1   **Role of theobromine in cocoa's metabolic properties in healthy rats**

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Abstract

Cocoa is rich in polyphenols and methylxanthines and it has been reported that its consumption, among other properties, has beneficial effects on metabolism. This study aimed to investigate the role of theobromine in cocoa's metabolic properties in healthy rats. In addition to morphometric measurements, biochemical markers of lipids and glucose metabolism and gene expression of molecules related to immune cells in adipose and hepatic tissues were assessed after 7 or 18 days of diet. Additionally, a metabolomic analysis was carried out at day 7. This study revealed the presence of six discriminant metabolites in plasma due to the diets. Moreover, the results showed that theobromine is the main responsible factor for cocoa's effects on body weight gain as well as on lipid and glucose metabolism. The effects on body weight and lipids appeared as early as after 7 days of diet, whereas those affecting glucose metabolism required a longer intervention.

INTRODUCTION

Cocoa is a product obtained from the seeds of the *Theobroma cacao* L. tree, which contains fiber, proteins, carbohydrates, lipids, minerals, vitamins, polyphenols and methylxanthines[1,2]. Within polyphenols, cocoa mainly contains flavonoids, the most important ones being the flavan-3-ols, such as epicatechin, catechin and procyanidins. Otherwise, theobromine (3,7-dimethylxanthine) is the methylxanthine found in the highest concentration in cocoa[3].

In recent years, cocoa has increasingly attracted attention because of its potential health effects[4]. Cocoa's anti-inflammatory, antioxidant, anti-allergy and anti-obesity properties have been reported; its intake also influences mood, intestinal microbiota, the cardiovascular system and, interestingly, the metabolic profile[4–9]. Within the role of cocoa in metabolic properties, it has been shown that cocoa consumption has a beneficial effect on obesity as well as in the improvement of glucose, lipids and insulin levels[8,10,11]. Preclinical studies evidenced that the consumption of a 10% cocoa-enriched diet for 9 weeks in diabetic Zucker rats lowered glucose and insulin levels, and improved glucose tolerance and insulin resistance[12]. Similarly, the administration of 8% cocoa powder to mice with high-fat-diet-induced obesity reduced body weight (BW) gain, and insulin resistance[11]. Likewise, mice fed with high-fat-diet treated with a cocoa polyphenol concentrate decreased adipose tissue mass and plasma triglycerides (TG), and consequently the BW gain, without modifications in plasma glucose and cholesterol levels[13]. Otherwise, obese diabetic rats fed a cocoa extract enriched with polyphenols and methylxanthines for 4 weeks showed reduced plasma total cholesterol, TG and low-density lipoprotein cholesterol[14]. Apart from the cocoa metabolic effects in rodents, interventional studies in humans have reinforced the role of cocoa in body metabolism. Thus, overweight/obese premenopausal women consuming a sugar-free natural cocoa beverage and dark chocolate twice a day for 18 weeks, decreased plasma glucose and insulin concentrations[15], and a similar population consuming dark chocolate for 7 days had higher HDL cholesterol (HDL-c) and reduced the abdomen circumference[16]. Moreover, Martínez-López et al. reported that the

57 consumption of a soluble cocoa product increased serum HDL-c whereas anthropometric

58 parameters were unaffected[17].

59 In our previous studies developed in rats, we detected that the administration of a diet containing

60 10% cocoa was associated with a lower BW gain[18–21]. More recently, we have shown that the

61 attenuating effect on antibody production and the lowering of BW gain in rats fed cocoa were

62 similar to that observed in rats receiving theobromine alone[22]. These results prompted us to consider

63 whether theobromine may also contribute to the effects of cocoa intake on glucose and lipid

64 metabolism. For this reason, the present study aimed to establish the role of theobromine in

65 metabolic cocoa's properties. Apart from comparing the metabolic profile induced by cocoa and

66 theobromine diets, rat physical activity was measured and an untargeted metabolomics study was

67 carried out.

MATERIALS AND METHODS

68

Reagents and biological material

69

70 Cocoa powder was obtained from Idilia Foods S.L. (formerly Nutrexpa S.L., Barcelona, Spain),

71 theobromine was purchased from Sigma-Aldrich (Madrid, Spain) and AIN-93M diet and basal mix

72 by Harlan Teklad (Madison, USA). Ketamine was from Merial Laboratories S.A. (Barcelona,

73 Spain) and xylazine from Bayer A.G. (Leverkusen, Germany). Polymerase Chain Reaction (PCR)

74 TaqMan® primers were provided by Applied Biosystems (Weiterstadt, Germany). Rats were

75 obtained from Janvier Labs (Saint-Berthevin Cedex, France).

76

Experimental design

77

78 Forty 3-wk-old Lewis rats were maintained at the animal facilities of the Faculty of Pharmacy and

79 Food Science (University of Barcelona) and kept under controlled conditions of temperature and

80 humidity in a 12h:12h light:dark cycle. This study was conducted in accordance with institutional

81 guidelines for the Care and Use of Laboratory Animals and was approved by the Ethical Committee

82   for Animal Experimentation of the University of Barcelona and the Catalonia Government

83   (CEEA/UB ref. 380/13 and DAAM 5988, respectively). The rats were distributed according to diet

84   into three groups (n=12−14 each one): reference group (RF group) fed standard diet (AIN-93M),

85   cocoa group (CC group) fed a diet containing 10% (w/w) cocoa powder (with a content of 2.5%

86   theobromine), and the theobromine group (TB group) fed a 0.25% theobromine diet, which had the

87   same amount of theobromine as that provided in the CC group. Diets containing cocoa and

88   theobromine were prepared in our own laboratory, mixing ingredients (Teklad diets, Envigo,

89   Indianapolis, USA) in the required quantities to provide the same amount of energy, micronutrients

90   and macronutrients as the standard diet (Supporting Table 1). After mixing, food pellets were made

91   and dried. The animals had access to food and water *ad libitum* throughout the study.

92   Rats were housed in cages (2−3 rats in each cage). During some periods (days 5 to 7 and days 9 to

93   14), animals were individually housed in order to establish their motor activity. The nutritional

94   intervention lasted for 7 or 18 days (**Figure 1**). After these periods, rats were deprived of food for

95   18 h and were then euthanized for sample collection.

96   **Data and sample collection**

97   Food and water intake (FI and WI, respectively) were monitored throughout the study. BW, body

98   length (BL), and rectal body temperature were determined just before euthanasia. Body mass index

99   (BMI), Lee index and food efficiency were also calculated. On days 8 and 19, inguinal and

100   retroperitoneal adipose tissues, heart, liver, right kidney, small intestine and large intestine (colon

101   and caecum) were collected and weighted. Moreover, plasma was obtained and stored at -80 ℃

102   until processing. At day 8, inguinal adipose tissue and liver were kept at -80 ℃ until mRNA

103   quantification and/or biochemical variables determination.

104   **Biochemical determinations in plasma and liver**

105   Plasma total cholesterol, HDL-c, TG, and glucose concentrations were determined by specific

106   colorimetric kits (Química Clínica Aplicada S.A., Spain), following the manufacturer's instructions

107   adapted to a microplate. Absorbance was measured at 505 nm (microplate Tecan photometer, Tecan

108 Group Ltd, Männedorf, Switzerland). LDL cholesterol (LDL-c) concentration was estimated by

109 means of the Friedewald formula (LDL-c = total cholesterol − (HDL-c - TG/5)). In order to measure

110 hepatic TG, frozen liver tissue samples were homogenized with a Polytron® (Kinematica, Lucerne,

111 Switzerland) in isopropanol (1 mL/50 mg liver), kept for 1 h at 4 ºC and then centrifuged (1313 g, 4

112 ºC, 5 min). Supernatants were used for TG determination.

Metabolic hormones determination

114 Plasma ghrelin, glucagon, glucagon-like peptide (GLP-1) and leptin were evaluated after fasting in

115 19-day samples, with the Bio-Plex Pro™ Diabetes Assay (Bio-Rad Laboratories, Madrid, Spain).

116 The lower and upper limits of quantification were: 159.58−155595.25 pg/mL (ghrelin),

117 23.43−1316.37 pg/mL (glucagon), 8.44−1999.39 pg/mL (GLP-1) and 123.73−130750.77 pg/mL

118 (leptin). Plasma insulin was measured using an ultra-sensitive ELISA kit (Ultra Sensitive Rat

119 Insulin ELISA kit) from Crystal Chem (Downers Grove, IL, USA) according to the manufacturer's

120 instructions.

Fecal fat content and pH determinations

122 Feces collected from the whole of day 7 were humidified and submitted to near infrared

123 spectroscopy to determine the fat content by means of an InfraAlyzer 500 (Bran+Luebbe,

124 Norderstedt, Germany). Furthermore, fecal pH determination was performed in feces from days 7

125 and 18 using a surface pH electrode (Crison Instruments, SA, Barcelona, Spain).

Gene expression quantification

127 In liver and adipose tissue samples, we determined the gene expression of molecules expressed in

128 particular immune cells, such as CD11b (monocytes, neutrophils, natural killer cells, granulocytes

129 and macrophages), CD11c (monocytes, granulocytes, some B cells, dendritic cells and

130 macrophages), CD68 (monocytes and macrophages), CD4 (predominant on T helper cells, but also

131 in macrophages, monocytes and dendritic cells), CD8α (predominant on cytotoxic T cells and in

132 natural killer cells and dendritic cells) and Foxp3 (regulatory T cells). In addition, we quantified the

133 gene expression of pro-inflammatory cytokines, such as interleukin (IL)-1β, TNFα, IL-6, MCP-1

134 and IFNγ, anti-inflammatory cytokines, such as IL-4 and IL-10, and the transcription factors NF-κB

135 (involved in inflammation) and PPAR-γ (present in adipose tissue and macrophages and involved in

136 inflammation regulation). For this, liver and adipose tissue samples were homogenized in a lysing

137 matrix tube (MP Biomedicals, Illkirch, France) by a FastPrep-24 equipment (MP Biomedicals, 30 s,

138 at 6.5 m/s). Total RNA was extracted using the RNeasy® mini kit (Qiagen, Madrid, Spain)

139 following the manufacturer's directions. The NanoPhotometer (BioNova Scientific, CA, USA) was

140 used to determine RNA concentration. A thermal cycler PTC-100 Programmable Thermal

141 Controller with TaqMan® Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt,

142 Germany) was applied to obtain cDNA. Subsequently, a PCR quantitative assay was carried out

143 (ABI Prism 7900 HT, AB) using the following specific PCR TaqMan® primers: CD11b

144 (Rn00709342_m1, Inventoried (I)), CD11c (Rn01511082_m1, I), CD68 (Rn01495634_m1, I), CD4

145 (Rn00562286_m1, I), CD8α (Rn00580577_m1, I), FoxP3 (Rn01525092_m1, I), IL-1β

146 (Rn00580432_m1), IL-10 (Rn00563409_m1, I), IL-6 (Rn01410330_m1, I), TNFα

147 (Rn99999017_m1, I), NF-κB (Rn01399572_m1, I), IFNγ (Rn00694078_m1, I) PPARγ

148 (Rn00440945_m1, I), MCP-1 (Rn00580555_m1, I). The relative amount of target mRNA was

149 normalized using β-actin (Rn00667869_m1, I) or HPRT1 (Rn01527840_m1, I) as housekeeping

150 genes and the $2^{-\Delta\Delta Ct}$ method, as previously described[23]. The gene expression obtained was

151 calculated giving the 1 value to the mean results from the RF group.

Metabolomic analyses

153 The untargeted metabolomics analyses were performed on plasma samples collected after 7 days of

154 intervention with a high-performance liquid chromatography coupled to electrospray ionization and

155 quadrupole time-of-flight mass spectrometry (HPLC-ESI-QToF-MS) as previously published[24].

156 Detailed description is given as Supporting Information (Supporting Table 2).

Motor activity measurement

158 Spontaneous motor activity of each animal was continuously recorded on days 5 to 7 and 9 to 14 by

159 means of activity meters placed outside the cage that used two perpendicular crossed infrared beams

160   situated 6 cm above the floor of the cage. Each beam interruption represented an activity count that

161   was registered and stored in 15-min data bins for further analysis. Total daily activity was

162   calculated as well as the nocturnal and diurnal activity separately. Results are expressed as the mean

163   values (counts every 15 minutes).

164   **Statistical analysis**

165   Data were evaluated with the software package SPSS 22.0 (IBM Statistical Package for the Social

166   Sciences, version 22.0, Chicago, IL, USA) by using the parametric test one-way ANOVA followed

167   by Bonferroni's post hoc test or the nonparametric tests Kruskal–Wallis and Mann–Whitney U in

168   accordance with its homogeneity of variance (Levene test) and distribution (Shapiro-Wilk test).

169   Significant differences were established when $P \leq 0.05$.

170   Significant mass features selected after multi- and univariate statistical analyses of the

171   metabolomics dataset were identified by a multistep procedure as specified in Supporting

172   Information. Untargeted metabolomics data were analyzed by multivariate statistical methods using

173   SIMCA-P+ 13.0 software (Umetrics, Umea, Sweden). Particular statistical analysis for these data is

174   expanded in the Supporting Information.

175   The metabolites identified by the untargeted metabolomics analysis were correlated with the results

176   from BW gain, BMI, Lee index, FI, WI, inguinal, retroperitoneal, small and large intestine tissue

177   weights, fecal fat, plasma total cholesterol, HDL-c and LDL-c concentrations and hepatic TG

178   levels. For this, after a log-transformation of results, Spearman's correlation analyses were

179   performed. The Benjamini–Hochberg method was used to adjust p-values for multiple testing

180   considering a 5% false discovery rate.

181   # RESULTS

182   **Morphometric variables**

183   Morphometric changes were already detected after 7 days of the experimental diets (**Table 1**).

184   Specifically, the animals from the CC and TB groups had lower BW gain (P<0.001). Moreover, the

185   rats fed CC and TB diets for 18 days presented lower body length than those of the RF group

186   (P<0.001). Decreased BMI was found in the CC and TB groups (P<0.05) on days 8 and 19 and,

187   additionally, on day 8 the Lee index in both interventional groups was lower than in the RF animals

188   (P<0.05). In any case, no differences were detected between CC and TB groups, the changes being

189   almost identical between them.

190   In spite of the lower values in morphometric changes, mean FI did not decrease in the CC or TB

191   groups, but mean WI increased in both interventional groups compared to the RF one (P<0.001)

192   (**Table 1**). With regard to food efficiency, the CC and TB groups showed lower values than the RF

193   animals, which was already evidenced after one single week of diet (P<0.05) (**Table 1**).

Body temperature and spontaneous physical activity

195   Rectal temperature was measured at days 7 and 18. There were no changes in body temperature

196   between the considered groups at any of the studied time points (**Table 1**). Moreover, motor activity

197   was individually measured between days 5 to 7 and days 9 to 14. In the first period, cocoa diet

198   decreased diurnal motor activity compared to the RF group (**Figure 2a−b**) without statistically

199   significant changes when considering the whole day. In the second period, higher activity was

200   observed in animals fed cocoa diet than in those from the RF group, both in diurnal and nocturnal

201   determinations. The activity in the TB group did not achieve a significant modification compared to

202   RF animals (**Figure 2c−d**).

Relative organ and tissue weights

204   After euthanasia on days 8 or 19, some organs and adipose tissues were collected and weighed

205   (**Table 1**). Animals from the CC and TB groups showed lower relative retroperitoneal fat weight at

206   both time points (P<0.05), although the relative inguinal fat weight was lower only in the TB group

207   after 18 days of diet. The rats fed cocoa for 18 days had higher relative heart weight (P<0.05). In

208   the two studied time points no changes in the relative kidney weight were observed. However, the

209   CC and TB groups had lower relative liver weight than the RF group (P<0.01), the TB value being

210   even lower than that of the CC group after 18 days of diet (P<0.05). On the other hand, the relative

211   small and large intestinal weights were higher after 7 and 18 days of the two experimental diets in

212   comparison to the RF group (P<0.05) (**Table 1**).

213   **Fecal fat and pH values**

214   Fecal fat content, determined after 7 days of diet, was lower in cocoa- and theobromine-fed animals

215   in comparison to the RF group (P<0.01), and even the rats from the CC group showed less fecal fat

216   levels than the TB group (P<0.05) (**Figure 3**). Otherwise, cocoa diet lowered fecal pH detected at

217   day 18 in comparison to the RF and TB groups (P<0.05) (**Figure 3**).

218   **Cholesterol and triglyceride concentrations**

219   After 7 days of diet, the total plasma cholesterol concentrations in both CC and TB groups were

220   about 15% lower than those detected in the RF group (P<0.05) (**Table 2**). Nevertheless, no

221   differences were detected later. Similar results were observed in LDL-c content. Interestingly,

222   HDL-c values increased by 24−41% in the CC and TB groups at both studied time points (P<0.05)

223   (**Table 2**). Moreover, plasma TG concentrations from the TB group were significantly lower after

224   18 days of diet (P<0.01) (**Table 2**) while hepatic TG concentrations decreased after both cocoa and

225   theobromine diets, this already observable just after one week of diet (P<0.001) (**Table 2**).

226   **Liver and adipose tissue gene expression**

227   The influence of diets containing cocoa or theobromine on the gene expression of some immune

228   molecules in hepatic and adipose tissues was quantified after one week of diet (**Figures 4** and **5**).

229   With regard to the liver (**Figure 4**), cocoa-enriched diet reduced the gene expression of CD11c and

230   CD4 without inducing significant changes in CD11b, CD68, Foxp3, TNF-α, NF-κB and PPAR-γ.

231   Moreover, both CC and TB groups showed lower mRNA levels of CD8α, IL-10 and IL-1β with

232   respect to the RF group. The TB group had higher TNF-α and NF-κB hepatic gene expressions than

233   the RF and CC groups (P<0.05). In this tissue, the mRNA levels of IL-4, IL-6, MCP-1 were

234   undetectable in any of the considered groups.

235   With regard to adipose tissue (**Figure 5**), the gene expression of CD11b, CD11c, Foxp3, IFN-γ,

236   TNF-α, IL-4, and IL-6 was undetectable. Both nutritional interventions induced an upregulation of

237　the CD68 and MCP-1 gene expression and a downregulation of the CD4 expression with respect to

238　the RF group (P<0.05). Moreover, animals fed cocoa had higher gene expression of IL-10 and

239　IL-1β whereas the TB group showed lower gene expression of CD8α in comparison to the RF

240　animals. No changes in NF-κB and PPAR-γ expressions were found in the CC and TB groups.

241　**Glucose metabolism**

242　After 7 days of diet, the plasma glucose concentrations in fasting conditions were similar within

243　groups, however, after 18 days, the CC and TB groups had higher plasma glucose concentrations

244　than the RF group (**Table 2**). At this later time point, plasma ghrelin, glucagon, GLP-1, leptin and

245　insulin concentrations were determined (**Figure 6**). The rats from CC and TB groups showed higher

246　ghrelin concentrations and lower glucagon levels than the RF group (P<0.01 and P<0.05,

247　respectively). In fasting conditions, GLP-1 and leptin levels were below the limit of detection in the

248　three experimental groups. There was no difference in plasma insulin levels among the studied

249　groups, although a tendency to decrease (P=0.092) was observed after both experimental diets.

250　**Metabolomic analyses**

251　The metabolomic analyses are detailed in the supporting information (Supporting Information

252　Figure S1 and Tables S3 and S4). As mentioned in the Material and Methods section, only features

253　showing high correlation coefficients (|p(corr)| > 0.75) in all models developed during the leave-

254　one-out procedure were included in the list of discriminating metabolites explaining the differences

255　between groups, which were then submitted to the metabolite identification procedure. A total of

256　185 features fulfilled this criteria, 135 features from the ESI(+) data set and 50 features from the

257　ESI(−) data set. The scores plot of the three-group PLS-DA models with non-OSC filtered data

258　using the selected features revealed higher differences (displayed through PC1) between the REF

259　group and the other two groups, followed by differences between the animals supplemented with

260　cocoa and those supplemented with theobromine (PC2), indicating that each diet induced a different

261　metabolomic pattern (**Figure 7**). Among the features selected as significant, eight of them were

262　assigned to the six compounds that were identified as discriminant metabolites. They were related

263 to at least one of the study groups: theobromine, pantothenic acid, glycocholic acid, citrulline, 5-(2'-

264 carboxyethyl)-4,6-dihydroxypicolinate (CEDHP), and 5-hydroxyindoleacetic acid (HIAA) (**Figure**

265 **8**). Mass features of these compounds are listed in the Supporting Information Table S5.

266 Theobromine was an exogenous metabolite only found in the CC and TB groups and, as expected,

267 there were no differences between the two experimental diets. As endogenous metabolites, the CC

268 and TB groups showed higher concentrations of pantothenic acid and glycocholic acid than the RF

269 group ($p<0.05$). Moreover, the TB group exhibited higher levels of citrulline compared with the RF

270 group, and higher levels of CEDHP in comparison to both the RF and CC groups ($P<0.05$). Finally,

271 the intake of both cocoa- and theobromine-enriched diets induced lower levels of HIAA than those

272 in the RF group.

273 **Correlations between morphometric and biochemical data, and the discriminant metabolites**

274 In order to determine the relationship between the discriminant metabolites and morphometric and

275 biochemical significant results obtained at the same time point, correlations between both data were

276 carried out (**Figure 9**). Theobromine plasma concentration correlated negatively with BMI,

277 retroperitoneal fat weight and hepatic TG, whereas it correlated positively with WI, small and large

278 intestine weights as well as with plasma HDL-c levels. Almost similarly, the plasma concentrations

279 of pantothenic acid negatively correlated with BW gain, BMI, food efficiency and hepatic TG while

280 it correlated directly with higher WI and small intestine weight.

281 Moreover, the more plasma concentration of glycocholic acid detected, the lower the fecal fat

282 observed. Likewise, higher citrulline concentrations correlated with higher levels of HDL-c. The

283 amount of the metabolite CEDHP correlated positively with the large intestinal relative weight and

284 negatively with plasma TG. Regarding the metabolite HIAA, found in lower concentrations in the

285 CC and TB groups, it positively correlated with lower BW gain, BMI, fecal fat content, total

286 cholesterol, LDL-c and plasma TG. Otherwise, HIAA correlated negatively with WI and small and

287 large intestinal relative weights.

288   Regarding the Lee index, FI and inguinal fat weight, no significant correlation was detected with

289   any of the studied metabolites.

DISCUSSION

291   Previous studies have reported the effect of cocoa on metabolism[8,11–13,25,26]. However, the exact

292   compound responsible for such effect and the length needed to achieve such benefits still remain

293   unknown and are a topic of debate. Although polyphenols and methylxanthines are recognized

294   bioactive compounds in cocoa[3], a vast number of studies have associated the cocoa role in

295   metabolism only with its polyphenol content. In the current study, we evidence the importance of

296   theobromine in the metabolic properties of cocoa as well as its rapid effects.

297   Rats fed with 10% cocoa that consumed a similar or even higher amount of food than the RF group,

298   underwent a lower body weight gain as early as after 7 days of diet, which was accompanied by

299   lower BMI and Lee's index. Importantly, theobromine on its own produced similar actions. Cocoa

300   effects on body weight increase have already been reported both in animal models and humans[11–

301   13,15,27] and it has been postulated that cocoa is a weight loss accelerator[28]. Our results concerning

302   body temperature and motor activity demonstrated that, at least after the first week of diet, there

303   was no increase in the metabolic rate. The metabolomic analyses carried out after 7 days of diet

304   showed that BMI inversely correlated with the plasma concentrations of theobromine, which is in

305   line with a previous study showing a negative association of BMI with the amount of theobromine

306   in urine of rats fed cocoa for 3 weeks[29]. On the other hand, we found a higher water intake in

307   animals fed cocoa or theobromine, data that correlated directly with the plasma concentrations of

308   theobromine. This higher water intake could be a consequence of the theobromine's diuretic

309   actions[30] in order to compensate the high volume of excreted urine. Overall, these results allow us

310   to conclude that cocoa theobromine content is an important cocoa metabolite involved in the body

311   weight homeostasis.

312 In line with a reduced body weight gain and BMI, rats fed cocoa or theobromine diets had a lower

313 amount of retroperitoneal fat after 7 and 18 days of diet. These results agree with those reported in

314 high-fat-fed obese mice fed 8% cocoa powder[11] and with a study carried out in European

315 adolescents reporting that higher chocolate consumption was related with lower fatness[31]. After 7

316 days of diet, as observed by correlations after metabolomic analyses, the higher the plasma

317 theobromine concentration, the lower the amount of retroperitoneal fat found. Thus, it can be

318 suggested that these cocoa effects on adipose tissue could be due to its theobromine content.

319 However, the proportion of inguinal fat in the theobromine group at day 19 was lower than that in

320 the cocoa group, suggesting that other cocoa compounds (polyphenols and/or fiber) could attenuate

321 this particular effect. On the other hand, adipose tissue is an immune organ that links metabolism

322 and immunity[32]. In this sense, it has been described that cocoa intake decreased macrophage

323 infiltration in adipose tissue in obese mice[11]. However, in this tissue, we have detected a higher

324 gene expression of CD68 and MCP-1 (molecules present in macrophages and involved in their

325 recruitment, respectively) after cocoa and theobromine intake, and higher IL-1β gene expression in

326 cocoa-fed rats. These discrepancies could be due to the status of the animals − healthy status in the

327 current study and obese status in the first described case[11] − which had already shown an increased

328 macrophage infiltration before cocoa intake. Nevertheless, although some macrophage-related

329 molecules increased, other transcriptional factors related to macrophage or immune activation, such

330 as NF-κB and PPAR-γ, were not modified by cocoa or theobromine diets, and, interestingly, the

331 cocoa diet increased the adipocyte gene expression of the anti-inflammatory cytokine IL-10.

332 Similarly, the gene expression of CD4 was down-regulated by cocoa and theobromine intake, and

333 that of CD8 only by the theobromine diet. Both CD4 and CD8 are mainly related to lymphocytes

334 and their decrease suggests that there was lower lymphocyte infiltration into the fat. In short, results

335 of adipocyte gene expression suggest that cocoa, partially due to its theobromine content, may be

336 involved in avoiding the low-grade inflammatory status observed in obesity[32]. Nevertheless,

337 because the effect in fat gene expression of both cocoa- and theobromine-enriched diets differed in

338　　some molecules, the role of other cocoa bioactive compounds such as polyphenols or fiber cannot

339　　be discarded.

340　　Fecal samples obtained after 7 days of cocoa or theobromine diet also provided significant results

341　　regarding fat content. We found that the fecal fat content in animals fed theobromine and cocoa

342　　decreased. These results do not agree with those reported in obese mice fed a cocoa diet[11] or in

343　　obese animals fed with other phytotherapeutic diets[33]. This disagreement could again be due to the

344　　different status of the animals or to the different technique used. Nevertheless, we observed that the

345　　two experimental diets exerted a similar effect, although it was more obvious in the cocoa group,

346　　suggesting the role of other compounds present in the whole cocoa diet in fat excretion.

347　　A relevant outcome observed in the current study was the influence of cocoa and theobromine on

348　　plasma lipids such as cholesterol. Although we found that cocoa diet decreased plasma

349　　concentrations of total cholesterol and LDL-c, after 7 days of diet, this effect was not longer

350　　detected. In fact, it has been reported that in rats fed a high-fat diet with 12.5% cocoa for 21 days[34]

351　　no decrease was seen in either plasma cholesterol or LDL-c levels, which also agree with studies

352　　carried out in humans[35,36]. Nevertheless, both the cocoa diet and the diet containing only

353　　theobromine increased plasma HDL-c levels. Similar effects have been reported in humans after a

354　　nutritional intervention with cocoa[35,36] and with rats fed a high-fat diet plus cocoa[34]. The increase in

355　　HDL-c concentration correlated with the plasma levels of theobromine. In partial agreement with

356　　this, a recent study in humans taking 500 mg/day theobromine for 4 weeks[37] demonstrates that

357　　theobromine consumption tended to increase fasting plasma HDL-c concentrations. Overall, the

358　　current results indicate that in the reported beneficial effects of cocoa intake on plasma lipids,

359　　theobromine from cocoa content plays a relevant role. Although the results obtained from the cocoa

360　　diet agree with a previous study showing a downregulation of colonic *Adipoq* gene expression,

361　　which is involved in lipid metabolism[38], further studies must be carried out to establish the effects

362　　of both cocoa and theobromine on key enzymes related to lipid metabolism.

363 On the other hand, cocoa diet and its methylxanthine alone influence the hepatic tissue. After one

364 week of both diets there was a reduction in hepatic triglycerides and changes in the gene expression

365 of several molecules. These results on hepatic triglycerides are in line with those reported in adult

366 mice fed with a high-fat diet plus 8% cocoa[11], although the effects obtained here were more

367 obvious, which could be due to the use of younger animals fed a standard diet, the higher cocoa

368 proportion and/or the cocoa's composition. Anyway, despite the fact that the effect of cocoa in the

369 suppression of hepatic fatty acid synthesis and transport systems in mesenteric white adipose tissue

370 has been reported[34], the influence of cocoa on hepatic triglycerides deserves a more in-depth study.

371 We also studied the hepatic gene expression of molecules involved in promoting or inhibiting

372 inflammation. Although both diets decreased the gene expression of the pro-inflammatory cytokine

373 IL-1β and cocoa intake down-regulated CD11c expression, there was a decrease in the expression

374 of the anti-inflammatory IL-10 and theobromine intake increased the gene expression of TNF-α and

375 NF-κB, promoting inflammation. On the other hand, CD4 and CD8 molecule gene expressions

376 were down-regulated by cocoa and/or theobromine, suggesting lower lymphocyte presence in the

377 liver. Overall, the results of the hepatic genes studied do not clarify whether immune mechanisms

378 were involved in the effects of cocoa on the liver. Moreover, the intake of cocoa and theobromine

379 did not show identical effects on the molecules considered, indicating that other bioactive

380 compounds included in the whole cocoa diet can enhance or attenuate that of theobromine alone.

381 As previous studies demonstrated the influence of a cocoa diet on glucose metabolism[12], we also

382 tested plasma glucose concentrations, which did not vary after 7 days of diet but increased in the

383 18-day samples in a similar way in both cocoa- and theobromine-fed animals. Therefore, we

384 determined insulin concentrations, as well as hormones associated with glucose metabolism at this

385 time point. The current study revealed that cocoa and theobromine on its own increased ghrelin

386 levels and decreased glucagon concentration, as already reported in rats fed 10% cocoa[29]. Ghrelin,

387 also known as the hunger hormone, is a multifaceted hormone secreted by the stomach, which is

388 influenced by the BMI, glucose and insulin levels, among other factors[39]. Ghrelin is involved in the

389   inhibition of insulin secretion and contributes to glucose homeostasis, apart from regulating energy

390   homeostasis[40]. Otherwise, glucagon is a hormone produced in the α cells of the pancreatic islets

391   stimulated by low levels of glucose[41]. From our results obtained in fasting samples after 18 days of

392   cocoa or theobromine diets, we can suggest that the higher ghrelin secretion might be due to

393   stimulation from the lower BMI and could increase plasma glucose concentration, whereas it

394   decreased glucagon levels and tended to decrease plasma insulin. The discrepancy between the

395   current results and previous studies that show the antidiabetic effects of a cocoa diet could be due to

396   the age of the rats at the beginning of the nutritional intervention as well as the use of healthy rather

397   than diabetic animals. In fact, a study in humans taking dark chocolate found decreased plasma

398   glucose concentrations when BMI$\geq$25 kg/m^2 but no changes when BMI<25 kg/m^2 [42]. Although

399   further studies are required to check the importance of age and the existence of diabetes/overweight

400   in the cocoa/theobromine effects, our findings suggest that theobromine plays an important role in

401   the influence of cocoa on glucose metabolism. In this sense, it has been reported that cocoa intake

402   produces a downregulation of colonic *Adipoq* gene expression involved in glucose metabolism as

403   well as that of lipids, as has been mentioned above[38].

404   Finally, it is important to highlight the levels of endogenous discriminant metabolites found after

405   cocoa and/or theobromine intake and their correlations with some outcomes obtained after 7 days of

406   diet. Higher plasma pantothenic acid was correlated with lower body weight gain, BMI, food

407   efficiency and hepatic triglycerides, and higher water intake and small intestine weight. The

408   pantothenic acid (vitamin B5) is a precursor for the coenzyme A (CoA) and acyl carrier protein,

409   involved in the metabolism of steroids, fatty acids and phosphatides[43]. CoA is found in the pathway

410   of cholesterol synthesis, amino acid catabolism, and is a crucial substrate in the oxidation of fatty

411   acids[44]. The higher levels of pantothenic acid in cocoa and theobromine groups might be associated

412   with an enhanced fatty-acid metabolism that would partially explain the lower body weight gain and

413   the decrease in hepatic triglycerides as well as plasma cholesterol levels. Also related to the lipid

414   metabolism, we found a negative correlation between the elevated glycocholic acid levels in cocoa

415 and theobromine groups and their reduced fecal fat content. As glycocholic acid is an indirect

416 cholesterol-derived bile acid[45], it can be suggested that a high amount of glycocholic in plasma

417 could promote lipid metabolism.

418 On the other hand, plasma citrulline concentrations were directly associated with those of HDL-c.

419 Citrulline is a nonessential amino acid, precursor of arginine. In particular, from arginine and

420 different cofactors NO-synthase produced NO with release of citrulline[46]. Although with the current

421 results we cannot evidence the exact pathway affected by the citrulline increase, it has been

422 reported, in agreement with the current results, that the supplementation of L-citrulline is associated

423 with increased HDL-c levels[47].

424 The metabolite 5-(2'-carboxyethyl)-4,6-dihydroxypicolinate (CEDHP) was found in higher plasma

425 concentrations in those animals receiving theobromine. This compound was derived from the

426 tryptophan metabolism and comes from the kynurenine pathway. Our results are in line with the

427 increased kynurenine production observed after black tea consumption[48]. In particular, CEDHP

428 levels inversely correlated with plasma triglycerides, suggesting the involvement of this pathway,

429 which is beneficial for the triglyceridemia status. Another metabolite indicating the implication of

430 this pathway is the HIAA, which is the only one that was found in lower concentrations after cocoa

431 and theobromine intake in comparison with the animals fed standard diet. HIAA was associated

432 with a lot of changes found after cocoa or theobromine consumption, such as body weight increase,

433 water intake, small and large intestine weights, fecal fat content and with plasma cholesterol and

434 hepatic triglyceride levels. HIAA is involved in the tryptophan metabolism system, as it is a

435 breakdown product of serotonin and considered as a marker for the endogenous serotonin

436 turnover[49]. In fact, higher urinary HIAA excretion in rats has been reported after cocoa intake and

437 has been related to cocoa tryptophan degradation by gut microbiota[29]. Therefore, it can be suggested

438 that the changes in the microbiota induced by cocoa and theobromine would reduce the absorption

439 of tryptophan that will be found in lower plasma concentrations after one week of diets containing

440 both products. Interestingly, such lower absorption seems related to lower plasma cholesterol and

441   triglyceride concentrations. In this line, it has been shown that severe tryptophan restriction

442   decreased body weight, body fat and lean mass in obese-prone rats[50].

443   In conclusion, from the results obtained in rats fed cocoa or only theobromine, we can conclude that

444   this methylxanthine is the main factor responsible for cocoa's effects on body weight gain as well as

445   on the lipid and glucose metabolism. These effects on body weight and lipids appeared as early as

446   after 7 days of diet, whereas those affecting glucose metabolism require a longer intervention.

Abbreviations

AB, Applied Biosystems; BL, body length; BMI, body mass index; BW, body weight; CC, cocoa group; CEDHP, 5-(2'-carboxyethyl)-4,6-dihydroxypicolinate; CoA, coenzyme A; FI, food intake; GLP-1, glucagon-like peptide; HDL-c, high-density lipoprotein cholesterol; HIAA, hydroxyindoleacetic acid; HPLC-ESI-QToF-MS, high-performance liquid chromatography coupled to electrospray ionization and quadrupole time-of-flight mass spectrometry; IL, interleukin; LDL-c, low-density lipoprotein cholesterol; OSC-PLS-DA, partial least squares discriminant analysis with orthogonal signal correction; PCA, principal components analyses; PCR, polymerase chain reaction; QC, quality control; RF, reference group; TB; theobromine group; TG, triglycerides; WI, water intake

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606 Figure captions

607 **Figure 1.** Experimental design. The nutritional intervention lasted for 7 or 18 days. After these

608 periods, rats were deprived of food for 18 h and were then euthanized.

609 **Figure 2.** Mean motor activity from the three groups of study (white bars represent reference group

610 (RF), grey bars represent cocoa group (CC), and striped bars represent theobromine group (TB));

611 during days 5 to 7 (a−b) and days 9 to 14 (c−d). Values are expressed as mean ± standard error (n =

612 6−7). Statistical difference: * $p \leq 0.05$ vs RF group by one-way ANOVA.

613 **Figure 3.** Fecal fat content and pH from the three groups of study (white bars represent reference

614 group (RF), grey bars represent cocoa group (CC), and striped bars represent theobromine group

615 (TB)). Values are expressed as mean ± standard error (n = 6−7). Statistical difference: * $p \leq 0.05$ vs

616 RF group, and δ $p \leq 0.05$ vs CC group by one-way ANOVA.

617 **Figure 4.** Liver gene expression. Liver gene expression from the three groups of study (white bars

618 represent reference group (RF), grey bars represent cocoa group (CC); and striped bars represent

619 theobromine group (TB)) in the 7 days-lasting experimental design. Values are expressed as mean ±

620 standard error (n = 6−7). Statistical difference: * $p \leq 0.05$ vs RF group, and δ $p \leq 0.05$ vs CC group by

621 Mann-Whitney U test.

622 **Figure 5.** Adipose tissue gene expression. Liver gene expression from the three groups of study

623 (white bars represent reference group (RF); grey bars represent cocoa group (CC); and striped bars

624 represent theobromine group (TB) in the 7-day experimental design. Values are expressed as mean

625 ± standard error (n = 6−7). Statistical difference: * $p \leq 0.05$ vs RF group by Mann-Whitney U test.

626 **Figure 6.** Metabolic variables. Ghrelin, glucagon and insulin concentration from the three groups of

627 study (white bars represent reference group (RF), grey bars represent cocoa group (CC), and striped

628 bars represent theobromine group (TB)) in the 19-day experimental design. Values are expressed as

629 mean ± standard error (n = 6−7). Statistical difference: * $p \leq 0.05$ vs RF group by one-way ANOVA.

630   **Figure 7.** OSC-PLS-DA scores plots (PC1 versus PC2) of study samples. Plasma samples from

631   reference group are indicated in blue, cocoa group in yellow and theobromine group in red.

632   **Figure 8.** Tentatively identified metabolites. Plasma concentrations of exogenous (theobromine)

633   and endogenous (pantothenic acid, glycocholic acid, citrulline, CEDHP and HIAA) metabolites in

634   the three groups of study (white bars represent reference group (RF), grey bars represent cocoa

635   group (CC); and striped bars represent theobromine group (TB)) in the 7-day experimental design.

636   Values are expressed as mean ± standard error (n = 6−7). Statistical difference: * $p \leq 0.05$ vs RF

637   group by one-way ANOVA.

638   **Figure 9.** Heatmap representation of the association between metabolites (in rows) and some

639   metabolic and biochemical variables (in columns). Blue color indicates a positive correlation and

640   red color displays a negative correlation. Color intensity and circle size are proportional to the

641   correlation coefficients. All displayed correlations were statistically significant (FDR<0.05).

Table 1. Morphometric measurements. Initial body weight, body weight gain, body length (with and without tail), body mass index, Lee's index, mean food and water intake, food efficiency, rectal temperature and the relative weight of inguinal and retroperitoneal fat, heart, liver, kidney, small intestine and colon and caecum in the two study time points and from the three groups of study (reference group (RF);, cocoa group (CC), and theobromine group (TB)). Values are expressed as mean ± standard error (n = 6−7). Statistical difference: * $p \leq 0.05$ vs RF group, and δ $p \leq 0.05$ vs CC group by one-way ANOVA.

Variable	7-days diet group			18-days diet group		
	RF	CC	TB	RF	CC	TB
Initial body weight (g)	80.08 ± 2.25	79.98 ± 1.86	80.62 ± 2.17	80.23 ± 2.81	79.82 ± 1.99	79.22 ± 1.14
Body weight gain (g)	35.30 ± 1.61	23.43 ± 0.60*	23.03 ± 0.88*	80.90 ± 2.98	51.53 ± 0.65*	52.11 ± 5.31*
Body length (cm)	28.22 ± 0.32	27.90 ± 0.29	27.70 ± 0.12	29.51 ± 0.28	26.91 ± 0.23*	27.10 ± 0.52*
Body length without tail (cm)	15.03 ± 0.16	15.23 ± 0.16	14.98 ± 0.14	16.50 ± 0.14	14.96 ± 0.17*	14.97 ± 0.23*
Body mass index	0.13 ± 0.00	0.11 ± 0.00*	0.12 ± 0.00*	0.11 ± 0.01	0.10 ± 0.00*	0.10 ± 0.00*
Lee's index	3.11 ± 0.00	2.93 ± 0.00*	2.99 ± 0.00*	2.81 ± 0.00	2.78 ± 0.00	2.79 ± 0.00
Mean food intake (g/100 g rat/day)	15.34 ± 0.24	16.27 ± 0.24*	15.11 ± 0.14δ	13.75 ± 0.15	13.77 ± 0.22	13.46 ± 0.20
Mean water intake (g/100 g rat/day)	12.20 ± 0.70	30.10 ± 1.88*	27.93 ± 2.78*	11.28 ± 0.18	20.54 ± 0.41*	17.67 ± 1.17*δ
Food efficiency	0.34 ± 0.01	0.30 ± 0.01*	0.31 ± 0.01*	0.35 ± 0.01	0.30 ± 0.01*	0.30 ± 0.00*
Rectal temperature (ºC)	35.85 ± 0.13	36.12 ± 0.11	36.03 ± 0.17	36.91 ± 0.34	37.3 ± 0.26	36.49 ± 0.30
Relative weight (%)						

Inguinal fat	0.15 ± 0.02	0.13 ± 0.01	0.11 ± 0.01	0.29 ± 0.03	0.29 ± 0.03	$0.19 \pm 0.02*^{\delta}$
Retroperitoneal fat	0.13 ± 0.02	$0.05 \pm 0.02*$	$0.07 \pm 0.02*$	0.11 ± 0.01	$0.04 \pm 0.01*$	$0.03 \pm 0.00*$
Heart	0.55 ± 0.02	0.57 ± 0.02	0.54 ± 0.02	0.58 ± 0.01	$0.65 \pm 0.02*$	0.59 ± 0.02
Liver	3.82 ± 0.05	3.66 ± 0.09	3.66 ± 0.05	4.12 ± 0.06	$3.80 \pm 0.05*$	$3.46 \pm 0.17*^{\delta}$
Kidney (one)	0.48 ± 0.02	0.57 ± 0.03	0.52 ± 0.01	0.49 ± 0.01	0.51 ± 0.01	0.52 ± 0.03
Small intestine	3.31 ± 0.03	$3.67 \pm 0.07*$	$3.62 \pm 0.05*$	4.19 ± 0.12	$4.80 \pm 0.20*$	$4.83 \pm 0.14*$
Colon and caecum	1.49 ± 0.05	$2.42 \pm 0.13*$	$2.66 \pm 0.13*$	0.98 ± 0.03	$1.36 \pm 0.03*$	$1.61 \pm 0.06*^{\delta}$

Table 2. Biochemical variables measurements. Fold increase of total cholesterol, HDL-c, LDL-c, serum and hepatic TG, and glucose from the three groups of study (reference group (RF), cocoa group (CC), theobromine group (TB)) at day 8 and 19. Values are expressed as mean ± standard error (n = 6-7). Statistical difference: * $p \leq 0.05$ vs RF group by one-way ANOVA.

|	7-days effect			18-days effect		
Variable	RF	CC	TB	RF	CC	TB
Total cholesterol	1.00 ± 0.06	$0.85 \pm 0.03*$	$0.86 \pm 0.02*$	1.00 ± 0.01	1.04 ± 0.03	0.98 ± 0.02
HDL-c	1.00 ± 0.08	$1.41 \pm 0.07*$	$1.39 \pm 0.08*$	1.00 ± 0.05	$1.34 \pm 0.05*$	$1.24 \pm 0.09*$
LDL-c	1.00 ± 0.07	$0.76 \pm 0.04*$	$0.78 \pm 0.03*$	1.00 ± 0.01	1.00 ± 0.03	0.95 ± 0.03
Plasma TG	1.00 ± 0.08	1.17 ± 0.09	1.13 ± 0.12	1.00 ± 0.11	0.83 ± 0.07	$0.54 \pm 0.05*$
Hepatic TG	1.00 ± 0.02	$0.35 \pm 0.10*$	$0.12 \pm 0.06*$	1.00 ± 0.10	$0.35 \pm 0.02*$	$0.34 \pm 0.03*$
Glucose	1.00 ± 0.06	1.10 ± 0.07	1.02 ± 0.06	1.00 ± 0.23	$2.09 \pm 0.40*$	$2.45 \pm 0.43*$
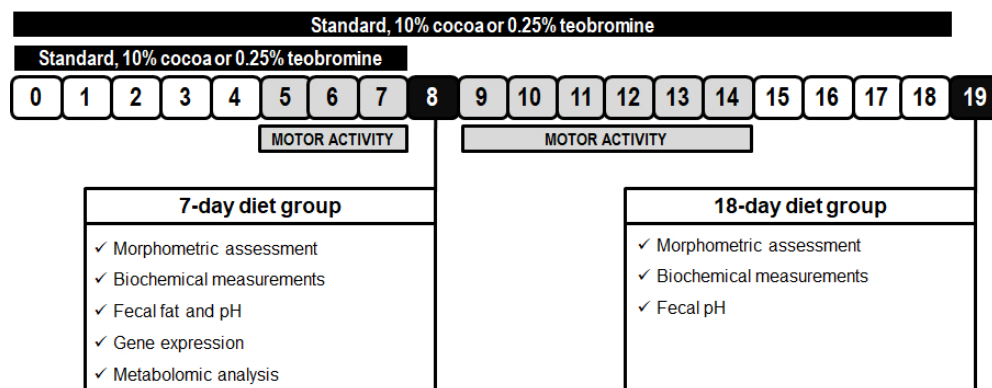
Figure 1
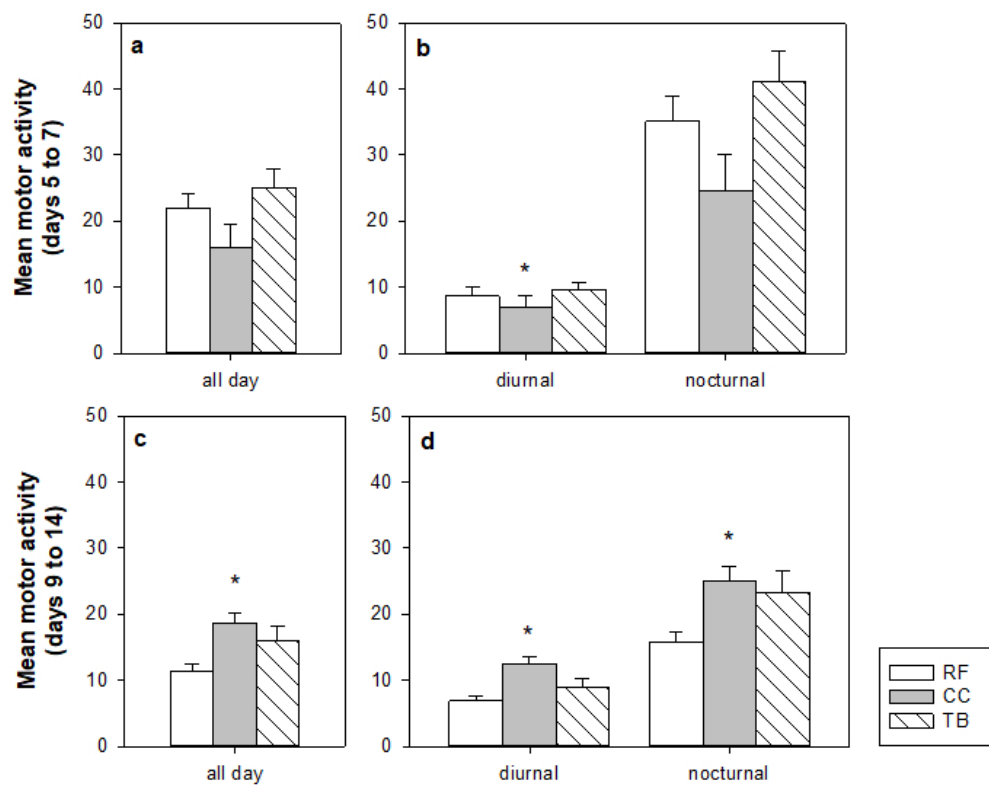
321x143mm (72 x 72 DPI)

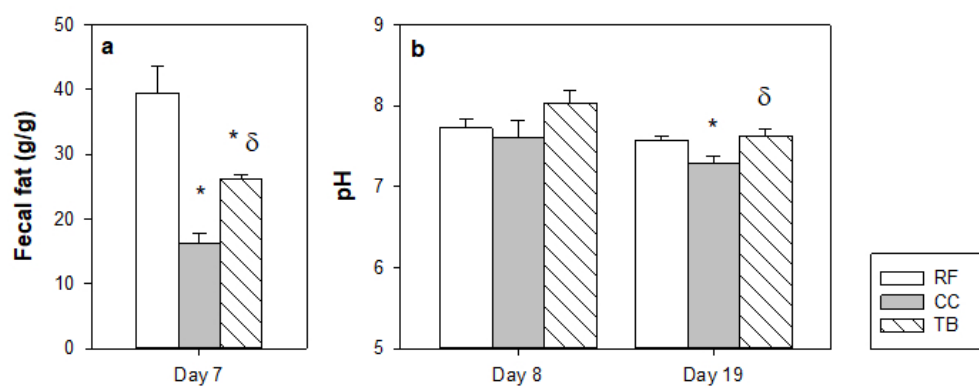Figure 2

242x200mm (72 x 72 DPI)
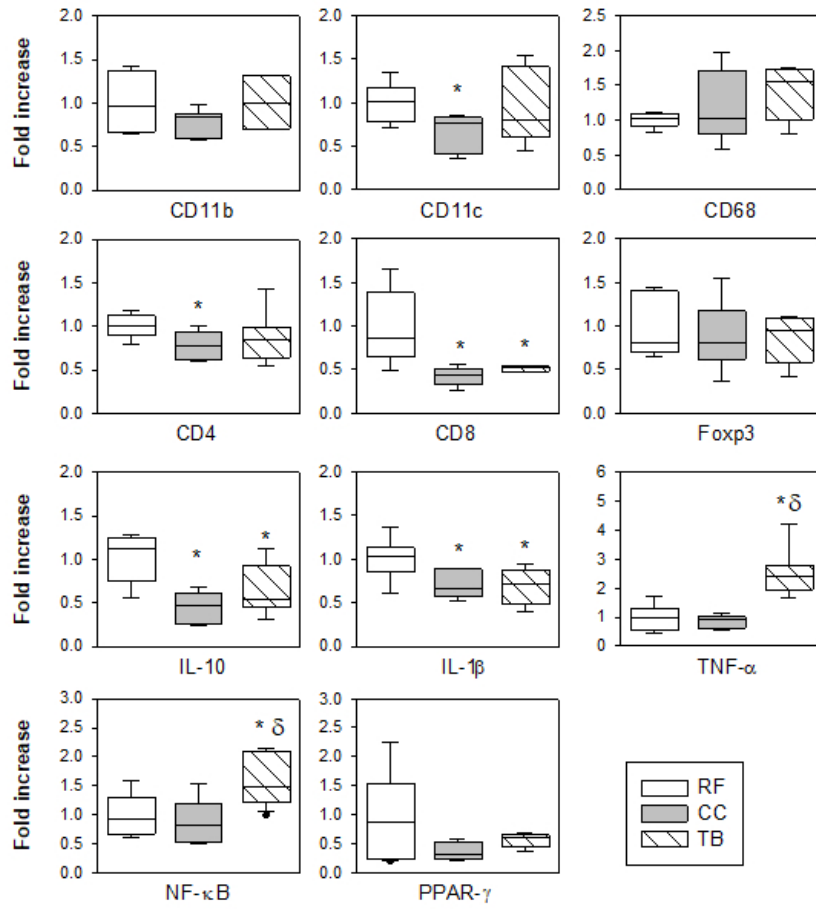
Figure 3

247x104mm (72 x 72 DPI)
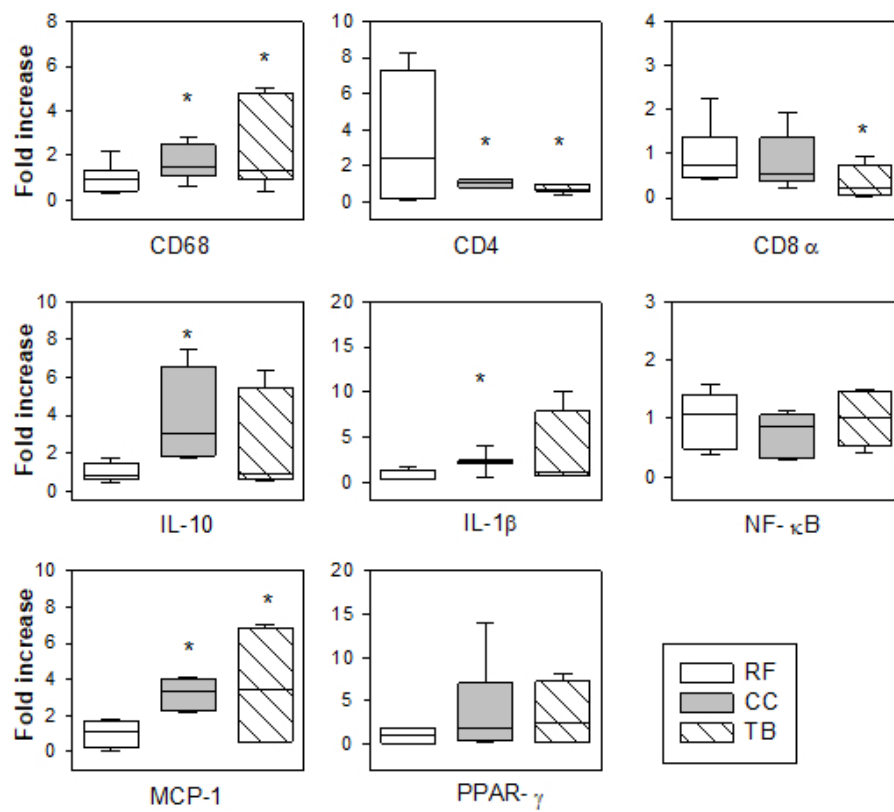
Figure 4

230x222mm (72 x 72 DPI)
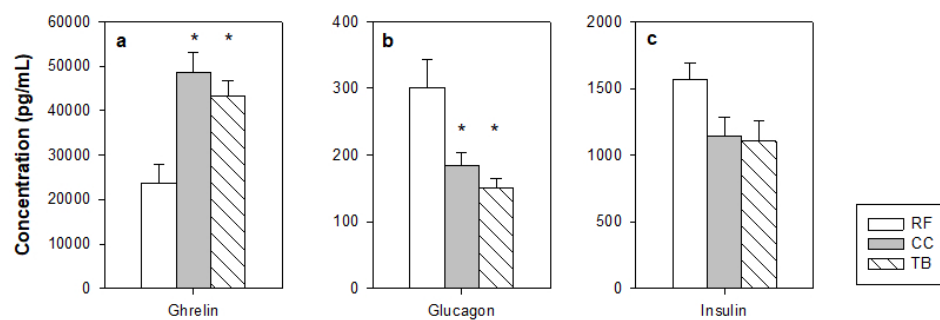
Figure 5

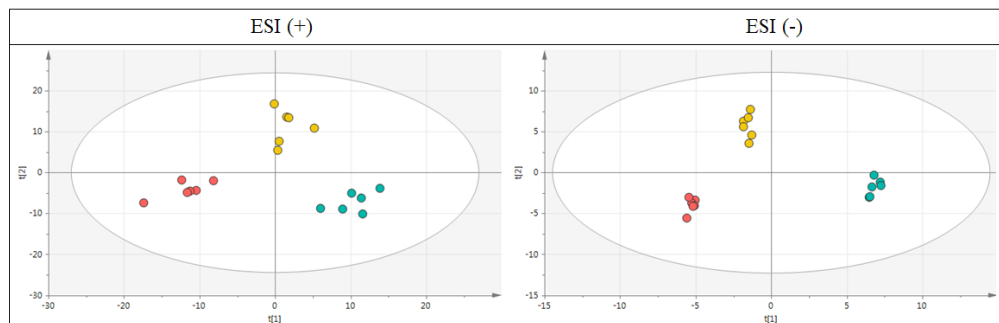201x164mm (72 x 72 DPI)

Figure 6

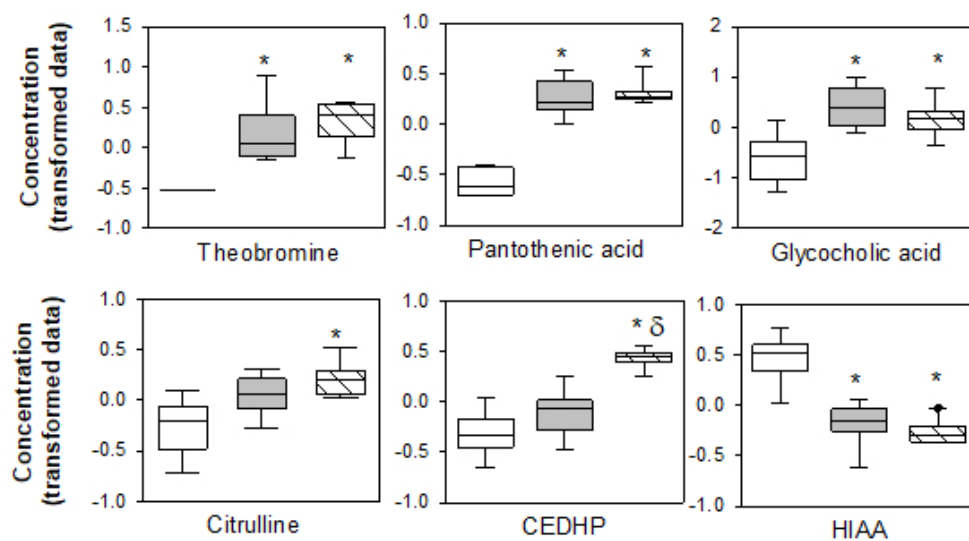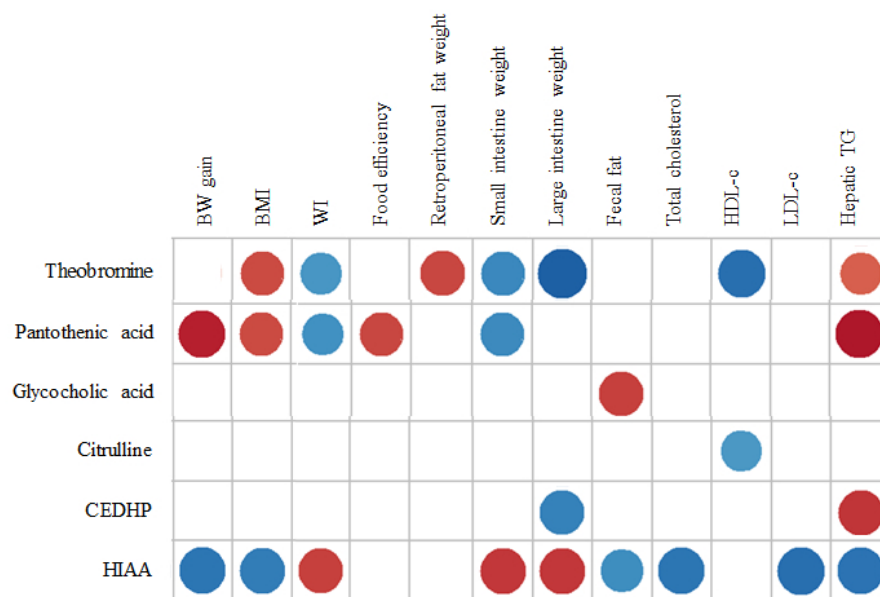299x128mm (72 x 72 DPI)

Figure 7

408x130mm (72 x 72 DPI)

Figure 8

196x109mm (72 x 72 DPI)

Figure 9

273x178mm (72 x 72 DPI)