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

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Abbreviation list

αKMV, α-keto-β-methyl-*n*-valerate; **αKIC**, α-ketoisocaproate; **1-MX**, 1-methylxanthine;**2-HIB**, 2-hydroxyisobutyrate; **2-OG**, 2-oxoglutarate; **3-HIB**, 3-hydroxyisobutyrate; **3-IS**, 3-indoxyl-sulfate;**3-MX**, 3-methylxanthine; **4-GB**, 4-guanidinobutanoic acid; **-CS**, 4-cresol sulfate; **4-CG**, 4-cresol glucuronide; **HMB**, β-hydroxy-β-methylbutyrate; **4-HPA**, 4-hydroxypropionic acid;**7-MX**, 7-methylxanthine; **C10**, 10% cocoa diet; **CF**, cocoa fiber diet; **DF**, dietary fiber; **DMA**, dimethylamine; **DMG**, dimethylglycine; **DMU**, dimethyluric acid; **GLP-1**, glucagon-like peptide-1; **IAA**, indoleacetic acid; **NAG**, *N*-acetylglycoprotein; **NMN**, nicotine mononucleotide; **NMNA**, *N*-methyl-nicotinic acid; **NMND**, *N*-methyl-nicotinamide; **OPLS-DA**, Orthogonal projection to latent structures-discriminant analysis; **PAG**, phenylacetylglycine; **REF**, Reference diet; **RD**, recycle delay; **SCFA**, short chain fatty acids;TMAO, trimethylamine *N*-oxide **TSP**, 3-trimethylsilyl-1-[2,2,3,3-$^{2}H_{4}$] propionate.

Keywords: Cocoa / Hormones / IgA / Metabolomics / Microbiota

Abstract

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

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

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

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1 Introduction

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

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

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

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

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

2 Materials and methods

2.1 Animals and diets

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

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

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

2.2 Sample collection and processing

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

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

2.3 Sample preparation for ^{1}H NMR analysis

Urine samples were defrosted and prepared for ^{1}H NMR spectroscopy by combining 400 µL of sample with 200 µL of phosphate buffer (pH 7.4; 100% D_2O) containing 1 mM of 3-trimethylsilyl-1-[2,2,3,3-$^{2}H_4$] propionate (TSP) as an external standard and 2 mM sodium azide as a bacteriocide. Samples were vortexed to mix and particules were removed by centrifugation (13000 *g* for 10 min) prior to transferring 550 µL to a 5 mm NMR tube. Standard one-dimensional ^{1}H NMR spectra of the urine samples were acquired on a 500 MHz Bruker NMR spectrometer using a standard noisy experiment incorporating a pre-saturation pulse to attenuate the water signal. This experiment consisted of [recycle delay (RD)-90°-t_1-90°-t_m-90°-acquire free induction decay]. The water signal was suppressed by irradiation during the RD of 2 s, with a mixing time (t_m) of 10 µs. The acquisition time was set to 2.91 s and the 90° pulse length was 15.87 µs. For each sample, 8 dummy scans were followed by 128 scans and collected in 64K data points using a spectral width of 16 ppm. Prior to data analysis, NMR spectra were phased, corrected for baseline distortions and calibrated using the reference standard TSP. ^{1}H NMR spectra (δ 0.2-10.0) were digitized into consecutive integrated spectral regions (~20,000) of equal width (0.00055 ppm) using Matlab (Mathworks). The regions containing signals from urea (δ 5.5 – 6.0) and the residual water (δ 4-7 – 5.2) were removed to minimize baseline effects arising from

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

2.4 Quantification of metabolic hormones in plasma

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

2.5 Fecal IgA quantification

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

2.6 Statistical analysis

Statistical analysis for body weight, chow intake, fecal IgA and metabolic hormones was performed using the software package IBM SPSS Statistics 22.0 (SPSS, Inc. USA). Levene's and Kolmogorov–Smirnov tests were applied to assess variance 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, ^{1}H 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

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

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

3 Results

3.1 Body weight and chow intake

Body weight and chow intake were monitored weekly throughout the study (**Fig. 1**). Although the initial body weight was similar among the groups (44.4 ± 0.7 g), a statistically slower body weight gain was observed already at day 7 in cocoa-fed animals in comparison to the REF group ($p < 0.05$) and lasted until the end of the 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-

methylxantine (3-MX), 7-methylxanthine (7-MX), imidazole, dimethyluric acid (DMU), and catechin derivatives. The excretion of cocoa polyphenol microbial-derived metabolites such as 4-hydroxypropionic acid (4-HPA), hippurate, and phenylacetylglutamine (PAG) was also increased in the urine of C10 animals. Alternatively, the higher excretions of 4-HPA, PAG and indole-3-acetate could be derived from tyramine, 2-phenylethylamine, and tryptamine, respectively, which are the main moamines contained in cocoa. Moreover, the urine from these rats had higher amounts of 2-hydroxyisobutyrate (2-HIB), another microbial-derived metabolite, as well as taurine On the other hand, those animals receiving the C10 diet excreted lower amount of energy metabolism-related metabolites (acetone, citrate, 2-oxoglutarate (2-OG) and *N*-methylnicotinamide (NMND) compared to the REF group. Other metabolites excreted in lower amounts include metabolites related to endogenous (α-keto-isocaproate (αKIC), α-keto-methylvalerate (αKMV), hydroxymethylbutyrate (HMB), 3-hydroxyisobutyrate (3-HIB) and glycine) and microbial-derived (3-indoxyl-sulfate (3-IS), 4-cresol sulfate (4-CS) and 4-cresol glucuronide (4-CG)) aminoacid metabolism; metabolites related to choline metabolism (dimethylamine (DMA), dimethylglycine (DMG) and choline), and metabolites related to dietary metabolism (sucrose, glucose, tartrate). The C10 diet also resulted in a lower excretion of others metabolites such as sebacate, 4-guanidinobutanoate, creatinine, allantoin, and pseudouridine compared to the REF diet.

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

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

found in the urine of rats receiving the CF diet but not those receiving the REF diet. Moreover, rats following a CF diet excreted greater amounts of 2-HIB (microbial metabolism-derived metabolite), IAA (metabolite derived from the amino acid metabolism), NMN (cocoa derived metabolite) and citrate, acetone and NMND all of them derived from the energy metabolism as well as sugars (sucrose and glucose), acetate and tartrate, derived from the dietary metabolism compared to those fed the REF diet. Lower amounts of microbial metabolism- (4-HPA, hippurate, 3-IS and PAG), amino acid- (αKI), choline- (DMG) and others- (sebacate, 4-guanidinobutanoate, ethanol, creatinine, allantoin and pseudouridine) related compounds were found in the CF group when compared to REF group.

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

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3.5 Correlations between urine metabolites and studied variables

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

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

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

3.6 Correlations between metabolic hormones and studied variables

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

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

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

4 Discussion

After ingestion of cocoa, both its polymeric flavanols and fiber reach the colon intact, 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 the10%
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.

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

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

Furthermore, it have been reported that the effects of body weight on the gut microbiota may be mediated, in part, by changes in circulating leptin concentration [38] since this hormone stimulates the mucin production in the intestine which could affect the composition of the microbiota [39]. Concurrently, the microbiota is able to 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

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

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

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

Author contributions

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

Acknowledgments

The authors would like to thank the Genomic Services of the 'Centres Científics i Tecnològics' of the University of Barcelona (CCiT-UB) and the NMR Laboratory of the Chemical Analysis Facility technicians from the University of Reading their

technical assistance. We want also thank Idilia Foods S.L. (formerly Nutrexpa S.L.) and InnovaFood 2005 S.L. for providing the conventional cocoa and cocoa fiber powders and the inulin extract, respectively. The present study was supported by a grant from the Spanish Ministry of Economy and Competitivity (AGL2011-24279). M. M.-C. holds a fellowship from the Generalitat de Catalunya (grant no. 2014FI_B2 00048).

Disclosures: All authors declare no conflict of interest. None of the funders had a role in the design or analysis of the study or in the writing of this article.

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Figure legends

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

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

Figure 3. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) comparing the urinary metabolic profiles of rats receiving different dietary regimens. Coefficient plots extracted from the OPLS-DA models comparing rats receiving REF diet with C10 diet (A); REF diet with CF diet (B); and C10 diet with CF diet (C). **αKMV**, α-keto-β-methyl-*n*-valerate; **αKIC**, α-ketoisocaproate; **1-MX**, 1-methylxanthine;**2-HIB**, 2-hydroxyisobutyrate; **2-OG**, 2-oxoglutarate; **3-HIB**, 3-hydroxyisobutyrate; **3-IS**, 3-indoxyl-sulfate;**3-MX**, 3-methylxanthine; **4-GB**, 4-guanidinobutanoic acid; **-CS**, 4-cresol sulfate; **4-CG**, 4-cresol glucuronide; **HMB**, β-hydroxy-β-methylbutyrate; **4-HPA**, 4-hydroxypropionic acid;**7-MX**, 7-methylxanthine; **C10**, 10% cocoa diet; **CF**, cocoa fiber diet;**DMA**, dimethylamine; **DMG**, dimethylglycine; **DMU**, dimethyluric acid; **IAA**, indoleacetic acid; **NAG**, *N*-acetylglycoprotein; **NMN**, nicotine mononucleotide; **NMNA**, *N*-methyl-nicotinic acid; **NMND**, *N*-methyl-nicotinamide; **PAG**, phenylacetylglycine; **REF**, Reference diet; **TMAO**, trimethylamine *N*-oxide.

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

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

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

655 **Tables**

656 **Table 1.** Metabolic hormones in plasma after three weeks of nutritional intervention.
657 Results are expressed as mean ± 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 ± 3.86	98.63 ± 18.73*‡	43.59 ± 4.27*
GLP-1 (pg/mL)	29.4 ± 16.4	7.4 ± 3.1*	4.2 ± 0.2*
Glucagon (pg/mL)	180.1 ± 25.3	100.4 ± 3.1*‡	166.2 ± 26.9
Leptin (pg/mL)	647.7 ± 135.5	335.4 ± 127.5	968.7 ± 177.6*†