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

ABSTRACT

SCOPE: To establish the role of cocoa theobromine on gut microbiota composition and fermentation products after cocoa consumption in rats.

METHODS AND RESULTS: Lewis rats were fed either a standard diet (RF diet), a diet containing 10% cocoa (CC diet) or a diet including 0.25% theobromine (TB diet) for 15 days. Gut microbiota (fluorescence *in situ* hybridization coupled to flow cytometry and metagenomics analysis), SCFA and IgA-coated bacteria were analyzed in fecal samples. CC and TB diets induced lower counts of *E. coli* whereas TB diet led to lower counts of *Bifidobacterium* spp., *Streptococcus* spp. and *Clostridium histolyticum-C. perfingens* group compared to RF diet. Metagenomics analysis also revealed a different microbiota pattern among the studied groups. The SCFA content was higher after both CC and TB diets, which was mainly due to enhanced butyric acid production. Furthermore, both diets decreased the proportion of IgA-coated bacteria.

CONCLUSION: Cocoa's theobromine plays a relevant role in some effects related to cocoa intake, such as the lower proportion of IgA-coated bacteria. Moreover, theobromine modifies gut microbiota although other cocoa compounds could also act on intestinal bacteria, attenuating or enhancing the theobromine effects.

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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68 **2.3. Quantification of fecal microbiota by fluorescence *in situ* hybridization (FISH)**

69 **coupled to flow cytometry (FCM)**

70 Quantification of representative groups of gut microbiota was carried out in feces from day 15

71 by FISH coupled to FCM (FISH–FCM), as described by Massot-Cladera et al. [15]. Briefly,

72 fixed fecal suspensions were incubated with Cy5-labeled probes targeting specific diagnostic

73 regions of 16S rRNA from different gut bacterial groups (*Bacteroidaceae-Prevotellaceae*

74 group, Bac303; *Bifidobacterium* spp., Bif164; *Clostridium histolyticum-C. perfringens* group,

75 Chis150; *Escherichia coli*, Ec1531; *Clostridium coccoides-Eubacterium rectale* group,

76 Erec482; *Lactobacillus-Enterococcus* group, Lab158, *Staphylococcus* spp., Staphy;

77 *Streptococcus* spp., Strept) (**Supplementary Table 1**). In the case of *Lactobacillus*, samples

78 were permeabilized with lysozyme (Serva, Heidelberg, Germany) prior to the hybridization

79 process [16]. All samples were hybridized at the specific probe hybridization temperature, as

80 described [15], and kept in the dark at 4 ºC overnight until FCM analysis.

81 To determine the total bacteria number, the samples were mixed with propidium iodide (PI,

82 1 mg/mL; Sigma-Aldrich, Madrid, Spain) prior to FCM analysis [5].

83

84 **2.4. Determination of the proportion of bacteria coated with IgA**

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

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87 **2.5. Flow cytometry analysis**

88 For FISH and IgA-coated bacteria quantification, FCM analysis was performed using a

89 FacsAria SORP sorter (BD, San José, CA, USA) as previously described [5]. Commercial

90 Flow Check™ Fluorospheres (Beckman Coulter, Inc. FL, USA) were used to determine total

91 counts combined with PI. Analysis was performed using Flowjo v7.6.5 software (Tree Star,

Inc.). Microbiota composition results are expressed as the $\log_{10}$ of specific probe labeled bacteria counts/g of feces in each sample. Moreover, the *Firmicutes* to *Bacteroidetes* (*F/B*) ratio was calculated taking into account the analyzed bacterial groups belonging to the *Firmicutes* phylum (those hybridized by Chis150, Erec482, Lab158, Staphy and Strept probes) and those belonging to the *Bacteroidetes* phylum (those hybridized by the Bac303 probe). IgA-coated bacteria results are expressed as the percentage of bacteria coated with IgA with respect to the total bacteria.

2.6. Lactic acid and SCFA analysis

After thawing fecal samples, homogenates were centrifuged to remove any particulate matter. Supernatants were filtered using Millex® filters (0.22 µm, Merck Millipore, Darmstadt, Germany). Supernatant (200 µL) was added to 50 µL of the internal standard (2-ethylbutyric 100 mM in isopropanol) in a Chromacol VALK vial (Thermo Scientific, Langerwehe, Germany) with a Fisher brand adaptor (Fisher Scientific, Loughborough, UK). Each sample was injected into a 1050 series HPLC System (HP, Crawley, West Sussex, UK) equipped with UV detection. The column used was an ion-exclusion REZEX-ROA organic acid column (Phenomenex, Macclesfield, UK) and a SecurityGuard pre-cartridge (Phenomenex) maintained at 85 °C in a 7981 model oven (Jones Chromatography, Lakewood, USA). Sulfuric acid (2.6 mM) was used as the eluent, and the flow rate was 0.5 mL/min. Peaks were integrated using Agilent ChemStation software (Agilent Technologies, Oxford, UK). Quantification of the samples was obtained through calibration curves of lactic, acetic, propionic, butyric and formic acids (12.5-100 mM). Results were expressed as mM (for total SCFA) and relative increases of the total and individual SCFA with respect to those values found in the RF group.

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 ≥ 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

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

3.3. Quantitative metagenomics analysis of gut bacterial populations

After the FISH-FCM analysis of microbiota, a metagenomics approach was carried out in representative feces, in order to get an idea about the most modified species. The metagenomics analysis allowed the relative abundance of the OTUs to be obtained (**Figure 3**).

The CC group showed a higher proportion of the *Firmicutes* and a lower proportion of *Bacteroidetes* phylum members than the RF group, which was associated with a significantly higher F/B ratio than the RF and TB groups. The TB group displayed no changes in *Firmicutes* and *Bacteroidetes* phyla but showed a higher proportion of the *Tenericutes* phylum than the RF and CC groups. A further analysis also revealed changes in the relative abundance of some species (**Table 2**). Regarding *Bacteroidetes* phylum, the proportion of the *Bacterioidales* order and particularly of the *Bacteroides* genus, e.g. *B. acidifaciens,* decreased with CC intake, whereas the percentage of the *Prevotella* genus increased, which was not observed in the TB group. Moreover, in the *Cyanobacteria* phylum, CC diet led to a higher proportion of the *Streptophyta* order. With regard to the *Firmicutes* phylum, CC diet led to a higher proportion of the SHA-98 and *Clostridiales* order, *Butyrivibrio* genus (*Lachnospiraceae* family) and *Ruminococcaceae* family, and a lower proportion of other *Clostridiales* (*Peptococcaceae* family and *Anaerotruncus* sp.) species. On the other hand, the TB group showed an increase in the proportion of the *Erysipelotrichaceae* family (*Firmicutes* phylum), *Ralstonia* sp. (*Proteobacteria* phylum) and one bacterium of the *Mollicutes* class (*Tenericutes* phylum) (**Table 2**).

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

increased by more than seven times the content of butyric acid compared to the RF diet. The CC diet also led to an increase in acetic acid concentration.

3.6 Percentage of fecal bacteria coated with IgA

The percentage of IgA-coated bacteria was determined before and at 8 and 15 days of the nutritional intervention (**Figure 5**). The CC group and, to a lesser extent, the TB group showed lower percentages of fecal IgA-coated bacteria compared to the RF group at days 8 and 15.

4. Discussion

Cocoa-enriched diets have demonstrated their influence on the gut microbiota and the intestinal immune system, which could be partially attributed to the cocoa's polyphenol and fiber content [4, 5, 7, 17]. As far as we are concerned, no data about the effect of theobromine on gut microbiota and immunity have been published before. In the present study, we have established the role of theobromine in the effects of cocoa on gut microbiota composition, SCFA, bacteria coated with IgA and on body weight increase.

In vitro, *in vivo* and clinical studies demonstrate that cocoa is able to modulate the growth of gut microbiota [5, 7, 15, 18]. Previous studies in rats show that the intake of cocoa-enriched diets for at least three weeks modifies the intestinal microbiota pattern [5, 7, 15]. In the present study, the ingestion of the cocoa diet for two weeks was not able to significantly modify most of the bacterial groups analyzed by FISH–FCM, probably because of the shorter length of this nutritional intervention. However, some changes were observed when theobromine was ingested alone, indicating that theobromine by itself is able to directly or indirectly modify gut microbial populations. The metagenomics analysis, even though it was

carried out in a small number of samples, allows to have an idea of particular genera and/or species from gut microbiota modified by CC and TB diets and thus, by using both techniques in a complementary manner, we obtained a wider approach of the gut microbiota changes.

According our FISH–FCM results, theobromine seems to exert an inhibitory effect on gut microbiota, mainly on bacteria belonging to the *Firmicutes* phylum (*Clostridium histolyticum-C. perfringens* group and *Streptococcus* spp.), *Bifidobacterium* spp. and *E.coli*. The effect of TB partially agrees with previously reported effects of a cocoa diet [5, 7] on *Clostridium* spp. and *Streptococcus* spp. In addition, according to the metagenomics analysis, the decrease in *Firmicutes* could be associated with the disappearance of *Ruminococcus flavefaciens*, a cellulolytic bacterium found to be increased by a flavonoid-enriched diet [19, 20]. The disappearance of *R. flavefaciens* after the TB diet, although it contained the same cellulose amount as the RF and CC diets, may reflect a particular effect of theobromine on this species that could be counteracted by the flavonoid content in the cocoa diet. Conversely, theobromine alone seems to be able to increase other bacteria from the same family (*Erysipelotrichaceae*). This family is decreased by a diet rich in flavonoids [21], which would explain the current changes observed only in the TB group. Furthermore, from the two samples analyzed in the TB group, it can be suggested that theobromine ingested alone induced the presence of "*Candidatus* Arthomitus", another member of the *Firmicutes* phylum. This is a segmented filamentous bacterium able to induce adaptive immune responses in the gut [22], and it can adhere to the epithelial cells in the ileum and Peyer's patches, contributing to the prevention of the colonization of the enteropathogenic *E.coli* O103, *Salmonella*, and others [23, 24].

The cocoa diet, including theobromine, seems to induce the growth of bacteria belonging to *Firmicutes*, according to the metagenomics analysis. This increase could be associated with a

higher relative abundance of one species from the *Lachnospiraceae* (*Butyrivibrio* genus) and another from the *Ruminococcaceae* families, all of them belonging to the *Clostridia* class. Moreover, the cocoa diet seems to lead to the appearance of new species belonging to the *Clostridia* class (*Dehalobacteriaceae* spp., *Roseburia faecis* and SHA-98 spp), which is in line with the increase of *Lachnospiraceae*, *Clostridiales*, and *Ruminococcaceae* found in pigs fed a grape seed extract [25], and therefore, it could be related to an effect of the cocoa's polyphenol content.

In the results of total *Bacteroidetes* phylum by FISH–FCM and metagenomics analyses, discrepancies were observed, which could be due to the low representation of bacterial members of this phylum in the first analysis and/or the low sample size in the second one. Nevertheless, the metagenomics analysis allowed us to suggest changes inside this phylum. For example, one species from the *Paraprevotellaceae* family disappeared with both diets, and the cocoa diet decreased in particular the number of species belonging to the *Bacteroidales* order (*Bacteroides* sp. and *Bacteroides acidifaciens*). The *B. acidifaciens* has been described to be the predominant bacteria responsible for promoting IgA production in the large intestine [26]. This agrees with our current results regarding IgA-coated bacteria and with previous studies showing lower intestinal IgA with a cocoa diet [5, 17, 27]. On the other hand, CC diet increased the relative abundance of *Prevotella* sp., which could be due to its polyphenol content since higher numbers in the *Prevotella* group have been associated with the daily consumption of red wine polyphenols [28].

One important finding of our study is that theobromine (both in the CC and TB groups) lowered the counts of *E. coli*. This agrees with the reported inhibitory effects of theobromine on Gram-negative bacteria [29], suggesting an inhibitory effect on the growth of potential gut 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

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

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

and the writing of the final manuscript; F.J.P.-C and M.C. contributed to the critical revision of the manuscript. All authors have read and approved the final version of the manuscript for 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.

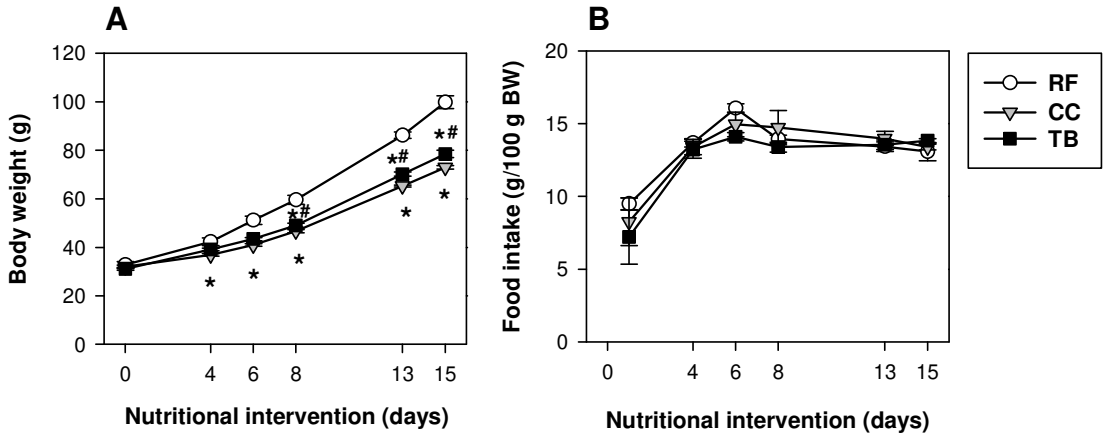


Figure 2: Total bacteria counts, total *Firmicutes* counts, *Firmicutes/Bacteroidetes* ratio, and bacteria counts detected with selected probes indicated in the top determined by FISH–FCM from fecal samples. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % 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.

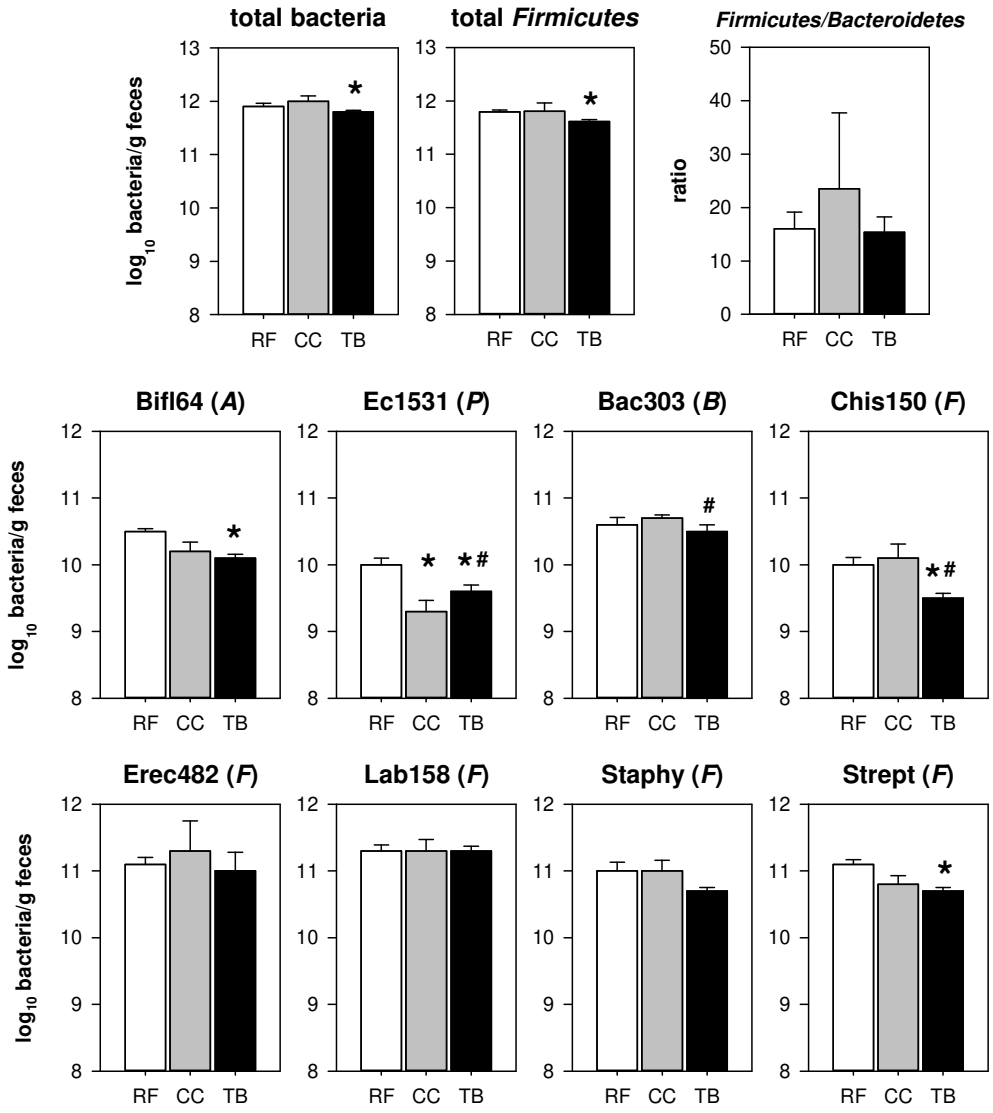


Figure 3. Abundance of phyla found in feces by metagenomics analysis. *Firmicutes*/*Bacteroidetes* ratio and relative abundance (%) of each phylum with respect to the total bacterial DNA for each experimental group. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. Values are given as means ± SEM (n=2). * P<0.05 *vs* RF group; # P<0.05 *vs* CC group.

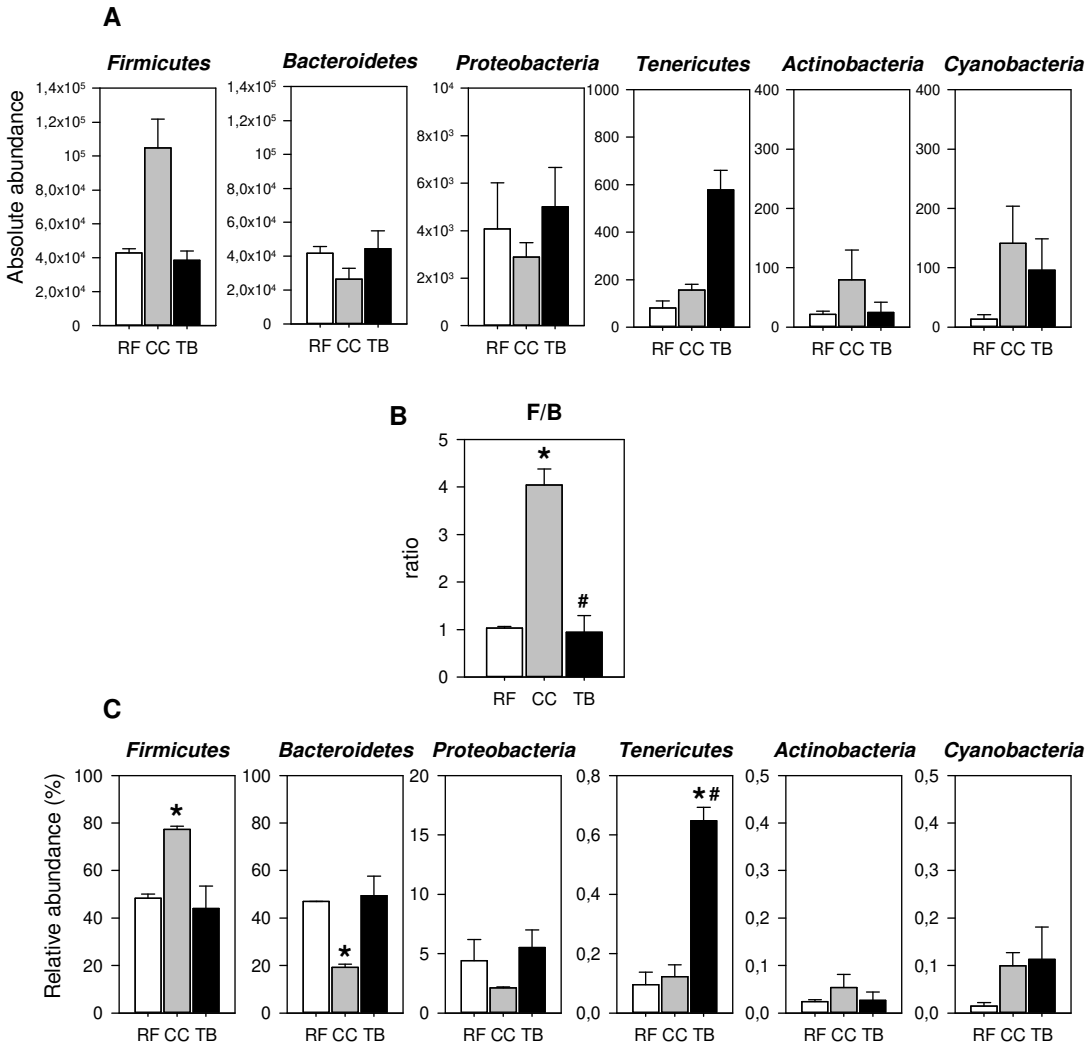


Figure 4. A) Fecal pH. **B**) Fold change of the total and the individual SCFA analyzed compared to the RF diet which was considered as 1. Values are expressed as mean ± SEM (n=7). RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. * P<0.05 *vs* RF group; # P<0.05 *vs* CC group.

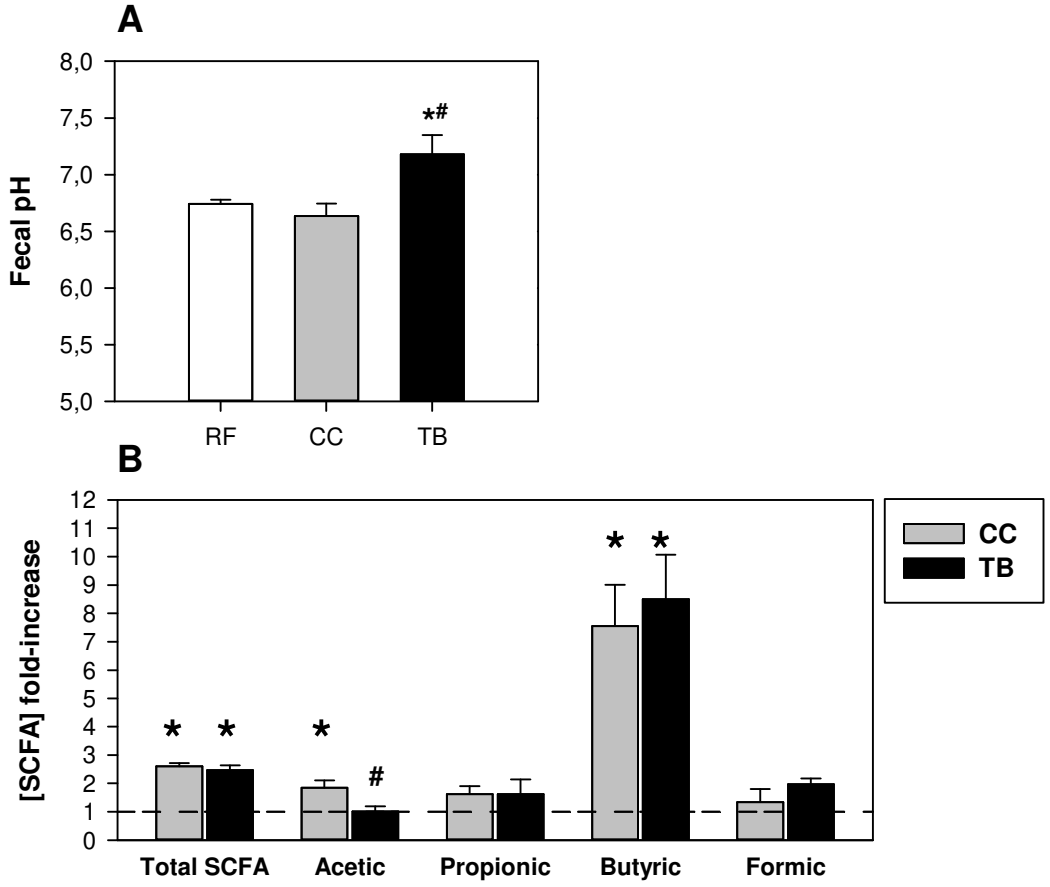


Figure 5. Fecal IgA-coated bacteria throughout the study. Values are expressed as percentage of IgA-coated bacteria (mean ± SEM, n=7). RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25% theobromine. * P<0.05 *vs* RF group; # P<0.05 *vs* CC group.

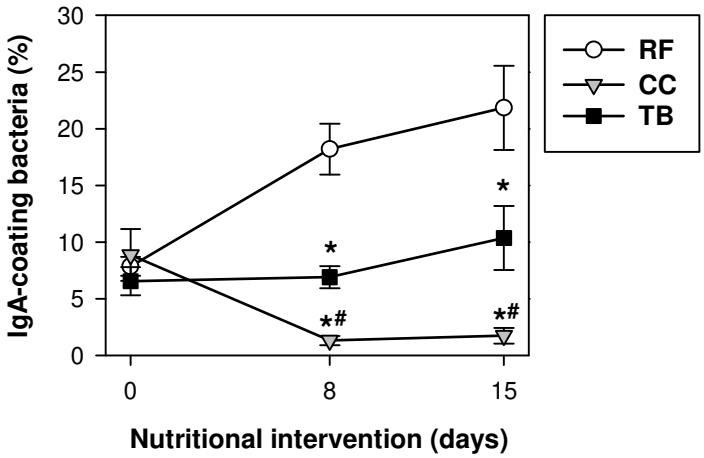


Table 1. Composition of diets used in the study

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.

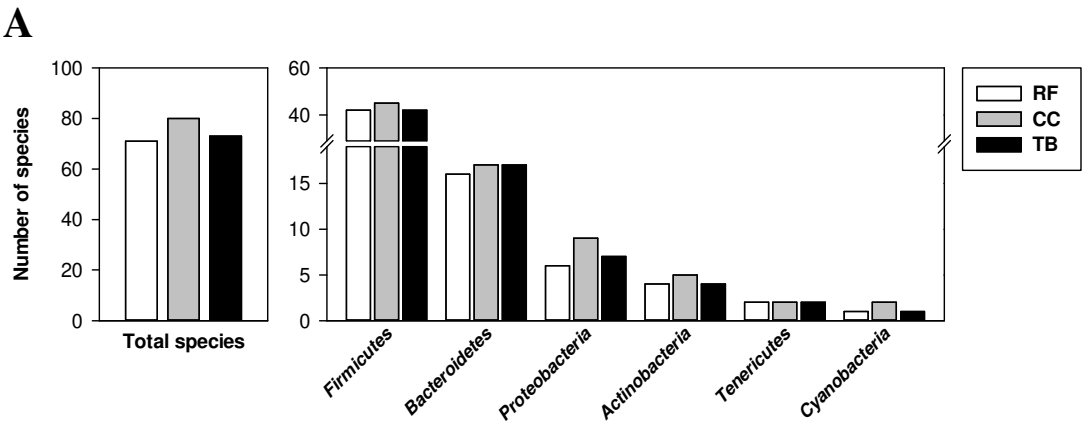
Table 2: Summary of the results found after analysis of OTU relative abundance in samples belonging to the three studied groups. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25% theobromine. Arrows indicate significant changes (P<0.05) for each pairwise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

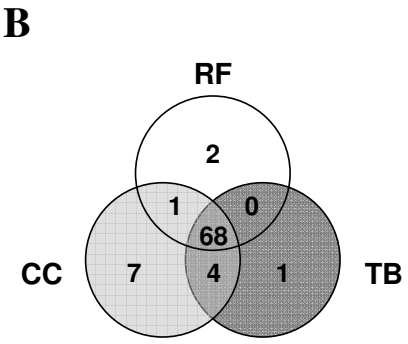
phylum	**class**	**order**	**family**	**genera (species)**	**CC *vs* RF**	**TB *vs* RF**	**TB *vs* CC**
Bacteroidetes	*Bacteroidia*	*Bacteroidales*			↓	↓	
			Bacteroidaceae	*Bacteroides*	↓		
				Bacteroides acidifaciens	↓		
			Prevotellaceae	*Prevotella*	↑		↓
Cyanobaceria	*Chloroplast*	*Streptophyta*			↑		↓
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.





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
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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*				