

1 **Cocoa diet modulates gut microbiota composition and improves intestinal health in**
2 **Zucker diabetic rats**

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Abbreviations used: AUC, area under the curve; GM, gut microbiota; GTT, Glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein 1; PCNA, anti-proliferating cell nuclear antigen; SCFA, short chain fatty acids; STZ, streptozotocin; T2D, type 2 diabetes; TNF- α , tumour necrosis factor- α ; ZDF, Zucker diabetic fatty; ZL, Zucker lean; ZO-1, Zonula occludens-1

22 **Abstract**

23 Cocoa supplementation improves glucose metabolism in Zucker diabetic fatty (ZDF) rats via
24 multiple mechanisms. Furthermore, cocoa rich-diets modify the intestinal microbiota composition both
25 in humans and rats in healthy conditions. Accordingly, we hypothesized that cocoa could interact with
26 the gut microbiota (GM) in ZDF rats, contributing to their antidiabetic effects. Therefore, here we
27 investigate the effect of cocoa intake on gut health and GM in ZDF diabetic rats.

28 Male ZDF rats were fed with standard (ZDF-C) or 10% cocoa-rich diet (ZDF-Co) during 10
29 weeks. Zucker Lean animals (ZL) received the standard diet. Colon tissues were obtained to determine
30 the barrier integrity and the inflammatory status of the intestine and faeces were analysed for microbial
31 composition, short-chain fatty acids (SCFA) and lactate levels. We found that cocoa supplementation
32 up-regulated the levels of the tight junction protein Zonula occludens-1 (ZO-1) and the mucin
33 glycoprotein and reduced the expression of pro-inflammatory cytokines such as tumour necrosis factor-
34 α (TNF- α), interleukin-6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1) in the colon of ZDF
35 diabetic animals. Additionally, cocoa modulated the microbial composition of the ZDF rats to values
36 similar to those of the lean group. Importantly, cocoa treatment increased the relative abundance of
37 acetate-producing bacteria such as *Blautia* and prevented the increase in the relative amount of lactate-
38 producing bacteria (mainly *Enterococcus* and *Lactobacillus* genera) in ZDF diabetic animals.
39 Accordingly, the total levels of SCFA (mainly acetate) increased significantly in the faeces of ZDF-Co
40 diabetic rats. Finally, modified GM was closely associated with improved biochemical parameters
41 related to glucose homeostasis and intestinal integrity and inflammation.

42 These findings demonstrate for the first time that cocoa intake modifies intestinal bacteria
43 composition towards a healthier microbial profile in diabetic animals and suggest that these changes
44 could be associated with the improved glucose homeostasis and gut health induced by cocoa in ZDF
45 diabetic rats.

46

47 **Keywords:** Diabetes type 2; Cocoa flavanols; Gut microbiota; Gut barrier; Gut inflammation; Glucose

48 homeostasis.

49

50 **Highlights.**

- 51 - Intestinal barrier integrity is improved in diabetic rats submitted to cocoa diet
- 52 - Cocoa diet prevents intestinal inflammation in diabetic rats
- 53 - Cocoa diet modifies gut microbiota to a healthier microbial profile in diabetic rats
- 54 - Total levels of short-chain fatty acids increases in diabetic rats fed on cocoa
- 55

56 **1.- Introduction**

57 Diabetes is a complex metabolic disorder characterized by hyperglycaemia resulting from
58 defects in insulin secretion and insulin action (ADA, 2017). At present, prevalence of type 2 diabetes
59 (T2D) is reaching epidemic proportions, becoming a serious threat to public health worldwide mainly
60 due to the associated complications (WHO, 2016). The growing prevalence of T2D is positively
61 related to harmful lifestyles, in particular the reduced levels of physical activity and increasingly
62 unhealthy eating habits, indicating that diet plays a crucial role in the onset and progression of T2D
63 (WHO, 2016). More importantly, an increasing body of evidence suggests that certain dietary
64 compounds may attenuate the risk of T2D by their ability to modulate gut microbiota (GM)
65 composition (Nie et al., 2019).

66 The GM comprises a complex community of bacteria that colonizes the surface and the lumen
67 of the gastrointestinal tract. Microbiota transform food components and produce a wide range of
68 derived metabolite that impact host's physiology and health in many ways, from the maintenance of
69 intestinal homeostasis to energy metabolism (Van Treuren & Dodd, 2020). Indeed, many disease states
70 have been associated with alterations in microbiota composition and consequently on their
71 functionality, indicating that GM **could be** involved in the development of numerous pathologies
72 (Danneskiold-Samsoe et al., 2019). In particular, microbiota function has been identified as a relevant
73 and potentially modifiable factor that contributes to the development of metabolic diseases, including
74 T2D (Li, Watanabe & Kimura, 2017). Therefore, interventions targeting GM are emerging as
75 promising effective strategies for the prevention and management of T2D.

76 Polyphenols are a large group of phytochemical compounds that have attracted much interest
77 due to their beneficial properties. Accumulating evidences suggest that dietary polyphenols may
78 interact with GM (Nash et al., 2018; Tomás-Barberán & Espín, 2019). Bioavailability of dietary

79 polyphenols in the digestive tract is highly variable. Aglycones, monomeric and dimeric structures can
80 be absorbed in the small intestine. However, most of polymeric structures reach the colon intact where
81 they are metabolized by GM producing small microbial derived metabolites which are absorbed more
82 efficiently and therefore may contribute to the beneficial health effects of polyphenols (González-
83 Sarrías, Espín, & Tomás-Barberán, 2017). In addition, these natural compounds modulate the
84 composition and function of GM exhibiting prebiotic effects and antimicrobial action against
85 pathogenic intestinal microbiota (Marchesi et al., 2016; Singh et al., 2019). In this way, polyphenols
86 can influence the bacterial production of fermented or degraded metabolites (short chain fatty acids –
87 SCFA-) which can modulate multiple physiological pathways in several tissues, affecting gut health,
88 glycaemic control, lipids profile and insulin resistance (Morrison, & Preston, 2016). Therefore, a more
89 complete understanding of this bidirectional interaction between polyphenols and GM should help to
90 explain the beneficial health effects of these natural compounds.

91 Cocoa is considered a rich source of dietary polyphenols, mainly flavanols such as epicatechin
92 and procyanidins. Cocoa flavanols can exert antidiabetic effects via multiple mechanisms, including
93 antioxidant and anti-inflammatory effects, as well as by increasing insulin secretion and insulin action
94 (Martín, Goya, & Ramos, 2016). In addition, a cocoa rich diet has been described as able to modify the
95 intestinal microbiota composition in healthy rats (Massot-Cladera et al., 2012; Massot-Cladera et al.,
96 2014), pigs (Jang et al., 2016; Magistrelli et al., 2016) and humans (Tzounis et al., 2011). Therefore, it
97 is probable that interaction of cocoa components with GM actively contributes to the antidiabetic
98 effects of cocoa. However, to date, the influence of cocoa feeding on intestinal health and on the
99 composition of GM in diabetes remains to be considered. Accordingly, the aim of the present study was
100 to investigate whether cocoa supplementation modulate intestinal dysbiosis induced by diabetes in an
101 *in vivo* model of T2D, using Zucker diabetic fatty (ZDF) rats. To this end, the effect of a cocoa rich-

102 diet on glucose homeostasis and biomarkers of gut health in diabetic ZDF rats was evaluated. In
103 addition, the compositional changes in GM and SCFAs induced by cocoa in diabetic animals were also
104 determined.

105

106 **2. Material and Methods**

107 **2.1. Diets, animals and experimental design**

108 Diets were prepared from an AIN-93G formulation (Panlab S.L., Barcelona, Spain). Cocoa rich-
109 diet (10%) was produced by adding 100 g/Kg of natural Forastero cocoa powder (a kind gift from Idilia
110 Foods, Barcelona, Spain) to AIN-93G diet. It contains epicatechin (382 mg/100 g), catechin (115
111 mg/100 g) and procyanidins (167 mg/100 g) and non-flavonoid compounds such as theobromine
112 (742mg/100 g). The resulting cocoa diet was isoenergetic and its composition is given in Table 1.

113 Male Zucker diabetic fatty (ZDF) rats (n=16) and their Zucker lean controls (ZL) (n=6) were
114 obtained from Charles River Laboratories (L'arbresle, France) at 9 weeks of age. Animals were placed
115 under standard controlled conditions (21 °C ± 1 °C; 12 h day/night cycle). After one week of
116 acclimation, ZDF diabetic rats were randomly divided into two groups of eight animals that received
117 the standard AIN-93G diet (ZDF-C) or the same control diet supplemented with 10 % of cocoa (ZDF-
118 Co) for 10 weeks. The lean Zucker rats (ZL) received the standard AIN-93G diet. During the
119 experiment, food and water were available *ad libitum*. Food intake was monitored daily and animal
120 weight and glycaemia was weekly followed. Animals were treated according to the European
121 (2010/63/EU) and Spanish (RD 53/2013) legislation on Care and Use of Experimental Animals and the
122 experiments were approved by the Ethics Committee from Comunidad de Madrid (PROEX 304/15).

123

124 **2.2. Biochemical determinations**

125 At 20 weeks of age, animals were fasted overnight and were scarified by exsanguination under
126 anaesthesia ketamine/xylazine (80 mg/8 mg Kg⁻¹, i.p.). Blood samples were collected for biochemical
127 analysis. Glucose was determined using an Accounted Glucose Analyzer (LifeScan España, Madrid,
128 Spain) and insulin and glycosylated haemoglobin (HbA1c) were quantified by ELISA kits (Rat Insulin,

129 Mercodia, Uppsala, Sweden; HbA1c Kit Spinreact, BioAnalitica, Madrid, Spain). Fasting plasma
130 concentrations of both glucose and insulin were used to calculate indices of insulin resistance
131 [homeostasis model assessment (HOMA)-IR] and secretion (HOMA-B) using the following formulae:
132 $HOMA-IR = \text{fasting insulin (mU/ml)} \times \text{fasting glucose (mM)} / 22.5$; $HOMA-B = 20 \times \text{fasting insulin}$
133 $(\text{mU/ml}) / [\text{fasting glucose (mM)} - 3.5]$. Triacylglycerols (TG), HDL-Cho and LDL-Cho were determined
134 in serum by kits (BioSystems, Madrid, Spain) as described elsewhere (Álvarez-Cilleros et al., 2019).
135 Tumour necrosis factor- α (TNF- α) and interleukin-6 (IL-6) levels were quantified in serum samples by
136 specific rat TNF-alpha Quantikine ELISA Kit (RTA00, R&D System, USA) and IL-6 DuoSet ELISA
137 Kit (DY506, R&D System, USA) according to the manufacturer's instruction.

138

139 **2.3. Glucose tolerance test (GTT)**

140 GTTs were performed one week before the end of the study. Briefly, an overload of glucose
141 (2g/Kg body weight) (Sigma Chemical, Madrid, Spain) was ip administered in animals subjected to
142 overnight fasting. Blood samples were collected from the tail vein at five different time points (0, 30,
143 60, 90, 120 and 180 min) and glucose levels were measured using an Accounted Glucose Analyzer
144 (LifeScan España, Madrid, Spain). The integrated glucose response (area under the curve, AUC) over a
145 period of 180 min after glucose overload was also calculated.

146

147 **2.4. Histologic and immuno-histochemical analysis**

148 Rats were sacrificed at 20 weeks of age and the entire colon was resected and cleaned with PBS.
149 Sections (2 cm) from the most distal portion of the colon were routinely processed and paraffin
150 embedded for histological and immune-histochemical analyses. Sections were cut, stained with
151 haematoxylin–eosin (H&E) or periodic-acid-Schiff (PAS) according to the manufacturer's instructions.

152 Images were obtained under light microscopy (Leica DM LB2) and a digital Leica DFC 320 camera
153 (Leica, Madrid, Spain) and quantified with ImageJ software (Fiji image J; 1.52i, NIH, USA). The crypt
154 depth was measured from H&E slices and was determined as number of cells per hemi-crypt. Only
155 crypts with an open longitudinal crypt axis were analysed. The tissue expression level of the neutral
156 mucin glycoprotein was determined by means of PAS and it was calculated as the number of PAS
157 positive cells per crypt.

158 For the immuno-histochemical staining, antibodies against monoclonal anti-proliferating cell
159 nuclear antigen (PCNA; PC-10) (Lab Vision Corporation and Bionova- Científica SL), Cyclin E (sc-
160 247, Santa Cruz Biotechnology), p21 (sc-6246, Santa Cruz Biotechnology), Zonula occludens-1 (ZO-
161 1) (sc-10804, Santa Cruz Biotechnology), tumour necrosis factor- α (TNF- α) (sc-133192, Santa Cruz
162 Biotechnology), interleukin-6 (IL-6) (sc-57315, Santa Cruz Biotechnology), monocyte chemoattractant
163 protein 1 (MCP-1) (sc-52701, Santa Cruz Biotechnology) and CD45 (ab-10558, Abcam) were used.
164 After deparaffinization and endogenous peroxidase quenching, serial colonic sections were incubated
165 with the primary antibodies overnight at 4°C. Secondary antibodies were used to detect primary
166 antibodies, followed by streptavidin-tagged horseradish peroxidase and visualized by 3,3'-
167 diaminobenzidine (DAB) substrate (Sigma Chemical, Madrid Spain). The sections were counterstained
168 with Harris's haematoxylin, dehydrated and mounted. Brown colour indicates specific protein
169 immunostaining and light blue colour indicates nuclear haematoxylin staining. Positive and negative
170 controls were used during the optimization of the methods.

171 At least 20 perpendicular well-oriented crypts were examined in each animal under light
172 microscopy at x400 magnification. The proliferative labelling index (LI) (%) was calculated as the
173 number of positive nuclei \times 100/total number of cells per crypt column height. ZO-1, TNF- α , IL-6,

174 MCP-1 and CD45 protein expression level was evaluated as percentage of the stained area to the total
175 area per crypt by using the colour deconvolution plug-in from ImageJ v1.52j software.

176

177 **2.5. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.**

178 Apoptotic colonic epithelial cells were labelled *in situ* by identifying DNA fragmentation on
179 paraffin embedded sections using the terminal deoxynucleotidyl transferase UTP nick end labelling
180 (TUNEL) assay. After deparaffinization and rehydration tissue sections were permeabilized with
181 proteinase K (20 µg/mL) for 15 min at 37°C, and then treated with 3% hydrogen peroxide for 5 min to
182 quench endogenous peroxidase activity. After sections were incubated with equilibration buffer for 10
183 min, followed by immediate application of TdT-enzyme working for 1 h at 37°C. Slices were incubated
184 with peroxidase conjugated streptavidin and subsequent staining with DAB and counterstaining with
185 methyl green. The apoptotic index represents the proportion of cells undergoing apoptosis within a
186 crypt column (x400) and was calculated as the ratio of TUNEL-positive cells to the total number of
187 cells counted within 50 full-length well orientated crypts.

188

189 **2.6. Faecal samples.**

190 Fresh faecal samples were collected at the end of the intervention period, early in the morning,
191 by abdominal massage in sterilized tubes and immediately frozen at –80°C for future analyses.

192

193 **2.7. DNA extraction, and 16S gene PCR amplification. Illumina Mi-Seq sequencing.**

194 DNA was extracted from faecal samples using G-spin columns (INTRON Biotechnology).
195 DNA concentration was determined using Quant-IT PicoGreen reagent (ThermoFisher Scientific, Inc.,
196 Waltham, MA, USA) and around 3 ng were used to amplify the V3-V4 region of 16S rRNA gene

197 (Caporaso et al., 2011). PCR products (approx. 450 bp) included extension tails, which allowed sample
198 barcoding and the addition of specific Illumina sequences in a second low-cycle number PCR.
199 Individual amplicon libraries were analysed using a Bioanalyzer 2100 (Agilent Technologies, Palo
200 Alto, CA, USA) and a pool of samples was made in equimolar amounts. The pool was further cleaned,
201 quantified and the exact concentration estimated by real time PCR (Kapa Biosystems). Finally, DNA
202 samples were sequenced on an Illumina MiSeq instrument with 2 x 300 paired-end read sequencing at
203 the Unidad de Genómica (Parque Científico de Madrid, Spain).

204 We used the BIPES pipeline to process the raw sequences (Zhou et al., 2011) and we performed
205 UCHIME (implemented in USEARCH, version 6.1) to screen out and remove chimeras in the *de novo*
206 mode (Edgar, & Flyvbjerg, 2015). In each sample between 90,000 and 220,000 sequences were
207 identified. All subsequent analyses were performed using 16S Metagenomics (Version: 1.0.1.0) from
208 Illumina. The sequences were then clustered to an operational taxonomic unit (OTU) using USEARCH
209 with default parameters (USERACH61). The threshold distance was set to 0.03. Hence, when the
210 similarity between two 16S rRNA sequences was 97%, the sequences were classified as the same OTU.
211 QIIME-based alignments of representative sequences were performed using PyNAST, and the
212 Greengenes 13_8 database was used as the template file. The Ribosome Database project (RDP)
213 algorithm was applied to classify the representative sequences into specific taxa using the default
214 database (Edgar, & Flyvbjerg, 2015). The Taxonomy Database (National Center for Biotechnology
215 Information) was used for classification and nomenclature. Bacteria were classified based on the SCFA
216 end-product as previously described (Wang, Garrity, Tiedje, & Cole, 2007).

217

218 **2.8. Measure of SCFAs and lactate in faeces**

219 Faecal samples were weighed and suspended in 1 mL of water with 0.5% phosphoric acid per
220 0.1 g of sample and frozen at -20°C immediately after collection. Once thawed, the faecal suspensions
221 were homogenized with a vortex for about 2 min and centrifuged for 10 min at 17949 g. Aliquots of
222 400 μL of supernatans were diluted with 100 μL of 4-methyl valeric acid used as internal standard at a
223 final concentration of 788 μM and then the suspension was extracted with 1 mL of n-butanol for 21
224 min and centrifuged for 105 min at 16000 g. A stock solution containing the mixture of standards
225 (WSFA-2; Sigma-Aldrich, Madrid, Spain) was treated as samples and diluted to obtain a calibration
226 curve ranging from 2 to 10000 μM . Internal standard was also added to the mixture of standards.

227 The analytes (2 μL) were injected in the splitless mode into an Agilent 7890A gas
228 chromatography (GC) system (Agilent Technologies, Palo Alto, CA, USA) equipped with a 5975C
229 mass spectrometer (MS) detector and an Agilent DB-WAXtr column (100% polyethilen glycol, 60 m,
230 0.325 mm, 0.250 μm). Helium was used as a carrier gas at 1.5 mL/min. The column temperature was
231 initially 50°C , then increased to 150°C at $15^{\circ}\text{C}/\text{min}$, to 200°C at $5^{\circ}\text{C}/\text{min}$, and finally to 240°C at
232 $15^{\circ}\text{C}/\text{min}$ and kept at this temperature for 20 min (total time 41.3 min). The MS was tuned during all
233 experiments; the signal acquisition for quantification was done in the single-ion monitoring (SIM)
234 mode. The temperature of the ionization source and the quadrupole were 230°C and 150°C ,
235 respectively. The electron-impact ionization energy was 70 eV. Concentrations of acetate, propionate
236 and butyrate were expressed as $\mu\text{M}/\text{g}$ of sample.

237 For lactate determination faecal samples were suspended in MilliQ water. After homogenization
238 with an ultrasonic liquid processor Vibra-Cell CV18 (Sonics & Materials, Connecticut, Unites States),
239 faecal suspensions were centrifuged at 1000 g for 5 min. The aqueous phase was filtered through a 0.2
240 μm cellulose acetate syringe filter (VWR International, Barcelona, Spain). After filtration, lactate was
241 measured with an Advanced Compact Ion Chromatographic instrument IC867 (Metrohm AG, Herisau,

242 Switzerland). Sodium L-lactate (Sigma-Aldrich, Madrid, Spain) was used as standard. Concentrations
243 of lactate were expressed as $\mu\text{M/g}$ of sample.

244 **2.9. Statistical Analysis**

245 Data from biochemical and immune-histochemical parameters, relative abundances of taxa and
246 SCFA levels were tested for normality and homogeneity of variances by the D'Agostino and Pearson
247 and Levene tests, respectively; for multiple comparisons, one-way ANOVA was followed by a Tukey
248 test when variances were homogeneous or by the Tamhane test when variances were not homogeneous.
249 The level of significance was $P < 0.05$. A GraphPad Prism version 7.00 (GraphPad software, Inc., La
250 Jolla, California) was used.

251 Shannon, Chao and Simpsons indexes were calculated to analyse α -diversity using QIIME.
252 Reads in each OTU were normalized to total reads in each sample. Unsupervised classification studies
253 with Principal Components Analysis (PCA) were carried out to analyse the differences between groups.
254 Relationship strength between parameters was assessed using the two tailed Pearson's correlation test.
255 The correlation was considered significant only when the absolute value of Pearson's correlation
256 coefficient r was > 0.5 .

257

258 **3. Results**

259 **3.1. Physiological parameters**

260 At the beginning of the study, ZDF animals showed marked increases in body weight as
261 compared to ZL rats (210.0 ± 9.0 vs 284.4 ± 10.6 g, respectively; $P < 0.05$). However, fasted glycaemia
262 was not significantly different between ZL and ZDF animals (84.6 ± 6.0 vs 102.2 ± 13.1 mg/dL,
263 respectively; $p < 0.05$), which indicates that at this time point animals were at a pre-diabetic stage.
264 Then, ZDF rats were randomly assorted to ZDF-C or ZDF-Co groups. The administration of cocoa for
265 10 weeks reduced body weight in diabetic ZDF rats while daily food intake remained constant (Figures
266 1A and 1B). Moreover, the increase in glycaemia, insulinaemia and HbA1c that is characteristic of
267 ZDF rats was significantly reduced in those feed with cocoa diet (ZDF-Co) (Figures 1C-1D). Likewise,
268 there was a significant reduction in insulin resistance state (HOMA-IR) and a significant increase in
269 beta-cell function (HOMA-B) in ZDF-Co rats as compared to ZDF (Fig. 1D). Finally, the glucose
270 tolerance test showed that diabetic ZDF-Co rats were less intolerant than ZDF-C rats and,
271 consequently, their AUC value was significantly reduced by the cocoa rich diet (Figure 1G). In
272 contrast, HDL-Cho, LDL-Cho and TG levels were significantly elevated in both diabetic ZDF groups
273 in comparison to the ZL group and cocoa diet only was able to partly reduce the levels of LDL-Cho in
274 ZDF animals (Figure 1H). Altogether, these results indicated that cocoa intake was able to improve
275 glycemic control but not lipid profile in diabetic and obese ZDF rats.

276

277 **3.2. Intestinal integrity and inflammation**

278 Next, we investigated the effect of cocoa treatment in the intestinal barrier integrity and colonic
279 inflammation in diabetic rats. Morphological modifications at a scale larger than crypts were not
280 observed. As shown in Figure 2A, diabetic ZDF-C rats revealed a similar crypt depth to the ZL lean

281 group whereas rats fed with cocoa presented a significantly larger crypt depth. Accordingly, colonocyte
282 proliferation and apoptosis (Figures 2B and 2C) were similar in diabetic and lean animals while both
283 processes were significantly increased in ZDF-Co animals, indicating that cocoa intake induced a faster
284 renewal rate of the colonic epithelium. To deepen the mechanism by which cocoa intake increases the
285 colon mucosa and its renewal, we also evaluated the expression patterns of cell cycle proteins such as
286 cyclin E, a late G1 phase cyclin and CDK p21 inhibitors as indicator of cell proliferation activity in
287 colonic mucosa. ZDF-Co rats showed increases in the cytosolic and diffuse distribution of positive
288 nuclei expression of cyclin E, compared to ZL and ZDF-C groups. The high levels of cyclins E induced
289 by cocoa might be explained, at least in part, by a coordinate decrease in p21, reducing their association
290 with cyclins and finally aiding the progression of cell cycle (Supplementary Figure 1). Likewise, levels
291 of the mucin glycoprotein, a crucial component of the mucus layer expressed in goblet cells, were also
292 slightly but significantly increased in the diabetic animals fed with cocoa (Figure 2D). In addition,
293 control diabetic animals showed a significant decrease in the levels of one of the main tight junction
294 proteins Zonula occludens-1 (ZO-1) in comparison with control lean group; interestingly, diabetic
295 animals fed with cocoa not only prevented the ZO-1 decrease induced by diabetes but also showed ZO-1
296 levels significantly higher than those of the lean group (Figure 2E). Finally, the expression of pro-
297 inflammatory cytokines (TNF α , IL-6, and MCP-1) and the levels of CD45 (marker of immune cell
298 infiltration) were significantly increased in the colonic mucosa of diabetic rats and they were partly
299 prevented in those fed with cocoa (Figure 3A-D). The circulating levels of the cytokines IL-6 and TNF-
300 α in serum were not significantly different among ZL, ZDF-C and ZDF-Co rats (Figure 3E). Overall,
301 these results indicate that cocoa diet to a large degree greatly maintains the intestinal integrity and
302 generally reduced intestinal inflammation in diabetic rats.

303

304 3.3. Bacterial diversity and taxa composition

305 To analyse the effect of diabetes on gut microbial composition and the influence of a cocoa rich
306 diet, we performed a metagenomic DNA sequencing of bacterial 16S rRNA gene regions V3-V4 of
307 faecal samples. The total number of species identified was higher in ZDF-C rats when compared with
308 ZL or ZDF-Co rats (Figure 4A). Likewise, Shannon, Simpsons and Chao indexes representing the
309 richness and evenness of species diversity within each sample (α -diversity), were markedly augmented
310 in control diabetic rats (Figures 4B-D). We also performed a bi-dimensional PCA of the bacterial
311 community, which measures microorganism diversity between samples (β -diversity) in an
312 unsupervised manner. The analysis revealed distinct clustering in each group (Figure 4E).

313 Microbiota in diabetic animals (ZDF-C) were characterized by a significant increase in the
314 relative abundance of *Proteobacteria* (3.6 fold increase), *Tenericutes* (2.8 fold increase) and
315 *Actinobacteria* (2.6 fold increase) phyla and a reduction of *Verrucomicrobia* phylum (by 76.9%) when
316 compared with ZL non-diabetic animals (Figure 4F). Notably, with the exception of *Verrucomicrobia*
317 phylum, the microbial changes induced by diabetes were totally prevented in animals that were fed
318 with cocoa. In addition, cocoa significantly increased the relative percentages of *Firmicutes* (1.4 fold
319 increase) and *Deferribacteres* phyla (9.3 fold increase) and decreased the relative abundance of
320 *Cyanobacteries* phylum (by 74.9%) when compared to the ZDF group. Overall, colonic microbiota was
321 dominated by the *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Verrumicrobia* phylum which
322 accounted for over 96.8%, 96.7% and 95.8% of total bacteria in ZL, ZDF-C and ZDF-Co groups,
323 respectively. Interestingly, the most important and recognized biomarker of dysbiosis, the
324 *Firmicutes/Bacteroidetes* (F/B) ratio, was significantly increased in both ZDF-C and ZDF-Co rats
325 (Figure 4G).

326 At the family level, twenty families were identified with a relative abundance greater than 1%
327 (Figure 5A). Eleven of these families significantly modified their relative abundance with diabetes or
328 cocoa (Figure 5B). In particular, in ZDF-C diabetic animals, the relative abundance of
329 *Enterobacteriaceae* (a family of the *Proteobacteria* phylum) and of *Enterococcaceae* and
330 *Lactobacillaceae* (families of the *Firmicutes* phylum) increased whereas the abundance of
331 *Ruminococcaceae* (of the *Firmicutes* phylum) and *Verrucomicrobiaceae* (of the *Verrucomicrobia*
332 phylum) decreased. Interestingly, except for the *Verrucomicrobiaceae* family, cocoa diet significantly
333 prevented all these microbial changes induced by diabetes. Moreover, cocoa supplementation increased
334 the abundance of *Flavobacteraceae*, *Prevotellaceae* and *Sphingobacteriaceae* (families from
335 *Bacteroidetes* phylum) and *Lachnospiraceae* (*Firmicutes* phylum) in diabetic animals.

336 Figure 6 shows the changes found at genus and species level. Twenty one genera were
337 identified with abundance greater than one per cent and eleven of these were significantly modified by
338 either diabetes or the cocoa diet. The major differences found in diabetic ZDF-C animals were
339 observed in the genera *Escherichia* (mainly *E. alberti* species), *Tepidibacter*, *Lactobacillus* (with *L.*
340 *antri*, *L. hayakitensis* and *L. johmsonii* as the most prevalent species) and *Enterococcus* (mainly *E.*
341 *lactis* specie) that where significantly higher compared with non-diabetic ZL. Conversely,
342 *Faecalibacterium* and *Oscillospira* genera were significantly lower in the ZDF-C group. Once again,
343 cocoa diet was able to prevent all these intestinal microbiota changes in diabetic animals. However,
344 both ZDF-C and ZDF-Co showed significantly decreased levels of *Akkermansia* genus when compared
345 with ZL group, a finding that was also noticeable at the species levels (*A. Muciniphila*). Finally, cocoa
346 treatment induced *Blautia* (mainly *B. hansenii* and *B. wexleare* species) and *Flavobacterium* and
347 reduced *Parabacteroides* (mainly *P. goldsteinii* and *P. distasonis*) and *Sutterella* genera in diabetic
348 animals.

349

350 **3.4. SCFA- and lactate-producing bacteria and levels of lactate and SCFA in faeces**

351 Next, we analysed the changes in the relative abundance of lactate- and SCFA-producing
352 bacteria (Figures 7A and 7C-F) as well as the lactate and SCFA levels in faeces (Figure 7B). The
353 relative abundance of butyrate-producing bacteria was essentially unchanged in all groups (Figure 7A).
354 However, we found that cocoa feeding significantly increased the relative abundance of acetate-
355 producing bacteria (mainly due to an induction in *Blautia* genus) (Figure 7C) that was accompanied by
356 higher levels of acetate in faeces (Figure 7A). On the other hand, diabetic animals showed a significant
357 increase in the amount of lactate-producing bacteria that was driven primarily by changes in
358 *Enterococcus* and *Lactobacillus* genera (Figure 7E) and a significant reduction in the relative amount
359 of propionate-producing bacteria that was reproduced for *Akkermansia* genus (Figure 7F). Accordingly,
360 the levels of lactate were significantly higher in the faeces of diabetic animals while the levels of
361 propionate were significantly lower. Notably, cocoa treatment partly prevented the increase in lactate-
362 producing bacteria and as a result the levels of lactate and propionate were over than those found in
363 diabetic animals.

364

365 **3.5. Correlation of gut microbiota and disease biomarkers**

366 Finally, to further explore the relationship between the significantly altered genera in the
367 microbiota of diabetic animals and clinical parameters related to glucose and lipid metabolism as well
368 as intestinal integrity and inflammation, we performed a Person correlation analysis (Figure 8). A
369 strong positive association was found between the relative abundance of *Enterococcus*, *Escherichia*,
370 *Lactobacillus* and *Tepidibacter* genera and the increase in body weight and in several biomarkers of
371 glucose homeostasis and inflammation. However, the genera *Oscillospira* and *Akkermansia* showed

372 significantly negative correlations with all these parameters. Likewise, *Fecalibacterium* showed
373 significantly negative correlations with increase in body weight, insulinaemia, HbA1c, LDL, TG, IL-6,
374 CD45 and MCP-1. Finally, the presence of *Flavobacterium* was also negatively associated with,
375 glycaemia, TNF- α and CD45. On the other hand, increased ZO-1 levels were positively associated with
376 *Blautia* and *Flavobacterium* and negatively linked with *Enterococcus*, *Escherichia*, *Parabacteroides*,
377 *Sutterella* and *Tepidibacter*.

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381 **4. Discussion**

382 In the present study, we show for the first time that cocoa intake modifies the intestinal bacteria
383 composition in ZDF diabetic animals towards a healthier microbial profile. Interestingly, some of the
384 gut microbiota modifications induced by cocoa are closely associated with improved glucose
385 homeostasis and gut health, suggesting that the beneficial effects of cocoa in diabetes could be
386 mediated, at least in part, by modulation of the microbiota.

387 Early reports have revealed the potential anti-diabetic properties of cocoa both *in vivo* and *in*
388 *vitro* (Martin, Goya, & Ramos, 2016). In agreement with this, here we show that cocoa
389 supplementation partially alleviated glucose and lipid control. More importantly, in this study we
390 found that dietary cocoa was able to improve the structure and the barrier integrity of the colon mucosa
391 in diabetic rats. The expression of the intestinal tight junction protein ZO-1 and the intestinal mucus
392 levels are extremely important in maintaining intestinal barrier function (Dhar & McAuley, 2019;
393 Andrade et al., 2015). Accordingly, in the present study, ZO-1 and mucin levels were significantly
394 reduced in the colon of control ZDF rats. However, diabetic animals fed on cocoa showed significantly
395 higher expression of mucin and ZO-1 than those of the lean group. These results suggest that cocoa diet
396 may improve the barrier function and integrity of diabetic animals through enhancing the protein
397 expressions of mucin and ZO-1. Supporting this it has been shown that the intake of *Salvia*
398 *miltiorrhiza*, a natural source of phenolic acids, ameliorates the damaged barrier function of diabetic
399 mice through enhancing the expressions of tight junction proteins decreased by streptozotocin (STZ)
400 (Gu et al., 2017). Likewise, it has been proved that long term intake of anthocyanins promoted
401 intestinal integrity in healthy mouse (Peng et al, 2019). Notably, the tight junction of intestinal mucosal
402 cells prevents excessive entrance of endotoxins and other noxious agents into the circulation system
403 and therefore attenuates the activation of local and systemic inflammatory responses (Balakumar et al.,

404 2018). Accordingly, diabetic animals showed a significantly increase in inflammatory cytokines such
405 as IL-6, TNF α and MCP-1 as well as in the levels of CD45 (marker of immune cell infiltration) in the
406 colonic mucosa that were significantly reduced by cocoa intake. Although the levels of LPS could not
407 be assayed in the plasma of these animals, it could be highlighted that no significant differences in the
408 levels of IL-6 and TNF α were found in plasma, which is consistent with previous data in ZDF rats at
409 this age and fed on standard diet (Morales-Cano et al., 2019). Therefore, it could be suggested that the
410 altered intestinal barrier function we described in ZDF rats is not sufficiently damaged to cause an
411 overt metabolic endotoxemia. Overall, here we show that cocoa intake has the potential to improve gut
412 barrier integrity in diabetic animals and to reduce colon inflammation emerging as an additional tool to
413 ameliorate diabetes. Although the precise molecular mechanism behind this protective effect is still
414 unclear, we hypothesize that it could be related in part to the potential changes induced by cocoa in
415 intestinal bacteria of diabetic animals due to its prebiotic activity (Singh et al., 2019).

416 Metabolic disorders such as diabetes have been associated with altered microbiota composition
417 (Li, Watanabe & Kimura, 2017). In this study, the composition of the GM in diabetic rats (ZDF-C) was
418 significantly different compared with lean animals (ZL). At the phylum level, the main changes
419 observed in diabetic animals were an increase in *Proteobacteria* and a decrease in *Verrucomicrobia*
420 phyla whereas no significant differences were found in the relative abundance of *Firmicutes* and
421 *Bacteroidetes*. Interestingly, microbiota in diabetic animals treated with cocoa differed between the
422 diabetic control and the lean groups, suggesting that cocoa may have specific effects on the microbial
423 community of diabetic animals. The reduction in *Proteobacteria* abundance in the ZDF-Co group can
424 be attributed to the potential of cocoa polyphenols to modulate intestinal microbiota, These results are
425 in concordance with those reported by Araujo et al. (Araujo et al., 2019), who observed a decrease in
426 the abundance of the phyla *Proteobacteria* in the faeces of obese rats fed with an ethanolic extract of

427 bacupari rich in phenol derivatives.. However, cocoa supplementation failed to correct the increase in
428 the *Firmicutes/Bacteroidetes* (F/B) ratio that is characteristic of murine genetic obese models
429 (Vallianou et al., 2019). It has been recently suggested that the regulation of microbiota by polyphenols
430 could be independent of the decrease in the F/B ratio (Yang et al., 2019). More importantly, a
431 systematic review has revealed that this relationship between F/B ratio and obesity is not always
432 consistent, suggesting changes in specific microbiota as main responsible for metabolic outcomes (Sze,
433 & Schloss, 2016). According to that, we found that cocoa intake modified some key bacterial groups
434 which may be related to the beneficial improvement on the glucose homeostasis and gut health induced
435 in diabetic animals.

436 The relative abundance of *Proteobacteria* in diabetic ZDF-C animals was partly ascribed to a
437 significant increase in bacteria that belong to *Escherichia* genus (*Enterobacteriaceae* family) that are
438 widely known to cause intestinal pathologies in humans and animals (Shin, Whon, & Bae, 2016; Allen-
439 Vercoe & Jobin, 2014). Likewise, although the absolute abundance of the *Firmicutes* phylum was
440 unchanged in diabetic rats, certain genera were significantly modified when compared with the control
441 lean group. Particularly, *Enterococcus* and *Lactobacillus* genera were increased in diabetic animals
442 whereas *Oscillospora* genus was decreased. *Enterococcus* is a bacterial group which includes potential
443 pathogens that have been associated with gut dysfunction and inflammatory diseases (Lo Presti et al.,
444 2019). In contrast, *Lactobacillus* is classically considered a beneficial group of bacteria for their
445 favourable effects on host metabolism; however, recent studies have indicated that the increase of this
446 genus might be related to obesity and inflammatory conditions (Zeng et al., 2013; Ge et al., 2018),
447 which is in concordance with our findings in diabetic animals. Similarly, *Oscillospira* genus is
448 associated with anti-inflammatory effects in the gut and it has been reported to be less abundant in T2D
449 and obese patients (Del Chieirico et al., 2017; Liu et al., 2018). Interestingly, cocoa diet strongly

450 prevented all these harmful changes observed in the gut microbiota of diabetic animals. These findings
451 are in agreement with previous studies showing that quercetin and resveratrol attenuate serum
452 inflammatory cytokines and improve glucose metabolism in high-fat diet-fed rats by modulating
453 bacterial species associated with diseases and inflammation (Zhao et al., 2017). In the same way,
454 modifications induced by cocoa intake on the GM of diabetic animals may play a key role in the
455 beneficial effects on glucose metabolism and gut health.

456 It is interesting to note that the abundance of *Akkermansia* genus (phylum *Verrucomicrobia*) is
457 low during obesity and diabetes (Vallianou et al., 2019). In addition, it has been demonstrated that
458 polyphenol treatment induces the expression of *A. muciniphila* which correlated with improved body
459 weight and glucose tolerance (Anhê et al., 2017). However, we found that cocoa supplementation failed
460 to restore the reduced abundance of *A. muciniphyla* in diabetic animals even though glucose
461 metabolism and gut health were significantly improved. Similar results have been reported in obese
462 mice treated with resveratrol (Sung et al., 2017) or with polyphenol-containing extracts from cinnamon
463 bark and grape pomace (Van Hul et al., 2018) showing improved glucose tolerance with reduced
464 abundance of *Akkermansia*. As suggested by the authors (Van Hul et al., 2018), it is possible that
465 polyphenols have varied prebiotic potential for *A. muciniphila*, In addition, several study design
466 differences (origin of polyphenols, type of diet, animal model, age or pathological status) may also
467 contribute to explain the divergence in results regarding the gut microbiota composition. Thus, further
468 studies are required to explore the role of this bacterial group in type 2 diabetes.

469 Microbiota modulate the production of SCFA (mainly acetate, butyrate and propionate) which
470 can modify the concentrations of several gut peptides involved in glucose metabolism, gut barrier
471 function and energy homeostasis (Parada et al., 2019). Accordingly, cocoa as a prebiotic food can
472 positively affect the growth of beneficial bacterial species (Singh et al., 2019). In particular, it has been

473 shown that a cocoa rich-diet promotes the growth of butyrogenic-type bacteria such as *Roseburia* in
474 pigs (Solano-Aguilar et al., 2018). However, we found that the levels of butyrate-producing bacteria
475 were essentially unchanged among all experimental groups while the abundance of acetate producing
476 bacteria was significantly increased in cocoa fed rats. In addition, cocoa feeding increased the relative
477 abundance of *Blautia*, a bacterial group that has been negatively correlated with obesity and T2D
478 (Rondanelli, et al., 2015; Inoue et al., 2017). This different prebiotic effect could be attributed to
479 significant modifications in the gut environment during the diabetic milieu which lead to the dissimilar
480 composition and distribution of the intestinal microbiota in healthy compared to diabetic animals.

481 Acetate can improve gut barrier function either by stimulating goblet cell differentiation or by
482 the reinforcement tight junctions of epithelial cells (Morrison & Preston, 2016). In this regard, it has
483 been recently shown, in an animal model of intestinal inflammation that the supplementation with
484 polyphenols from grape peel significantly increased the production of SCFA (mainly acetate and
485 butyrate) and the colonic protein levels of ZO-1 (Maurer et al., 2019). Similarly, in this study, the
486 relative abundance of *Blautia* was directly associated with increased levels of ZO-1 and mucin.
487 Moreover, cocoa diet also decreased the abundance of lactate producing bacteria (mainly *Lactobacillus*
488 and *Enterococcus*) and thus the level of lactate (precursor of SCFAs) in faeces. Interestingly,
489 correlation analysis showed that both genera were positively associated with body weight and
490 biochemical parameters related to glucose homeostasis and intestinal integrity and inflammation.
491 Increased lactate levels have been observed in both humans and animal models of T2D and obesity and
492 have been also associated with inflammation (Wu et al., 2016; Nishitsuji et al, 2017). However,
493 microbial produced lactate is generally converted into propionate or butyrate by a subset of lactate-
494 utilizing bacteria and it is unclear whether bacterial derived lactate contributes to the high levels of
495 plasma lactate in diabetics. Additionally, it should be taken into account that gut microbiota produces

496 many other classes of metabolites such as SCFAs, bile acids and amino acid derivatives that may also
497 have essential signaling functions (Van Treuren & Dodd, 2019). Altogether, here we show that cocoa
498 could modulate GM and SCFAs production, contributing to the recovery of colon barrier function,
499 attenuating inflammation and eventually improving glucose metabolism. Therefore, these results
500 suggest that the modulation of GM might be one of the mechanisms involved in the antidiabetic effects
501 of cocoa. Although we do not know whether the changes observed in fecal SCFAs concentration in
502 both ZDF and ZDF-Co reflect similar variations in circulating SCFAs, it is reasonable to hypothesize
503 that potential changes in systemic SCFAs levels could also contribute to the observed phenotypes of
504 the animals.

505 **One limitation of this study is the different levels of starch and fibre between control and cocoa**
506 **diet. However, it has been shown that** the intake of polyphenols improve the health effects of the
507 intestinal microbiota by activating SCFA excretion, intestinal immune function, and other
508 physiological processes (Kawabata et al., 2019). Therefore, **after cocoa intake, the increase in SCFAs is**
509 **not only due to its fibre content but also to other bioactive compounds mainly polyphenols and**
510 **theobromine (Martín-Peláez et al., 2017).** Further studies will clarify if the effect of cocoa on intestinal
511 microbial populations can be ascribed to cocoa polyphenols and/or theobromine, to dietary fibre or to a
512 possible synergistic activity of all of these dietary components.

513 In summary, the present study demonstrates for the first time that cocoa supplementation
514 improves intestinal integrity and inflammation in ZDF diabetic rats. Moreover, cocoa intake modifies
515 gut microbiome in ZDF diabetic rats towards a healthier profile and these changes have been closely
516 associated with the improved glucose homeostasis and gut health found in the diabetic animals.
517 Consequently, we suggest that modulation of GM by cocoa may be an important mechanism that could
518 partly mediate beneficial metabolic effects in diabetic animals. Future studies using faecal

519 transplantation from cocoa fed donors could help to address whether or not the alterations in the gut
520 microbiota found in ZDF-Co rats play a pivotal role in mediating the beneficial metabolic effects of
521 cocoa. Likewise, the application of metabolomics to microbiota could provide a more complex analysis
522 to finally advance in the knowledge of the ultimate causality.

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536

537 **Conflict of interest**

538 The authors declare that there are no conflicts of interest.

539

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715 **Figure captions**

716

717 **Figure 1.- Effect of cocoa diet in glucose and lipid homeostasis in Zucker lean (ZL), Zucker**
718 **Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co).** (A)
719 Food intake. (B) Body weight. (C) Plasma glucose levels. (D) Plasma insulin levels. (E) Glycosylated
720 haemoglobin (HbA1c). (F) Homeostasis model assessment (HOMA)-IR and HOMA-B. (G) Plasma
721 glucose levels during GTT and total area under the curve calculated from the GTT data. (H) Levels of
722 HDL, LDL and TG in serum. Data represent the means \pm SD of 6-8 animals. Means sharing the same
723 letter are not significantly different from each other ($P < 0.05$)

724

725 **Figure 2.- Effects of cocoa diet on colon mucosa of Zucker lean (ZL), Zucker Diabetic rats fed**
726 **control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co).** (A) Representative
727 haematoxylin–eosin (H&E) stained sections and crypts depth measured as cells number per hemicrypt
728 of the distal colon mucosa (scale bar 10 μm). (B) Colonic epithelial apoptosis as revealed by terminal
729 deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (brown-positive nuclei) (scale
730 bar 10 μm) and quantification of apoptotic cells by TUNEL labelling index (%). (C) Representative
731 photographs for immunohistochemical staining of proliferating cell nuclear antigen (PCNA) (brown-
732 positive nuclei) (scale bar 10 μm) and PCNA labelling index (%) in colonic mucosa. (D)
733 Representative images of mucine glycoprotein by PAS staining (magenta) and quantitative analysis of
734 positive PAS staining cells (%) (scale bar 10 μm). (E) Representative IHC photographs of ZO-1 protein
735 expression and the positive area staining (%) (brown-stained) (scale bar 10 μm). Means sharing the
736 same letter are not significantly different from each other ($P < 0.05$)

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739 **Figure 3.- Effects of cocoa diet on intestinal inflammation of Zucker lean (ZL), Zucker Diabetic**
740 **rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co).** Representative
741 immunohistochemistry photomicrograph of TNF- α (A), IL-6 (B), MCP-1 (C) and CD45 (D) (brown-
742 stained) in distal colon and immunoreactive score (scale bar 10 μ m). Levels of TNF- α and IL-6 in
743 plasma (E). Values are expressed as mean \pm SD (n = 6-8). Means sharing the same letter are not
744 significantly different from each other (P<0.05)

745

746 **Figure 4.- Bacterial diversity and taxa composition in Zucker lean (ZL), Zucker Diabetic rats fed**
747 **control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co).** (A) Number of species
748 identified. (B) Shannon, (C) Simpsons and (D) Chao indexes were measured to evaluate the α -
749 diversity. (E) Unsupervised PCA were carried out to analyse the β -diversity. Each principal component
750 describes most of the variation between samples. (F) Composition of the most abundant bacterial phyla
751 (>0.1%) expressed as a percent of total bacteria and pie graphs of most abundant phyla. (G) *Firmicutes*
752 to *Bacteroidetes* ratio (F/B). Data represent means \pm SD of 6–8 animals per condition. Means sharing
753 the same letter are not significantly different from each other (P<0.05)

754

755 **Figure 5.- Bacterial families composition in Zucker lean (ZL), Zucker Diabetic rats fed control**
756 **diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co).** (A) Distribution bar-plot of
757 families with relative abundance greater than 1%. (B) Composition of the most abundant families
758 modified with diabetes or with cocoa expressed as a percent of total bacteria. Data represent means \pm
759 SD of 6–8 animals per condition. Means sharing the same letter are not significantly different from
760 each other (P<0.05)

761

762 **Figure 6.- Genera and species composition in Zucker lean (ZL), Zucker Diabetic rats fed control**
763 **diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet (ZDF-Co).** (A) Distribution bar-plot of genera
764 with relative abundance greater than 1%. (B) Composition of the most abundant bacterial genera
765 modified with diabetes or with cocoa expressed as a percent of total bacteria. (B) Composition of the
766 most abundant bacterial species modified with diabetes or with cocoa expressed as a percent of total
767 bacteria. Data represent means \pm SD of 6–8 animals per condition. Means sharing the same letter are
768 not significantly different from each other ($P < 0.05$)

769

770 **Figure 7.- SCFA- and lactate-producing bacteria and SCFA and lactate faeces levels in Zucker**
771 **lean (ZL), Zucker Diabetic rats fed control diet (ZDF-C) and Zucker Diabetic rats fed cocoa diet**
772 **(ZDF-Co).** (A) Sum of all SCFA- and lactate-producing genera expressed as a percent of total bacteria.
773 (B) Acetate, butyrate, propionate and lactate levels in faeces. (C–F) Most abundant acetate-, butyrate-,
774 propionate- and lactate-producing genera expressed as a percent of total bacteria. Data represent means
775 \pm SD of 6–8 animals per condition. Means sharing the same letter are not significantly different from
776 each other ($P < 0.05$)

777

778 **Figure 8.- Correlation analysis between gut microbiota and host parameters in diabetic rats.** (A)
779 Heatmap of correlation between the main significantly altered genera in the gut microbiota and host
780 parameters related to diabetes and intestinal integrity and inflammation. Pearson correlation values
781 were used for the matrix. The intensity of the colour represents the degree of association. *Denotes
782 adjusted $P < 0.05$.

783

784 **Table 1.** Composition of the experimental control and cocoa-rich diets.
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 786

Component (g/Kg dry weight)	Control	Cocoa
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
t-BHQ (<i>tert</i> -butylhydroquinone)	0.008	0.008
Mineral mix.	35	35
Vitamin mix.	10	10
L-Cys	1.8	1.8
Cholin bitartrate	2.5	2.5
Cellulose	100	66
Starch	415.7	349.7
Cocoa podwer	-	100
Energy (kJ/kg diet)	15048	15048

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