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Anthocyanin bioaccessibility and anti-inflammatory activity of a grape-based 3D printed food for dysphagia

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Alan Giovanini de Oliveira Sartori, Ana Sofia Martelli Chaib Saliba, Bruna Sousa Bitencourt, Jaqueline Souza Guedes, Larissa Catelli Rocha Torres, et al.. Anthocyanin bioaccessibility and anti-inflammatory activity of a grape-based 3D printed food for dysphagia. *Innovative Food Science & Emerging Technologies / Innovative Food Science and Emerging Technologies*, 2023, 84, pp.103289. 10.1016/j.ifset.2023.103289 . hal-03965827

HAL Id: hal-03965827

<https://hal.science/hal-03965827>

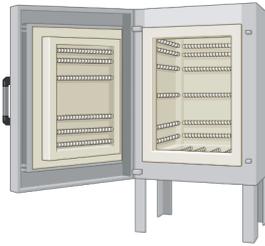
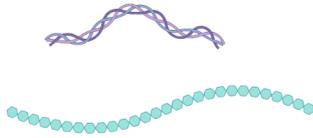
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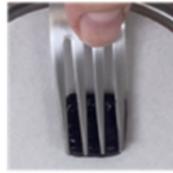
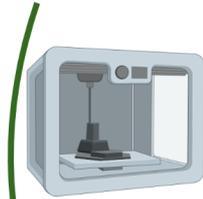
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Sartori, A. G. O., Saliba, A. S. M. C., Bitencourt, B. S., Guedes, J. S., Torres, L. C. R., de Alencar, S. M., & Augusto, P. E. D. (2023). Anthocyanin bioaccessibility and anti-inflammatory activity of a grape-based 3D printed food for dysphagia. *Innovative Food Science & Emerging Technologies*, 84, 103289.

<https://doi.org/10.1016/j.ifset.2023.103289>



Different gelatin/cassava starch (native or modified by **dry heating treatment**) ratios and total solid contents



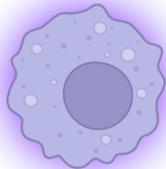
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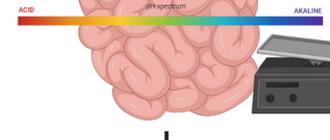
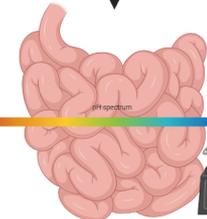
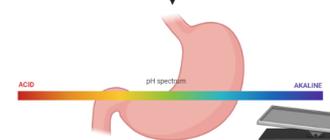
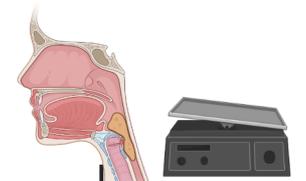
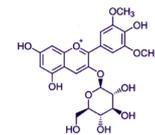
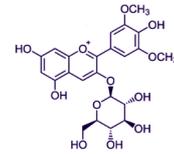
Grape juice gels for patients with dysphagia



↓ **NF-κB activation**
↓ **TNF-α release**

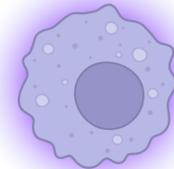


Anthocyanin bioaccessibility



Digested grape juice gels

↓ **NF-κB activation**
↓ **TNF-α release**
↓ **CXCL2/MIP-2**



Highlights:

- 3D printable grape juice gels made with gelatin/starch to reduce choking risk
- Dry heat-treated starch increased anthocyanin bioaccessibility of grape juice gels
- Grape juice gels deactivated NF- κ B after simulated gastrointestinal digestion
- Digested grape juice gels with dry heat-treated starch decreased TNF- α secretion
- Digested grape juice gels with dry heat-treated starch lowered CXCL2/MIP-2 secretion

25 5% solids) were used to produce grape juice gels. The gels with DHT-starch showed the
26 highest anthocyanin bioaccessibility (between 22.9 and 28.7%). After *in vitro* digestion, all
27 gels decreased NF- κ B activation, whereas the secretion of TNF- α and CXCL2/MIP-2
28 decreased in cells treated with the native or DHT_2h gels. Therefore, the DHT could enhance
29 chemical and biological properties of 3D printable foods containing grape juice tailored to
30 suit dysphagia diets.

31 *Keywords:* dry heating treatment; starch modification; simulated gastrointestinal digestion;
32 texture-modified foods; NF- κ B; TNF- α

33

34 *Industrial relevance text:*

35 Although there is rising number of works with 3D printing of foods, the evaluation
36 of new ingredients obtained by emerging technologies and their impact on the interaction of
37 bioactive compounds with the human body is still missing. In this work, the developed
38 ingredients were used not only to obtain 3D printable grape juice gels, but also to tailor them
39 for diets of people with dysphagia. Moreover, the effects of gelling ingredients were
40 evaluated not only in relation to the printability of the gels, but also on anthocyanin
41 bioaccessibility and anti-inflammatory activity based on cell assays. The obtained results
42 expand the knowledge of 3D printing of foods towards industrial application.

43

44 *Abbreviations:*

45 CJ: cooked grape juice; DHT: dry heating treatment; DHT_2h: cassava starch dry heat-
46 treated at 130 °C for 2 h; DHT_4h: cassava starch dry heat-treated at 130 °C for 4 h; FJ:
47 fresh grape juice; IDDSI: International Dysphagia Diet Standardisation Initiative; LPS:
48 lipopolysaccharide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

49 NF- κ B: Nuclear Factor kappa B; RPMI: Roswell Park Memorial Institute; SSF: simulated
50 salivary fluid; SGF: simulated gastric fluid; SIF: simulated intestinal fluid; TMF: texture-
51 modified foods; TNF- α : Tumor necrosis factor- α ; TSC: total solid content.

52

53 **1. Introduction**

54 Dysphagia is the clinical term for difficulty in swallowing, and is a common
55 complication after stroke, head and neck cancer, as well as a common comorbidity of
56 Parkinson's disease and Alzheimer's disease (Cichero, 2013; Mira et al., 2022). In moderate
57 to severe Alzheimer's disease, e.g., the prevalence of dysphagia ranges from 84 to 93% (Mira
58 et al., 2022). Moreover, it is well known that the global population is ageing, which increases
59 the prevalence of the aforementioned diseases, and, consequently, of dysphagia.

60 When eating and drinking, people with dysphagia struggle to control the liquid flow
61 through the pharynx and to avoid the airway, which increases their susceptibility to
62 asphyxiation from choking and malnutrition due to reduced oral intake (Cichero, 2013). One
63 strategy to make mastication and swallowing safer for people with dysphagia is the
64 consumption of texture-modified foods (TMF), since they slow down the transit of the bolus
65 through the pharynx (Beck et al., 2018).

66 The International Dysphagia Diet Standardisation Initiative (IDDSI) has developed
67 standardized terminologies and definitions for TMF to individuals with dysphagia worldwide
68 (Cichero et al., 2017). Briefly, the IDDSI Framework classifies foods and drinks that can be
69 eaten by people with dysphagia into categories based on their hardness, cohesiveness,
70 adhesiveness, and particle size, and includes simple tests to simulate how these foods and
71 drinks behave during mastication and swallowing (Cichero et al., 2017; The International
72 Dysphagia Diet Standardisation Initiative, 2019a, 2019b).

73 In this context, the three-dimensional (3D) printing technology may contribute
74 towards the development of texture customized and healthy food products for people with
75 dysphagia (Dick et al., 2020; Mirazimi et al., 2022; Portanguen et al., 2019). Extrusion-based
76 3D printing is the most common technology to print foods, where a syringe connected with
77 a motor extrudes food-grade “inks” onto a platform to build layer by layer 3D foods with
78 customized textures, in geometric forms based on digital models (Ma & Zhang, 2019).

79 Starches are widely available as raw materials and important gelling ingredients that
80 can be used as food-grade “inks” for 3D printing (Demei et al., 2022). During the extrusion
81 process, the starch molecules are compressed, increasing the formation of hydrogen bonds,
82 and consequently forming a denser and possibly more stable gel network structure (Goldstein
83 et al., 2010). Cassava (*Manihot esculenta* Crantz) is an important source of starch, being a
84 highly drought-tolerant culture, and quite productive when planted in poor soils, and
85 marginal lands (Zhu, 2015). However, native cassava starch makes weak gels even after 3D
86 printing, and its modification is an interesting alternative to tailor new functionalities. The
87 dry heating treatment (DHT), which is a simple, safe, clean, and chemical-free modification
88 process, increased the firmness and improved 3D printability of cassava starch gels (Maniglia
89 et al., 2020).

90 Another strategy to improve the structural properties of starch-based gels is to add
91 proteins. Gelatin, a mixture of peptides derived from collagen mainly from porcine skin,
92 bovine hides, and bovine bones, stands out as an abundant and affordable protein source (Liu
93 et al., 2015). Gelatin can form strong gels and is commonly used as a main structural
94 ingredient added with other ingredients to improve the hardness, chewiness, consistency, and
95 elasticity of 3D printing materials (Demei et al., 2022).

96 Furthermore, 3D printable foods must contain nutrients as well as bioactive
97 compounds, such as phenolic compounds. For instance, by containing anthocyanins, those
98 foods can help to improve the quality of life of people with dysphagia by decreasing the risk
99 of cardiovascular events (Del Bo' et al., 2016; Maleki et al., 2019), and improving brain
100 activation and cerebral perfusion (Feng et al., 2022). Grape juice is a commercial source of
101 dietary phenolic compounds, including anthocyanins, that can be easily added in food
102 formulations (Mohamedshah et al., 2020). However, the addition of protein and starch may
103 impair the absorption of phenolic compounds by the body, which consequently reduces their
104 bioactivities (Terán & Blanco-Lizarazo, 2021). Moreover, it remains unclear the effect of
105 starch modification by DHT on biological activities of food formulations containing it.

106 Although the number of works with 3D food printing is rising, the evaluation of new
107 ingredients obtained by emerging technologies and their impact on the interaction of
108 bioactive compounds with the human body is still missing. Concomitantly, the development
109 of texture-modified foods containing gelling ingredients to meet the needs of the growing
110 population with dysphagia is helpful to combat malnutrition, especially of the elderly
111 (Cichero, 2013). Consequently, the purposes of this study were to: a) develop 3D printable
112 grape juice gels made with gelatin/cassava starch for people with dysphagia; and b) assess
113 for the first time the effect of starch modification by DHT on anthocyanin bioaccessibility
114 and anti-inflammatory activity of the obtained gels. The obtained results expand the
115 knowledge of 3D printing of foods towards industrial application.

116

117 **2. Material and methods**

118 *2.1. Material*

119 Gelatin extracted from bovine hides (225 bloom and 40 mesh) was provided by
120 Rousselot (Irving, TX, USA). Regular native cassava starch was provided by Podium
121 Alimentos (Tamboara, Brazil), showing pasting properties and morphology similar to the
122 cassava starch reported in a previous study of our research group (Maniglia et al., 2020).
123 Pasteurized whole grape juice (14.7 °Brix, 140 g sugar/L, 5.5 g/L total acidity, pH of 3.4,
124 85.03 ± 0.18% moisture) of a blend of Bordô, Isabel and Concord grapes from Garibaldi,
125 Brazil (S 29°12'8.23'' W 51°38'46.04'', 640 m), obtained by thermo-maceration, and
126 containing sulfur dioxide was purchased in a local market in Piracicaba, Brazil.

127 The following reagents were used: pancreatic amylase, pepsin, pancreatin and
128 pancreatic lipase (all from porcine), ethanol, formic acid, bile extract, CaCl₂(H₂O)₂, HCl,
129 NaOH, KCl, KH₂PO₄, NaHCO₃, NaCl, MgCl₂(H₂O)₆, (NH₄)₂CO₃, lipopolysaccharide from
130 *Escherichia coli* 0111:B4, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
131 (MTT), fetal bovine serum (FBS), dimethylsulfoxide (DMSO), Roswell Park Memorial
132 Institute (RPMI) 1640, amphotericin-B, petunidin-3-*O*-glucoside chloride (Sigma-Aldrich,
133 St. Louis, MO, USA); luciferin (Promega, Madison, WI, USA); penicillin/streptomycin
134 (Gibco, Grand Island, NE, USA); and lysis buffer TNT (Amresco, West Chester, PA, EUA).

135

136 2.2. Starch modification and gels production

137 The DHT was carried out in a hot-air convective oven (Marconi, Piracicaba, Brazil)
138 at 130 °C for 2 h and 4 h (designated as DHT_2h and DHT_4h, respectively), as previously
139 reported (Maniglia et al., 2020). Briefly, before being placed in the oven, the starch (50 g)
140 was distributed over an aluminum foil sheet, which was covered with another aluminum foil
141 sheet. The lateral parts of the sheets were folded and taped to form a closed envelope. Starch
142 moisture (12.15 ± 0.73%) decreased to 5.86 ± 0.48% after 2 h of DHT, and to 5.16 ± 0.21%

143 after 4 h of DHT, and the actual moisture was used to calculate the amount of starch added
144 in the gels.

145 For the hydrogels screening, the gelatin/cassava starch hydrogels were prepared as
146 aqueous solutions with 5 g or 10.7 g total solid content (TSC) in 100 mL water (5% and
147 10.7%, respectively). Pastes were produced by analytically weighing gelatin and cassava
148 starch (native, DHT_2h and DHT_4h) in amounts corrected to 14% moisture basis, at ratios
149 of 1:0, 3:1, 1:1, 1:3, 0:1 (w/w) in deionized water. The glass flasks were covered with
150 aluminum foil, and mechanically homogenized at 500 rpm at 90 ± 3 °C for 20 min in a water
151 bath (Marconi, Piracicaba, Brazil).

152 Pastes were added in plastic molds (3.8 cm ϕ , 2.2 cm height), which were placed in a
153 desiccator with water at the bottom to avoid moisture changes in a BOD chamber (Marconi,
154 Piracicaba, Brazil) at 5 °C for 24 h for hydration and gelation. Thereafter, hydrogels were
155 evaluated considering their performances in the 3D printing test with the star model and
156 IDDSI fork pressure test (sections 2.3. and 2.4., respectively). The formulations with the best
157 performances were used to produce gels with grape juice, which was used to replace the
158 deionized water. The grape juice gels were evaluated through pH, total soluble solids, and
159 moisture content (Maniglia et al., 2020), as well as for the IDDSI fork pressure test and 3D
160 printing (sections 2.3. and 2.4., respectively).

161

162 *2.3. 3D printing*

163 A WiiboxSweetin 3D Food Printer (Wiibox, Nanjing, China) was used. For
164 screening, the hydrogels were placed in 60 mL disposable feed tubes just after the pasting,
165 which were closed using plastic caps and then kept in the BOD chamber at 5 °C. After 24h,
166 the syringes were removed and processed in the 3D printer using 0.84 mm metal printing

167 needles. A five-point regular star of ten equal sides with dimensions of 5 x 5 cm and 6 layers-
168 height was used for this initial screening (Maniglia et al., 2020), and printed with travel and
169 infill speed of 5 mm/s.

170 The grape juice gels were also printed as parallelepiped with dimensions of 1.5 x 1.5
171 x 0.75 cm, with travel and infill speed of 10 mm/s. The dimension of 1.5 cm is recommended
172 to reduce risk of choking for people with dysphagia (Cichero et al., 2017). Printing was
173 conducted in a room at 20 °C. Both the star and the parallelepiped models were created using
174 the Cura software, version 15.02.1.

175

176 *2.4. IDDSI fork pressure test*

177 The fork pressure test was chosen to measure texture-modified foods level 6 (soft &
178 bite-sized), based on the expected physical characteristics of hydrogels made with gelatin
179 and starch. Hydrogels were placed upon a paper in a surface, and pressed with the tines of a
180 common fork up to a pressure where the thumbnail of the researcher blanched to white (The
181 International Dysphagia Diet Standardisation Initiative, 2019b). A single researcher, which
182 was previously trained to minimize thumb pressure variations, performed the test in all
183 samples. The pressure exerted by the thumb when the thumbnail blanches to white is similar
184 to the regular tongue pressure during swallowing, and blanching indicate that pressure has
185 surpassed average arterial blood pressure, which has been measured to occur at
186 approximately 17 kPa (The International Dysphagia Diet Standardisation Initiative, 2019b).
187 The hydrogels were unmolded at the time the test was performed, as well as the printed grape
188 juice gels were printed at the time the test was performed. Pictures were taken and videos
189 were recorded when assessing each sample to further investigation and verification (Table
190 1).

191

192 2.5. *Texture analysis of hydrogels*

193 A puncture assay was used to determine gel firmness in the texture analyzer TA TX
194 Plus (Stable Micro Systems, Surrey, UK) with a load cell of 50 kgf (490 N). The cylindrical
195 probe (P/0.5R) directly penetrated the gels within the molds at 1 mm/s. Force was measured
196 as a function of the penetration depth. Gel firmness was assessed by the force (N) to penetrate
197 hydrogels at 6 mm.

198

199 2.6. *Three-stage simulated gastrointestinal digestion*

200 Digestion was performed for the fresh grape juice, the cooked grape juice prepared
201 under the same conditions for pasting the gels (90 ± 3 °C for 20 min in a bath), and the
202 selected grape juice gels. The static *in vitro* simulation of gastrointestinal digestion was
203 performed based on the Infogest protocol to assess the effects of the pasting temperature and
204 of the food matrix on the bioaccessibility of grape juice anthocyanins. The simulated salivary
205 fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) were
206 previously prepared as described in the Infogest protocol (Brodkorb et al., 2019).

207 Gel samples (5 g) were mixed with 4 mL of the SSF, pH was adjusted to 7.0 with
208 HCl 5 M; added with 25 μ L CaCl₂ solution (0.3 M) and amylase (75 U/mL). It was incubated
209 in a refrigerated bacterial incubator (Ethik technology, Vargem Grande Paulista, Brazil) at
210 37 °C under constant agitation promoted by a Rocker 25 platform (Labnet International,
211 Edison, NJ, USA) inside the incubator for 2 min to simulate the oral phase. The gastric phase
212 was simulated by mixing the bolus (10 mL from oral phase) with 8 mL of the SGF. After pH
213 was adjusted to 3.0, the solution was added with 5 μ L CaCl₂ solution (0.3 M) and pepsin
214 (2,000 U/mL), and incubated at 37 °C under constant agitation for 2 h.

215 For the small intestinal phase, the chyme (20 mL from gastric phase) was mixed with
216 8 mL of the SIF. After pH was adjusted to 7.0 with NaOH 5 M, 40 μ L CaCl₂ (0.3 M),
217 pancreatin (trypsin activity of 100 U/mL), pancreatic lipase (2000 U/mg), and bile extract
218 from porcine were added, and it was incubated at 37 °C under constant agitation for 2 h.
219 Thereafter, samples were centrifuged at 10,000 g for 10 min in a centrifuge (5810R,
220 Eppendorf, Hamburg, Germany), and the supernatant, which was considered the
221 bioaccessible fraction, was filtered in filter paper, and frozen at -20 °C until further analyzes.

222 *In vitro* bioaccessibility of anthocyanins was calculated using the Equation 1:

223

$$224 \text{ In vitro bioaccessibility (\%)} = \frac{\text{Concentration of anthocyanins after gastrointestinal digestion}}{\text{Concentration of anthocyanins before gastrointestinal digestion}} \times 100 \quad (1)$$

225

226 2.7. Tentative identification of anthocyanins

227 2.7.1. Sugar removal

228 A Discovery DSC-18 SPE cartridge (2 g, Sigma-Aldrich, St. Louis, MO, USA) was
229 conditioned with water:formic acid, 99:1 (v/v). Fresh juice (1 mL) was mixed with the
230 acidified water (4 mL), and the solution was injected in the cartridge. The acidified water
231 was injected abundantly to remove the sugars. Thereafter, methanol:formic acid, 99:1 (v/v)
232 was injected for anthocyanins recovery. The solvent was evaporated under N₂ flow up to a
233 volume of approximately 500 μ L. The sample was filtered and kept at -20 °C until analysis.

234

235 2.7.2. HPLC-ESI-UV/MS/MS analysis

236 A Shimadzu (Tokyo, Japan) high performance liquid chromatography (HPLC)
237 system with a CBM-20A controller, LC-30AD pumps, SPD-20A UV-Vis detector, CTO-

238 20A oven, and SIL 30AC auto injector coupled to an Amazon Speed ETD mass spectrometer
239 (Bruker Daltonics Corporation, Billerica, MA, USA) was used. Separation was achieved
240 using a C18 Luna column (250 x 4.6 mm; 5 μ m, Phenomenex, Torrance, CA, USA). The
241 mobile phase, its gradient, and flow rate are described elsewhere (Melo et al., 2015), with a
242 slight modification: solvent A of the mobile phase was water:formic acid, 99:1 (v/v). The
243 UV-Vis detector operated at 530 nm. Mass spectrometer was operated in the positive mode,
244 under the following conditions: nebulizer at 27 Psi, dry gas at 7 L/min, temperature of 325
245 $^{\circ}$ C, and HV of 4,500 V. Data was processed using the DataAnalysis software (Bruker, version
246 4.3). Compounds were tentatively identified by comparison of molecular mass, structural
247 formulas, and MSⁿ fragmentation patterns with available literature.

248

249 2.8. *Quantification of anthocyanins*

250 2.8.1. *Extracts production*

251 Homogenized juices or juice gels (1 g, fresh weight) were mixed with 15 mL
252 ethanol:water, 80:20 (v/v), vortexed for 1 min, sonicated for 20 min, vortexed for 1 min,
253 centrifuged at 10,000 g at 10 $^{\circ}$ C for 15 min, filtered in filter paper, rotaevaporated at 40 $^{\circ}$ C,
254 and freeze-dried. Thereafter, they were resuspended in Milli-Q water. The digested samples
255 were dissolved in Milli-Q water (1:2, v/v). All samples were filtered in PVDF (0.22 μ m) and
256 cellulose (0.20 μ m) membranes before injection in the HPLC system.

257

258 2.8.2. *HPLC-PDA analysis*

259 A Shimadzu (Tokyo, Japan) HPLC system with a SCL-10AVp controller, LC-6AD
260 pumps, SPD-M10AVp photodiode array detector (PDA), CTO-ASVp oven, and SIL-10AF
261 auto injector was used. Separation was achieved using an ODS-A column with the same

262 dimensions of the column used for the HPLC-ESI-UV/MS/MS analysis (250 x 4.6 mm, 5
263 μm , Shimadzu, Tokyo, Japan). Chromatographic conditions were the same described by
264 Melo et al. (2015). Chromatograms were analyzed using the Class-VP software (Shimadzu,
265 Tokyo, Japan). Anthocyanins were tentatively identified by comparison of retention times
266 with the retention times of the compounds detected by the HPLC-ESI-UV/MS/MS at 530 nm
267 (Tables S3 and S4). Anthocyanins were quantified as petunidin-3-*O*-glucoside chloride
268 equivalents, based on the building of a standard curve with solutions of different
269 concentrations of an authentic standard. Linearity ($R^2 = 0.9994$), and limits of detection (0.40
270 ng/mL) and quantification (1.21 ng/mL) for the range of 0.5 to 50 ng were calculated as
271 described by Melo et al. (2015).

272

273 *2.9. Anti-inflammatory activity using lipopolysaccharide (LPS)-induced macrophages*

274 The cell-based assays were carried out based on the procedures described by Lazarini
275 et al. (2022).

276 *2.9.1. Cell culture and cell viability*

277 RAW 264.7 murine macrophages transfected with the Nuclear Factor kappa B (NF-
278 κB)pLUC gene (BCRJ code 0212, Rio de Janeiro, Brazil) were cultured in RPMI 1640
279 supplemented with 10% FBS (v/v), 1% penicillin/streptomycin (v/v), and 0.12 %
280 amphotericin-B (v/v), and incubated at 37 °C in humidified atmosphere with 5% CO₂ in an
281 incubator (MCO-18AIC, Sanyo, Osaka, Japan).

282 Cell viability was tested by the MTT method. Cells were seeded in 96-well
283 microplates with 200 μL of suspension/well, and incubated for 24 h. The cells were treated
284 in incomplete RPMI with undigested samples (at 100, 250, 500 and 1000 $\mu\text{g}/\text{mL}$), digested
285 fresh grape juice (25, 50, 100 and 250 $\mu\text{g}/\text{mL}$), digested cooked grape juice (10, 25, 50, 100

286 and 250 $\mu\text{g}/\text{mL}$), digested grape juice gels (at 100, 250, 400, 500 and 1000 $\mu\text{g}/\text{mL}$), and
287 negative control. After 4 h of incubation, the supernatant was discarded and 0.03 mg/mL
288 MTT solution was added in each well. The microplates were incubated for 3 h, the
289 supernatant was discarded, each well was added with 100 μL of DMSO, and absorbance at
290 570 nm was measured in a microplate reader (SpectraMax M3, Molecular Devices, CA,
291 EUA).

292

293 2.9.2. *NF- κ B activation and secretion of Tumor Necrosis Factor- α (TNF- α) and* 294 *CXCL2/MIP-2 by macrophage cells*

295 Macrophages (6×10^5 cells/mL) treated with the samples at the concentrations
296 defined by the MTT method were incubated for 30 min, and then stimulated with 10 ng/mL
297 LPS (final concentration) for 4 h. Commercial ELISA kits (R&D Systems, Minneapolis, MN,
298 USA) were used to quantify the secretion of TNF- α and CXCL2/MIP-2 from the supernatant,
299 following manufacturer's instructions. The remaining cells stimulated for 4 h were used to
300 assess NF- κ B activation by luminescence quantification, as described elsewhere (Lazarini et
301 al., 2022). A positive control (LPS-induced macrophages without treatment) and a negative
302 control (macrophages not induced by LPS) were assessed under the same experimental
303 conditions.

304

305 2.10. *Statistical analysis*

306 All quantitative analyzes were performed in triplicate and results were expressed as
307 mean \pm standard deviation (SD). Data were previously verified for normality of distributions
308 by the Shapiro-Wilk's test and homoscedasticity by the Bartlett's test. Means were compared
309 by One-way ANOVA (analysis of variance) and the *post hoc* Tukey's test, using the R

310 software, version 4.1.1. Two-way ANOVA followed by the *post hoc* Bonferroni's test was
311 performed to compare the concentrations of anthocyanins before and after *in vitro* digestion
312 for each treatment using the Prism 5 software, version 5.00. All analyzes were performed at
313 a level of confidence of 0.05.

314

315 **3. Results and discussion**

316 *3.1. Screening of hydrogels*

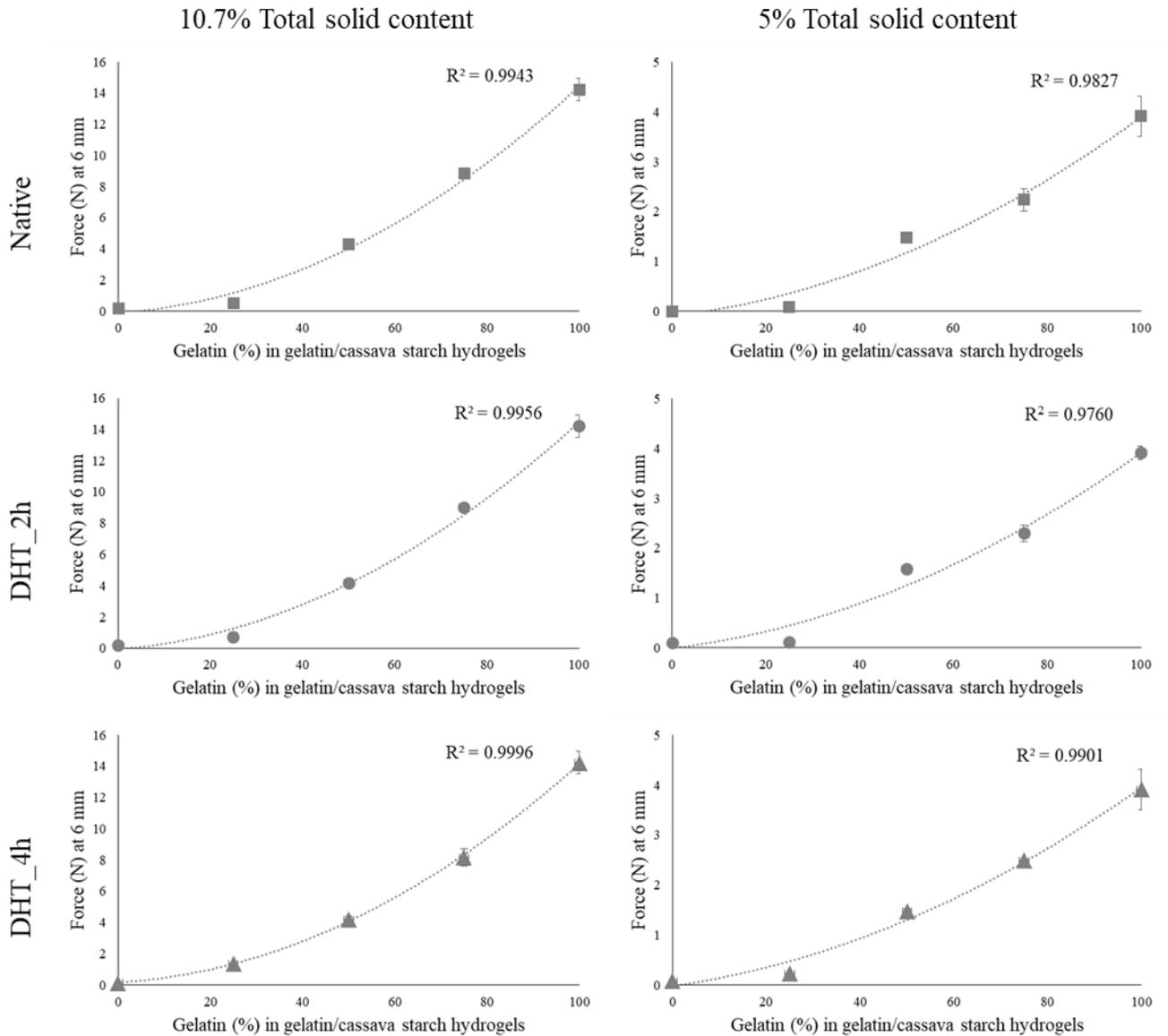
317 Firstly, the hydrogels made with different TSC and different ratios of gelatin:cassava
318 starch, whether native or modified by DHT, were assessed in relation to their firmness,
319 feasibility for dysphagia diets, and 3D printability.

320

321 *3.1.1. Hydrogel firmness*

322 The effect of adding gelatin at the ratios of 1:3 (25% of the TSC is gelatin), 1:1 (50%
323 of the TSC is gelatin) and 3:1 (75% of the TSC is gelatin) on the firmness of cassava starch
324 hydrogels is shown at Figure 1. Regardless the TSC (5% or 10.7%), gelatin increased
325 hydrogel firmness, with the obtained curves showing a quadratic trend.

326



327

328 Figure 1. Effect of adding gelatin in cassava starch hydrogels.

329 Cassava starch was native or submitted to dry heating treatment (DHT) for 2h (DHT_2h) or 4 h (DHT_4h) at
 330 130 °C. Moisture corrected to 14%.

331

332 The DHT process did not affected the firmness ($p > 0.05$) of the hydrogels made with
 333 gelatin:starch at the ratios of 1:1 (w/w) (~4.2 N for 10.7% TSC, and ~1.5 N for 5% TSC),
 334 and 3:1 (w/w) (~8.5 N for 10.7% TSC, and ~2.3 N for 5% TSC). These hydrogels formed

335 stable structures at room temperature after gelation. In a previous study, the DHT conducted
336 under the same temperature (130 °C) and times (2h and 4h) produced firmer cassava starch
337 hydrogels (10.7% TSC, with moisture corrected to 14%), with increased number of enlarged
338 granules, although the general molecular size decreased (Maniglia et al., 2020). Nonetheless,
339 in this study, adding gelatin at the tested concentrations overcame the effect promoted by the
340 DHT, probably due to crosslinking of gelatin with starch to form more compact and
341 physically stable food structures (Lu et al., 2016).

342 As expected, both gelatin hydrogels (5% and 10.7% TSC) also kept their structure
343 capacities. The same was observed for the hydrogels made with gelatin and DHT-starch at
344 the ratio of 1:3 (w/w), which showed gel firmness of 0.75 ± 0.02 N (DHT_2h) and $1.38 \pm$
345 0.01 N (DHT_4h). Although not statistically different ($p > 0.05$), the 1:3 (w/w) gelatin:native
346 starch hydrogel, with gel firmness of 0.49 ± 0.11 N, had its structure collapsed after being
347 removed from the mold. Therefore, 0.75 N at 6 mm of penetration (1 mm/s) may be the
348 minimum force to reach well-structured gelatin:cassava starch hydrogels, regardless they are
349 made with native or dry heated (2 h or 4 h at 130 °C) cassava starch.

350

351 *3.1.2. IDDSI fork pressure test*

352 The methods developed by the IDDSI were standardized worldwide in order to
353 evaluate foods and drinks that can be indicated for people with dysphagia, according to its
354 severity (Cichero et al., 2017). The results of the fork pressure test are shown at Table 1 for
355 all hydrogels. The IDDSI TMF level 6 (soft & bite-sized) must be: eaten with a fork;
356 mashed/broken down with fork pressure; cut with no knife, although it can be cut with fork
357 or spoon; soft, tender and moist throughout but with no separate thin liquid; chewed before
358 swallowing; ‘bite-sized’ pieces $\leq 1.5 \times 1.5$ cm for adults (The International Dysphagia Diet

359 Standardisation Initiative, 2019a). These characteristics could be reached by the gels made
 360 with gelatin and starch, which could not, e.g., fulfill the requirements for TMF level 5 (mince
 361 & moist), since they could not be scooped and shaped on a plate.

362

363 Table 1. Screening of gelatin:cassava starch hydrogels for the development of IDDSI level 6
 364 foods to people with dysphagia.

Cassava starch type	Gelatin:starch (w/w)	Structured gel.	Squashes within the tines of the fork.	Changes shape.	Returns to original shape.	Picture of when the thumbnail blanches.
10.7% of total solid content (moisture corrected to 14%)						
No starch added.	1:0	Yes	No	No	-	
Native	0:1	No	Yes	Yes	Yes	
	1:3	No	Yes	Yes	Yes	
	1:1	Yes	No	No	-	
	3:1	Yes	No	No	-	
DHT_2h	0:1	No	Yes	Yes	Yes	
	1:3	Yes	Yes	Yes	Yes	
	1:1	Yes	No	No	-	
	3:1	Yes	No	No	-	
DHT_4h	0:1	No	No	Yes	Yes	

	1:3	No	Yes	Yes	Yes	
	1:1	Yes	No	No	-	
	3:1	Yes	No	No	-	
5% of total solid content (moisture corrected to 14%)						
No starch added.	1:0	Yes	No	No	-	
Native	0:1	No	Yes	No	-	
	1:3	No	Yes	No	-	
	1:1	Yes	Yes	Yes	No	
	3:1	Yes	Partially	Yes	No	
DHT_2h	0:1	No	Yes	No	-	
	1:3	No	Yes	No	-	
	1:1	Yes	Yes	Yes	No	
	3:1	Yes	Partially	Yes	No	
DHT_4h	0:1	No	Yes	Yes	Yes	
	1:3	No	Yes	Yes	Yes	
	1:1	Yes	Yes	Yes	No	
	3:1	Yes	Partially	Yes	No	

365 According to the International Dysphagia Diet Standardisation Initiative (IDDSI), using the fork pressure test
366 (IDDSI, 2019a). Native: native cassava starch. DHT_2h: cassava starch submitted to dry heating treatment at
367 130 °C for 2h. DHT_4h: cassava starch submitted to dry heating treatment at 130 °C for 4h.

368

369 For screening purposes, we choose to assess hydrogels made with higher dimensions
370 (3.8 cm \varnothing \times 2.2 cm height) than that indicated by the IDDSI for the fork pressure test in order
371 to have a safer margin. No hydrogels visually released thin liquid (Table 1), which is a desired
372 characteristic following IDDSI recommendation.

373 Clearly, the gelatin/cassava starch hydrogels at a ratio of 1:1 (w/w) and 5% TSC
374 showed better performances at the fork pressure test, regardless the cassava starch was native
375 or dry heated. They were the only ones able to form structured gels, and to fulfill all the
376 IDDSI requirements: completely squashed within the tines of the fork when the thumbnail
377 blanched to white, changed shape, which did not return to its original form after thumbnail
378 pressure release.

379 The increase on gelatin from 1:1 (gelatin:cassava starch; w/w) to 3:1 (w/w) resulted
380 in stronger hydrogels with undesirable behavior. At 5% TSC, they could be partially
381 squashed within the tines of the fork, and not reach the bottom of the hydrogel due to
382 increased gel firmness, from ~1.5 N (1:1; w/w) to ~2.3 (3:1; w/w) (Figure 1). Moreover,
383 hydrogels with 10.7% TSC containing the same ratios of gelatin:cassava starch (1:1 and 3:1;
384 w/w) could not be squashed within the tines of the fork, and showed gel firmness of ~4.1 N
385 and ~8.5 N, respectively. Hydrogels with only gelatin – 3.91 ± 0.41 N (5% TSC) and 14.22
386 ± 0.72 N (10.7% TSC) (Figure 1) – formed structured gels, but unable to be squashed within
387 the tines of the fork (Table 1). Therefore, the maximum force for the development of

388 gelatin/cassava starch hydrogels for people with dysphagia may be up to 1.5 N at 6 mm of
389 penetration (1 mm/s).

390 It is worth noting that the hydrogel made only with DHT_4h cassava starch (10.7%
391 TSC) did not show the same structure capacity as previously reported (Maniglia et al., 2020).
392 The difference in behavior, even with the starches showing similar pasting properties and
393 morphology, may be explained by other factors that affect the chemical composition of
394 cassava starch such as crop genetics, planting season and year, as well as the starch extraction
395 procedure (Zhu, 2015). Only minor differences on physiochemical characteristics, including
396 amylose content, observed in starch gels made with cassavas from four cultivars harvested
397 during different planting seasons elicited considerable differences in gel firmness and
398 crystallinity (Asaoka et al., 1991). Nevertheless, mixing cassava starch, whether native or
399 modified by DHT, with gelatin overcame those cassava starch variations, making its use more
400 feasible for the development of customized foods that rely on texture as an important physical
401 feature. Furthermore, based on the IDDSI fork pressure test, the gelatin/cassava starch
402 hydrogels at a ratio of 1:1 (w/w) and 5% TSC showed the lowest risk of choking if eaten by
403 people with dysphagia.

404

405 *3.1.3. 3D printability test*

406 Almost all tested hydrogels were printable under the tested conditions, except for the
407 gelatin hydrogel with 10.7% TSC (Table 2). For the tested hydrogels with 10.7% TSC, the
408 regularity in the design of the stars was observed when the gelatin:starch ratio was at least
409 1:1 (w/w) for the native and DHT_2h treatments – the 10 sides of the stars were equal or at
410 least similar, and the geometry presented defined angles and connected lines. The
411 gelatin/DHT_4h hydrogels, however, showed regular printed stars from the 1:3 ratio (w/w)

412 on. Therefore, DHT for 4 h may have improved the 3D printability of cassava starch, as
413 previously observed (Maniglia et al., 2020).

414

415 Table 2. Screening of gelatin:cassava starch hydrogels for 3D printability of a star model.

Type of cassava starch	Gelatin:starch (w/w)	10.7% total solid content*		5% total solid content*	
		10 well-defined sides.	Continuous printed lines.	10 well-defined sides.	Continuous printed lines.
Native	0:1	No	Yes		
Native	1:3	No	Yes		
Native	1:1	Yes	Yes		
Native	3:1	Yes	Yes		
DHT_2h	0:1	No	Yes		
DHT_2h	1:3	No	Yes		
DHT_2h	1:1	Yes	Yes		
DHT_2h	3:1	Yes	No		
DHT_4h	0:1	No	Yes		
DHT_4h	1:3	Yes	Yes		
DHT_4h	1:1	Yes	Yes		
DHT_4h	3:1	Yes	No		

Gelatin	1:0	-	-	-	Yes	No	
---------	-----	---	---	---	-----	----	---

416 Only the gelatin hydrogel with 10.7% total solid content was not printable. *Moisture corrected to 14%. Native: native cassava starch.

417 DHT_2h: cassava starch submitted to dry heating treatment at 130 °C for 2h. DHT_4h: cassava starch submitted to dry heating treatment

418 at 130 °C for 4h.

419

420 The variation on TSC (5 or 10.7%) was sufficient to affect the opacity/transparency
421 of the gelatin/starch hydrogels, as seen in the photos at Table 2. Among the hydrogels with
422 5% TSC, only treatments with gelatin:cassava starch ratio of at least 1:1 (w/w) clearly
423 showed well-defined edges between the five points of the printed stars.

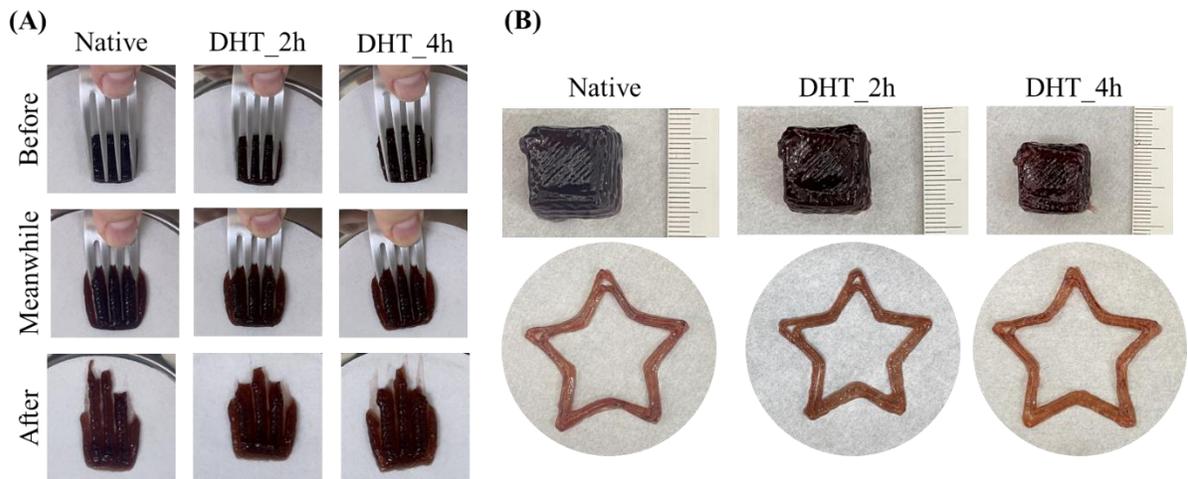
424 Therefore, based on the assessments of firmness, feasibility to a dysphagia diet by the
425 IDDSI fork pressure test, and 3D printability, the gelatin/starch hydrogels at the ratio of 1:1
426 (w/w) and 5% TSC were used for further investigation concerning the addition of grape juice,
427 and its effects.

428

429 *3.2. Production of grape juice gels with gelatin/cassava starch*

430 The 3D printed grape juice gels were made with gelatin/cassava starch focused on
431 people with dysphagia, as shown in Figure 2. Adding grape juice in the gels made with
432 gelatin/cassava starch (1:1, w/w) with 5% of gelling ingredients decreased pH from ~5.6 to
433 ~3.8, and affected gel firmness by reducing it from ~1.5 N (Figure 1) to 1.1 ± 0.01 N,
434 regardless the type of cassava starch used (native, DHT_2h or DHT_4h). This relatively low
435 effect of pH variation on gel firmness was expected, since gelatin gel strength is more
436 dependent on gelatin concentration than on pH or ionic strength (Marfil et al., 2012).

437



438

439 Figure 2. 3D printed grape juice gels made with gelatin/cassava starch.

440 (A) Before, meanwhile and after the fork pressure test of the International Dysphagia Diet Standardisation
 441 Initiative (IDDSI). (B) 3D printability test using the parallelepiped-shaped and star-shaped geometries. DHT:
 442 dry heating treatment at 130 °C for 2 h (DHT_2h) or for 4 h (DHT_4h). Gels were made with gelatin:cassava
 443 starch (1:1, w/w) and 5% of gelling ingredient (moisture of the gelling ingredients corrected to 14%).

444

445 As expected after selecting the composition of gelling ingredients, the three grape
 446 juice gels showed good 3D printability for both the parallelepiped and star geometries, as
 447 well as for the IDDSI fork pressure test (Figure 2). Therefore, the effect of this new matrix
 448 on grape anthocyanin bioaccessibility and anti-inflammatory activity was studied.

449

450 3.3. Anthocyanin content in grape juice gels made with gelatin/cassava starch

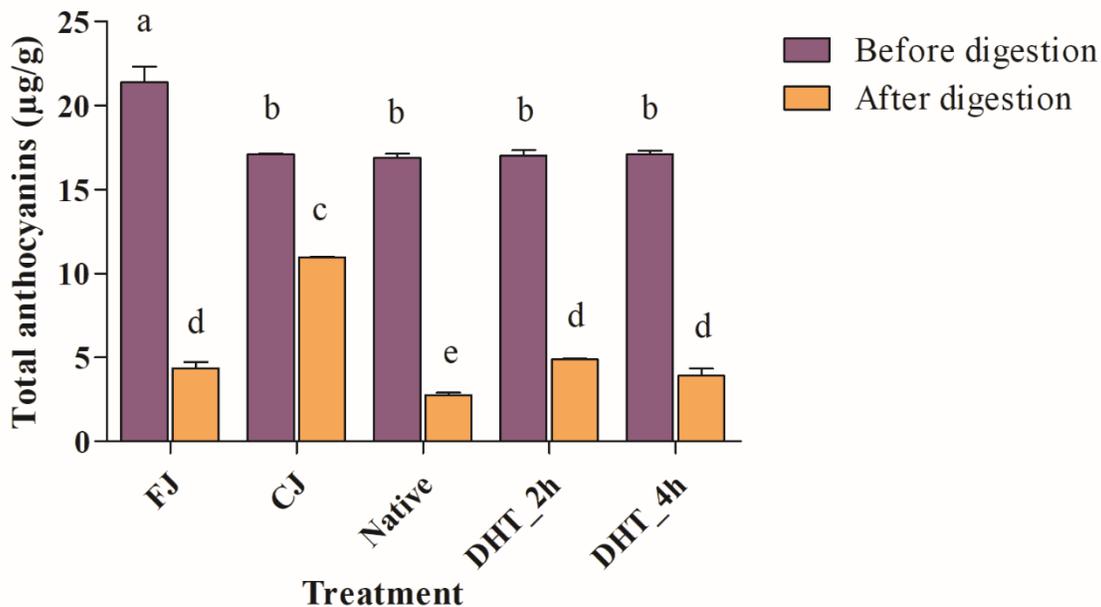
451 Grape juices are important dietary sources of phenolic compounds, including flavan-
 452 3-ols, anthocyanins, flavonols, and stilbenes. Among them, anthocyanins are known to show
 453 the lowest bioavailability (Mohamedshah et al., 2020), and therefore, their bioaccessibility
 454 was assessed.

455 Six anthocyanins were quantified in the commercial grape juice and all the other
456 undigested samples by the HPLC-PDA analysis: malvidin 3,5-diglucoside, malvidin 3-
457 glucoside, cyanidin 3-coumaroyl-5-diglucoside, delphinidin 3-coumaroylglucoside,
458 petunidin 3-coumaroylglucoside, and malvidin 3-coumaroylglucoside. These compounds
459 were tentatively identified by comparison of their MS spectra with those previously
460 determined in Concord grape juice anthocyanins by the same ion trap identification technique
461 (Wang et al., 2003) (Table 3). The concentrations of each anthocyanin in the samples are at
462 Table S1, and the total anthocyanin concentration is shown in Figure 3.
463

464 Table 3. Tentative identification by HPLC-ESI-UV/MS/MS of anthocyanins quantified by HPLC-PDA in all undigested samples.

Putative compound	RT (min)	Molecular ion [M-H] ⁺ (<i>m/z</i>)	Product ions (<i>m/z</i>)
Malvidin 3,5-diglucoside	12.3	655.42	493.2860, 331.1438
Malvidin 3-glucoside	15.1	493.26	331.1457
Cyanidin 3-coumaroyl-5-diglucoside	17.7	757.48	595.3137, 449.2713, 287.0278
Delphinidin 3-coumaroylglucoside	19.3	611.34	303.0425
Petunidin 3-coumaroylglucoside	20.0	625.35	317.0785
Malvidin 3-coumaroylglucoside	20.8	639.36	331.147

465



467

468 Figure 3. Total content of anthocyanins before and after *in vitro* gastrointestinal digestion.

469 Fresh (FJ) and cooked (CJ) grape juice. Grape juice gels made with gelatin and native cassava starch (Native)
 470 or with cassava starch submitted to dry heating treatment at 130 °C for 2h (DHT_2h) or 4h (DHT_4h). Different
 471 letters indicate statistically significant differences (One-way ANOVA and Tukey's test), at a level of confidence
 472 of 0.05.

473

474 The total anthocyanin concentration decreased after cooking the grape juice, as
 475 expected, due to thermal degradation (Oancea, 2021). The grape juice gels showed total
 476 anthocyanin concentration similar to cooked grape juice ($p < 0.05$). It is important to note
 477 that when adding the gelling ingredients, the juice content in the gels is lower than the juice
 478 itself, thereby the anthocyanin concentration should be lower in the gels as well. Therefore,
 479 the complexes formed by cassava starch and gelatin from bovine hides used in this study may
 480 have helped to avoid at a low extent the heating degradation of anthocyanins in the gels.

481 Phenolic compounds have been reported to form V-type complexes with starch, which in
482 turn tends to be encapsulated by protein in food formulations via formation of hydrogen
483 bonds, crosslinking, and electrostatic interactions with the amino acids of the peptides (Terán
484 & Blanco-Lizarazo, 2021).

485

486 *3.4. Effects of in vitro gastrointestinal digestion on anthocyanin concentration*

487 To exert their bioactivities, anthocyanins must first be released from the food matrix
488 within an aqueous phase in the intestine (bioaccessible fraction), to then become bioavailable,
489 which means that their bioaccessible fraction must be absorbed to reach the sites of action.
490 Although bioaccessibility only indicates the potential of a compound to be bioavailable,
491 generally compounds that are not bioaccessible are not bioavailable (Han et al., 2019).
492 Recently, the development of standardized protocols of *in vitro* gastrointestinal digestion,
493 with special regard to the Infogest protocol reproduced in this study, have made possible the
494 bioaccessibility assessment of several food formulations per experiment with good
495 repeatability, relatively ease of execution, and no ethical restrictions (Brodkorb et al., 2019).

496 In this study, the differences between the total anthocyanin concentration before and
497 after *in vitro* gastrointestinal digestion were significant, as calculated using both One-way
498 ANOVA with Tukey *post hoc* test to compare all treatments, and Two-way ANOVA with
499 Bonferroni *post hoc* test to compare within groups of undigested and digested for each
500 treatment ($p < 0.001$) (Figure 3). Only one anthocyanin, the tentatively identified malvidin
501 3-glucoside, was quantified by the HPLC-PDA method in the digested samples (Table S1).
502 This compound was found all in the undigested samples, and have been reported as a major
503 anthocyanin in different grape varieties (Colombo et al., 2019; Wang et al., 2003). *In vitro*
504 bioaccessibility of anthocyanins was 3-fold higher for cooked grape juice (64.2%) than fresh

505 grape juice (20.4%), which indicates that the heating may have affected cellular structures
506 and/or chemical bonds, increasing the release of this anthocyanin later during *in vitro*
507 digestion. To the best of our knowledge, the effect of further heating on grape juice
508 anthocyanin bioaccessibility have not being studied. However, it has been hypothesized that
509 the increased content of anthocyanins released within the bioaccessible fraction when heating
510 was employed during the juicing process overcomes the losses during the processing for
511 Concord grape juice (Mohamedshah et al., 2020).

512 Gelling the grape juice with the tested biopolymers affected the bioaccessibility of
513 anthocyanins. The grape juice gel made with native starch had the anthocyanin
514 bioaccessibility reduced (16.3%). The grape juice gels made with dry heated starch showed
515 anthocyanin bioaccessibility higher than fresh grape juice and grape juice gel made with
516 native starch (28.7% and 22.9% for DHT_2h and DHT_4h, respectively).

517 During gastrointestinal digestion, anthocyanins generally are stable in the acidic
518 conditions of the gastric phase, but are easily oxidized and degraded into smaller phenolic
519 compounds in the mild alkaline pH of the intestine, where they can also form insoluble
520 complexes with trypsin and bile salts (Liu et al., 2022). Moreover, the interactions between
521 protein and starch may generate a steric hindrance for amylases, decreasing starch hydrolysis
522 in the small intestine (Terán & Blanco-Lizarazo, 2021), and consequently hampering
523 anthocyanin release from the gelatin/starch matrix. In a previous study, it was observed that
524 DHT at 130 °C for 2 h and 4 h increased cleavage of glycosidic bonds, solubility of the
525 granules, and formation of carbonyl groups in cassava starch (Maniglia et al., 2020).
526 Therefore, the higher molecular breakdown in the dry heated starch granules may have: a)
527 allowed the release of higher contents of anthocyanins in the intestine by increasing the
528 interface where amylases act; and b) released the anthocyanins following a kinetics that

529 prevent their excessive degradation under the mild alkaline pH during the 2 h simulated small
530 intestinal digestion.

531 Hence, although eating grapes or drinking grape juice is the best option for healthy
532 people, strategies such as adding gelatin/DHT-cassava starch as gelling ingredients may
533 allow people with dysphagia to have the benefits related to proper anthocyanin
534 bioaccessibility rates.

535

536 *3.5. Anti-inflammatory activity before and after in vitro gastrointestinal digestion*

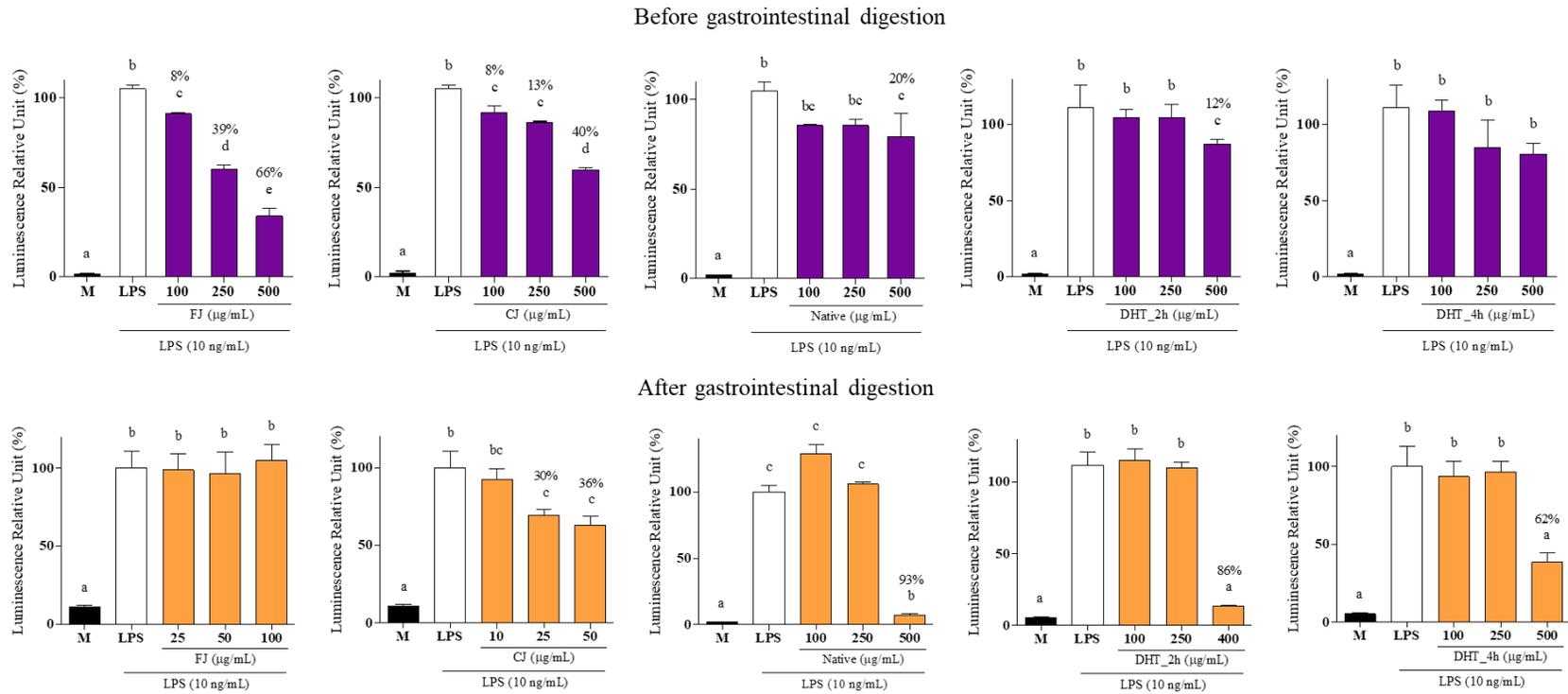
537 Systemic chronic inflammation is age-related, low-grade and persistent, typically
538 triggered in the absence of an acute infection by environmental factors across the life span
539 and metabolic dysfunction, leading to collateral damage to tissues and organs (Furman et al.,
540 2019). Healthy lifestyles, which include an adequate intake of plant foods containing
541 bioactive compounds, may downregulate the expression of proinflammatory genes, which
542 have been related to the onset and progression of noncommunicable diseases (Maleki et al.,
543 2019).

544 In this study, the RAW 264.7 cell line was used as a model of macrophages, since it
545 has been recognized as appropriate to confirm the anti-inflammatory effects of drugs and to
546 assess important signaling pathways related to inflammation (Seo et al., 2022). The viability
547 of macrophages treated with different concentrations of the samples was verified prior to the
548 anti-inflammatory activity assays, as shown in Figure S1. Only concentrations that did not
549 significantly reduce cell viability, when compared with the control ($p > 0.05$), were presented
550 and used for the anti-inflammatory assays (Figure S1). No reduced cell viability was
551 observed for macrophages treated with grape juices, whether fresh or cooked, up to 500
552 $\mu\text{g/mL}$, which means that the cell death rate was statistically the same as for cells in only

553 medium and no treatment ($p > 0.05$). After *in vitro* gastrointestinal digestion, cell viability
554 was maintained for macrophages treated with the cooked and the fresh grape juices up to 50
555 and 100 $\mu\text{g/mL}$, respectively. Higher concentrations of the grape juice gels (up to 500 $\mu\text{g/mL}$)
556 were well tolerated by the macrophages, indicating a protective effect of the gelatin/starch
557 matrices after *in vitro* digestion.

558 The NF- κ B forms a family of transcription factors that has been suggested as a
559 potential target for therapy of inflammation-related diseases (Yu et al., 2020). In this study,
560 cooking the grape juice had a negative effect on decreasing NF- κ B activation in the innate
561 immune cells, when compared to the fresh grape juice, as shown in Figure 4. Although at a
562 lower extent, the grape juice gels made with native or DHT_2h decreased NF- κ B activation
563 in the macrophages ($p < 0.05$), indicating the release of contents of phenolic compounds with
564 this anti-inflammatory activity from the gelatin/starch matrices. No significant decrease in
565 NF- κ B activation was observed in macrophages treated with grape juice gels made with
566 DHT_4h at any tested concentration ($p < 0.05$).

567



568

569 Figure 4. NF-κB activation induced by lipopolysaccharide (LPS) in RAW 264.7 macrophages treated with grape juices and grape juice
570 gels.

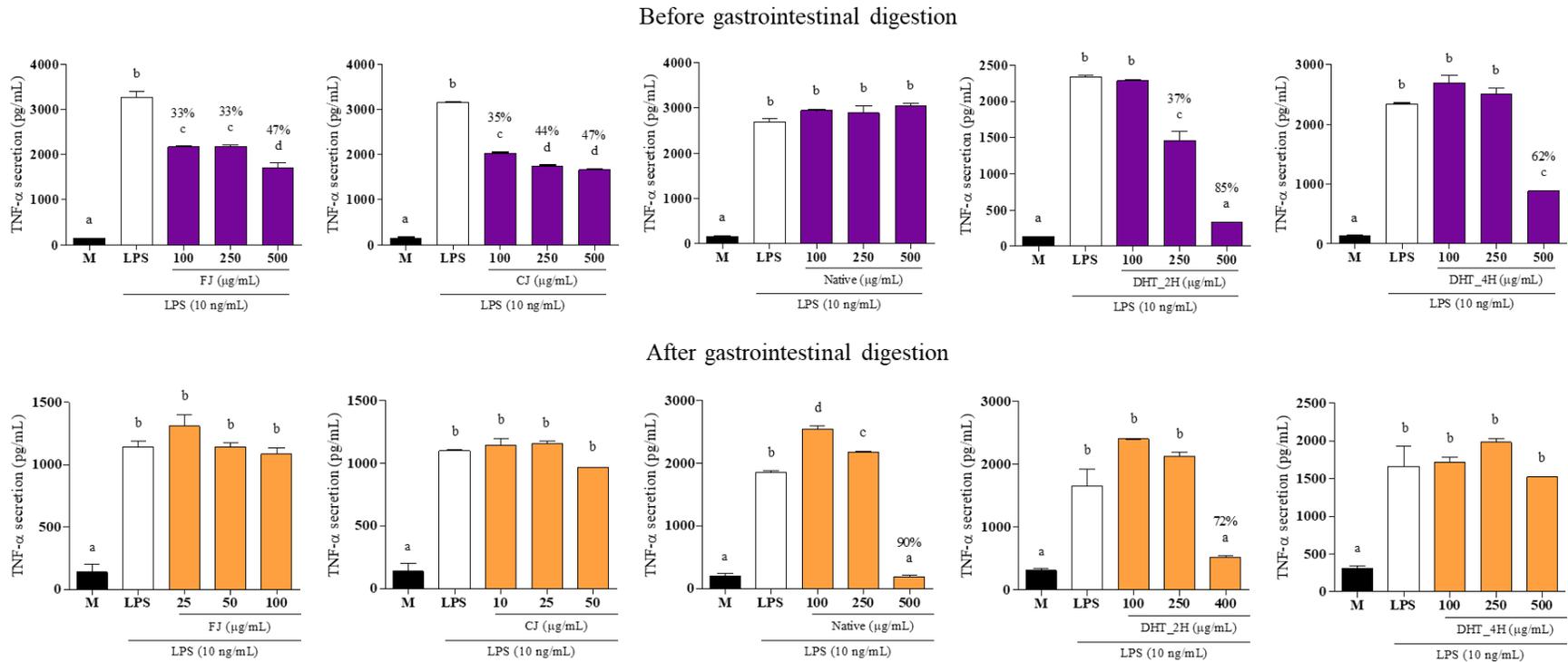
571 Fresh (FJ) and cooked (CJ) grape juice, and grape juice gels made with gelatin and native cassava starch (Native) or cassava starch submitted to dry heating treatment
572 at 130 °C for 2h (DHT_2h) or 4h (DHT_4h). M: negative control, which was non-induced macrophages. LPS: positive control, which was the LPS-induced untreated
573 macrophages. Different letters within each graphic indicate statistically significant differences (One-way ANOVA and Tukey's test), at a level of confidence of
574 0.05.

575 After *in vitro* gastrointestinal digestion, only the cooked grape juice, which showed
576 the highest anthocyanin bioaccessibility, significantly decreased NF- κ B activation, at 25 and
577 50 μ g/mL, when compared to the positive control ($p < 0.05$). Therefore, the malvidin 3-
578 glucoside at the concentration found in the digested cooked grape juice may have contributed
579 in decreasing NF- κ B activation, along with other grape juice phenolics such as procyanidins,
580 which have been reported to exert this anti-inflammatory activity (Colombo et al., 2019). It
581 is worth noting that, although no activity was observed for digested fresh grape juice at the
582 tested concentrations, it does not mean that it has no activity, since further metabolization
583 occurs within the enterocytes and activity can be detected after basolateral transport.

584 All grape juice gels decreased NF- κ B activation at the highest concentrations tested,
585 including the one made with DHT_4h (Figure 4). Therefore, further release of phenolic
586 compounds able to deactivate the NF- κ B from the grape juice gel matrices may have occurred
587 during gastrointestinal digestion.

588 TNF- α is a cytokine produced by LPS-induced macrophages, and its blockage has
589 been a therapeutic approach to reduce organ damage in inflammation-related diseases, and
590 potentially to COVID-19 (Del Valle et al., 2020). In this study, similar decreases in TNF- α
591 secretion were observed in macrophages treated with grape juice, whether fresh or cooked,
592 before *in vitro* digestion ($p < 0.05$), as shown in Figure 5, regardless the difference in
593 anthocyanin concentration (Figure 3). Therefore, the inhibition on TNF- α secretion may be
594 more related to other phenolic compounds found in red grape juice that have shown this effect
595 and not monitored in our study such as quercetin and epigallocatechin gallate (Magrone et
596 al., 2020).

597



598

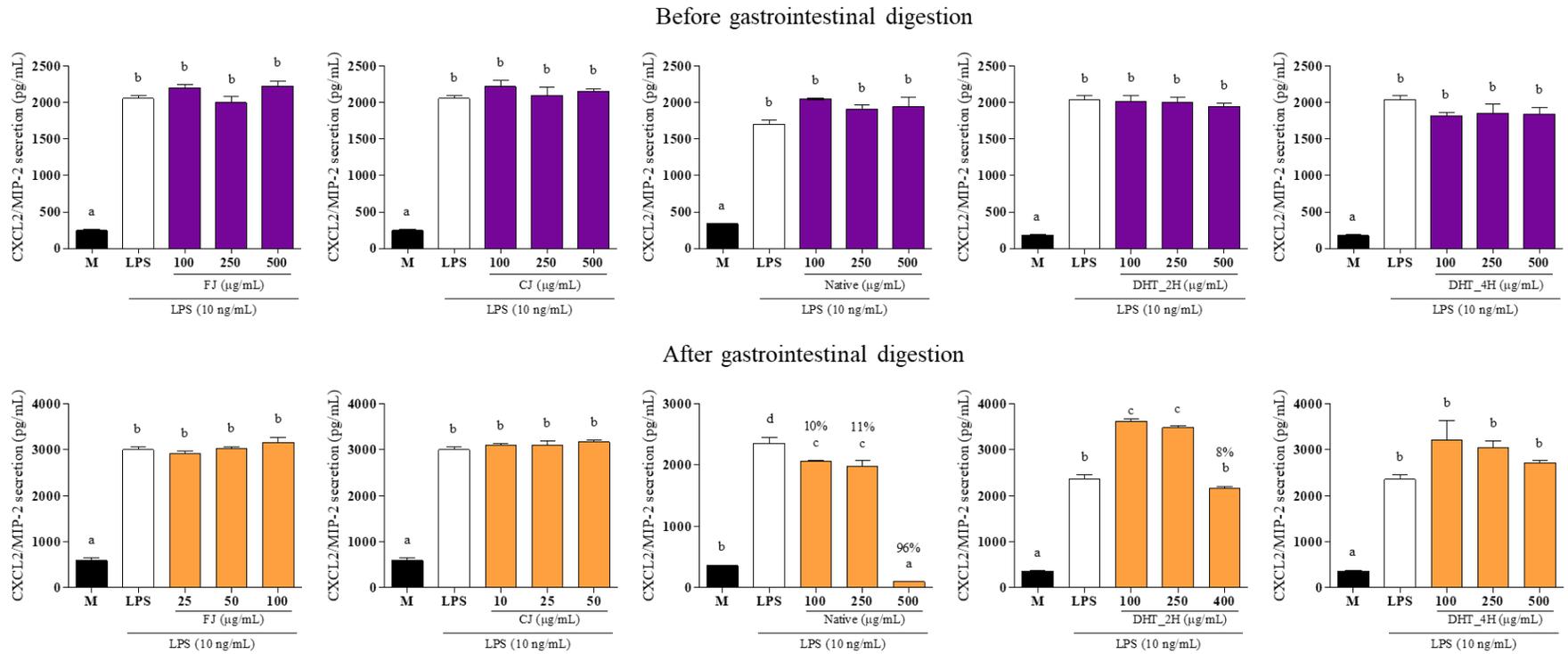
599 Figure 5. TNF- α secretion induced by lipopolysaccharide (LPS) from RAW 264.7 macrophages treated with grape juices and grape juice
600 gels.

601 Fresh (FJ) and cooked (CJ) grape juice, and grape juice gels made with gelatin and native cassava starch (Native) or cassava starch submitted to dry heating treatment
602 at 130 °C for 2h (DHT_2h) or 4h (DHT_4h). M: negative control, which was non-induced macrophages. LPS: positive control, which was the LPS-induced untreated
603 macrophages. Different letters within each graphic indicate statistically significant differences (One-way ANOVA and Tukey's test), at a level of confidence of
604 0.05.

605 No decrease in TNF- α secretion was observed in macrophages treated with grape
606 juice gels made with native cassava starch ($p < 0.05$), whereas the grape juice gels made with
607 DHT_2h or DHT_4h significantly decreased TNF- α secretion at 250 and 500 $\mu\text{g/mL}$ or 500
608 $\mu\text{g/mL}$, respectively ($p < 0.05$). However, after *in vitro* digestion, no significant decreases in
609 TNF- α secretion were observed for the grape juices and the grape juice gel made with
610 DHT_4h at all concentrations tested, when compared with the positive control ($p < 0.05$).
611 The grape juice gels made with native and DHT_2h at the highest concentrations tested, were
612 able to decrease the secretion of TNF- α for 90% and 72%, respectively ($p < 0.05$). Hence,
613 there is indication that the *in vitro* digestion releases compounds related to this anti-
614 inflammatory activity from the grape juice gel matrices made with native or DHT_2h,
615 although the dry heating at 130 °C for 4 h is excessive and does not modulate this release
616 properly under gastrointestinal conditions.

617 During inflammation, chemokines are secreted by macrophages to recruit blood
618 neutrophils to the damaged tissue, exacerbating inflammation, and the chemokine
619 CXCL2/MIP-2 outstands during this process (Sawant et al., 2021). In this study, the secretion
620 of CXCL2/MIP-2 by the LPS-induced macrophages was not affected by the treatment of any
621 undigested sample, as shown at Figure 6 ($p > 0.05$).

622



623

624 Figure 6. CXCL2/MIP-2 secretion induced by lipopolysaccharide (LPS) from RAW 264.7 macrophages treated with grape juices and
 625 grape juice gels.

626 Fresh (FJ) and cooked (CJ) grape juice, and grape juice gels made with gelatin and native cassava starch (Native) or cassava starch submitted to dry heating treatment
 627 at 130 °C for 2h (DHT_2h) or 4h (DHT_4h). M: negative control, which was non-induced macrophages. LPS: positive control, which was the LPS-induced untreated
 628 macrophages. Different letters within each graphic indicate statistically significant differences (One-way ANOVA and Tukey's test), at a level of confidence of
 629 0.05.

630 After *in vitro* digestion, similarly to what was observed for the secretion of TNF- α ,
631 significant decreases in the secretion of CXCL2/MIP-2 were observed only for the grape
632 juice gels made with native, at all concentrations tested, and with DHT_2h at the highest
633 concentration tested ($p < 0.05$). These anti-inflammatory activities (inhibition of the secretion
634 of TNF- α and CXCL2/MIP-2) may also be related to the bovine hide gelatin added in the
635 grape juice gels. Water extracts of donkey hide gelatin administered in mice decreased NF-
636 κ B activation as well as the secretion of proinflammatory cytokines and chemokines (Lee et
637 al., 2022). However, the interactions between dry heated cassava starch and phenolic
638 compounds of grape juice seem to affect this gelatin effect.

639 Therefore, the use of gelatin and cassava starch as gelling ingredients produced grape
640 juice gels with anti-inflammatory activity even after simulated gastrointestinal digestion,
641 which may be helpful to people with dysphagia that are unable to eat grapes or drink grape
642 juice.

643

644 **4. Conclusion**

645 This work evaluated the combination of cassava starch, native and modified by the
646 dry heating treatment (DHT), and gelatin, as gelling ingredients for grape juice. The obtained
647 gels were designed to be used for 3D printing of food for people with dysphagia.

648 The addition of gelatin from bovine hides showed a bigger effect than modifying
649 cassava starch in starchy hydrogels' firmness, feasibility to dysphagia diets and 3D
650 printability. Nonetheless, grape juice gels made with gelatin and DHT-starch showed slightly
651 increased anthocyanin bioaccessibility, when compared to fresh grape juice and grape juice
652 gel made with gelatin/native starch. Furthermore, all grape juice gels decreased NF- κ B
653 activation, whereas the grape juice gels made with native starch and DHT-starch at 130 °C

654 for 2 h suppressed TNF- α and CXCL2/MIP-2 secretion after *in vitro* gastrointestinal
655 digestion in LPS-induced murine macrophages.

656 Therefore, it was demonstrated that the simple, environmentally friendly, and low-
657 cost modification of cassava starch by dry heating treatment could enhance chemical and
658 biological properties of 3D printed grape juice gels, also containing gelatin, for people with
659 dysphagia.

660

661 **Acknowledgements**

662 The authors are grateful to:

- 663 • The “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil
664 (CAPES)” - for the B.S. Bitencourt PhD. scholarship (88887.636998/2021-00) and
665 L.C.R. Torres PhD. scholarship (Finance Code 001);
- 666 • The São Paulo Research Foundation (FAPESP, Brazil) for funding the projects n°
667 2019/05043-6 and 2020/08727-0, and the J.S. Guedes PhD. scholarship (2021/06398-
668 2);
- 669 • The PRPI/USP for funding the project PIPAE 2021.1.1024.1.9;
- 670 • The National Council for Scientific and Technological Development (CNPq, Brazil)
671 for the A.S.M.C. Saliba PhD. scholarship (311894/2020-8);
- 672 • Communauté urbaine du Grand Reims, Département de la Marne, Région Grand Est
673 and European Union (FEDER Champagne-Ardenne 2014-2020) are acknowledged
674 for their financial support to the Chair of Biotechnology of CentraleSupélec and the
675 Centre Européen de Biotechnologie et de Bioéconomie (CEBB).

676

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688 Data Curation; Writing - Review & Editing.

689 Severino Matias de Alencar: Conceptualization; Methodology; Formal Analysis; Resources;
690 Writing - Review & Editing; Supervision; Project administration; Funding acquisition.

691 Pedro Esteves Duarte Augusto: Conceptualization; Methodology; Formal Analysis;
692 Resources; Writing - Review & Editing; Supervision; Project administration; Funding
693 acquisition.

694

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842

Supplementary material

Anthocyanin bioaccessibility and anti-inflammatory activity of a grape-based 3D printed food for dysphagia

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Brief description:

This supplementary material encompasses:

- Table S1, with the concentration of individual and total anthocyanins by HPLC-PDA analysis in fresh and cooked grape juice and in grape juice gels made with gelatin:cassava starch (1:1; v:v), before and after *in vitro* gastrointestinal digestion.
- Figure S1, with viability of RAW 264.7 macrophages after 4 hours, determined by the MTT method, after treatment with different concentrations of fresh (FJ) and cooked (CJ) grape juice, and grape juice gels made with gelatin and native cassava starch (Native) or with cassava starch submitted to dry heating treatment at 130 °C for 2h (DHT_2h) or 4h (DHT_4h).

Table S1. Concentration of individual and total anthocyanins by HPLC-PDA analysis in fresh and cooked grape juice and in grape juice gels made with gelatin:cassava starch (1:1; v:v), before and after *in vitro* gastrointestinal digestion.

Putative compound	RT (min)	Concentration ($\mu\text{g/g}$ petunidin-3- <i>O</i> -glucoside chloride equiv.)				
		FJ	CJ	Native	DHT_2h	DHT_4h
Before <i>in vitro</i> gastrointestinal digestion						
Malvidin 3,5-diglucoside	12.443	1.12 ± 0.07	0.87 ± 0.06	1.15 ± 0.02	1.29 ± 0.04	1.17 ± 0.04
Malvidin 3-glucoside	15.695	7.41 ± 0.35	5.86 ± 0.13	5.75 ± 0.11	5.94 ± 0.13	5.90 ± 0.14
Cyanidin 3-coumaroyl-5-diglucoside	17.511	0.57 ± 0.06	0.33 ± 0.03	0.31 ± 0.03	0.31 ± 0.06	0.33 ± 0.04
Delphinidin 3-coumaroylglucoside	19.159	1.27 ± 0.13	0.79 ± 0.05	0.57 ± 0.01	0.50 ± 0.02	0.74 ± 0.04
Petunidin 3-coumaroylglucoside	21.235	1.32 ± 0.01	1.28 ± 0.05	1.18 ± 0.04	1.22 ± 0.09	1.12 ± 0.01
Malvidin 3-coumaroylglucoside	22.776	9.72 ± 0.46	7.95 ± 0.03	7.91 ± 0.17	7.75 ± 0.13	7.88 ± 0.23
Total anthocyanins		21.41 ± 0.91	17.08 ± 0.07	16.88 ± 0.25	17.02 ± 0.32	17.11 ± 0.19
After <i>in vitro</i> gastrointestinal digestion						
Malvidin 3-glucoside	15.701	4.36 ± 0.36	10.97 ± 0.05	2.75 ± 0.16	4.89 ± 0.03	3.92 ± 0.41

Fresh weight basis. FJ: fresh juice. CJ: cooked juice. Native: native cassava starch. DHT_2h: cassava starch submitted to dry heating treatment at 130 °C for 2h.

DHT_4h: cassava starch submitted to dry heating treatment at 130 °C for 4h.

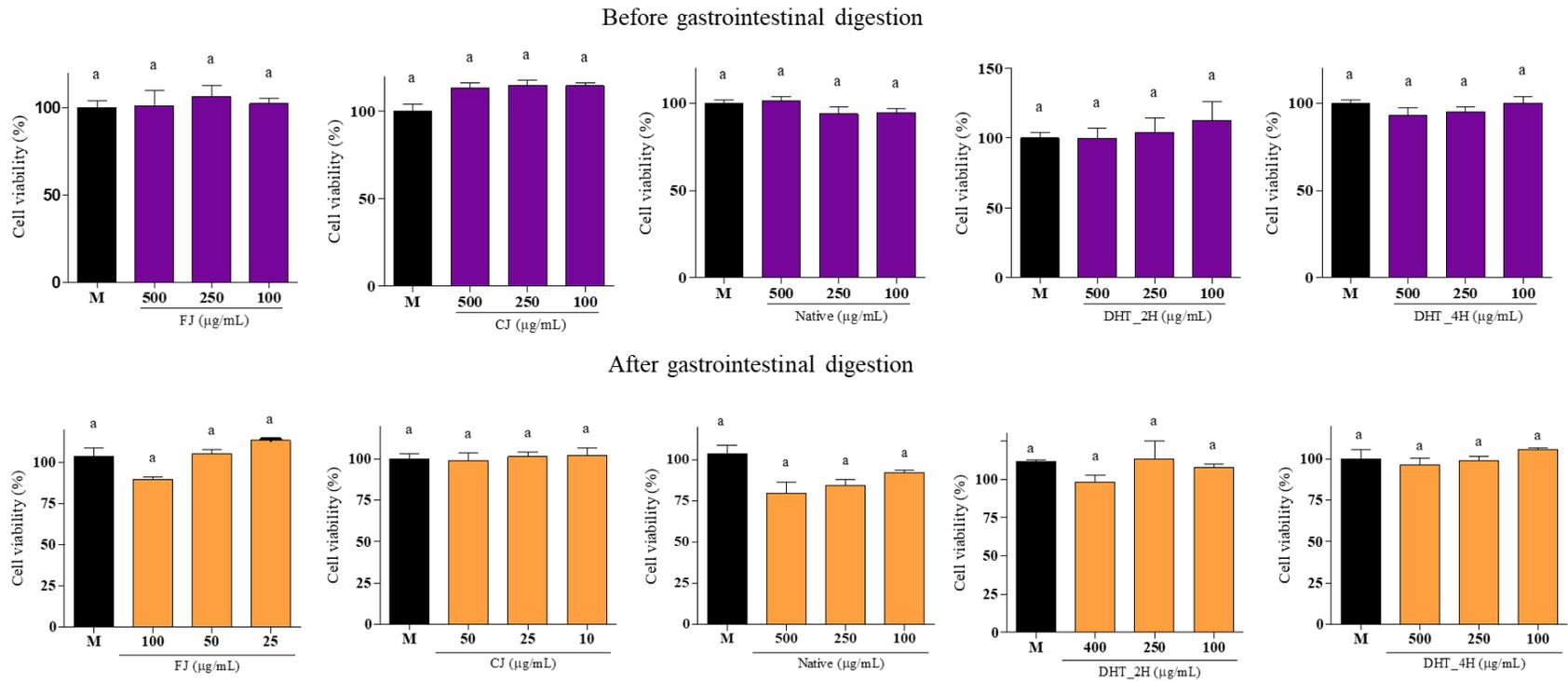


Figure S1. Viability of RAW 264.7 macrophages treated with grape juices and grape juice gels after 4 hours, determined by the MTT method.

Fresh (FJ) and cooked (CJ) grape juice, and grape juice gels made with gelatin and native cassava starch (Native) or cassava starch submitted to dry heating treatment at 130 °C for 2h (DHT_2h) or 4h (DHT_4h). M: negative control, which was untreated macrophages. Different letters within each graphic indicate statistically significant differences (One-way ANOVA and Tukey's test), at a level of confidence of 0.05.

