolysates could interfere with the Folin-Ciocalteu analysis. It was reported that free tyrosine, tryptophan and cysteine showed pronounced reactivity to the Folin-Ciocalteu method . Thus, more enzyme autolysis in water-blank digestion might lead to overestimating the actual polyphenol content released by the microalgae substrate. Secondly, polyphenol degradation might occur during intestinal digestion, especially for the digesta, which releases more polyphenols from the previous gastric phase. The loss of phenolic compounds might be attributed to the chemical conditions during pancreatic digestion, where polyphenols are highly sensitive to mild alkaline conditions. This can cause them to transform into unknown or undetected structures, thereby reducing their bioaccessibility . Therefore, more polyphenol degradation might account for the minor TPC level gap between microalgae and blank digestion. Regardless, the total phenolic content of the digesta increased as digestion proceeded, which may indicate the microalgae's potential antioxidant activity. However, it is important to note that certain phenolic compounds may act as anti-nutritional factors, adversely affecting protein absorption. For instance, tannins are reported to exhibit anti-carcinogenic and antimutagenic activities but are also known to inactivate digestive enzymes and form complexes with proteins .

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studied the anti-nutritional factor in *C.pyrenoidosa* and reported 2.7 mg/g of tannins in the biomass. Notably, some physical treatments have proven effective in reducing these anti-nutritional factors . Therefore, when considering dietary shifts, it is essential to identify and quantify the specific anti-nutritional factors present and apply suitable methods if necessary.

Conclusion

This research addressed the knowledge gap regarding the digestive behaviour of *C. vulgaris*, particularly in terms of its physical and step-wise chemical changes during digestion. Semi-dynamic *in vitro* digestion was employed to simulate the physiological relevance and acquire detailed kinetics results of the gastrointestinal digestion. This study examined physical and chemical changes occurring during the digestion of microalgae suspension containing 4 % protein (w/w).

The results indicated slow protein release in the gastric phase, likely due to reduced pepsin activity and low substrate solubility. A notable increase in the intensity of protein hydrolysis and polyphenol release was observed, starting from 40.5 min in gastric digestion. As digestion progressed to the intestinal phase, solubility increased, and amino acids were predominantly released. However, intact cell structures were still observable at the end of gastrointestinal digestion, suggesting that the rigid cell wall of *C. vulgaris* composed of chitosan-like polysaccharides, cellulose, rhamnose etc., serves as the protective barrier against digestive enzymes, thereby limiting nutrient release and leading to slower digestion.

With its milder taste and texture, honey *C. vulgaris* demonstrates considerable potential for functional food applications. While microalgae biomass has already been able to produce on a large scale, future research should focus on refining processing methods to enhance bioaccessibility and fully realise its potential as a sustainable protein source.

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Acknowledgements

This research was funded by the European Union's Horizon 2020 research and innovation program under grant agreement no.101059632 (project GIANT LEAPS). S.F. is supported by Teagasc Walsh Scholarship (2021232). A.B. and L.G. are funded by Science Foundation Ireland and DAFM Grant 16/RC/3835-VistaMilk. Graphic abstract was created with BioRender.com.

CRediT authorship contribution statement

S.F.: Writing–original draft, Writing-review & editing, conceptualization, methodology, formal analysis, visualization, data curation, investigation; **E.H.**: Writing–original draft, Writing-review & editing, conceptualization, formal analysis, data curation, investigation; **A.S.**: Writing-review & editing, conceptualization, formal analysis, supervision; **L.G.**: Writing-review & editing, conceptualization, formal analysis, supervision, funding acquisition and project administration; **A.B.**: Writing–original draft, Writing-review & editing, conceptualization, formal analysis, supervision, funding acquisition and project administration.

References

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