**Impact of a plant sterol- and galactooligosaccharide-enriched beverage on
colonic metabolism and gut microbiota composition using an *in vitro*
dynamic model**

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19 **ABSTRACT**

20 A beverage enriched with plant sterols (1 g/100 mL) and galactooligosaccharides (1.8
21 g/100 mL) was subjected to a dynamic gastrointestinal and colonic fermentation process
22 to evaluate the effect on sterol metabolism, organic acid production and microbiota
23 composition. Production of sterol metabolites (coprostanol, methylcoprostanol,
24 ethylcoprostanol, ethylcoprostanol and sitostenone) was observed in the transverse
25 (TC) and descending colon vessels (DC) in general, from 24 and 48 h, respectively.
26 Microbial activity was assessed through the production of organic acids, mainly acetate
27 in all colon vessels, lactate in the AC and butyrate and propionate in the TC and DC. A
28 higher diversity in the microbial community was found in the TC and DC, in accordance
29 with a higher sterol metabolism and organic acid production. Although the prebiotic
30 effect of galactooligosaccharides was not detected, changes in microbiota composition
31 (an increase in *Parabacteroides* genus and the *Synergistaceae* and *Lachnospiraceae*
32 families) indicated an enhancement of sterol metabolism.

33 **Keywords:** dynamic colonic fermentation, sterol metabolites, short-chain fatty acids,
34 lactate, 16S rRNA gene sequencing

35 INTRODUCTION

36 Diet is considered a modulator of the gut microbiota composition and function, and
37 could, therefore, influence the health of the host.¹ The major metabolites generated
38 during colonic fermentation of non-digestible carbohydrates by the microbiota are short
39 chain fatty acids (SCFA) (such as acetate, propionate and butyrate) that have been
40 related to beneficial effects including promotion of gut integrity and regulation of
41 glucose homeostasis, lipid metabolism, appetite, immune system and inflammatory
42 response.²

43 *In vitro* fermentation studies in batch³⁻⁵ or dynamic⁶⁻⁹ models, using fecal inocula from
44 healthy subjects, have demonstrated that galactooligosaccharides (GOS) stimulate the
45 growth of *Bifidobacterium* and *Lactobacillus* species and imply an increase on SCFA
46 production.

47 Likewise, plant sterols (PS) used for food enrichment due to their cholesterol-lowering
48 effect¹⁰ present a low absorption (0.1-15%) and reach the colon¹¹ where they are
49 subjected to the action of the gut microbiota, generating sterol metabolites, as in the
50 case of cholesterol.¹² Milk-based fruit beverages (MfB), which can be enriched with PS,¹³
51 are healthy foods due to their low fat content and the presence of antioxidant
52 compounds such as vitamin C, polyphenols and carotenoids. In previous studies of our
53 research group, an MfB enriched with PS had a cholesterol-lowering and anti-
54 inflammatory effect in post-menopausal women with mild hypercholesterolemia.¹⁴ As
55 far as we are aware, only two studies carried out by our research group have reported
56 the use of *in vitro* fermentation systems to evaluate the biotransformation of sterols
57 (cholesterol and PS). First, applying batch-culture fermentation to a residue from the

58 simulated gastrointestinal digestion of an MfB enriched with PS,¹⁵ and in a TIM-2 model
59 feeding the system with different formats of PS (ingredient or standard).¹⁶ In both
60 studies, a preference for PS, rather than for cholesterol, as substrate by the microbiota
61 was demonstrated (production of ethylcoprostanol in batch-culture and
62 methylcoprostanone in TIM-2 was observed). In addition, the presence of PS during the
63 batch-culture fermentation led to a decrease in *Erysipelotrichaceae* species and an
64 increment of *Eubacterium hallii*.¹⁵ Whereas, in the TIM-2 model, the presence of PS
65 increased the prevalence of some genera belonging to *Firmicutes* phylum using
66 microbiota of lean or obese subjects. Regarding SCFA production, an increase of mainly
67 acetate and butyrate was only reported in the fermentation with the lean microbiota.¹⁶

68 It has recently been demonstrated that the addition of GOS to a similar beverage
69 enriched with PS had no effect on the bioaccessibility of sterols.¹⁷ Thus, this combined
70 enrichment with GOS and PS could improve the beneficial effects previously mentioned
71 for both bioactive compounds, and could also modulate microbial genera implied in
72 sterol metabolism. In addition, the presence of GOS does not affect organoleptic
73 characteristics and show high stability against low pH and high temperature.¹⁸ Given the
74 lack of studies on the metabolism of sterols present in food matrices using dynamic
75 fermentation systems and the absence of literature regarding the evaluation of the
76 presence of GOS on this biotransformation, the present study was carried out with the
77 aim of shedding light on this issue. Thus, we have evaluated, for the first time, the impact
78 of the dynamic *in vitro* fermentation of a food matrix (MfB) enriched with PS and GOS
79 on sterol metabolism, organic acid production and microbiota composition.

80 MATERIAL AND METHODS

81 **Reagents.** The sterol standards used were cholestane ($\geq 97\%$) as internal standard (IS),
82 coprostanol (98%), cholesterol (99%), desmosterol (84%), stigmastanol (97%),
83 stigmasterol (97%), β -sitosterol (99%) and lathosterol (99%), all of them were purchased
84 from Sigma Chemical Co. (St Louis, Missouri). Campesterol (96%) was provided by
85 Chengdu Biopurify Phytochemicals Ltd (Sichuan, China) and brassicasterol (98%) and
86 coprostanone (98%) by Steraloids Inc. (Newport, RI, United States). The derivatization
87 reagents used were anhydrous pyridine from Acros Organics (Geel, Belgium) and N,O-
88 bis trimethylsilyl trifluoroacetamide (BSTFA) (1% of trimethylchlorosilane (TMCS)) from
89 Sigma Chemical Co. (St Louis, Missouri). For determination of organic acids, butyrate,
90 iso-butyrate, lactate, propionate and valerate were provided from Sigma Chemical Co.
91 (St Louis, Missouri), acetate from Janssen Chimica (Beerse, Belgium), formate from
92 Panreac (Barcelona, Spain) and iso-valerate and phosphoric acid from Fluka (Buch,
93 Switzerland). Butylhydroxytoluene (BHT), pancreatin from porcine pancreas (EC
94 232.468.9), pepsin from porcine gastric mucosa (E.C 3.4.23.1) were purchased from
95 Sigma Chemical Co. (St Louis, Missouri). Sodium hydroxide and thioglycolate were
96 provided by Merck (Whitehouse Station, New Jersey). Dehydrated fresh bile (Difco™
97 Oxgall) was purchased from BD Biosciences (Oxford, United Kingdom). Water was
98 purified using a Milli-Q system (Milford, Massachusetts).

99 **Experimental beverage.** A PS-enriched (1%, w/v) MfB with addition of GOS (1.8%, w/v)
100 was elaborated by the Hero Global Technology Center (Alcantarilla, Murcia, Spain)
101 according to the manufacturing process described by Blanco-Morales et al.¹⁷ This
102 beverage contained skimmed milk with the addition of milk fat and whey protein
103 concentrate enriched with milk fat globule membrane (MFGM) (49%), mandarin juice
104 from concentrate (45%), banana puree (4%), microencapsulated free microcrystalline PS

105 from tall oil (Lipophytol® 146 ME Dispersible, Lipofoods) and GOS syrup (Vivinal® GOS
106 from Friesland Campina Ingredients). The energy and nutritional information per 100 mL
107 was: energy (KJ, kcal) 359.0/85.8, protein (g) 3.0, carbohydrates (g) 12.2, fat (g) 2.8 and
108 fiber (g) 0.5. Cholesterol content was 13.6 mg/100 g beverage and the PS relative
109 abundance was β -sitosterol $\approx$ 80% (753 mg/100 g), sitostanol $\approx$ 12% (105 mg/100 g),
110 campesterol $\approx$ 7% (58 mg/100 g), campestanol $\approx$ 1% (9.2 mg/100 g), stigmasterol $\approx$ 0.7%
111 (5.9 mg/100 g).¹⁷

112 **Dynamic gastrointestinal and colonic fermentation model.** The Dynamic-Colonic
113 Gastrointestinal Digester (D-CGD) was developed by AINIA Technology Center (Valencia,
114 Spain) based on the one developed and validated by Molly et al.^{19,20} The equipment
115 consists of five vessels that simulate the stomach, small intestine, and ascending (AC),
116 transverse (TC) and descending (DC) colon, whose pH values, residence times,
117 temperature (37 °C) and volume capacities were controlled by a software specifically
118 developed by the aforementioned center (Figure 1). All vessels were connected by
119 peristaltic pumps working semi-continuously in the stomach and small intestine and
120 continuously in the colon stretch. The gastric digestion step was simulated by the
121 addition of 60 mL of a 0.03% (w/v) pepsin solution (2100 U/mg) to the stomach vessel,
122 whereas digestion on the small intestine was simulated by the addition of 240 mL of a
123 solution containing pancreatin (0.9 g/L), NaHCO₃ (12 g/L) and oxgall dehydrated fresh
124 bile (6 g/L) in distilled water. Anaerobiosis was maintained by flushing gaseous N₂ for
125 15 min twice a day.

126 A pool with the feces from 5 healthy volunteers who, in the previous month, had not
127 received any antibiotic, hormonal, anabolic or hypocholesterolemic treatments, and had

not consumed enriched foods or supplements with vitamins, carotenoids, probiotics/prebiotics, phytoestrogens or PS, herbal products, or foods high in fiber; and who had not followed weight loss diets, suffered acute inflammation or diseases of the gastrointestinal tract, taken chronic medication or were vegan or vegetarian, was used. A fecal solution at 20% (w/v) was prepared with regenerated thioglycolate, inoculated in the colon vessels (50, 80 and 60 mL for AC, TC and DC, respectively) and filled with culture medium up to a total volume of 1000, 1600 and 1200 mL, respectively. The culture medium, elaborated according to Molly et al.,^{19,20} provided all the necessary nutritional components to simulate the conditions of the human colon and allowed the growth of the intestinal microbiota.

An 11-day period was required to allow the stabilization of the human fecal microbiota in the colonic vessels, during which, 200 mL of culture medium were added to the stomach vessel three times a day. Samples of the fermentation liquids (FL) from the AC, TC and DC vessels corresponding to time 0 were taken at the end of the microbiota stabilization period. Since the beverage provided substrates (sugars and proteins) for the microbiota, a mixture (1:1, v/v)²¹ of the beverage and a modified culture medium was added to the system once per day. The modified culture medium was elaborated without carbohydrates and taking into account the content of proteins provided by the beverage. The other two additions were done only with culture medium. The maintenance of the microbial population after the stabilization (time 0) and during treatment (72 h) periods was checked by plate counts of total anaerobic bacteria (on Schaedler agar under anaerobic incubation, 37°C/72 h). No relevant differences were observed among both periods (6.5 vs. 6.9 log CFU for the AC, 7.4 vs. 8.7 log CFU for the TC and 7.9 vs. 8.3 log CFU for the DC). The 8-h samples from all colon vessels were taken

152 when the small intestinal contents had been poured into the AC (constituting the control
153 of sterols provided by the beverage without fermentation). However, the following
154 samples (from the AC, TC and DC) were collected once a day, 15 minutes before the
155 contents of the small intestine was transferred to the AC (corresponding to 24, 48, 72,
156 96, 120, 144 and 168 h). All collected FL were aliquoted and stored at -20 °C until sterols
157 were analyzed.

158 **Sample preparation.** Due to the complexity of the composition of the FL, different
159 sample preparations were evaluated: i) filtration with a Millex-FH filter unit of 0.45 µm
160 (Millipore, Milford, Massachusetts), ii) sonication (30 min) with subsequent
161 centrifugation at 18 °C for 10 min at 4000 rpm (Eppendorf® centrifuge 5810R, Hamburg,
162 Germany) to obtain the supernatant phase of the FL, and iii) sampling under continuous
163 stirring. Taking into account a previous screening applying the methodology of Cuevas-
164 Tena et al.,²² the FL from the TC compartment at 120 h of fermentation (from now,
165 experimental sample) was selected as a representative sample for these assays, due to
166 its larger quantity and diversity of sterol metabolites. Each sample preparation was
167 assayed in duplicate and the sterol content was determined in 150 µL of FL according to
168 the methodology described in the following section.

169 **Determination of sterols by GC-MS.** Contents of sterols and metabolites, from now on
170 sterols, in the samples of FL were determined in triplicate applying the methodology
171 validated by Cuevas-Tena et al.²² and applied to FL by our research group.^{15,16} The
172 amount of sterols quantified at 0 h were subtracted from the amounts measured at each
173 sampling point. Different volumes of FL (25 µL for the analysis of ethylcoprostanol,
174 campesterol, β-sitosterol and sitostanol and 150 µL for coprostanol, cholesterol,

175 methylcoprostanol, ethylcoprostanol, campestanol, stigmasterol and sitostenone) were
176 taken, and 20 μg of 5 α -cholestane (IS) and 5 μL of BHT 0.02% (w/v) ethanolic solution
177 were added. Then, hot saponification with 1 mL of 0.75M potassium hydroxide
178 (ethanolic solution) was carried out at 65 °C for 1 h and, subsequently, the
179 unsaponifiable fraction was extracted with 3x2 mL of n-hexane. After evaporation until
180 dryness under N₂, the unsaponifiable fractions were derivatized with 200 μL of BSTFA
181 (1% TMCS): pyridine 10:3 (v/v) at 65 °C for 1 h. The trimethylsilyl ether derivatives
182 obtained were dissolved and filtered (Millex-FH filter unit, 0.45 μm Millipore, Milford,
183 Massachusetts) with 3x1 mL of n-hexane, evaporated and dissolved in 50 μL of n-
184 hexane. One μL of derivatized samples was injected into a gas chromatography-mass
185 spectrometry (GC-MS) system (Thermo Science Trace® GC-Ultra with ion trap ITQ 900,
186 Waltham, Massachusetts) under the same conditions as those reported by Cuevas-Tena
187 et al.²² Moreover, two extraction methods of unsaponifiable fraction (with hexane,
188 describe above, and with diethyl ether used by Alvarez-Sala et al.²³ were applied to the
189 saponified experimental sample (150 μL) taken under continuous stirring.

190 For the identification of sterols, relative retention times and the ion fragmentation
191 patterns obtained from commercial standards, literature^{22,24-26} and data bases^{27,28} were
192 used (characteristic ions are shown in Table S1 of the Supporting Information).
193 Calibration curves for the quantification of sterols were performed with standards with
194 the addition of IS (20 μg) (Table 1). Coprostanol curves were used for coprostanol,
195 methylcoprostanol, ethylcoprostanol and ethylcoprostanol quantification, stigmasterol
196 for campestanol and sitostanol, and coprostanone for sitostenone. Moreover, the limits
197 of detection (LOD) and quantitation (LOQ) of sterols were determined from the
198 background response of 0-h samples (n = 6) in each colon compartment (see Table 2).

199 **Organic acid analysis.** The FL (1 mL) from 8, 24, 72, 120 and 168 h sample points were
200 centrifuged (12000 rpm /4 °C/ 5 min) (AccuSpin Micro 17R, Fisher scientific) and 100 µL
201 of the supernatant were dissolved up to 1 mL with phosphoric acid 0.1% (w/v) (pH 2.4).
202 Then, the mixture was filtered (Millex 0.45 µm filter unit, PTFE, Millipore, USA) and 20
203 µL were injected in a HPLC (Jasco Corporation, Japan) with a UV detector at 210 nm.
204 System conditions were specified by Sarmiento-Rubiano et al.²⁹ The determined organic
205 acids were: SCFA (acetate, butyrate, propionate and valerate), branched-chain fatty
206 acids (iso-butyrate and iso-valerate) and intermediate metabolites (lactate and
207 formate).

208 **Microbiota composition: DNA extraction, 16S rRNA, gene sequencing and**
209 **bioinformatics.** Total DNA from FL samples (0.25 g) were isolated using the DNeasy
210 PowerSoil Kit (Qiagen, Carlsbad, CA), according to the manufacturer's instructions. Then,
211 the variable V3 and V4 regions of the 16S rDNA gene were amplified following the 16S
212 rDNA gene Metagenomic Sequencing Library Preparation Illumina protocol (Cod.
213 15044223 Rev. A). Gene-specific primers 515F/806R containing Illumina adapter
214 overhang nucleotide sequences were selected according to Klindworth et al.³⁰ After 16S
215 rDNA gene amplification, the multiplexing step was performed using Nextera XT Index
216 Kit. DNA libraries were sequenced on the MiSeq platform using a paired-end 2 × 300-bp
217 reagent kit (Illumina, San Diego, CA, USA). Raw reads generated from the MiSeq run
218 were checked for quality and adapter trimmed using cutadapt 1.8.3³¹ and FastQC
219 v0.11.5.³² Resulting reads were analyzed using QIIME software V1.9.1³³ including
220 forward and reverse reads joining, quality filtering, chimera removal and taxonomic
221 annotation. Reads were clustered into 97% identity using an open-reference operational
222 taxonomic unit (OTU) picking protocol against the 13_8 revision of the Greengenes

223 database.³⁴ Taxonomic assignments was made using the RDP Naive Bayesian classifier.
224 Chimeric sequences were removed from the reads using the Usearch6.1 algorithm. A
225 biome table was constructed for downstream analyses, and OTUs represented by 10 or
226 less sequences were removed. Calypso v8.48³⁵ was applied to calculate alpha and beta
227 diversities, differences in community composition and results visualization. For alpha
228 diversity, Shannon index was applied and statistical comparisons were performed using
229 ANOVA test at genus level ($p < 0.001$). For analyzing beta diversity, Bray–Curtis
230 dissimilarities were calculated using normalized data (total-sum normalization
231 combined with square root transformation) and principal coordinates analysis (PCoA)
232 plot was used to visualize differences among compartments.

233 **Statistical analysis.** Statistically significant differences ($p < 0.05$) in sterol content in the
234 different sample preparations and in sterol or organic acid contents in FL at the different
235 fermentation times were assessed applying a one-way analysis of variance (ANOVA),
236 followed by Tukey's *post hoc* test. The Statgraphics® Centurion XVI.I statistical package
237 (Statpoint Technologies Inc., Virginia) was used.

238 RESULTS

239 **Assays of sample preparation and unsaponifiable extraction.** Regarding sample
240 preparation, a great loss of sterols was observed after filtration and
241 sonication+centrifugation compared to sampling under continuous stirring (Table 3). In
242 the latter, sterols present in the beverage as well as the metabolites coprostanol,
243 methylcoprostanol and ethylcoprostenol could be determined. It should be indicated
244 that ethylcoprostanol, campesterol, β -sitosterol and sitostanol were present in amounts
245 above the highest point of the calibration curve since. Although no significant

246 differences in sterol contents were observed between the evaluated methods using
247 sampling under continuous stirring, sitostenone could only be determined by means of
248 unsaponifiable extraction with hexane (method by Cuevas-Tena et al.)²² (see Table 3).
249 Therefore, and taking into account that the extraction by Cuevas-Tena et al.²² is less
250 time-consuming and requires a lower volume of solvents, compared to that by Alvarez-
251 Sala et al.,²³ sampling under continuous stirring and using hexane for unsaponifiable
252 extraction was chosen as the best option for sterol determination.

253 The accuracy of this method was tested, in triplicate, with 100 μ L (or 25 μ L in the case
254 of β -sitosterol) of the experimental sample. The samples were spiked with standards of
255 the main sterols at levels similar to their contents in FL; recoveries (%) were 116 ± 3 for
256 cholesterol, 109 ± 3 for campesterol, 90 ± 17 for β -sitosterol and 105 ± 3 for sitostanol. The
257 method is of good accuracy since the suitable recovery range proposed by the
258 Association of Official Analytical Chemists (AOAC) for analyte concentrations of μ g/g is
259 80-115%.³⁶

260 **Sterol metabolism.** The evolution of sterols throughout the fermentation experiment
261 from the AC, TC and DC is shown in Figure 2. In the Supporting Information, the mean
262 sterol contents $\pm$ standard deviation values (Tables S2, S3 and S4) as well as a
263 representative chromatogram of the sterols present in a sample from the fermentation
264 of the beverage (Figure S1) are shown. Non-quantifiable contents of desmosterol,
265 brassicasterol, lathosterol and methylcoprostanone (values below LOQ indicated in
266 Table 2) were observed in all colon vessels during beverage fermentation (data not
267 shown).

268 Cholesterol contents along the fermentation in the AC presented a marked increase until
269 48 h with an irregular evolution thereafter (Figure 2a). In the TC, the maximum amount
270 of cholesterol was detected at 72 h (when coprostanol started to be detected) reaching
271 a plateau. The coprostanol/cholesterol ratios increased along fermentation times from
272 0.2 (72 h) to 0.7 (for 120 h, 144 h and 168 h). In the case of the DC, cholesterol was
273 detected at 72 h and kept relatively stable along the entire assay (7-9 mg/compartment).
274 In this compartment, the evolution of the coprostanol contents is similar to that in the
275 TC with slightly higher coprostanol/cholesterol ratios (from 0.9 at 72 h to 1.3 at 120 h
276 and onwards).

277 A progressive increase in the β -sitosterol (Figure 2b) and sitostanol contents (Figure 2c)
278 was observed during fermentation, reaching maximum levels between 48 and 96 h and
279 remaining stable afterwards. Ethylcoprostanol (Figure 2b) was only detected in the TC
280 and DC from 48 and 24 h with a significant increase ($p < 0.05$) until 120 and 144 h,
281 respectively. This fact is reflected in the ethylcoprostanol/ β -sitosterol ratios which
282 increased during the fermentation from 0.01 (48h) to 0.46 (from 120 h onwards) in the
283 TC and from 0.2 (24 h) to 0.7 (144 h) in the DC (Figure 2b). Moreover, sitostenone
284 (another β -sitosterol metabolite) was only detected at 144 h in the TC (9.4 ± 0.3
285 mg/compartment) and from 72 h onwards in the DC (mean value of 7.7 ± 0.3
286 mg/compartment) (see Supporting Information Table S3 and S4).

287 In the case of the other plant sterols (campesterol, campestanol and stigmasterol)
288 (Figures 2d, 2e and 2f, respectively), a similar trend of accumulation was observed,
289 reaching, in general, their maximum amounts in all vessels between 96 and 120 h.
290 Regarding metabolites, a production of methylcoprostanol (from campesterol) was

291 detected from 72 and 48 h in the TC and DC, increasing until 120 h and 144 h,
292 respectively (Figure 2d). This tendency is also observed in the increments of the ratios
293 throughout fermentation: from 0.08 (72 h) to 0.26 (120 h) in the TC, and from 0.2 (48 h)
294 to 0.5 (168 h) in the DC. Additionally, the presence of ethylcoprostenol (from
295 stigmasterol) was detected from 96 h in the TC and from 72 h in the DC, remaining
296 constant until the end of the fermentation assay, whereas the ratios increased from 0.5
297 (96 h) to 0.6 (120 h) in the TC and decreased from 1.0 (72 h) to 0.8 (168 h) in the DC (see
298 Supporting Information Table S3 and S4).

299 **Analysis of organic acids.** Contents of organic acids through the fermentation in all colon
300 compartments (the AC, TC and DC) are shown in Figure 3. Moreover, the mean organic
301 acid contents are shown in Table S5 of the Supporting Information. Results showed that
302 the fermentation of the beverage led to a significant increment in total organic acid
303 production in all colon compartments (Figure 3) from 72 h in the AC and from 24 h in
304 the TC and DC. Higher concentrations of organic acids were determined in the distal
305 compartments with respect to the AC and increments at 168 h with respect to initial
306 values (8 h) were also higher (1.5- fold for the AC, 2.0- fold for the TC and 2.8- fold for
307 the DC).

308 The organic acid profile was compartment-dependent, differing the AC with respect to
309 the TC and DC (iso-butyrate > acetate > lactate > butyrate vs. acetate > propionate >
310 butyrate > iso-butyrate, respectively) (Figure 3). In the AC, a significant increase in
311 lactate contents at 24 h of fermentation was observed and maintained relatively stable
312 thereafter. Lactate contents in the TC and DC remaining stable during the fermentation
313 (0.27 mM) (see Table S5 of the Supporting Information). Iso-butyrate contents showed

314 a significant increase after 72 h of fermentation in the AC, whereas in the TC and DC,
315 low contents were detected (0.3-3.2 mM). Acetate contents in all colon compartments
316 increased significantly: 1.5- fold in the AC, 2.3- fold in the TC and 2.6- fold in the DC, at
317 the end of the fermentation with respect to 8 h. Relatively stable amounts of butyrate
318 were detected in the AC, ranging between 5.2 and 6.2 mM, whereas a similar
319 progressive increase were observed in the TC and DC (increments of 3.8- fold and 4.0-
320 fold, respectively). Although no contents of propionate were observed in the AC, a
321 significant increase was observed in the TC (from 24 h) and DC (from 72 h). However, its
322 contents remained stable in these compartments from 120 h, showing increments at
323 168 h, with respect to initial values, of 2.5- and 2.6- fold, respectively. Finally, iso-
324 valerate and valerate were minority in all colon compartments (0.5 – 2.7 mM and 0.3 –
325 1.3 mM, respectively), while formate was only detected in the AC (2.1 – 4.2 mM) (Table
326 S5 of the Supporting Information).

327 **Microbiota composition.** The Shannon diversity index (Figure 4a) showed no differences
328 between the TC and DC compartments, although significantly increased ($p < 0.001$) with
329 respect to the AC, thus demonstrating a higher species richness and evenness in the
330 distal compartments. Regarding to PCoA score plot (Figure 4b), the AC compartment
331 was grouped in a separate cluster than the TC and DC, which is indicative that the
332 different conditions of the compartments led to a distinctive microbial community. The
333 relative abundance of the genera during the fermentation of the beverage is shown in
334 Figure 5. In the AC, *Clostridium* genus is the most abundant during all the experiment
335 showing a maximum of abundance at 48 h (88%) and a minimum at 168 h (52%) with an
336 irregular evolution (Figure 5a). The unclassified genus of the family *Lachnospiraceae* and
337 the *Ochrobactrum* genus also presented an irregular trend, reaching their maximum

338 contents at 168 h (changes in its abundance were from 5 to 12% and from 3 to 5%,
339 respectively). A progressive decrease of *Turicibacter* genus over the time was observed,
340 from 15 to 0.1 %. Other genera, such as *Delftia* and *Pediococcus*, increased with respect
341 to their initial values (0.2 and 0%) during fermentation, presenting maximum contents
342 at 144 h (7 and 6%, respectively). Finally, *Stenotrophomonas*, an unclassified genus of
343 the family *Clostridiaceae*, *Pseudomonas* and *Enterococcus* genera tended, in general, to
344 decrease during fermentation, showing abundances lower than 5%.

345 In contrast, in the TC and DC (Figure 5b and 5c, respectively), the *Bacteroides* genus
346 showed a trend to decrease during the fermentation of the beverage from 72 h, ranging
347 their contents between 70 and 44% for the TC, and 65 and 41% for the DC. A marked
348 increase in the abundance of *Parabacteroides* genus was observed at 96 h in the TC and
349 at 120 h in the DC, maintaining their abundances relatively over time. Unclassified genus
350 belonging to *Synergistaceae* family tended to increase from 24 h in the TC (from 5.8 to
351 14.4%) and from 48 h in the DC (from 5.5 to 25.5%). In addition, the unclassified genus
352 of the family *Lachnospiraceae* presented an increase at 24 h in the TC (from 4.2 to 9.1%),
353 whereas in the DC the maximum abundance was observed at 48 and 72 h of
354 fermentation (from 3.4 to 7.3%), decreasing in both cases thereafter. The remaining
355 genera (*Sutterella*, *Bilophila*, unclassified *Rikenellaceae*, *Oscillospira*, *Akkermansia* and
356 unclassified *Clostridiales* and *Synergistaceae*) showed an irregular evolution and, in any
357 case, exceeded 5% of abundance.

358 DISCUSSION

359 In this study, for the first time, the metabolism of sterols present in a food matrix (MfB)
360 enriched with PS and GOS, which has demonstrated beneficial systemic biological

361 effects, has been assessed using a five-vessel dynamic simulator which includes a
362 complete process of gastric digestion and colonic fermentation. Moreover, the effect of
363 the fermentation of the beverage upon organic acid production and microbiota
364 composition was also evaluated in order to estimate other potential effects in the
365 colonic tract.

366 Sterol metabolism was only observed in the distal colon compartments (the TC and DC)
367 during the fermentation of the beverage. The presence of coprostanol,
368 methylcoprostanol and ethylcoprostanol has been described in other *in vitro* studies
369 through the formation of different metabolites such as phytostanone and phytostenone
370 intermediates.¹² Besides, a direct pathway has been suggested for coprostanol
371 formation.³⁷ In the present study, the non-detection of sterol intermediate metabolites
372 suggests their rapid bioconversion or, in the case of cholesterol, that the dominant route
373 for its degradation was the direct pathway of conversion to coprostanol. In contrast to
374 our results, a recent study¹⁵ reported the absence of cholesterol metabolism during the
375 batch fermentation of a residue from the gastrointestinal digestion of a PS-enriched
376 beverage during 48 h. Only a decrease in campesterol and an increment in
377 ethylcoprostanol contents (39% and 50%, respectively) at 48 h of fermentation were
378 statistically significant, suggesting that gut microbiota prefers PS as substrate. On the
379 other hand, the fermentation of approx. 2 g of PS (from an ingredient source of PS or a
380 commercial standard mixture of PS) during 72 h using a TIM-2 system and fecal inoculum
381 from lean subjects¹⁶ resulted in a drop of coprostanol contents (41-61%) and an increase
382 in coprostanone (109-136%) and cholestanol contents (27-29%) with respect the initial
383 values. Moreover, production of methylcoprostanone was observed from 24 to 72 h (8-
384 and 25-fold increases in PS-ingredient or commercial standard mixture of PS,

385 respectively). In contrast, a lack of both, cholesterol and PS metabolism was detected
386 when the assay was carried out with fecal inoculum from obese subjects. Moreover, it
387 is noteworthy that higher conversion ratios of the main sterols were detected in the DC
388 vs. TC: cholesterol > stigmasterol > β -sitosterol > campesterol, considering the maximum
389 ratios.

390 Regarding organic acid production, acetate increased in all colon compartments, while
391 butyrate and propionate only increased in the TC and DC. In fact, higher organic acid
392 production was detected in the DC and TC vs. AC. The similar microbiota in the TC and
393 DC support the above-mentioned results (for organic acids and also for sterol
394 metabolism), showing more similarity and a more diverse community with respect to
395 the AC. This is in accordance with the results reported in a SHIME model,³⁸ whose
396 authors suggested that the lower species richness observed in the AC was probably due
397 to the lower pH and the higher concentration of bile salts. Moreover, the different pH
398 conditions of the vessels favor the selective growth of microbial populations. Similar to
399 our results, in batch culture models,^{39,40} higher *Bacteroides* levels (up to 80% of total
400 eubacteria) were observed with the increase of pH from 5.5 (AC) to 6.5-6.7 (DC),
401 suggesting that a mild acidic pH might limit the *Bacteroides* population in the proximal
402 colon. In our study, a predominance of *Bacteroides* was observed in the colon
403 compartments with cholesterol metabolism (the TC and DC), although with a decreasing
404 tendency during the fermentation of the beverage. The genera *Bacteroides* and
405 *Eubacterium* have only been reported, in the studies carried out several years ago with
406 isolated cultures, to be cholesterol-reducing bacteria related to the direct as well as
407 indirect routes for cholesterol metabolism.^{12,41-43} An absence of *Eubacterium spp.*, the
408 only species associated with the PS biotransformation pathway,^{12,44} was observed in the

409 present study, being suggestive of the involvement of other bacterial species in the PS
410 metabolization pathway.

411 Production of organic acids has been studied in presence of GOS or PS using *in vitro*
412 studies. According to our results, in presence of both compounds an increase in acetate
413 and butyrate contents^{6-9,16,45,46} and also propionate and lactate was observed.^{8,16,46}
414 Butyrate- and propionate-producing bacteria (species belonging to *Lachnospiraceae* and
415 *Bacteroidetes*)⁴⁷ are most representative in the TC and DC. Moreover, accumulation of
416 lactate was only observed in the AC, being indicative of a faster fermentation of the
417 substrate,⁴⁸ and is in accordance with the carbohydrate-excess conditions of this
418 compartment due to the presence of GOS from the beverage. In the TC and DC, lactate
419 contents were low and remained stable, suggesting that lactate from the AC could be
420 used in the distal compartments for the production of butyrate and propionate (showing
421 their highest increments in this compartments).

422 Furthermore, in order to estimate other potential effects of the combined addition of
423 PS and GOS, changes in microbiota composition were also evaluated. Increments of
424 *Bifidobacterium* and *Lactobacillus* have been reported during the fermentation of GOS
425 in different models (three-stage continuous culture systems^{6,7,9} and TIM-2 model⁸) using
426 concentrations that ranged from 1 g to 10 g/day. However, in the present study, these
427 effects were not observed, thus, suggesting that the combination of both bioactive
428 compounds (PS and GOS) could influence the prebiotic function of GOS, besides
429 improving the fast biotransformation of PS, as mentioned above. In our work,
430 *Clostridium* genus presented an increasing tendency in the AC, which has also been
431 reported after the fermentation of a PS-ingredient in the TIM-2 model with microbiota

432 of obese subjects.¹⁶ However, after GOS fermentation, decreases in the *Clostridium*
433 *histolyticum* group were observed.⁷ On the other hand, *Bacteroides* genus tended to
434 decrease, while *Parabacteroides* tended to increase in the TC and DC (both belonging to
435 *Bacteroidetes* phylum). Fermentation of PS in batch culture is related with the increase
436 of some members of the uncharacterized S-24 family of *Bacteroidetes*,¹⁵ and in a study
437 carried out with hamsters, the addition of PS to a high-fat diet showed an increase of
438 *Bacteroides*.⁴⁵ However, *in vitro* fermentation of GOS has shown the decrease of
439 *Bacteroides-Prevotella spp.*^{7,8} In our study, unclassified *Lachnospiraceae* genera
440 increased in the AC and at short fermentation times in the TC and DC. Similarly,
441 increments of different species belonging to this family have been reported after the
442 fermentation of PS in batch culture (*Eubacterium hallii*),¹⁵ in a TIM-2 model carried out
443 with microbiota from lean subjects (*Coprococcus*)¹⁶ and in an *in vivo* study with hamsters
444 (*Coprococcus*)⁴⁵ and also after GOS fermentation (*Eubacterium rectale-Clostridium*
445 *coccoides* group).^{6,7} Thus, in general, changes in microbiota composition seem to be
446 more influenced by the presence of PS than GOS.

447 Therefore, the results of the current study provide valuable novel insights into the
448 impact of the fermentation of a complex food matrix (MfB) with GOS on sterol
449 metabolism. It has been demonstrated that sterol metabolism is colon compartment-
450 dependent and that the presence of GOS enhances their rapid biotransformation (with
451 higher ratios for cholesterol than PS). Likewise, organic acid production was higher in
452 the distal compartments, being in accordance with a higher diversity of the microbial
453 community. In addition, effects on microbiota composition during beverage
454 fermentation could be determined by the higher PS presence, showing an increase in
455 *Bacteroidetes* phylum and *Lachnospiraceae* family (related with PS fermentation).

456 However, as *in vitro* fermentation models are only an approximation of the *in vivo*
457 situation, the results of this study should be considered a first stage for future human
458 trials, taking into a count the inter-individual variability in gut microbiota composition.

459 **Abbreviations.** Ascending colon, AC; Association of Official Analytical Chemists, AOAC;
460 Butylhydroxytoluene, BHT; Descending colon, DC; Dynamic-Colonic Gastrointestinal
461 Digester, D-CGD; Fermentation liquid, FL; Gas chromatography-mass spectrometry, GC-
462 MS; Galactooligosaccharides, GOS; Internal standard, IS; Limit of detection, LOD; Limit
463 of quantitation, LOQ; Milk fat globule membrane, MFGM; Milk-based fruit beverage,
464 MfB; *N,O*-Bis(trimethylsilyl) trifluoroacetamide, BSTFA; Plant sterols, PS; Short chain
465 fatty acids, SCFA; Transverse colon, TC; Trimethylchlorosilane, TMCS.

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476 **SUPPORTING INFORMATION**

477 A GC-MS representative chromatogram, characteristic MS ions, as well as sterol and
478 organic acid contents are available as supporting information.

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- 635

636 **FIGURE CAPTIONS**

637 **Figure 1.** Schematic representation of the Dynamic-Colonic Gastrointestinal Digester (D-
638 CGD).

639 **Figure 2.** Evolution of sterol (continuous line) and metabolite (dashed line) contents
640 (mg/compartment) in the ascending colon (AC), transverse colon (TC) and descending
641 colon (DC).

642 **Figure 3.** Total and individual organic acid contents (mM) in the **a)** ascending colon, **b)**
643 transverse colon and **c)** descending colon.

644 **Figure 4. a)** Shannon diversity index and **b)** PCoA score plot of microbiota composition
645 based on colon compartment (ascending colon (AC), transverse colon (TC) and
646 descending colon (DC)).

647 **Figure 5.** Changes in microbiota composition at genus level (relative abundance) in the
648 **a)** ascending colon, **b)** transverse colon and **c)** descending colon.

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Table 1. Calibration curves with sterol standards obtained by GC-MS.

Sterol	Range (μg)	Calibration equation	Linear correlation coefficient
Coprostanol	0.002 – 0.42	$y = 0.0539x - 0.0181$	0.9973
Coprostanone	0.50 – 8.00	$y = 0.0211x - 0.0150$	0.9921
Cholesterol	0.50 – 6.03	$y = 0.0366x - 0.0175$	0.9927
Desmosterol	0.04 – 0.84	$y = 0.0152x - 0.0008$	0.9930
Brassicasterol	0.10 – 1.57	$y = 0.0851x - 0.0141$	0.9803
Lathosterol	0.10 – 1.19	$y = 0.0744x - 0.0076$	0.9958
Campesterol	0.25 – 4.00	$y = 0.0289x - 0.0100$	0.9959
Stigmasterol	0.25 – 2.01	$y = 0.0317x - 0.0061$	0.9872
β -Sitosterol ^a	0.50 – 5.00	$y = 0.0321x - 0.0121$	0.9931
	5.0 – 20.09	$y = 0.0589x - 0.1794$	0.9952
Stigmastanol	0.10 – 4.07	$y = 0.0831x - 0.0258$	0.9813

y = sterol area/internal standard area, x = μg of sterol. ^a Two sets of calibration curves at different ranges of concentrations were prepared due to the great differences in the contents present in the sampling points

Table 2. Limits of detection (LOD) and quantitation (LOQ) in each colon compartment.

Sterol	Compartment	LOD		LOQ	
		ng in assay	mg/ compartment	ng in assay	mg/ compartment
Coprostanol	AC	5.2	0.03	17.3	0.12
	TC	2.6	0.03	8.6	0.09
	DC	50.4	0.40	168.0	1.34
Cholesterol	AC	253.1	1.69	843.5	5.62
	TC	59.4	0.63	197.9	2.11
	DC	18.5	0.15	61.5	0.49
Methyl-coprostanol	AC	7.0	0.05	23.5	0.16
	TC	1.5	0.02	5.0	0.05
	DC	6.6	0.05	22.0	0.18
Desmosterol	AC	7.8	0.05	26.1	0.17
	TC	2.0	0.02	6.5	0.07
	DC	4.7	0.04	15.5	0.12
Brassicasterol	AC	2.1	0.01	7.0	0.05
	TC	1.0	0.01	3.3	0.04
	DC	1.0	0.01	3.2	0.03
Lathosterol	AC	0.9	0.01	3.0	0.02
	TC	0.7	0.01	2.5	0.03
	DC	1.2	0.01	4.2	0.03
Methyl-coprostanone	AC	3.7	0.02	12.3	0.08
	TC	1.6	0.02	5.2	0.06
	DC	4.4	0.04	14.7	0.12
Ethyl-coprostenol	AC	3.0	0.02	10.1	0.07
	TC	3.3	0.04	11.1	0.12
	DC	3.0	0.02	9.9	0.08
Ethyl-coprostanol	AC	6.0	0.04	19.9	0.13
	TC	3.8	0.04	12.6	0.13
	DC	3.8	0.03	12.7	0.10
Campesterol	AC	9.1	0.06	30.2	0.20
	TC	16.6	0.18	55.3	0.59
	DC	3.7	0.03	12.3	0.10
Campestanol	AC	10.3	0.07	34.3	0.23
	TC	8.4	0.09	27.9	0.30
	DC	4.2	0.03	14.0	0.11
Stigmasterol	AC	37.6	0.25	125.2	0.83
	TC	42.2	0.45	140.6	1.50
	DC	51.4	0.41	171.4	1.37
β -Sitosterol	AC	30.2	0.20	100.6	0.67
	TC	53.8	0.57	179.2	1.91
	DC	54.4	0.44	181.4	1.45
Sitostanol	AC	2.4	0.02	8.1	0.05
	TC	10.5	0.11	35.1	0.37
	DC	7.4	0.06	24.6	0.20
Sitostenone	AC	11.5	0.08	38.4	0.26
	TC	11.3	0.12	37.5	0.40
	DC	11.8	0.09	39.3	0.31

LOD = 3*SD/ slope of calibration curve for each sterol, LOQ = 10*SD/ slope of calibration curve for each sterol

Table 3. Assays of sample preparation and method for unsaponifiable extraction.

Sterol	Filtered	Sonication + centrifugation	Sampled in continuous stirring	
			Unsaponifiable extraction	
			With hexane (Cuevas-Tena et al.) ²²	With diethyl ether (Alvarez-Sala et al.) ²³
Coprostanol	-	4.15 ± 0.03 x	14.68 ± 2.01 y	13.77 ± 1.55 y
Cholesterol	a	a	20.54 ± 2.97 x	24.34 ± 2.17 x
Methylcoprostanol	-	4.58 ± 0.04 x	29.97 ± 4.57 y	23.63 ± 1.32 y
Ethylcoprostenol	-	-	4.46 ± 0.23 x	4.72 ± 0.11 x
Ethylcoprostanol	a	18.44 ± 0.64	b	b
Campesterol	4.91 ± 0.09 x	7.37 ± 0.07 y	b	b
Campestanol	a	a	13.03 ± 1.70 x	9.84 ± 0.80 x
Stigmasterol	-	-	8.14 ± 1.12 x	7.54 ± 1.04 x
β-Sitosterol	a	68.40 ± 0.82	b	b
Sitostanol	5.47 ± 0.15 x	9.74 ± 0.27 y	b	b
Sitostenone	-	-	8.70 ± 0.36	a

Values expressed as mean ± SD (n = 2) in mg/compartment. Assays performed with fermentation liquids (150 µL) from transverse colon at 120 h of beverage fermentation. ^a Value below the lowest point of the quantification curve; ^b Value above the highest point of the quantification curve (see Table 1). Different letters (x, y) denote statistically significant differences (p < 0.05), in the same sterol in each sample preparation

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

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