Prebiotic effects of cocoa fibre on rats

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Abbreviations: AB, applied biosystems; cDNA, complementary deoxyribonucleic acid; CF, a diet based on cocoa fibre; C10, a diet containing 10% cocoa; DF, dietary fibre; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; FCM, flow cytometry; FISH, fluorescence *in situ* hybridization; *FSC/SSC*, forward scatter and side scatter; F/B, *Firmicutes* to *Bacteroidetes* ratio; HB, hybridization buffer; I, diet containing inulin; In, inventoried; Ig, immunoglobulin; *Ocldn*, occludin; PBS, phosphate buffered saline; PI, propidium iodine; REF, REF group which received a standard diet; RNA, ribonucleic acid; RT-PCR, real-time PCR; SCFA, short chain fatty acids; SDS, sodium dodecyl sulfate; TLR, toll like receptor.

Abstract

The impact of cocoa on microbiota composition, its crosstalk with the immune system and the SCFA production, focusing on the involvement of cocoa fibre were investigated Wistar rats were fed for 3-weeks a standard diet, a diet containing 10%-cocoa (C10), cocoa fibre (CF) or inulin (I). Faecal and serum samples were collected before and after the intervention and caecal content and colon sample were collected at the end. Microbiota composition and IgA-coated bacteria and the SCFA content was quantified. The colonic expression of immune-related genes was studied. The CF diet increased *Bifidobacterium* and *Lactobacillus* counts, the proportion of IgA-coated bacteria, the SCFA concentrations and the TLR2, TLR5, TLR7 and occludin expression. With the exception on *Lactobacillus* counts, the I diet modified the other variables in a more modest way than the CF diet. The CF, unlike from the C10 diet, has prebiotic effects and modulates intestinal immune markers.

Keywords: rat, cocoa, cocoa fibre, microbiota, SCFA, TLR

1. Introduction

Cocoa consumption has been suggested to correlate with an amelioration of cardiovascular alterations such as hypertension and atherosclerosis, and with protective effects on proliferative disorders and neurodegenerative diseases (Ellam & Williamson, 2013). In addition, cocoa has been reported to modulate the immune response both at systemic and intestinal levels (Massot-Cladera et al. 2012; Pérez-Berezo et al. 2011; Pérez-Cano et al. 2013), but understanding of the mechanisms and specific components within cocoa which exert these effects is limited. Cocoa contains bioactive compounds such as polyphenols, mainly flavonoids: (+)-catechin and (–)-epicatechin as monomers and procyanidins as polymers, among others (Shahidi & Ambigaipalan, 2015), but cocoa is also a good source of dietary fibre (Lecumberri et al. 2007).

Some of the polyphenols present in cocoa are able to pass intact through the small intestine, reaching the colon (Etxeberria et al. 2013; Monagas et al. 2010), where they are subject to metabolism by the intestinal microbiota. This conversion is essential for their absorption and generates new compounds with potentially greater biological activities than the original compounds (Clifford, 2004; Monagas et al. 2010; Neilson & Ferruzzi, 2011; Selma et al. 2009; Tzounis et al. 2008). Both the dietary polyphenols and their microbially-derived phenolic metabolites modulate the gut microbiota composition (Etxeberria et al. 2013; Lee et al. 2006; Massot-Cladera et al. 2014; Selma et al. 2009; van Duynhoven et al. 2013). Therefore, there is a reciprocal relationship between bacteria and polyphenols: bacteria can be involved in the polyphenol metabolism, and flavonoids can influence microbiota growth and composition (Hayek, 2013).

Dietary fibre (DF) present in cocoa consists of complex carbohydrates which resist hydrolysis and digestion in the stomach and the small intestine, and thus reach the colon intact. DF is then available to be metabolized to oligosaccharides by the colonic microbiota, forming short chain fatty acids (SCFA) during the fermentation and resulting in the production of gases (CO_2, CH_4 and H_2) and heat (Jakobsdottir et al. 2013; Puertollano et al. 2014; Wong & Jenkins, 2007). Acetate, propioniate and butyrate (in a ratio of 3:1:1) are the main SCFAs produced, and are well known to possess beneficial properties for host health (Cook & Sellin, 1998; Wong et al. 2006) through their effects on immunoregulation, colonic gene expression, cancer, obesity, insulin resistance, metabolic regulation as well as maintaining gut and overall health (Fukuda et al. 2011; Gao et al. 2009; Maslowski & Mackay, 2011; Peng et al. 2009; Puertollano et al. 2014). SCFA, especially butyrate, are also used as energy sources for colonocytes (Jakobsdottir et al. 2013; Puertollano et al. 2014). SCFA production is liable to be modified depending, amongst others factors, on the fibre source

and the microbiota composition. In addition to a direct influence of fibre as a substrate for SCFA production, it may also exert an indirect modulatory effect on SCFA production by altering the composition of the gut microbiota (Claesson et al. 2012; Turnbaugh et al. 2009; Wu et al. 2011) primarily by means of a bifidogenic shift (Meyer & Stasse-Wolthuis, 2009). Thus, the proportion of different SCFA producers and, as a result, the SCFA production could be modified by fibre intake (Puertollano et al. 2014).

It has recently been suggested that the phenolic fraction of cocoa could not be the main responsible of the health-related outcomes and may be other cocoa components such as cocoa fibre can be, or at least in part, responsible of such effects (Massot-Cladera et al. 2014). This is also the case for other complex foods, such as grapes (Touriño et al. 2009). On this basis, the aim of the current study was to evaluate the contribution of fibre *vs* phenolics in cocoa on the gut microbiota composition, the production of SCFA and on immune-related markers in rats.

2. Material and Methods

2.1. Animals and diets. Female Wistar rats (3-week-old, 40 animals) were obtained from Janvier Labs (Saint-Berthevin, France) and housed in cages under conditions of controlled temperature and humidity in a 12:12 light-dark cycle. The rats were randomly distributed into four dietary groups (n=10/each). The reference group (REF) was fed with a standard diet AIN-93M (Harlan, Barcelona, Spain); the cocoa group (C10) received chow with 10% cocoa providing a final proportion of 0.4% of polyphenols, 0.85% soluble fibre and 2.55% of insoluble fibre; the cocoa fibre group (CF) was fed a diet with the same soluble and insoluble fibre proportion from cocoa as C10 diet (0.85% and 2.34%, respectively) but with a very low amount of polyphenols (<0.02%), provided by 5.22% cocoa fibre powder; and the reference fibre group (I) received 0.85% of soluble fibre as inulin (**Tables 1 and 2**). The diets, provided *ad libitum*, were fed for three weeks. Natural Forastero cocoa and cocoa fibre (mainly rich in cellulose, hemicellulose and pectic substances) powders (Nutrexpa Group S.L.Barcelona, Spain) were used to elaborate the C10 and CF diets, respectively. Inulin obtained from chicory roots (Fibruline® Instant; InnovaFood 2005, S.L, Barcelona, Spain) was used for the I diet. The three experimental diets were elaborated on basis of the AIN-93M formula by subtracting the amount of carbohydrates, proteins, lipids and insoluble fibre provided by the corresponding cocoa and cocoa fibre. The resulting chows were isoenergetic and had the same

proportion of macronutrients (carbohydrates, proteins and lipids) and insoluble fibre as the standard diet.

Table 1 Composition of experimental diets (g/kg diet). Reference diet (REF); cocoa diet (C10); cocoa fibre diet (CF); inulin diet (I).

Components	REF (g/kg) AIN-93M	C10 (g/kg)	CF (g/kg)	I (g/kg)
Casein	121.5	97.1	109.7	118.7
L-Cystine	1.8	1.4	1.4	1.6
Corn Starch	418.1	423.7	437.2	426.4
Maltodextrin	148.5	118.7	120.4	131.5
Sucrose	102.6	108.7	110.9	110.9
Soybean oil	38.2	26.2	33.5	38.9
Cellulose	50	24.5	26.5	50.0
Minerals	35.3	27.7	27.9	31.3
Vitamins	9.1	7.2	7.2	8.1
Choline bitartrate	2.5	2.0	2.0	2.2
tert-Butylhydroquine	0.008	0.006	0.006	0.006
Water	72.4	63	71.1	72
Cocoa powder	-	100	-	-
Cocoa fiber powder	-	-	52.3	-
Inulin powder	-	-	-	8.5

Body weight and food intake were monitored throughout the study. Experiments were performed according to the Guide for the Care and Use of Laboratory Animals, and experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 358/12).

Table 2 Composition of nutrients provided by the cocoa, cocoa fibre and inulin extracts (g/kg diet).

Components	Cocoa powder (g/kg)	Cocoa fiber powder (g/kg)	Inulin powder (g/kg)
Protein	22	8	-
Carbohydrate	16	0.5	-
Lipid	11	4.8	-
Fiber (insoluble/soluble)	34 (25.5/8.5)	31.9 (23.4/8.5)	8.5 (- /8.5)
Minerals	6	7	-
Total Polyphenols[1]	4	0.02	-
Water	7	-	-

[1] Total polyphenol compounds were quantified by Folin-Ciocalteu.

2.2. Sample collection and processing. At the beginning and end of the study, blood samples were collected and serum was kept at -20 ºC until SCFA analysis. Faecal samples were collected at the same time points and were processed for bacterial characterization and SCFA analysis. After 3 weeks of nutritional intervention, the colon (CO) were excised and immediately immerse in RNAlater® (Ambion, Life Technologies, Austin, TX, USA), incubated at 4 ºC overnight and stored at -20 ºC until PCR analysis. At the same time point, caecum content was obtained and processed for SCFA analysis. Faecal and caecal homogenates were obtained following procedures previously described (Massot-Cladera et al. 2012) with some modifications. Faeces were weighed, diluted 1:10 (w/v) in phosphate-buffered saline (PBS, pH 7.2) and homogenized using a Polytron (Kinematica, Lucerne, Switzerland). The homogenates obtained were then centrifuged (300 *g*, 1 min, 4 ºC) and kept at -20 ºC until the SCFA analysis. Faecal supernatant was used for the bacterial characterization and IgA-coated bacteria determination as described previously (Massot-Cladera, et al. 2012). pH of faecal samples and caecal content was determined using a surface electrode (Crison Instruments, S.A., Barcelona, Spain).

2.3. Short Chain Fatty Acid analysis. Serum SCFA were measured by gas chromatography as previously reported with some modifications (Fernandes et al. 2011; Vogt & Pencharz, 2004). A 200 µL aliquot of serum was filtered through a 30-kDa micropartition system (Vivaspin RC VS02H22 filters, Sartorius Inc., Mississauga, ON, Canada) by centrifugation (14000 *g*, 4 °C, 90 min). The protein-free filtrate supernatant was mixed with 25 µL of internal standard solution consisting of 100 mM ethyl-butyrate and 100 mM formic acid in a 2 mL Hichrom vial (Agilent Technologies, South Queensferry, West Lothian, UK). One microliter of each sample was injected into a 5890 Series II GC system (HP, Crawley, West Sussex, UK) fitted with a NukolTM Capilllary Column (30 m × 0.53 mm × 1.0 µm, SUPELCO^{TM} Analytical, Gilligham ,UK) and flame ionisation detector. The carrier gas, helium, was delivered at a flow rate of 14 mL/min. The head pressure was set at 10 psi with split injection. Run conditions were: initial temperature 60 °C, 1 min; + 20 °C / min to 145 °C; + 4 °C / min to 200 °C, hold 25 min. Peaks were integrated using Agilent ChemStation software (Agilent Technologies, Oxford, UK) and SCFA content quantified by single point internal standard method. Peak identity and internal response factors were determined using a 1 mM calibration cocktail including acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, ethyl-butyric and caproic acids.

To prepare the faecal and caecal diluted samples at 1:10 (w/v) for gas chromatography, 500 µL of faecal and caecal homogenates were centrifuged (18000 *g* at room temperature for 10 min), and the

supernatant was filtered using a sterile Millex® syringe-driven sterile filter unit (0.22 µm; Merck Millipore, Eschborn, Germany) and then analyzed for SCFA determination following the same protocol as that used for the serum samples. From the same animal, SCFA were also determined in serum and faecal samples obtained before the nutritional intervention. Therefore, results from these samples are expressed as the difference in SCFA concentration after three weeks of nutritional intervention (µM). Results from caecum content are presented as final SCFA concentration (µM).

2.4. Lactic acid determination. D-lactic acid concentration in caecal and faecal homogenates and serum were quantified by an ELISA kit following the manufacturer's instructions (BioNova Científica, S.L., Barcelona, Spain). Absorbance was measured in a microplate photometer (Labsystems Multiskan, Helsinki, Finland) at 450 nm. Data were interpolated by means of Ascent v.2.6 software (Thermo Fisher Scientific, S.L.) into the standard curves and expressed as the mean ± SEM (µg/mL).

2.5. *Fluorescence in situ hybridization (FISH) of gut microbiota.* The bacterial groups present in faeces were characterized by means of FISH technique using group- or genus-specific fluorochrome-conjugated probes (Sigma-Aldrich, Madrid, Spain), as previously established (Massot-Cladera et al. 2014), with some modifications. The specific probes and controls used in this study, as well as the hybridization conditions, are included as supplementary material **(See supplementary Table S1)**. In order to express the results with respect to total bacteria, the samples were mixed with propidium iodine (PI, Sigma-Aldrich, Madrid, Spain) prior to FCM analysis (Massot-Cladera et al. 2012).

2.6. Immunoglobulin-coated bacterial staining. The proportion of IgA-coated bacteria was determined as previously established (Massot-Cladera et al. 2012) with minor modifications. In the present study, faecal homogenates were diluted in 1% (v/v) FBS/PBS and centrifuged (8000 *g*, 5 min, 4 ºC).

2.7. *Flow cytometry bacterial analysis.* FCM analysis for bacterial characterization was performed using a FacsAria SORP sorter (BD, San José, CA, USA) as previously described (Massot-Cladera et al. 2012). 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, Inc.). Microbiota composition results were expressed as the faecal positive cells/g of faeces in each sample. Taking into account the studied genera, the *Firmicutes* (F) to

Bacteroidetes (B) ratio (F/B ratio) for each group was also expressed. Ig-coated bacteria results were expressed as previously described (Massot-Cladera et al. 2012).

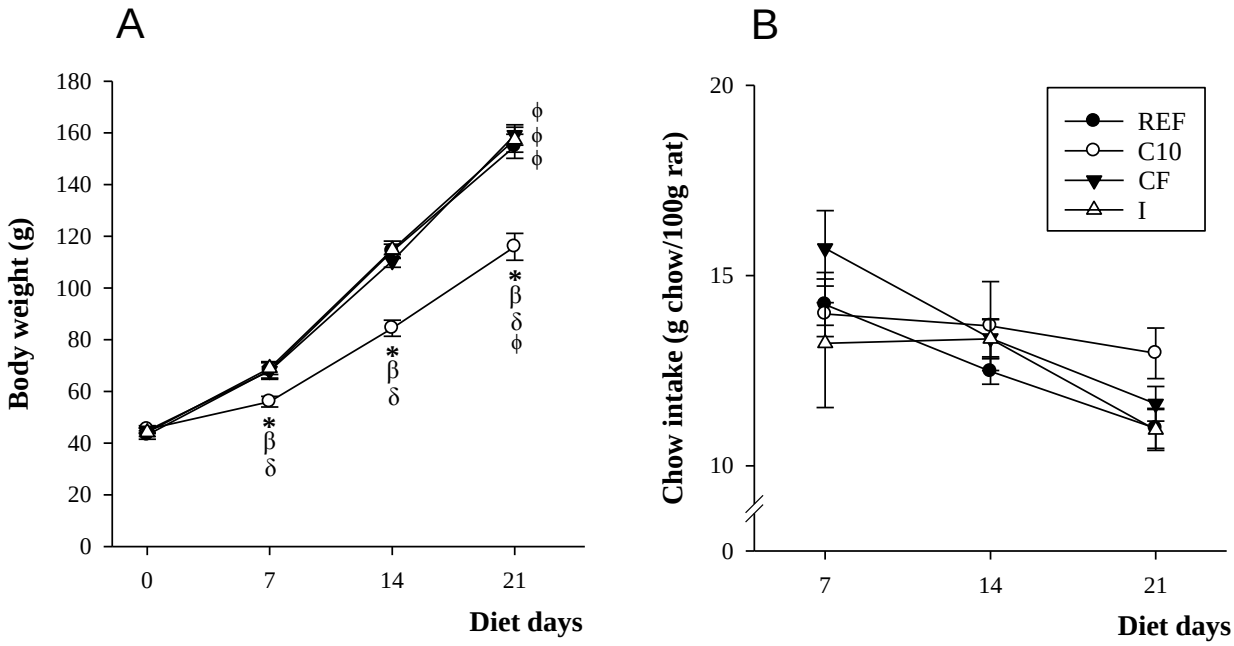
2.8. Assessment of RNA gene expression by RT-PCR. The RNA was isolated from colonic tissue samples in RNAlater® by the RNAeasy® mini Kit (Qiagen, Madrid, Spain) following the manufacturer's recommendations. The NanoDrop spectrophotometer and NanoDrop IVD-1000 V.3.1.2 software (NanoDrop Technologies, Wilmington, DE, USA) were used to quantify the amount of RNA obtained. The Agilent 2100 Bioanalyzer with the RNA 6000 LabChip 1 kit (Agilent Technologies, Madrid, Spain) was used to assess the RNA integrity for each sample. Two μg of total RNA was converted to cDNA. Specific PCR TaqMan® primers and probes (Applied Biosystems, AB, Weiterstadt, Germany) were used to measure selected targets: *tlr2* (Rn02133647_s1, inventoried (In)), *tlr4* (Rn00569848_m1, In), *tlr5* (Rn04219239_s1, In), *tlr7* (Rn01771083_s1, In), *tlr9* (Rn01640054_m1, In) and *Ocldn* (Rn00580064_m1, In). Quantitative real-time PCR assays were performed in duplicate for each sample using an ABI PRISM 7900HT Sequence Detection System (AB). Quantification of the studied genes was normalized to the housekeeping *Gusb* (Rn00566655_m1, In). The SDS v2.4 software (AB) was used to analyze the expression data. The amount of target mRNA relative to the endogenous control expression was calculated for the three nutritional intervention groups relative to values from the REF group which represents 100% gene expression, using the standard $2^{-\Delta\Delta Ct}$ method, as previously described (Pérez-Cano et al. 2009). Results are expressed as the mean ± SEM of the percentage of these values.

2.9. *Statistical analysis.* Levene's and Kolmogorov–Smirnov tests were applied to assess variance equality and normal distribution, respectively. Conventional one-way ANOVA followed by the Bonferroni *post hoc* significance test was performed when the assumptions of normality and equal variance were met. Where this was not the case, non-parametric tests (Kruskal-Wallis and Mann–Whitney *U* rank-sum test) were used to assess significance and in some cases the non-parametric Friedman test was used to compare three or more matched groups. The Spearman's correlation test was used to calculate the correlations between bacterial count changes and either faecal or caecal pH, SCFAs production or Ig-coated bacteria changes as a result of the dietary intervention. Statistical analysis was performed using the software package SPSS 22.0 (SPSS, Inc.) and significant differences were established at $p < 0.05$.

3. Results

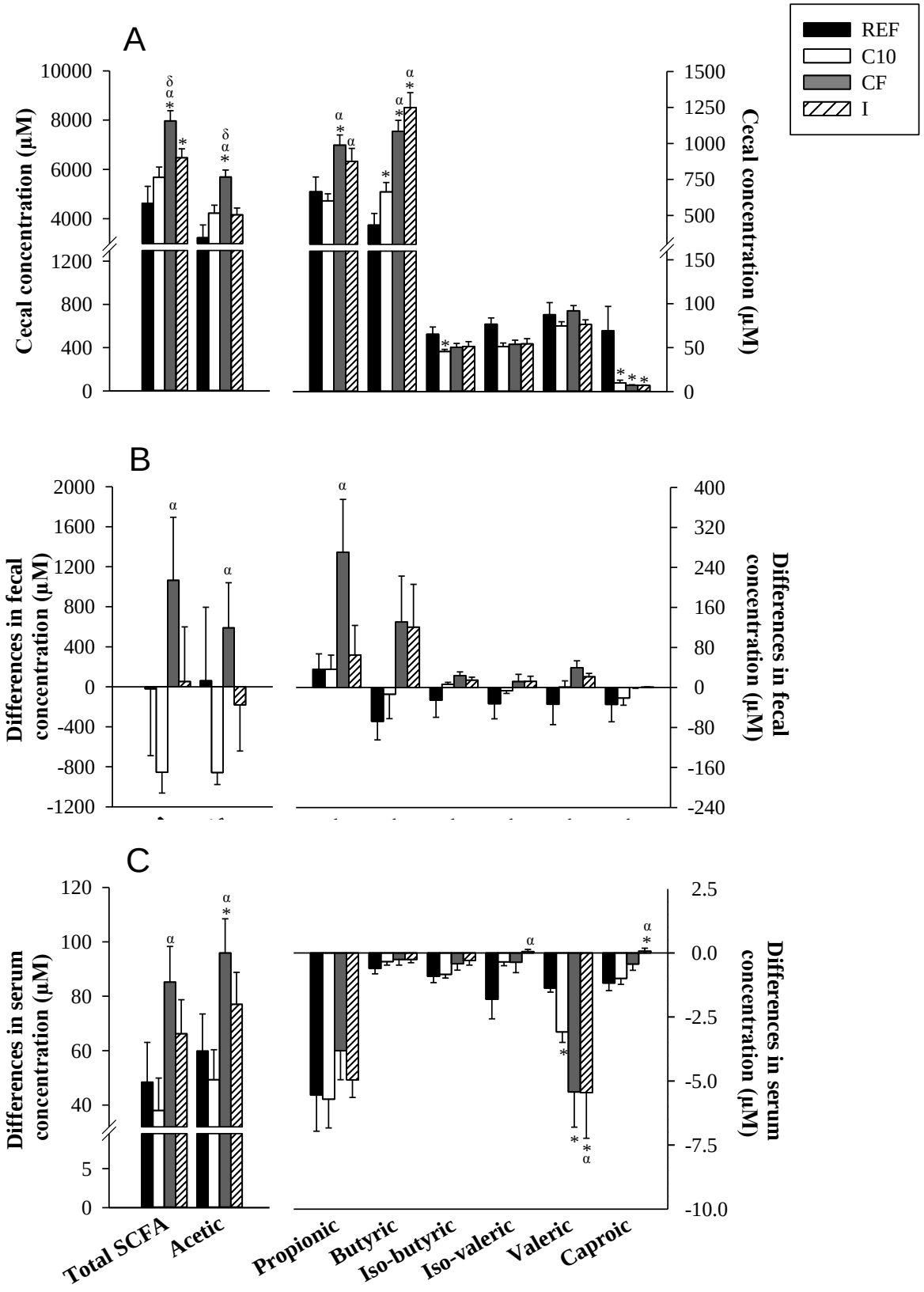
*3.1. Body weight and chow intake***.** Body weight and chow intake were monitored throughout the study (**Fig. 1**). Although the initial body weight was similar among the groups, a statistically slower body weight gain was observed in cocoa-fed animals in comparison to the other groups ($p < 0.01$ day 7, 14, 21) (**Fig. 1A**). This effect was not related to lower chow intake, which was similar throughout the study among all experimental groups (**Fig. 1B**).

Fig. 1 Body weight (A) and chow intake (g/100 g rat/day) (B) monitored throughout the nutritional intervention. Values are expressed as mean ± SEM (n=10). Reference diet (REF); Cocoa diet (C10); Cocoa fibre diet (CF); Inulin diet (I). Statistical differences: *p < 0.05 *vs* REF; α p < 0.05 *vs* C10; β p < 0.05 *vs* CF and δ p < 0.05 *vs* I.



3.2. SCFA production. At the end of the study, for all groups, the highest concentration of SCFA was detected in the content of the caecum (6184.5 ± 305.2 µM), which was approximately twofold higher than that quantified in faecal samples (3053 ± 1451.4 µM). The lowest concentration of SCFA was detected in serum samples (216.9 ± 6.2 µM).

Fig. 2 Effects of cocoa fibre on SCFA production. Caecal SCFA concentration at the end of the study (A, B); and difference in faecal (C,D) and serum (E,F) SCFA concentration compared to the initial values after three weeks of nutritional intervention. Values are expressed as mean ± SEM (n=10). Reference diet (REF); Cocoa diet (C10); Cocoa fibre diet (CF); Inulin diet (I). Statistical differences: *p < 0.05 *vs* REF; α p < 0.05 *vs* C10; β p < 0.05 *vs* CF and δ p < 0.05 *vs* I.



Regarding caecum SCFA content (**Fig. 2A and 2B**), the C10-fed animals presented higher butyric acid and lower iso-butyric and caproic acid concentrations than the animals fed with a standard diet ($p = 0.028$, 0.049 and 0.002, respectively). The CF diet resulted in higher concentration of the total caecal SCFA in comparison to the other dietary groups ($p = 0.0001$ *vs* REF, 0.012 *vs* C10 and 0.010 *vs* I). This increase was reflected in all three SCFA: acetic ($p = 0.0001$ *vs* REF, 0.043 *vs* C10 and 0.031 *vs* I), propionic ($p = 0.045$ *vs* REF and $p = 0.010$ *vs* C10) and butyric ($p < 0.0001$ *vs* REF and $p = 0.006$ *vs* C10) acids. The I diet decreased the caproic concentration compared to the REF group ($p < 0.0001$) whereas it increased the proportion of butyric acid compared to the REF ($p < 0.0001$) and C10 ($p < 0.0001$) diets and the concentration of propionic in comparison with the C10 diet ($p = 0.010$). All these changes were reflected in a higher concentration of total SCFA in comparison to the REF group ($p = 0.05$). Similar caecal lactic acid concentration was detected for all groups (1.17 ± 0.52 µg/mL).

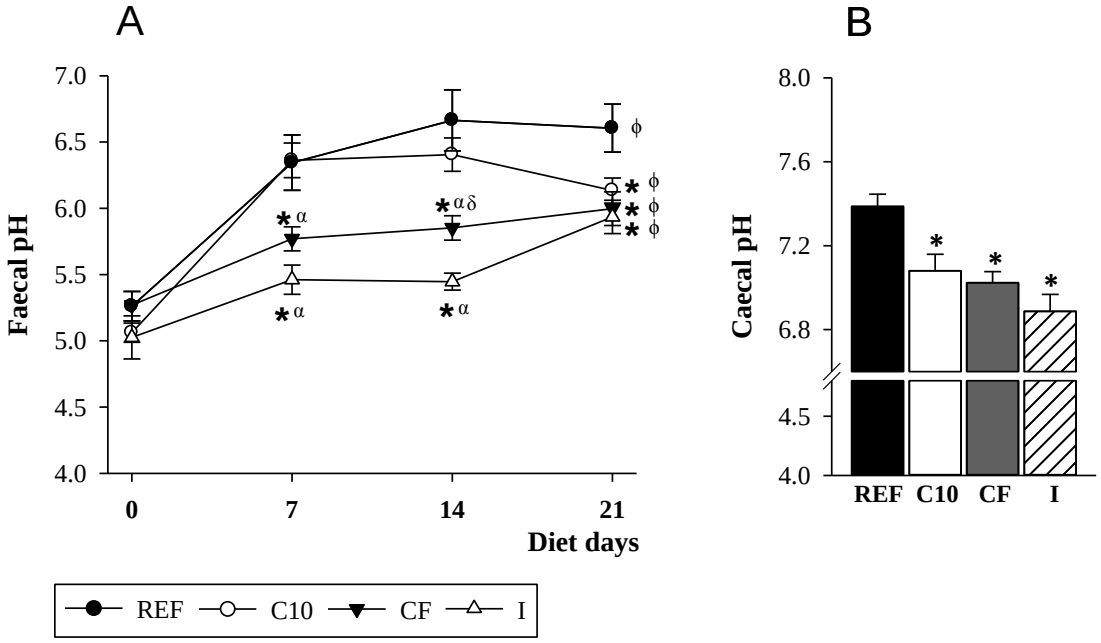
Concerning faecal SCFA, all groups showed a similar pattern at baseline (data not shown). However, after three weeks of nutritional intervention, some significant differences were observed among groups (**Fig. 2C and 2D**). Faecal SCFA were not significantly modified by the C10 diet. The CF diet resulted in an increase in acetic and propionic acid concentrations, which were significantly higher than those in the C10 group ($p = 0.019$ and 0.050, respectively), but did not differ from those observed in the REF group. These increases were reflected in the total SCFA proportion compared to the C10 group ($p = 0.031$). The diet containing inulin tended to reduce acetic acid and to increase butyric and propionic acids proportions with respect to their initial values, although these changes did not differ from the other groups. Regarding lactic acid, all groups showed a similar concentration in faecal samples at the baseline (5.34 ± 1.73 µg/mL) and also after three weeks of nutritional intervention (4.38 ± 0.96 µg/mL) without significant differences among them.

There was a ~ 50 µM increase in total serum SCFA concentration in the REF group due to a rise in acetic acid, while the concentrations of other SCFA were reduced (**Fig. 2E and 2F)**. The C10 diet resulted in a significant reduction in serum valeric acid ($p = 0.0001$ *vs* REF). The CF diet produced the highest increase in serum total SCFA concentration after three weeks of nutritional intervention ($p = 0.034$ *vs* C10) due to a higher proportion of acetic ($p = 0.05$ *vs* REF and $p = 0.019$ *vs* C10). Nevertheless, CF diet also decreased serum valeric acid ($p < 0.0001$). The inulin-fed animals showed an increase in serum caproic and iso-valeric acid concentrations which differed from those quantified in the REF and C10 groups ($p = 0.002$ *vs* REF and $p = 0.007$ and 0.041 *vs* C10,

respectively). In addition, I diet resulted in stronger valeric acid decrease than that produced by the REF ($p = 0.004$) and C10 diets ($p = 0.05$) and similar to that seen in the CF diet. Before nutritional intervention, acid lactic was not detected in serum from any group and three weeks later all groups showed a similar concentration (0.75 ± 0.17 µg/mL).

3.3. Faecal and caecal pH. All groups showed a similar faecal pH at baseline (~5 - 5.5) which significantly increased up to ~6-6.5 three weeks later ($p < 0.05$) (**Fig. 3A**). The C10 and REF groups had a similar faecal pH pattern until day 14, after that C10 diet reduced it ($p = 0.035$). CF and I diets resulted in a significant lower increase in the faecal pH from day 7 of diet in comparison to the REF ($p = 0.015$ and 0.002) and C10 groups ($p = 0.001$ and 0.0003). Significance remained until the end of the study with respect to the REF group ($p = 0.009$ in both cases). The reduction produced in the first two weeks by the I diet was stronger than that produced by the CF diet (day 14; $p = 0.001$). Similarly to faecal pH, at the end of the study, the caecal pH was significantly reduced by C10, CF and I diets when compared to the REF group ($p = 0.020$, 0.004 and < 0.0001, respectively) (**Fig. 3B**).

Fig. 3 Effects of cocoa fibre on faecal (A) and caecal pH (B) after three weeks of nutritional intervention. Results are expressed as mean ± SEM (n = 10). Reference diet (REF); Cocoa diet (C10); Cocoa fibre diet (CF); Inulin diet (I). Statistical differences: *p < 0.05 *vs* REF; α p < 0.05 *vs* C10; β p < 0.05 *vs* CF, δ p < 0.05 *vs* I and Φ p < 0.05 *vs* initial values.



3.4. Microbiota characterization by FCM-FISH. After three weeks of nutritional intervention, there were significant differences in the gut microbiota composition between groups (**Table 3**).

Although the total counts of bacteria were not affected by the diets, the C10-fed animals had lower counts of *Bifidobacterium, Staphylococcus* and *Streptococcus* groups and higher of *Bacteroides* and *Clostridium coccoides*/*Eubacterium rectale* in comparison to the REF group ($p < 0.05$ in all cases). Similar effects on *Bacteroides* spp. were observed in the CF and I groups compared to the REF group ($p = 0.021$ and 0.05, respectively). However, both groups fed with different types of fibre without polyphenols (CF and I) resulted in a different gut microbiota composition compared to the REF diet for other genera. Whereas the C10 diet did not modify *Lactobacillus* group counts, CF and I diets enhanced its counts, being higher than that observed in REF group ($p = 0.037$ and 0.001, respectively). This enhancement was stronger in the I group than in the cocoa fibre-fed animals ($p = 0.005$). In contrast to the effects on *Bifidobacterium* spp. in animals fed the C10 diet, both fibre diets significantly enhanced their presence in the intestinal microbiota, in comparison with the REF group ($p < 0.05$ in all cases). Similarly, both diets based on fibre led to higher counts of *Staphylococcus* and *Streptococcus* groups than the C10 diet ($p < 0.05$ in all cases), with only the number of *Streptococcus* after CF intake being statistically higher than that in REF animals ($p = 0.021$). *Clostridium histolitycum*/*C. perfringens* counts in the CF and I groups were also higher than those in the REF group ($p = 0.025$ and 0.005, respectively), also being this increase observed in I-fed animals superior than those observed in the C10 group ($p = 0.034$). Finally, faecal samples from the I group had also higher numbers of *Clostridium coccoides*/*Eubacterium rectale* groups compared to the REF group ($p = 0.016$).

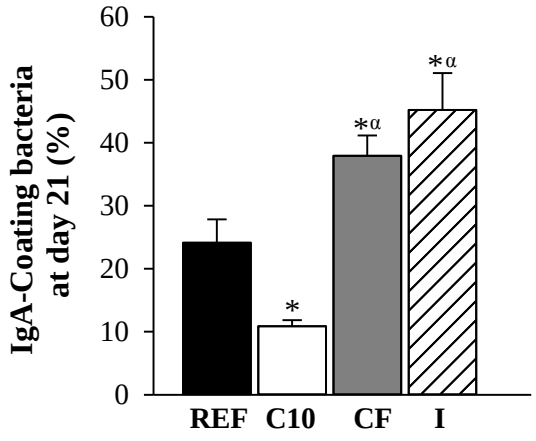
There was an inverse correlation between the faecal pH and the number of *Clostridium histolyticum/C. perfringens* and *Bacteroides* genera at the end of the study ($r = -0.38$, $p = 0.03$ and $r = -0.37$, $p = 0.03$; respectively). Moreover, the C10 decreased the *Firmicutes* to *Bacteroidetes* ratio, and as a result was significantly different from the REF diet ($p = 0.025$), CF ($p = 0.049$) and I groups ($p = 0.023$).

Table 3 Bacterial counts of each genus relative to total bacteria determined by FISH-FCM in faeces. Microbiota composition data are expressed as the mean ± SEM of bacterial cells x10^7/g of faeces. *Firmicutes* to *Bacteroidetes* ratio is expressed for each experimental group as the mean ± SEM. **A**: *Actinobacteria*; **B**: *Bacteroidetes*; **F**: *Firmicutes;* **P***: Proteobacteria*. Reference diet (REF); Cocoa diet (C10); Cocoa fibre diet (CF); Inulin diet (I). Statistical differences: *p < 0.05 *vs* REF; α p < 0.05 *vs* C10; β p < 0.05 *vs* CF.

	After intervention							
Groups	**REF**		**C10**		**CF**		**I**	
	Mean (x10^7 cells/g)	SEM	Mean (x10^7 cells/g)	SEM	Mean (x10^7 cells/g)	SEM	Mean (x10^7 cells/g)	SEM
Total Bacteria	143.43	46.30	146.71	33.25	193.06	26.94	210.78	22.62
Bacteroides (**B**)	3.46	1.14	7.45*	1.40	7.85*	1.63	7.36*	1.64
Bifidobacterium (**A**)	3.71	0.71	1.68*	0.33	10.90*$^{\alpha}$	2.69	16.54*$^{\alpha}$	5.09
Lactobacillus (**F**)	4.21	0.94	7.25	2.65	12.20*	2.77	24.38*$^{\alpha\beta}$	4.23
E. coli (**P**)	2.51	1.10	1.57	0.69	2.49	0.62	2.90	0.61
Staphylococcus (**F**)	5.44	1.74	2.22*	0.61	12.26$^{\alpha}$	3.68	4.99$^{\alpha}$	1.06
Streptococcus (**F**)	5.94	1.83	1.27*	0.21	18.30*$^{\alpha}$	5.20	6.55$^{\alpha}$	0.74
Clostridium histolitycum/ C. perfringens (**F**)	3.40	1.22	6.53	1.69	11.69*	2.94	12.40*$^{\alpha}$	2.24
Clostridium coccoides/ Eubacterium rectale (**F**)	3.41	1.11	9.99*	3.64	9.90	2.61	8.92*	1.39
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
F/B ratio	7.94	1.29	3.15*	0.67	8.06$^{\alpha}$	1.83	9.11$^{\alpha}$	2.82

3.5. IgA-coated bacteria. The proportion of faecal IgA-coated bacteria after the nutritional intervention was ~10 % lower in the C10 group compared to the REF group ($p = 0.007$) (**Fig. 4**). In contrast, the CF and I diets led to a significantly higher proportion of IgA-coated bacteria in comparison with the REF and C10 diets ($p < 0.02$ in all cases).

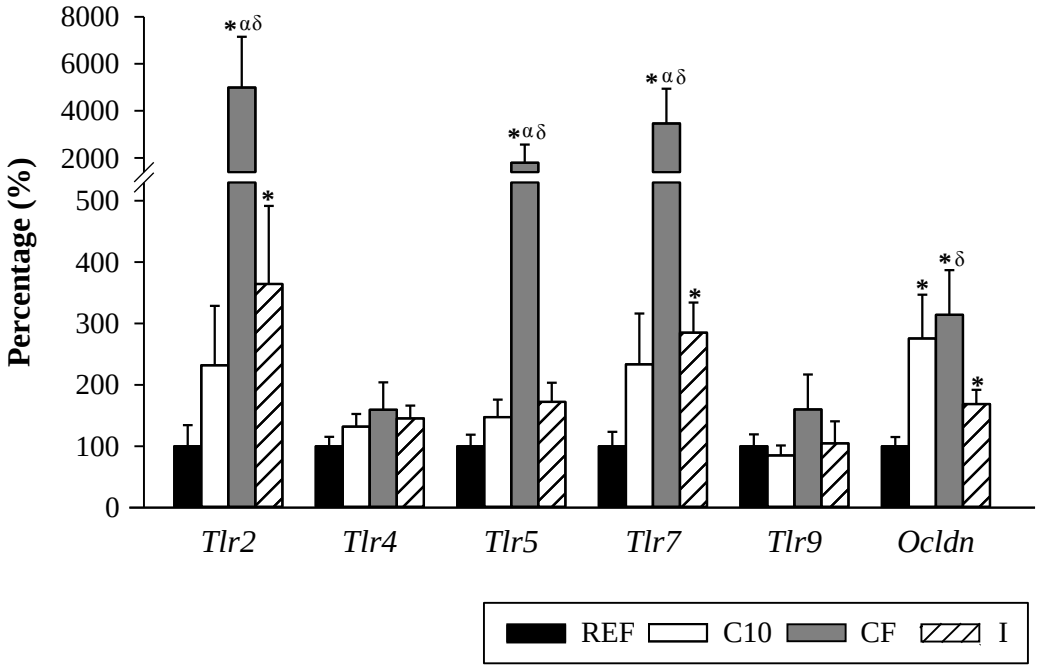
Fig. 4 Effects of cocoa fibre on percentage of faecal bacteria coated with IgA after 3 weeks of nutritional intervention. Results are expressed as mean percentage ± SEM (n = 10). Reference diet (REF); Cocoa diet (C10); Cocoa fibre diet (CF); Inulin diet (I). Statistical differences: *p < 0.05 *vs* REF; α p < 0.05 *vs* C10.



When samples from all groups were considered together, the proportion of IgA-coated bacteria positively correlated with the numbers of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* genera at the end of the study (r = 0.44, p = 0.01 for *Lactobacillus*; r = 0.35, p = 0.04 for *Bifidobacterium* and r = 0.41, p = 0.02 for *Streptococcus*).

3.6. Colonic TLR gene expression. Gene expression of TLR2, TLR4, TLR5, TLR7, TLR9 and occludin was assessed in the colon tissue at the end of the study (**Fig. 5**). The C10 diet did not modify TLR gene expression, but it induced a significant twofold increase in occludin mRNA levels in comparison with the REF group (p = 0.008). Colonic TLR4 and TLR9 gene expression was not modified in the CF-fed animals, whereas TLR2, TLR5, TLR7 and occludin expressions were ~ fiftyfold, twentyfold, thirty-fivefold and threefold up-regulated compared to the REF group, respectively (p < 0.0000, p = 0.002, 0.001 and 0.008, respectively). These significant increases in TLR2, TLR7 and occludin expression were also observed in the inulin-fed animals, but were more modest (p = 0.016, 0.004 and 0.041, respectively).

Fig. 5 TLR gene expressions in the colon after nutritional intervention with cocoa fibre diet. Expression values are normalized using the expression of *Gusb* as the endogenous housekeeping gene. Each bar represents the mean ± SEM of the percentage of the C10 group (white bars), CF group (grey bars) and I group (striped bars) with respect to the REF group (black bars), which represents 100% gene expression. Reference diet (REF); Cocoa diet (C10); Cocoa fibre diet (CF); Inulin diet (I). Significant differences: *p < 0.05 *vs* REF; α p < 0.05 *vs* C10; and δ p < 0.05 *vs* I.



4. Discussion

After ingestion of cocoa, both its polymeric flavonoids and fibre reach the colon intact, where commensal bacteria have an opportunity to metabolize them (Monagas et al. 2010; Selma et al. 2009) and the resulting metabolites can influence the intestinal environment and the immune system. Here we demonstrate that fibre from cocoa, consumed by rats for three weeks, exerts stronger prebiotic effects than inulin by increasing *Bifidobacterium* and *Lactobacillus* genera, SCFA production, and the gene expression of microbial receptors and tight junction molecules while decreasing both caecal and faecal pH.

It is well known that non-digestible complex carbohydrates are metabolized by the microbiota to oligosaccharides and then fermented to SCFA (Puertollano et al. 2014), but fermentation of fibre from cocoa has not been investigated. In the current study, consumption of cocoa fibre led to the profound changes in SCFA production both in faeces and caecum, which were greater than the effects of the well-characterized prebiotic, inulin, but this did not occur after cocoa consumption. Cocoa fibre resulted in higher production of all three of the main SCFA (acetic, propionic and butyric acids) in caecum content, which play an important role in host health (Puertollano et al. 2014; Tan et al. 2014). Nevertheless, the intake of whole cocoa, containing the same proportion of fibre as CF diet, but also the other components present in cocoa, such as flavonoids, did not result in significant changes in all three of the main SCFA, with the exception of a higher butyric acid concentration in the caecum. This suggests that phenolics and other compounds in cocoa may inhibit colonic fibre metabolism. This lack of effect of cocoa could contribute to the lower energy harvest and therefore the lower weight gain observed in animals fed with C10 diet both in the current and in previous studies (Massot-Cladera et al. 2013; Massot-Cladera et al. 2012; Pérez-Berezo et al. 2009; Ramiro-Puig et al. 2007; 2008). Inulin diet did not modify the production of so many SCFA as CF diet did in the current study. However, inulin is a well-known prebiotic extensively metabolized which leads to significant SCFA production (Meyer & Stasse-Wolthuis, 2009; Roberfroid, 2002). These distinguishing results related to the inulin could be attributed to several factors such as the methodologies, population groups, length of intervention as well as the type and dose of inulin used (Kolida et al. 2007). Our results show that cocoa fibre diet has been more metabolized than the inulin one and, therefore higher energy harvest and functional benefits from those generated SCFA.

In order to classify a food ingredient as a prebiotic not only must it be selectively fermented, but it must also be able to alter the colonic microflora towards a healthier composition (Kolida et al.

2002). In the present study, concurrently with changes in the intestinal SCFA production described above, the CF modified the faecal microbiota composition. Specifically, the CF diet resulted in a significantly higher proportion of acetic acid-producing bacteria such as *Bifidobacterium* and *Lactobacillus*, similarly to what happened in I group. The growth of both genera after prebiotic ingestion is commonly related to positive benefits on health. Whole cocoa did not increase *Bifidobacterium* counts, suggesting that, as with SCFA production, other compounds present in cocoa could be suppressing the prebiotic effect exerted by its fibre. The major fraction of cocoa fibre is insoluble and rich in cellulose, followed by highly fermented pectic substances and hemicellulose, which is less fermentable than the former (Lecumberri et al. 2007) and contains a lesser amount of other cocoa compounds (i.e. polyphenols) (Cullen & Oace, 1989). In addition, polyphenols have been also described to possess antimicrobial effects (Lee et al. 2006; Puupponen-Pimiä et al. 2005). Overall, cocoa fibre composition lacking polyphenols allows promoting the growth of beneficial bacteria.

Regarding cocoa polyphenols, controversial results have been reported concerning their capacity to enhance the growth of beneficial bacteria in *in vitro*, *in vivo* and clinical studies (Etxeberria et al. 2013; Fogliano et al. 2011; Massot-Cladera et al. 2014; Tzounis et al. 2008). Some studies suggest that cocoa flavanols promote the growth of *Bifidobacterium*, *Lactobacillus* and *Eubacterium rectale–C. coccoides* genera (Tzounis et al. 2008; 2011). However, we have previously reported a decrease in the proportion of these using different types of cocoa polyphenol extracts (Massot-Cladera et al. 2014). Moreover, cocoa polyphenols have been associated with an inhibitory effect on the growth of *C. histolyticum/C. perfringens*, *Bacteroides*, *Staphylococcus* and *Streptococcus* genera in *in vitro,* preclinical and clinical studies (Massot-Cladera et al. 2012; 2014; Tzounis et al. 2008; 2011). These differential results could be attributed to several factors such as cocoa composition (i.e. polyphenols, fibre, methylxanthines), dose and dissimilar composition and distribution intestinal ecosystem (rats *vs* human gut).

The enhancement of the growth of beneficial bacteria produced by both fibre diets could be partially due to and/or caused by their effects on faecal pH. The faecal content acidification, which is closely related to the concentration of SCFA, could be the consequence of the prebiotic effects of fibre, promoting the growth of specific SCFA-producing bacteria, which are able to resist in this environment. These conditions suppress at the same time the growth of certain opportunistic bacteria. C10 diet also produced more acidic faeces than those from reference group, but less acidic than those observed in CF group. The faecal pH reduction by the C10 diet did not appear to be

associated with the SCFA concentration. Thus, the prebiotic effects of CF showed here may be hindered by other cocoa compounds such as polyphenols or methylxanthines which could reduce the pH through different mechanisms. Further studies should be carried out in order to evaluate the effects of the main methylxanthine present in cocoa (theobromine) on microbiota, SCFA and pH are recommended.

The C10 diet significantly decreased the proportion of IgA-coated bacteria, which is consistent with previous data (Massot-Cladera et al. 2012; 2014). In contrast, the CF and inulin diets increased the proportion of IgA-coated bacteria, once again suggesting that non-fibre components of cocoa counteract the effects of the cocoa fibre. Moreover, the proportion of IgA-coated bacteria was associated with the number of faecal *Lactobacillus*, *Bifidobacterium* and *Streptococcus* genera. These results may suggest that the changes in these bacteria genera influence the differentiation of IgA^+ B cells and/or IgA synthesis in the intestinal compartment, processes in which TLR transduction signals are involved.

For this reason, the gene expression of selected TLRs and molecules involved in intestinal barrier function, and therefore in the paracellular crossing of antigens through epithelial barrier, such as occludin, were also examined in colon specimens. There was greater expression of TLR2, TLR5, TLR7 and occludin in the CF-fed animals than in REF animals, whereas the C10 diet tended to increase the expression of TLR4 and reduce that of TLR9, in agreement with previous studies using a 10% cocoa diet with similar experimental design (Massot-Cladera et al. 2012). Both TLRs and occludin have been positively associated with the appropriate functionality of the intestinal compartment. The principal role of TLRs is defense against opportunistic bacteria by means of promoting signals for IgA and antimicrobial peptide expression, barrier fortification and proliferation of epithelial cells after the bacterial molecules (Abreu et al. 2005; 2010). In particular, TLR2 recognizes a wide variety of molecules (peptidoglycan, lipopeptides and lipoproteins of Gram-negative bacteria, mycoplasma lipopeptides and fungal zymosan); TLR4 interacts with bacterial lipopolysaccharide; TLR5 recognizes bacterial flagellin; nucleic acids are essential to activate TLR7 whereas TLR9 recognizes motifs commonly present in bacterial and viral genomes (Pérez-Cano et al. 2014). On the other hand, occludin together with the large family of claudins and tight junction adhesion molecules, have been reported to be essential for the correct morphology and functionality of the epithelial barrier (Wang et al. 2013). Therefore, it is plausible that the preventive effect of cocoa and/or cocoa fibre on certain intestinal chronic/inflammatory diseases may at least in part result from effects on TLRs and occludin. The present results suggest that, with

the exception of occludin, cocoa fibre is chiefly responsible for this modulation, which may reflect the changes in the intestinal microbiota and/or its direct relationship with intestinal epithelial and immune cells.

Although there was no difference in food intake between dietary groups, animals in the C10 group showed lower body weight gain, in agreement with previous studies (Massot-Cladera et al. 2012; 2013; Pérez-Berezo et al. 2009; Ramiro-Puig et al. 2007). The ability of flavonoids to inhibit lipid digestion and absorption (Gu et al. 2011), to reduce fat deposition ; Min et al., 2013; Cherniack, 2011; Faisal et al. 2015) and/or to down-regulate the expression of genes involved in lipid metabolism (unpublished own data from current analysis) are proposed mechanisms by which cocoa intake may affect body weight gain. Another possible mechanism is the effects of polyphenols on the gut microbiota composition. A number of studies have addressed the association between the ratio of the two main phyla (*Firmicutes* and *Bacteroidetes*) and obesity or weight loss (Sanz et al. 2013). Specifically, it has been reported that *Bacteroidetes* possess fewer genes for enzymes involved in lipid and carbohydrates metabolism than *Firmicutes* (Jumpertz et al. 2011; Kallus & Brandt, 2012). Therefore, the lower body weight gain in the C10 group could be partially attributed to both the lower counts of *Staphylococcus* and *Streptococcus* (belonging to the *Firmicutes* phylum) and the increase in *Bacteroides* (included in *Bacteroidetes* phylum) leading to a lesser ratio of *Firmicutes* to *Bacteroidetes*. Neither this lower F/B ratio nor a lower body weight increase was observed in animals fed the CF diet, which suggests that other cocoa compounds must be involved in such regulation. A previous study including the intake of diets containing high proportions of cocoa polyphenols (Massot-Cladera et al. 2014) also reported no effects on body weight. Therefore, methylxanthines present in cocoa may be involved, as suggested by Dulloo (Dulloo, 2011), who demonstrated a synergistic thermogenic interaction between methylxanthines and flavonoids (Dulloo, 2011).

5. Conclusions

Although previous studies showed that the intake of whole cocoa modulates the intestinal compartment, the results from the present study shed light on the contribution of cocoa fibre to these effects. In particular, fibre contained in cocoa modulates the microbiota composition, enhancing the growth of beneficial bacteria (*Lactobacillus* and *Bifidobacterium* genera) that are associated with a beneficial SCFA profile and, as a result, with a lower caecal and faecal pH. The

CF also reinforces intestinal immune defenses and barrier function by means of IgA-CB and occludin enhancement. Fibre from cocoa therefore possesses prebiotic properties. In addition, cocoa fibre in the colon modulated the expression of some TLR genes. Since whole cocoa did not replicate the effects of the CF, it is likely that other cocoa compounds mask the effects of its fibre.

Fundings

The present study was supported by a grant from the Spanish Ministry of Economy and Competitivity (AGL2011-24279). M. M.-C. holds a fellowship from the Generalitat de Catalunya (grant no. 2014FI_B2 00048).

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

The authors would like to thank the flow cytometry and genomic services of the 'Centres Científics i Tecnològics' of the University of Barcelona (CCiT-UB) and the Senior Technician, Analytical and Research Services from the University of Reading, Christopher J. Humphrey, his technical assistance. We also thank Nutrexpa Group S.L. and InnovaFood 2005 S.L. for providing the conventional cocoa powder and the inulin extract, respectively. M. M.-C., À. F., F.J.P.-C and M. C. conceived and designed the research; M. M.-C., A.C. and C.E.C carried out the GC experiments under the supervision of P.Y., and M.M.-C the rest of the experiments; M. M.-C., A.C. and C.E.C. carried out the GC data analysis whereas M.M.-C, M.C. and F. J. P.-C. carried out the rest of the data analysis; M. M.-C., F.J.P.-C and M. C. were involved in the interpretation of the data; M. M.-C. contributed to the initial draft of the manuscript; A.C., C.E.C., P.Y., À. F., F. J. P.-C and M. C. contributed to the critical revision of the manuscript; F. J. P.-C. has primary responsibility for the final content. All authors have read and approved the final version of the manuscript for publication.

References

Abreu, M. T., Fukata, M., & Arditi, M. (2005). TLR signaling in the gut in health and disease. *Journal of Immunology*, *174*(8), 4453–4460.

Abreu, M. T. (2010). Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function. *Nature Reviews. Immunology*, *10*(2), 131–144.

Cherniack, E. P. (2011). Polyphenols: planting the seeds of treatment for the metabolic syndrome. *Nutrition*, *27*(6), 617–623.

Claesson, M. J., Jeffery, I. B., Conde, S., Power, S. E., O'Connor, E. M., Cusack, S., Harris, H.M., Coakley, M., Lakshminarayanan, B., O'Suvillan, O., Fitzgerald, G.F., Deane, J., O'Connor, M., Harnedy, N., O'Connor, K., O'Mahony, D., van Sinderen, D.,Wallace, M., Brennan, L., Stanton, C., Marchesi, J. R., Fitzgerald, A. P., Shanahan, F., Hill, C., Ross, R. P., & O'Toole, P.W. (2012). Gut microbiota composition correlates with diet and health in the elderly. *Nature*, *488* (7410), 178–184.

Clifford, M. N. (2004). Diet-derived phenols in plasma and tissues and their implications for health. *Planta Medica*, *70*(12), 1103–1114.

Cook, S. I., & Sellin, J. H. (1998). Review article: short chain fatty acids in health and disease. *Alimentary Pharmacology & Therapeutics*, *12*(6), 499–507.

Cullen, R. W., & Oace, S. M. (1989). Fermentable dietary fibers elevate urinary methylmalonate and decrease propionate oxidation in rats deprived of vitamin B-12. *The Journal of Nutrition*, *119*(8), 1115–1120.

Dulloo, G. (2011). The search for compounds that stimulate thermogenesis in obesity management: from pharmaceuticals to functional food ingredients. *Obesity Reviews*, *12*(10), 866–883

Ellam, S., & Williamson, G. (2013). Cocoa and Human Health. *Annual Review of Nutrition*, *33*, 105–128.

Etxeberria, U., Fernández-Quintela, A., Milagro, F. I., Aguirre, L., Martínez, J. A., & Portillo, M. P. (2013). Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. *Journal of Agricultural and Food Chemistry*, *61*(40), 9517–9533

Faisal, A., Amin, I., Norhaizan, M. E., Chong, P. P., & Sander, K. (2015). Hepatic genome-wide expression of lipid metabolism in diet-induced obesity rats treated with cocoa polyphenols. *Journal of Functional Foods*, *17*, 969–978.

Fernandes, J., Vogt, J., & Wolever, T. M. (2011). Inulin increases short-term markers for colonic fermentation similarly in healthy and hyperinsulinaemic humans. *European Journal of Clinical Nutrition*, *65*(12), 1279–1286.

Fogliano, V., Corollaro, M. L., Vitaglione, P., Napolitano, A., Ferracane, R., Travaglia, F., Arlorio, M., Costabile, A., Klinder, A., & Gibson, G. (2011). In vitro bioaccessibility and gut

biotransformation of polyphenols present in the water-insoluble cocoa fraction. *Molecular Nutrition and Food Research*, *55* Suppl 1, 44S–55S

Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobet, T., Clarke, J. M., Topping, D. L., Suzuki, T., Taylos, T. D., Ithoh, K., Kikuchi, J., Morita, H., Hattori, M., & Ohno, H. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature*, *469*(7331), 543–547.

Gao, Z., Yin, J., Zhang, J., Ward, R. E., Martin, R. J., Lefevre, M., Cefalu, W.T., & Ye, J. (2009). Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes*, *58*(7), 1509–1517.

Gu, Y., Hurst, W. J., Stuart, D. A., & Lambert, J. D. (2011). Inhibition of key digestive enzymes by cocoa extracts and procyanidins. *Journal of Agricultural and Food Chemistry*, *59*(10), 5305–5311.

Hayek, N. (2013). Chocolate, gut microbiota, and human health. *Frontiers in Pharmacology*, *4,* 11.

Jakobsdottir, G., Blanco, N., Xu, J., Ahrné, S., Molin, G., Sterner, O., & Nyman, M. (2013). Formation of short-chain Fatty acids, excretion of anthocyanins, and microbial diversity in rats fed blackcurrants, blackberries, and raspberries. *Journal of Nutrition and Metabolism*, *2013*, 202534

Jumpertz, R., Le, D. S., Turnbaugh, P. J., Trinidad, C., Bogardus, C., Gordon, J. I., & Krakoff, J. (2011). Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. *American Journal of Clinical Nutrition*, *94*(9), 58–65.

Kallus, S. J., & Brandt, L. J. (2012). The intestinal microbiota and obesity. *Journal of Clinical Gastroenterology*, *46*(1), 16–24.

Kolida, S., Meyer, D., & Gibson, G. R. (2007). A double-blind placebo-controlled study to establish the bifidogenic dose of inulin in healthy humans. *European Journal of Clinical Nutrition*, *61*, 1189–1195.

Kolida, S., Tuohy, K., & Gibson, G. R. (2002). Prebiotic effects of inulin and oligofructose. *The British Journal of Nutrition*, *87* Suppl 2, 193S–197S.

Lecumberri, E., Mateos, R., Izquierdo-Pulido, M., Rupérez, P., Goya, L., & Bravo, L. (2007). Dietary fibre composition, antioxidant capacity and physico-chemical properties of a fibre-rich product from cocoa (Theobroma cacao L.). *Food Chemistry*, *104*, 948–954.

Lee, H. C., Jenner, A. M., Low, C. S., & Lee, Y. K. (2006). Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Research in Microbiology*, *157*(9), 876–884.

Maslowski, K. M., & Mackay, C. R. (2011). Diet, gut microbiota and immune responses. *Nature Immunology*, *12*(1), 5–9.

Massot-Cladera, M., Pérez-Berezo, T., Franch, À., Castell, M., & Pérez-Cano, F. J. (2012). Cocoa modulatory effect on rat faecal microbiota and colonic crosstalk. *Archives of Biochemistry and Biophysics*, *527*(2), 105–112.

Massot-Cladera, M., Franch, À., Castellote, C., Castell, M., & Pérez-Cano, F. J. (2013). Cocoa flavonoid-enriched diet modulates systemic and intestinal immunoglobulin synthesis in adult lewis rats. *Nutrients*, *5*(8), 3272–3286.

Massot-Cladera, M., Abril-Gil, M., Torres, S., Franch, À., Castell, M., & Pérez-Cano, F. J. (2014). Impact of cocoa polyphenol extracts on the immune system and microbiota in two strains of young rats. *The British Journal of Nutrition*, *112*(12), 1944–1954.

Meyer, D and Stasse-Wolthuis, M. (2009). The bifidogenic effect of inulin and oligofructose and its consequences for gut health. *European Journal of Clinical Nutrition*, *63*(11), 1277–1289.

Min, S. Y., Yang, H., Seo, S. G., Shin, S. H., Chung, M. Y., Kim, J., Lee, S. J., Lee, H. J., & Lee, K. W. (2013). Cocoa polyphenols suppress adipogenesis in vitro and obesity in vivo by targeting insulin receptor. *International Journal of Obesity*, *37*(4), 584–592

Monagas, M., Urpi-Sarda, M., Sánchez-Patán, F., Llorach, R., Garrido, I., Gómez-Cordovés, C., Andrés-Lacueva, C., & Bartolomé, B. (2010). Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food & Function*, *1*(3), 233–253.

Neilson, A. P., & Ferruzzi, M. G. (2011). Influence of formulation and processing on absorption and metabolism of flavan-3-ols from tea and cocoa. *Annual Review of Food Science and Technology*, *2*, 125–151.

Peng, L., Li, Z.-R., Green, R. S., Holzman, I. R., & Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *The Journal of Nutrition*, *139*(9), 1619–1625.

Pérez-Berezo, T., Ramiro-Puig, E., Pérez-Cano, F. J., Castellote, C., Permanyer, J., Franch, À., & Castell, M. (2009). Influence of a cocoa-enriched diet on specific immune response in ovalbumin-sensitized rats. *Molecular Nutrition and Food Research*, *53*, 389–397.

Pérez-Berezo, T., Franch, À., Ramos-Romero, S., Castellote, C., Pérez-Cano, F. J., & Castell, M. (2011). Cocoa-enriched diets modulate intestinal and systemic humoral immune response in young adult rats. *Molecular Nutrition and Food Research*, *55* Suppl 1, 56S–66S.

Pérez-Cano, F. J., Ramírez-Santana, C., Molero-Luís, M., Castell, M., Rivero, M., Castellote, C., & Franch, À. (2009). Mucosal IgA increase in rats by continuous CLA feeding during suckling and early infancy. *Journal of Lipid Research*, *50*(3), 467–476.

Pérez-Cano, F. J., Massot-Cladera, M., Franch, À., Castellote, C., & Castell, M. (2013). The effects of cocoa on the immune system. *Frontiers in Pharmacology*, *4*, 71.

Pérez-Cano, F. J., Massot-Cladera, M., Rodríguez-Lagunas, M. J., & Castell, M. (2014). Flavonoids Affect Host-Microbiota Crosstalk through TLR Modulation. *Antioxidants*, *3*, 649–670

Puertollano, E., Kolida, S., & Yaqoob, P. (2014). Biological significance of short-chain fatty acid metabolism by the intestinal microbiome. *Curr Opin Clin Nutr Metab Care, 17*(2), 139-144.

Puupponen-Pimiä, R., Nohynek, L., Hartmann-Schmidlin, S., Kähkönen, M., Heinonen, M., Määttä-Riihinen, K., & Oksman-Caldentey, K. M. (2005). Berry phenolics selectively inhibit the growth of intestinal pathogens. *Journal of Applied Microbiology, 98*(4), 991–1000.

Ramiro-Puig, E., Pérez-Cano, F. J., Ramírez-Santana, C., Castellote, C., Izquierdo-Pulido, M., Permanyer, J., Franch, À., & Castell, M. (2007). Spleen lymphocyte function modulated by a cocoa-enriched diet. *Clinical and Experimental Immunology, 149*(3), 535–542

Ramiro-Puig, E., Pérez-Cano, F. J., Ramos-Romero, S., Pérez-Berezo, T., Castellote, C., Permanyer, J., Franch, À., Izquierdo-Pulido, M., & Castell, M. (2008). Intestinal immune system of young rats influenced by cocoa-enriched diet. *Journal of Nutritional Biochemistry*, *19*(8), 555–565.

Roberfroid, M. B. (2002). Functional foods: concepts and application to inulin and oligofructose. *The British Journal of Nutrition*, *87* Suppl 2, 139S–143S.

Sanz, Y., Rastmanesh, R., & Agostonic, C. (2013). Understanding the role of gut microbes and probiotics in obesity: How far are we? *Pharmacological Research*, *69*(1), 144–155

Selma, M. V, Espín, J. C., & Tomás-Barberán, F. A. (2009). Interaction between phenolics and gut microbiota: role in human health. *Journal of Agricultural and Food Chemistry*, *57*(15), 6485–6501.

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects: A review. *Journal of Functional Foods*, 1–78. doi:10.1016/j.jff.2015.06.018.

Tan, J., McKenzie, C., Potamitis, M., Thorburn, A. N., Mackay, C. R., & Macia, L. (2014). The role of short-chain fatty acids in health and disease. *Advances in immunology, 121*, 91-119.

Touriño, S., Fuguet, E., Vinardelu, M. P., Cascante, M., & Torres, J. L. (2009). Phenolic metabolites of grape antioxidant dietary fiber in rat urine. *Journal of Agricultural and Food Chemistry*, *57*(23), 11418–11426.

Turnbaugh, P. J., Ridaura, V. K., Faith, J. J., Rey, F. E., Knight, R., & Gordon, J. I. (2009). The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Science Translational Medicine, 1*(6), 6ra14.

Tzounis, X., Vulevic, J., Kuhnle, G. G., George, T., Leonczak, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. (2008). Flavanol monomer-induced changes to the human faecal microflora. *The British Journal of Nutrition*, *99*(4), 782–792.

Tzounis, X., Rodriguez-Mateos, A., Vulevic, J., Gibson, G. R., Kwik-Uribe, C., & Spencer, J. P. E. (2011). Prebiotic evaluation of cocoa-derived flavanols in healthy humans by using a randomized, controlled, double-blind, crossover intervention study. *The American Journal of Clinical Nutrition*, *93*(1), 62–72.

van Duynhoven, J., Vaughan, E. E., van Dorsten, F., Gomez-Roldan, V., de Vos, R., Vervoort, J., van der Hooft, J. J., Roger, L., Draijer, R., & Jacobs, D. M. (2013). Interactions of black tea polyphenols with human gut microbiota : implications for gut and cardiovascular health. *American Journal Clinical Nutrition*, *98* Suppl 6, 1631S–1641S.

Vogt, J. A., & Pencharz, P. B. (2004). L -Rhamnose increases serum propionate in humans. *American Journal Clinical Nutrition*, *80*(1), 89–94.

Wang, L., Jin, X., Lin, D., Liu, Z., Zhang, X., Lu, Y., Liu, Y., Wang, M., Yang, M., Li, J., & Quan, C. (2013). Clinicopathologic significance of claudin-6, occludin, and matrix metalloproteinases -2 expression in ovarian carcinoma. *Diagnostic Pathology*, *8*, 190.

Wong, J. M. W., de Souza, R., Kendall, C. W. C., Emam, A., & Jenkins, D. J. a. (2006). Colonic health: fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology*, *40*(3), 235–243.

Wong, J. M. W., & Jenkins, D. J. (2007). Carbohydrate digestibility and metabolic effects. *The Journal of Nutrition*, *137* Suppl 11, 2539S–2546S.

Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.-Y., Keilbaugh, S. A., Bewta, M., Knights, D., Walters, W. A., Knights, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D.,& Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. *Science*, *334*(6052), 105–108.