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## Fermentation-driven modulation of protein, mineral and peptide bioaccessibility in SC-CO<sub>2</sub>-defatted *spirulina* during *in vitro* digestion

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Supercritical carbon dioxide-defatted-*spirulina* (SC-D-*Sp*) produced under the following conditions (25.0 MPa, 50.0 °C, CO<sub>2</sub> flow rate: 16.0 mL min<sup>-1</sup>, CO<sub>2</sub>/EtOH = 90:10, 60.0 min) was used as the substrate for fermentation with lactic acid bacteria (LAB) *Lactiplantibacillus plantarum* (LP) and *Lactiacaseibacillus casei* (LC). After fermentation, the solid and supernatant fractions were subjected to *in vitro* simulated gastrointestinal digestion. The resulting digesta were then evaluated for protein content, protein bioaccessibility, bioactive peptides (BPs), mineral content and mineral bioaccessibility, and total antioxidant capacity (TAC). Protein concentrations and protein bioaccessibility in digest-supernatants (control, 5% LP, and 5% LC) were significantly higher than those measured in the corresponding digest-solid fractions. The identified peptide sequences were associated with previously reported bioactivities, including potential angiotensin-converting enzyme's (ACE's) inhibitory, antioxidant, antibacterial, hypotensive, antithrombotic, and calcium-binding activities. Mineral (Mg, P, K, Fe, Cu, and Se) bioaccessibility was also significantly higher in the digested supernatants. Notably, the digested supernatants from the 5% LP and 5% LC fermentations showed elevated TAC values. Principal component analysis (PCA) further indicated that the fermented digested supernatants (5% LP and 5% LC) were associated with variables related to bioactive peptides, antioxidant capacity, mineral bioaccessibility, and protein bioaccessibility.

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### Sustainability spotlight

This study advances sustainable food bioprocessing through the integrated valorization of *Spirulina* biomass using supercritical CO<sub>2</sub> extraction followed by lactic acid bacteria fermentation. Rather than focusing on single-compound recovery, the approach enables dual valorization of both the lipid fraction and the remaining protein-rich biomass within a biorefinery framework. Fermentation enhances nutrient bioaccessibility and promotes the release of functional peptides during *in vitro* digestion, improving the nutritional potential of the processed material without intensive chemical treatments. By maximizing the functional yield of microalgal biomass—recognized as a low-resource, climate-efficient protein source—this work supports resource-efficient food production systems. The strategy contributes to SDG 2 (Zero Hunger), SDG 12 (Responsible Consumption and Production), and SDG 13 (Climate Action).

## Introduction

*Arthrospira platensis* (*Spirulina*), a blue-green microalga, has gained attention over the last few decades due to its potential for intensive cultivation and wider availability in commercial products.<sup>1</sup>

*Spirulina* is a great source of high-added-value compounds, including 50–70% (dry weight) protein, 5–10% lipids, 15–20% carbohydrates, *etc.*<sup>2</sup> Although *Spirulina* is one of the few species of microalgae accepted for human consumption by the European Food Safety Authority (EFSA),<sup>3</sup> there is still reluctance on the part of the population to consume it as is. Some of the limitations, such as degradation or leaving toxic residual solvent in the product,<sup>4</sup> are related to the lack of consumer

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acceptance at the organoleptic level. That is why, for a few years now, various extraction technologies have been used to recover the compounds from *Spirulina*.

Apart from the conventional solid–liquid or liquid–liquid solvent extractions, one of the technologies that has drawn more interest is supercritical carbon dioxide (SC-CO<sub>2</sub>),<sup>5–7</sup> as it allows the recovery of oil, and the remaining cakes are of special interest due to their protein and carbohydrate composition, which makes them an interesting matrix for lactic acid bacteria fermentation.

For instance, SC-CO<sub>2</sub> extraction has been successfully applied to defat microalgae species, with the resulting defatted biomass subsequently subjected to novel extraction techniques, such as pulsed electric fields (PEF) and fermentation with lactic acid bacteria (LAB), to recover valuable bioactive compounds.<sup>8</sup> Inspired by this approach, SC-CO<sub>2</sub> was used to defat *spirulina* in this study, and the resulting SC-D-*Sp* was fermented to obtain both solid and supernatant fractions for further analysis.

Microalgae are well-known sources of bioactive peptides (BPs), which often exhibit antioxidant activity through free radical scavenging and enhancement of the body's defense systems.<sup>9</sup> LAB are known to secrete proteases that hydrolyze larger proteins into smaller peptides during fermentation.<sup>10</sup> Previous research has investigated LAB fermentation of *spirulina*, reporting reductions in crude protein content (46.6% in non-fermented broth (NFB) and 47.4% in fermented broth (FB)) and an increase in the ratio of non-protein nitrogen to total nitrogen (24.8% for NFB and 28.4% for FB), indicating protein breakdown and release of smaller nitrogenous compounds.<sup>11</sup> Other studies have also demonstrated that fermentation with LAB and *Bacilli* strains (*Lactiplantibacillus plantarum*, *Lactobacillus acidophilus*, *Bacillus subtilis*, and *Bacillus coagulans*) results in reduced phycocyanin and allophycocyanin content, along with an increased ratio of free essential to non-essential amino acids, suggesting improved nutritional profiles.<sup>12</sup> Nevertheless, the literature lacks comprehensive data on the impact of LAB fermentation, specifically on SC-D-*Sp*, particularly regarding the comparative analyses of the fermented solid and supernatant fractions. In this study, LAB were used to ferment SC-D-*Sp*, and both the fermented solids and supernatants were collected and then subjected to *in vitro* simulated gastrointestinal digestion. The goal was to explore the generation of BPs and antioxidant compounds, and to evaluate mineral bioaccessibility from these fermented fractions.

For instance, nutritional composition, bioavailability, and digestibility are critical factors in the development of novel functional foods.<sup>13,14</sup> Protein digestibility, which refers to the proportion of ingested protein that is absorbed into the bloodstream, can be assessed through *in vitro* or *in vivo* gastrointestinal models.<sup>15</sup> *In vitro* digestion systems, in particular, offer a reliable starting point for evaluating the structural changes, release, and bioactivity of food components under simulated gastrointestinal conditions.<sup>16–18</sup> These models also allow assessment of nutrient bioaccessibility and potential metabolic interactions at the intestinal level.<sup>19</sup>

This study aimed to investigate nutrient release from SC-D-*Sp* fermented fractions (solids and supernatants) using *in vitro*

simulated gastrointestinal digestion. Post-digestion, protein concentration, BPs, mineral content, mineral bioaccessibility, and total antioxidant capacity (TAC) were assessed. We hypothesized that digestion of LAB-fermented SC-D-*Sp* would release functional bioactive compounds and enhance mineral bioaccessibility. These findings offer valuable insight into optimizing *spirulina* fermentation for functional food development and underscore the importance of *spirulina* as a sustainable source of health-promoting ingredients.

## Materials and methods

### Samples

*Spirulina* (*Arthrospira platensis*) whole cell powder was provided by A4F (Algae for Future, Portugal). Lactic acid bacteria (LAB) strains *Lactiplantibacillus plantarum* (LP) and *Lactocaseibacillus casei* (LC) were sourced from the Spanish Type Culture Collection (CECT) in Valencia, Spain. Supercritical carbon dioxide (SC-CO<sub>2</sub>) was used to defat *spirulina*. The solid matrix and the supernatants (in dry weight) were obtained following defatted *Spirulina* LAB fermentation with 5% *L. plantarum* and 5% *L. casei* at 30.0 °C for 18 h. *In vitro* simulated gastrointestinal digestion was then performed on the freeze-dried SC-D-*Sp* fermented fraction (solid and supernatant). All the schematic processes are shown in Fig. 1.

### Chemicals and reagents

2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate (K<sub>2</sub>S<sub>2</sub>O<sub>8</sub>), sodium nitrate (≥99% purity), ammonium carbonate ((NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>), sodium bicarbonate (NaHCO<sub>3</sub>), potassium chloride (KCl), sodium chloride (NaCl), potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>), calcium chloride dihydrate (CaCl<sub>2</sub>·2H<sub>2</sub>O), and analytical-grade enzymes including pepsin (975 units/protein, porcine), pancreatin (8× USP, porcine), and porcine bile extract were all purchased from

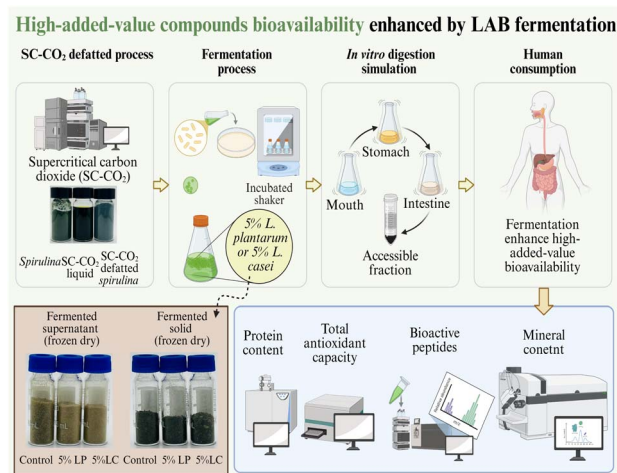


Fig. 1 Schematic representation of the experimental workflow applied to SC-CO<sub>2</sub> defatted *spirulina* (SC-D-*Sp*) fermented fractions (solid and supernatant) subjected to *in vitro* simulated gastrointestinal digestion (created in <https://www.BioRender.com>).



Sigma-Aldrich (St. Louis, MO, USA). Additionally, 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (Trolox®) was sourced from Sigma-Aldrich (Steinheim, Germany). MRS agar and broth were obtained from Condalab (Laboratorios Conda S.A., Spain). Deionized water was prepared using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA).

Besides, the simulated digestion fluids, simulated salivary fluids (SSF, pH in 7), simulated gastric fluids (SGF, pH in 3), and simulated intestinal fluids (SIF, pH in 7) were prepared using potassium chloride (KCl), potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ), sodium bicarbonate ( $\text{NaHCO}_3$ ), sodium chloride (NaCl), magnesium chloride hexahydrate ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ), ammonium carbonate ( $(\text{NH}_4)_2\text{CO}_3$ ), 1 M sodium hydroxide (NaOH), and 6 M hydrochloric acid (HCl).

### Supercritical carbon dioxide (SC-CO<sub>2</sub>) defatting process

According to previous research, SC-CO<sub>2</sub> was applied to defat *spirulina*, yielding the SC-CO<sub>2</sub> extraction liquid of *spirulina* and SC-CO<sub>2</sub> defatted *spirulina* (SC-D-*Sp*). The whole process was referenced to the previous methods,<sup>5</sup> using 25 MPa, 50 °C, and a flow rate of 16 mL min<sup>-1</sup> with CO<sub>2</sub>/EtOH = 90 : 10, and an extraction time of 60 min.

### Lactic acid bacteria (LAB) fermentation

LAB strains LP and LC were chosen for the SC-D-*Sp* fermentation. More importantly, the SC-D-*Sp* was chosen as a sole carbon source to support LAB growth, referenced to previous research with slight modifications.<sup>12</sup> The pasteurized (at 95 °C for 15 min) SC-D-*Sp* broth was then inoculated with 5% (of the total volume) LAB strain broth, which had been cultivated for 18 h, and the LAB broth was adjusted to an optical density at 600 nm (OD<sub>600</sub>) between 1.8 and 2.3. Referenced to the previous LAB fermentation, the SC-D-*Sp* fermentation was conducted at 30 °C with agitation at 190 rpm for 18 h.<sup>8</sup>

### *In vitro* simulated gastrointestinal digestion

A total of 2.5 g of fermented solids and supernatants (freeze-dried) were mixed with 25 g of distilled water. The *spirulina* fermented fractions underwent *in vitro* digestion according to the standardized INFOGEST methodology,<sup>20</sup> including oral, gastric, and intestinal stages, as outlined below. The procedure followed a previously established method,<sup>21,22</sup> including the oral, gastric, and intestinal stages. For that purpose, the SSF (pH = 7), SGF (pH = 3), and SIF (pH = 7) were prepared prior to the digestion process.

### Oral stage (chewing) digestion

$\alpha$ -Amylase was omitted during this phase. A 2.5 g sample previously prepared was combined with 2.0 mL of SSF and shaken for 1 min. Then, 12.5  $\mu\text{L}$  of CaCl<sub>2</sub> and distilled water were added to reach a final volume of 5.0 mL. The mixture was incubated in a water bath at 37 °C for 2 min.

### Gastric stage digestion

To the oral digest, 4.55 mL of SGF was added, and the mixture was vortexed for 1 min. Then, 8 mg of porcine pepsin (2000 U mL<sup>-1</sup>) and 2.5  $\mu\text{L}$  of CaCl<sub>2</sub> were incorporated, followed by another 1 min of vortexing. The pH was adjusted to 3 using 6 M HCl or 1 M NaOH, and the volume was brought to 10 mL with distilled water. The sample was incubated in brown bottles at 37 °C for 2 h.

### Intestinal stage digestion

Following gastric digestion, 5.5 mL of SIF was added and vortexed for 1 min. Then, 2.5 mL of porcine pancreatin (800 U mL<sup>-1</sup>), 1.25 mL of porcine bile extract (0.16 M), and 20  $\mu\text{L}$  of CaCl<sub>2</sub> were added, followed by another 1 min of vortexing. The pH was adjusted to 7, and distilled water was added to a final volume of 20 mL. Samples were incubated at 37 °C for 2 h, then centrifuged at 2504 × *g* for 40 min at 4 °C. Finally, the supernatant was collected for further analysis.

### Protein content tests

Protein content in the digest-solid and digest-supernatant fractions was determined by the Dumas method using a Euro-Vector elemental analyzer (EuroVector SpA, Milan, Italy).<sup>23</sup> Prior to analysis, samples were freeze-dried and ground into a fine powder. The nitrogen-to-protein conversion factor for microalgae is 4.44.<sup>24</sup> Additionally, the results of protein bioaccessibility were expressed as the ratio of the concentration of protein content in the digested fractions, calculated using the following equation (eqn (1)) referenced to a previous study.<sup>21</sup>

Protein bioaccessibility (%) =

$$\left( \frac{\text{protein content in digested fraction}}{\text{total protein content}} \right) \times 100 \quad (1)$$

### Liquid chromatography-tandem mass spectrometry (LC-MS/MS) Q-TOF analysis of bioactive peptide sequences

An LC-MS/MS Q-TOF system (6600plus Triple TOF, AB SCIEX, Framingham, MA, USA) was used to determine the bioactive peptides.<sup>25</sup> Only the maximum confidence score (a value equal to 1) for the sequence was chosen for the later analyses with the specific activity sequences using the BIOPEP-UWM database to identify potential bioactive properties based on relevant literature.<sup>26</sup>

### Inductively coupled plasma mass spectrometry (ICP-MS) of mineral content and mineral bioaccessibility assay

ICP-MS (7900) was used to assess the mineral concentration in the SC-D-*Sp* fermented fraction (solid and supernatant) subjected to *in vitro* simulated gastrointestinal digestion. The results of mineral bioaccessibility were expressed as the ratio of the concentration of mineral compounds in the bioaccessible fraction calculated using the following equation (eqn (2)) referenced to a previous study.<sup>21</sup>



Mineral bioaccessibility (%) =

$$\left( \frac{\text{mineral content in digested fraction}}{\text{total mineral content}} \right) \times 100 \quad (2)$$

### Total antioxidant capacity (TAC) tests

TAC, measured by trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays, was determined in the SC-D-*Sp* fermented fractions (solids and supernatants) before and after *in vitro* simulated gastrointestinal digestion. For TEAC, the specific process used was referenced to previously reported literature with some modifications.<sup>12,27</sup> The stock solution was prepared by mixing 25 mL of ABTS at 7 mM, and 440  $\mu$ L  $K_2S_2O_8$  at 140 mM, and was stored in the dark for 16 h before use. The working solution was then prepared at an absorbance of  $0.70 \pm 0.02$  at 734 nm by diluting the stock solution in ethanol. The calibration curve was prepared with 5 mM Trolox and ethanol. All the absorbances were measured at 734 nm with 100  $\mu$ L sample/standard and 2.5 mL of ABTS working solution using a PerkinElmer UV/Vis Lambda 2 spectrophotometer (PerkinElmer, Jügesheim, Germany).

For the ORAC assay, the whole process was referenced to ref. 27. One liter of working buffer was prepared with 61.6 mL  $Na_2HPO_4$ , 38.9 mL  $KH_2PO_4$ , and Milli-Q water. 1 mM Trolox, 1 mM fluorescein, and 221.25 mM AAPH were then prepared with the working buffer. 96-well black plates were employed in the measurement with 50  $\mu$ L fluorescein, 50  $\mu$ L standard (trolox) or sample, and 50  $\mu$ L of 221.25 mM AAPH in each well. Measurements were carried out in a plate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany).

### Statistical analysis

All tests were conducted in triplicate, and the average (mean) with standard deviation (SD) is presented. A one-way analysis of variance (ANOVA) and Duncan's multiple range test were carried out, considering significant differences when  $p < 0.05$ . GraphPad Prism (GraphPad Software Company, Boston, MA, USA) was used for graph painting, and SPSS 20.0 analysis software (IBM, Armonk, NY, USA) was used to perform statistical analyses. The bioactive peptide sequences were identified with various activities using the BIOPEP-UWM database.<sup>26</sup>

## Results and discussion

### Protein content

In a previous study, the SC-D-*Sp* was subjected to LAB fermentation to obtain both fermented solid and supernatant fractions (article under review). These fractions were then evaluated using an *in vitro* simulated gastrointestinal digestion model (INFOGEST).

Protein content in the digestion liquid fraction was measured, and the results are shown in Fig. 2(A). The protein concentrations were as follows: 25.2 g/100 g, 25.2 g/100 g, and 25.6 g/100 g for the digest-solid (control, 5% LP, and 5% LC, respectively), and 29.5 g/100 g, 29.4 g/100 g, and 30.3 g/100 g for

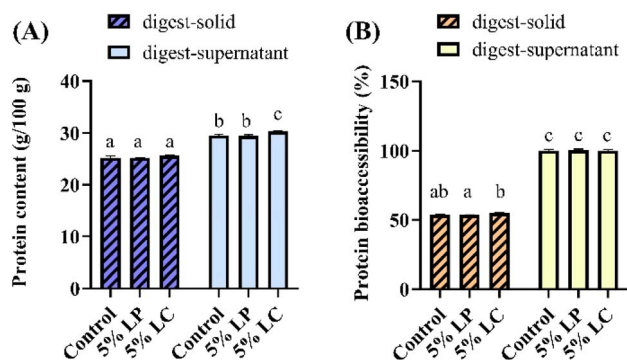


Fig. 2 Protein content (A) and protein bioaccessibility (B) in the digested liquid of SC-CO<sub>2</sub>-defatted *spirulina* (SC-D-*Sp*) fermented fractions (solid and supernatant) after *in vitro* simulated gastrointestinal digestion. LP: *Lactiplantibacillus plantarum*; LC: *Lactocaseibacillus casei*. Results are presented as mean  $\pm$  SD of three independent replicates.

the digest-supernatant (control, 5% LP, and 5% LC, respectively). Significant differences ( $p < 0.05$ ) were observed among the different treatments within the digest-solid fraction or the digest-supernatant fraction.

However, the protein content in the digest-supernatant fraction (control, 5% LP, and 5% LC) was significantly higher ( $p < 0.05$ ) than that in the digest-solid fraction. Moreover, although the initial protein content of the fermented solids fraction was higher than that of the fermented supernatant fraction, as previously reported,<sup>23</sup> this trend was reversed after *in vitro* simulated gastrointestinal digestion. This result may suggest that proteins and peptides in the fermented supernatant fraction were more readily released into the soluble bioaccessible fraction during *in vitro* digestion. Protein digestibility and protein bioaccessibility are closely related parameters commonly used to evaluate protein release during gastrointestinal digestion.<sup>15</sup> However, it should be noted that *in vitro* digestion models estimate bioaccessibility rather than actual intestinal absorption or *in vivo* bioavailability.

As can be seen in Fig. 2(B), the protein bioaccessibility was as follows: 53.7%, 53.6%, and 54.8 for the digest-solid (control, 5% LP, and 5% LC, respectively), and 100.0%, 100.4%, and 100.0% for the digest-supernatant (control, 5% LP, and 5% LC, respectively). Protein bioaccessibility in the digest-supernatant fractions approached 100%, whereas values in the digest-solid fractions remained close to 50%. The digest-supernatant showed a significant increase in protein bioaccessibility after *in vitro* digestion compared to the digest-solid. This behavior could be associated with the presence of more soluble low-molecular-weight peptides and partially hydrolyzed proteins in the fermented supernatant fraction, which may facilitate their release during gastrointestinal digestion.

As a consequence, the protein content and protein bioaccessibility in the digest-solid (control, 5% LP, and 5% LC) and digest-supernatant (control, 5% LP, and 5% LC) have indicated that the digest-supernatant in SC-D-*Sp* may represent an interesting ingredient for further exploration in functional food formulations.



## Bioactive peptide sequences

*Spirulina* is rich in proteins and amino acids, making it an ideal substrate for LAB fermentation. LAB fermentation has been shown to enhance protein breakdown and promote the release of bioactive peptides.<sup>10</sup> In this study, peptide sequences associated with potential bioactive properties were identified in the SC-D-*Sp* fermented fractions (solid and supernatant) following *in vitro* simulated gastrointestinal digestion. Previous research has demonstrated that fermentation alters the nutritional profile and antioxidant properties of *Spirulina*. Notably, proteolysis during fermentation significantly reduced phycocyanin and allophycocyanin content while increasing the ratio of free essential to non-essential amino acids.<sup>12</sup>

All peptide sequences identified through comparison with the BIOPEP-UWM database<sup>26</sup> are summarized in Table S1. It should be noted that the reported bioactivities were predicted based on sequence similarity and were not experimentally validated in the present study. As shown in the table, a significant reduction ( $p < 0.05$ ) in the number of bioactive peptide sequences was observed post-digestion compared with the non-digested SC-D-*Sp* fermented fractions (solid and supernatant), suggesting possible peptide degradation or transformation during digestion.

The six types of active sequences with (i) antioxidant (active sequences include WG, GW, LY, YQ, LK, LY, NEN, GGE, and GSH), (ii) hypouricemic (active sequence contains TL), (iii) antibacterial (active sequence contains AA), (iv) hypotensive (active sequence includes AA), (v) antithrombotic (active sequence contains PG), and (vi) binding activity (calcium binding) (active sequence contains EG) are shown in Fig. 3(A), and (vii) angiotensin-converting enzyme's (ACE's) inhibitory activity is presented in Fig. 3(B). Besides, the decrease in sequence abundance post-digestion might result from structural changes to proteins or peptides during gastrointestinal simulation. Investigating these changes further could provide insights into the mechanisms of peptide transformation and stability.

Peptide sequences potentially associated with hypouricemic activity included TL (Fig. 3A). Hyperuricemia (HUA, plasma uric acid  $> 7 \text{ mg dL}^{-1}$ ) is a metabolic syndrome caused by excessive production and/or decreased excretion of uric acid due to purine metabolic disorders in the body.<sup>28</sup> Previous researchers have illustrated that marine-derived bioactive peptides could exhibit antihyperuricemic properties associated with the preparation methods, mechanism of action, and potential application.<sup>29</sup> In a similar study, the water extracts of *Cyclocarya paliurus* leaves (CPE) could exhibit hypouricemic effects by improving purine metabolism and attenuate kidney injury by improving arachidonic acid metabolism and alleviating kidney inflammation.<sup>30</sup>

The antibacterial activity sequences include AA (Fig. 3A). Previous studies showed that the antibacterial peptide AQ-1766 (LWFYTMWH), extracted from the marine microalgae *Tetraselmis suecica*, has been previously reported to exhibit activity against Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*), as well as against

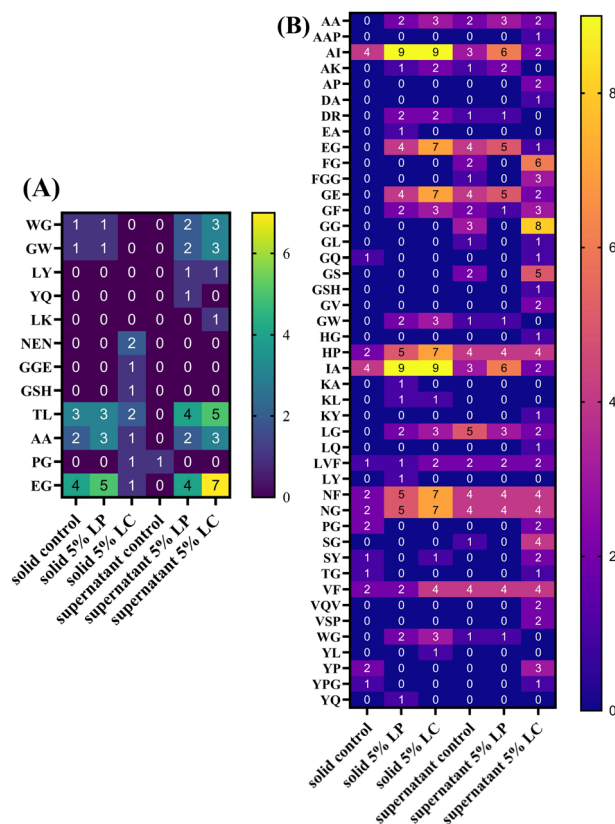


Fig. 3 Peptide sequences identified in the digested liquid of SC-CO<sub>2</sub>-defatted *spirulina* (SC-D-*Sp*) fermented fractions (solid and supernatant) after *in vitro* simulated gastrointestinal digestion. LP: *Lactiplantibacillus plantarum*; LC: *Lactocaseibacillus casei*. (A) Peptide sequences associated with potential antioxidant activity (WG, GW, LY, YQ, LK, LY, NEN, GGE, GSH), hypouricemic activity (TL), antibacterial and hypotensive activity (AA), antithrombotic activity (PG), and calcium-binding activity (EG). (B) Peptide sequences associated with potential ACE inhibitor activity sequences.

Gram-positive bacterial strains (*Bacillus cereus*, Methicillin-resistant *Staphylococcus aureus*, *Listeria monocytogenes*, and *Micrococcus luteus*).<sup>31</sup> Ref. 32 summarized the mechanism of antibacterial peptides with a clear cationic character from microalgae, rather similar to those peptides described in higher eukaryotes, which involved the disruption of the cell membrane after specific insertion into the bacterial cell membrane. Peptide fractions of the microalga *Chlorella vulgaris* have also been evaluated for their antibacterial activity, with the protein fraction with 62 kDa hydrolyzed by pepsin digestion, and the antibacterial activity determined against *E. coli* CECT 434. (ref. 33)

The sequences of antithrombotic activity include PG (Fig. 3A). *Spirulina* extracts showed strong antithrombotic properties with washed rabbit platelets.<sup>34</sup> Calcium-binding peptide sequences were also identified in the digested fractions (Fig. 3A).

The hypotensive activity sequences include AA (Fig. 3A). Hypertension is regarded as a widespread disease that has a direct correlation with the risk of other cardiovascular



diseases.<sup>35</sup> The identification of peptide sequences previously associated with ACE inhibitory activity may be related to the proteolytic action of LAB during fermentation, particularly *L. plantarum*.<sup>10</sup> The relationship between *in vitro* ACE-inhibitory activity and amino acid sequence has been established by previous studies. For instance, amino acid residues with bulky side chains and hydrophobic side chains were preferred for ACE inhibition by measuring the *in vitro* ACE inhibitory activity of 168 types of dipeptides.<sup>36</sup>

Several peptide sequences identified in the digested fractions have been previously associated with antioxidative activity, including WG, GW, LY, YQ, LK, LY, NEN, GGE, and GSH (Fig. 3A). Reactive oxygen species (ROS) play a significant role as growth factors and intercellular signaling regulators in maintaining the metabolic balance between oxidation and antioxidants.<sup>37</sup> The hydrophobic amino acids can easily cross the cell membrane lipid bilayer to scavenge the ROS in cells.<sup>38</sup> Moreover, hydrophobic amino acids could enhance the affinity and reactivity of peptides to cell membranes and increase their accessibility to lipid-soluble ROS, thereby terminating lipid peroxidation.<sup>39</sup>

Similarly, the application of fermentation to enhance the production of bioactive peptides has been investigated by many researchers, for instance, the investigation of solid-state fermentation of red seaweed (*Pyropia* spp.) with lactic acid bacteria on the effects on protein profiles and *in vitro* digestibility.<sup>40</sup> In that study, where red seaweed was fermented with *Limosilactobacillus reuteri* and *Lacticaseibacillus paracasei* (at a 1:2 ratio) for 33 days, the soluble protein content and 58 peptide segments were determined, and their biological functions were predicted.

Our study demonstrated that *in vitro* simulated gastrointestinal digestion of fermented SC-D-*Sp* released peptide

sequences potentially associated with antioxidant, hypouricemic, antibacterial, hypotensive, antithrombotic, and calcium-binding activities. Besides, the digest-supernatant fraction (control, 5%LP, and 5%LC) showed a higher abundance of predicted bioactive peptide sequences. This finding may support further investigation of SC-D-*Sp* fermented fractions as potential ingredients for functional food applications.

### Analysis of mineral content

The mineral composition in the digested liquid of SC-D-*Sp* fermented fractions (solid and supernatant) after *in vitro* simulated gastrointestinal digestion was analyzed. Mineral concentrations in the digested fraction are presented in Fig. 4, and the corresponding mineral bioaccessibilities are shown in Fig. 5.

As illustrated in Fig. 4, the major minerals identified include calcium (Ca), phosphorus (P), magnesium (Mg), and potassium (K), with these data consistent with a previous study.<sup>41</sup> Additionally, the trace minerals iron (Fe), selenium (Se), and copper (Cu) were also detected in the digested liquid. The measured concentrations ranged from 16.0 to 22.7 mg L<sup>-1</sup> for Mg, 105.1 to 173.0 mg L<sup>-1</sup> for P, 424.0 to 477.0 mg L<sup>-1</sup> for K, 33.1 to 97.0 mg L<sup>-1</sup> for Ca, 0.523 to 0.766 mg L<sup>-1</sup> for Fe, 66.8 to 117.7 μg L<sup>-1</sup> for Cu, and 23.4 to 33.9 μg L<sup>-1</sup> for Se. Notably, the highest concentrations of Mg, K, Fe, Cu, and Se were observed in the digest-supernatants (5% LP and 5% LC). In contrast, Ca levels were consistently higher in the digest-solid (control, 5% LP, and 5% LC) compared to their respective supernatants.

Fig. 5 further demonstrated that mineral bioaccessibility for Mg, P, K, Fe, Cu, and Se was significantly higher in the digest-supernatants (5% LP and 5% LC) than in the corresponding solid fractions. These findings suggested that the SC-D-*Sp* fermented supernatant fraction was more bioaccessible after

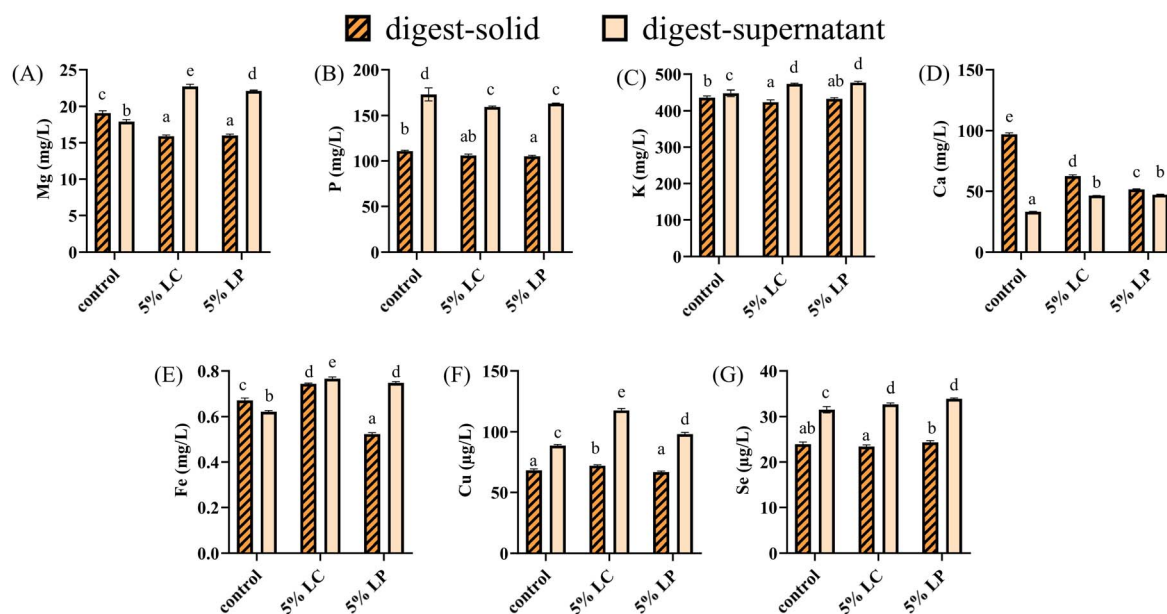


Fig. 4 Mineral composition in the digested liquid of SC-CO<sub>2</sub>-defatted *spirulina* (SC-D-*Sp*) fermented fractions (solid and supernatant) after *in vitro* simulated gastrointestinal digestion. LP: *Lactiplantibacillus plantarum*; LC: *Lacticaseibacillus casei*. Results are presented as mean ± SD of three independent replicates.



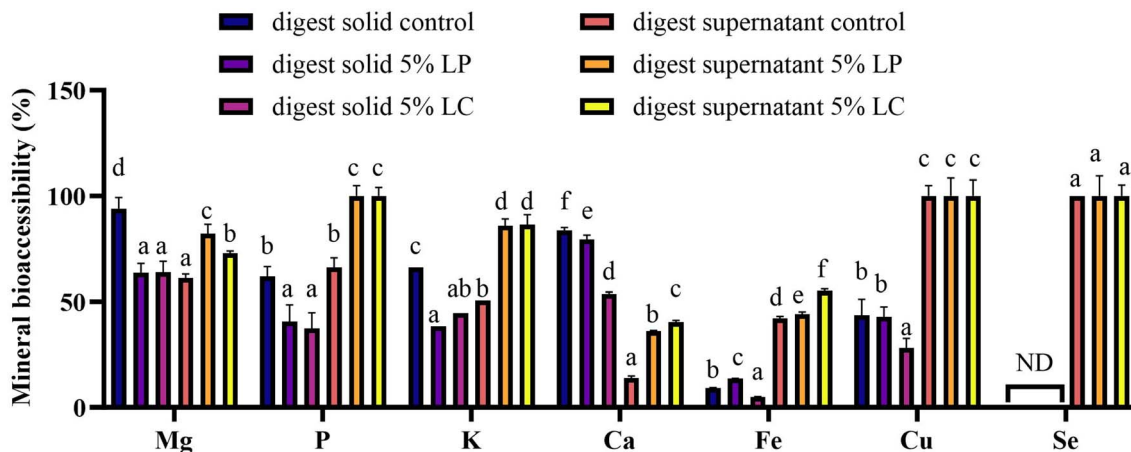


Fig. 5 Mineral bioaccessibility in the digested liquid of SC-CO<sub>2</sub>-defatted *spirulina* (SC-D-*Sp*) fermented fractions (solid and supernatant) after *in vitro* simulated gastrointestinal digestion. LP: *Lactiplantibacillus plantarum*; LC: *Lacticaseibacillus casei*. Results are presented as mean  $\pm$  SD of three independent replicates.

digestion, implying its potential for enhanced mineral absorption in the human digestive system. This indicates promising applications for the SC-D-*Sp* fermented fraction, particularly its supernatant fraction, in functional food development and human health promotion.

However, Ca bioaccessibility exhibited the opposite trend, with the digest-supernatant (control, 5% LP, and 5% LC) showing markedly lower values than the corresponding digest-solid fractions. This behavior may be related to the preferential association of calcium with insoluble or less soluble matrix components remaining in the solid fraction, as well as to possible interactions with proteins, peptides, or other negatively charged compounds generated during digestion. Regarding Fe bioaccessibility, the digest-solid fractions showed the lowest values: 9.0% for the control, 13.5% for 5% LP, and 4.8% for 5% LC. These results are consistent with earlier findings reporting low Fe bioaccessibility in *Spirulina*, contributing approximately 6% of the daily recommended intake.<sup>42</sup> The limited Fe bioaccessibility may be explained by interactions with dietary fiber, phenolic compounds, or other matrix constituents capable of forming insoluble complexes during digestion, thereby reducing its release into the bioaccessible fraction. Moreover, Salgado *et al.*, 2024 (ref. 42) also found that Cu and Se were among the most bioaccessible minerals in digested supernatant fractions.

Assessing mineral bioaccessibility after gastrointestinal digestion provides insight into mineral solubility and potential release from the food matrix. However, these results should be interpreted as bioaccessibility data and not as direct evidence of intestinal absorption or *in vivo* bioavailability. Overall, the mineral bioaccessibility observed in the digested fractions supports their potential value for the development of nutritionally enhanced food products.

### Analysis of total antioxidant capacity (TAC)

The *in vitro* antioxidant activity of peptides is typically evaluated using two main mechanisms: hydrogen atom transfer (HAT), as measured by the ORAC assay,<sup>9</sup> and single electron transfer

(SET), assessed through DPPH $\cdot$  or ABTS $^{\cdot+}$  assays, as well as scavenging activity against hydroxyl and superoxide radicals generated during oxidative processes.<sup>43</sup>

To evaluate the TAC of the digested liquid of SC-D-*Sp* fermented fractions (solid and supernatant) after digestion, both TEAC and ORAC assays were conducted. As shown in Fig. 6 (A), TEAC values for the digest-solid fraction were 194.3  $\mu$ M TE (control), 206.3  $\mu$ M TE (5% LP), and 209.3  $\mu$ M TE (5% LC), while the digest-supernatant fraction showed higher values: 249.1  $\mu$ M TE (control), 297.7  $\mu$ M TE (5% LP), and 307.7  $\mu$ M TE (5% LC). These results indicate that the digest-supernatant fractions (particularly for 5% LP and 5% LC) exhibited significantly higher antioxidant capacity than the corresponding digest-solid fractions.

Similarly, the results of ORAC (Fig. 6B) show a consistent trend. The antioxidant capacity of the digest-solid fraction was 17 359.7  $\mu$ M TE (control), 17 884.7  $\mu$ M TE (5% LP), and 20 161.0  $\mu$ M TE (5% LC), while the digest-supernatant fraction yielded 18 161.7  $\mu$ M TE (control), 20 764.7  $\mu$ M TE (5% LP), and 20 479.3  $\mu$ M TE (5% LC). Notably, the digest-supernatant fractions (5% LP and 5% LC) also showed higher ORAC values, consistent

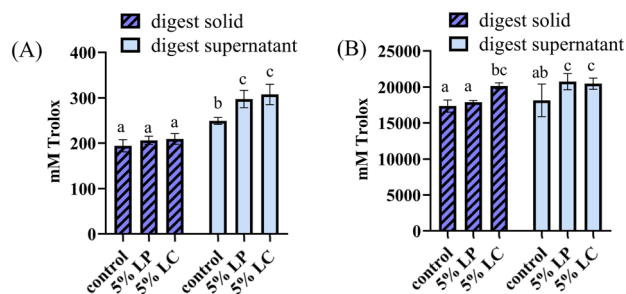


Fig. 6 TEAC (A) and ORAC (B) values of SC-CO<sub>2</sub>-defatted *spirulina* (SC-D-*Sp*) fermented fractions (solid and supernatant) after *in vitro* simulated gastrointestinal digestion. LP: *Lactiplantibacillus plantarum*; LC: *Lacticaseibacillus casei*. Results are presented as mean  $\pm$  SD of three independent replicates.



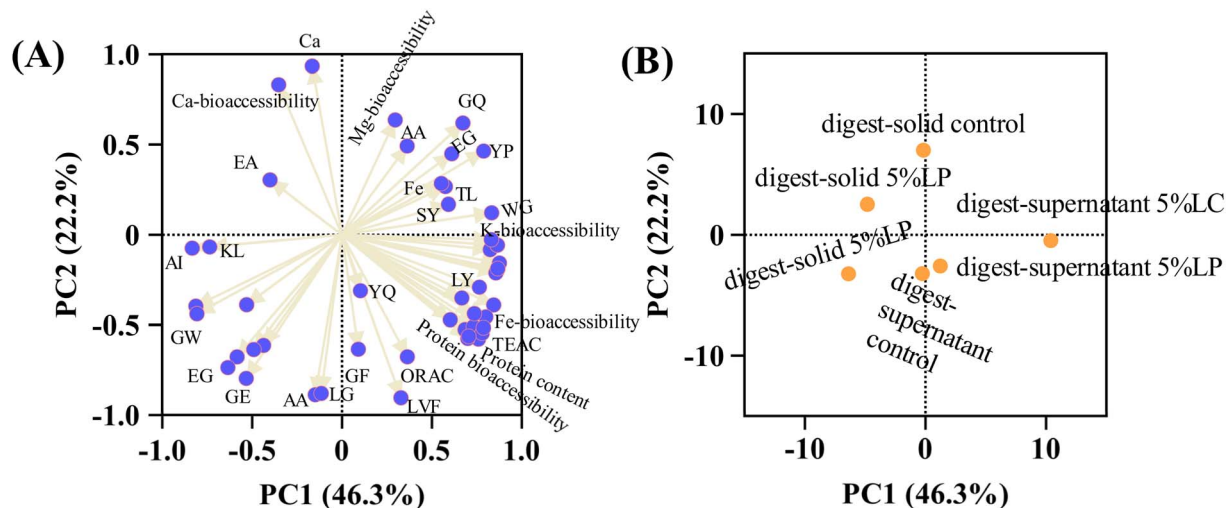


Fig. 7 Principal component analysis (PCA) showing loading plots (A) and PC score plots (B) of SC-CO<sub>2</sub>-defatted *spirulina* (SC-D-Sp) fermented fractions (solid and supernatant) after *in vitro* simulated gastrointestinal digestion. LP: *Lactiplantibacillus plantarum*; LC: *Lacticaseibacillus casei*.

with the TEAC results. The higher antioxidant capacity observed in the digest-supernatant fractions may be associated with the release of soluble antioxidant peptides and other low-molecular-weight compounds during fermentation and gastrointestinal digestion.

These findings are supported by previous studies.<sup>10</sup> For instance, the antioxidant capacity of the bioaccessible fractions of fermented *Spirulina* beverages produced with *Lactobacillus* spp. was evaluated using an *in vitro* system. The highest DPPH and ABTS values were observed in the intestinal > gastric > oral phases for both laboratory (L) and commercial (C) samples, respectively.<sup>10</sup> Similarly, the present study showed increased TAC values after gastrointestinal digestion, particularly in the digest-supernatant fractions. Previous studies have suggested that the increase in antioxidant capacity during intestinal digestion may be associated with the release of antioxidant compounds and peptides from the food matrix.<sup>44</sup> In that study, DPPH radical scavenging activity and total phenolic content increased by 79% and 320%, respectively, after fermentation and *in vitro* digestion processes.

In line with these findings, the results of ref. 40 showed that solid-state fermented red seaweed (*Pyropia* spp.) samples were more readily digested and exhibited significantly higher antioxidant activity compared to unfermented samples. Specifically, the ABTS scavenging activity in fermented samples increased by 25.98%, supporting the contribution of fermentation and digestion processes to the release of antioxidant compounds.

Overall, the results suggest that the digest-supernatant fractions corresponding to 5% LP and 5% LC presented higher antioxidant capacity after gastrointestinal digestion, supporting their further evaluation as ingredients for functional food applications.

#### Analysis of principal component analysis (PCA)

PCA was performed using protein content, bioactive peptides (BPs), mineral composition, mineral bioaccessibility, and

TAC data obtained from the digest-solid and digest-supernatant fractions of fermented SC-D-Sp after *in vitro* simulated gastrointestinal digestion. This analysis was used to evaluate the relationships among nutritional and bioactive variables associated with the bioaccessible fractions after gastrointestinal digestion. Two principal components (PCs) were selected based on their contribution to the variance. PC1 and PC2 explained 46.3% and 22.2% of the total variance, respectively, together accounting for 68.4% of the total variability.

In Fig. 7, both the loading plot and the PCA score plot are presented. In Fig. 7A, the variables were distributed across four quadrants, with PC1 and PC2 effectively separating them into distinct groups. Points located in the first quadrant, such as Mg-bioaccessibility, AA, GQ, Fe, YP, TL, SY, and WG, showed positive correlations with both PC1 and PC2. In contrast, points in the third quadrant displayed negative correlations with both PC1 and PC2.

Fig. 7B illustrates the PCA score plot for the six digested liquid fractions, including the digest-solid and digest-supernatant fractions for control, 5% LP, and 5% LC. Notably, digest-supernatant-5% LP and digest-supernatant-5% LC were positively associated with PC1, indicating a closer relationship with variables contributing positively to this component. The remaining four samples were positioned on the negative side of the X-axis. In contrast, digest-solid control and digest-solid 5% LP showed a positive association with PC2, whereas the remaining samples were negatively associated.

Overall, the digest-supernatant-5% LP and digest-supernatant-5% LC showed closer associations with variables related to bioactive peptides, antioxidant capacity, and mineral bioaccessibility. These findings support the potential relevance of the digest-supernatant fractions as sources of bioaccessible nutritional and bioactive compounds after *in vitro* gastrointestinal digestion.



## Conclusions

In this study, SC-D-*Sp* was systematically evaluated as a novel, sustainable fermentation substrate for LAB strains, specifically 5% LP and 5% LC. The fermentation process generated two distinct fractions (solid and supernatant), which were subsequently subjected to *in vitro* simulated gastrointestinal digestion. After digestion, the bioaccessible fractions obtained were analyzed for protein content, protein bioaccessibility, bioactive peptides, mineral composition, mineral bioaccessibility, and TAC. The digest-supernatant fractions exhibited significantly higher protein concentrations than the digest-solid fractions. Notably, several peptide sequences associated with potential bioactive properties were identified in the digested fractions, including sequences previously related to ACE inhibitory activity. Additional potential bioactivities were also associated, with the identified peptide sequences, including antioxidant-related sequences (WG, GW, LY, YQ, LK, NEN, GGE, and GSH), hypouricemic-associated sequences (TL), antibacterial and hypotensive-associated sequences (AA), antithrombotic-associated sequences (PG), and calcium-binding-associated sequences (EG). In total, multiple categories of potential bioactivities were identified based on the detected peptide sequences. Moreover, TAC measurements demonstrated that the digested supernatants, particularly those from the 5% LP and 5% LC treatments, had notably higher antioxidant capacities compared to the corresponding digest-solid fractions ( $p < 0.05$ ), which may be associated with the presence of antioxidant-related peptide sequences and other soluble compounds released during digestion.

PCA results delineated clear separation between treatments, with 5% LP and 5% LC supernatants showing associations with variables related to peptide content, mineral bioaccessibility, and antioxidant capacity after digestion. Overall, these findings suggest that SC-D-*Sp*, particularly when fermented with LAB strains and then subjected to *in vitro* simulated gastrointestinal digestion, may support further investigation of this substrate for fermented functional food applications.

## Author contributions

Yixuan Liu: investigation, formal analysis, methodology, data curation, writing – original draft. Noelia Pallarés: formal analysis, validation, writing – review and editing. Albert Sebastià: visualization, writing – review and editing. Pedro V. Martínez-Culebras: validation, writing – review and editing. Francisco J. Martí-Quijal: resources, writing – review and editing. Juan Manuel Castagnini: supervision, resources, writing – review and editing. Yuthana Phimolsiripol: funding acquisition, writing – review and editing. Houda Berrada: supervision, validation, writing – review and editing. Francisco J. Barba: supervision, funding acquisition, writing – review and editing.

## Conflicts of interest

There are no conflicts to declare.

## Data availability

The data used is provided in the manuscript. Supplementary information (SI): peptide and bioactive sequences evaluated. See DOI: <https://doi.org/10.1039/d6fb00048g>.

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