Effect of a cocoa-enriched diet on immune response and anaphylaxis in a food allergy model in Brown Norway rats

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Abbreviations: AB: Applied Biosystems; AR: anaphylactic response; AU: arbitrary units; AUC: area under the curve; βLG: β-lactoglobulin; BN: Brown Norway; CC: conventional cocoa; FA: food allergy; FA-CC: food allergy group fed with a conventional cocoa diet; FA-NFC: food allergy group fed with a non-fermented cocoa diet; FA-RF: food allergy group with a reference diet; FcεRI: high-affinity IgE receptor; GLP-1: glucagon-like peptide 1; H-RF: healthy group fed with a reference diet; i.p.: intraperitoneal; MLN: mesenteric lymph nodes; NFC: non-fermented cocoa: OVA: ovalbumin; RMCP-II: rat mast cell protease II; RF: reference; tBp: toxin from *Bordetella pertussis*; TGF-β: transforming growth factor-β; Th: T helper

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Abstract

Previous studies have demonstrated that cocoa intake decreased Th2 immune-related antibodies in rats. In consequence, we aimed to study in depth this cocoa action, particularly assessing its effect on a rat model of food allergy (FA) and also on an anaphylactic response. The involvement of the intestinal immune system was analyzed to allow the action mechanisms to be investigated. The role of cocoa flavonoids in the anti-allergic properties of cocoa was also established.

Brown Norway rats were fed either a reference diet or diets containing conventional cocoa (CC) or non-fermented cocoa (NFC). FA to ovalbumin (OVA) was induced and, later, an anaphylactic response was provoked.

As expected, the synthesis of anti-OVA IgE and other Th2-related antibodies was inhibited by CC diet. In addition, the release of mast cell protease II after anaphylaxis was partially prevented by CC, although other variables were not modified. The CC diet also attenuated the increase of some Th2-related cytokines released from mesenteric lymph node and spleen cells, and modulated the intestinal gene expression of molecules involved in allergic response. These results demonstrated the local and systemic influence of CC diet. The effects of the NFC diet were weaker than those of CC, suggesting that cocoa components other than flavonoids play a role in cocoa's action.

In conclusion, by acting on intestinal and systemic immune functions, a cocoa-enriched diet in rats exhibited a protective effect against FA and partially against anaphylaxis, making this a food of high interest to the fields of health and immunonutrition.

Keywords: anaphylaxis, cocoa, cytokines, flavonoids, IgE, mast cell protease

55 ## Highlights

56 - In a rat model of food allergy, we demonstrated here that a diet with conventional
57 cocoa prevented completely the synthesis of specific IgE, which play a key role in
58 allergy, and was able to modulate specific IgG antibodies associated with Th2-
59 immune response.

60 - Cocoa intake showed a protective role against the increase of Th2-cytokines, such as
61 IL-5 and IL-13, released from mesenteric lymph node cells, and also prevented the
62 increase of IL-4 in spleen cells, a result that could explain the high inhibition of IgE
63 synthesis by the this diet.

64 - After anaphylaxis induction, the release of mast cell protease II was greatly prevented
65 by the cocoa diet. In addition, this diet down-regulated intestinal and mast cell
66 protease and IgE receptor gene expression.

67 - Anaphylactic response assessed by body temperature, hematocrit, intestinal
68 permeability and motor activity was not prevented in cocoa fed animals, with the
69 exception of a faster recuperation of hematocrit.

70 - Results obtained with a diet with purer cocoa flavonoids allow the suggestion that
71 flavonoids were only partially responsible for the reported effects, other compounds in
72 cocoa must enhance its anti-allergy effect.

73

74 # 1. Introduction

75 Cocoa has a relatively high content of antioxidant flavonoids, mainly flavanols such as epicatechin,
76 catechin and procyanidins [1]. Its immunomodulatory effects in healthy rats have been demonstrated
77 by its modification of the composition and functionality of spleen B and T lymphocytes [2], its
78 promotion of T cell maturation [3] and its ability to decrease the intestinal IgM and IgA secretion [4].
79 Moreover, in ovalbumin-(OVA) immunized rats, the intake of a cocoa-enriched diet decreases serum
80 specific IgG1, IgG2a, IgG2c and IgM concentrations [5].

81 Allergy is an immune disease mainly mediated by IgE. After allergen intake and processing, specific T
82 lymphocytes differentiate and expand into T helper (Th) 2 cells characterized by producing cytokines
83 such as IL-4, IL-5, IL-10, and IL-13, which switch B cell antibody production against the allergen to
84 predominantly IgE [6]. IgE coats the surface of mast cells, binding to the high-affinity IgE receptors
85 (FcεRI), producing their sensitization. Later exposure to the same allergen triggers the mast cell
86 releasing of mediators such as histamine, proteases and cytokines, which result in allergic symptoms
87 involving the skin, respiratory and gastrointestinal systems or even the nervous and cardiovascular
88 systems [7]. Several studies suggest the preventive role of flavonoids in allergic reactions. Thus, in a
89 respiratory allergy model in rodent, chrysin, baicalin or quercitin can suppress the airway hyper-
90 responsiveness, decrease the inflammatory cells in the bronchoalveolar lavage fluid and mucus
91 production, as well as decrease the total or specific IgE synthesis [8–10]. In mice with allergic rhinitis,
92 the administration of KOB03, mainly containing baicalin, improves the rhinitis symptoms and inhibits
93 the mast cell activation, the release of allergic mediators and the production of inflammatory cytokines
94 [11]. In addition, we have previously reported the inhibitory effect of a cocoa-enriched diet on specific
95 IgE and other Th2-related antibodies in intraperitoneally (i.p.) sensitized allergic Brown Norway rats
96 [12]. In humans, a clinical trial in persistent allergic rhinitis patients demonstrates the ability of apple
97 polyphenols to ameliorate clinical symptoms [13]. Since these effects are mainly reported in
98 respiratory allergy, it is important to establish the influence of flavonoid intake on food allergy (FA),
99 which, with its increasing prevalence, is a major public health problem in developed countries. Food
100 allergy involves the intestinal immune system and the loss of oral tolerance, and can lead to an
101 anaphylactic response [14]. In this regard, the prevention of the development of FA in mice through
102 the intake of apple polyphenol has been described, providing protection against a decrease in body
103 temperature, inhibiting histamine release and decreasing the specific IgE antibody levels [15–17].
104 Based on these antecedents, the present study aimed to study in depth the anti-allergic properties of
105 cocoa, in particular assessing its effect on a rat model of FA and also its repercussions on an
106 anaphylactic response. The intestinal immune system was also analyzed to allow the action
107 mechanisms to be investigated. Finally, the role of cocoa flavonoids on the anti-allergic actions of
108 cocoa was established.

109 # 2. Material and methods

110 *2.1 Diets*

111 Three types of diets were elaborated (Table 1): reference diet with no polyphenols (RF) and two
112 cocoa-enriched diets either including conventional cocoa (CC) or cocoa flavonoids from non-
113 fermented cocoa (NFC), both containing 0.4% of polyphenols. The CC diet was made up from Natural
114 Forastero cocoa containing 40.18 mg/g of polyphenols provided by Idilia Foods SL (formerly
115 Nutrexpa S.L., Barcelona, Spain) The NFC diet was elaborated with an ethanol extract of non-
116 fermented and non-roasted cocoa beans containing 510 mg/g of polyphenols (Naturex, Avignon,
117 France). The addition of 100 g/kg of conventional Natural Forastero cocoa and 8.7 g/kg of NFC was
118 established in order to obtain final isocaloric diets with 0.4% of polyphenols and the same proportion
119 of macronutrients as the reference diet. The diets were prepared from a basal mix diet and particular
120 components were supplied by Teklad Global Diets (Harlan, Indianapolis, IN, USA) and provided *ad*
121 *libitum.*

122 *2.2 Induction of food allergy and anaphylaxis*

123 Dams with 14-day-old Brown Norway (BN) rat litters (50% male, 50% female) were obtained from
124 Janvier (Saint-Berthevin, France). After a one-week acclimation period, the weaned rats were
125 randomized into four groups: healthy sham rats fed RF diet (H-RF), FA rats fed RF diet (FA-RF), FA
126 rats fed CC diet (FA-CC) and FA rats fed NFC diet (FA-NFC) (Fig. 1). The FA induction was
127 performed as previously described [18]. Briefly, rats received i.p.50 µg of ovalbumin (OVA, grade V,
128 Sigma-Aldrich, Madrid, Spain) as allergen, 2.5 mg of alum (Imject®; Pierce, IL, USA) and 50 ng of
129 toxin from *Bordetella pertussis* (tBp; Sigma-Aldrich) as adjuvants. Fourteen days later, animals daily
130 received 1 mL of OVA solution in mineral water (1 mg per rat) by oral gavage for one week. H-RF
131 group received 1 mL of vehicle. Every 2-3 days water intake and food consumption were registered,
132 body weight was measured and blood samples were collected weekly (Fig. 1).

133 Five days after finishing the oral OVA administration, the animals were fasted overnight and then
134 received 1 mL of 200 mg/mL of OVA orally in order to induce an anaphylactic response (AR). Blood
135 was collected at 0, 30, 60, 90, and 120 min from the saphenous vein to determine hematocrit and
136 RMCP-II concentration. In order to assess the intestinal barrier integrity [19], 30 min after the
137 challenge each rat received 1 mL of β-lactoglobulin (βLG, Sigma-Aldrich) (100 mg/rat) by oral
138 gavage. During AR, rectal temperature was measured using a digital thermometer (Acorn® Temp TC
139 Thermocouple Thermometer, Oakton, IL, USA). Three days after the AR induction rats were
140 sacrificed and biological samples were obtained (Fig. 1).

141 The experimental design was repeated three times in order to acquire representative results from a
142 sufficient number of animals per group (n=4 per group in each experiment, n=12 per group at the end

143 of the study). Experimental procedures were approved by the Ethical Committee for Animal
144 Experimentation at the University of Barcelona (ref.494/14).

145 *2.3 Motor activity measurement*

146 Motor activity was determined as previously described with slight modifications [20]. Briefly, two
147 motor activity measurements were performed to establish the basal movements and the changes
148 induced by AR: the first was measured 24 h before anaphylactic induction, and the second
149 immediately after the oral challenge. Motor activity movement counts were recorded using time
150 frames from 1 min to 21 min. To stimulate rat movements, 10 min after the beginning of the
151 measurement, the lights were turned off for 6 min and then turned on until the end of the
152 measurement. The results refer to the movements in three time phases: pre-darkness, darkness and
153 post-darkness, as well as the entire period. The area under the curve (AUC) for the 21 min period, and
154 the percentage of motor activity decrease after AR induction with respect to the basal measurement in
155 each studied phase and the whole period were calculated.

156 *2.4 Quantification of mast cell mediators and intestinal permeability during anaphylaxis*

157 In serum samples obtained during the AR, RMCP-II concentration was quantified using an ELISA set
158 (Moredun Animal Health, Edinburgh, UK) as previously described [20]. To assess intestinal
159 permeability, βLG was quantified in serum obtained during AR by ELISA as previously reported [18].

160 *2.5 Sample processing*

161 Three days after the AR induction, mesenteric lymph nodes (MLN) and spleen were dissected for
162 immediate lymphocyte isolation. From the middle of the small intestine, a piece (0.5 cm) was kept in
163 RNA later® (Ambion, Life Technologies, Austin, USA) until gene expression analysis and another
164 little piece was separated for histology. Gut washes were obtained from the distal half of the small
165 intestine for quantification of total and specific IgA [18].

166 *2.6 Determination of cytokines released from MLN and spleen cells*

167 Spleen and MLN cell suspensions were obtained and cultured (2.5×10^6/mL) with or without OVA
168 (10 μg/mL) as previously described [18]. After 96 h, supernatants were collected to assess
169 representative Th1 and Th2 cytokine concentrations using the Bio-Plex Pro™ Rat Cytokine Th1/Th2
170 Assay (Bio-Rad, Madrid, Spain) according to the manufacturer's instructions. Analysis was carried
171 out with the Bio-Plex® MAGPIX™ Multiplex Reader and the Bio-Plex Data Pro™ software (Bio-
172 Rad). The limits of quantification can be found in the Supporting Information.
173

174 *2.7 Quantification of metabolic hormones in serum*

175 At the end of the study, serum concentrations of ghrelin, glucagon, glucagon-like peptide (GLP)-1 and
176 leptin were determined using the Bio-Plex Pro™ Diabetes Assay (Bio-Rad) as detailed above. The
177 limits of quantification can be found in the Supporting Information.

178 *2.8 Quantification of gene expression in small intestine*

179 For RNA isolation, small intestine pieces were processed as previously described [18]. Two
180 micrograms of total RNA were converted to cDNA using random hexamers (Applied Biosystems
181 (AB), CA, USA). The specific PCR TaqMan® primers and probes (AB) used were: *Iga* (331943,
182 made to order), *Fcer1a* (Rn00562369_m1, inventoried (I)), *Mcpt2* (Rn00756479_g1, I), *Tgfb1*
183 (Rn00572010_m1, I), *Muc2* (Rn01498195_m1, I) and *Ocldn* (Rn00580064_m1, I). Quantification of
184 the genes of interest was normalized to the endogenous control *actb* (Rn00667869_m1, I). Real-time
185 PCR assays were performed in duplicate using an ABI Prism 7900HT sequence detection system
186 (AB).

187 The amount of target mRNA relative to *actb* expression and relative to H-RF animals was calculated
188 using the $2^{-\Delta\Delta Ct}$ method, as previously described [21]. Ct is the cycle number at which the fluorescence
189 signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR.

190 *2.9 Small intestine histological analysis*

191 Histological samples were processed as previously described [4]. Next, sections were stained with
192 hematoxylin-eosin and goblet cells were counted from each complete villus observed using light
193 microscopy.

194 *2.10 Anti-OVA antibody quantification*

195 Anti-OVA IgG1, IgG2a, IgG2b and IgA antibody concentrations were quantified using an indirect
196 ELISA, and OVA-specific IgE concentration by an antibody-capture ELISA as previously described
197 [18]. The relative concentration of each anti-OVA antibody isotype is expressed in arbitrary units
198 (AU), which were assigned using a positive pool of OVA-immunized rat serum as standard. The
199 AU/mL assigned were 100,000 AU/mL for IgG1 and IgG2a, 10,000 AU/mL for IgG2b, and 10
200 AU/mL for IgE. For intestinal anti-OVA IgA, since no positive samples were available, data are
201 expressed by means of OD values. Intestinal IgA antibodies were determined by sandwich ELISA as
202 previously described [22].

203 *2.11 Statistical analysis*

204 The software package IBM SPSS Statistics 20 (SPSS Inc., USA) was used. Levene's and
205 Kolmogorov-Smirnov tests were applied to assess variance equality and normal distribution,

206 respectively. Two-way ANOVA tests were used to study the effect of group and group x time
207 interaction. The motor activity data were analyzed by two-way ANOVA for repeated measures
208 considering the group and time as the interacting factors followed by Bonferroni's *post hoc* test.

209 To analyze the results from antibodies, metabolic hormones, RMCP-II, βLG and cytokine
210 concentrations, body weight, food consumption, water intake, decrease of movements, AUC from
211 motor activity measurement, hematocrit, body temperature, relative gene expression, and number of
212 goblet cells, non-parametric tests (Kruskal–Wallis and Mann–Whitney U) were used due to non-
213 variance homogeneity. Differences were considered statistically significant for p values <0.05.

214

215 # 3. Results

216 *3.1 Body weight, food and water intake and metabolic hormones*

217 At the end of the study, body weight from the FA-RF group was similar to that of the H-RF (Table 2).
218 However, in the FA-CC group but not in the FA-NFC group the body weight was lower ($p<0.05$).
219 Food and water intake was monitored throughout the study and no significant differences among
220 groups were found (Table 2).

221 The last day of the study, serum ghrelin concentrations showed no significant differences between
222 groups (Table 2). However, GLP-1 and leptin levels were significantly increased by the FA induction
223 ($p<0.05$). Both increases were prevented by the CC diet, and it also produced a decrease in the
224 glucagon levels ($p<0.05$). The NFC diet prevented only the GLP-1 increase.

225 *3.2 Serum specific anti-OVA antibodies*

226 Sera from the H-RF group did not contain anti-OVA antibodies of any isotype (Fig. 2). The i.p. OVA
227 administration induced the synthesis of anti-OVA IgG1, IgG2a, IgG2b and IgE antibodies that were
228 already detectable on day 7. The oral administration of the allergen on days 14-21, boosted the
229 antibody synthesis of IgG1 and IgG2a in the FA-RF group by about 30 times (Fig. 2A and 2B). These
230 increases were completely prevented by the CC diet. In animals from the FA-NFC group, anti-OVA
231 IgG1 and IgG2a antibodies only increased 8-9 fold at day 21 and were significantly lower than those
232 in the FA-RF group.

233 The FA induction produced lower specific IgG2b antibody production than IgG1 and IgG2a isotypes
234 and the oral administration of OVA on days 14-21 did not increase it (Fig. 2C). Nevertheless, at day
235 14, their levels were significantly higher in the FA-CC group than in the FA-RF rats.

236 Regarding specific IgE antibodies (Fig. 2D), the oral administration of OVA from day 14 to day 21
237 increased anti-OVA IgE fourfold in the FA-RF group. The CC diet prevented the synthesis of
238 anti-OVA IgE, whereas the NFC diet reduced these antibodies when oral administration was finished.

239 *3.3 Intestinal IgA and specific anti-OVA IgA antibodies*

240 At the end of the study, the levels of total intestinal IgA were similar between the H-RF and FA-RF
241 groups (Fig. 2E). In both groups fed with cocoa flavonoids, total IgA was significantly decreased, the
242 strongest effect being found in FA-CC animals. Intestinal anti-OVA IgA (Fig. 2F), significantly rose
243 in the FA-RF group and this increase was prevented by the CC diet but not by the NFC diet.

244 *3.4 Assessment of anaphylaxis*

245 Changes in motor activity, body temperature, hematocrit, serum RMCP-II and βLG concentrations
246 were determined immediately after oral challenge to assess anaphylaxis.

247 Basal values of motor activity showed that FA-CC animals had a lower number of movements in
248 comparison with the rest of the groups ($p<0.05$; Fig. 3A-B). After AR induction, all the FA animals
249 showed a significant decrease in the number of movements ($p<0.05$; Fig. 3C-D). To avoid the effect of
250 lower basal motor activity, the percentage of decrease between basal and post-AR induction was
251 calculated for the whole period and for each phase (Fig. 3E). There was a significant and similar
252 decrease of almost 70% in the motor activity of all the FA groups (in the pre-darkness and the whole
253 studied period).

254 Before the induction of anaphylaxis, the basal body temperature was similar in all groups and around
255 37.8 ± 0.04 ºC (mean ± S.E.M). During AR, it dropped in the three groups with FA ($p<0.05$) without
256 differences due to diet (Fig. 4A). Inversely, hematocrit increased in the three FA groups in comparison
257 with H-RF animals ($p<0.05$; Fig. 4B), but after 2 h of the oral challenge, both cocoa groups partially
258 recovered healthy values ($p<0.05$). In parallel, serum RMCP-II concentration (Fig. 4C) rose after the
259 oral challenge in the FA-RF group, and this increase was partially prevented in the FA-CC group.
260 Nevertheless, intestinal permeability augmented in all animals with FA, regardless of the diet
261 (Fig. 4D).

262 *3.5 Intestinal structure and small intestine gene expression*

263 Intestinal histology (Fig. 5A) in the animals of the FA-RF group did not reveal a clear inflammation
264 with the exception of certain crypt elongation. Both cocoa flavonoid diets induced slight villous
265 atrophy (Fig. 5A) and a lower number of goblet cells (Fig. 5B).

266 The relative gene expression of IgA, FcεRI, RMCP-II, TGF-β1, occludin and mucin 2 was quantified
267 in the small intestine (Fig. 6). In the FA-RF group, the FA induction produced a significant increase in
268 the IgA gene expression in comparison with the H-RF group ($p<0.05$), whereas both cocoa diets
269 prevented this increase ($p<0.05$); the FA-CC group even reduced the IgA mRNA levels with respect to
270 the H-RF animals ($p<0.05$). In addition, the FA-CC group, but not the FA-NFC group, also had lower
271 gene expression of FcεRI, RMCP-II and TGF-β1 in comparison with H-RF and FA-RF animals
272 ($p<0.05$). With regard to the mRNA levels of occludin and mucin 2, no significant changes were
273 observed due to FA or cocoa diets.

274 *3.6 Cytokine production by MLN and spleen cells*

275 MLN cells from the FA-RF group produced significantly higher levels of IL-4, IL-5 and IL-13 in
276 comparison with cells from the H-RF group ($p<0.05$) (Fig. 7A). Both cocoa diets prevented the
277 increase in IL-5 and IL-13 ($p<0.05$ *vs.* FA-RF group). In addition, the MLN cytokine pattern from

278 animals fed cocoa diets underwent several other changes. Cells from the FA-CC group decreased the
279 secretion of IL-1α, IL-1β and IFN-γ with respect to the H-RF and FA-RF group. The FA-NFC group
280 decreased IL-1α and GM-CSF secretion in comparison with the two RF groups ($p<0.05$).

281 Regarding the cytokine production from splenocytes (Fig. 7B), FA induction significantly increased
282 the release of IL-2, IL-4 and IL-13 in the FA-RF group ($p<0.05$ *vs.* H-RF group). However, cells from
283 FA-CC rats did not increase IL-2 and IL-4, and showed higher IL-6 production ($p<0.05$ *vs.* RF
284 groups). Splenocytes from the FA-NFC group showed a similar increase in IL-4 and IL-13 and higher
285 levels of IL-6, IL-10 and IFN-γ ($p<0.05$) than the FA-RF group.

286 Cytokines released from MLN and spleen cells in non-stimulated conditions were lower than those
287 observed after OVA-stimulation and did not differ between groups (data not shown).

288

4. Discussion

Previous studies concerning the effect of a cocoa diet on the immune system in rats reveal the ability of a cocoa diet to attenuate serum and intestinal immunoglobulin synthesis [2–5,23,24]. In addition, a diet containing 10% of cocoa in a rat allergy model induced by only an i.p. immunization demonstrates an important inhibition in Th2-specific antibodies [12]. These results prompted us to ascertain both the effect of this diet on a FA model and its repercussions on anaphylactic response [18]. In addition, the study of local (intestine and MLN) and systemic (spleen) lymphoid tissues allowed the action mechanisms to be investigated. Finally, in order to learn about the role of cocoa flavonoids, a diet containing more pure polyphenols obtained from unfermented cocoa was included.

In a rat model of FA, we demonstrated here that a diet with CC that started at the same time as allergy induction was also able to modulate those specific IgG antibodies associated with Th2-immune response (IgG1 and IgG2a), without affecting those related to Th1 cell activation (IgG2b). More interestingly, CC diet in FA rats prevented completely the synthesis of specific IgE that play a key role in allergy. Although some studies using polyphenol-enriched diets show a similar effect [25,26], our results with the NFC diet on specific antibodies allow the suggestion that flavonoids are only partially responsible for the attenuating action on Th2-antibody synthesis and other compounds in CC must enhance this anti-allergy effect. In agreement with this suggestion, a previous study in healthy Lewis rats shows that a diet containing 0.8% of polyphenols from NFC produced the same immunomodulatory effect as a diet containing 0.2% of polyphenols from CC [23]. Therefore, the cocoa content of fiber and methylxanthines, which was higher in CC than in NFC, could add to or enhance the action of polyphenols. In this sense, CC contains fiber such as cellulose, hemicelluloses and pectic substances [27], that are able to modify microbiota [28] and this could eventually affect to the food sensitization process as has been described [29].

On the other hand, FA induction caused an increase in intestinal anti-allergen IgA synthesis, as quantified by protein levels and also by IgA gene expression. Although IgA is considered the first specific line of defense in protecting the intestine, it has been shown that the induction of tolerance in FA mice is associated with an inhibition of specific intestinal IgA [30]. The CC diet reduced total intestinal IgA protein and gene expression in agreement with previous studies [4,22], thus avoiding the synthesis of the specific IgA. A comparison of these results with the NFC diet again suggests that other compounds and/or particular flavonoids in the CC, which are known to change during the fermentation process [31], must play a role in its attenuating effects on the gut immune system. With regard to the mechanisms involved in CC-induced intestinal IgA decrease, we studied TGF-β1 gene expression and found that CC, but not NFC, down-regulated this cytokine that promotes IgA synthesis [32,33].

323 In order to establish the action mechanisms of cocoa diets in FA, Th1 and Th2 cytokines were
324 assessed in OVA-stimulated MLN and spleen cells, as representative intestinal and systemic tissues.
325 Consistently, FA increased the release of Th2 cytokines such as IL-4, IL-5 and IL-13 from MLN cells.
326 Although IL-4 plays an essential role in the antibody class-switching to IgE synthesis [34,35], it was
327 not lowered in MLN from cocoa-fed animals, which agrees with previous studies on cocoa-fed allergic
328 rats [12]. Interestingly, both cocoa diets showed a protective role against the increase of other Th2-
329 cytokines such as IL-5 and IL-13, and the CC diet also decreased the release of the Th1-cytokines
330 IL-1α, IL-1β and IFN-γ from MLN cells. The modulatory activity of cocoa on these Th1 cytokines
331 could be beneficial in FA because IL-1 has been involved in the inflammatory process of allergic
332 diseases [36]. In spleen cells, FA also increased the release of IL-4 and IL-13. Although cocoa diets
333 were unable to avoid the increase in IL-13, the CC diet, but not the NFC diet, prevented the increase of
334 IL-4, a result that could explain the high inhibition of IgE synthesis by the CC diet. These results
335 partially agree with decreased splenic IL-4 production in immunized mice fed with chrysin and
336 apigenin [37] and suggest that particular flavonoids have different immunomodulatory properties. In
337 addition, it must be added that the NFC diet increased the production of IL-10 in both tissues and
338 IFN-γ in splenocytes, whereas it decreased the production of GM-CSF in MLN cells; on the other
339 hand, both cocoa diets increased the levels of IL-6 in spleen cells. Taken together, these results
340 evidence the complex modulatory effects of flavonoids and also other compounds present in cocoa on
341 immune function. Some cytokines that promote Th2 response, IL-5 and IL-13, were inhibited by both
342 cocoa diets in MLN, but importantly only CC was able to inhibit the IL-4 release from spleen cells,
343 which may shed some light on the IgE-regulatory role of this diet.

344 In this study we also focused on the intestinal changes induced by FA in the gene expression of
345 epithelial barrier proteins, such as mucin 2 and occludin. The FA, regardless of the cocoa diets, did not
346 modify the gene expression of either epithelial barrier proteins involved in luminal protection of the
347 intestine. This fact must be attributed to the method of FA induction, which, unlike other models [38],
348 does not use adjuvants such as cholera toxin. Mucin 2 is secreted by goblet cells [39] and, from the
349 histology sections, we counted less goblet cells after CC intake, although this was not confirmed by
350 PCR. More research should be focused on confirming these results and the mechanism involved.

351 The most severe response of FA is anaphylaxis after allergen intake, induced by the fast degranulation
352 of mast cells. In our FA model this can be quantified by high serum RMCP-II concentrations. We have
353 found that this increase in RF-FA animals was greatly prevented by the CC diet, whereas NFC intake
354 only showed a certain effect. This protective response by cocoa could be a consequence of lower
355 specific IgE levels and also to the down-regulation of FcεRI gene expression, both effects found here.
356 *In vitro* studies have demonstrated the inhibitory effects of flavonoids on FcεRI surface molecule or
357 gene expression [40,41]. Although mast cells were not determined histologically in the intestine, from

358 these results we can hypothesize that cocoa intake would reduce mast cell accumulation in the
359 intestine and/or down-regulate FcεRI, producing, in any case, a lower RMCP-II release after allergen
360 intake. As the NFC diet did not produce the same effects, compounds of cocoa other than flavonoids
361 must be responsible for, or enhance, such actions.

362 Anaphylactic response was also assessed by a drop in body temperature, and an increase in hematocrit
363 and intestinal permeability. Surprisingly, these alterations were not prevented by either the CC or the
364 NFC diet, with the exception of a faster recuperation of hematocrit. As body temperature fall is due to
365 a massive vasodilatation induced by mast cell mediators [42], the recognized vasodilator properties of
366 cocoa [43] could explain the lack of effect of cocoa intake on this variable. The anaphylactic response
367 was also assessed through the reduction in motor activity. In fact, rats fed with CC showed a lower
368 motor activity in basal conditions which merits further studies. Nevertheless, when the decrease
369 percentage of motor activity after the induction of anaphylaxis was quantified, CC rats showed a
370 similar pattern to that of FA and NFC groups. Therefore, the overall effects on anaphylactic response
371 suggest that, although CC diet reduced the production of anti-allergen IgE, RMCP-II synthesis and
372 down-regulated FcεRI and RMCP-II gene expression, these actions were not enough to prevent the
373 anaphylactic response after oral challenge with the allergen.

374 Finally, although food intake did not differ among groups, the CC diet produced a decrease in the
375 body weight without modifying food intake. The slower body weight increase was similar to that
376 reported in healthy rats [24,28], and this fact prompted us to determine some metabolic hormones. The
377 FA process and later induction of anaphylaxis caused increased levels of leptin and GLP-1, both
378 prevented by CC diet. The interaction between obesity, asthma and leptin has long been studied [44],
379 and some studies have reported the association of higher serum leptin levels with some allergic status
380 [45,46]. In agreeing with these studies we show here that FA can also increase serum leptin in rats
381 together with GLP-1, which has not been described in previous studies. The CC diet prevented these
382 increases and also produced lower glucagon levels, effects that deserve further studies in line with
383 those demonstrating the influence of cocoa diet on metabolism [47].

384 In conclusion, a cocoa-enriched diet suppresses the Th2 immune response, both in intestinal and
385 systemic compartments, as evidenced in Th2-related cytokines and antibodies, and is also able to
386 attenuate the release of mast cell mediators. These effects are stronger in CC than NFC, suggesting
387 that components other than flavonoids take part in this response after the induction of anaphylaxis.
388 Further studies to clarify which components present in cocoa are responsible for this action should be
389 developed. Although cocoa seems not to have a protective role in the face of anaphylaxis, the
390 inhibition of serum allergen-specific antibodies as well as the RMCP-II release supposes the
391 cornerstone of its protective properties, becoming a potential nutrient in the prevention of FA and
392 making this a food of high interest in the field of health and immunonutrition.

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396 their technical assistance.

397 ## Conflict of interests

398 None declared
399

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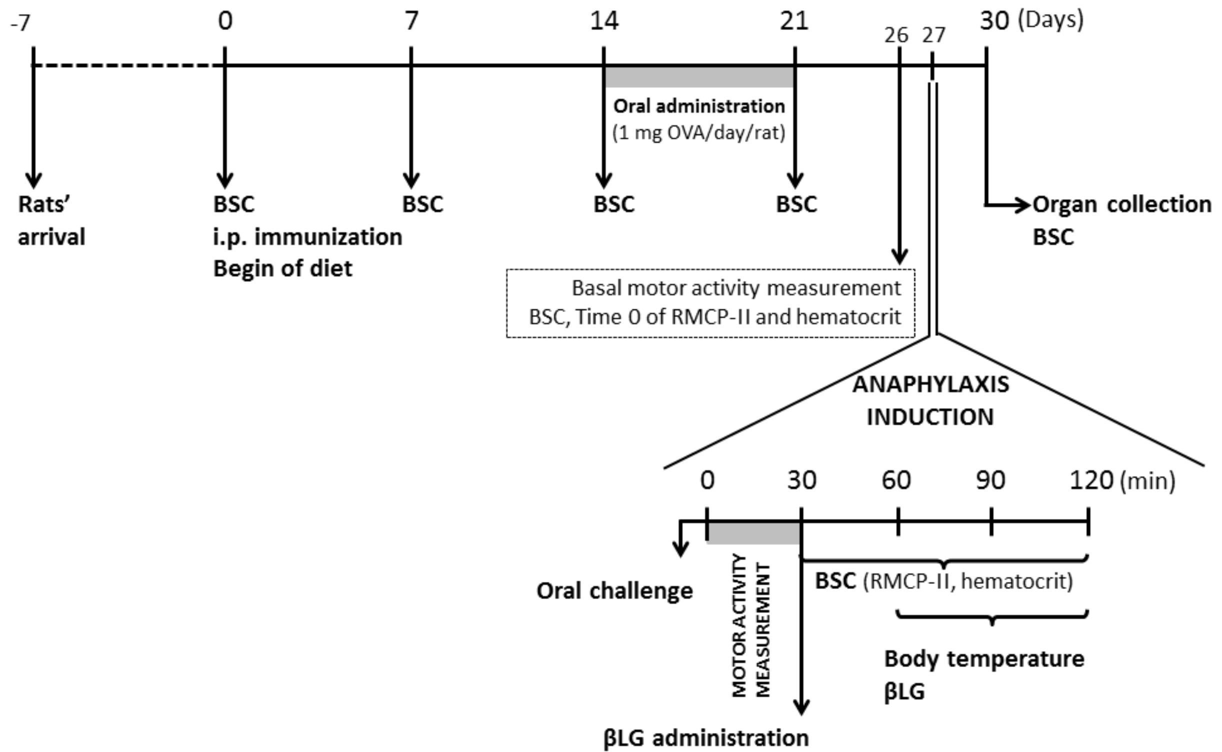
527 # Figures

528 **Figure 1.** Experimental design. Time-course of the experimental protocol including the points of

529 sample collection and the anaphylaxis induction. BSC: blood sample collection.



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531

532 **Figure 2.** Concentrations of serum anti-OVA antibodies during the study: A) IgG1, B) IgG2a,

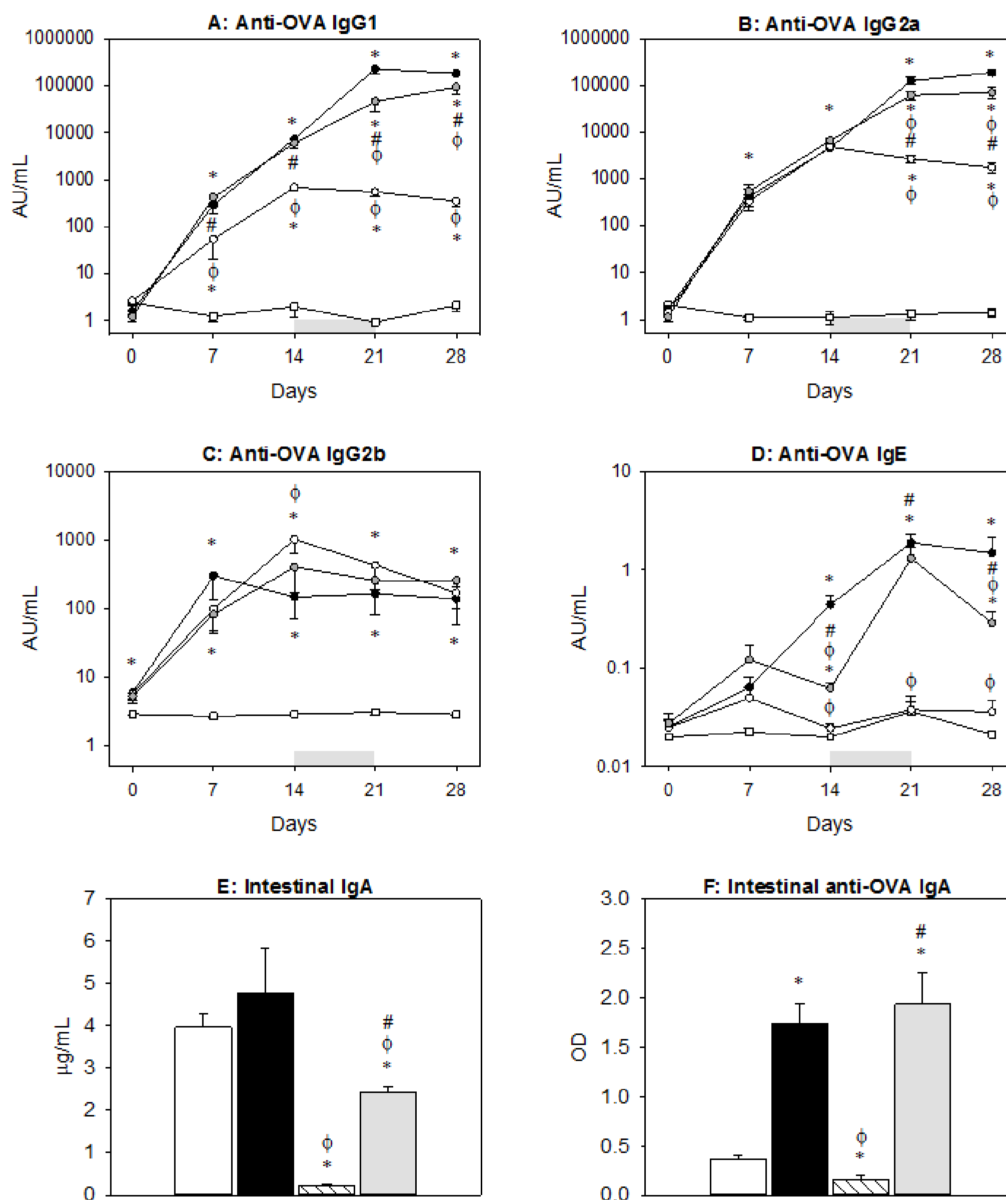
533 C) IgG2b, D) IgE, and E) intestinal IgA and F) intestinal anti-OVA IgA. White bars or □ represent

534 H-RF group, black bars or ● represent FA-RF group, white-striped bars or ○ represent FA-CC group

535 and grey bars or ○ represent FA-NFC group. Shadow period corresponds to oral administration of

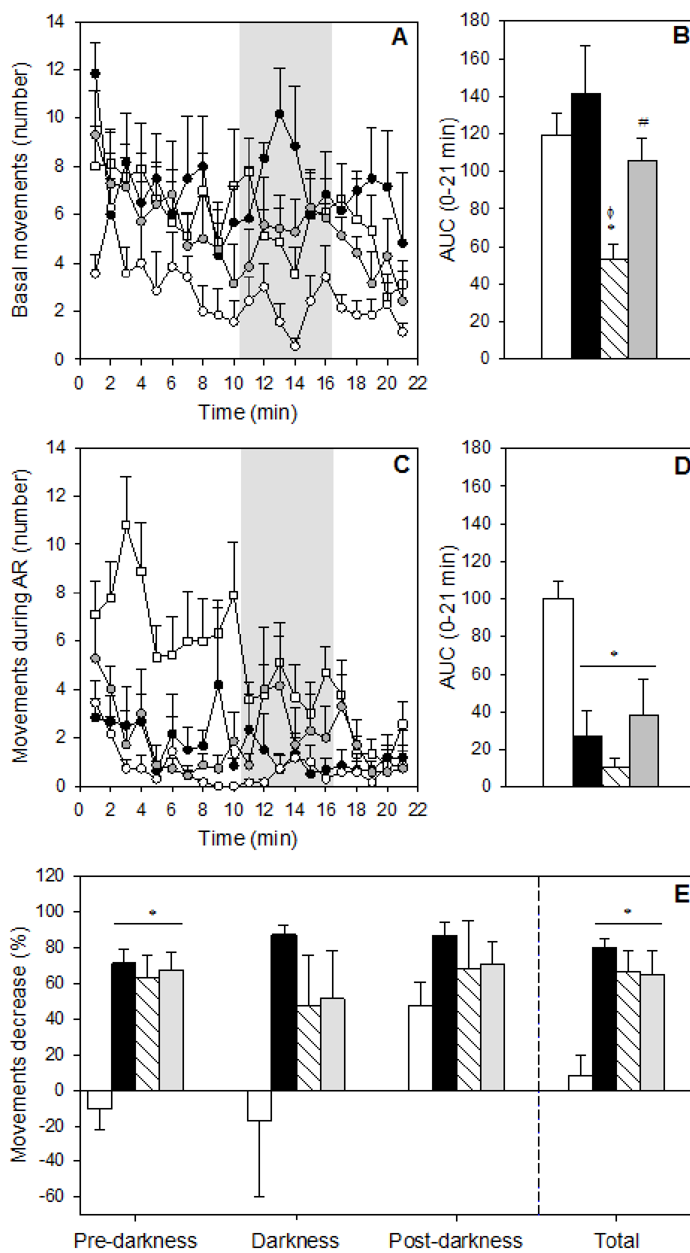
536 OVA in FA groups. Results are expressed as mean ± SEM (n = 12). *p<0.05 *vs*. H-RF group, and

537 $^{\phi}p$<0.05 *vs*. FA-RF group and $^{\#}p$<0.05 *vs*. FA-CC group.



538

539 **Figure 3.** Motor activity for 21-min period: A) basal motor activity and B) AUC assessed 24 h before
540 the AR induction; C) motor activity and D) AUC assessed immediately after AR induction; E)
541 percentage of motor activity decrease after AR induction referring to pre-darkness, darkness, post-
542 darkness and the whole period. White bars or □ represent H-RF group, black bars or ● represent FA-
543 RF group, white-striped bars or ○ represent FA-CC group and grey bars or ○ represent FA-NFC
544 group. In A and C, shadow period corresponds to darkness. Results are expressed as mean ± SEM (n =
545 12). *p<0.05 *vs*. H-RF group, and $^{\phi}p$<0.05 *vs*. FA-RF group and $^{\#}p$<0.05 *vs*. FA-CC group.

546 

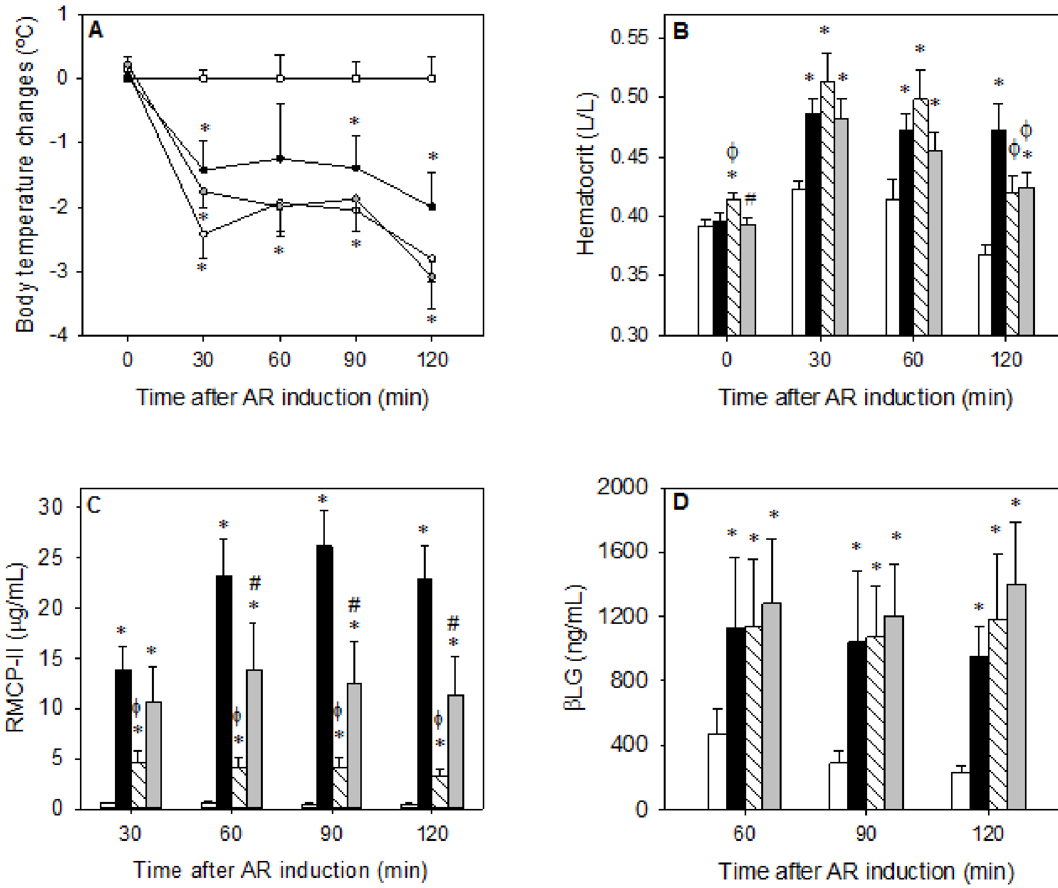
547 **Figure 4**. Variables measured during 2 h after anaphylaxis induction: A) changes in body temperature,

548 B) hematocrit, C) serum RMCP-II and D) βLG concentrations. White bars or □ represent H-RF

549 group, black bars or ● represent FA-RF group, white-striped bars or ○ represent FA-CC group and

550 grey bars or ○ represent FA-NFC group. Results are expressed as mean ± SEM (n = 12). *p<0.05 *vs.*

551 H-RF group, and $^{\phi}p$<0.05 *vs.* FA-RF group and $^{\#}p$<0.05 *vs.* FA-CC group.



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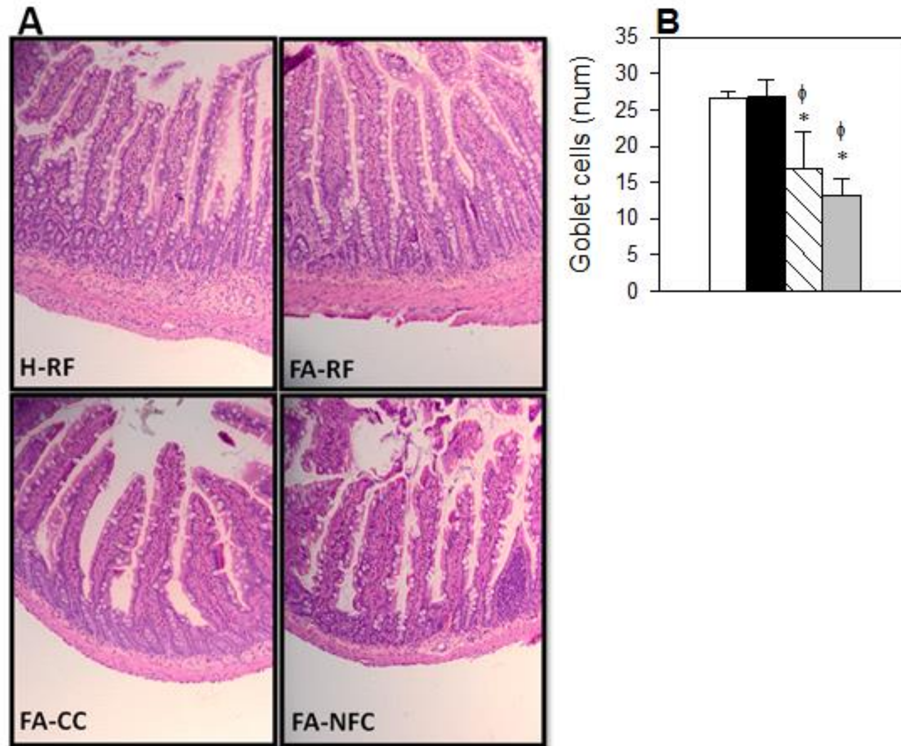
553

554 **Figure 5.** Intestinal structure. A) Representative intestinal sections from H-RF, FA-RF, FA-CC and
555 FA-NFC groups stained with hematoxilin-eosin (100x magnification) and B) number of goblet cells.
556 Results are expressed as mean ± SEM (n = 6). *p<0.05 *vs*. H-RF group, and $^{\phi}p$<0.05 *vs.* FA-RF group.



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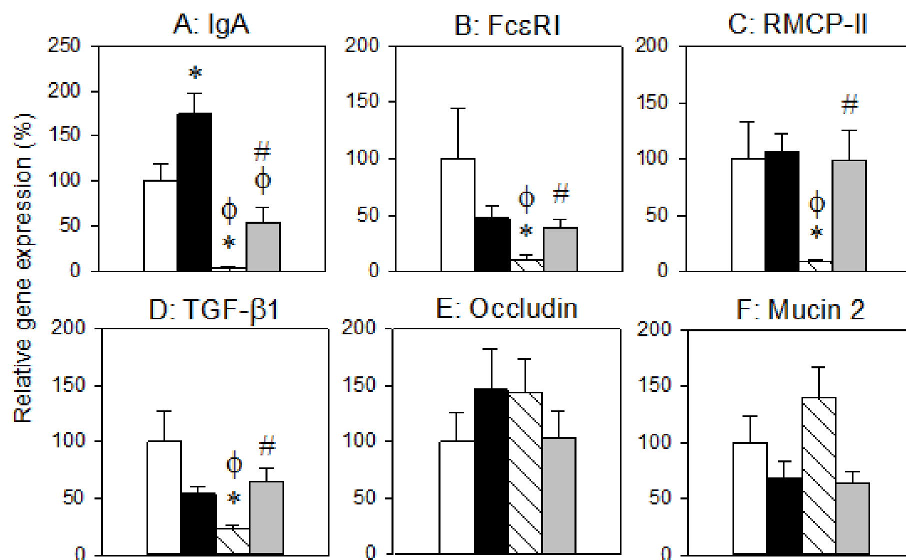
558

559 **Figure 6**. Relative gene expression of several molecules in small intestine. Expression levels were
560 normalized using *actb* as the endogenous housekeeping gene and were expressed as percentage in
561 comparison with the H-RF group, which was considered as 100% gene expression. White bars
562 represent H-RF group, black bars represent FA-RF group, white-striped bars represent FA-CC group
563 and grey bars represent FA-NFC group. Results are expressed as mean ± SEM (n = 12). $^{*}p<0.05$ *vs.*
564 H-RF group, and $^{\phi}p<0.05$ *vs.* FA-RF group and $^{\#}p<0.05$ *vs.* FA-CC group.



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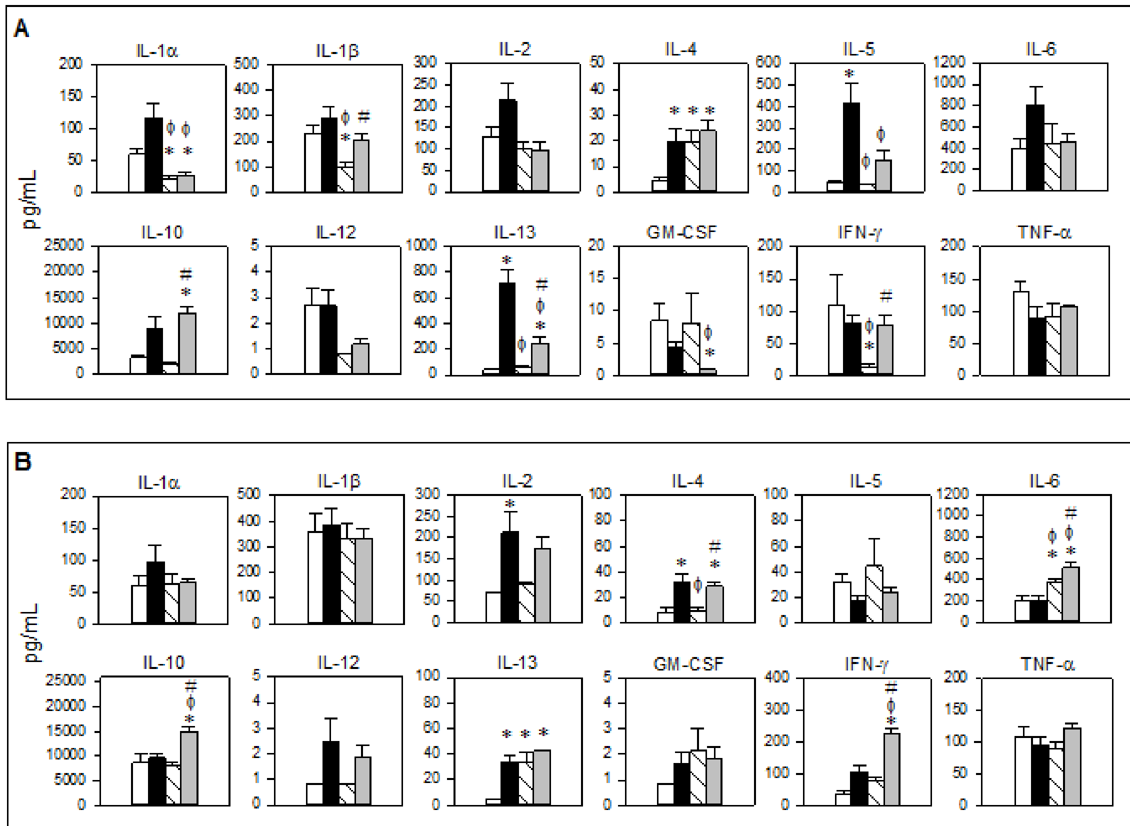
566

567 **Figure 7**. Cytokine production from A) mesenteric lymph nodes and B) spleen cells after OVA-

568 stimulation. White bars represent H-RF group, black bars represent FA-RF group, white-striped bars

569 represent FA-CC group and grey bars represent FA-NFC group. Results are expressed as mean ± SEM

570 (n = 6-8). *p<0.05 *vs*. H-RF group, and $^{\phi}p$<0.05 *vs*. FA-RF group and $^{\#}p$<0.05 *vs*. FA-CC group.



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572

573 **Table 1.** Composition of reference (RF), conventional cocoa (CC) and non-fermented (NFC) cocoa

574 diets

Components	RF diet (g/kg)	CC diet (g/kg)	NFC diet (g/kg)
Casein	124.3	99.5	124.3
L-Cystine	1.8	1.4	1.8
Corn starch	419.5	432.1	419.5
Maltodextrin	148.6	116.8	148.6
Sucrose	102.7	108.7	102.7
Soybean oil	38.3	26.2	38.3
Cellulose	50	24.5	50
Minerals	35.3	27.8	35.3
Vitamins	2.0	7.2	2.0
Choline bitartrate	9.1	2.0	9.1
Cocoa powder	-	100	8.7
Protein	-	22	1.13
Carbohydrate	-	16	0.82
Lipid	-	11	0.56
Fiber (insoluble/soluble)	-	34 (25.5/8.5)	1.75 (1.31/0.44)
Total polyphenols	-	4	4
Polyphenols provided by cocoa powder:			
Catechin	-	0.073	0.040
Epicatechin	-	0.204	0.689
Isoquercetin	-	0.0053	n.d.
Quercetin	-	0.0029	n.d.
Procyanidin B1	-	n.d.	0.127
Procyanidin B2	-	0.167	0.356
Total procyanidins	-	n.d.	3.897

575 n.d. means non-determined.

576

577 **Table 2.** Body weight, food consumption, water intake and metabolic hormones in H-RF, FA-RF, FA-

578 CC and FA-NFC groups. Results are expressed as mean ± SEM (n = 6-12).

	H-RF	FA-RF	FA-CC	FA-NFC
Weight day 0 (g)	36.4 ± 1.01	35.8 ±0.76	36.1 ± 0.79	35.1 ± 0.86
Weight day 30 (g)	116.0 ± 9.31	103.0 ± 4.59	81.3 ± 4.23*ϕ	110.7 ± 4.03#
Chow consumption (g/rat)[a]	204.5± 20.36	185.3 ± 10.17	176.1 ± 15.16	202.1 ± 10.79
Water intake (mL/rat)[a]	306.0 ± 14.09	297.3 ± 23.50	325.0 ± 28.92	333.8 ± 16.82
Metabolic hormones				
Ghrelin (ng/mL)	19.61 ± 4.30	10.27 ± 2.03	12.98 ± 3.72	13.94 ± 2.12
Glucagon (pg/mL)	115.08 ± 7.82	127.70 ± 10.75	92.04 ± 2.99*ϕ	126.65 ± 12.72#
GLP-1 (pg/mL)	79.67 ± 9.03	137.55 ± 10.99*	91.35 ± 4.71ϕ	80.95 ± 16.60ϕ
Leptin (pg/mL)	367.62 ± 80.22	586.91 ± 88.74*	367.24 ± 53.45ϕ	569.68 ± 75.45#

*$p<0.05$ *vs*. H-RF group

ϕ$p<0.05$ *vs*. FA-RF group

#$p<0.05$ *vs*. FA-CC group.

[a] Food and water intake corresponds to the total consumption per animal during the entire experimental procedure.

579
580

581 **Appendix A. Supplementary data**

582 Lower (LLOQ) and upper limits of quantification (ULOQ) for cytokines and metabolic hormones

583 according to the Bioplex-Pro assay applied

Cytokines (pg/mL)		
	LLOQ	**ULOQ**
IL-1α	7.82	31953.56
IL-1β	8.26	31999.96
IL-2	145.58	32188.31
IL-4	0.84	15954.37
IL-5	7.79	8068.31
IL-6	35.36	32600.93
IL-10	32.31	31999.8
IL-2p70	1.64	32202.1
IL-13	6.87	34692.34
GM- CSF	1.71	30927.75
IFN-γ	8.51	33143.52
TNF-α	1.55	8171.98
Metabolic hormones (pg/mL)		
Ghrelin	159.58	155595.25
Glucagon	23.43	1316.37
GLP-1	8.44	1999.39
Leptin	123.73	130750.77

584