TITLE

Effect of cocoa's theobromine on intestinal microbiota of rats

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ABBREVIATIONS

CC: cocoa

F/B: Firmicutes/Bacteroidetes ratio

FBS: fetal bovine serum

FCM: flow cytometry

FISH: fluorescence *in situ* hybridization

OTU: operational taxonomic units

PI: propidium iodide

RF: reference

TB: theobromine

1 **ABSTRACT**

2 SCOPE: To establish the role of cocoa theobromine on gut microbiota composition and

3 fermentation products after cocoa consumption in rats.

4 METHODS AND RESULTS: Lewis rats were fed either a standard diet (RF diet), a diet

5 containing 10% cocoa (CC diet) or a diet including 0.25% theobromine (TB diet) for 15 days.

6 Gut microbiota (fluorescence *in situ* hybridization coupled to flow cytometry and

7 metagenomics analysis), SCFA and IgA-coated bacteria were analyzed in fecal samples.

8 CC and TB diets induced lower counts of *E. coli* whereas TB diet led to lower counts of

9 *Bifidobacterium* spp., *Streptococcus* spp. and *Clostridium histolyticum-C. perfingens* group

10 compared to RF diet. Metagenomics analysis also revealed a different microbiota pattern

11 among the studied groups. The SCFA content was higher after both CC and TB diets, which

12 was mainly due to enhanced butyric acid production. Furthermore, both diets decreased the

13 proportion of IgA-coated bacteria.

14 CONCLUSION: Cocoa's theobromine plays a relevant role in some effects related to cocoa

15 intake, such as the lower proportion of IgA-coated bacteria. Moreover, theobromine modifies

16 gut microbiota although other cocoa compounds could also act on intestinal bacteria,

17 attenuating or enhancing the theobromine effects.

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1. Introduction

Although cocoa powder was initially used for medical purposes by Mesoamerican civilizations [1], it is only recently that cocoa has come to be considered a valuable product with healthy properties [2]. Among these beneficial effects, it has been reported that cocoa-enriched diets modulate the immune system and the gut microbiota [3]. In particular, a cocoa-enriched diet is able to attenuate secretory IgA (S-IgA) in several intestinal compartments [4–6] and also the IgA-coated bacteria proportion in the gut [5]. Moreover, a diet containing 10% cocoa for 6 weeks modifies the intestinal microbiota composition in rats by decreasing the proportion of the *Bacteroides* spp., the *Staphylococcus* spp., and the *Clostridium histolyticum* subgroup [5], and thus causing a different short-chain fatty acid (SCFA) production [7]. Similarly, a cocoa diet modulates the intestinal microbiota in orally sensitized rats, as determined by a metagenomics analysis [8].

Cocoa powder contains macronutrients, fiber, minerals, polyphenols (flavonoids, mainly flavanols) and methylxanthines [9]. The most abundant xanthine found in cocoa is theobromine, followed by caffeine. In fact, cocoa is the richest natural source of theobromine [10, 11]. While the effects of flavonoids present in cocoa have been thoroughly studied, less attention has been paid to the presence of theobromine in cocoa. Even so, a few studies have related its content to a variety of properties attributed to cocoa powder [10, 12]. As theobromine is able to reach the gut [13, 14], we hypothesized that this methylxanthine could contribute to the effects of cocoa intake on gut microbiota. Therefore, the purpose of the present work was to establish the role of cocoa theobromine in the composition of gut microbiota and fermentation products after cocoa consumption in rats.

2. Material and methods

2.1. Animals and diets

Lewis rats (3 week old) obtained from Janvier Labs (Saint-Berthevin Cedex, France) were

housed in cages (2-3 animals/cage on days 0-8, and individually on days 8-15) under

controlled temperature and humidity in a 12:12 h light:dark cycle. The rats were randomly

distributed into three dietary groups (n=7 per group): the reference (RF) group ingested a

standard diet AIN-93M (Teklad, Madison, USA), the cocoa (CC) group ingested a standard

diet with 10% of natural Forastero cocoa (Idilia Foods S.L., Barcelona, Spain) containing

2.5% theobromine, and the theobromine (TB) group ingested a standard diet including 0.25 %

of theobromine (Sigma-Aldrich, Madrid, Spain), i.e. the content of theobromine present in the

CC diet. The two experimental diets were elaborated on the basis of the AIN-93M formula by

subtracting the amount of carbohydrates, proteins, lipids and insoluble fiber provided by the

corresponding supplements. The resulting diets were isoenergetic and contained the same

proportion of macronutrients and insoluble fiber as the RF diet (**Table 1**). Animals were

provided with feed and water *ad libitum* for 2 weeks. Animal procedures were approved by

the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 5988).

2.2. Fecal samples collection and pre-analytical procedures

Fresh feces were collected at days 0, 8 and 15 and processed according to the specific

variables to be analyzed. Some fresh fecal samples were used to determine fecal pH, using a

surface electrode (Crison Instruments, S.A., Barcelona, Spain). The rest of the fecal samples

were directly frozen either at -20 ºC until the metagenomics analysis, the bacterial

characterization by fluorescence *in situ* hybridization, and the IgA-coated bacteria

quantification, or at -80 ºC until SCFA analysis. For these determinations, fecal homogenates

were later obtained following procedures previously described [5].

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2.3. Quantification of fecal microbiota by fluorescence *in situ* hybridization (FISH) coupled to flow cytometry (FCM)

Quantification of representative groups of gut microbiota was carried out in feces from day 15 by FISH coupled to FCM (FISH–FCM), as described by Massot-Cladera et al. [15]. Briefly, fixed fecal suspensions were incubated with Cy5-labeled probes targeting specific diagnostic regions of 16S rRNA from different gut bacterial groups (*Bacteroidaceae-Prevotellaceae* group, Bac303; *Bifidobacterium* spp., Bif164; *Clostridium histolyticum-C. perfringens* group, Chis150; *Escherichia coli*, Ec1531; *Clostridium coccoides-Eubacterium rectale* group, Erec482; *Lactobacillus-Enterococcus* group, Lab158, *Staphylococcus* spp., Staphy; *Streptococcus* spp., Strept) (**Supplementary Table 1**). In the case of *Lactobacillus*, samples were permeabilized with lysozyme (Serva, Heidelberg, Germany) prior to the hybridization process [16]. All samples were hybridized at the specific probe hybridization temperature, as described [15], and kept in the dark at 4 ℃ overnight until FCM analysis.

To determine the total bacteria number, the samples were mixed with propidium iodide (PI, 1 mg/mL; Sigma-Aldrich, Madrid, Spain) prior to FCM analysis [5].

2.4. Determination of the proportion of bacteria coated with IgA

Quantification of IgA-coated bacteria was carried out as previously described [15].

2.5. Flow cytometry analysis

For FISH and IgA-coated bacteria quantification, FCM analysis was performed using a FacsAria SORP sorter (BD, San José, CA, USA) as previously described [5]. Commercial Flow Check[TM] Fluorospheres (Beckman Coulter, Inc. FL, USA) were used to determine total counts combined with PI. Analysis was performed using Flowjo v7.6.5 software (Tree Star,

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92 Inc.). Microbiota composition results are expressed as the $\log_{10}$ of specific probe labeled

93 bacteria counts/g of feces in each sample. Moreover, the *Firmicutes* to *Bacteroidetes* (*F/B*)

94 ratio was calculated taking into account the analyzed bacterial groups belonging to the

95 *Firmicutes* phylum (those hybridized by Chis150, Erec482, Lab158, Staphy and Strept

96 probes) and those belonging to the *Bacteroidetes* phylum (those hybridized by the Bac303

97 probe). IgA-coated bacteria results are expressed as the percentage of bacteria coated with

98 IgA with respect to the total bacteria.

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100 **2.6. Lactic acid and SCFA analysis**

101 After thawing fecal samples, homogenates were centrifuged to remove any particulate matter.

102 Supernatants were filtered using Millex® filters (0.22 µm, Merck Millipore, Darmstadt,

103 Germany). Supernatant (200 µL) was added to 50 µL of the internal standard (2-ethylbutyric

104 100 mM in isopropanol) in a Chromacol VALK vial (Thermo Scientific, Langerwehe,

105 Germany) with a Fisher brand adaptor (Fisher Scientific, Loughborough, UK). Each sample

106 was injected into a 1050 series HPLC System (HP, Crawley, West Sussex, UK) equipped

107 with UV detection. The column used was an ion-exclusion REZEX-ROA organic acid

108 column (Phenomenex, Macclesfield, UK) and a SecurityGuard pre-cartridge (Phenomenex)

109 maintained at 85 °C in a 7981 model oven (Jones Chromatography, Lakewood, USA).

110 Sulfuric acid (2.6 mM) was used as the eluent, and the flow rate was 0.5 mL/min. Peaks were

111 integrated using Agilent ChemStation software (Agilent Technologies, Oxford, UK).

112 Quantification of the samples was obtained through calibration curves of lactic, acetic,

113 propionic, butyric and formic acids (12.5-100 mM). Results were expressed as mM (for total

114 SCFA) and relative increases of the total and individual SCFA with respect to those values

115 found in the RF group.

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2.7. Metagenomics analysis

DNA was extracted from two randomly selected samples from each group using a FastDNA®
SPIN Kit (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol.
Amplicons of 16S rDNA were purified and diluted in equal concentrations prior to
sequencing in Ion Torrent platforms by the Genetic Diagnostic Bioarray facilities (Bioarray,
Alicante, Spain), as previously described [8]. Briefly, a massive sequencing using the QIIME
software package v1.8.0. and USEARCH v7.0.1090 was carried out and the obtained
sequences were assigned into operational taxonomic units (OTUs; sequences that share $\geq 97\%$
similarity) using the UCLUST algorithm and Greengenes reference database (v13_8). Results
are expressed as absolute and relative abundance of phyla and number of detected species.
The bacterial species found among the experimental conditions, in common or not, were also
considered and represented through a Venn diagram.

2.8. Statistical analysis

The normality of continuous variables was assessed by normal probability plots and the
Shapiro–Wilk test, and the variance equality by Levene's test. Non-normally distributed
variables were analyzed by non-parametric tests, specifically Kruskal–Wallis and Mann–
Whitney U tests. Normally distributed variables were analyzed by one-way ANOVA followed
by Bonferroni post hoc significance test. Student T-test was used to analyze the
metagenomics study. $P \leq 0.05$ was considered statistically significant. Statistical analysis was
performed using the software package SPSS 22.0 (IBM Statistical Package for the Social
Sciences, version 22.0, Chicago, IL, USA).

3. Results

3.1. Body weight and food intake

Although the initial body weight was similar among the groups, a statistically slower body weight gain was observed during the study for both the CC and TB groups (**Figure 1A**). The measurement of the food intake revealed that, even though there was not lower food intake when considering the relative amount per body weight (in all cases it was about 12 g/100g of BW), lower absolute food intake per rat in both CC and TB groups than in RF group was found from the first day of diet (**Figure 1B**).

3.2. Gut bacterial populations by FISH–FCM

After 15 days of dietary intervention, significant differences in the gut microbiota composition were observed (**Figure 2**). Concerning total bacteria counts, the CC diet caused the elimination of higher number of bacteria per day than the RF diet. This increase could be associated with the stool amount per day, which was higher in CC rats (3.07 g ± 0.11 g) than that from RF rats (1.78 g ± 0.10 g) (P<0.05). Nevertheless, the total bacteria counts relative to fecal weight from CC fed rats were similar to those in the RF group, whereas the TB group showed lower counts than the other groups (P=0.021 and P=0.055 compared to the RF and CC groups, respectively).

Regarding particular bacterial groups, both the CC and TB groups presented lower counts of *E. coli* than the RF group, with the counts being even lower in the CC group than in the TB one. The TB diet also led to significantly lower counts of *Bifidobacterium* spp., *Streptococcus* spp. and *Clostridium histolyticum-C. perfringens* than the RF group. The decrease in the *Clostridium* group, together with a reduction in the *Bacteroidaceae-Prevotellaceae* group, was also significant compared to the CC group. As a result, the *Firmicutes* counts were lower

164 in feces from the TB group than those from RF rats (P=0.005). Even so, the *F/B* ratio was not

165 significantly modified in the feces of the studied groups.

166

167 **3.3. Quantitative metagenomics analysis of gut bacterial populations**

168 After the FISH-FCM analysis of microbiota, a metagenomics approach was carried out in

169 representative feces, in order to get an idea about the most modified species. The

170 metagenomics analysis allowed the relative abundance of the OTUs to be obtained (**Figure**

171 **3)**.

172 The CC group showed a higher proportion of the *Firmicutes* and a lower proportion of

173 *Bacteroidetes* phylum members than the RF group, which was associated with a significantly

174 higher F/B ratio than the RF and TB groups. The TB group displayed no changes in

175 *Firmicutes* and *Bacteroidetes* phyla but showed a higher proportion of the *Tenericutes*

176 phylum than the RF and CC groups. A further analysis also revealed changes in the relative

177 abundance of some species (**Table 2**). Regarding *Bacteroidetes* phylum, the proportion of the

178 *Bacterioidales* order and particularly of the *Bacteroides* genus, e.g. *B. acidifaciens,* decreased

179 with CC intake, whereas the percentage of the *Prevotella* genus increased, which was not

180 observed in the TB group. Moreover, in the *Cyanobacteria* phylum, CC diet led to a higher

181 proportion of the *Streptophyta* order. With regard to the *Firmicutes* phylum, CC diet led to a

182 higher proportion of the SHA-98 and *Clostridiales* order, *Butyrivibrio* genus

183 (*Lachnospiraceae* family) and *Ruminococcaceae* family, and a lower proportion of other

184 *Clostridiales* (*Peptococcaceae* family and *Anaerotruncus* sp.) species. On the other hand, the

185 TB group showed an increase in the proportion of the *Erysipelotrichaceae* family (*Firmicutes*

186 phylum), *Ralstonia* sp. (*Proteobacteria* phylum) and one bacterium of the *Mollicutes* class

187 (*Tenericutes* phylum) (**Table 2**).

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3.4. Qualitative metagenomics analysis of gut bacterial populations

A total of 71, 80 and 73 different species were detected by metagenomics analysis in feces from the RF, CC and TB groups, respectively (**Supplementary Figure 1A**). To determine the relation among bacterial species present in each group, a Venn diagram was created (**Supplementary Figure 1B**). From all the fecal-detected species, 68 were common to all three studied groups. CC intake led to 11 new species; of these, four species were also found in the TB group (species belonging to *Bacteroidetes, Firmicutes* and *Proteobacteria* phyla) and seven were exclusively detected in the CC group (including species belonging to the *Actinobacteria, Cyanobacteria, Firmicutes* and *Proteobacteria* phyla) (**Table 3**). Only "*Candidatus* Arthromitus" (*Firmicutes* phylum*, Clostridia* class) was found exclusively in the TB group. Two species were only detected in the RF group, which belonged to the *Paraprevotellacea* family (*Bacteroidetes* phylum) and *Coprobacillus* genus (**Table 3**). In addition *Ruminicoccus flavefaciens* (*Firmicutes* phylum) disappeared in the theobromine-fed animals.

3.5 Fecal pH, lactic acid and SCFA

The TB diet led to higher pH values than those found after the RF and CC diets (**Figure 4A**). Fecal concentrations of lactic acid were not significantly affected by the experimental diets (4.26 ± 1.54 mM in RF group; 1.96 ± 0.41 mM in CC group; 2.69±0.73 mM in TB group).

Figure 4B shows the fold-increase of the total and the individual fecal SCFA analyzed (acetic, propionic, butyric and formic acids) in the CC and TB groups compared to the RF group. The intake of CC and TB led to the detection of significantly higher amounts of total SCFA (sum of acetic, propionic, butyric and formic acid) compared to the RF diet (37.8 ± 3.85 mM and 35.9 ± 5.98 mM *vs* 14.5 ± 8.31 mM, respectively). Both CC and TB diets

213   increased by more than seven times the content of butyric acid compared to the RF diet. The

214   CC diet also led to an increase in acetic acid concentration.

215

216   **3.6 Percentage of fecal bacteria coated with IgA**

217   The percentage of IgA-coated bacteria was determined before and at 8 and 15 days of the

218   nutritional intervention (**Figure 5**). The CC group and, to a lesser extent, the TB group

219   showed lower percentages of fecal IgA-coated bacteria compared to the RF group at days 8

220   and 15.

221

222   **4. Discussion**

223   Cocoa-enriched diets have demonstrated their influence on the gut microbiota and the

224   intestinal immune system, which could be partially attributed to the cocoa's polyphenol and

225   fiber content [4, 5, 7, 17]. As far as we are concerned, no data about the effect of theobromine

226   on gut microbiota and immunity have been published before. In the present study, we have

227   established the role of theobromine in the effects of cocoa on gut microbiota composition,

228   SCFA, bacteria coated with IgA and on body weight increase.

229   *In vitro*, *in vivo* and clinical studies demonstrate that cocoa is able to modulate the growth of

230   gut microbiota [5, 7, 15, 18]. Previous studies in rats show that the intake of cocoa-enriched

231   diets for at least three weeks modifies the intestinal microbiota pattern [5, 7, 15]. In the

232   present study, the ingestion of the cocoa diet for two weeks was not able to significantly

233   modify most of the bacterial groups analyzed by FISH–FCM, probably because of the shorter

234   length of this nutritional intervention. However, some changes were observed when

235   theobromine was ingested alone, indicating that theobromine by itself is able to directly or

236   indirectly modify gut microbial populations. The metagenomics analysis, even though it was

237 carried out in a small number of samples, allows to have an idea of particular genera and/or

238 species from gut microbiota modified by CC and TB diets and thus, by using both techniques

239 in a complementary manner, we obtained a wider approach of the gut microbiota changes.

240 According our FISH–FCM results, theobromine seems to exert an inhibitory effect on gut

241 microbiota, mainly on bacteria belonging to the *Firmicutes* phylum (*Clostridium histolyticum-*

242 *C. perfringens* group and *Streptococcus* spp.), *Bifidobacterium* spp. and *E.coli*. The effect of

243 TB partially agrees with previously reported effects of a cocoa diet [5, 7] on *Clostridium* spp.

244 and *Streptococcus* spp. In addition, according to the metagenomics analysis, the decrease in

245 *Firmicutes* could be associated with the disappearance of *Ruminococcus flavefaciens*, a

246 cellulolytic bacterium found to be increased by a flavonoid-enriched diet [19, 20]. The

247 disappearance of *R. flavefaciens* after the TB diet, although it contained the same cellulose

248 amount as the RF and CC diets, may reflect a particular effect of theobromine on this species

249 that could be counteracted by the flavonoid content in the cocoa diet. Conversely,

250 theobromine alone seems to be able to increase other bacteria from the same family

251 (*Erysipelotrichaceae*). This family is decreased by a diet rich in flavonoids [21], which would

252 explain the current changes observed only in the TB group. Furthermore, from the two

253 samples analyzed in the TB group, it can be suggested that theobromine ingested alone

254 induced the presence of "*Candidatus* Arthomitus", another member of the *Firmicutes* phylum.

255 This is a segmented filamentous bacterium able to induce adaptive immune responses in the

256 gut [22], and it can adhere to the epithelial cells in the ileum and Peyer's patches, contributing

257 to the prevention of the colonization of the enteropathogenic *E.coli* O103, *Salmonella*, and

258 others [23, 24].

259 The cocoa diet, including theobromine, seems to induce the growth of bacteria belonging to

260 *Firmicutes*, according to the metagenomics analysis. This increase could be associated with a

261   higher relative abundance of one species from the *Lachnospiraceae* (*Butyrivibrio* genus) and

262   another from the *Ruminococcaceae* families, all of them belonging to the *Clostridia* class.

263   Moreover, the cocoa diet seems to lead to the appearance of new species belonging to the

264   *Clostridia* class (*Dehalobacteriaceae* spp., *Roseburia faecis* and SHA-98 spp), which is in

265   line with the increase of *Lachnospiraceae*, *Clostridiales*, and *Ruminococcaceae* found in pigs

266   fed a grape seed extract [25], and therefore, it could be related to an effect of the cocoa's

267   polyphenol content.

268   In the results of total *Bacteroidetes* phylum by FISH–FCM and metagenomics analyses,

269   discrepancies were observed, which could be due to the low representation of bacterial

270   members of this phylum in the first analysis and/or the low sample size in the second one.

271   Nevertheless, the metagenomics analysis allowed us to suggest changes inside this phylum.

272   For example, one species from the *Paraprevotellaceae* family disappeared with both diets,

273   and the cocoa diet decreased in particular the number of species belonging to the

274   *Bacteroidales* order (*Bacteroides* sp. and *Bacteroides acidifaciens*). The *B. acidifaciens* has

275   been described to be the predominant bacteria responsible for promoting IgA production in

276   the large intestine [26]. This agrees with our current results regarding IgA-coated bacteria and

277   with previous studies showing lower intestinal IgA with a cocoa diet [5, 17, 27]. On the other

278   hand, CC diet increased the relative abundance of *Prevotella* sp., which could be due to its

279   polyphenol content since higher numbers in the *Prevotella* group have been associated with

280   the daily consumption of red wine polyphenols [28].

281   One important finding of our study is that theobromine (both in the CC and TB groups)

282   lowered the counts of *E. coli*. This agrees with the reported inhibitory effects of theobromine

283   on Gram-negative bacteria [29], suggesting an inhibitory effect on the growth of potential gut

284   pathogens. This inhibition was enhanced with the CC diet, suggesting the role of polyphenols

285 in this effect [30]. In the same phylum, *Ralstonia* sp. seems to appear due to the CC and TB

286 diets. *Ralstonia* sp. was formerly included in the *Pseudomonas* genera, which includes species

287 able to degrade methylxanthines [31, 32]. Therefore, its presence may reflect the adaptation of

288 gut microbiota to diets rich in methylxanthines.

289 The impact of theobromine on gut microbiota was also patent in the *Tenericutes* phylum,

290 which increased almost fourfold with theobromine ingested alone. This was associated with a

291 higher number of bacteria belonging to the *RF39* order (*Mollicutes* class). A study reported a

292 similar effect with the ingestion of cocoa for 4 weeks [8]. The absence of effects on

293 *Tenericutes* with the CC diet suggests that other cocoa compounds delayed the theobromine

294 effect on this phylum.

295 With regard to *Actinobacteria*, a prebiotic effect of cocoa polyphenols in humans [33] and of

296 cocoa fiber in rats [7] by increasing the counts of *Bifidobacterium* group has been reported.

297 As TB diet, but not CC diet, decreased the proportion of *Bifidobacterium* spp., it can be

298 suggested that theobromine is counteracting the prebiotic effects of cocoa fiber. However, the

299 metagenomics results suggested no changes in the relative abundance of *Actinobacteria*

300 species, either in the TB or CC diet, although it seems that CC diet leads the appearance of

301 one species of the *Actinomycetales* order. In line with these results, blueberries increased the

302 relative abundance of *Actinomycetales* order in rats, which allows us to suggest the role of

303 polyphenols in such an effect [34]. Finally, the appearance of one species of the *Streptophyta*

304 order (*Cyanobacteria* phylum) with the ingestion of cocoa, in agreement with the reported

305 effect of a CC diet for 4 weeks [8], must be related to the cocoa's polyphenol or fiber content.

306 Nevertheless, the role of such bacteria in the intestinal microbiota remains to be elucidated.

307 Overall, this study reveals the impact of theobromine on gut microbiota. The effects were

308 different depending on whether theobromine was ingested alone or when forming part of

15

309 cocoa, although few common characteristics were found. Some changes observed exclusively

310 in the TB group would have been due to the action of this methylxanthine, which were

311 counteracted by other cocoa compounds, such as fiber and polyphenols. Other changes in the

312 TB group agree with previous results reported with a longer CC diet, suggesting that these

313 other compounds included in the CC diet could delay the TB effect. The modifications

314 exclusively found in the CC group must be related to the cocoa's fiber or polyphenol content.

315 The effect of theobromine on gut microbiota has also been reflected by the changes observed

316 in SCFA in both theobromine-containing diets. The enhanced generation of SCFA was

317 mainly due to the butyric acid. Butyrate is considered the main energy source for colonocytes,

318 and is also important for the regulation of gene expression, the intestinal barrier and the

319 immune system, among others [35, 36]. However, whereas butyric acid increased with both

320 diets, the increase in the proportion of acetic acid was only observed after cocoa ingestion.

321 This disagreement could be due to the fermentation of different substrates with both

322 interventions. After cocoa intake, SCFA would come directly from polyphenol and/or fiber

323 fermentation [7], whereas for the TB diet, changes in the generation of SCFA would be

324 indirectly due to the inhibition of some bacterial populations and thus contribute to enhancing

325 the amount of substrate available for other bacteria. The differential patterns in the SCFA

326 generated support the idea that the ingestion of theobromine alone or as part of cocoa has a

327 different impact on gut microbiota. Furthermore, the unexpected higher fecal pH when

328 theobromine was ingested alone deserves further studies focusing on microbial metabolites

329 which could explain the observed fecal pH changes.

330 The current results evidence that theobromine (both in the TB and CC diets) contributes to the

331 lower proportion of bacteria coated with IgA found after the cocoa diet, in line with previous

332 results [5, 7, 15]. As rats fed the CC diet even showed a lower proportion of IgA-coated

16

bacteria, the combination of cocoa polyphenols with theobromine in the CC diet could have

an additive or a synergistic effect on reducing their proportion. On the other hand, the effect

of cocoa fiber must be discarded because it was associated with an increase in the percentage

of IgA-coated bacteria [7].

Results regarding body weight suggest that theobromine present in cocoa was the main reason

for a slower body weight increase produced by the 10% cocoa diet. In fact, there was a lower

food intake per animal already in the first day of diet, which could affect the body weight

increase and it can also influence gut microbiota. On the other hand, body growth could be

affected by TB influence on metabolism. In this sense, it has been demonstrated that caffeine

has a stimulatory effect on thermogenesis [37] and has been associated with bone mass loss

[38].

In conclusion, here we demonstrate that cocoa theobromine plays a relevant role in some

effects related to cocoa intake, such as lower body weight increase and the proportion of IgA-

coated bacteria. In addition, theobromine modifies gut microbiota, although other cocoa

compounds –such as cocoa polyphenols or fiber– also act on the intestinal bacteria,

attenuating or enhancing the theobromine effects, that overall leads to the global effect of

cocoa on microbiota which differs from that of each particular cocoa component.

Author contributions

The authors' contributions were as follows: À.F., F.J.P.-C. and M.C. conceived and designed

the study; M.C.-B. and M.M.-C. were responsible for the animal experiments and sampling;

S.M.-P. carried out the FISH–FCM and IgA-coated bacteria analyses; S.M.-P, M.C.-B.,

M.M.–C., F.J.P.-C. and M.C. carried out the metagenomics data analysis; M.R.-A. analyzed

the SCFA; S.M –P. and M.C.-B were mainly responsible for the interpretation of the results

357 and the writing of the final manuscript; F.J.P.-C and M.C. contributed to the critical revision

358 of the manuscript. All authors have read and approved the final version of the manuscript for

359 publication.

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FIGURE LEGENDS

Figure 1. Body weight (**A**) and food intake (**B**) throughout the study. The amount of food intake showed in each day was calculated considering the amount fed in each interval divided into the number of days in each period. Values are expressed as mean ± SEM (n=7). RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. Statistical differences between groups and days of study are shown with different letters.
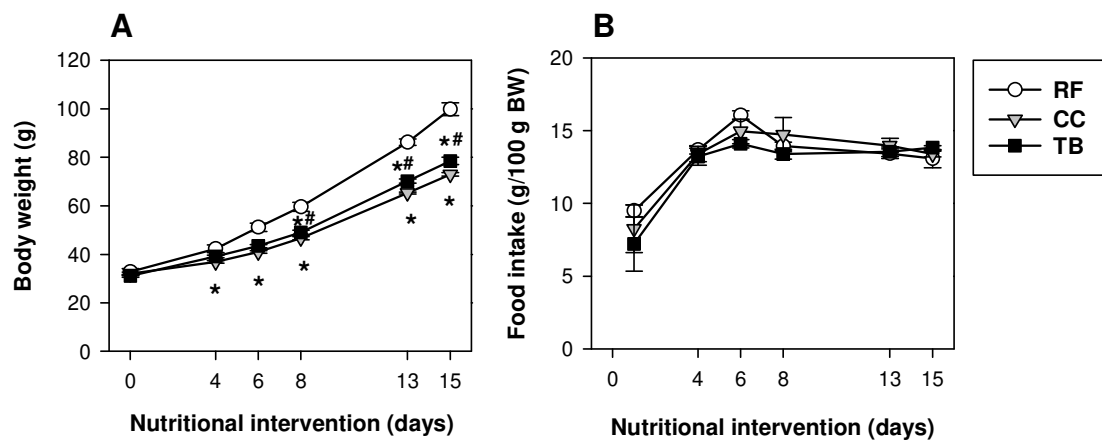
Figure 2: Total bacteria counts, total *Firmicutes* counts, *Firmicutes/Bacteroidetes* ratio, and bacteria counts detected with selected probes indicated in the top determined by FISH–FCM from fecal samples. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. *A: Actinobacteria, B: Bacteroidetes; F: Firmicutes, P: Proteobacteria*. Total bacteria counts are expressed as bacteria/day and bacteria/g feces. Bacterial groups and phylum counts are given as means of $\log_{10}$ bacteria/g feces ± SEM (n=7). * P<0.05 *vs* RF group; # P<0.05 vs CC group.
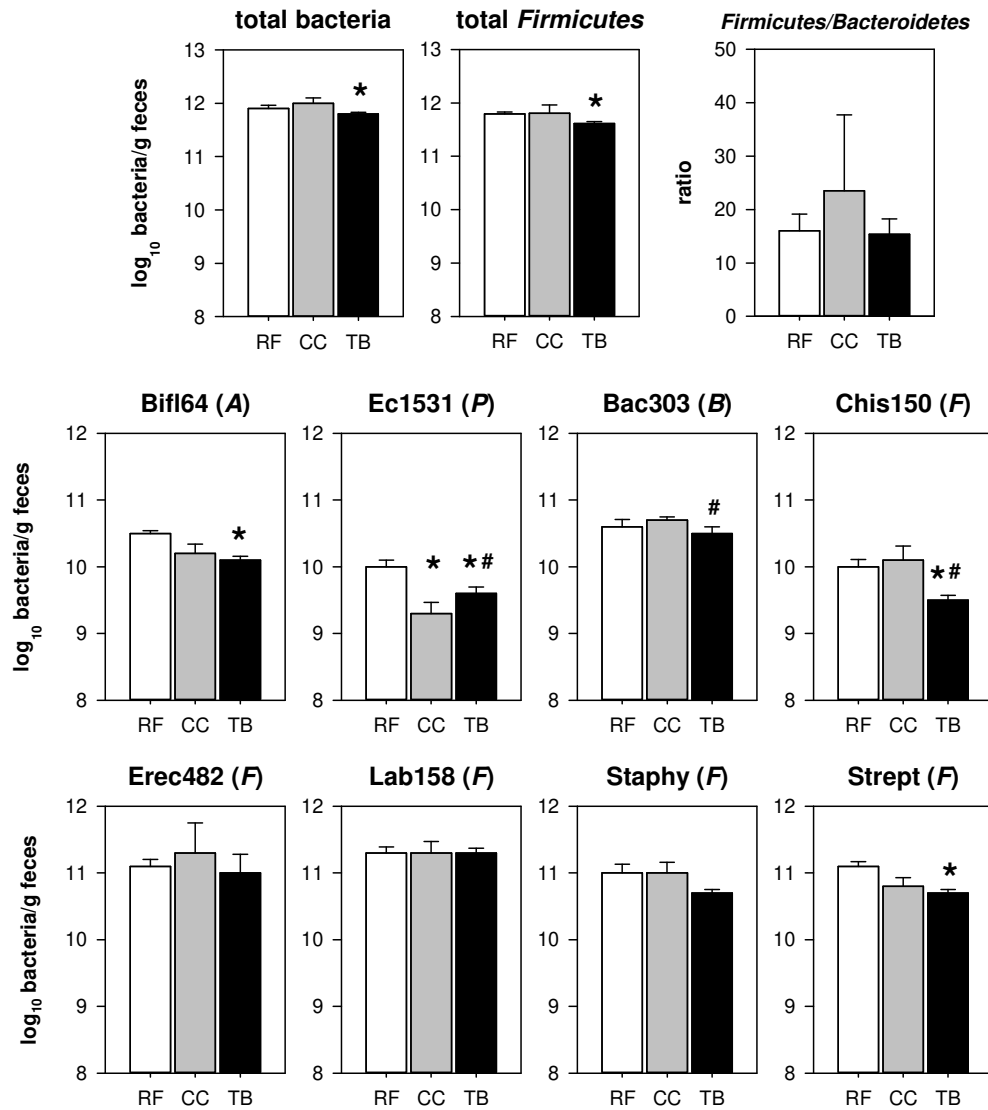
Figure 3. Abundance of phyla found in feces by metagenomics analysis.
Firmicutes/Bacteroidetes ratio and relative abundance (%) of each phylum with respect to the total bacterial DNA for each experimental group. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. Values are given as means ± SEM (n=2). * P<0.05 *vs* RF group; # P<0.05 *vs* CC group.
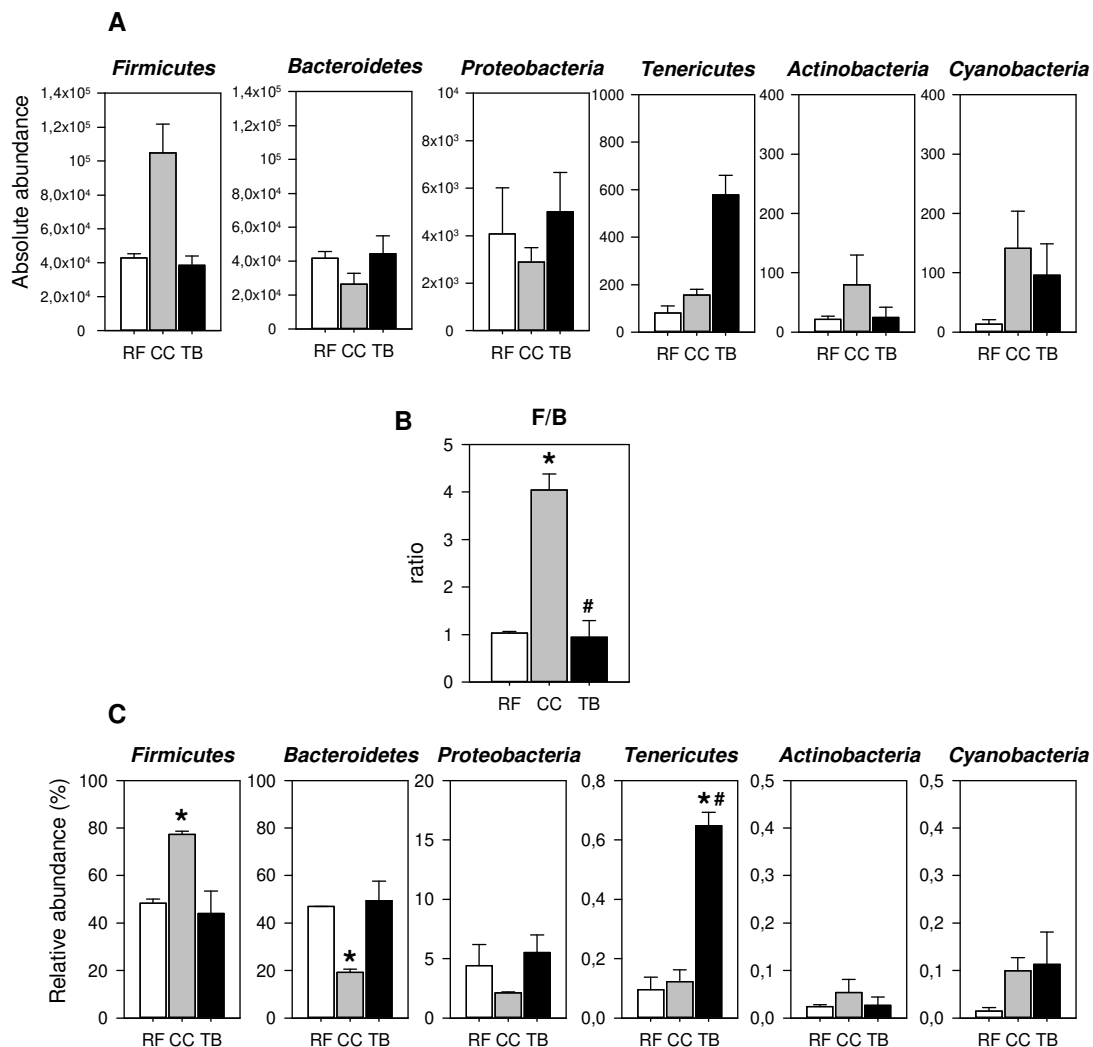
Figure 4. A) Fecal pH. **B**) Fold change of the total and the individual SCFA analyzed compared to the RF diet which was considered as 1. Values are expressed as mean ± SEM (n=7). RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. * P<0.05 *vs* RF group; # P<0.05 *vs* CC group.
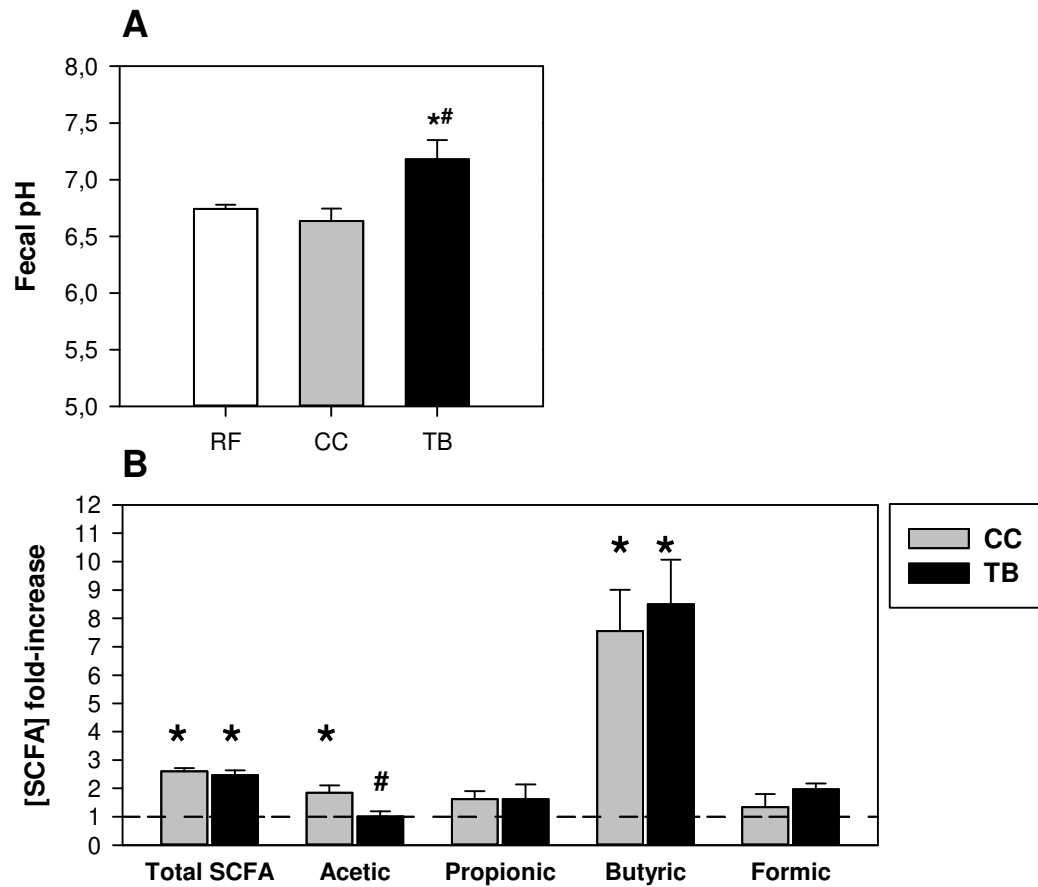
Figure 5. Fecal IgA-coated bacteria throughout the study. Values are expressed as percentage of IgA-coated bacteria (mean ± SEM, n=7). RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25% theobromine. * P<0.05 *vs* RF group; # P<0.05 *vs* CC group.
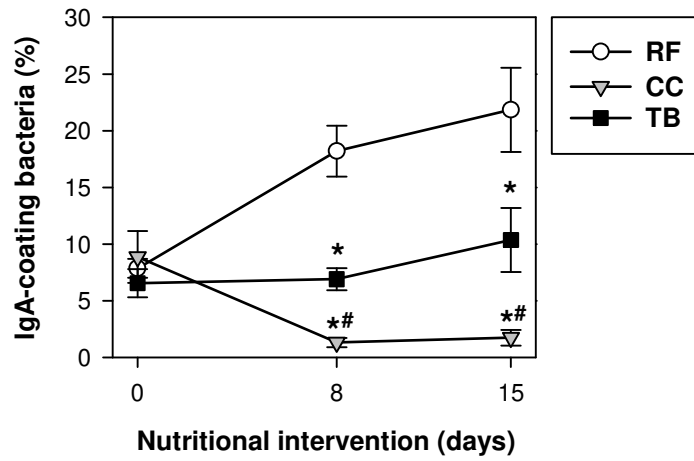
Table 1. Composition of diets used in the study

Components	Diets (g/kg)[a]		
	RF	**CC**	**TB**
Carbohydrates	721.9	709.5	720.1
Proteins	140.8	141.3	140.4
Lipids	38.7	38.5	38.6
Insoluble fiber	50.0	51.2	49.9
Soluble fiber	-	8.9	-
Micronutrients	48.6	44.1	48.5
Theobromine	-	2.5	2.5
Phenolic compounds	-	4.0	-
Total	**1000.0**	**1000.0**	**1000.0**

[a] RF, reference diet; CC, diet containing 10% cocoa; TB, diet containing 0.25% theobromine.

Table 2: Summary of the results found after analysis of OTU relative abundance in samples belonging to the three studied groups. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25% theobromine. Arrows indicate significant changes (P<0.05) for each pairwise comparison.

phylum	class	order	family	genera (species)	CC *vs* RF	TB *vs* RF	TB *vs* CC
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			↓	↓	
			Bacteroidaceae	*Bacteroides*	↓		
				Bacteroides acidifaciens	↓		
			Prevotellaceae	*Prevotella*	↑		↓
Cyanobaceria	*Chloroplast*	*Streptophyta*			↑		↓
Firmicutes	*Clostridia*	*Clostridiales*			↑		↓
			Lachnospiraceae	*Butyrivibrio*	↑		↓
			Peptococcaceae	*rc4-4*	↓		
			Ruminococcaceae		↑		
				Anaerotruncus	↓		
		SHA-98			↑		↓
	Erysipelotrichi	*Erysipelotrichales*	*Erysipelotrichaceae*			↑	
Preoteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Ralstonia*		↑	
Tenericutes	*Mollicutes*	*RF39*				↑	↑

Table 3: Bacteria detected in one or two of the studied groups. Grey color indicates bacteria presence. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25% theobromine.

Phylum	Class	Order	Family	Genus	Specie	RF	CC	TB
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*			▦	▦	
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Coprobacillus*		▦	▦	
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	*flavefaciens*	▦	▦	
Actinobacteria	*Actinobacteria*	*Actinomycetales*	Other	Other	Other		▦	
Cyanobacteria	*Chloroplast*	*Streptophyta*					▦	
Firmicutes	*Clostridia*	*Clostridiales*	*Dehalobacteriaceae*				▦	
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*	*faecis*		▦	
Firmicutes	*Clostridia*	*SHA-98*					▦	
Proteobacteria	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	Other	Other		▦	
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomona*			▦	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	Other		▦	▦
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*			▦	▦
Firmicutes	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	Other		▦	▦
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Ralstonia*				▦
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	"*Candidatus* Arthromitus"				▦

Supplementary Figure 1. Diversity of bacterial species found in feces by metagenomics analysis. **A**) Richness of bacterial species; **B**) Venn diagram of differentially detected species. The diagram shows the absolute number of detected species that belonged to each of the individual nutritional interventions, the detected species common to each pair of groups and the detected species in common to all the three nutritional interventions (in the center of the representation). RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine.

A

B
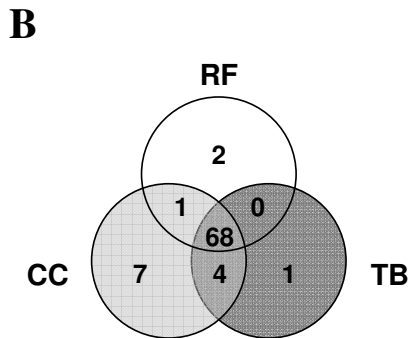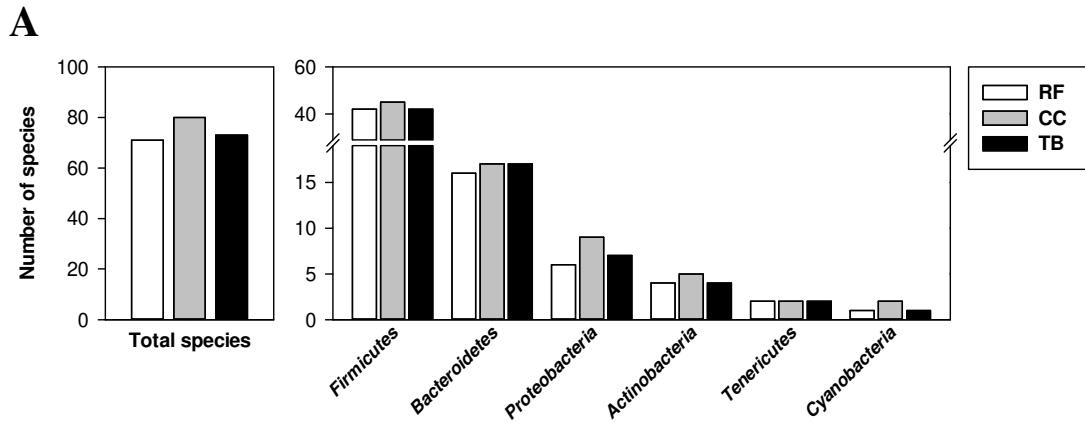
Supplementary Table 1: Bacteria specific probes for the FISH analyses.

Bacterial group	Probe	Sequence (5'-3')	References
Bacteroidaceae-Prevotellaceae	Bac303	CCAATGTGGGGGACCTT	[1]
Bifidobacterium spp.	Bif164	CATCCGGCATTACCACCC	[2]
Clostridium histolyticum-C. Perfringens	Chis150	TTATGCGGTATTAATCTYCCTTT	[3]
Escherichia coli	Ec1531	CACCGTAGTGCCTCGTCATCA	[4]
Clostridium coccoides-Eubacterium rectale	Erec482	GCTTCTTAGTCARGTACCG	[5]
Lactobacillus-Enterococcus	Lab158	GGTATTAGCAYCTGTTTCCA	[6]
Staphylococcus spp.	Staphy	TCCTCCATATCTCTGCGC	[7]
Streptococcus spp.	Strept	CACTCTCCCCTTCTGCAC	[7]

Y= (C/T), R= (A/G)

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Supplementary Table 2: Bacteria detected in one or two of the studied groups. Grey color indicates bacteria presence. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25% theobromine.

Phylum	Class	Order	Family	Genus	Specie	RF	CC	TB
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*			■		
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Coprobacillus*		■		
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	*flavefaciens*	■	■	
Actinobacteria	*Actinobacteria*	*Actinomycetales*	Other	Other	Other		■	
Cyanobacteria	*Chloroplast*	*Streptophyta*					■	
Firmicutes	*Clostridia*	*Clostridiales*	*Dehalobacteriaceae*				■	
Firmicutes	*Clostridia*	*Clostridiales*	*Lachnospiraceae*	*Roseburia*	*faecis*		■	
Firmicutes	*Clostridia*	*SHA-98*					■	
Proteobacteria	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	Other	Other		■	
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomona*			■	
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*	Other			■
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Prevotellaceae*	*Prevotella*				■
Firmicutes	*Bacilli*	*Bacillales*	*Staphylococcaceae*	*Staphylococcus*	Other			■
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Ralstonia*				■
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Candidatus Arthromitus*				■