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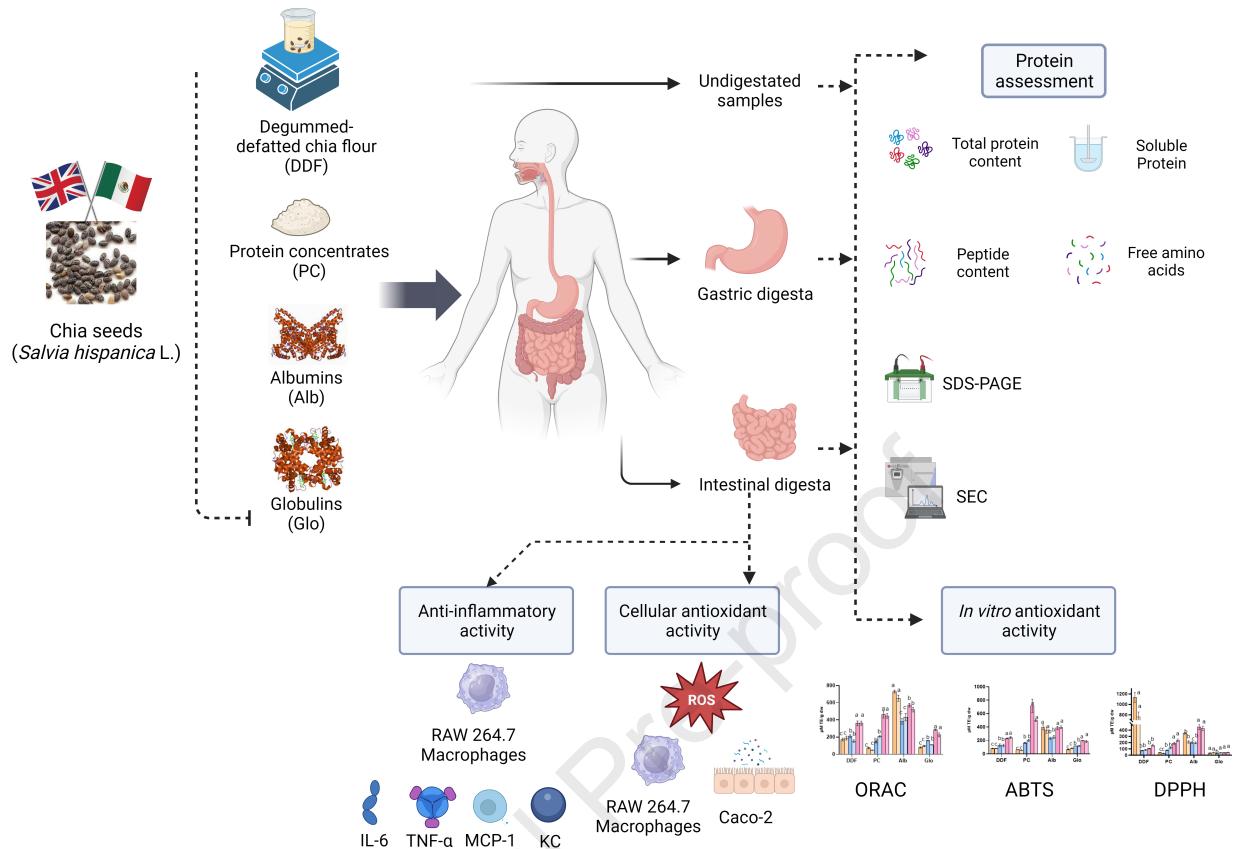
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## CRediT author statement

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**A comparative study of the digestion behavior and functionality of protein from chia (*Salvia hispanica L.*) ingredients and protein fractions**

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1    **Abstract**

2    Protein derived from chia (*Salvia hispanica* L.), characterized by a balanced amino acid  
3    composition, represents a potentially healthier and environmentally friendly alternative poised  
4    for innovation within the plant-based food sector. It was hypothesized that the growing location  
5    of chia seeds and processing techniques used might influence protein digestion patterns, which  
6    in turn could affect the biological functions of the digestion products. To examine this  
7    hypothesis, we assessed the gastrointestinal fate of degummed-defatted flour (DDF), protein  
8    concentrate (PC), and isolated albumin (Alb) and globulin (Glo) fractions. Furthermore, we  
9    compared the antioxidant and anti-inflammatory activities of the resulting digesta by means of  
10   *in vitro* and cellular assays. Post-gastrointestinal digestion, the PC exhibited elevated levels of  
11   soluble protein (7.6 and 6.3% for Mexican and British PC, respectively) and peptides (24.8 and  
12   27.9%, respectively) of larger molecular sizes compared to DDF, Alb, and Glo. This can be  
13   attributed to differences in the extraction/fractionation processes. Leucine was found to be the  
14   most prevalent amino acids in all chia digesta. Such variations in the digestive outcomes of chia  
15   protein components significantly influenced the bioactivity of the intestinal digestates. During  
16   gastrointestinal transit, British Glo exhibited the best reactive oxygen species (ROS) inhibition  
17   activity in oxidative-stressed RAW264.7 macrophages, while Mexican digesta outperformed  
18   British samples in terms of ROS inhibition within the oxidative-stressed Caco-2 cells.  
19   Additionally, both Mexican and British Alb showed effectively anti-inflammatory potential,  
20   with keratinocyte chemoattractant (KC) inhibition rate of 82 and 91%, respectively.  
21   Additionally, Mexican PC and Alb generally demonstrated an enhanced capacity to mitigate  
22   oxidative stress and inflammatory conditions *in vitro*. These findings highlight the substantial

23 potential of chia seeds as functional food ingredients, resonating with the shifting preferences

24 of health-conscious consumers.

25 **Keywords:** anti-inflammatory, antioxidant, chia, *in vitro* digestion, protein concentrate,

26 globulins

27

28    **1. Introduction**

29    Gastrointestinal digestion, a complex interplay of physical and chemical actions, is essential  
30    for breaking down food via various enzymes, thereby releasing nutrients for organismal  
31    absorption and utilization (Santos-Hernández et al., 2020). Ideally, the nutritional quality of  
32    foods, particularly its protein fraction, is best assessed through *in vivo* studies in humans or  
33    animals. However, these studies often present challenges such as high costs, technical  
34    complexities, time constraints, and ethical considerations (Dupont et al., 2019, Sousa et al.,  
35    2020). Consequently, the development of *in vitro* digestion models that accurately replicate  
36    human digestion processes has become a necessary and efficient alternative to *in vivo*  
37    experiments. Recognizing this need, a harmonized *in vitro* digestion protocol, reflecting human  
38    physiological conditions, was formulated by an international collaboration of scientists from  
39    over 35 countries, under the COST Action INFOGEST initiative (Brodkorb et al., 2019). The  
40    biological relevance and efficacy of the protocol has been validated for a wide range of proteins,  
41    particularly for milk, oat, sorghum, peanut and bean proteins through comparative studies with  
42    porcine and human digests (Egger et al., 2017, Sanchón et al., 2018, Sánchez-Velázquez et al.,  
43    2021, Sousa et al., 2020).

44    Chia (*Salvia hispanica L.*) seed, a pseudocereal grain originally from Southern Mexico and  
45    Northern Guatemala, has become a popular food due to its excellent nutritional composition  
46    consisting of 30–34% dietary fiber, 26–41% carbohydrates, and 29-39% lipids rich in  
47    polyunsaturated fatty acids (Sánchez-Velázquez et al., 2023). As compared to most consumed  
48    cereal grains, chia seeds are characterized by a higher protein content (18-25% dry weight, dw)  
49    and present a balanced amino acid composition that fulfills the dietary recommendations

50 (Muñoz et al., 2012, FAO/WHO, 1991, FAO/WHO, 2011). Moreover, *in vitro* and *in silico*  
51 approaches have revealed that proteins from chia have a relevant role in health promotion  
52 through the release of bioactive peptides during digestion (Orona-Tamayo et al., 2015, Chim-  
53 Chi et al., 2018, Grancieri et al., 2019c, Martínez Leo and Segura Campos, 2020). In addition  
54 to macronutrients, chia seeds are a rich source of minerals (4–6% dw, with 6 times more Ca  
55 than milk and 1.6 times more Fe than chickpea), vitamins (A, B, K, E, and D) and  
56 phytochemicals such as polyphenols and sterols (Cahill, 2003, Segura-Campos et al., 2014,  
57 Ullah et al., 2016, Coorey et al., 2012, Gu et al., 2021). Among polyphenols, phenolic acids  
58 (e.g., caffeic, chlorogenic, ferulic and rosmarinic acids) and flavanols (e.g., myricetin, quercetin  
59 and kaempferol) are particularly abundant in chia seeds. The outstanding nutritional  
60 composition of chia seeds have positioned this pseudocereal as a healthier and added-value  
61 alternative for food innovation considering the new consumer preferences for low-sugar,  
62 gluten-free alternatives, high fiber and protein, or mineral-enriched products, among others  
63 (Mesías et al., 2023). However, it is important to highlight that chia nutritional composition can  
64 vary based on external factors like climate, geographical location, soil characteristics, and year  
65 of cultivation (Grancieri et al., 2019a). Ayerza (2009) specifically demonstrated that factors  
66 such as temperature, climate, and altitude significantly influence the protein and oil content and  
67 fatty acid composition in chia seeds. This variability underlines the importance of considering  
68 environmental variables when evaluating the nutritional quality of chia seeds from different  
69 locations.

70 Generally, chia flour is obtained from seeds after partial oil extraction, resulting in a fiber-  
71 protein rich ingredient, suitable to enhance the nutritional properties of several food products

72 (Aranibar et al., 2018, Mas et al., 2020). Extraction and fractionation processes are often applied  
73 to remove chia mucilage and produce protein concentrates from defatted chia flour (Zettel and  
74 Hitzmann, 2018). Degumming is performed to extract and/or remove chia mucilage by different  
75 procedures. Chia mucilage is a hydrocolloid with interesting technological properties used in  
76 the food industry as thickener, emulsifier, gelling agent, etc (Zettel and Hitzmann, 2018). Food  
77 processing of defatted and degummed chia flour (DDF) may continue with the wet protein  
78 extraction and isoelectric precipitation to yield chia protein concentrates (PC) and/or protein  
79 isolates that could find different applications in the food industry to develop plant-based foods.  
80 Plant-based foods are generally lacking in certain essential amino acids and have lower  
81 digestibility due to the presence of antinutritional factors, thus, consequently regarded as of  
82 lower quality as compared to conventional sources of animal protein (Tachie et al., 2023). In a  
83 previous study, we studied the effects of defatting, degumming, and protein  
84 extraction/fractionation of chia flour on protein quality (Wang et al., 2023). The outcomes of  
85 the study demonstrated that defatting and degumming of chia flour followed by further wet  
86 protein extraction and isoelectric precipitation increased *in vitro* protein digestibility corrected  
87 amino acid score (IVPDCAAS) and reduced the concentration of certain antinutrients  
88 (phenolics and phytic acid) in PC as compared to DDF. In this work, it was hypothesized that  
89 the differences in the protein content and composition between chia seeds grown in different  
90 locations and chia seed processing can affect the protein digestion pattern and ultimately the  
91 bioavailability and biological functions of chia protein digestion products. To test this  
92 hypothesis, DDF, PC, albumin (Alb) and globulin (Glo) fractions were prepared from chia  
93 flours from two different locations (United Kingdom and Mexico). Samples were digested

94 using the harmonized static INFOGEST 2.0 method (Brodkorb et al., 2019). The objective of  
95 this study was to: 1) assess and compare the protein digestion patterns of defatted chia seed  
96 fractions (DDF), protein concentrates (PC), albumins (Alb), and globulins (Glo) derived from  
97 British and Mexican chia seeds; 2) establish how the distribution of protein digestion products  
98 correlates with *in vitro* antioxidant activity through biochemical and cellular assays; and 3)  
99 assess the anti-inflammatory properties of the gastrointestinal digestates by evaluating the  
100 inhibition of nitric oxide (NO), interleukin-6 (IL-6), keratinocyte chemoattractant (KC),  
101 monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-alpha (TNF- $\alpha$ ).  
102 Accomplishing these objectives will significantly contribute to the existing knowledge on chia  
103 seed protein digestibility and aims to provide crucial insights into the impact of chia protein  
104 digestion on health-promoting properties. This understanding is essential for advancing the  
105 application of chia seeds in health-centric dietary solutions and innovative food products.

106 **2. Materials and Methods**

107 **2.1. Preparation of chia ingredients and protein fractions**

108 Chia seeds (*Salvia hispanica* L.) were obtained from United Kingdom (British seeds grown at  
109 Great Tey in Essex in 2019, Hodmedod's company, Essex, UK) and Mexico (provided by  
110 producers located in Guadalajara, Mexico, harvest of January 2019). Degummed-defatted chia  
111 flour (DDF), protein concentrates (PC), albumin (Alb) and globulin (Glo) fractions were  
112 prepared as described in a previous study (Wang et al., 2023). Briefly, the mucilage from the  
113 samples was removed by sonication at 50% amplitude, 750W for 4 min in an ultrasonic bath  
114 (Sonics, Tacoma, WA, USA), and manually separated from the seeds with the aid of a sieve  
115 (200 mm/30 mesh). The degummed seeds were ground using a commercial coffee grinder

116 (De'Longhi KG200, Treviso, Italy) and the oil was extracted using hexane (1:5, w:v) under  
117 constant stirring for 2 h. The slurry was centrifuged (4,816 x g, 20 min, 4 °C) and DDF was left  
118 overnight under the fume cupboard and stored at 4 °C in vacuum-sealed bags. PC were  
119 produced from DDF by alkaline solubilization (pH 10) coupled to isoelectric precipitation (pH  
120 4.5) and final centrifugation at 8,288 x g, 15 min at 4 °C (Avanti J-30I, Beckman Coulter, Brea,  
121 CA, USA). For Alb and Glo fractionation, DDF was dispersed in distilled water (1:40, w/v),  
122 stirred for 1 h at 4 °C and centrifuged at 13,000 x g for 20 min at 4 °C. Supernatant (Alb fraction)  
123 was collected and freeze-dried (Labconco, Kansas, MO, USA), ground and stored at 4 °C in  
124 vacuum-sealed bags. The pellet was resuspended in 50 mM Tris containing 0.4 M NaCl at pH  
125 8.0 (1:10, w/v), stirred and centrifuged as above. The supernatant (Glo fraction) was collected  
126 and freeze-dried, ground, and stored at 4 °C in vacuum-sealed bags.

127 **2.2. *In vitro* simulated gastrointestinal digestion (SGID)**

128 Chia samples were digested using a two-phase gastro-intestinal *in vitro* digestion model  
129 following a modified consensus INFOGEST 2.0 protocol (Brodkorb et al., 2019) with minor  
130 modifications. Briefly, all samples were mixed with a pre-warmed simulated salivary fluid (SSF)  
131 at a final ratio of 1:1 (w/v), intentionally excluding human salivary  $\alpha$ -amylase due to the  
132 predominantly protein content in the samples studied. Immediately, the mixture was combined  
133 with simulated gastric fluid (SGF) and freshly prepared porcine pepsin from gastric mucosa  
134 (E.C. 3.4.23.1, Merck, Darmstadt, Germany) to a final ratio of food to SGF of 1:1 (v/v) and  
135 enzyme activity of 2000 U/mL, respectively. The pH of the mixture was adjusted to 3 with 1 M  
136 HCl before the gastric digestion was simulated by incubating sample tubes at 37 °C for 120  
137 min. Thereafter, the gastric chyme was mixed with warmed simulated intestinal fluid (SIF) in

138 a final ratio of 1:1 (v/v) to simulate the intestinal phase, respectively. Fresh porcine bile extract  
139 and pancreatin from porcine pancreas (Merck, Darmstadt, Germany) solution were added by  
140 considering the final concentration of 2.5 mM and trypsin enzymatic activity of 100 U/mL,  
141 respectively. The chyme pH was adjusted to 7 with 1 M NaOH and incubated at 37 °C for 120  
142 min to complete 240 min *in vitro* digestion. The intestinal phase was stopped by heating in a  
143 boiling water bath for 5 min and immediately cooled in ice water. The digestates from both  
144 gastric (GP) and intestinal (IP) phases were stored at -80 °C and then freeze-dried (Labconco,  
145 Kansas City, MO, USA), ground and stored at 4 °C in vacuum-sealed bags until use.

146 **2.3. Total protein content**

147 The total protein content of protein samples was measured by the Dumas combustion method  
148 (AOAC, 1995) using the Trumac nitrogen analyzer (Leco Corporation, St Joseph, MA, USA).  
149 A conversion factor of 6.25 was used to convert nitrogen values to protein content. Results were  
150 expressed as g protein/100 g dw.

151 **2.4. Soluble protein, peptides, and free amino acids analysis**

152 Chia samples were dispersed at a final concentration of 1% (w/v) in 2 mL Milli-Q water. Flour  
153 dispersions were stirred on thermomixer C (Thermo Fisher Scientific, Waltham, MA, USA) for  
154 1 h and centrifuged (Eppendorf 5424R, Thermo Fisher Scientific, Waltham, MA, USA) at  
155 10,000 x g and 4 °C for 5 min. The supernatant was collected, and protein concentration was  
156 measured using the Pierce 660 nm protein assay (Thermo Fisher Scientific, Waltham, MA, USA)  
157 according to the manufacturer's instructions. Bovine serum albumin (Merck, Darmstadt,  
158 Germany) was used as standard. Samples were measured in a Synergy HT microplate (BioTek  
159 Instruments, Winooski, VT, USA) at 660 nm.

160 Total peptide content was measured by Pierce Quantitative Colorimetric Peptide Assay Kit  
161 (Thermo Fisher Scientific, Waltham, MA, USA) in filtrates obtained from supernatant  
162 ultrafiltration using 10 kDa molecular weight (MW) cut-off membranes (Thermo Fisher  
163 Scientific, Waltham, MA, USA). Absorbance was read at 480 nm using a Synergy HT  
164 microplate reader (BioTek Instruments, Winooski, VT, USA). The results were expressed as  
165 g/100 g dw.

166 For extraction of free amino acids, 200 mg from each sample were taken and homogenized in  
167 2 mL of 0.01 M HCl solution containing 10 µmol/mL norvaline as internal standard. This  
168 suspension was then centrifuged at 2,500 x g for 15 min and the supernatant was collected for  
169 subsequent analysis by reversed-phase high-performance liquid chromatography (RP-HPLC)  
170 and diode array detection (DAD) using an Agilent 1200 chromatographic system (Agilent  
171 Technologies, Inc., Wilmington, DE, USA) equipped with an G1329A automatic sampler.  
172 Separation of amino acids was performed into an Agilent Zorbax Eclipse Plus C18 column (4.6  
173 × 250 mm, with particle size of 5 µm) at 40 °C. Two solvents (A and B) were used as the mobile  
174 phase. Solvent A consisted of 10 mM Na<sub>2</sub>HPO<sub>4</sub>:10 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, pH 8.2:5 mM NaN<sub>3</sub> and  
175 solvent B was acetonitrile:methanol:water (45:45:10, v:v:v). The injection volume was 20 µL  
176 and the mobile phase flow rate was 1.5 mL/min. The gradient flow for chromatographic  
177 separation started from 2% B for 0.5 min followed by 57% B for 30 min then 100% B for 10  
178 min. Initial chromatographic conditions were set out for column re-equilibration between  
179 sample injections. Amino acid detection was performed using automated derivatization in the  
180 autosampler. Derivatization reagents: Borate buffers (0.4 M in water, pH 10.2), *o*-  
181 phthaldialdehyde (OPA, 10 mg/mL in 0.4 M borate buffer and 3-mercaptopropionic acid) and  
182 fluorenylmethoxycarbonyl (FMOC, 2.5 mg/mL in acetonitrile) were ready-made solutions  
183 supplied by Agilent. They were transferred from their container into an autosampler vial. DAD  
184 was set up for collecting two channels (Signal A 338 nm, to detect OPA derivatized amino acids  
185 and Signal B 262 nm, to detect FMOC-lys derivatized amino acids). Peak identification was

186 performed by retention time comparison with amino acid standards. Standard solutions of 20  
187 amino acids available from Agilent (1 nmol/µL) were used to prepare calibration curves.  
188 Calibration curves with standard concentration range from 10 to 1,000 nmol/mL of individual  
189 free amino acids were determined, with each concentration measured in triplicate. The linearity  
190 was evaluated by the calibration curves for each standard and least-squares regression lines  
191 relating the absorbance peak area.

192 **2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

193 To assess the distribution of polypeptides in chia samples as well as in their corresponding  
194 gastric digestates, SDS-PAGE was performed using reagents and equipment purchased from  
195 Lifescience Technologies (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, 10 µL of  
196 supernatant (**section 2.4.**) were mixed with 20 µL NuPAGE® lithium dodecyl sulfate (LDS)  
197 sample buffer (without  $\beta$ -mercaptoethanol), pH 8.4, making a total volume of 30 µL. Samples  
198 were heated at 70 °C for 10 min using a thermomixer C (Thermo Fisher Scientific, Waltham,  
199 MA, USA) and then centrifuged at 4 °C and 10,000 x g for 5 min (Eppendorf 5424R, Thermo  
200 Fisher Scientific, Waltham, MA, USA). For analysis, 13 µg protein/well and 5 µL of Novex®  
201 Sharp Prestained Protein Standard were loaded onto a 1.0 mm x 10 well 4–12% gradient Bis-  
202 Tris gel. The protein separation was run in a Mini Gel Tank at 200 V for 35 min using  
203 NuPAGE® 2-(N-morpholino) ethanesulfonic acid-SDS running buffer. For the albumin  
204 fraction with low MW protein fragments, aliquots of 10 µL were mixed in 20 µL Tricine SDS  
205 sample buffer (1X). For analysis, 13 µg peptide/well and 5 µL of PageRuler Unstained Low  
206 Range Protein Ladder Standard onto 1.0 mm x 10 well Novex 16% Tricine Gels. Protein  
207 separation was performed at 125 V for 90 min with Tricine SDS running buffer. Bands were  
208 stained using SimplyBlue and images were analyzed using ChemiDoc XRS+ and Quantity One

209 software (Bio-Rad, Hercules, CA, USA).

210 **2.6. Size exclusion chromatography**

211 To assess changes in peptide size distribution of chia samples during gastric and intestinal  
212 phases, supernatants (**section 2.4.**) of sample suspensions, were analyzed by size exclusion  
213 chromatography as described by Rieder et al. (2021) with modifications. A solvent made up of  
214 30% acetonitrile and 0.05% trifluoracetic acid (TFA) in water was used as the mobile phase.  
215 Supernatants (**section 2.4.**) were diluted 1:5 with mobile phase and filtered using a syringe filter  
216 (0.45 µm polyvinylidene difluoride (PVDF) membrane). Sample volumes of 10 µL were  
217 injected in HPLC-DAD (Waters, Milford, MA, USA) controlled by Empower (Waters, Milford,  
218 MA, USA) and eluted at 0.5 µL /min. Peptides were separated using a TSKgel column  
219 G2000SWXL (7,600 × 7.5 mm; Tosoh Bioscience GmbH, Stuttgart, Germany) over 30 min and  
220 detected at a wavelength of 214 nm. Peptide MW was estimated based on elution time of a  
221 standard peptide mixture that included angiotensin II (1,046 Da), met-enkephalin (573.611 Da),  
222 Leu-enkephalin (555.6 Da), Val-Tyr-Val (379.5 Da) and Gly-Tyr (235.24 Da).

223 **2.7. *In vitro* antioxidant activity**

224 Antioxidant activity was determined in flours before and after gastric and intestinal digestion  
225 using four methods: Oxygen Radical Absorbance Capacity (ORAC), 2,2-azino-bis-3-  
226 ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay, and 2,2-diphenyl-1-  
227 picrylhydrazyl (DPPH) radical scavenging assay and inhibition of intracellular reactive oxygen  
228 species (ROS) in *tert*-butyl hydroperoxide (*t*-BHP) challenged cell lines.

229 The ORAC method was executed according to Bautista-Expósito et al. (2021). The  
230 fluorescence of the samples was assessed using a battery of dilutions prepared in 75 mM sodium  
231 phosphate buffer (pH 7.4). The measurements were taken at 485 and 520 nm every 2 min for  
232 2.5 h, using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA). Data

233 were obtained using a Trolox standard curve (0 to 160 µM) and results were expressed as µM  
234 Trolox equivalents (TE)/g of sample.

235 The ABTS analysis was performed as described by (Martín-Diana et al., 2021). A series of  
236 sample dilutions was prepared in 0.1 M phosphate buffer with 0.15 M NaCl. Absorbances were  
237 measured at 734 nm every minute for 30 min, using the microplate reader. Data were obtained  
238 from a Trolox calibration curve (0 to 800 µM) and results were expressed as µM TE/g of  
239 sample.

240 The DPPH assay was conducted as previously reported by Brand-Williams et al. (1995). In this  
241 case, various dilutions of sample were prepared using Milli-Q water and absorbance was  
242 measured at 515 nm after 30 min of incubation. Data were obtained from a Trolox standard  
243 curve (0 to 200 µM) and results were expressed as µM TE/g of sample.

244 Cellular antioxidant activity was determined in two cell lines: murine RAW 264.7 macrophages  
245 (ATCC, TIB-71, Rockville, MD, USA) and human adenocarcinoma Caco-2 cells (ATCC,  
246 Rockville, MD, USA) cultured in Dulbecco's modified Eagle's Medium (DMEM) and  
247 Minimum Essential Medium Alpha (MEM- $\alpha$ ), respectively, both containing 10% fetal bovine  
248 serum (FBS) and 1% penicillin-streptomycin (P/S). Cells were maintained in an oven incubator  
249 with a 5% CO<sub>2</sub> atmosphere at 37 °C. For experiments, cells were seeded into black 96-well  
250 plates ( $5 \times 10^4$  cells/well) and allowed to attach overnight at 37 °C with 5% CO<sub>2</sub>. Cells were  
251 treated with intestinal digests (0.5 and 3 mg/mL) previously dissolved in complete DMEM with  
252 0.1% of FBS and filtered using sterile filters of 0.22 µm (Sarstedt AG & Co KG, Nümbrecht,  
253 Germany). Treated cells were incubated at 37 °C with 5% CO<sub>2</sub> for 18 h, washed twice with 100  
254 µL of ice-cold phosphate buffer saline (PBS) and incubated for 30 min at 37 °C and 5% CO<sub>2</sub> in

255 darkness with 150 µL of 10 µM dichloro-dihydro-fluoresceine diacetate (H<sub>2</sub>DCFDA) in PBS.  
256 After incubation, cells were washed again with PBS followed by treatment with 200 µL of 2.5  
257 mM *terc*-butylhydroperoxide (tBHP). Fluorescence intensity was recorded at  $\lambda_{\text{exc}} = 485$  nm and  
258  $\lambda_{\text{em}} = 535$  nm wavelengths on a Synergy HT microplate reader (BioTek Instruments Inc.,  
259 Winooski, VT, USA). Results were expressed as percentages concerning untreated cells  
260 (tBHP-). Data represents the mean and the standard deviation of eight biological replicates.

261 **2.8. *In vitro* anti-inflammatory activity**

262 Murine RAW264.7 macrophages were cultured in complete DMEM supplemented by 10% FBS  
263 and 1% P/S in a humidified incubator at 37 °C and 5% CO<sub>2</sub>. Cells were seeded in 96-well plates  
264 at a density of  $2.5 \times 10^4$  cells/well. After overnight attachment, the cells were treated with  
265 intestinal digests (0.5 and 3 mg/mL complete growth medium) for 1 h and challenged with 1  
266 µg/mL of lipopolysaccharide (LPS) for 23 h. After incubation, the cell spent media was  
267 collected for determination of nitric oxide (NO) via Griess reagent assay (Martinez-Villaluenga  
268 et al., 2009) and cytokine/chemokine (IL-6, KC, MCP-1, and TNF-α) were determined by flow  
269 cytometry using the Mouse Cytokine Magnetic kit (MCYTOMAG-70K, Merck KGaA,  
270 Darmstadt, Germany). Analysis was performed on a Luminex XYP flow cytometer (Luminex  
271 Co., Austin, TX, USA) using the Belysa™ Data Analysis Software (version 1.2, Merck KGaA,  
272 Darmstadt, Germany). The spent medium was replaced with 100 µL of serum-free medium  
273 with CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay solution (ratio 9:1, v/v)  
274 for 2 h at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. Absorbance was read at 490 nm on a  
275 Synergy HT microplate reader (Biotek Instruments, Winooski, VT, USA). Untreated  
276 RAW264.7 were considered as negative control (LPS-). Cell viability was expressed as a

277 percentage of untreated cells (LPS–). Data represents the mean and the standard deviation of  
 278 five biological replicates.

279 **2.9. Statistical Analysis**

280 All the performed analyses were done in triplicates, except for the simulated digestion which  
 281 was carried out in duplicates. The results were presented as mean  $\pm$  standard deviation. Two-  
 282 way ANOVA statistical tests (Tukey's HSD multiple comparison test) were performed on the  
 283 collected data where appropriate with a 95% confidence interval (GraphPad Prism v.9.0  
 284 software, Domatics, Stortford, UK). Statistical comparisons between two groups were  
 285 determined by an unpaired two-tailed *t* test.  $P < 0.05$  was considered statistically significant for  
 286 all comparisons.

287 **3. Results and Discussion**

288 **3.1. Total protein content in Mexican and British chia ingredients, Alb and Glo fractions**

289 Total protein content of undigested chia DDF, PC, Alb, and Glo is presented in **Figure 1S**  
 290 (**Supplementary Material**). The total protein content in Mexican (MDDF) and British (BDDF)  
 291 chia degummed and defatted flour was 35 and 37 g/100 g dw, respectively (data not shown).  
 292 While, Mexican (MPC) and British (BPC) chia protein concentrates showed protein contents  
 293 of 88 and 89 g/100 g dw, respectively, in line with previous reported values (Malik and Riar,  
 294 2022, Timilsena et al., 2016). The protein content in Alb fractions from MDDF and BDDF  
 295 reached 38 and 57 g/100 g dw, respectively, whereas the protein content in the Glo fraction  
 296 from MDDF and BDDF was 44 and 39 g/100 g dw. However, a previous study by Sandoval-  
 297 Oliveros and Paredes-López (2013) determined the protein content in chia protein fractions  
 298 from Colima, Mexico, finding 17 g/100 g dw in albumin and 52 g/100 g dw in globulin fractions.

299 In contrast, Segura-Campos (2019) reported 21 g/100 g dw in albumin and 17 g/100 g dw in  
 300 globulin fractions from chia seeds sourced from Yucatan, Mexico. These findings indicated that  
 301 the origin of chia seeds influenced significantly ( $p < 0.05$ ) the protein content of DDF, PC, Alb  
 302 and Glo fractions.

303 **3.2. Changes in soluble protein in Mexican and British chia ingredients, Alb and Glo**  
 304 **fractions during gastrointestinal transit**

305 Soluble protein (SP) content was measured during SGID to understand the variations in protein  
 306 digestibility among chia ingredients and protein fractions. As shown in **Table 1**, prior to SGID  
 307 and at neutral pH, MDDF and BDDF exhibited SP contents of 28 and 34 g/100 g dw,  
 308 respectively. Among all samples, PC displayed the highest SP value, with 62 g/100 g dw for  
 309 MPC and 75 g/100 g dw for BPC, in coherence to their higher protein content. The SP content  
 310 in Mexican (MALb) and British (BALb) albumin was 32.5 and 56 g/100 g dw, respectively, while  
 311 in Mexican (MGlo) and British (BGlo) globulin, it was 35 and 33 g/100 g dw, respectively, with  
 312 statistically significant differences ( $p < 0.05$ ) observed between locations.

313 At the end of gastric digestion, a pronounced reduction in SP content (over 80%;  $p < 0.05$ ) was  
 314 observed in all samples. This finding can be attributed to changes in pH manifesting as an acidic  
 315 milieu or fluctuations in ionic strength that occurred during food digestion. These alterations  
 316 are recognized to impede the interactive dynamics between proteins and water, thereby eliciting  
 317 an elevation in protein-protein interactions that ultimately induce protein aggregation and thus  
 318 precipitation (Culbertson, 2005, Yuliana et al., 2014, Ivanova et al., 2013). Previous studies  
 319 demonstrated that chia proteins have the isoelectric point at pH 3 in which protein solubility  
 320 ranges between 5-10% (Timilsena et al., 2016, Coelho and de las Mercedes Salas-Mellado,

321 2018). Among chia protein fractions, MGlo exhibited the highest SP content at 8.7 g/100 g dw,  
322 while BDDF showed the lowest content at 3.3 g/100 g dw. Similarly, Julio et al. (2019) showed  
323 a higher protein solubility of the Glo fraction from Mexican chia seeds at pH 3. This  
324 phenomenon may be attributed to the augmented presence of hydrophilic amino acid residues  
325 on the surface of Glo, facilitating interactions with water molecules and consequently  
326 enhancing solubility (Zeng et al., 2013).

327 At the endpoint of intestinal digestion, a substantial reduction of approximately 90% in SP  
328 content was observed in all samples as compared to undigested counterparts ( $p < 0.05$ , **Table**  
329 **1**). Although chia protein solubility increases at neutral pH values reached 35% (Khushairay et  
330 al., 2023), lower soluble protein values at the end of intestinal digestion may be attributed to  
331 the extensive proteolysis caused by pancreatic proteases (e.g. trypsin and chymotrypsin) and  
332 peptidases producing smaller peptide fragments and free amino acids. Among the studied  
333 samples, the MPC and BPC displayed the highest SP content (7.6 g/100g dw and 6.4 g/100g  
334 dw, respectively), while MDDF and BDDF exhibited the lowest content (1.6 g/100g dw and  
335 1.4 g/100g dw, respectively).

336 Chia processing (extraction/fractionation) affected the remaining SP content at the end of  
337 gastrointestinal digestion. Regardless of chia seed origin, PC and Alb fraction showed higher  
338 SP content in intestinal digesta than DDF and Glo fractions (**Table 1**,  $p < 0.05$ ) suggesting a  
339 lower protein digestibility of the former. These results are coherent with the higher  
340 concentration of antinutrients (trypsin inhibitors and polyphenols) observed in PC and Alb  
341 fraction as compared to DDF and Glo fraction (Wang et al., 2023).

342 Notably, there was a significant influence of chia seeds origin on the content of SP at the  
 343 beginning and the end of gastric and duodenal digestion (**Table 1**,  $p < 0.05$ ). Generally, British  
 344 chia samples exhibited higher SP content than Mexican samples before and at the end of  
 345 digestion. These results indicated that proteins in ingredients prepared from British chia seeds  
 346 have a higher resistance to digestion than those prepared from Mexican chia seeds. Therefore,  
 347 the origin of chia seed may influence protein digestibility.

348 **3.3. Changes in peptide content in Mexican and British chia ingredients, Alb and Glo**  
 349 **fractions during gastrointestinal transit**

350 Except for Alb fraction, peptide content in undigested chia samples was low varying between  
 351 2.5 and 5.6 g/100 g dw (**Table 1**). The lowest peptide content was observed in MGlo, followed  
 352 by BDDF < MDDF < BGlo < MPC < BPC ( $p > 0.05$ , **Table 1**). Alb exhibited the highest peptide  
 353 content before digestion, with values of 31 and 24 g/100 g dw in in MAlb and BAlb,  
 354 respectively ( $p < 0.05$ ). This higher peptide content observed in Alb could potentially be  
 355 attributed to the use of water as a solvent during extraction of Alb, as Alb are water soluble  
 356 proteins with low MW (Orona-Tamayo et al., 2017, Orona-Tamayo et al., 2015). Water, being  
 357 a polar solvent, possesses the ability to efficiently dissolve and interact with the polar and  
 358 hydrophilic constituents of peptides (Wolfenden, 1978).

359 During SGID, peptide content increased rapidly in all samples (**Table 1**,  $p < 0.05$ ) as a result of  
 360 the action of digestive enzymes (pepsin and pancreatin) which selectively cleave peptide bonds,  
 361 breaking down proteins into peptides. Regardless of chia seed samples, Alb followed by PC  
 362 gastric and intestinal digestates showed a significantly higher peptide content as compared to  
 363 DDF and Glo ( $p < 0.05$ ). A higher content of digestion resistant peptides in PC and Alb gastric

364 and intestinal digestates was indicative of a lower degree of proteolysis, in consistence with the  
 365 higher concentration of antinutrients reported for these samples (Wang et al., 2023).  
 366 Considering the origin of chia seeds, the peptide content of DDF and Alb derived from Mexican  
 367 chia seeds was higher than British before SGID. However, after SGID, protein fractions (Alb  
 368 and Glo) isolated from Mexican chia seeds exhibited higher peptide content indicating a higher  
 369 digestibility, which was confirmed by a higher degree of hydrolysis (data not shown).

370 **3.4. Changes in free amino acid content in Mexican and British chia ingredients, Alb and  
 371 Glo fractions during gastrointestinal transit**

372 FAA in undigested chia samples was low ranging from 0.42 to 5.06 g/100 g dw. Alb fraction  
 373 showed the highest FAA content as compared to DDF, PC and Glo (**Table 1**,  $p < 0.05$ ).  
 374 Regarding chia seed origin, statistical differences were observed particularly in MAlb (5.06  
 375 g/100 g dw) that showed higher FAA levels than BALb (4.02 g/100 g dw).  
 376 FAA content increased in a greater extent during intestinal digestion phase in all studied chia  
 377 samples (**Table 1**,  $p < 0.05$ ). At the end of gastrointestinal digestion, no statistical differences  
 378 were observed in the FAA content when comparisons were performed by chia seed location or  
 379 type of processing. **Table 2** shows the FAA composition of undigested DDF, PC, Alb, and Glo  
 380 and their corresponding digestates after *in vitro* gastric and intestinal digestion. The data  
 381 analysis revealed that, in most cases, there were no significant differences ( $p < 0.05$ ) between  
 382 DDF and PC prior to SGID. This suggests that the technique used for PC extraction has minimal  
 383 impact on the AA composition (Wang et al., 2023).  
 384 Among the non-essential amino acids (NEAA), arginine and tyrosine emerged as the most  
 385 abundant FAA in chia DDF, PC, Alb, and Glo. Regarding the influence of chia seeds origin on

386 the free arginine content of chia ingredients, it was observed that Mexican chia samples showed  
387 higher arginine contents in comparison to their British counterparts before SGID. Conversely,  
388 the results exhibited the opposite trend after SGID. Among them, PC presented a substantial  
389 increase in arginine content in comparison to the undigested samples (19-fold for MPC and 21-  
390 fold for BPC), while MALb and BALb exhibited a relatively minor rise in arginine content after  
391 SGID, with fold changes of 1.4 and 1.8, respectively. Arginine is known for its potential role in  
392 preventing heart disease (Pszczola, 2000). Therefore, chia, being rich in arginine, could be  
393 considered as a beneficial dietary ingredient with potential to contribute to cardiovascular  
394 health. Likewise, a similar trend was observed for tyrosine with its content also showing an  
395 increase after digestion. Specifically, MPC and BPC demonstrated considerable changes,  
396 indicating 13-fold and 14-fold increases, respectively, compared to their respective undigested  
397 equivalents. In contrast, the Alb fraction, MALb and BALb, displayed more modest variations  
398 (1.4-fold and 1.7-fold increases, respectively). Relevant to this observation, Kowalska et al.  
399 (2022) conducted a study on chickpea and soy flours and reported significant levels of free  
400 tyrosine, with values of 0.024 and 0.023 g/100 g dw, respectively. These values were found to  
401 be lower than the results obtained in our study for the undigested MDDF and BDDF, with  
402 respective values of 0.042 and 0.04 g/100 g dw. The amino acids that appear to exert a limiting  
403 influence on protein content are NEAA, especially free cysteine and proline. Concentrations  
404 for these amino acids were the smallest before and after SGID. Cysteine, as one of the sulfur-  
405 containing amino acids, plays a significant role in preserving the tertiary and quaternary  
406 structure of proteins (Paredes-Lopez, 1991). On the other hand, proline functions as a signaling  
407 molecule, capable of modulating mitochondrial functions, influencing cell proliferation, cell

408 death, and triggering specific gene expression.

409 Regarding essential amino acids (EAA), leucine is considered as the most abundant amino acid

410 in chia samples before and after SGID, followed by lysine and phenylalanine. The leucine

411 content varied among samples before digestion, ranging from 0.014 to 0.37 g/100 g dw, but

412 reached similar values (0.454 to 0.517 g/100 g dw) after SGID in all samples. Particularly, MPC

413 and BPC showed significant increases of 34-fold and 35-fold, respectively, after SGID, while

414 DDF and Glo from both locations displayed over 10-fold increases. Alb showed minor increases

415 of 1.2-fold and 1.5-fold for the Mexican and British samples, respectively. A similar trend was

416 observed for phenylalanine and lysine from both Mexican and British samples after SGID, with

417 PC, Glo, DDF, and Alb displaying substantial increases and reaching relatively consistent

418 values ranging from 0.265 to 0.424 g/100 g dw for phenylalanine and 0.454 to 0.518 g/100 g

419 dw for lysine. Remarkably, digested Mexican and British Alb exhibited the highest content for

420 most of the EAA. In comparison to previous studies, the undigested DDF in this study showed

421 higher levels of free phenylalanine (0.051 g/100 g dw for MDDF and 0.050 g/100 g dw for

422 BDDF) than peanut (0.011g/100 g dw) and pumpkin flours (0.009 g/100 g dw) (Kowalska et

423 al., 2022). Furthermore, attention should be given to tryptophan, a precursor of serotonin, as its

424 synthesis in the brain depends on the availability of dietary precursors (Friedman, 2018, Jenkins

425 et al., 2016). Chia seeds displayed significant differences in individual FAA content compared

426 to traditional wheat and cereals, as lysine and tryptophan are commonly limiting EAAs in corn,

427 oats, and rice (Sytar et al., 2018, Mustafa et al., 2007, Sánchez-Velázquez et al., 2021). However,

428 in this study, it was observed that chia proteins presented the lowest content in histidine and

429 threonine before and after SGID. Histidine plays a crucial role in various physiological

430 processes, including enzyme catalysis and metal ion coordination (Schneider, 1978,  
 431 Fullenkamp et al., 2013), while threonine is essential for protein synthesis and contributes to  
 432 immune function and intestinal health (Feng et al., 2013, Abbasi et al., 2014).

433 **3.5. Changes in the protein profile of Mexican and British chia ingredients, Alb and Glo**  
 434 **fractions during gastrointestinal transit**

435 The changes in the protein profile of Mexican and British DDF, PC, Alb and Glo fractions  
 436 during *in vitro* digestion was analyzed using SDS-PAGE as summarized in **Figure 1**. Prior to  
 437 digestion, both MDDF and BDDF showed similar band distributions. Two prominent bands  
 438 were evident in MDDF and BDDF (**Figure 1A**), with MW of 20 and 30 kDa, indicating the  
 439 presence of albumin and/or glutelin (Orona-Tamayo et al., 2015, López et al., 2018b, Malik and  
 440 Riar, 2022). More intense bands were observed in the range of 50-60 kDa, attributed to the  
 441 presence of 11S globulin. Under non-reducing conditions, the 11S globulin can be resolved into  
 442 monomers with MW from 50 to 60 kDa (Sandoval-Oliveros and Paredes-López, 2013,  
 443 Kačmárová et al., 2016). It has been confirmed that 11S globulin is the most abundant protein  
 444 in chia seeds (López et al., 2018a, Wang et al., 2023). Additionally, DDF profiles exhibited  
 445 bands of low MW (less than 20 kDa), likely originating from both Alb and the prolamin fraction  
 446 (Orona-Tamayo et al., 2015). Furthermore, the presence of trypsin inhibitors also could  
 447 contribute to the appearance of low MW bands (approximately 10, 15 and 22 kDa) (Jiménez-  
 448 Munoz et al., 2022). Recently, the existence of ShT1 (trypsin inhibitor) in chia seeds has been  
 449 confirmed, presenting a MW of ~11 kDa under non-reducing conditions. ShT1 exhibited  
 450 resistance to high temperature (100 °C) and acid-alkaline (pH 2-10) conditions (de Souza et al.,  
 451 2022). Thus, enabling its protein extraction during sample preparation steps used in this study.

452 Similarly, undigested PC (**Figure 1B**) exhibited a similar band distribution profile as DDF,  
453 indicating PC encloses all the prominent proteins present in DDF.

454 According to previous studies (Sandoval-Oliveros and Paredes-López, 2013, Orona-Tamayo et  
455 al., 2015), undigested Alb does not show high intensity bands, particularly in the MW range  
456 between 60 to 220 kDa. Therefore, a tricine gel was employed for a higher electrophoretic  
457 resolution of low MW proteins present in Alb fraction. Differences in the protein profile  
458 between MAlb and BAlb were observed (**Figure 1C**), similar to the findings reported by  
459 Grancieri et al. (2019c). In particular, the protein band of 20 kDa showed higher intensity in  
460 BAlb compared to MAlb. Additionally, MAlb displayed four intense bands at 3.5, 7, 10, and 13  
461 kDa, while BAlb presented three intense bands at 3.5, 5, and 11 kDa. The variations in Alb  
462 protein profile related to chia seed origin could be attributed to genetic and environmental  
463 factors (Malik and Riar, 2022). The 13 kDa band observed in MAlb is similar in size to the  
464 'high cysteine' 2S albumin found in many oilseeds (Youle and Huang, 1981, Srđić et al., 2020).  
465 It is probable that 2S albumins with MW between 7-9 kDa also exist in MAlb (Shewry and  
466 Pandya, 1999). Regarding Glo fraction, protein profile (**Figure 1D**) closely resembled that of  
467 DDF and PC, indicating that Glo is the most abundant protein fraction in the chia samples.  
468 All chia samples showed an extensive protein hydrolysis after the *in vitro* gastric digestion  
469 (**Figure 1**). In fact, most high MW polypeptides were not visible at the end of the gastric stage  
470 in DDF, while low MW bands  $\leq$  20 kDa were persistent in the gastric and intestinal digestates  
471 regardless of chia seed origin (**Figure 1A**). For PC, it was possible to conclude that under *in*  
472 *vitro* gastric conditions, only low MW polypeptides ( $\leq$  10 kDa) persisted in the gastric digesta  
473 whereas polypeptides ranging from 3.5 to 60 kDa persisted at the end of intestinal stage (**Figure**

474 **1B).** This observation may be attributed to the neutral pH during intestinal stage, which  
475 potentially facilitates the solubility of higher MW proteins, while at gastric stage the pH is  
476 acidic, thus promoting protein aggregation. Comparison of the bands under intestinal conditions  
477 between DDF and PC clearly demonstrate a difference in digestibility between both ingredients  
478 while no differences were observed related to chia seed origin. Alb fraction (**Figure 1C**) showed  
479 persistent bands at 80 kDa and below 10 kDa in both Mexican and British samples after the  
480 gastric phase whose intensity was notably reduced at the end of the intestinal stage. For Glo  
481 fraction (**Figure 1D**), two intense bands between 3.5 to 15 kDa persisted in the gastric digesta,  
482 while digestion resistant polypeptides with MW ranging from 5 to 30 kDa, with higher intensity  
483 in MGlo than BGlo were observed at the end of intestinal stage. Although the SDS-PAGE  
484 profile of chia proteins has been previously investigated, this study performs for the first time  
485 a characterization of protein digestion products from chia ingredients and protein fractions, thus,  
486 making comparisons with other studies not feasible.

487 **3.6. Changes in the size distribution of peptides in Mexican and British chia ingredients,**  
488 **Alb and Glo fractions during gastrointestinal transit**

489 To better understand possible differences in the peptide distributions of the undigested samples,  
490 as well as the gastric and intestinal digestates, supernatants from the Mexican and British chia  
491 ingredients and protein fractions were analyzed by size exclusion chromatography.  
492 Representative elution profiles are shown in **Figure 2S**. Under experimental conditions, the  
493 peptides present in the chia samples eluted in a MW range from 0.2 to 1 kDa. Four regions were  
494 quantified as percentage over total elution area (**Figure 2**), reporting an elution of peptides in  
495 four different groups: > 1 kDa (between 9.1 and 9.9 min), 0.5–1 kDa (10.0–13.6 min), 0.2–0.5

496 kDa (13.7–15.9 min), and <0.2 kDa (over 16 min). The distribution percentages of the different  
497 peptide sizes in chia samples are shown in **Table 1S (Supplementary Material)** and visualized  
498 in **Figure 2**. Except for BPC, the undigested chia samples showed a predominant population of  
499 soluble peptides between 0.5 and 1 kDa, constituting 48-75% of the total peptide fraction.  
500 Conversely, BPC mainly consisted of peptides exceeding 1 kDa, accounting for 50% of total  
501 peptide fraction. While MALb and BALb presented a significantly high proportion of smaller  
502 peptides (<0.5 kDa) in comparison to other undigested chia samples, comprising 43% and 39%  
503 (**Table 1S**,  $p < 0.05$ ), respectively. Additionally, a clear trend was observed, characterized by a  
504 decline in signals within the lower elution volume range (9-10 min) and a concurrent increase  
505 in signals within the higher elution volume range (10-16 min) relative to digestion time across  
506 most samples. This trend aligns well with the increasing presence of small peptides and  
507 decreasing amounts of high MW proteins and larger peptides.  
508 At the end of gastric digestion, a discernible increase in the proportion of larger peptides (> 1  
509 kDa) and a reduction in the percentage of smaller peptide sizes (< 0.2, 0.2-0.5, and 0.5-1 kDa)  
510 were observed (**Figure 2S**, **Figure 2**). Alb exhibited a distinctive attribute, characterized by its  
511 lower representation of larger peptides, constituting 21% and 40% for Mexican and British  
512 samples, respectively ( $p < 0.05$ , **Table 1S**). However, other samples exhibited analogous SEC  
513 profiles, defined by a reduced proportion of small peptide sizes, with samples from Mexico  
514 showing higher abundance of small peptides. This finding suggests a potentially elevated  
515 protein digestibility for Alb during gastric phase. After intestinal digestion, higher percentages  
516 of smaller peptides and a reduction in the proportion of peptides exceeding 1 kDa was observed  
517 across all samples. The intestinal digestates of PC and Alb from both locations exhibited a

518 higher proportion of large peptides (25% and 15.6% for Mexican samples, 28% and 16.2% for  
519 British samples, respectively), while the digested DDF and Glo samples were characterized by  
520 a lack of larger peptides. These results imply an enhanced digestibility during the intestinal  
521 phase. Moreover, when considering the growing locations of both chia seeds, it becomes  
522 evident that the Mexican samples demonstrated a superior protein digestibility when compared  
523 to their British counterparts. This result differs from previous findings (Wang et al., 2023), in  
524 which British chia samples showed higher *in vitro* digestibility. This could be due to the  
525 different methods employed. Specifically, the INFOGEST, offers a digestive milieu more  
526 closely aligned with human metabolic process (Brodkorb et al., 2019), thus yielding a more  
527 precise and reliable outcome in the current study; while the *in vitro* protein digestibility method  
528 by Tinus et al. (2012), represents a more simple and rapid method to determine protein  
529 digestibility, this complicates the comparison of results since enzymatic conditions vary  
530 significantly across different methods.

531 **3.7. Changes in antioxidant activity of Mexican and British chia ingredients, Alb and Glo**  
532 **fractions during gastrointestinal transit**

533 To elucidate the impact of chia processing, chia seed origin and gastrointestinal digestion on  
534 antioxidant activity, three *in vitro* assays were used: ORAC, ABTS and DPPH. ORAC measures  
535 the ability of a substance to scavenge free radicals and protect against oxidative damage  
536 (Kulczyński et al., 2019). As shown in **Figure 3A**, prior to digestion, ORAC values of MDDF  
537 and BDDF were 172 and 189 µM TE/g dw, respectively. These values were lower compared  
538 to that reported for defatted Chilean chia seeds (517 µM TE/g) (da Silva Marineli et al., 2014).  
539 These differences in the antioxidant activity of chia seeds could be attributed to the influence

of genetic and environmental factors as well as chia processing (Fernandes et al., 2023). In this regard, the removal of mucilage during processing could have reduced the antioxidant properties of chia flour (Fernandes et al., 2023). Among the samples, PC displayed the lowest ORAC values of 73 and 48 µM TE/g dw for Mexican and British samples, respectively. MALb exhibited the highest ORAC activity of 731 µM TE/g dw, followed by BALb (652 µM TE/g dw). While MGlo and BGlo showed 79 and 96 µM TE/g dw, respectively. The highest antioxidant activity observed for Alb was consistent with its higher phenolic content (TPC) (Wang et al., 2023). Phenolic compounds are potent free radical scavengers, effectively donating electrons to neutralize free radicals (Rizvi et al., 2010, Roy et al., 2010). Thus, ORAC values showed an increase in most samples as a function of digestion time. On the contrary, Alb fractions (MALb and BALb) showed a decrease of 48% and 34%, respectively, after gastric digestion. However, after intestinal digestion, MALb exhibited a 49% increase, and BALb showed a 20% increase. This phenomenon appears to be linked to the exposure of hydrophobic amino acids and the subsequent release of peptides resulting from intestinal proteolysis. Amino acids such as tryptophan exhibited a marked increase of 109% and 48% for MALb and BALb, respectively. Similarly, tyrosine showed increases of 24% and 34% for MALb and BALb, respectively. Moreover, phenylalanine presented an increase of 3.6% and 13% for MALb and BALb, while methionine displayed enhancements of 2.1% and 12% for MALb and BALb, respectively (**Table 2**) (Je et al., 2015, Sánchez-Velázquez et al., 2021). Despite the lower ORAC values in digested MALb and BALb compared to their undigested states, Alb still maintained the highest value compared to other chia samples (DDF, Alb, and Glo). In chia flours (DDF), ORAC values increased by 22% in the MDDF but decreased by 19% in BDDF.

562 after gastric digestion. After intestinal digestion, ORAC values of MDDF and BDDF increased  
563 by 101% and 91%, respectively, compared to the undigested samples. After digestion, PC  
564 exhibited a marked increase anti-inflammatory activity, with a 537% rise for MPC and 828%  
565 rise for BPC. In a similar manner, digested MGlo displayed a 259% increase in ORAC, and a  
566 136% increase for BGlo. For comparison purposes between the two seeds locations, it was  
567 observed that Mexican chia seeds exhibited higher ORAC values for the protein fractions (Alb  
568 and Glo), while both protein ingredients (DDF and PC) showed overall almost equal ORAC  
569 values.

570 ABTS assay is shown in **Figure 3B**. Similar to ORAC assay, the highest ABTS value was  
571 observed in Alb samples before digestion, with values of 395 µM TE/g dw for MAlb and 355  
572 µM TE/g dw for BALb, respectively. While the lowest ABTS values were found in PC (69 and  
573 54 µM TE/g dw for MPC and BPC, respectively). With the exception of Alb, ABTS activity  
574 increased in all samples during SGID. After gastric digestion, MAlb and BALb exhibited a  
575 decrease of 41% and 29%, respectively, followed by an increase after intestinal digestion,  
576 reaching values of 396 and 397 µM TE/g dw, respectively. Alb, as previously mentioned,  
577 contains a significant amount of TPC before undergoing SGID. These TPC could donate  
578 electrons, neutralize free radicals, and halt further oxidative reactions (Schaich et al., 2015, Pé  
579 rez-Jiménez and Saura-Calixto, 2008, Osman et al., 2006). The observed decline in antioxidant  
580 activity during the gastric phase can be attributed to diminished protein solubility under acidic  
581 pH conditions. The acidic conditions promotes protein dissociation, exposing binding sites for  
582 potential interactions with polyphenols through electrostatic affinities (Ozdal et al., 2013).  
583 Several globulin proteins, including albumins, form aggregates that interact with polyphenols,

584 thereby reducing polyphenol solubility (Le Bourvellec and Renard, 2012, Quan et al., 2019),  
585 subsequently resulting in diminished antioxidant activity. On the contrary, the neutral pH in the  
586 intestinal phase enhances protein solubility, making proteins more accessible to digestive  
587 enzymes. Protein degradation in the intestinal phase facilitates the release of phenolics that  
588 were initially bound to proteins during the gastric phase, thus, contributing to the potential  
589 antioxidant properties of the metabolites formed in the digestive process (Stojadinovic et al.,  
590 2013, Quan et al., 2019). On the other hand, tryptophan, tyrosine, and sulphur amino acids  
591 (cysteine and methionine) are released during SGID and can exhibit enhanced antioxidant  
592 properties, as detected by the ABTS assay (Meucci and Mele, 1997). These amino acids'  
593 antioxidant capacities contribute to the observed increase in ABTS values at the end of  
594 digestion.

595 In contrast to ORAC results, the highest ABTS values were found for MPC (760 µM TE/g dw)  
596 and BPC (508 µM TE/g dw) following the completion of digestion (**Figure 3B**). Digested Alb  
597 and Glo demonstrated a significant increase of over 2-fold compared to their undigested  
598 counterparts ( $p < 0.05$ , **Figure 3B**). This enhanced antioxidant ability of digested chia samples  
599 suggests the generation of new antioxidant peptides during SGID. In a study by Phongthai et  
600 al. (2018) on rice bran protein hydrolysates, it was found that peptides with lower MW (< 3  
601 kDa) exhibited higher ABTS activity. Similarly, this applies to all chia samples after SGID,  
602 where higher content of small peptides (0.2-1 kDa) was observed (**section 3.6**) with highest  
603 ABTS activity. These findings are in accordance to Foh et al. (2010), which demonstrated that  
604 protein hydrolysates from Tilapia (*Oreochromis niloticus*) with MW < 1 kDa exhibited higher  
605 efficiency in scavenging ABTS radicals. Similarly, Feng et al. (2018) confirmed the presence

606 of two antioxidant peptides (VYTE and VSAFLA) from Chinese chestnut (*Castanea*  
607 *mollissima Blume*) protein hydrolysate with MW of 590.2 Da and 606.3 Da, respectively, which  
608 displayed the highest ABTS radical scavenging capacity.

609 The ability of chia samples to scavenge DPPH radicals was assessed, and the results are  
610 presented in **Figure 3C**. Undigested MDDF and BDDF displayed the highest DPPH values  
611 before SGID (1132 and 759 µM TE/g dw, respectively,  $p < 0.05$ ). This may be attributed to the  
612 presence of phenolics in DDF (Wołosiak et al., 2022). The previous study confirmed that  
613 MDDF and BDDF contains 629 and 580 mg gallic acid/100 g dw of phenolic compounds,  
614 respectively (Wang et al., 2023). Furthermore, phytic acid is recognized as a natural plant  
615 antioxidant that has the capacity to chelate metal ions like iron and copper, forming stable  
616 complexes (Graf and Eaton, 1990, Graf et al., 1987). In the prior study, MDDF and BDDF  
617 exhibited the highest phytic acid content among the chia samples, with values of 2.8 and 2.4  
618 g/100 g dw, respectively (Wang et al., 2023). The abundance of phytic acid in these samples  
619 might have contributed to the high DPPH scavenging activity of DDF. In line with this, Khattab  
620 et al. (2010) reported that phytic acid demonstrated superior DPPH-scavenging activity  
621 compared to tannins in canola (*Brassica napus L.*) and Indian mustard (*Brassica juncea L.*).  
622 The DPPH radical scavenging ability of MPC and BPC (40 and 31 µM TE/g dw) was lower as  
623 compared to DDF, respectively. MGo and BGlo exhibited similar DPPH scavenging activity  
624 (39 and 36 µM TE/g dw, respectively) to PC, whereas Alb showed higher scavenging capacity  
625 than Glo and PC, with values of 350 and 212 µM TE/g dw for MAlb and BAlb, respectively ( $p$   
626  $< 0.05$ , **Figure 3C**). After gastric digestion, the DPPH radical scavenging ability greatly  
627 decreased in DDF (93% for MDDF and 89% for BDDF). Once digestion was completed,

628 MDDF and BDDF displayed increases of 43% and 86%, respectively. Similarly, MALb and  
629 BALb antioxidant activity decreased after gastric digestion (42% and 6%, respectively).  
630 Subsequently, after intestinal digestion, an evident increase of 121% and 115% was observed  
631 for MALb and BALb, respectively, indicating their enhanced DPPH radical scavenging ability.  
632 This finding aligns with the results reported by (Grancieri et al., 2019b). A similar trend was  
633 observed in Glo, with a decrease of 24% and 9% in Mexican and British samples, respectively,  
634 after gastric digestion, and subsequent increases of 40% and 19% after intestinal digestion were  
635 observed. Furthermore, the DPPH values of PC increased during SGID, with digested MPC  
636 and BPC showing 378% and 668% increases compared to undigested sample. This could be  
637 attributed to the high content of aromatic amino acids (phenylalanine, tryptophan, and tyrosine)  
638 present in PC (Wang et al., 2023). The cleavage of peptide bonds between hydrophobic and  
639 preferably aromatic amino acids during SGID, results in increased hydrophobicity, allowing  
640 PC released peptides to react effectively with DPPH radicals (Phongthai et al., 2018, Gulcin,  
641 2020).  
642 Two cellular models were also used to assess the antioxidant effect of intestinal digestates of  
643 chia ingredients and protein fractions. The effect of DDF, PC, Alb and Glo intestinal digestates  
644 on the modulation of intracellular ROS production in oxidative stressed RAW264.7  
645 macrophages and Caco-2 intestinal cells is presented in **Figure 4**. The addition of *t*-BHP  
646 triggered oxidative stress in macrophages (*t*BHP+), causing an increase in intracellular ROS  
647 production in comparison to the untreated cells (*t*BHP-) after 3 h of exposure (**Figure 4A**). The  
648 results indicated that most of the tested intestinal digestates reduced dose-dependently oxidative  
649 stress in RAW264.7 cells, with the exception of MGlo. These results confirm that the protein

650 digestion products maintain their antioxidant activity after gastrointestinal digestion. At the  
651 highest concentration tested (3 mg/mL), British intestinal digestates demonstrated a markedly  
652 superior ROS scavenging capability ( $p < 0.05$ , **Figure 4A**) than the Mexican counterparts. BGlo  
653 showed the highest inhibition of intracellular ROS production (32%), followed by BPC and  
654 BDDF (30% and 27%, respectively) whereas BAlb exerted the lowest reduction of ROS  
655 intracellular levels (18%). A recent study from Villanueva-Lazo et al. (2022) showed that chia  
656 protein hydrolysates, derived from Mexican chia protein isolate after treatment with Alcalase  
657 exhibited ROS scavenging capability in human monocyte-macrophage plasticity response.  
658 Regarding Caco-2 intestinal cells, the exposure to *t*-BHP for 3 h (tBHP+) increased ROS levels  
659 as compared to non-treated cells (tBHP-). All the digested Mexican and British (**Figure 4B**)  
660 chia protein ingredients and protein fractions scavenged ROS, presenting similar ROS levels at  
661 varying concentrations (1 and 3 mg/mL). The findings suggest that both digested Mexican and  
662 British chia protein ingredients and protein fractions can effectively inhibit oxidative stress by  
663 reducing ROS production, especially at the highest tested concentrations (3 mg/mL). The  
664 observed antioxidant effects of the digested chia ingredients and protein fractions are likely  
665 attributable to the collective presence of phenolic compounds and specific peptides/amino acids.  
666 Previous studies highlighted the capacity of phenolic compounds to modulate cellular redox  
667 states and safeguard cells against oxidative damage by scavenging free radicals and chelating  
668 metal ions (Martemucci et al., 2022). These compounds also have the capacity to influence the  
669 activity of antioxidant enzymes and regulate signaling pathways involved in the cellular  
670 response to oxidative stress (Kučera et al., 2014). Amino acids such as cysteine serves as a  
671 precursor for glutathione, a potent intracellular antioxidant (Guru et al., 2021). Other amino

672 acids, such as methionine, tryptophan, tyrosine, and proline are other amino acids known for  
 673 their antioxidant capabilities (Levine et al., 2000, Atmaca, 2004, Brandelli et al., 2015).  
 674 Moreover, certain peptides derived from chia proteins have been recognized as antioxidants.  
 675 Madrazo and Campos (2022) demonstrated that the antioxidant activity of peptides, KLLKKYL,  
 676 KKLLKI, YACLKVK, and KLKKNL, derived from a chia peptide fraction F < 1kDa, obtained  
 677 by *in silico* approaches, indicated that both optimized and chia-derived peptides contain amino  
 678 acid residues associated with antioxidant activity, notably YACLKVK. These findings  
 679 emphasize the beneficial influence of the bioactive compounds in chia seeds on managing  
 680 oxidative stress and underscore their potential as therapeutic agents for diseases associated with  
 681 oxidative damage.

682 **3.8. Changes in the anti-inflammatory activity of Mexican and British chia ingredients,**

683 **Alb and Glo fractions during gastrointestinal transit**

684 Macrophages are implicated in inflammatory responses, as these secrete an array of mediators  
 685 such as cytokines, chemokines, and adhesion molecules (Grancieri et al., 2019c). Upon  
 686 exposure to certain stimuli, such as lipopolysaccharides (LPS), macrophages activate through  
 687 Toll-like receptor 4, the secretion of cytokines including IL-6, KC, Monocyte Chemoattractant  
 688 Protein-1 (MCP-1), and Tumor Necrosis Factor-alpha (TNF- $\alpha$ ) (Tucureanu et al., 2018, Araiza-  
 689 Calahorra et al., 2022). To assess the putative anti-inflammatory capacity of Mexican and  
 690 British chia digested proteins and fractions, these were assessed at different concentrations in  
 691 LPS-challenged RAW264.7 macrophages. **Figure 5A** shows cell viability after cell exposure  
 692 to different doses of intestinal digestates. The results revealed that digested chia samples did  
 693 not show cytotoxic effects.

694 Nitric oxide (NO) can activate tissue damage and DNA injury at sites of inflammation (Conforti  
695 and Menichini, 2011). In response to LPS exposure, most samples reduced NO release in RAW  
696 264.7 cells in a dose-dependent manner. As shown in **Figure 5B**, for Mexican digests, treatment  
697 with MPC (3 mg/mL) yielded the lowest NO production (0.33 µg/mL), thus showing a  
698 maximum NO inhibition of 84%, followed by MDDF and MAlb (79.4% and 78.9%) ( $p < 0.05$ ,  
699 **Figure 5B**), respectively. Similar findings were observed for British chia digests; BPC  
700 exhibited the maximum NO inhibition of 66% at 3 mg/mL, followed BDDF (54%) and BAlb  
701 (35%) ( $p < 0.05$ , **Figure 5B**). However, the maximum NO inhibition for BGlo was observed at  
702 1 mg/mL with a value of 38%. Given that 3 mg/mL was the most effective concentration to  
703 reduce NO production, therefore, this concentration was employed to evaluate cytokine  
704 production (IL-6, KC, MCP-1, and TNF- $\alpha$ , **Figure 5C-F**). IL-6 production was significantly  
705 reduced by both Mexican and British chia samples ( $p < 0.05$ , **Figure 5C**), being MAlb and  
706 BGlo the samples exerting maximum inhibitions of 65% and 78%, respectively (**Figure 5C**).  
707 Additionally, BDDF exhibited comparable IL-6 inhibition to BGlo, displaying a significant  
708 reduction of 74% ( $p > 0.05$ , **Figure 5C**). Both MAlb and BAlb yielded the lowest KC  
709 production (**Figure 6D**), thus, showing the highest inhibitions of 82% and 91%, respectively.  
710 MCP-1 is recognized as a chemotactic cytokine, functioning as a pivotal driver of monocyte  
711 chemotaxis by recruiting additional monocytes to the site of inflammation (Bianconi et al.,  
712 2018). Simultaneously, TNF- $\alpha$  serves as a leading inflammatory mediator that macrophages  
713 secrete when stimulated by LPS (Takashiba et al., 1999, Grancieri et al., 2022). Moreover, a  
714 different behavior was observed in MCP-1 and TNF- $\alpha$  inhibition between Mexican and British  
715 chia samples (**Figure 5E and 5F**, respectively). Among the Mexican samples, only PC showed

716 inhibition of MCP-1 secretion (25% inhibition, **Figure 5E**), while all other samples did not  
717 contribute positively to the reduction of MCP-1 production. Similarly, MALb was the only  
718 digestate capable to inhibit slightly TNF- $\alpha$  production (2.6%) (**Figure 5F**). Intestinal digests of  
719 British chia demonstrated a positive effect on the inhibition of MCP-1 production, with BGlo  
720 being the most potent, reducing this cytokine by 32%. However, they did not effectively inhibit  
721 TNF- $\alpha$  production. Grancieri et al. (2022) reported different findings when examining the  
722 impact of digested Brazilian chia protein, which notably resulted in a reduction of TNF- $\alpha$   
723 expression (data not shown).

724 The anti-inflammatory efficacy of plant-derived bioactive peptides is intricately influenced by  
725 their MW and amino acid sequence. Previous reviews have demonstrated that peptides with  
726 lower MW (< 1 kDa) exhibit higher anti-inflammatory activity (Liu et al., 2022). Notably,  
727 investigations have identified plant-derived bioactive peptides with MW of approximately 0.5  
728 kDa (~5 amino acid residues) as possessing the most potent anti-inflammatory attributes (Craik  
729 et al., 2013). This observation aligns with the findings presented in **section 3.6**, where it was  
730 observed that digestion of MPC and BPC released peptides of smaller MW (< 0.6 kDa),  
731 correlating with superior NO inhibition capacity. Similar conclusions were observed by  
732 Saisavoei et al., 2021, in which a bee pollen hydrolysate with a MW below 0.65 kDa showed  
733 the highest NO inhibitory activity.

734 In addition to MW, the anti-inflammatory properties of food-derived peptides are closely linked  
735 to their amino acid composition. Hydrophobic amino acids have been consistently identified as  
736 key contributors to the anti-inflammatory effects of peptides (Guha and Majumder, 2019). The  
737 mechanism underlying this effect involves the binding of hydrophobic amino acids to LPS

738 molecules, resulting in the formation of peptide-LPS complexes that counteract LPS-induced  
739 inflammatory responses. Moreover, these hydrophobic amino acids are capable of scavenging  
740 LPS by inducing cell membrane charge reversal, further mitigating inflammation (Singh et al.,  
741 2017). Among these amino acids, leucine plays a pivotal role in enhancing the anti-  
742 inflammatory activity, with the presence of tryptophan and phenylalanine further augmenting  
743 this effect (Liu et al., 2022). Consequently, as elucidated in **section 3.4**, the high content of  
744 these hydrophobic amino acids in chia seeds is potentially responsible for their enhanced anti-  
745 inflammatory properties, distinguishing them from other traditional cereals and oilseeds.  
746 Beyond hydrophobic amino acids, positively charged amino acids, such as lysine and arginine,  
747 have emerged as important contributors to the improved anti-inflammatory activity of plant-  
748 derived peptides (**section 3.4**). For instance, pure peptides, NSPGPHDVALDQ and  
749 RMVLPEYELLYE, isolated from Brazil chia seeds, have shown notable inhibitory effects on  
750 NO, PGE<sub>2</sub>, and TNF- $\alpha$  in RAW264.7 cells, with one of these peptides containing arginine in its  
751 amino terminal (Grancieri et al., 2021). Lysine has been found to be prevalent in most anti-  
752 inflammatory peptides derived from various plant sources. Oligopeptides KLRSRNLLHPT and  
753 TNGRHSAKKH, derived from bee pollen, have been demonstrated to inhibit the expression of  
754 COX-2, iNOS, IL-6, and TNF- $\alpha$  in RAW264.7 macrophages (Saisavoey et al., 2021). Likewise,  
755 green tea peptides (LAEQAER, VECTIPK, DAYVGDEAQSK, and MASLALK) have been  
756 shown to reduce iNOS and TNF- $\alpha$  in diabetic mice, with three of these peptides containing  
757 lysine (Chen et al., 2022).

758 Given the complexities governing the anti-inflammatory potential of plant-derived bioactive  
759 peptides, it is crucial to conduct further research to explore and identify such peptides in chia  
760 seeds, aiming for a comprehensive understanding of their anti-inflammatory mechanisms.

761 **4. Conclusion**

762 Our study undertook a thorough exploration of chia proteins' behavior during gastrointestinal  
763 transit, considering critical factors such as the geographic origin of chia seeds and the effects  
764 of various processing techniques. The investigation revealed distinctive digestion patterns for  
765 proteins derived from chia seeds sourced from different locations (UK and Mexico) and  
766 subjected to diverse extraction and fractionation methods. While similarities were observed in  
767 the breakdown of certain proteins in DDF, PC, and Glo, significant variations emerged in the  
768 bioaccessible content of protein, peptides and free amino acids as well as the size peptide  
769 distribution. These differences carry significant implications for the antioxidant and anti-  
770 inflammatory activities of the resultant intestinal digestates. Our study showcased the capability  
771 of digested chia protein ingredients and fractions to mitigate reactive oxygen species (ROS)  
772 production and reduce pro-inflammatory cytokine levels. Notably, British chia samples  
773 exhibited pronounced anti-inflammatory properties, emphasizing their promising role as  
774 healthier ingredients. In summary, the favorable protein content, digestibility, and potent  
775 antioxidant and anti-inflammatory attributes of chia, position this emergent seed as a  
776 compelling option for developing dietary strategies targeting oxidative damage and  
777 inflammation-related disorders. The insights gained from this investigation open avenues for  
778 further research, including the identification of specific bioactive peptides responsible for these  
779 health benefits and their innovative applications in functional foods and nutraceuticals.

780    **Declaration of Competing Interest**

781    The authors declare that they have no known competing financial interests or personal  
782    relationships that could have appeared to influence the work reported in this paper.

783    **Data availability**

784    Data will be made available on request.

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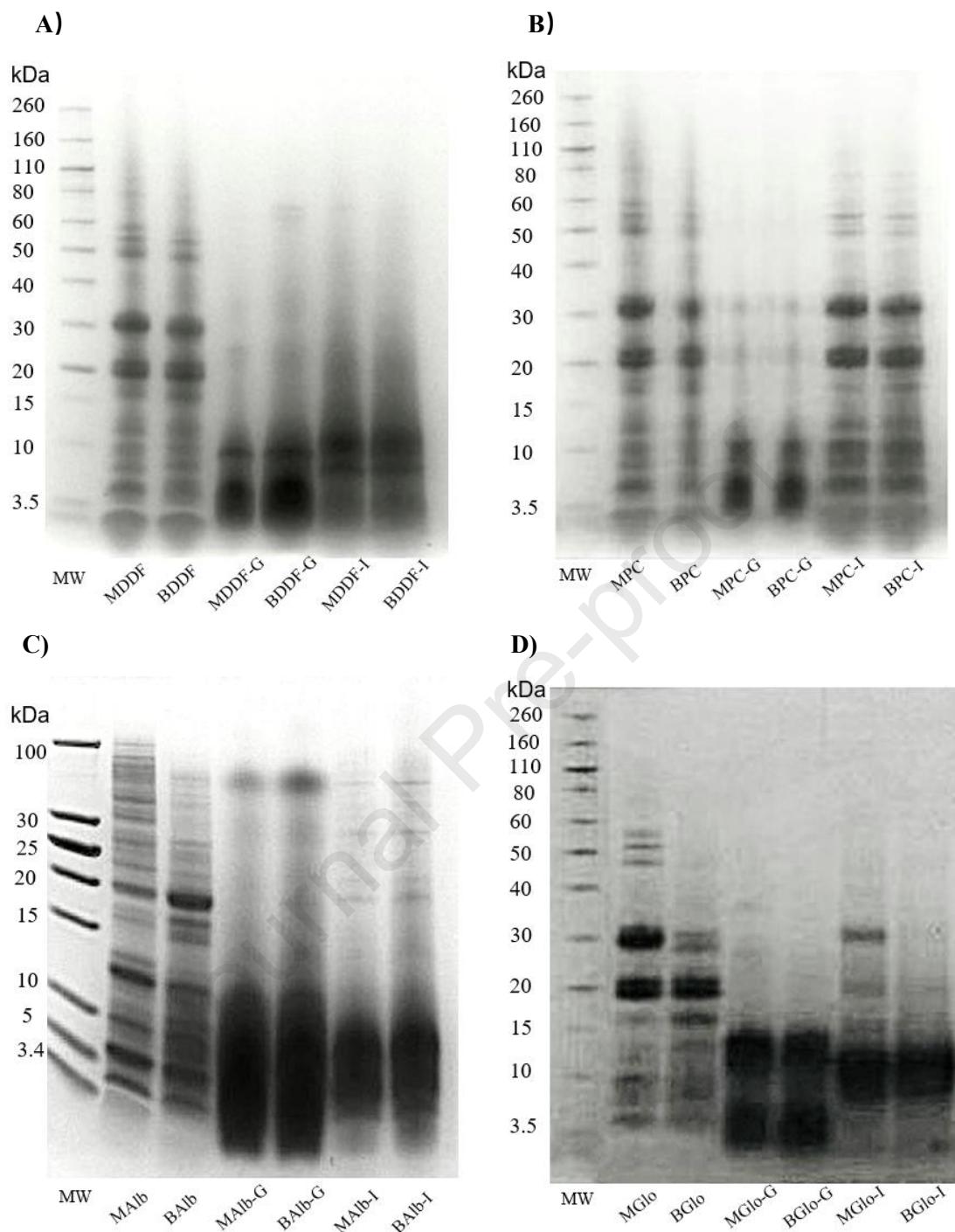
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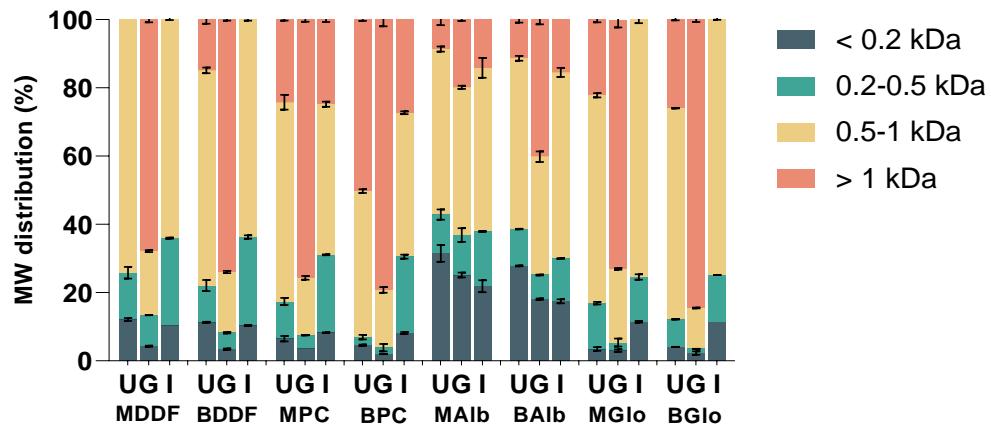
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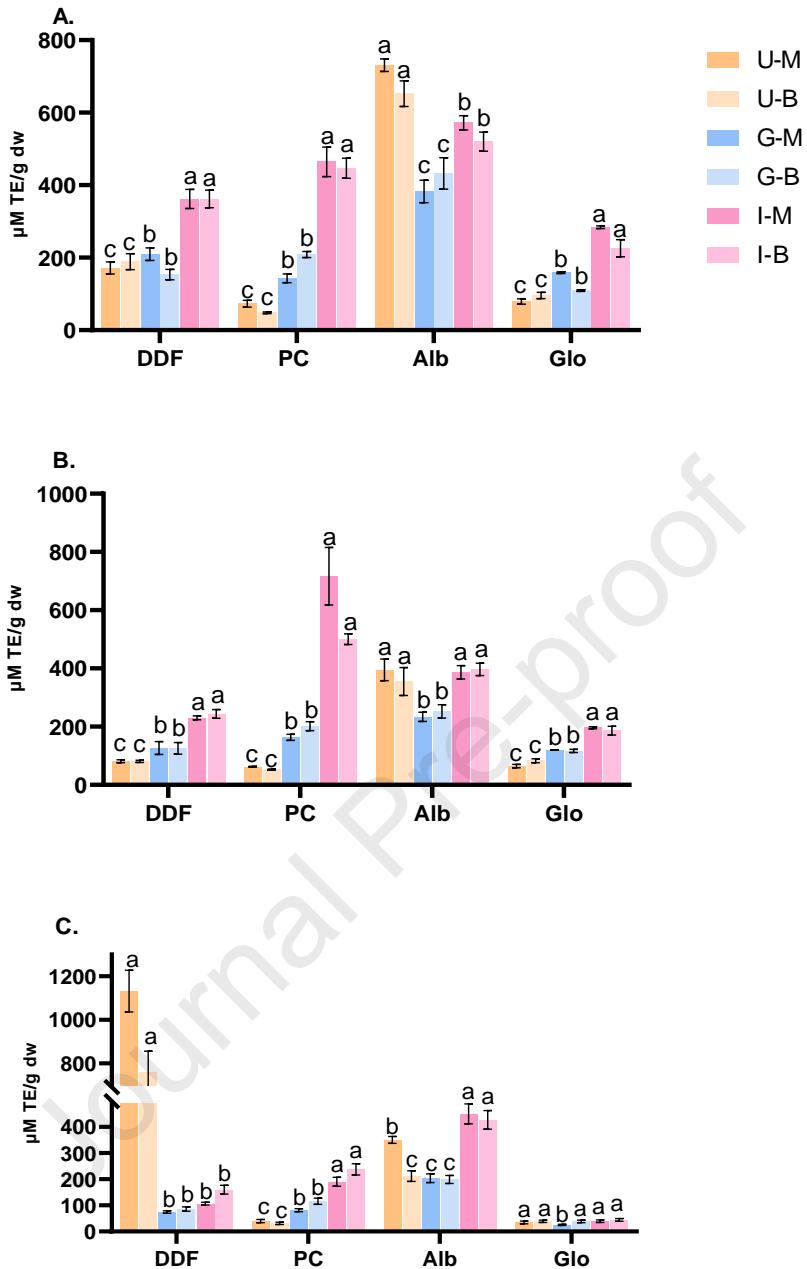


**Figure 1.** SDS-PAGE profiles of four different chia (*Salvia hispanica L.*) samples before and after gastric and intestinal digestion under non-reducing conditions: **A)** degummed-defatted flour; **B)** protein concentrates; **C)** albumin fraction; and **D)** globulin fraction. Abbreviations: MDDF, Mexican degummed-defatted flour; BDDF, British degummed-defatted flour; MPC, Mexican protein concentrates; BPC, British protein concentrates; MALb, Mexican chia albumin; BALb, British chia albumin; MGlo, Mexican chia globulin; BGlo, British chia globulin. G, I

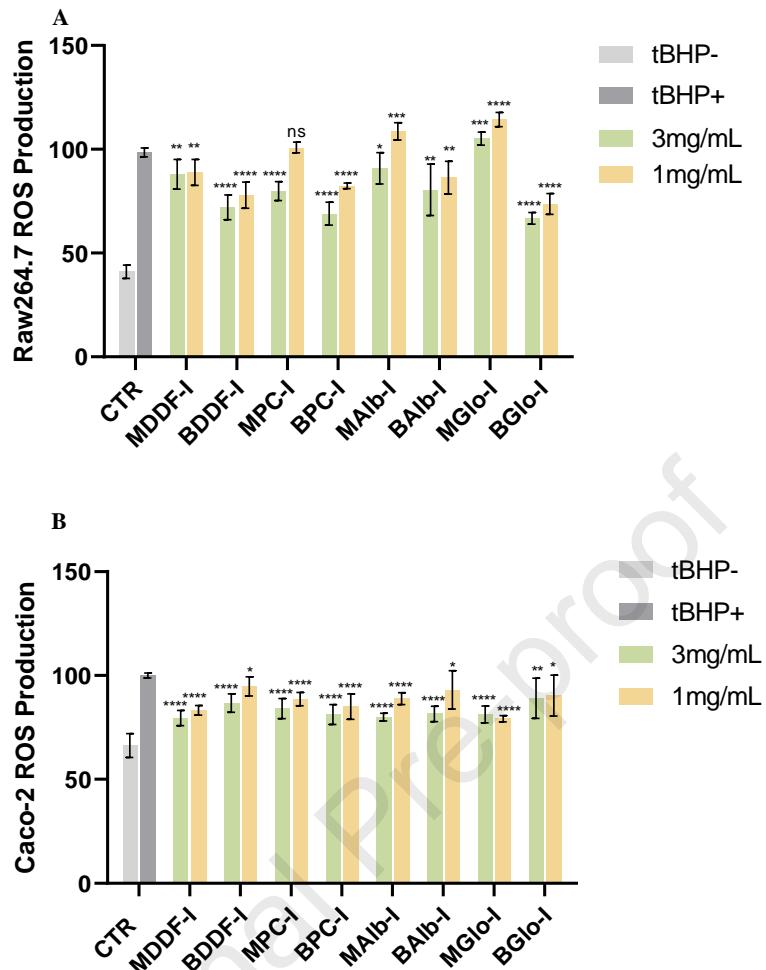
gastric digestion endpoint; I, intestinal digestion endpoint. MW, molecular weight standard (kDa).



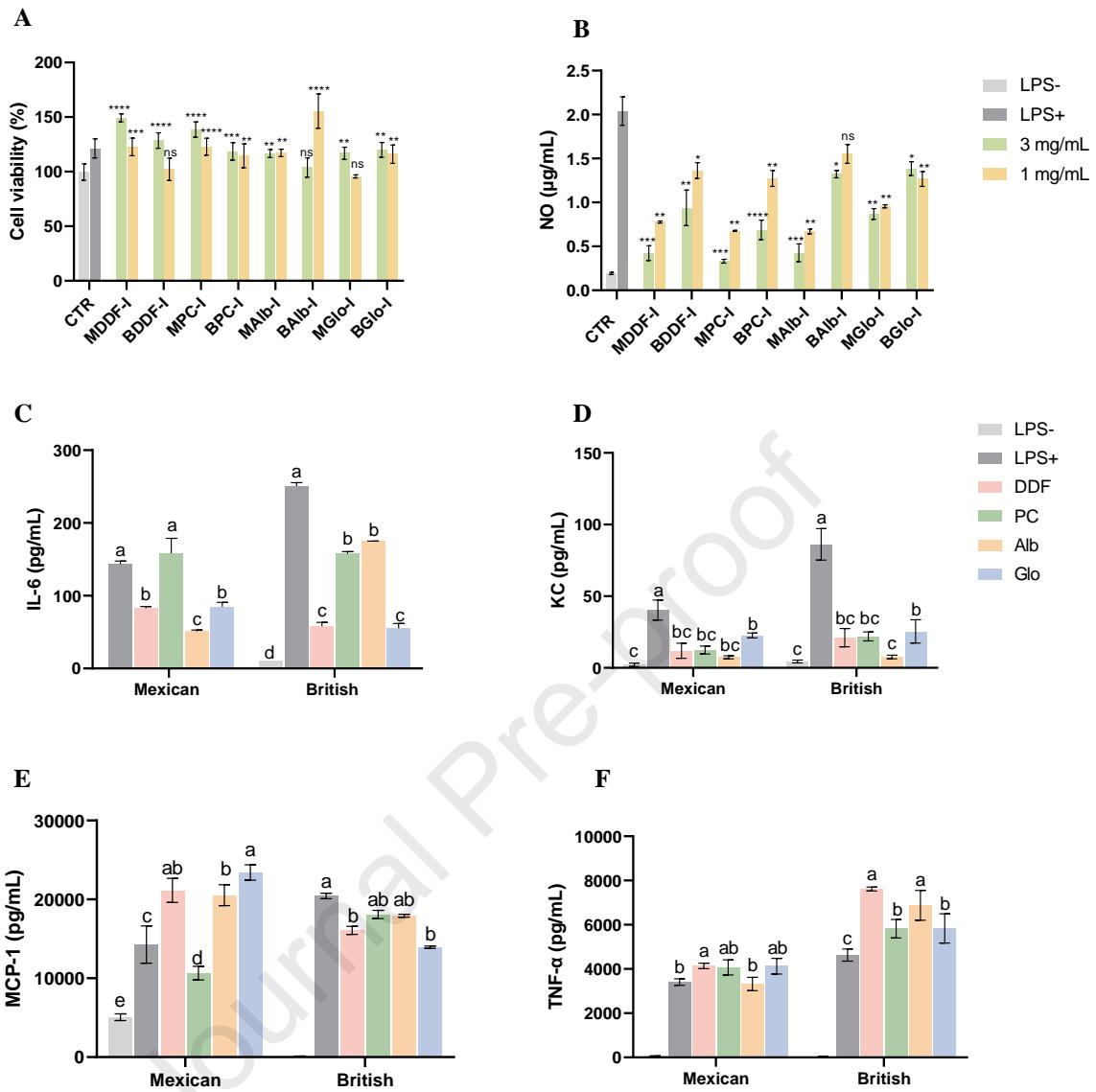
**Figure 2.** Peptide distribution (as ratio of total area eluted peak (%)) of soluble fractions of chia (*Salvia hispanica* L.) ingredients and protein fractions before and after SGID. Abbreviations: MDDF, Mexican degummed-defatted flour; BDDF, British degummed-defatted flour; MPC, Mexican protein concentrates; BPC, British protein concentrates; MAlb, Mexican chia albumin; BALb, British chia albumin; MGlo, Mexican chia globulin; BGlo, British chia globulin; U, undigested sample; G, gastric digestion endpoint; I, intestinal digestion endpoint.



**Figure 3.** *In vitro* antioxidant activity measured in undigested chia (*Salvia hispanica* L.) ingredients and protein fractions and digestates after *in vitro* intestinal digestion. (A) Oxygen radical absorbance capacity (ORAC), (B) 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, and (C) 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Abbreviations: DDF, degummed-defatted flour; PC, protein concentrates; Alb, chia albumin; Glo, chia globulin; M, Mexican chia samples; B, British chia samples; U, undigested; G, gastric phase; I, intestinal phase. Different lowercase letters within the groups indicates statistical differences among different digestion phases of the same chia sample ( $p < 0.05$ , Tukey test).



**Figure 4.** Cellular antioxidant activity measured in chia samples digestates after *in vitro* intestinal digestion. A) Production of intracellular reactive oxygen species (ROS) in *t*-BHP challenged RAW264.7 macrophages pretreated with intestinal digests. B) Production of intracellular ROS in *t*-BHP challenged Caco-2 cells pretreated with intestinal digests. Cells were induced into an oxidative stress (*t*BHP+) after the exposure at different concentrations of intestinal digests (1 and 3 mg/mL) for 20 h. Non-challenged cells (*t*BPH-) were used as negative control. Results are presented as mean  $\pm$  SD (n = 8).  $p < 0.05$  is considered significant (significance is denoted as follows: ns no significance; \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$ ) vs. *t*BHP+ group. Abbreviations: MDDF, Mexican degummed-defatted flour; BDDF, British degummed-defatted flour; MPC, Mexican protein concentrates; BPC, British protein concentrates; MAIb, Mexican chia albumin; BAIIb, British chia albumin; MGlo, Mexican chia globulin; BGlo, British chia globulin; I, intestinal phase; *t*BHP+, cells treated with *t*-BHP; *t*BPH-, untreated cells.



**Figure 5.** Anti-inflammatory activity measured in chia samples digestates after *in vitro* intestinal digestion. Effect of different concentrations (1 and 3 mg/mL) of intestinal digests from Mexican and British chia samples over the viability of RAW264.7 macrophage (A) Concentration of nitric oxide (B) and pro-inflammatory cytokines interleukin (IL)-6 (C), chemokine (C-X-C motif) ligand 1 (KC) (D) monocyte Chemoattractant Protein-1 (MCP-1) (E) Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) (F) released in the extracellular media by activated cells with LPS. Cells were induced into an inflammatory state (LPS+) in the presence of different concentrations of intestinal digests (1 and 3 mg/mL for NO and 3 mg/mL was selected to measure KC, IL-6, MCP-1, and TNF- $\alpha$ ). Non-activated cells (LPS-) were used as negative control. Results are presented as mean  $\pm$  SD (n = 5). P < 0.05 is considered significant

(significance is denoted as follows: ns no significance;  $*p \leq 0.05$ ;  $**p \leq 0.01$ ;  $***p \leq 0.001$ ;  $****p \leq 0.0001$ ); (A) vs. LPS- group; (B) vs. LPS+ group (unpaired two-tailed *t* test). Different lowercase letters within the groups in (C, D, E, and F) indicates statistical differences among different digestion phases of the same chia sample ( $p < 0.05$ , Tukey test). Abbreviations: MDDF, Mexican degummed-defatted flour; BDDF, British degummed-defatted flour; MPC, Mexican protein concentrates; BPC, British protein concentrates; MALb, Mexican chia albumin; BALb, British chia albumin; MGlo, Mexican chia globulin; BGlo, British chia globulin; I, intestinal phase.

**Table 1.** Soluble protein, peptides and free amino acids content (g/100 g dw) during gastrointestinal transit of ingredients and protein fractions prepared from British and Mexican chia (*Salvia hispanica* L.) seeds.

Chia samples	Location	Digestion phase	Soluble protein	Peptides	Free amino acids
DDF	M	U	27.64±0.09 <sup>a,E</sup>	3.46±0.25 <sup>c,C</sup>	1.20±0.01 <sup>b,C</sup>
		G	4.09±0.08 <sup>b,A</sup>	14.43±0.61 <sup>b,C</sup>	1.22±0.06 <sup>b,C</sup>
		I	1.55±0.08 <sup>b,B</sup>	26.31±0.82 <sup>a,D</sup>	3.72±0.12 <sup>a,AB</sup>
	B	U	33.59±2.48 <sup>a,D</sup>	3.11±0.06 <sup>c,C</sup>	0.96±0.05 <sup>b,C</sup>
		G	3.29±0.11 <sup>b,A</sup>	16.56±1.55 <sup>b,C</sup>	1.15±0.01 <sup>b,C</sup>
		I	1.43±0.07 <sup>b,B</sup>	27.10±1.88 <sup>a,D</sup>	3.87±0.14 <sup>a,AB</sup>
PC	M	U	62.44±0.21 <sup>a,B</sup>	5.08±0.11 <sup>c,C</sup>	0.42±0.01 <sup>b,C</sup>
		G	6.17±0.07 <sup>b,A</sup>	36.52±1.51 <sup>b,BC</sup>	0.30±0.02 <sup>b,C</sup>
		I	7.57±0.26 <sup>b,A</sup>	44.34±2.16 <sup>a,B</sup>	3.38±0.91 <sup>a,B</sup>
	B	U	75.34±0.96 <sup>a,A</sup>	5.62±0.30 <sup>c,C</sup>	0.43±0.02 <sup>b,C</sup>
		G	6.78±0.18 <sup>b,A</sup>	32.56±1.93 <sup>b,C</sup>	0.28±0.01 <sup>b,C</sup>
		I	6.33±0.21 <sup>b,AB</sup>	45.55±2.63 <sup>a,B</sup>	3.50±0.09 <sup>a,AB</sup>
Alb	M	U	32.54±0.70 <sup>a,D</sup>	31.02±3.83 <sup>c,A</sup>	5.06±0.69 <sup>a,A</sup>
		G	6.001±0.18 <sup>b,A</sup>	52.46±8.57 <sup>b,A</sup>	4.02±0.05 <sup>b,B</sup>
		I	6.16±0.15 <sup>b,AB</sup>	60.09±1.17 <sup>a,A</sup>	4.26±0.85 <sup>b,A</sup>
	B	U	55.68±0.95 <sup>a,C</sup>	24.08±3.96 <sup>b,B</sup>	4.02±0.41 <sup>b,B</sup>
		G	3.58±0.04 <sup>b,A</sup>	39.60±2.06 <sup>a,B</sup>	5.71±0.28 <sup>b,A</sup>
		I	5.97±0.16 <sup>b,AB</sup>	41.98±1.78 <sup>a,B</sup>	4.04±0.61 <sup>a,AB</sup>
Glo	M	U	35.46±0.47 <sup>a,D</sup>	2.52±0.27 <sup>c,C</sup>	0.64±0.01 <sup>b,C</sup>
		G	8.69±0.11 <sup>b,A</sup>	23.73±3.35 <sup>b,D</sup>	0.59±0.03 <sup>b,C</sup>
		I	3.30±0.11 <sup>c,AB</sup>	32.47±2.38 <sup>a,C</sup>	3.71±0.43 <sup>a,AB</sup>
	B	U	33.26±0.24 <sup>a,D</sup>	3.79±0.57 <sup>b,C</sup>	0.74±0.01 <sup>b,C</sup>
		G	6.05±0.11 <sup>b,A</sup>	25.71±2.32 <sup>a,D</sup>	0.69±0.04 <sup>b,C</sup>
		I	1.56±0.03 <sup>b,AB</sup>	29.21±2.37 <sup>a,CD</sup>	4.06±0.63 <sup>a,AB</sup>

Data are the mean ± standard deviation of three replicates. Different lowercase letter within the column indicates statistical differences among different digestion phases of the same chia sample ( $p < 0.05$ , Tukey test). Different uppercase letter within the column indicates statistical differences among chia samples at the same digestion phases ( $p < 0.05$ , Tukey test). Abbreviations: DDF, degummed-defatted chia flour; PC, protein concentrate; Alb, albumin; Glo, globulin; M, Mexican chia samples; B, British chia samples; U, undigested; G, endpoint of gastric digestion; I, endpoint of intestinal digestion.

**Table 2.** Free amino acids measured in undigested chia (*Salvia hispanica* L.) ingredients and protein fractions and the digestates after *in vitro* gastric and intestinal digestion.

Amino acids	Location	Digestion Phase	Chia ( <i>Salvia hispanica</i> L.) samples			
			DDF	PC	Alb	Glo
Non-essential amino acids (g/100 g dw)						
Asparagine	M	U	0.015±0 <sup>a,B</sup>	0.0025±0.004 <sup>a,B</sup>	0.105±0.030 <sup>ab,A</sup>	0.008±0 <sup>a,B</sup>
		G	0.019±0.001 <sup>a,B</sup>	0.001±0 <sup>a,B</sup>	0.086±0.002 <sup>b,A</sup>	0.004±0 <sup>a,B</sup>
		I	0.034±0 <sup>a,AB</sup>	0.021±0.002 <sup>a,B</sup>	0.043±0.011 <sup>c,A</sup>	0.023±0.004 <sup>a,AB</sup>
	B	U	0.015±0.001 <sup>a,B</sup>	0.006±0 <sup>a,B</sup>	0.084±0.013 <sup>b,A</sup>	0.01±0 <sup>a,B</sup>
		G	0.017±0.001 <sup>a,B</sup>	0.002±0 <sup>a,B</sup>	0.125±0.006 <sup>a,A</sup>	0.006±0 <sup>a,B</sup>
		I	0.032±0.001 <sup>a,A</sup>	0.022±0 <sup>a,A</sup>	0.035±0.006 <sup>c,A</sup>	0.027±0.004 <sup>a,A</sup>
Glutamic acid	M	U	0.102±0.001 <sup>a,B</sup>	0.02±0 <sup>b,B</sup>	0.763±0.132 <sup>a,A</sup>	0.036±0.001 <sup>a,B</sup>
		G	0.055±0.001 <sup>a,B</sup>	0.017±0.001 <sup>b,B</sup>	0.599±0.006 <sup>b,A</sup>	0.031±0.001 <sup>a,B</sup>
		I	0.127±0.002 <sup>a,B</sup>	0.081±0.011 <sup>b,B</sup>	0.296±0.071 <sup>c,A</sup>	0.098±0.011 <sup>a,B</sup>
	B	U	0.103±0.001 <sup>a,B</sup>	0.017±0.001 <sup>b,B</sup>	0.617±0.071 <sup>b,A</sup>	0.048±0.001 <sup>a,B</sup>
		G	0.057±0.001 <sup>a,B</sup>	0.018±0.001 <sup>b,B</sup>	0.848±0.145 <sup>a,A</sup>	0.042±0.001 <sup>a,B</sup>
		I	0.125±0.001 <sup>a,AB</sup>	0.086±0.001 <sup>a,C</sup>	0.219±0.037 <sup>c,A</sup>	0.112±0.016 <sup>a,B</sup>
Serine	M	U	0.046±0.001 <sup>b,B</sup>	0.014±0 <sup>b,C</sup>	0.234±0.021 <sup>a,A</sup>	0.025±0.001 <sup>b,BC</sup>
		G	0.045±0.001 <sup>b,B</sup>	0.010±0.001 <sup>b,C</sup>	0.170±0.004 <sup>b,A</sup>	0.021±0.001 <sup>b,BC</sup>
		I	0.123±0.001 <sup>a,B</sup>	0.091±0.011 <sup>a,C</sup>	0.162±0.033 <sup>b,A</sup>	0.114±0.012 <sup>a,BC</sup>
	B	U	0.038±0.003 <sup>b,B</sup>	0.016±0.001 <sup>b,B</sup>	0.182±0.011 <sup>b,A</sup>	0.033±0 <sup>b,B</sup>
		G	0.038±0.001 <sup>b,B</sup>	0.009±0.001 <sup>b,C</sup>	0.245±0.013 <sup>a,A</sup>	0.030±0.001 <sup>b,BC</sup>
		I	0.12±0.001 <sup>a,A</sup>	0.090±0.001 <sup>a,B</sup>	0.125±0.017 <sup>c,A</sup>	0.125±0.015 <sup>a,A</sup>
Glutamine	M	U	0.047±0.001 <sup>b,B</sup>	0.016±0 <sup>b,B</sup>	0.278±0.032 <sup>a,A</sup>	0.021±0 <sup>b,B</sup>
		G	0.049±0.001 <sup>b,B</sup>	0.012±0.001 <sup>b,B</sup>	0.150±0.001 <sup>c,A</sup>	0.022±0.001 <sup>b,B</sup>
		I	0.228±0.002 <sup>a,A</sup>	0.207±0.009 <sup>a,A</sup>	0.201±0.041 <sup>b,A</sup>	0.237±0.023 <sup>a,A</sup>
	B	U	0.038±0.001 <sup>b,B</sup>	0.014±0.001 <sup>b,B</sup>	0.192±0.015 <sup>bc,A</sup>	0.025±0.001 <sup>b,B</sup>
		G	0.039±0.001 <sup>b,B</sup>	0.011±0.001 <sup>b,B</sup>	0.237±0.008 <sup>ab,A</sup>	0.025±0 <sup>b,B</sup>
		I	0.242±0.001 <sup>a,A</sup>	0.210±0.001 <sup>a,A</sup>	0.174±0.024 <sup>bc,B</sup>	0.238±0.033 <sup>a,A</sup>
Glycine	M	U	0.023±0 <sup>b,B</sup>	0.005±0.001 <sup>b,C</sup>	0.115±0.004 <sup>b,A</sup>	0.009±0.001 <sup>b,BC</sup>
		G	0.021±0.001 <sup>b,B</sup>	0.003±0 <sup>b,C</sup>	0.070±0.001 <sup>c,A</sup>	0.007±0 <sup>b,BC</sup>
		I	0.1±0.001 <sup>a,B</sup>	0.076±0 <sup>a,C</sup>	0.15±0.021 <sup>a,A</sup>	0.091±0.006 <sup>a,B</sup>
	B	U	0.021±0.001 <sup>b,B</sup>	0.006±0 <sup>b,B</sup>	0.075±0.003 <sup>c,A</sup>	0.01±0 <sup>b,B</sup>
		G	0.019±0.001 <sup>b,B</sup>	0.003±0 <sup>b,C</sup>	0.112±0.006 <sup>b,A</sup>	0.008±0.001 <sup>b,BC</sup>
		I	0.105±0.002 <sup>a,A</sup>	0.083±0.001 <sup>a,B</sup>	0.103±0.006 <sup>b,AB</sup>	0.096±0.006 <sup>a,AB</sup>
Arginine	M	U	0.138±0.002 <sup>b,B</sup>	0.037±0.001 <sup>b,B</sup>	0.398±0.034 <sup>b,A</sup>	0.109±0.001 <sup>b,B</sup>
		G	0.120±0.006 <sup>b,B</sup>	0.027±0.001 <sup>b,B</sup>	0.350±0.002 <sup>b,A</sup>	0.105±0.004 <sup>b,B</sup>
		I	0.722±0.004 <sup>a,A</sup>	0.723±0.037 <sup>a,A</sup>	0.554±0.113 <sup>ab,B</sup>	0.772±0.008 <sup>a,A</sup>
	B	U	0.114±0.006 <sup>b,B</sup>	0.036±0 <sup>b,B</sup>	0.351±0.023 <sup>b,A</sup>	0.126±0.001 <sup>b,B</sup>
		G	0.103±0.001 <sup>b,B</sup>	0.026±0.001 <sup>b,B</sup>	0.432±0.021 <sup>b,A</sup>	0.119±0.002 <sup>b,B</sup>
		I	0.755±0.003 <sup>a,B</sup>	0.755±0.001 <sup>a,B</sup>	0.631±0.087 <sup>a,B</sup>	0.833±0.114 <sup>a,A</sup>
Alanine	M	U	0.063±0 <sup>b,B</sup>	0.049±0.001 <sup>c,B</sup>	0.369±0.045 <sup>ab,A</sup>	0.027±0.006 <sup>b,B</sup>
		G	0.069±0.001 <sup>b,B</sup>	0.014±0.001 <sup>b,B</sup>	0.325±0.006 <sup>b,A</sup>	0.028±0.001 <sup>b,B</sup>

		I	0.142±0 <sup>a,B</sup>	0.122±0.001 <sup>a,B</sup>	0.238±0.044 <sup>c,A</sup>	0.139±0.009 <sup>a,B</sup>
B	U	0.059±0.003 <sup>b,B</sup>	0.070±0.002 <sup>c,B</sup>	0.321±0.026 <sup>b,A</sup>	0.042±0.001 <sup>b,B</sup>	
		0.065±0 <sup>b,B</sup>	0.013±0.001 <sup>b,C</sup>	0.409±0.021 <sup>a,A</sup>	0.035±0.001 <sup>b,BC</sup>	
		0.144±0.001 <sup>a,AB</sup>	0.114±0.006 <sup>a,B</sup>	0.187±0.021 <sup>c,A</sup>	0.151±0.032 <sup>a,AB</sup>	
Tyrosine	M	U	0.042±0.001 <sup>b,B</sup>	0.013±0 <sup>b,B</sup>	0.234±0.040 <sup>b,A</sup>	0.031±0 <sup>b,B</sup>
		G	0.063±0.002 <sup>b,B</sup>	0.016±0.001 <sup>b,B</sup>	0.271±0.002 <sup>b,A</sup>	0.042±0.004 <sup>b,B</sup>
		I	0.21±0.001 <sup>a,B</sup>	0.168±0.011 <sup>a,B</sup>	0.335±0.077 <sup>ab,A</sup>	0.225±0.042 <sup>a,B</sup>
	B	U	0.04±0.003 <sup>b,B</sup>	0.013±0 <sup>b,B</sup>	0.238±0.027 <sup>b,A</sup>	0.045±0.001 <sup>b,B</sup>
		G	0.055±0.001 <sup>b,B</sup>	0.017±0.002 <sup>b,B</sup>	0.302±0.009 <sup>b,A</sup>	0.061±0 <sup>b,B</sup>
		I	0.223±0.004 <sup>a,BC</sup>	0.178±0.001 <sup>a,C</sup>	0.406±0.074 <sup>a,A</sup>	0.294±0.074 <sup>a,B</sup>
Cysteine	M	U	0.007±0 <sup>bc,A</sup>	0.005±0.002 <sup>b,A</sup>	0.007±0.001 <sup>b,A</sup>	0.004±0 <sup>b,A</sup>
		G	0.015±0.002 <sup>bc,B</sup>	0.021±0.001 <sup>a,AB</sup>	0.019±0.006 <sup>b,AB</sup>	0.029±0.001 <sup>a,A</sup>
		I	0.064±0.004 <sup>a,A</sup>	0.018±0.001 <sup>ab,BC</sup>	0.014±0.01 <sup>b,C</sup>	0.028±0 <sup>a,B</sup>
	B	U	0.003±0 <sup>c,B</sup>	0.006±0 <sup>b,AB</sup>	0.018±0.002 <sup>b,A</sup>	0.006±0.001 <sup>b,AB</sup>
		G	0.017±0.004 <sup>b,A</sup>	0.012±0 <sup>ab,A</sup>	0.012±0.008 <sup>b,A</sup>	0.021±0 <sup>a,A</sup>
		I	0.073±0.001 <sup>a,A</sup>	0.017±0 <sup>ab,C</sup>	0.033±0.013 <sup>a,B</sup>	0.034±0.004 <sup>a,B</sup>
Proline	M	U	0.165±0 <sup>a,A</sup>	0.067±0.003 <sup>a,B</sup>	0.011±0.011 <sup>c,C</sup>	0.07±0.001 <sup>a,B</sup>
		G	0.023±0.014 <sup>c,B</sup>	0.01±0.001 <sup>b,B</sup>	0.090±0.018 <sup>b,A</sup>	0.002±0.001 <sup>b,B</sup>
		I	0.130±0.003 <sup>a,B</sup>	0.092±0.002 <sup>a,C</sup>	0.168±0.035 <sup>a,A</sup>	0.084±0.008 <sup>a,C</sup>
	B	U	0.082±0.008 <sup>b,A</sup>	0.062±0.019 <sup>a,A</sup>	0.017±0.009 <sup>c,B</sup>	0.063±0.006 <sup>a,A</sup>
		G	0.109±0.006 <sup>ab,A</sup>	0.007±0.006 <sup>b,B</sup>	0.140±0.006 <sup>a,A</sup>	0.008±0.005 <sup>b,B</sup>
		I	0.128±0.004 <sup>a,B</sup>	0.092±0 <sup>a,C</sup>	0.170±0.006 <sup>a,A</sup>	0.088±0.003 <sup>a,C</sup>
<b>Essential amino acids (g/100 g dw)</b>						
Histidine	M	U	0.023±0.001 <sup>b,B</sup>	0.005±0 <sup>b,C</sup>	0.052±0.014 <sup>bc,A</sup>	0.012±0.001 <sup>b,BC</sup>
		G	0.019±0 <sup>b,B</sup>	0.010±0.001 <sup>b,B</sup>	0.065±0.001 <sup>b,A</sup>	0.019±0.001 <sup>b,B</sup>
		I	0.070±0.001 <sup>a,B</sup>	0.072±0.002 <sup>a,AB</sup>	0.050±0.011 <sup>bc,C</sup>	0.081±0.006 <sup>a,A</sup>
	B	U	0.019±0.002 <sup>b,B</sup>	0.005±0 <sup>b,C</sup>	0.048±0.010 <sup>c,A</sup>	0.003±0 <sup>c,C</sup>
		G	0.022±0.001 <sup>b,B</sup>	0.011±0 <sup>b,B</sup>	0.090±0.001 <sup>a,A</sup>	0.022±0 <sup>b,B</sup>
		I	0.065±0.001 <sup>a,B</sup>	0.067±0.001 <sup>a,B</sup>	0.050±0.005 <sup>bc,C</sup>	0.087±0.008 <sup>a,A</sup>
Threonine	M	U	0.029±0 <sup>b,B</sup>	0.008±0 <sup>b,B</sup>	0.136±0.028 <sup>b,A</sup>	0.014±0.002 <sup>b,B</sup>
		G	0.032±0.003 <sup>ab,B</sup>	0.004±0 <sup>b,C</sup>	0.119±0.004 <sup>bc,A</sup>	0.012±0.001 <sup>b,BC</sup>
		I	0.057±0 <sup>ab,B</sup>	0.042±0 <sup>a,B</sup>	0.078±0.018 <sup>d,A</sup>	0.049±0.006 <sup>ab,B</sup>
	B	U	0.026±0.001 <sup>b,B</sup>	0.007±0.002 <sup>b,B</sup>	0.107±0.015 <sup>c,A</sup>	0.024±0 <sup>b,B</sup>
		G	0.030±0.001 <sup>b,B</sup>	0.004±0.001 <sup>b,C</sup>	0.196±0.011 <sup>a,A</sup>	0.017±0 <sup>b,BC</sup>
		I	0.056±0.001 <sup>a,A</sup>	0.042±0 <sup>a,A</sup>	0.054±0.008 <sup>d,A</sup>	0.054±0.008 <sup>a,A</sup>
Valine	M	U	0.052±0.001 <sup>b,B</sup>	0.015±0 <sup>b,B</sup>	0.343±0.049 <sup>a,A</sup>	0.030±0.001 <sup>b,B</sup>
		G	0.076±0.005 <sup>b,B</sup>	0.011±0.001 <sup>b,C</sup>	0.252±0.002 <sup>b,A</sup>	0.033±0.001 <sup>b,BC</sup>
		I	0.161±0.001 <sup>a,B</sup>	0.125±0.006 <sup>a,B</sup>	0.235±0.040 <sup>b,A</sup>	0.153±0.019 <sup>a,B</sup>
	B	U	0.049±0.003 <sup>b,B</sup>	0.014±0 <sup>b,B</sup>	0.249±0.026 <sup>b,A</sup>	0.038±0.002 <sup>b,B</sup>
		G	0.068±0.001 <sup>b,B</sup>	0.012±0 <sup>b,C</sup>	0.382±0.021 <sup>a,A</sup>	0.037±0.001 <sup>b,BC</sup>
		I	0.164±0 <sup>a,A</sup>	0.126±0.001 <sup>a,A</sup>	0.167±0.019 <sup>c,A</sup>	0.160±0.022 <sup>a,A</sup>
Methionine	M	U	0.008±0 <sup>b,B</sup>	0.005±0 <sup>b,B</sup>	0.091±0.008 <sup>a,A</sup>	0.004±0.001 <sup>b,B</sup>
		G	0.033±0.001 <sup>ab,B</sup>	0.012±0.001 <sup>b,B</sup>	0.097±0.001 <sup>a,A</sup>	0.016±0.001 <sup>b,B</sup>

		I	0.125±0.001 <sup>a,A</sup>	0.123±0.005 <sup>a,A</sup>	0.099±0.021 <sup>a,B</sup>	0.124±0.020 <sup>a,A</sup>
Tryptophan	M	B	U	0.010±0.001 <sup>b,B</sup>	0.003±0 <sup>b,B</sup>	0.101±0.007 <sup>a,A</sup>
		G		0.032±0 <sup>b,B</sup>	0.010±0 <sup>b,B</sup>	0.101±0.005 <sup>a,A</sup>
		I		0.115±0.003 <sup>a,A</sup>	0.125±0.001 <sup>a,A</sup>	0.113±0.016 <sup>a,A</sup>
Phenylalanine	M	B	U	0.031±0.001 <sup>b,B</sup>	0.012±0 <sup>b,B</sup>	0.120±0.016 <sup>b,A</sup>
		G		0.031±0.005 <sup>b,AB</sup>	0.012±0.004 <sup>b,B</sup>	0.053±0 <sup>a,A</sup>
		I		0.099±0.001 <sup>a,A</sup>	0.085±0.008 <sup>a,A</sup>	0.111±0.023 <sup>a,A</sup>
	B	B	U	0.02±0.001 <sup>b,B</sup>	0.009±0.001 <sup>b,B</sup>	0.07±0.006 <sup>b,A</sup>
		G		0.021±0.001 <sup>b,B</sup>	0.013±0.001 <sup>b,B</sup>	0.067±0.005 <sup>a,A</sup>
		I		0.097±0.001 <sup>a,AB</sup>	0.089±0 <sup>a,B</sup>	0.099±0.014 <sup>a,AB</sup>
Isoleucine	M	B	U	0.050±0.004 <sup>b,B</sup>	0.019±0 <sup>b,B</sup>	0.246±0.028 <sup>b,A</sup>
		G		0.127±0 <sup>b,B</sup>	0.061±0.001 <sup>b,B</sup>	0.275±0.004 <sup>b,A</sup>
		I		0.325±0.004 <sup>a,B</sup>	0.388±0.006 <sup>a,A</sup>	0.285±0.059 <sup>b,A</sup>
	B	B	U	0.027±0.001 <sup>b,B</sup>	0.006±0 <sup>b,B</sup>	0.194±0.034 <sup>ab,A</sup>
		G		0.040±0.002 <sup>b,B</sup>	0.003±0 <sup>b,C</sup>	0.147±0.002 <sup>b,A</sup>
		I		0.120±0.001 <sup>a,A</sup>	0.125±0.001 <sup>a,A</sup>	0.120±0.028 <sup>b,A</sup>
Leucine	M	B	U	0.025±0.001 <sup>b,B</sup>	0.006±0.001 <sup>b,B</sup>	0.148±0.021 <sup>b,A</sup>
		G		0.032±0.001 <sup>b,B</sup>	0.003±0.001 <sup>b,B</sup>	0.221±0.01 <sup>a,A</sup>
		I		0.110±0.003 <sup>a,B</sup>	0.125±0.001 <sup>a,AB</sup>	0.111±0.020 <sup>b,B</sup>
	B	B	U	0.048±0.004 <sup>b,B</sup>	0.014±0 <sup>b,B</sup>	0.320±0.023 <sup>b,A</sup>
		G		0.118±0.001 <sup>b,B</sup>	0.022±0.004 <sup>b,B</sup>	0.414±0.021 <sup>ab,A</sup>
		I		0.496±0.006 <sup>a,A</sup>	0.493±0.004 <sup>a,A</sup>	0.485±0.081 <sup>a,A</sup>
Lysine	M	B	U	0.048±0.002 <sup>b,B</sup>	0.013±0.001 <sup>b,B</sup>	0.243±0.020 <sup>b,A</sup>
		G		0.059±0.002 <sup>b,B</sup>	0.015±0.001 <sup>b,B</sup>	0.226±0.001 <sup>b,A</sup>
		I		0.409±0.012 <sup>a,A</sup>	0.287±0.013 <sup>a,A</sup>	0.376±0.105 <sup>a,A</sup>
	B	B	U	0.043±0.009 <sup>b,B</sup>	0.015±0.001 <sup>b,B</sup>	0.236±0.039 <sup>b,A</sup>
		G		0.059±0.004 <sup>b,B</sup>	0.014±0 <sup>b,B</sup>	0.273±0.013 <sup>b,A</sup>
		I		0.397±0.013 <sup>a,A</sup>	0.309±0.004 <sup>a,B</sup>	0.339±0.070 <sup>ab,AB</sup>

Mean ± standard deviation of three replicates. Different lowercase letter within the column indicates statistical differences among different digestion phases of the same chia fractions in each amino acid ( $p < 0.05$ , Tukey test). Different uppercase letter within the same row indicates statistical differences among chia samples at the same digestion phases in each amino acid ( $p < 0.05$ , Tukey test). Abbreviations: DDF, degummed-defatted flour; PC, protein concentrates; Alb, albumin; Glo, globulin; M, Mexican chia sample; B, British chia sample; U, undigested samples; G, endpoint of gastric digestion; I, endpoint of intestinal digestion.

**Highlights:**

- Chia protein concentrates showed elevated peptide content post-digestion.
- Protein concentrates and albumins showed the highest antioxidant activity.
- British chia globulins exhibited higher cellular reactive oxygen species inhibition.
- Mexican and British protein concentrates led in macrophages nitric oxide inhibition.
- Digested chia proteins fractions lowered IL-6 and KC pro-inflammatory cytokines.

**Declaration of interests**

- The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.
- The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: