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Valorisation of Persimmon and Blueberry By-Products to Obtain Functional Powders: *in vitro* Digestion and Fermentation by Gut Microbiota.

Claudia Bas-Bellver[1], Cristina Andrés[1], Lucía Seguí[1], Cristina Barrera[1], Nuria Jimenéz-Hernandéz[2,3], Alejandro Artacho[2], Noelia Betoret[1*], María José Gosalbes[2,3*]

1 Instituto de Ingeniería de Alimentos para el Desarrollo (IIAD), Universitat Politècnica de València, Camí de Vera s/n, 46022 Valencia, Spain.

2 Unitat Mixta d'Investigacióen Genòmica i Salut, Fundacióper al Foment de la InvestigacióSanitària i Biomèdica de la Comunitat Valenciana (FISABIO-Salut Pública)/Institut de Biologia Integrativa de Sistemes, Universitat de València, 46010 València, Spain

3 CIBER en Epidemiología y Salud Pública, 28029 Madrid, Spain

*Corresponding authors:

Noelia Betoret, email: noebeval@tal.upv.es; phone: +34 963877000 (ext. 83624)

María José Gosalbes, email: maria.jose.gosalbes@uv.es; phone: +34 961925965

1 ABSTRACT

2 Globalization of fruit and vegetable market generates overproduction, surpluses and

3 potentially valuable residues. The valorisation of these by-products constitutes a

4 challenge, to ensure sustainability and reintroduce them into the food chain. This work

5 focuses on blueberry and persimmon residues, rich in polyphenols and carotenoids, to

6 obtain powders with high added value to be used **as ingredients** in food formulation.

7 These powders have been characterized and the **changes** of the bioactive compounds in

8 *in vitro* gastrointestinal digestion have been evaluated. The results indicated that the type

9 of residue, the drying process, as well as the content and type of fibre determine the

10 release of antioxidants during digestion. *In vitro* colonic fermentations were also

11 performed, and it was observed that the characteristics of digested powders had an effect

12 on the composition of the growing microbial community. Thus, carotenoids and

13 anthocyanins maintain an interplay with microbiota that could be beneficial for human

14 health.

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17 **Keywords:** bioactive compounds, fruit by-products, *in vitro* digestion, colonic

18 fermentation, gut microbiota, metagenomics

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INTRODUCTION

The fruit and vegetable processing industry generates a large amount of waste with an important ecological footprint.[1] The concept of sustainable diets, recently introduced by the Food and Agriculture Organization (FAO, http://www.fao.org), combines the challenges of healthy diets for a growing population while reducing its environmental impacts. In recent years, fruit and vegetable powders have become a new way of consuming these products in the diet and to account for an adequate intake of phytochemicals, such as polyphenols and carotenoids, which have been associated with beneficial effects on human health.[2] These powders are presented in a stable concentrated form and could be used directly or as an ingredient in food formulation. Thus, the manufacture of powders from fruit and vegetable processing wastes could be applied with similar purposes since the bioactive compounds (polyphenols, carotenoids and fibre) are also present in these by-products, in most cases in a higher percentage. To ensure functionality of the manufactured powders and the final products, it is of capital importance to understand the impact of the digestion process on the compounds of interest, thus elucidating the relationship between food composition and structure, processing and digestion steps. Also, it is crucial to investigate into the interactions between bioactive compounds and other components, such as fibre, present in the whole product. It is known that some specific phenolic constituents remain associated to solid matrix macromolecules, they having been referred as non-extractable polyphenols in contrast to solvent-soluble ones.[3]. Nevertheless, as a more open concept, extractability depends on several factors such as the physico-chemical characteristics of the solid matrix, processing or cooking methods applied, as well as the digestion process itself, which has an impact on phenolics extractability and its physiological effect. In this sense, polyphenols will be solubilized and released to the liquid phase along digestion, part of

49   them reaching the colon in a solubilized form as being present in the liquid phase retained

50   by the pellets. During digestion, polyphenols may interact with sugars and dietetic fibre,

51   affecting their stability and bioavailability.[4] Phenolic compounds can also act as digestive

52   enzyme inhibitors reducing the glycaemic response from starch, at least *in vitro*, but this

53   inhibitory activity could be reduced by the interactions between polyphenols and soluble

54   fibre.[5]

55   In recent years, the study of the human microbiome has been promoted through

56   massive sequencing techniques and "omics" approaches, considerably increasing

57   knowledge about the structure, metabolic functions and interactions of the intestinal

58   microbiota, as well as its role in human health.[6] The gut microbiota performs a wide

59   variety of beneficial functions such as the synthesis of essential vitamins and amino acids,

60   the development of the immune system, the proliferation, differentiation and maintenance

61   of the intestinal epithelium and protection against pathogens. Additionally, the microbiota

62   plays a fundamental role in nutrition by intervening in metabolic processes such as the

63   degradation of complex polysaccharides and fibre and the metabolism of phenolic

64   compounds and carotenoids from foods of plant origin.[7] Thus, there is a dual interaction

65   as the diet is one of the factors that most influence the composition of the intestinal

66   microbiota and gut bacterial population degrades food constituents producing metabolites

67   with potential beneficial effect on human health.

68   There is extensive evidence supporting that the polyphenols show strong antioxidant

69   and anti-inflammatory properties with an important role in the human health, although

70   the mechanisms of action are not entirely clear. However, the interactions between plant-

71   origin bioactive compounds and microbiota has not been deeply studied and has been,

72   mainly, focus on polyphenols.[8,9] Several i*n vivo* and *in vitro* works have showed that the

4

anthocyanins produce an increase of *Lactobacillus* and *Bifidobacterium* in the microbiota.[8-10]

Carotenoids have been largely studied in terms of health effects in epidemiological and human intervention studies, focusing on the part of phytochemicals that after being ingested enter the circulatory system. However, only a small fraction of the carotenoids ingested in the diet is absorbed in the intestine and reach our tissues, the rest arrive to the colon where they are metabolized by the microbiota.[11] Despite this fact, there is a lack of information regarding the interactions between the carotenoids and the gut microbiota. The carotenoids, β-carotene, β-cryptoxanthin, and α-carotene, are the preferential source of vitamin A and they are provided in the diet. After ingestion, provitamin A carotenoids are converted into vitamin A, mainly retinoic acid. In mice, deficiency in retinoic acid reduced the numbers of segmented filamentous bacteria (SFB), contributing to the decreased number of T helper 17 (TH17) cells observed.[12] Recently, a fucoxanthin supplementation study in mice showed significantly changes in the composition of both the cecal and faecal microbiota.[13] Another marine carotenoid, astaxanthin, has been used in eight-week supplementation study in mice detecting a change in the microbiota composition.[14]

Persimmon and blueberry by-products are rich in carotenoids and polyphenols, respectively. The purpose of the present study was to understand the effect of processing on the physicochemical ~~and functional~~ properties of persimmon and blueberry waste powders, as well as to investigate the impact of *in vitro* digestion steps on antioxidant properties and specific bioactive compounds. In addition, colonic fermentations of the digested powders were undergone in order to determine the effect of polyphenols and carotenoids on colonic bacteria growth and the associations between physicochemical properties of powders and fermentative bacterial community.

MATERIALS AND METHODS

Raw material

Whole persimmon fruits (*Diospyros kaki* L. var. Rojo Brillante) were supplied by the agricultural cooperative of Benaguasil (Valencia, Spain), after undergoing a CO_2 de-astringent treatment. Pulp was separated so that resulting peels and chalices (external whorl of the flowers that remain at the top of persimmon fruits after being collected) were grinded at 5000 rpm for 4 s in a TM31 Thermomix® food processor (Vorwerk, Madrid, Spain), and stored in plastic bags at -20 ºC until further processing. Frozen organic blueberries (*Vaccinium corymbosum* L. cv. Duke) were supplied by Samanes S.L (Navarra, Spain) and processed to obtain juice as described by Castagnini et al.[15] and pomace obtained as a by-product.

Obtaining powders from persimmon and blueberry residues

Persimmon and blueberry by-products powders were obtained by dehydration (freeze-dried or air-dried) and milling. Freeze drying (FD) consisted of a freezing stage at -40 ºC during 12 h, and further sublimation during 24 h at -45 ºC and 0.1 mbar in a Telstar Lioalfa 6-80 freeze drier. Air-drying (AD) was conducted in a CLW 750 TOP+ Pol-Eko-Aparatura SPJ transverse flow tray dryer with air at 2 m/s at 60 and 70 ºC for persimmon and blueberry residues, respectively and according to preliminary experiences. Dried residues were milled at 10,000 rpm for 2 min at 30 s intervals in a TM31 Thermomix® food processor (Vorwerk, Madrid, Spain). Powders were stored at room temperature in closed glass jars covered with aluminium foil until further analysis.

Powders *in vitro* simulation of gastrointestinal digestion

122   *In vitro* static digestions of 1 g of sample were carried out according to the

123   standardised INFOGEST protocol[16], human saliva was used as salivary fluid, while

124   simulated gastric and intestinal fluids were prepared from the corresponding electrolyte

125   stock solutions. Human saliva supplied by a single healthy donor was mixed with fruit

126   powders in a ratio 1:1 (v/w) in falcon tubes, homogenized and incubated for 2 min at

127   37 °C. Porcine pepsin (Sigma-Aldrich, Madrid, Spain) was added to the corresponding

128   gastric phase solution (2000 UmL^{-1} in the final digestion mixture), together with the

129   required HCl (1 M) to reduce the pH to 3.0. The obtained simulated gastric fluid was

130   mixed in a ratio 1:1 (v/w) with the oral bolus, in the falcon tubes, and were flipped top to

131   bottom at 55 rpm for 120 min at 37 °C in an Intell-Mixer RM-2 (Elmi Ltd, Riga, Latvia)

132   placed in the incubation chamber. For the intestinal phase, pancreatin from porcine

133   pancreas (Sigma-Aldrich, Madrid, Spain) was added to achieve 100 UmL^{-1} in the final

134   digestion mixture, together with 1 M NaOH to reach pH 7.0. The simulated intestinal

135   fluid containing the porcine pancreatin was then added in a ratio 1:1 (v/w) to each falcon

136   tube, and samples were flipped top to bottom at 55 rpm for another 120 min at 37 °C. *In*

137   *vitro* digestion of inulin from chicory (SaludViva, Elx (Alicante), Spain) was performed

138   as a control. Each powder was digested in duplicate in order to obtain the amount of

139   substrate required in the further *in vitro* colonic fermentation. Additional *in vitro*

140   gastrointestinal digestions were performed in order to measure in triplicate the content in

141   specific compounds (carotenoids in persimmon waste samples and anthocyanins in

142   blueberry bagasse samples), total phenols, total flavonoids and the ability to scavenge

143   DPPH and ABTS radicals at the end of the gastric and intestinal phases in both the

144   supernatant and the pellet.

145

Analytical determinations of moisture content (x_w), water activity (a_w), soluble solids content (x_{ss}), solubility and fibre content

Moisture content was obtained by drying 5 g of sample at 95 ºC on an infrared balance until constant weight. Water activity was directly measured at 25 ºC in a dew point hygrometer (Decagon devices Inc., Pullman WA, USA). Brix degrees were measured in a 1:10 (w/v) aqueous solution using a table refractometer (AbbeAtago, 3-T, Tokyo, Japan), at 20 ºC. Total soluble solids content was calculated from the moisture content and the Brix degrees value. Solubility, i.e. the ratio between total soluble solids content and total solids content, was determined on a 1:50 (w/v) aqueous solution following the method described by Mimouni et al.[17], after vacuum drying to constant weight a filtered and a non-filtered 20 g of the previous solution at 60 ºC and 200 mbar. The fibre content was analysed following the Van Soest method describe in Mertens[18], which provides the neutral-detergent fibre (NDF or total fibre, that corresponds to the lignin, cellulose and hemicellulose content), the acid-detergent fibre (ADF, that corresponds to the lignin and cellulose content) and lignin with acid detergent (LAD, that corresponds to the pure lignin content). From these values, the insoluble fibre content (that is the content of both cellulose and lignin) and the soluble fibre content (considered to be equivalent to the hemicellulose content) were obtained.

Antioxidant (AO) properties

Antioxidant properties were measured in the powders before digestion and after the gastric and intestinal phases. Prior to antioxidant determinations, the digested samples were centrifuged (10,000 rpm, 5 min) in order to distinguish between supernatant and precipitate. Antioxidant properties of supernatant were determined directly, whereas antioxidants present in the undigested powders and digested precipitate were extracted

171   with an 80% (v/v) methanol in water solution (1 h stirring and further centrifugation at

172   10,000 rpm for 5 min).

173   Total phenolic and flavonoid content were measured by the colorimetric methods of

174   Folin–Ciocalteu and aluminium chloride, respectively, as described in Seguí et al.[19]

175   Likewise, antioxidant activity was assessed by measuring the ability to scavenge DPPH

176   and ABTS radicals (purity $\geq$ 98%) relative to that of the reference antioxidant Trolox

177   (purity $\geq$ 97%)[19]. For the former, 0.1 mL of extract were mixed with 0.9 mL of methanol

178   and 2 mL of a 0.025 mg/mL solution of DPPH in methanol and kept in darkness for 2 h

179   before measuring the absorbance at 515 nm (Thermo Scientific Helios Zeta UV/Vis

180   spectrophotometer). For the latter, 0.1 mL of extract were mixed with 2.9 mL of an

181   ABTS+ in phosphate buffer solution (0.7 absorbance at 734 nm) and kept in darkness for

182   7 min before measuring the absorbance at 734 nm in a Thermo Scientific Helios Zeta

183   UV/Vis spectrophotometer. Distilled water replacing the extract was used as a blank.

184

185   **Carotenoids extraction and quantification by HPLC**

186   Extraction of carotenoids from persimmon by-product powders was carried out

187   following the procedure described by Bunea et al.[20] with some modifications. 1 g of

188   undigested powder (or alternatively the result of digesting 1 g of powder) and 25 mL of

189   a 1:1:1 (v/v/v) methanol/ethyl acetate/petroleum ether solution were mixed using a T25

190   digital ULTRA TURRAX®. After centrifugation (10,000 rpm, 5 min), the precipitate was

191   re-extracted with the same solvent mixture until colour exhaustion. All the extracts were

192   collected in a separation funnel and washed several times with 100 mL of a saturated

193   saline solution until both aqueous and etheric phase were clear. Etheric phase containing

194   the compounds of interest was dried over anhydrous sodium sulphate and evaporated at

195   35 ºC under vacuum conditions in a Heidolph rotary evaporator. The dried extract was

196 recovered in 5 mL of diethyl ether and 5 mL of a 30% methanolic potassium hydroxide

197 solution and kept in darkness overnight at room temperature. Then, the mixture was

198 washed alternating 70 mL of a saturated saline solution and 70 mL of distilled water until

199 both aqueous and etheric phase were clear, and the pH was neutralized. Once again, the

200 organic phase containing the carotenoids was dried over anhydrous sodium sulphate and

201 evaporated until dryness. The evaporated residue was finally re-dissolved in 1.5 mL of

202 diethyl ether and filtered with 0.45 μm pore size Whatman® PTFE membrane filters

203 before analysis by HPLC. An Agilent 1100 HPLC system equipped with a quaternary

204 pump, an automatic injector and a diode array detector was used for carotenoids

205 quantification. The chromatographic separation of the compounds was achieved at 25 ºC

206 by using a Waters reverse phase C30 YMC column (5 μm, 250 mm × 4.6 mm) and a

207 linear gradient of acetonitrile: water: triethylamine (90:10:0.25, v/v/v) and ethyl acetate:

208 triethylamine (100:0.25, v/v) from 90:10 to 10:90 in 20 min as mobile phase. All

209 chromatograms were monitored at 450 nm and external standard curves of HPLC purified

210 standards supplied by Merck Laboratories (Madrid, Spain) were used for carotenoids

211 identification and quantification. Calibration curves were made using seven different

212 concentrations (0-125 μg/mL) of pure β-cryptoxanthin (CAS No. 472-70-8, purity ≥ 97%

213 purity, Sigma-Aldrich), α-carotene (CAS No. 7488-99-5, purity ≥ 95.0%, Sigma-Aldrich)

214 and (9Z)-β-carotene (CAS No. 13312-52-2, purity ≥ 90.0%, Sigma-Aldrich).

215

216 **Anthocyanins extraction and quantification by HPLC**

217 Extraction of anthocyanins from blueberry pomace powders was carried out following

218 the procedure described by Cătunescu et al.[21] with some modifications. 0.1 g of powder

219 (or alternatively the result of digesting 1 g of powder) was mixed with 10 mL of acidified

220 methanol (0.1% HCl), sonicated for 30 s and centrifuged at 5,000 rpm and 4 ºC for 5 min.

221 This process was repeated until complete discoloration occurred. The combined

222 supernatants were then dried over anhydrous sodium sulphate and evaporated at 35 ºC

223 under vacuum conditions in a Heidolph rotary evaporator. The evaporated residue was

224 finally dissolved in 1.5 mL of solvent and filtered with 0.45 μm pore size Whatman®

225 PTFE membrane filters before analysis by high-performance liquid chromatography. An

226 Agilent 1100 HPLC system equipped with a quaternary pump, an automatic injector and

227 a diode array detector was used for anthocyanins quantification. The chromatographic

228 separation of the compounds was achieved at 35 ºC by using a Waters reverse phase C18

229 column (5 μm, 4.6 mm × 250 mm) and 4.5% formic acid in water (solvent A) and

230 acetonitrile (solvent B) as mobile phase. The gradient elution system started with 10%

231 acetonitrile for 9 min, increased linearly to 12% acetonitrile at 17 min and continued up

232 to 25% acetonitrile at 30 min, remaining constant at 35% acetonitrile between 30 and 40

233 min. Chromatograms were monitored at 520 nm and external standard curves of HPLC

234 purified standards supplied by Merck Laboratories (Madrid, Spain) were used for

235 anthocyanins identification and quantification. Calibration curves were made using seven

236 different concentrations (0-125 μg/mL) of cyanin chloride (CAS No. 2611-67-8, purity $\geq$

237 90%, Sigma-Aldrich), delphinidin chloride (CAS No. 528-53-0, analytical standard,

238 Supelco) and malvidin chloride (CAS No. 643-84-5, purity $\geq$ 95.0%, Sigma-Aldrich)

239 HPLC standards.

240

241 **Faecal samples and processing**

242 Faecal samples were collected from 7 healthy donors (male n=3 and female n=4). All

243 subjects gave their informed consent before they participated in the study. The protocol

244 was approved by the Ethics Committee of the Public Health Department and the Centre

245   for Public Health Research (DGSP-CSISP), Valencia, Spain (approval number:

246   20190301/03).

247   Volunteers transported the samples to the laboratory in a container with a gas

248   generator. To prepare the slurries, faecal samples were weighted and diluted in a ratio

249   1:10 (w/v) with 0.1 M anaerobic sterile sodium phosphate buffer at pH 7,0 containing 0.4

250   g/L of cysteine hydrochloride and 15% of glycerol as reducing agent and cryoprotector,

251   respectively. The 7 resulting faecal slurries were stored at -80 ºC until further use as

252   inoculum in the batch cultures. Frozen and cryoprotected samples have been used in other

253   studies[22,23], allowing the performance of all the fermentation experiments with the same

254   inoculum.

255

256   ***In vitro* colonic fermentation**

257   The *in vitro* static batch culture fermentation procedure was applied according to

258   Olano-Martin et al.[24] with slight modifications. In detail, a series of 50 mL tubes with 45

259   mL of basal medium and the substrate under investigation were inoculated with 5 mL of

260   faecal slurry and incubated at 37 ºC in anaerobic jars with carbon dioxide generator during

261   24 h. Pre-digested powders were used as the substrate for colonic fermentations at 1%

262   (w/v). We used the 7 slurries as individual inoculum to ferment the powders obtained

263   from each investigated substrate: freeze-dried persimmon waste (P.FD); air-dried

264   persimmon waste (P.AD): freeze-dried blueberry bagasse (B.FD); air-dried blueberry

265   bagasse (B.AD) and from inulin as control positive for bacterial growth (35 *in vitro*

266   fermentations). Moreover, a control fermentation without substrate was performed using

267   as inoculum a mix of the 7 slurries previously obtained from the faecal samples.

268   Aliquots were removed from the fermenters at baseline (t = 0 h) and after 24 h for

269   further analysis. The incubation and processing procedures were carried out under

270 anaerobic conditions in an anaerobic jars or anaerobic chamber. High purity H_2 was used

271 for initially purging the anaerobic chamber and the working gas mixture employed was

272 N_2:H_2:CO_2 proportioned at 80:10:10 (v/v/v).

273

274 **Total DNA extraction, sequencing analysis and characterization of microbiota**

275 Total DNA was extracted from fermentation aliquots and faecal samples in the robotic

276 workstation MagNA Pure LC Instrument (Roche, San Cugat (Barcelona), Spain) using

277 the MagNA Pure LC DNA isolation kit III (Bacteria, Fungi) (Roche, San Cugat

278 (Barcelona), Spain). Total DNA was quantified with a Qubit Fluorometer (ThermoFisher,

279 Alcobendas (Madrid), Spain). Next, the V3-V4 region of the 16S rRNA gene was

280 amplified using as template total DNA obtained of bacterial population from fermentation

281 batches and faecal samples. Amplicon libraries were constructed following Illumina

282 instructions. Sequencing were performed with the Kit v3 (2×230 cycles) in a MiSeq

283 platform (Illumina, Eindhoven, The Netherlands) at FISABIO-Salud Pública. All the

284 sequences have been deposited in the EBI database under the number PRJEB36995.

285 16S rRNA gene reads with low-quality score and short read length as well as potential

286 chimeras were removed using DADA2 pipeline in R package (R). DADA2 pipeline was

287 used to create the amplicon sequence variants (ASV). The taxonomic information of the

288 16S rDNA sequences was obtained by similarity comparison using BLAST algorithm

289 against SILVA database (v.132).

290 To analyse bacterial composition of the fermentations, box plots and canonical

291 correspondence analysis (CCA) at genus level were generated with in-house R scripts.

292 The box plot represented the average of the abundance of those genera that are at least

293 present in the 60% of the samples of the group. The linear discriminant analysis (LDA)

294 effect size (LEfSe) algorithm was applied to identify biomarkers of the microbiota

295   composition from the different groups.[25] Default parameters were used for significance

296   (p-value < 0.05) and linear discriminant analysis threshold (> 2.0).

297

298   **Statistical analysis**

299   To statistically assess the effect of the dehydration technique and the *in vitro* digestion

300   on the physicochemical properties of the powders, including antioxidant properties and

301   specific compounds, analysis of variance (simple and multifactor) with a 95% confidence

302   level were carried out using Statgraphics Centurion (XVI.I version, StatPoint

303   Technologies, Inc.).

304   Multivariate analysis of variance based on dissimilarity test (ADONIS) was applied

305   to evaluate the effect of the external factors on the bacterial composition using the vegan

306   library from the R package (adonis function). To statistically evaluate differences

307   between groups in continuous variables, the Kruskal–Wallis test was used. The pairwise

308   comparisons of continuous variables were analysed using the Wilcoxon rank-sum test.

309   The alpha values for both tests were set to 0.05 or 0.1.

310   To establish correlations between powders and digested-powders properties of

311   persimmon waste and blueberry bagasse and microbiota abundance at 24 h fermentations,

312   multivariate analysis Sparse Partial Least Squared (sPLS)[26] applying the "mixOmics"

313   package from R was used. For this analysis, we set the properties of the fermentation

314   substrates (antioxidant activity, content of cellulose, antioxidants, polyphenols,

315   flavonoids, hemicellulose, lignin, total fibre and insoluble fibre) as predictors and the

316   microbiota composition as response variable.

317

318   **RESULTS AND DISCUSSION**

319   **Physicochemical properties of persimmon and blueberry residues powders**

320   Physicochemical properties of persimmon and blueberry residues powders are

321   summarized in Table 1. Water activity (a_w), moisture content (grams of water/g total

322   grams), soluble solids content (x_{ss}), as well as fibre fractions values are given. Drying

323   processes allowed to reduce a_w to safe values (≤ 0.3), thus granting stability to the

324   powders.[27] Corresponding moisture contents were also very low as compared to original

325   peel and bagasse, in which water is present in more than a 90%, the drying process having

326   contributed to reduce most of the free water content responsible for spoilage reactions.

327   Statistically significant differences between the persimmon and blueberry powders reveal

328   the different composition and characteristics of both raw materials. Soluble solids content

329   in persimmon waste powders was higher than in blueberry bagasse powders. The fact that

330   the peeling process takes out some of the persimmon flesh would have contributed to this

331   difference. Nevertheless, soluble solids content was relatively low as compared to fruit

332   (pulp, juice) powders, in which caking or stickiness may become a problem during storage

333   due to sugars content[28]. With regard to processing, freeze-dried powders showed a

334   significant higher soluble solids content for both types of residue. Freeze-drying usually

335   results in a finer powder[29] because of the more porous structure of freeze-dried

336   materials[30], which are therefore more easily milled than air dried. On the one hand, finer

337   powders would facilitate the extraction of soluble solids, since more surface area is

338   available for the extraction of soluble compounds; on the other hand, a more effective

339   milling implies breaking fibres into smaller molecules.

340   Total fibre content was significantly different among samples, blueberry powders

341   having a significant higher content than persimmon ones. In general, fibre values obtained

342   were lower than the reported by other authors (for persimmon by-products[31] and for

343   blueberry bagasse[32]). This could be due to difference in the cultivar, maturity or analytical

344   procedures being used. As for the different fibre fractions, it is generally accepted that

345   fibre sources suitable to be used as food ingredients should have a ratio soluble dietary

346   fibre to insoluble dietary fibre close to 1:2, so as to provide adequate physiological

347   effects[33]. This criterion is met by all the powders obtained in the present work, persimmon

348   and blueberry, air-dried and freeze-dried. On the other hand, the fact that both freeze-

349   dried powders presented lower fibre values than their respective air-dried ones could be

350   explained by the effect of milling and particle size reduction[31]. Freeze-dried matrices are

351   more open and porous as a consequence of the water sublimation process, this leading to

352   a particular capillary structure which is more easily milled.[34] This would imply more

353   breakage of fibres during milling and, consequently, an increase in the soluble solids

354   content, which agrees with soluble solids results.

355

356   **Antioxidant properties of persimmon and blueberry residues powders along *in vitro***

357   **gastrointestinal digestion**

358   Response of the obtained fruit waste powders to *in vitro* gastrointestinal digestion

359   regarding their antioxidant properties is summarized in Table 2. Results evidence that

360   blueberry bagasse powders exhibited significantly better antioxidant properties than

361   persimmon peel ones, which is due to differences in the raw materials. Blueberries are

362   particularly rich in anthocyanins, compounds which confer this material excellent

363   antioxidant properties[32]. With regard to persimmon residue, it is important to note that

364   persimmon postharvest techniques include de-astringency with CO_2 during 48 h, which

365   insolubilizes tannins, which is known to reduce total phenol content and corresponding

366   antioxidant capacity[35]. Martínez-las Heras et al.[36] reported similar results for persimmon

367   peel fibre powder.

368   The drying process being used (FD *vs.* AD) did not have a significant effect on phenol

369   and flavonoid contents or AO capacity in the case of persimmon waste powder, but

16

370 significantly affected blueberry bagasse powder. ~~As shown further, this different response~~

371 ~~to processing might have an impact on the microbiota.~~ FD especially produced a powder

372 with significant higher content in phenols and flavonoids that might affect to the bacterial

373 community composition that carried out the colonic fermentation. This had a significant

374 impact on AO properties only in the case of the ABTS measurements. This would suggest

375 that some of the compounds present in the blueberry bagasse are especially sensitive to

376 the AD process, which applies higher temperatures. In fact, the already mentioned

377 anthocyanins are known to be less stable at higher temperatures, having been reported

378 significant reductions of their concentration on berries treated at 35 ºC *vs.* berries

379 subjected to 25 ºC[37].

380   Table 2 also shows the evolution of the AO properties of powders along the *in vitro*

381 simulated digestion process. Measurements were performed on the supernatant and pellet

382 phases, after each phase, i.e. gastric and intestinal. Only antioxidants released from the

383 food matrix by the action of digestive enzymes (small intestine) and bacterial community

384 (large intestine) are bioaccessible in the gut and therefore potentially bioavailable[38].

385 Results evidence that the amount of antioxidants collected at the supernatant phase is

386 significantly lower than the amount present in the pellet phase. This implies that most of

387 polyphenols solubilized during digestion remained in the pellets, which consist of solid

388 matrix and liquid phase retained on it. When analyzing the pellet fraction, authors are

389 determining solubilized antioxidant components, the initially soluble and the solubilized

390 by digestion media and digestion conditions, as affected by previous processing.

391   In general, antioxidant compounds and activity increased after the gastric phase, and

392 decreased during the intestinal one. Breaking down of the solid matrix due to gastric fluids

393 might have solubilized phenolic constituents previously linked to it, increasing the

394 availability of reactive antioxidants, for which a rise in the phenols and flavonoid

395   contents, as well as the DPPH and ABTS antioxidant activities were observed in most

396   cases. This was especially evidenced in the case of blueberry powders. With regard to

397   antioxidants reactivity, Chen et al.[39] reported that the pH of a substance may modify

398   compounds reactivity and alter their biological reactivity, for which they could become

399   more reactive at acidic pH in the gastric phase and less reactive in the duodenal one. As

400   said, most of the solubilized antioxidant components remained in the precipitate (pellets).

401   In fact, the bioavailability of polyphenols such as anthocyanins has been said to be

402   generally low, for which it has been proposed that metabolites resulting during colonic

403   fermentation may be the components responsible for their health benefits[30].

404   As for interactions with other compounds, fibrous particles are known to interfere

405   with the release and absorption of antioxidants in different ways. On the one hand, a

406   fibrous matrix may limit antioxidant release and increase the viscosity of luminal

407   contents, thus reducing rates of antioxidant absorption by trapping the antioxidant within

408   the fibre matrix in the chyme. Accordingly, the rate of release of antioxidants from fibrous

409   particles to the surrounding intestinal fluid is inversely related to particle size[38]. Ortega et

410   al.[4] have reported that polyphenols may interact with sugars and dietetic fibre, which

411   would exert a protective role for phenolic constituents during the digestion process, thus

412   affecting their stability and bioavailability. In their study performed on washed and non-

413   washed carob flours, they concluded that the soluble components (soluble sugars and

414   soluble dietary fibre) exerted a protective role for polyphenols against pH changes or

415   enzymatic activities during the digestion process. In addition, disruption of the food

416   matrix under digestion conditions may not only release polyphenols, but also other

417   soluble components which establish interactions with certain polyphenol compounds

418   enhancing their recovery and stability during digestion. In contrast, other components

419   such as polysaccharides or insoluble dietary fibre may be responsible for phenol losses

420   during digestion.

421   In this sense, the results of the present work suggest that a smaller content of fibre,

422   mainly insoluble fibre (which was the case of persimmon powders), would allow the

423   antioxidant components to be more accessible. This is deduced from the bioaccessibility

424   index (Supplementary Figure 1), obtained as the amount of antioxidant component or

425   corresponding antioxidant activity in the liquid phase of the digested sample (after

426   intestinal phase), with respect to the same component/activity as measured in the non-

427   digested sample. On the other hand, bioaccessibility was in general higher for fine

428   powders (freeze-dried), although this was not evident for blueberry. As said previously,

429   anthocyanins are generally considered to be very unstable compounds and their contents

430   significantly reduced during digestion due to pH changes[30]. Results of this part suggest

431   that the type of residue, the drying process, the resulting particle size and the fibre content

432   and characteristics determines the release of antioxidants during digestion.

433

434   **Carotenoids and anthocyanins response to *in vitro* gastrointestinal digestion**

435   Figure 1 shows the carotenoid and anthocyanin content of persimmon residue and

436   blueberry bagasse powders before and after gastric and intestinal digestion. α-

437   cryptoxanthin was the most abundant carotenoid in persimmon powders. Total carotenoid

438   content was slightly higher in the freeze-dried powders than in the air-dried ones, as

439   expected from the temperatures applied. Degradation of all carotenoids analysed during

440   the *in vitro* simulated digestion process was evidenced. This had also been confirmed by

441   other authors in different samples such as orange juice[40] or carrot powder[41]. ~~Nevertheless,~~

442   ~~there was a slight but statistically significant increase of each individual carotenoid~~

443   ~~analysed after the intestinal stage, a phenomenon also observed by Tydeman et al.[42] in~~

19

444 ~~carrot samples. During digestion, carotenoids are passively absorbed in the small intestine~~

445 ~~along with lipids, the efficiency of absorption depending on dissolving lipophilic~~

446 ~~molecules into dietary lipids in the form of micelles[36]. Accordingly, the increase in the~~

447 ~~carotenoid content after intestinal digestion has been attributed to lipid phase~~

448 ~~emulsification[41]~~, which would imply an increased solubilization of carotenoids in the lipid

449 ~~phase. Regarding the interaction with fibre, it has been suggested to interfere with micelle~~

450 ~~formation[36],~~ apart from increasing viscosity of the intestinal content.

451   The three anthocyanins identified in blueberry samples (cyanidin, delphinidin,

452 malvidin) were found in higher concentrations in the freeze-dried powders, in accordance

453 with AO results. The fact that these differences were only statistically significant for

454 ~~delphinidin~~ cyanidin ~~and malvidin~~ suggests that ~~cyanidin~~ delphinidin and malvidin could

455 be more resistant to heating and oxidation induced by drying air. As reported by Zorić et

456 al.[42], increasing temperature accelerates anthocyanins and their conjugated sugars

457 breaking down into small molecules such as aldehydes and benzoic acid derivatives or

458 their corresponding anthocyanidins. Regarding *in vitro* digestion, anthocyanins content

459 was significantly affected by the dehydration technique used to obtain the powder.

460 Despite providing a powder with higher anthocyanin content, freeze-dried samples were

461 more significantly affected by digestion. On the one hand, it seems that temperature

462 sensitive anthocyanins, which had been preserved during lyophilization, would be yet

463 degraded during digestion; on the other hand, structural changes promoted by freeze-

464 drying (cells breakage and cells content release, increased porosity and particle size)

465 could enhance anthocyanins exposure to digestive fluids, resulting in an increased

466 depletion of anthocyanins. Although not statistically significant, total anthocyanin

467 content in the air dried blueberry bagasse powder increased after both the gastric and the

468 intestinal stages of *in vitro* simulated digestion, what could be due to a more effective

469 release of fibre-bound anthocyanins. ~~Total anthocyanin content in the freeze-dried~~

470 ~~blueberry bagasse powder decreased by 20%, while that in the air-dried powder increased~~

471 ~~by 26%.~~ Thus, despite being lower after processing, anthocyanins content in the air-dried

472 powders would be higher after digestion.

473

474 **Fermentative microbiota composition**

475 To assess the metabolic capacity of faecal microbiota (7 samples), *in vitro*

476 fermentations with persimmon waste and blueberry bagasse powders, both air-dried and

477 freeze-dried, was performed. All the fermentations and the 7-initial inoculum (faecal

478 samples) were sequenced. After sequencing and taxonomy classification, no significant

479 differences were detected in the number of ASVs and genera (p = 0.432) for all the

480 fermentations (t = 24 h) and the initial inoculum (t = 0 h).

481 As it is shown in Figure 2, 10 bacterial genera presented higher relative abundance in

482 the five fermentations than those in faeces control (Supplementary Table 1). An increase

483 of different genera of Actinobacteria (*Cornisella*, *Bifidobacterium)* and Verrumicrobia

484 (*Akkermansia)* phyla were observed in some fermentations. These genera have been

485 described as fibre-degrader with beneficial effects on immune system and health

486 status[43,44]. The lactic acid bacteria, *Streptococcus* and *Lactobacillus,* also showed higher

487 abundance after fermentations including the digested powders (B.FD, B.AD, P.FD, P.AD

488 and inulin) than in the faeces control. Moreover, butyrate-producing bacteria such as

489 *Faecalibacterium* and Ruminococcaceae UCG-014 also presented higher abundance after

490 the fermentations. Butyrate is a short-chain fatty acid (SCFA) produced by the intestinal

491 bacteria as a result of the fermentation of non-digestible polysaccharides. This metabolite

492 is a critical mediator of the colonic inflammatory response and an immune system

493 contributor. In fact, butyrate has an important role in the colon homeostasis providing

494   about 70% of the energy to the colonocytes. Two genera of Veillonellaceae family,

495   *Megamonas* and *Dialister*, which grew in the five fermentations, could produce other

496   SCFA such as propionate[45]. Recently, Christensenellaceae family has been related with

497   low body mass index, with low triglyceride levels and elevated levels of high density

498   lipoprotein[46]. On the other hand, high abundance of Proteobacteria, mainly

499   *Escheria/Shigella*, at 24 h fermentations was also detected (Figure 2). This overgrowth

500   could be due to residual nutrients in the faecal samples and it has been described in other

501   studies on *in vitro* fermentations.[45-47]

502   The compositional differences at genus level among the fermentations carried out with

503   the 4 substrates: air-dried blueberry bagasse (B.AD), freeze-dried blueberry bagasse

504   (B.FD), air-dried persimmon waste (P.AD) and freeze-dried persimmon waste (P.FD),

505   were studied applying Canonical Correspondence Analysis (CCA) (Figure 3). Although

506   the permutational multivariable analysis, ADONIS test, did not reveal significant

507   differences in the microbial community structures, this ordination technique showed that

508   the first axis, explaining 45.63% of variability, separated the blueberry bagasse and the

509   persimmon waste fermentative microbiota on the base of abundance and composition,

510   while the second axis, explaining 36.85% of variability, separated B.AD and B.FD. As

511   other authors indicated[45-49], the substrates affected the microbial community growing

512   during the fermentation. Additionally, it was observed that the dehydration technique

513   applied had a higher effect on blueberry bagasse than on persimmon waste, thus

514   conditioning the composition of the *in vitro* resulting microbial community.

515   To assess which bacteria were preferentially growing in persimmon waste and

516   blueberry bagasse fermentations, LefSe analysis was performed (Figure 4). It was found

517   that *Streptococcus* ($p = 0.0064$) and *Veillonella* ($p = 0.0152$) were significantly more

518   abundant in persimmon than in blueberry fermentation with high discriminant values.

519 These results suggested that these genera grew better on persimmon waste, rich in

520 carotenoids, mainly in α-cryptoxanthin. Other bacteria such as *Odoribacter* (p = 0.0101)

521 and *Butyricimonas* (p = 0.0072) also presented higher abundance in persimmon than in

522 blueberry fermentation. On the contrary, the Firmicutes genera, Ruminococcaceae

523 GCA_900066225 (p = 0.0129), *Pygmaiobacter* (p = 0.0251) and *Lactobacillus* (p =

524 0.0027) showed significant higher abundance in blueberry fermentation. Since

525 Ruminococcaceae genera have been described as degrader bacteria from complex

526 polysaccharides and fibre[43,44], the higher abundance of Ruminococcaceae biomarkers in

527 blueberry fermentation is in accordance with the result of a higher total dietary fibre

528 content in blueberry bagasse than in persimmon waste. In a previous work, Vendrame et

529 al.[98] showed that the consumption of a blueberry powder drink, which was rich in

530 anthocyanins, increased *Lactobacillus acidophilus* and *Bifidobacterium spp.* as compared

531 to the placebo drink.

532 Finally, Sparse Partial Least Squared (SPLS) multivariate analysis was performed to

533 relate the physicochemical properties of digested and undigested persimmon waste and

534 blueberry bagasse powders with the microbial community composition after

535 fermentations. The results were presented as two association networks (Figure 5). The

536 genera *Streptococcus*, *Faecalibacterium* and *Bifidobacterium* were the core of both

537 networks, presenting high association value with almost all the properties of the substrates

538 (Supplementary Table 2). However, all the correlations with *Streptococcus* were

539 negative, thus indicating that high content of antioxidant compounds and fibre is

540 associated with a low abundance of this genus. As *Streptococcus* appeared as a biomarker

541 of persimmon fermentation, namely this genus is higher abundant in persimmon than in

542 blueberry fermentation, we suggest that a high content of anthocyanins and fibre affect to

543 *Streptococcus* growth. In fact, different studies have pointed out the selective

544   bacteriostatic or bactericide effect of polyphenols on faecal microbiota, mainly potential

545   pathogen[49]. In contrast, other polyphenols such as anthocyanins, can promote the growth

546   of beneficial bacteria *Bifidobacterium*, *Lactobacillus*, *Akkermansia muciniphila* and

547   *Faecalibacterium prausnitzii*.[50] Our results were in accordance with these previous data

548   since in both digested and undigested powder networks high positive associations

549   between polyphenols and flavonoids with *Bifidobacterium*, *Akkermansia, Collinsella,*

550   *Faecalibacterium* Ruminococcaceae_UCG-014 were observed (Figure 5).

551   As conclusions, temperature sensitive anthocyanins content in the air-dried blueberry

552   powder would be higher after digestion than in freeze-dried powder due to the resulting

553   particle size and porosity produced by the later drying process. On other hand, total

554   carotenoid content was higher in the freeze-dried persimmon powder than in the air-dried

555   and its behaviour during the *in vitro* digestion depends on the fibre and lipid content and

556   characteristics. In the colonic fermentation, high content of anthocyanins and fibre has a

557   negative impact on *Streptococcus* but promotes Ruminococcaceae genus and

558   *Lactobacillus* growth. Also, positive correlations were detected between polyphenols and

559   Actinobacteria genera (*Collinsella* and *Bifidobacterium*), *Akkermansia* and

560   Ruminococcaceae_UCG−014. Moreover, the content of fibre is positively associated

561   with *Faecalibacterium* and *Bifidobacterium*.

ABBREVIATIONS USED

DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); HPLC, high performance liquid chromatography; BLAST, Basic Local Alignment Search Tool.

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SUPPORTING INFORMATION DESCRIPTION

Supporting information Figure 1. Bioaccessibility index (%) of phenols and ABTS-DPPH- antioxidant activities of persimmon and blueberry residues powders (freeze dried and air dried) subjected to in vitro simulated digestion. P.FD: persimmon freeze-dried, P.AD: persimmon air-dried, B.FD: blueberry freeze-dried, B.AD: blueberry air-dried.

Supporting information Table 1. Wilcoxon rank-sum test of relative abundance of the bacterial genera in feces control fermentation versus blueberry, persimmon and inulin fermentations.

Supporting information Table 2. Statistical parameters of sparse partial least squared analysis and lineal model

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FIGURE CAPTIONS

742 **Figure 1.** Carotenoid and anthocyanin quantification (µg/g of dried sample) in A)

743 persimmon peel and B) blueberry bagasse powders, air-dried and freeze-dried, before

744 digestion (BD) and after gastric (GD) and intestinal (ID) digestion. Mean ± standard

745 deviation of three repetitions. P.FD: persimmon freeze-dried, P.AD: persimmon air-dried,

746 B.FD: blueberry freeze-dried, B.AD: blueberry air-dried. [a, b, c...] Different letters for the same

747 compound indicate statistically significant differences (p-value < 0.05)

748

749 **Figure 2.** Relative abundance at genus level after *in vitro* fermentations of the assessed

750 substrates and faeces control. The box plot represented the average of the abundance of

751 those genera that are at least present in the 60% of the samples of the group. B.FD,

752 lyophilized blueberry bagasse; B.AD, air dried blueberry bagasse; P.FD, lyophilized

753 persimmon waste; P.AD, air dried persimmon waste.

754

755 **Figure 3.** Canonical correspondence analysis at genus level of the bacterial community

756 after fermentations. B.FD, lyophilized blueberry bagasse; B.AD, air dried blueberry

757 bagasse; P.FD, lyophilized persimmon waste; P.AD, air dried persimmon waste.

758

759 **Figure 4.** LefSe analysis of genera between blueberry bagasse and persimmon waste

760 fermentations. Linear discriminative analysis (LDA) scores (log 10) for the most

761 discriminative genera in persimmon waste were represented on the positive scale; LDA-

762 negative scores indicated enriched genera in blueberry bagasse.

763

764 **Figure 5.** Networks representing the associations between microbiota composition and

765 A) physicochemical properties of powders of blueberry bagasse and persimmon waste,

766   B) physicochemical properties of digested-powder blueberry bagasse and persimmon

767   waste.

768

769   **FUNDING SOURCES**

TABLE 1. Physicochemical Properties of Persimmon (P) Waste and Blueberry (B) Bagasse Powders, Air (AD) or Freeze-Dried (FD). Moisture Content (x_w, $g_w/100\ g_{total}$), Water Activity (a_w), Soluble Solids Fraction (x_{ss}, $g_{ss}/100\ g_{total}$), and Fibre Content ($g_{fibre}/100\ g_{total}$) and Fractions % ($g_{fibre}/100\ g_{dry\ matter}$). Mean ± Standard Deviation of Three Replicates.

property	B.AD	B.FD	P.AD	P.FD
x_w (%)	3.67±0.03 a	4.60±0.16 b	5.2±0.3 bc	5.6±0.5 c
a_w	0.196±0.010 a	0.208±0.010 ab	0.302±0.003 c	0.235±0.003 b
x_{ss} (%)	33.2±1.1 a	43.0±1.1 b	65.7±1.3 c	77.2±1.0 d
total fibre (%)	29.28±0.12 b	30.1±1.0 b	17.1±0.2 a	17.0±0.2 a
fibre (%dm)	**B.AD**	**B.FD**	**P.AD**	**P.FD**
hemicellulose	9.44±0.06 c	9.56±0.04 c	7.63±0.10 b	7.1±0.2 a
cellulose	14.09±0.07 b	14.5±0.7 b	6.91±0.10 a	6.212±0.014 a
lignin	6.9±0.2 c	7.5±0.4 c	3.456±0.003 b	2.75±0.11 a
insoluble fibre	21.0±0.2 b	22.0±1.0 b	10.37±0.11 a	8.96±0.13 a
total fibre	30.40±0.12 b	31.5±1.0 b	18.0±0.2 a	18.0±0.2 a

a, b, c… Different letters within the same row indicate statistically significant differences (p-value < 0.05)

TABLE 2. Total Phenols (mg Gallic Acid Equivalents/g non-Digested Powder), Flavonoid Content (mg Quercetin Equivalents/g non-Digested Powder) and Antioxidant (AO) Capacity by the DPPH and ABTS Methods (mg TE/g non-Digested Powder), of Persimmon (P) Waste and Blueberry (B) Bagasse Powders, Air (AD) or Freeze-Dried (FD). Before Digestion, and After Gastric and Intestinal Phases. Content in the Supernatant Phase (SP) and Pellet (PP) Phases. Mean ± Standard Deviation of Three Repetitions.

	before digestion			
powder	**total phenols (mg GAE/g)**	**flavonoids (mg QE/g)**	**AO-DPPH (mg TE/g)**	**AO-ABTS (mg TE/g)**
B.AD	35±2 b	18.3±0.8 b	66.8±0.8 b	576±2 b
B.FD	51.3±0.8 c	26.4±0.7 c	66.8±0.7 b	654±11 c
P.AD	3.30±0.07 a	1.10±0.05 a	9.0±0.2 a	31.3±1.1 a
P.FD	2.70±0.13 a	1.04±0.11 a	9.3±0.2 a	30.0±0.4 a
	after gastric phase			
powder (fraction)	**total phenols (mg GAE/g)**	**total flavonoids (mg QE/g)**	**AO-DPPH (mgTE/g)**	**AO-ABTS (mg TE/g)**
B.AD (SP)	3.36±0.02 d	1.258±0.008 a	9.0±0.5 c	37.1±0.3 d
B.FD (SP)	3.11±0.05 c	1.451±0.005 b	7.2±0.2 b	32.9±0.5 c
P.AD (SP)	2.59±0.02 b	n.d.	0.82±0.02 a	2.59±0.03 b
P.FD (SP)	0.0750±0.0010 a	n.d.	0.277±0.013 a	0.73±0.02 a
B.AD (PP)	55.3±0.3 d	27.2±0.2 d	192.2±1.8 d	607±12 d
B.FD (PP)	43.1±0.8 c	20.5±0.4 c	128.6±1.6 c	450±5 c
P.AD (PP)	3.63±0.03 b	0.920±0.004 a	14.7±0.2 b	34.3±0.2 b
P.FD (PP)	2.15±0.13 a	2.30±0.03 b	3.4±1.2 a	16.6±1.2 a
B.AD (total)	58.7±0.3 d	28.47±0.22 d	201±2 d	644±11 d
B.FD (total)	46.2±0.8 c	22.16±0.15 c	135.8±1.5 c	483±5 c
P.AD (total)	6.22±0.05 b	0.920±0.004 a	15.5±0.2 b	36.9±0.2 b
P.FD (total)	2.23±0.13 a	2.30±0.03 b	4.3±0.3 a	17.3±1.2 a
	after intestinal phase			
powder (fraction)	**total phenols (mg GAE/g)**	**total flavonoids (mg QE/g)**	**AO-DPPH (mgTE/g)**	**AO-ABTS (mg TE/g)**
B.AD (SP)	6.58±0.02 c	3.278±0.007 a	13.9±0.6 c	89.3±0.7 d
B.FD (SP)	6.79±0.18 c	3.03±0.02 b	16.8±0.4 d	74.5±1.2 c
P.AD (SP)	1.232±0.012 b	n.d.	2.69±0.12 a	15.2±0.2 b
P.FD (SP)	0.94±0.17 a	n.d.	3.6±0.2 b	11.51±0.08 a
B.AD (PP)	27.2±0.7 d	12.48±0.04 c	82±2 b	313±3 b
B.FD (PP)	14.6±0.4 c	14.5±0.2 d	103±5 c	369±4 c
P.AD (PP)	1.57±0.04 a	0.542±0.011 a	2.73±0.06 a	20.3±0.2 a
P.FD (PP)	2.59±0.10 b	4.8±0.2 b	0.8±0.3 a	15.8±0.6 a
B.AD (total)	33.8±0.7 d	15.76±0.05 c	96±3 b	402±3 c
B.FD (total)	21.4±0.2 c	17.62±0.11 d	120±5 c	443±3 d
P.AD (total)	2.80±0.04 a	0.542±0.011 a	5.42±0.06 a	35.47±0.04 b
P.FD (total)	3.5±0.2 b	4.8±0.2 b	4.51±0.11 a	27.4±0.6 a

a,b,c… Different letters within the same row an type of powder (fraction) at each stage of the *in vitro* digestion indicate statistically significant differences (p-value < 0.05)
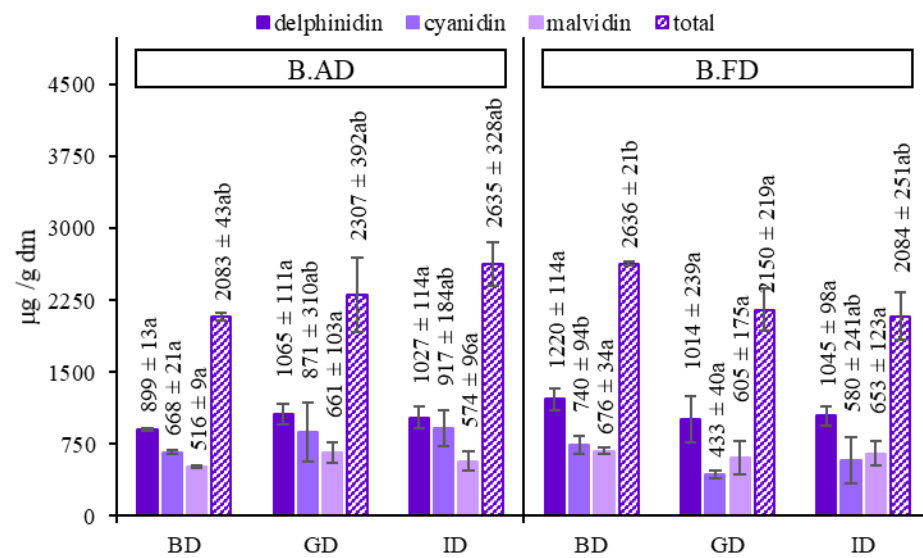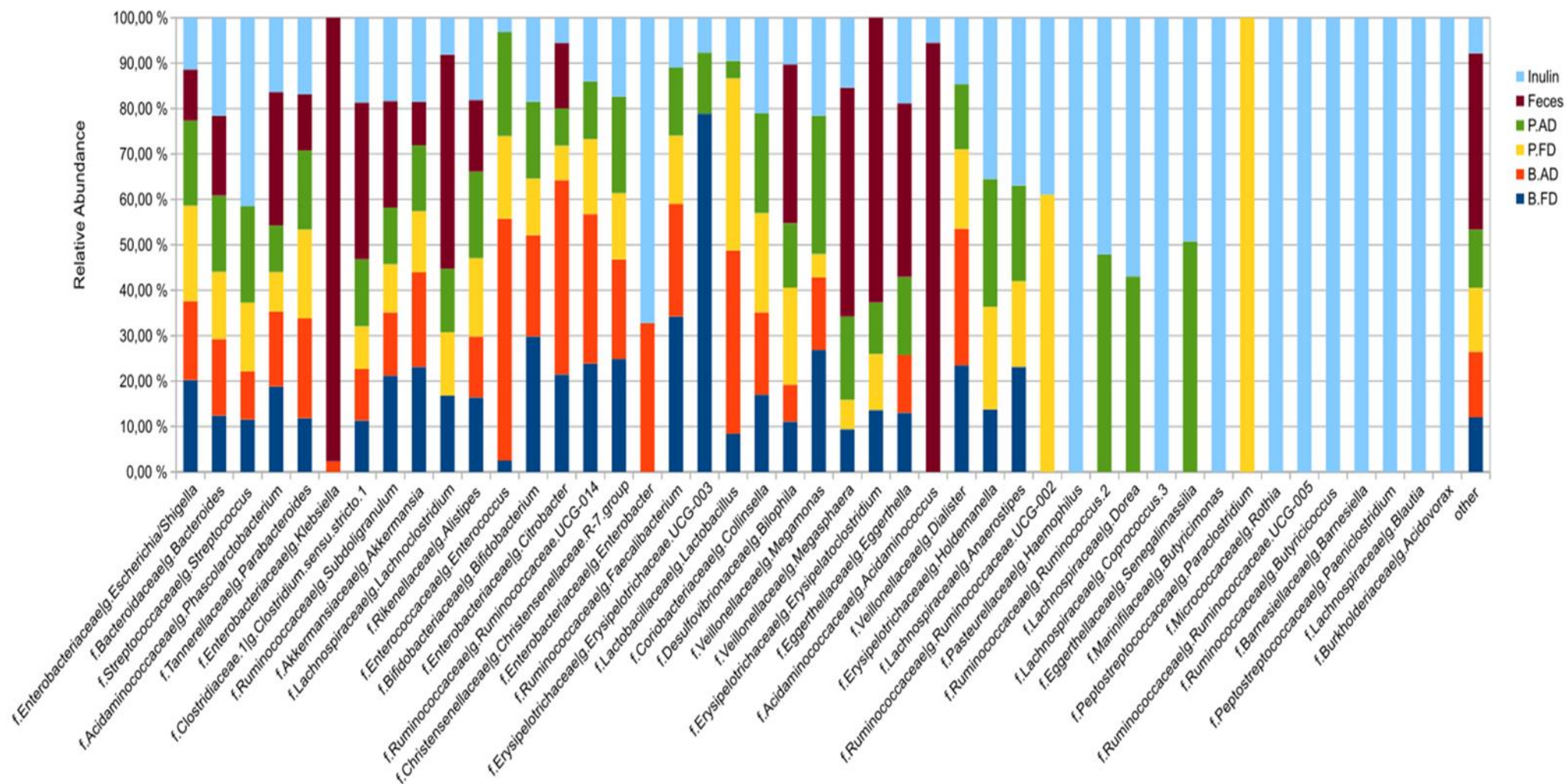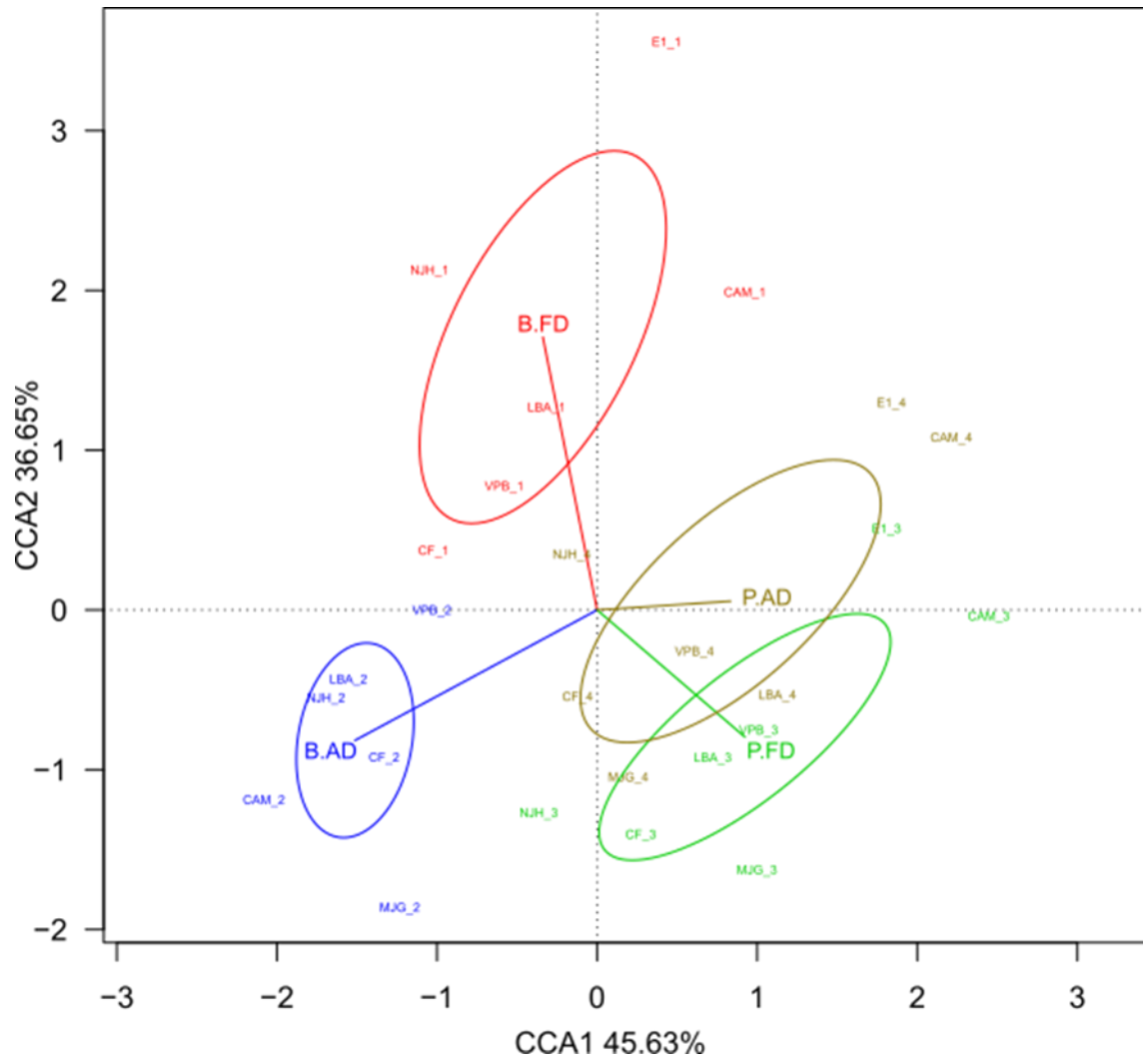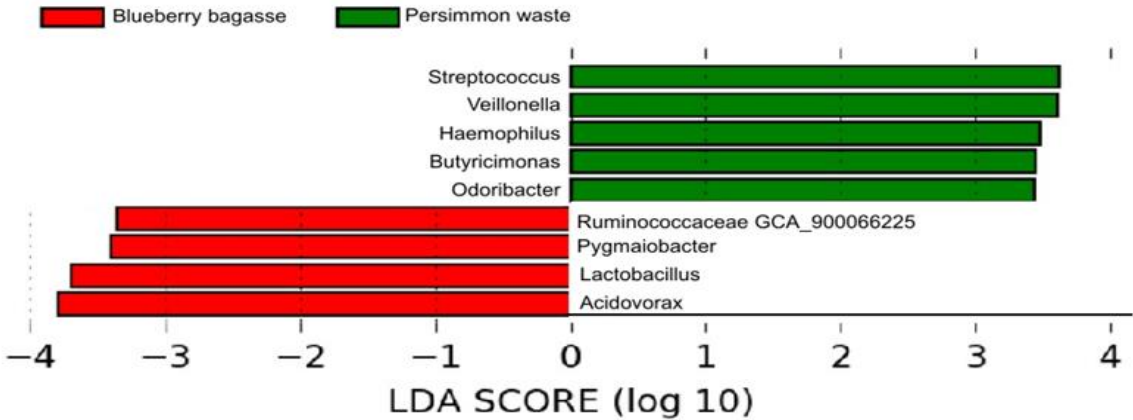
Figure 1

A
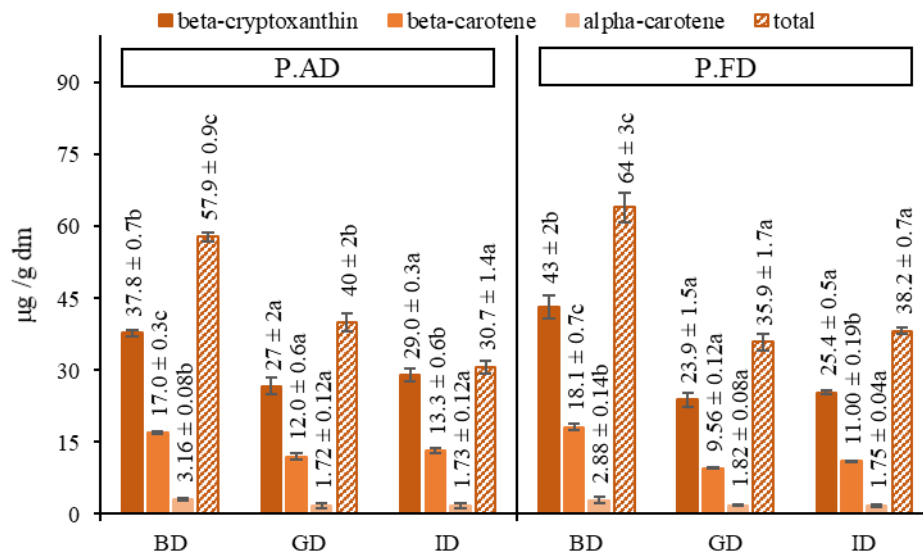
B

Figure 2

Figure 3

Figure 4

Figure 5