

1 **Association between cocoa diet effects on rats' body weight, microbiota and**
2 **intestinal immunity and their urine metabolomic profile**

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27 **Abbreviation list**

28 **α KMV**, α -keto- β -methyl-*n*-valerate; **α KIC**, α -ketoisocaproate; **1-MX**, 1-
29 methylxanthine; **2-HIB**, 2-hydroxyisobutyrate; **2-OG**, 2-oxoglutarate; **3-HIB**, 3-
30 hydroxyisobutyrate; **3-IS**, 3-indoxyl-sulfate; **3-MX**, 3-methylxanthine; **4-GB**, 4-
31 guanidinobutanoic acid; **-CS**, 4-cresol sulfate; **4-CG**, 4-cresol glucuronide; **HMB**, β -
32 hydroxy- β -methylbutyrate; **4-HPA**, 4-hydroxypropionic acid; **7-MX**, 7-
33 methylxanthine; **C10**, 10% cocoa diet; **CF**, cocoa fiber diet; **DF**, dietary fiber; **DMA**,
34 dimethylamine; **DMG**, dimethylglycine; **DMU**, dimethyluric acid; **GLP-1**, glucagon-
35 like peptide-1; **IAA**, indoleacetic acid; **NAG**, *N*-acetylglycoprotein; **NMN**, nicotine
36 mononucleotide; **NMNA**, *N*-methyl-nicotinic acid; **NMND**, *N*-methyl-nicotinamide;
37 **OPLS-DA**, Orthogonal projection to latent structures-discriminant analysis; **PAG**,
38 phenylacetyl glycine; **REF**, Reference diet; **RD**, recycle delay; **SCFA**, short chain
39 fatty acids; **TMAO**, trimethylamine *N*-oxide **TSP**, 3-trimethylsilyl-1-[2,2,3,3-²H₄]
40 propionate.

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43 **Keywords:** Cocoa / Hormones / IgA / Metabolomics / Microbiota

44 **Abstract**

45 **Scope:** The aim of this study was to find out the relationship between the urine
46 metabolomics fingerprints with the effects of cocoa and cocoa fiber on body weight
47 and metabolism, microbiota composition and intestinal immunity.

48 **Methods and results:** Wistar rats were fed, for two weeks, either a diet containing
49 10% cocoa (C10, providing a final proportion of 0.4% polyphenols, 0.85% soluble
50 fiber and 2.55% insoluble fiber), or two other diets with same proportion of soluble
51 fiber: one based on cocoa fiber (CF, with a very low amount of polyphenols) and
52 other containing inulin as a reference diet (REF). Twenty-four hours urine samples
53 were collected after two weeks of diet and metabolomics analysis by ¹H NMR
54 spectroscopy was carried out. Concentration of fecal IgA and metabolic hormones in
55 plasma were also quantified. Clear differences were observed between the urine from
56 the C10 group and those from the CF group ($Q^2Y=0.89$; $p=0.001$). The C10 diet
57 decreased the fecal IgA, GLP-1 and glucagon concentrations. Urine metabolites
58 mainly derived from cocoa catechin and methylxanthines were correlated with their
59 effects on body weight, microbiota and immunity.

60 **Conclusions:** These results allow us to establish a relationship between metabolomics
61 of cocoa compounds and their effects.

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It must be shortened.

64 **1 Introduction**

65 Cocoa is considered a great source of bioactive compounds such as polyphenols and
66 dietary fiber (DF) to whom consistent positive health effects have been attributed [1–
67 6]. Regarding its polyphenols, cocoa contains monomeric flavonoids such as the
68 flavanols (+)-catechin and (–)-epicatechin, and mainly its oligomers and polymers
69 known as procyanidins. These polymeric compounds are able to pass intact through
70 the small intestine and to reach the colon [7], where they are metabolised by the
71 intestinal microbiota. This conversion is crucial for their absorption and also for the
72 generation of new compounds which are biologically more active than the original
73 ones [7–11]. Thus, there is a growing body of evidence on the reciprocal relationship
74 between bacteria and polyphenols that may help understand the documented benefits
75 of polyphenols consumption: bacteria can be involved in the polyphenol metabolism
76 and, at the same time flavonoids influence microbiota growth and composition [12].
77 In this sense, it has been extensively reported that both dietary polyphenols and the
78 corresponding microbially-derived phenolic metabolites modulate the gut microbiota
79 composition in *in vitro*, *in vivo* and clinical studies [10,13–15]. Cocoa flavonoids-
80 enriched diets have also shown microbiota modulatory effects [15,16].

81 Similar ability to modulate the microbiota composition has been attributed to the DF
82 which has been described to indirectly lead to a different short chain fatty acids
83 (SCFA) production [17–21]. This ability to modulate the microbiota composition as
84 well as the SCFA production has also recently been associated to cocoa fiber-enriched
85 intake in rats [20]. The DF fraction in cocoa is mainly rich in cellulose, followed by
86 highly fermented pectic substances and hemicellulose, which is less fermentable than
87 the former [22].

88 After microbial transformation, the cocoa metabolites either from flavonoids or cocoa
89 fiber (i.e. SCFA) are absorbed into the bloodstream, providing another source of
90 potentially bioactive compounds [23,24]. To date, the microbial metabolites from
91 flavanols, included in the term of food metabolome [25], are mainly metabolized by
92 liver phase-II enzymes to hepatic conjugated derivatives that are subsequently
93 eliminated in urine [11].

94 Previous preclinical studies carried out in our laboratory have evidenced that cocoa,
95 cocoa flavanols and cocoa fiber modify some aspects of the intestinal and systemic
96 immune response [3,16,20,26]. On the other hand, cocoa diet, but not its flavonoids or
97 its fiber, is able to reduce the body weight gain [16,20,27]. Given that this effect on
98 weight is not associated with a lower chow intake, it is necessary to deep into the
99 mechanism involved in such effect.

100 On the basis of this background, the aim of this study was to find out the relationship
101 between the urine metabolomics fingerprints by ¹H NMR spectroscopy with the
102 effects of cocoa on body weight and metabolism, microbiota composition and
103 intestinal immunity. The particular involvement of cocoa fiber in such effects has also
104 been studied.

105 **2 Materials and methods**

106 **2.1 Animals and diets**

107 Female Wistar rats (3-week-old) were obtained from Janvier (Saint-Berthevin,
108 France) and housed in cages under conditions of controlled temperature and humidity
109 in a 12:12 light-dark cycle. The rats were randomly distributed into three dietary
110 groups: cocoa (C10), cocoa fiber (CF) and reference (REF) groups (*n*=10/each). The

111 C10 group received chow containing 10% cocoa that finally provided a 0.4% of
112 polyphenols, 0.85% soluble fiber and 2.55% of insoluble fiber; the CF group received
113 a diet with the same cocoa soluble and insoluble fiber proportions as the C10 group
114 but with a very low amount of polyphenols (<0.02%); and the REF group received the
115 same amount of soluble fiber as the C10 group (0.85%) but as inulin in order to
116 distinguish the particular effect of cocoa fiber. Natural Forastero cocoa and cocoa
117 fiber powders (provided by Idilia Foods S.L., formerly Nutrexa S.L.), Barcelona,
118 Spain) with 4.02% and 0.35% of polyphenols, respectively, were used to elaborate the
119 C10 and CF diets. Inulin from chicory roots (Fibruline® Instant; InnovaFood 2005,
120 S.L., Barcelona, Spain) was used as a reference soluble fiber. The three experimental
121 diets were elaborated on basis of the AIN-93M formula by subtracting the amount of
122 carbohydrates, proteins, lipids and insoluble fiber provided by the corresponding
123 supplement. The resulting chows were isoenergetic and had the same proportion of
124 macronutrients (carbohydrates, proteins and lipids) and insoluble fiber as the REF diet
125 as has been previously reported [20]. Animals were given free access to water and
126 chow. The diets lasted for three weeks.

127 Body weight and food intake were monitored throughout the study. Experiments were
128 performed according to the Guide for the Care and Use of Laboratory Animals, and
129 experimental procedures were approved by the Ethical Committee for Animal
130 Experimentation of the University of Barcelona (ref. 358/12).

131 **2.2 Sample collection and processing**

132 The 24 h urine samples were collected at 15 days after beginning with the nutritional
133 intervention by means of metabolic cages. Urines were then centrifuged and were
134 kept at – 80 °C until rat urine metabolic fingerprint profile analysis using ¹H NMR

135 spectroscopy. Moreover, blood samples were collected after three weeks of diet using
136 EDTA-treated tubes (Sardstedt AG & Co, Nümbrecht, Germany) and plasma was
137 kept at - 20 °C prior to metabolic hormones determination. Fecal samples were also
138 collected at the third week of diet and the homogenates were obtained as previously
139 described [20] and frozen at - 20 °C until analysis.

140 2.3 Sample preparation for ^1H NMR analysis

141 Urine samples were defrosted and prepared for ^1H NMR spectroscopy by combining
142 400 μL of sample with 200 μL of phosphate buffer (pH 7.4; 100% D_2O) containing
143 1 mM of 3-trimethylsilyl-1-[2,2,3,3- $^2\text{H}_4$] propionate (TSP) as an external standard and
144 2 mM sodium azide as a bactericide. Samples were vortexed to mix and particules
145 were removed by centrifugation (13000 g for 10 min) prior to transferring 550 μL to a
146 5 mm NMR tube. Standard one-dimensional ^1H NMR spectra of the urine samples
147 were acquired on a 500 MHz Bruker NMR spectrometer using a standard noisy
148 experiment incorporating a pre-saturation pulse to attenuate the water signal. This
149 experiment consisted of [recycle delay (RD)- 90° - t_1 - 90° - t_m - 90° -acquire free induction
150 decay]. The water signal was suppressed by irradiation during the RD of 2 s, with a
151 mixing time (t_m) of 10 μs . The acquisition time was set to 2.91 s and the 90° pulse
152 length was 15.87 μs . For each sample, 8 dummy scans were followed by 128 scans
153 and collected in 64K data points using a spectral width of 16 ppm. Prior to data
154 analysis, NMR spectra were phased, corrected for baseline distortions and calibrated
155 using the reference standard TSP. ^1H NMR spectra (δ 0.2-10.0) were digitized into
156 consecutive integrated spectral regions (\sim 20,000) of equal width (0.00055 ppm) using
157 Matlab (Mathworks). The regions containing signals from urea (δ 5.5 – 6.0) and the
158 residual water (δ 4-7 – 5.2) were removed to minimize baseline effects arising from

Comment [JM2]: Did you use normal NMR tubes instead of capillary tubes Jon?

Comment [JM3]: Jon, can you provide this values? I do not have TopSpin here to open the Spectra and check these values.

159 imperfect water suppression. Chemical shift variation was minimized across the
160 dataset by applying a recursive segment-wise peak alignment (RSPA) algorithm to
161 each spectrum. Each spectrum was normalized to unit area to account for variation in
162 sample concentration.

163 **2.4 Quantification of metabolic hormones in plasma**

164 Plasma concentrations of ghrelin, glucagon, glucagon-like peptide (GLP)-1 and leptin
165 were determined in plasma using the Bio-Plex Pro™ Diabetes Assay (Bio-Rad,
166 Madrid, Spain) according to the manufacturer's instructions. Analysis was carried out
167 with the Bio-Plex® MAGPIX™ Multiplex Reader and the Bio-Plex Data Pro™
168 software (BioRad) as in previous studies [28]. The limits of quantification can be
169 found as Supporting information.

170 **2.5 Fecal IgA quantification**

171 The concentration of IgA in feces was quantified by ELISA following the
172 manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, TX, USA).
173 Absorbance was measured in a microplate photometer (LabSystems Multiskan) and
174 data were interpolated using ASCENT version 2.6 software (Thermo Fisher
175 Scientific, Barcelona, Spain) into the standard curves, and expressed as ng/mg of
176 feces.

177 **2.6 Statistical analysis**

178 Statistical analysis for body weight, chow intake, fecal IgA and metabolic hormones
179 was performed using the software package IBM SPSS Statistics 22.0 (SPSS, Inc.
180 USA). Levene's and Kolmogorov–Smirnov tests were applied to assess variance
181 equality and normal distribution, respectively. Conventional one-way ANOVA was

182 performed when normal distribution and equality of variance existed. The Bonferroni
183 test was applied when specific cocoa intake had a significant effect on the dependent
184 variable. Non-parametric Mann–Whitney U and Wilcoxon tests were used in order to
185 assess significance for independent and related samples, respectively.

186 Multivariate modeling was performed in Matlab using in-house scripts. This included
187 principal components analysis using pareto scaled data and orthogonal projection to
188 latent structures-discriminant analysis (OPLS-DA) using a unit variance scaling
189 approach. Pairwise OPLS-DA models were constructed to aid model interpretation
190 and identify discriminatory metabolites between the study groups. Here, ^1H NMR
191 spectroscopic profiles served as the descriptor matrix (X) and the experimental groups
192 (REF, C10, CF) were used pairwise as the response variable (Y). Orthogonal signal
193 correction filters were used to remove variation in the descriptor matrix unrelated to
194 the response variable to assist model interpretation. Loading coefficient plots were
195 generated by back-scaling transformation where covariance is plotted between the Y-
196 response matrix and the signal intensity of the metabolites in the NMR data (X).
197 These plots are colored based on the correlation coefficient (r^2) between each
198 metabolite and the Y-response variable, with red indicating strong significance and
199 blue indicating weak significance. The predictive performance (Q^2Y) of the model
200 was calculated using a seven-fold cross validation approach and model validity was
201 established by permutation testing (1.000 permutations).

202 *Clustering analysis.* Unsupervised hierarchical clustering analysis (HCA) was
203 performed to identify general patterns of metabonomic variation between samples. To
204 do so, we used the normalized levels of metabolites identified to contribute to class
205 separation through the OPLS-DA models. For comparative analysis across different

206 metabolites, data were standardized as z-scores across samples for each metabolite
207 before clustering, so that the mean is 0 and the standard deviation is 1. This
208 standardized matrix was subsequently used in unsupervised HCA for samples and
209 metabolites using Euclidean distance and average linkage, by means of the pdist and
210 linkage functions in the MATLAB bioinformatics toolbox. Heatmaps and
211 dendrograms following HCA were generated with MATLAB imagesc and
212 dendrogram functions, respectively. In the heatmaps, a red-blue color scale is used so
213 that shades of red and blue represent higher and lower values, respectively, compared
214 with the mean. Different diet groups are color-coded and shown under the
215 dendrogram for each sample.

216 *Correlation analysis.* To explore the functional correlation between the changes on
217 gut microbiome, body weight, metabolism and intestinal immunity and metabonome
218 perturbations, Spearman's correlation analyses were performed. The Benjamini-
219 Hochberg method was used to adjust p -values for multiple testing considering a 5%
220 false discovery rate (FDR).

221

222 **3 Results**

223 **3.1 Body weight and chow intake**

224 Body weight and chow intake were monitored weekly throughout the study (**Fig. 1**).
225 Although the initial body weight was similar among the groups (44.4 ± 0.7 g), a
226 statistically slower body weight gain was observed already at day 7 in cocoa-fed
227 animals in comparison to the REF group ($p < 0.05$) and lasted until the end of the
228 study (**Fig. 1A**). This effect was not related to lower chow intake, which was similar

229 throughout the study among all experimental groups (**Fig. 1B**). No changes in body
230 weight gain were found as a result of CF diet intake.

231 **3.2 Metabolic hormones**

232 The metabolic hormones quantified in plasma after three-week dietary intervention
233 for all groups are summarized in **Table 1**. Both the C10 and the CF diets increased the
234 concentration of ghrelin compared to that of the REF animals ($p < 0.05$). This
235 increase was higher in the C10 group compared to the CF group ($p < 0.05$). Both
236 diets also resulted in a lower plasma GLP-1 concentration in comparison with the
237 REF group ($p < 0.05$). Moreover, the C10 diet reduced the glucagon concentration in
238 plasma compared to the REF and CF diets ($p < 0.05$). The leptin concentration was
239 not significantly affected after the C10 diet but it was up-regulated as a result of the
240 CF diet intake compared to the rest of the groups ($p < 0.05$) (**Table 1**).

241 **3.3 Fecal IgA**

242 The C10 diet intake resulted in a significantly attenuation of the IgA concentration
243 compared to the rest of the groups ($p < 0.05$) (**Fig. 2**). The CF diet did not produce
244 any change in the IgA concentration, which was similar to that quantified in the REF
245 group.

246 **3.4 Urinary metabolic profile**

247 An OPLS-DA model with strong predictive ability ($Q^2Y = 0.93$; $p = 0.001$) was
248 returned comparing the metabolic profiles from rats receiving the C10 diet and the
249 REF diet (**Fig. 3A and 4**). Rats fed the C10 diet excreted higher amounts of [cocoa-](#)
250 [derived metabolites. These include](#) *N*-methylnicotinic acid (NMNA; trigonelline),
251 [nicotine mononucleotide \(NMN\)](#), theobromine, xanthine, 1-[methylxantine \(1-MX\)](#), 3-

252 [methylxantine \(3-MX\)](#), [7-methylxanthine \(7-MX\)](#), imidazole, dimethyluric acid
253 (DMU), and catechin derivatives. The excretion of [cocoa polyphenol microbial-](#)
254 [derived metabolites](#) such as 4-hydroxypropionic acid (4-HPA), hippurate, and
255 phenylacetylglutamine (PAG) was also increased in the urine of C10 animals.

256 [Alternatively, the higher excretions of 4-HPA, PAG and indole-3-acetate could be](#)
257 [derived from tyramine, 2-phenylethylamine, and tryptamine, respectively, which are](#)
258 [the main moamines contained in cocoa](#). Moreover, the urine from these rats had
259 higher amounts of [2-hydroxyisobutyrate \(2-HIB\)](#), another [microbial-derived](#)
260 [metabolite, as well as](#) taurine [On the other hand](#), those animals receiving the C10 diet
261 excreted lower amount of energy metabolism-related metabolites (acetone, citrate,
262 2-oxoglutarate (2-OG) and *N*-methylnicotinamide (NMND) compared to the REF
263 group. [Other metabolites excreted in lower amounts include](#) metabolites related to
264 [endogenous](#) (α -keto-isocaproate (α KIC), α -keto-methylvalerate (α KMV),
265 hydroxymethylbutyrate (HMB), 3-hydroxyisobutyrate (3-HIB) and glycine) and
266 [microbial-derived](#) ([3-indoxyl-sulfate \(3-IS\)](#), [4-cresol sulfate \(4-CS\)](#) and [4-cresol](#)
267 [glucuronide \(4-CG\)](#)) [aminoacid metabolism](#); metabolites related to choline
268 metabolism (dimethylamine (DMA), dimethylglycine (DMG) and choline), and
269 metabolites related to dietary metabolism (sucrose, glucose, tartrate). The C10 diet
270 also resulted in a lower excretion of others metabolites such as sebacate, 4-
271 guanidinobutanoate, creatinine, allantoin, and pseudouridine compared to the REF
272 diet.

Comment [JM4]: Maybe I will put them together since we use the same color to classify them in teh clustergram as metabolites derived from cocoa. Since caffeine is part of cocoa, but it is up to you if you think that it is better to show them separately.

Comment [JM5]: I found this when Reading about cocoa, but probably you will know it better than me.

273 Regarding CF diet, a clear metabolic variation was observed in the urine from rats fed
274 the CF diet compared to those fed the REF diet (**Fig. 3B and 4**; OPLS-DA model
275 $Q^2Y = 0.65$; $p = 0.001$). Cocoaderived metabolite NMNA and [caffeine-related](#)
276 metabolites such as theobromine, xanthine, 1-[MX](#), 3-[MX](#), 7-[MX](#), and DMU were

Comment [JM6]: Again, we could put them together. Otherwise it can seem that caffeine is something a part from cocoa.

277 found in the urine of rats receiving the CF diet but not those receiving the REF diet.

278 Moreover, rats following a CF diet excreted greater amounts of 2-HIB (microbial
279 metabolism-derived metabolite), IAA (metabolite derived from the amino acid
280 metabolism), NMN (cocoa derived metabolite) and citrate, acetone and NMND all of
281 them derived from the energy metabolism as well as sugars (sucrose and glucose),
282 acetate and tartrate, derived from the dietary metabolism compared to those fed the
283 REF diet. Lower amounts of microbial metabolism- (4-HPA, hippurate, 3-IS and
284 PAG), amino acid- (α KI), choline- (DMG) and others- (sebacate, 4-
285 guanidinobutanoate, ethanol, creatinine, allantoin and pseudouridine) related
286 compounds were found in the CF group when compared to REF group.

287 Finally, the OPLS-DA model contrasting the urinary metabolic phenotypes from rats
288 receiving the C10 diet and those fed the CF diet ($Q^2Y = 0.89$; $p = 0.001$) also showed
289 some clear differences. As expected, rats consuming the C10 diet excreted higher
290 levels of methylxanthines and its metabolites theobromine, xanthine, 1- and 3-
291 methylxanthine, imidazole, DMU as well as the cocoa (NMNA, NMN) and catechin
292 derivatives compared to those fed the CF diet. The C10 diet fed animals also excreted
293 higher amount of metabolites derived from the microbial metabolism (4-HPA,
294 hippurate and PAG) and from the amino acid metabolism (IAA and taurine).
295 However, the C10 diet fed animals eliminated lower amounts of amino acid
296 metabolism- (α KMV, HMB), energy metabolism- (acetone, 2-OG, NMND and
297 citrate); choline (DMA and choline); microbial metabolism derivate (3-IS); dietary
298 metabolism-related metabolites (sucrose and glucose) among others (sebacate, 4-
299 guanidinobutanoate, fumarate, allantoin and pseudouridine) compared to those fed the
300 CF diet.

Comment [JM7]: Maybe, I would not repeat again the origin of the metabolites, since it has already been stated in the previous paragraph. I would just say that there is an increase in 2HIB, IAA, NMN and a decrease in...but without classifying them. It will make it easier to read.

Comment [JM8]: It can come from tryptophan or tryptamine.

Comment [JM9]: Same comment. Not necessary to classify them again.

301 **3.5 Correlations between urine metabolites and studied variables**

302 The correlation analysis between the urine metabolic fingerprints with the effects of
303 cocoa on body weight, microbiota composition (reported previously [20,29]) and
304 intestinal immunity was also studied (**Fig. 5**). Regarding body weight and metabolic
305 hormones, when samples from all the groups were considered together, the body
306 weight showed a significant inverse correlation with the detected amounts of the
307 cocoa metabolism- and amino acid metabolism-related metabolites in urine.
308 Moreover, a significant positive correlation was found between ghrelin concentration
309 and the amount of cocoa derivate metabolites as well as between the concentration of
310 plasma glucagon and the amount of choline.

311 In a previous study we characterized the microbiota composition after both the C10
312 and the CF diets intake [20]. Using these results we found that, the proportion of
313 *Streptococcus* genus presented a significant inverse correlation with the amount of
314 epicatechin (derived from the cocoa metabolism) and 4-HPA (from the amino acid
315 metabolism) determined in urine. In addition, a positive correlation was found
316 between *Bifidobacterium* counts and the amount of amino acid metabolism-related 3-
317 IS. When the *Firmicutes/Bacteroidetes* ratio was considered, a strong positive
318 correlation was observed between its values and those from the choline-related
319 metabolites.

320 Concerning the immunological parameters, the fecal IgA concentration had a
321 significant positive correlation with the amount of DMA and DMG (from choline
322 metabolism) and allantoin at the same time that had an inverse correlation with the
323 amount of cocoa derivate metabolites (theobromine, xanthines and DMU).

324 **3.6 Correlations between metabolic hormones and studied variables**

325 The correlation analysis between the effects of cocoa on the metabolic hormones,
326 body weight, microbiota composition [20] and intestinal immunity was also studied
327 (Fig. 6). When samples from all the groups were considered together, the body weight
328 showed a significant positive correlation with the proportion of *Bifidobacterium*,
329 *Clostridium histolyticum/C. perfringens*, *Streptococcus* genus, the
330 *Firmicutes/Bacteroidetes* ratio as well as with the concentration of butyric acid in
331 cecum content. There was also a positive correlation between the body weight and
332 both the glucagon and leptin plasma concentrations whereas it was negatively
333 correlated with the plasma ghrelin concentration.

334 The metabolic hormones also showed association with the microbiota composition
335 and functionality. Particularly, the ghrelin concentration was negatively correlated
336 with the *Bifidobacterium* spp., *Lactobacillus* spp., *Clostridium histolyticum/C.*
337 *perfringens*, *Streptococcus* spp. proportion as well as with the
338 *Firmicutes/Bacteroidetes* ratio, and the cecal butyric and the fecal IgA concentrations.
339 Moreover, the leptin concentration in plasma was positively correlated with the fecal
340 counts of *Bifidobacterium* spp. and *Streptococcus* spp., which, together with the
341 *Firmicutes/Bacteroidetes* ratio and the cecal butyric concentration, were also
342 positively correlated with the glucagon concentration.

343 Regarding the fecal IgA, its concentration was positively associated with the fecal
344 *Streptococcus* spp. counts and the rat's body weight.

345 **4 Discussion**

346 After ingestion of cocoa, both its polymeric flavanols and fiber reach the colon intact,
347 where commensal bacteria have an opportunity to metabolize them [7,10] resulting in

348 more active metabolites which can influence the intestinal immune system and lipid
349 metabolism. Previous studies showed that diets containing either 10% cocoa, cocoa
350 polyphenols or cocoa fiber modulate the microbiota composition and the intestinal
351 immune system in rats [16,20,29]. Here we demonstrate that both the whole cocoa
352 and the fiber from cocoa resulted in distinct urinary metabolome patterns which are
353 differentially correlated with the effects of cocoa on body weight, metabolic
354 hormones and the immunological status determined here and also with the microbiota
355 composition reported previously [20]. There are also significant correlations between
356 body weight, metabolic hormones profile and IgA concentration with the microbiota
357 composition and functionality.

358 The main differences observed in the urine metabolomic fingerprints from all the
359 experimental diets, and in line with previous controlled cocoa dietary intervention
360 studies [30,31], was the expected greater amounts of cocoa-derived metabolites.
361 These include the caffeine metabolites and the catechin derivatives, produced by the
362 gut microbiota, that have been identified in the urine from the animals fed the 10%
363 cocoa diet but not in those fed the cocoa fiber diet. These results confirm the lower
364 concentrations of polyphenols and methylxantines in the cocoa fiber powder used in
365 the present study to elaborate the CF diet.

366 Agreeing with previous studies [20,29,32–34], a significant lower body weight gain
367 has been observed in the animals fed the 10% cocoa diet. Although different feasible
368 mechanisms involved in cocoa effect on body weight and lipid metabolism have been
369 already proposed [20,35], we aimed to evaluate whether the cocoa intake affects the
370 metabolic hormones. In this sense, we have observed that the effect of cocoa on body
371 weight was accompanied by a tendency to reduce the leptin concentration in plasma.

372 Leptin is secreted by adipocytes and it provides the central nervous system with a
373 signal of the state of the body energy balance, which helps to control the appetite and
374 food intake, and to maintain a stable body weight [36]. However, given that there was
375 no difference in food intake between dietary groups along the study, the leptin
376 pathway can be discarded as the main mechanism by which cocoa influences the body
377 weight. Likewise, although the cocoa fiber intake increased the concentration of leptin
378 in plasma at the end of the study, it did modify neither the chow intake nor the body
379 weight gain in those animals. On the contrary we expected the C10-fed animals had
380 the highest ghrelin concentration compared to the rest of the groups. Although it is
381 known that ghrelin stimulates the appetite and food intake, increases fat mass
382 deposition and weight gain and influences glucose and lipid metabolism [37], in the
383 present study it did not provoked changes in chow intake. In fact, the ghrelin
384 concentration was negatively correlated with the body weight when all samples were
385 considered together.

386 We have also evaluated the impact on glucagon, a peptide hormone involved in the
387 glucose metabolism. In this report it can be observed that the diet containing the
388 whole cocoa, but not the one containing only cocoa fiber, significantly reduced the
389 glucagon level. These results, which are in line with previous studies [28], brings to
390 light the lack of contribution of cocoa fiber to these effects.

391 Furthermore, it have been reported that the effects of body weight on the gut
392 microbiota may be mediated, in part, by changes in circulating leptin concentration
393 [38] since this hormone stimulates the mucin production in the intestine which could
394 affect the composition of the microbiota [39]. Concurrently, the microbiota is able to
395 partially mediate the appetite control by regulating the level and type of

396 autoantibodies targeting the appetite-regulating hormones [40,41]. In the present
397 study we also evaluated the association between the metabolic hormones and the
398 microbiota composition characterized after both the C10 and the CF diets intake. In
399 this sense, a negative correlation was observed when all samples were considered
400 together between the ghrelin concentration and the proportion of *Lactobacillus*, which
401 agrees with other authors [36], as well as *Clostridium histolyticum*/*C. perfringens*
402 genera. Besides showing a negative correlation with the ghrelin plasma
403 concentration, the proportions of *Bifidobacterium* and *Streptococcus* genera as well as
404 the *Firmicutes/Bacteroidetes* ratio showed a positive correlation with the leptin
405 concentration, results which are partially in line with previous studies [36].

406 Associated to the microbiota composition, microbiota functionality can better explain
407 its pivotal role in host metabolism. In this regard, and in disagreement with our
408 results, it has been reported a food intake inhibition caused by two of the main SCFA
409 (butyrate and propionate) through stimulating gut hormones [42]. In our study, the
410 ghrelin concentration has been positively correlated with the butyric concentration in
411 cecum content. Moreover, a positive correlation has been observed between ghrelin
412 concentration and the amounts of theobromine, a methylxanthine present in cocoa in a
413 high concentration. Nonetheless, these theobromine metabolites were also negatively
414 associated with body weight, so oppositely to what it was observed, it would be
415 expected higher chow intake together with a lower body weight gain in the 10%
416 cocoa-fed animals. Thus these results evidence the strong contribution of theobromine
417 and its metabolites to the effect of cocoa on body weight. Additionally, an inverse
418 association was found between the body weight and the presence of metabolites
419 derived from the microbial metabolism, especially with PAG and 4-HPA. Thus, it
420 might be proposed that the direct modulatory effects of cocoa on microbiota

421 composition [29] are, among others, a key factor of this lower body weight gain. In
422 fact, studies carried out in our laboratory showed that the C10 diet intake decreased
423 the counts of *Staphylococcus* and *Streptococcus* (belonging to the *Firmicutes* phylum)
424 and increased those of *Bacteroides* (included in *Bacteroidetes* phylum) leading to a
425 lesser ratio of *Firmicutes* to *Bacteroidetes* [20,29], which has been extensively
426 associated with obesity or weight loss [35]. In line with these results, here an inverse
427 correlation has also been found between the counts of *Streptococcus* and the excreted
428 amounts of 4-HPA and epicatechin in urine.

429 Although the attenuating effect of the whole cocoa diet and the cocoa polyphenols-
430 enriched diet on the Ig synthesis has already been reported [3,16,32], less is known
431 about the impact of a cocoa fiber diet on the IgA synthesis. Here, the C10 diet resulted
432 in a significantly lower fecal IgA concentration at the end of the study, which
433 reinforces this cocoa effect while the cocoa fiber intake did not produce changes on
434 IgA concentration. In fact, it has been already reported the cocoa interaction with the
435 mechanisms involved in the IgA synthesis in the small intestine [26]. Moreover, a
436 significant inverse correlation was found between the fecal IgA concentration and the
437 amounts of cocoa-derived metabolites, including the caffeine metabolites and the
438 catechin derivatives, resulting from the gut microbiota metabolism. Overall it suggests
439 the role of both cocoa polyphenols and methylxanthines which after microbiota
440 metabolism may lead to the formation of compounds that interact with the
441 mechanisms involved in the IgA secretion and/or synthesis. Indeed, not only the
442 microbiota-generated metabolites are important, but also the microbiota composition.
443 In this sense, the lower counts of *Streptococcus* genus are positively well correlated
444 with the lessening of the fecal IgA concentration.

445 In summary, we have demonstrated that a cocoa diet led to a higher excretion of
446 metabolites related to its main bioactive components in young Wistar rats. Moreover,
447 cocoa diet intake resulted in a lower fecal IgA secretion as well as differential
448 metabolic hormones profile. In addition, most of the effects caused by the cocoa
449 intake are well correlated among them as well as with the amount of excreted
450 metabolites in urine derived from the cocoa metabolism. Therefore it suggests the
451 contribution of others cocoa compounds, but not the cocoa fiber, on such effects.
452 Further studies should be carried out in order to evaluate the precise contribution of
453 methylxanthines present in cocoa to such effects.

454 **Author contributions**

455 The authors' contributions were as follows: M. M. -C., À. F., F. J. P. -C and M. C.
456 conceived and designed the research; M. M.-C. and J. M.-P. carried out the
457 metabolomics experiments whereas M. M.-C. carried out the rest of the experiments;
458 J.M.-P. and J.R.S. and A.D. carried out the metabolomics data analysis and were
459 involved in the interpretation of these data whereas M. M. -C., F. J. P.-C. and M. C.
460 carried out the luminex and IgA data analysis and interpretation of the data; M. M. -C.
461 and J. M.-P. contributed equally to the initial draft of the manuscript; A.C., J.R.S., À.
462 F., F. J. P. -C and M. C. contributed to the critical revision of the manuscript; F.J.P.-
463 C. has primary responsibility for the final content. All authors have read and approved
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477

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607 free fatty acid receptor 3-independent mechanisms. *PLoS One*. 2012, 7,
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- 609

610 **Figure legends**

611 **Figure 1.** Body weight increase (%) compared to the baseline (A) and chow intake
612 (%) compared to the REF diet which represents 100% (B) monitored throughout the
613 nutritional intervention. Values are expressed as mean \pm SEM (n=10). * $p < 0.05$ vs
614 REF diet; and ‡ $p < 0.05$ vs CF diet.

615 **Figure 2.** Fecal IgA concentration determined after three weeks of nutritional
616 intervention. Results are expressed as mean \pm SEM (n = 9 -10). * $p < 0.05$ vs REF diet;
617 ‡ $p < 0.05$ vs CF diet.

618 **Figure 3.** Orthogonal projection to latent structures-discriminant analysis (OPLS-DA)
619 comparing the urinary metabolic profiles of rats receiving different dietary regimens.
620 Coefficient plots extracted from the OPLS-DA models comparing rats receiving REF
621 diet with C10 diet (A); REF diet with CF diet (B); and C10 diet with CF diet (C).

622 **α KMV**, α -keto- β -methyl-*n*-valerate; **α KIC**, α -ketoisocaproate; **1-MX**, 1-
623 methylxanthine; **2-HIB**, 2-hydroxyisobutyrate; **2-OG**, 2-oxoglutarate; **3-HIB**, 3-
624 hydroxyisobutyrate; **3-IS**, 3-indoxyl-sulfate; **3-MX**, 3-methylxanthine; **4-GB**, 4-
625 guanidinobutanoic acid; **-CS**, 4-cresol sulfate; **4-CG**, 4-cresol glucuronide; **HMB**, β -
626 hydroxy- β -methylbutyrate; **4-HPA**, 4-hydroxypropionic acid; **7-MX**, 7-
627 methylxanthine; **C10**, 10% cocoa diet; **CF**, cocoa fiber diet; **DMA**, dimethylamine;
628 **DMG**, dimethylglycine; **DMU**, dimethyluric acid; **IAA**, indoleacetic acid; **NAG**, *N*-
629 acetylglycoprotein; **NMN**, nicotine mononucleotide; **NMNA**, *N*-methyl-nicotinic
630 acid; **NMND**, *N*-methyl-nicotinamide; **PAG**, phenylacetyl glycine; **REF**, Reference
631 diet; **TMAO**, trimethylamine *N*-oxide.

632 **Figure 4.** Dendrogram and heatmap representation of unsupervised hierarchical
633 clustering (HCA) of the metabonome for all rats. Each column corresponds to a single

634 rat and each row corresponds to a specific metabolite. Metabolites identified to
635 contribute to the separation between diets through OPLS-DA models were used for
636 sample clustering. Metabolite z-score transformation was performed on the levels of
637 each metabolite across samples, with blue denoting a lower and red a higher level
638 compared to the mean. Metabolites and samples are clustered using correlation
639 distance and average linkage and color coded by diet (*Brown*, cocoa diet; *Orange*,
640 cocoa fiber; *Blue*, REF) or pathway (*Red*: aminoacid metabolism; *Purple*: microbial
641 metabolism; *Orange*: dietary; *Green*: energy metabolism; *Yellow*: choline
642 metabolism; *Blue*: miscellaneous; *Pink*: cocoa metabolites), respectively. HCA
643 grouped the urinary metabolic profiles from the C10-fed animals together and distinct
644 from the other studied animals. Profiles from animals receiving the CF diet clustered
645 together and were separate from the REF diet.

646 **Figure 5.** Correlations between metabolites and responses. The intensity of the colors
647 represents the degree of correlation, with red and blue indicating positive and negative
648 correlations, respectively. Metabolites identified to contribute to the separation
649 between diets through OPLS-DA models were to obtain the correlations. The order of
650 metabolites is the same obtained from the unsupervised hierarchical clustering in
651 Figure 4. Only significant correlations after applying a Benjamini and Hochberg
652 procedure for controlling for a false discovery rate of 5% are shown. Correlation
653 coefficients were based on Spearman's correlation.

654

655 **Tables**

656 **Table 1.** Metabolic hormones in plasma after three weeks of nutritional intervention.

657 Results are expressed as mean \pm SEM (n = 7). * $p < 0.05$ vs REF diet; † $p < 0.05$ vs

658 C10 diet and ‡ $p < 0.05$ vs CF diet.

659

	REF	C10	CF
Ghrelin (ng/mL)	30.57 \pm 3.86	98.63 \pm 18.73*‡	43.59 \pm 4.27*
GLP-1 (pg/mL)	29.4 \pm 16.4	7.4 \pm 3.1*	4.2 \pm 0.2*
Glucagon (pg/mL)	180.1 \pm 25.3	100.4 \pm 3.1*‡	166.2 \pm 26.9
Leptin (pg/mL)	647.7 \pm 135.5	335.4 \pm 127.5	968.7 \pm 177.6*‡