Cocoa and cocoa fibre differentially modulate the IgA and IgM production at mucosal sites

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Abstract

Previous studies show that a 10% cocoa diet, containing polyphenols and fibre among others, modifies intestinal and systemic immunoglobulin (Ig) production. The present study was aimed at evaluating the impact of cocoa on IgA and IgM production in the intestinal and extra-intestinal mucosal compartments, establishing the involvement of cocoa fibre in such effects. Mechanisms by which cocoa intake may affect the IgA synthesis in the salivary glands were also studied. To this effect, rats were fed either a standard diet, a diet containing cocoa (C10), cocoa fibre (CF) or inulin (I). Intestinal (the gut wash (GW), Peyer's patches (PP) and mesenteric lymph nodes (MLN)) and extra-intestinal (salivary glands) mucosal tissues and blood samples were collected for IgA and IgM quantification. The IgA production- and homing-related molecules gene expression was studied in the salivary glands. The C10 diet decreased IgA and IgM intestinal production. Although CF diet decreased the GW IgA concentration, it increased PP, MLN and serum IgA concentration. Both the C10 and the CF diets produced a down-regulatory effect on the IgA secretion in the extra-intestinal tissues. The C10 diet interacted with the mechanisms involved in the IgA synthesis, whereas the CF showed particular effects on the homing and transcytosis of IgA across the salivary glands. Overall, CF is able to up-regulate IgA production in the intestinal-inductor compartments whereas it down-regulates its production at the mucosal-effector ones. Further studies must be directed to ascertain the mechanisms involved in the effect of particular cocoa components on gut-associated lymphoid tissue (GALT).

Introduction

The gut-associated lymphoid tissue (GALT) is the first line of defence against a variety of antigens (Ag) in the intestine. Structurally, the GALT is divided into organized and diffuse compartments. The organized or inductor GALT is formed by isolated lymphoid follicles (ILF) and Peyer's patches (PP), whereas the diffuse or effector GALT is formed by lymphocyte populations scattered across the epithelial or in the lamina propria. Moreover, the mesenteric lymph nodes (MLN) are part of the intestinal immune system, although they are not referred to as GALT as they do not sample intestinal antigens directly[1].

The main humoral immune product in the GALT, as well as in the other mucosal tissues, is the polymeric immunoglobulin (Ig) A (IgA), which represents around 80-90% of the total secreted Ig (S-Ig) of the body[2,3]. In a lower proportion, IgM (6-19%) and IgG (4-5%) are also present in the mucosal surfaces[4]. The high content of S-IgA in the intestine plays a key role in the maintenance of gut homeostasis and oral tolerance[5].

M cells from PP are specialized cells in luminal Ag uptake that transport luminal Ags from the intestinal lumen towards Ag-presenting cells. These cells interact with interfollicular T lymphocytes[5], which once activated bring about the differentiation and maturation of B cells, inducing them to become IgA+ cells and later IgA-secreting cells (IgA-SCs). This process depends on determined cytokines, such as transforming growth factor β1 (TGF-β1), interleukin (IL)-5, IL-6, IL-10 and IL-21[6–9]. B-cell differentiation occurs in PP and, to a lesser extent, in ILF and MLN[2]. From those inductive sites, the activated B cells reach peripheral blood and migrate to secretory effector sites where they extravasate depending on adhesion molecules and chemokine-chemokine receptor pairs[1,10]. The interaction between several regulated endothelial adhesion molecules and the corresponding ligands expressed on the memory/effector B cells' surface provokes a successful homing.

Although GALT is thought to constitute the major part of mucosal-associated lymphoid tissue (MALT), the induction of mucosal immune responses can take place in other mucosal compartments that also belong to the MALT[3]. In fact, studies point to the possibility that other mucosal compartments may be even more important than the GALT ones for S-IgA production. In this sense, salivary glands are considered important effector sites in the mucosal immune network[11].

Previous studies carried out in our laboratory have demonstrated the ability of a cocoa diet to modulate the immune system *in vitro* and *in vivo*[12]. In particular, a down-regulation of the serum and intestinal Ig content was observed in young rats fed with a cocoa diet[13–16]. As cocoa contains mainly flavonoids such as (−)-epicatechin, (+)-catechin and their polymeric forms called procyanidins[17], most of these effects of cocoa have been attributed to its high content of polyphenols[18]. However, it must be added that cocoa is also an important source of dietary fibre (DF) (26-40% of its content, mostly insoluble fibre) and, therefore, DF seems to strengthen or to be in part responsible for the effects described above[19].

On the basis of this background, the aim of this study was to investigate in depth the impact of cocoa on IgA and IgM production in the intestinal and extra-intestinal (salivary glands) mucosal compartments, establishing the particular involvement of cocoa fibre in such effects. Moreover, some of the mechanisms by which cocoa intake may affect the expression of molecules involved in IgA+ B cell homing and IgA synthesis in the salivary glands were also studied.

Material and Methods

Animals and diets

Female Wistar rats (3-week-old) 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: the reference (REF), cocoa (C10), cocoa fibre (CF) and inulin (I) groups (n=10/each group). The REF group was fed with the standard diet AIN-93M (Harlan, Barcelona, Spain); the C10 group received chow containing 10% cocoa, which finally provided 0.4% of polyphenols, 0.85% soluble fibre and 2.55% of insoluble fibre; the CF group received a diet with the same proportions of soluble and insoluble cocoa fibre as the C10 group but with a very low amount of polyphenols (<0.02%); and the I group received the AIN-93M standard diet, to which the same amount of soluble fibre as that given to the C10 group (0.85%) was added, but as inulin, in order to distinguish the particular effect of soluble cocoa fibre (Table 1). Natural Forastero cocoa and cocoa fibre powders (provided by Idilia Foods S.L. (formerly Nutrexpa S.L.), Barcelona, Spain) with 4.02% and 0.35% of polyphenols, respectively, were used to elaborate the C10 and CF diets. Inulin from chicory roots (Fibruline® Instant; InnovaFood 2005, S.L., Barcelona, Spain) was used as a reference soluble fibre. The three experimental diets were elaborated on the basis of the AIN-93M formula by subtracting the amount of carbohydrates, proteins, lipids and insoluble fibre provided by the corresponding supplements.

Table 1. Composition and content of macro- and micronutrients of experimental diets (g/kg diet).

Components	REF (g/kg) AIN-93M	C10 (g/kg) **(Cocoa)**	CF (g/kg) **(Cocoa fibre)**	I (g/kg) **(Inulin)**
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
Extract	-	100	52.3	8.5
Protein	-	22	8	-
Carbohydrate	-	16	0.5	-
Lipid	-	11	4.8	-
Fibre (insoluble/soluble)	-	34 (25.5/8.5)	31.9 (23.4/8.5)	8.5 (- /8.5)
Minerals	-	6	7	-
Total Polyphenols[1]	-	4	0.2	-
Water	-	7	-	-

[1] Total polyphenol compounds were quantified using the Folin-Ciocalteu method.

The resulting chows were isoenergetic and had a similar proportion of macronutrients (carbohydrates, proteins and lipids) and insoluble fibre as the reference diet (Table 1). Animals were given free access to water and chow. The diets lasted for three weeks. In addition, taking into account the obtained results, a similar design but with just one week of diet duration was executed which results are included as a supplementary material.

Studies were performed according to the criteria outlined by the Guide for the Care and Use of Laboratory Animals. Experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 358/12).

Sample collection and preparation

At the end of the nutritional intervention, mucosal and blood samples were collected for Ig quantification. Briefly, animals were anaesthetized intramuscularly with ketamine (90 mg/kg) (Merial Laboratorios, S.A., Barcelona, Spain) and xylazine (10 mg/kg) (Bayer A.G, Leverkusen,

Germany). The small intestine, mesenteric lymph nodes (MLN), and the left submaxillary salivary gland (SMG) and the left parotid salivary gland (PSG) were collected. A small piece of SMG was immediately immersed in RNAlater® (Ambion, Life Technologies, Austin, TX, USA), incubated at 4 ºC overnight and stored at -20 ºC until PCR analysis. The distal half of the small intestine was carefully rinsed with cold 0.9% NaCl saline solution in order to remove faecal content, it was opened lengthwise and visible Peyer's patches (PP) were excised. The intestinal tissue without PP was used to obtain the gut wash (GW) for Ig quantification by ELISA, as established previously in our laboratory[13]. The MLN, PP and both salivary glands were processed for Ig quantification. For this purpose, MLN, PP, SMG and PSG homogenates were obtained as described previously[16].

Immunoglobulin quantification

IgA and IgM concentrations in GW, MLN, PP, SMG and PSG homogenates and serum IgA, IgM and IgG were quantified at the end of the nutritional intervention by ELISA following the manufacturer's instructions for IgA (Bethyl Laboratories, Inc., Montgomery, TX, USA) and IgM (BD Biosciences, Heidelberg, Germany). Absorbance was measured in a microplate photometer (LabSystems Multiskan) and data were interpolated using ASCENT version 2.6 software (Thermo Fisher Scientific) into the standard curves, and expressed as ng/mg of tissue in homogenates and GW, and as µg/mL for the serum results.

Assessment of RNA gene expression by RT-PCR

To assess the gene expression of IgA, TGF-β1, RARα, RARβ, CCL28, pIgR and IL-6, the RNA was isolated from SMG samples by the RNeasy® 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. All samples used for further experiments showed an RNA integrity number (RIN) ≥ 9 and purity between 1.814 and 2.033 using the A_{260}/A_{280} ratio. 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: *Iga* (331943, made to order), *Ccl28* (Rn00586715_m1, I), *Tgfb1* (Rn00572010_m1, I), *Rara* (Rn00580551_m1, inventoried (I)), *Rarb* (Rn01537835_m1, I), *Pigr* (Rn00562362_m1, I) and *Il6* (Rn01410330_m1, I). 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* (beta glucuronidase gene) (Rn00566655_m1, I), which showed constant level of expression in our experimental conditions being similar to the level of expression of studied genes. The SDS v2.4 software (AB) was used to analyse 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[20]. Results are expressed as the mean ± SEM of the percentage of these values.

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 applied when normal distribution and equality of variance existed. Otherwise, non-parametric tests (Kruskal-Wallis and Mann-Whitney *U* rank-sum tests) were used to assess significance. Statistical analysis was performed using the software package SPSS 22.0 (SPSS, Inc. USA) and significant differences were established at $P<0.05$.

Results

Body weight and chow intake

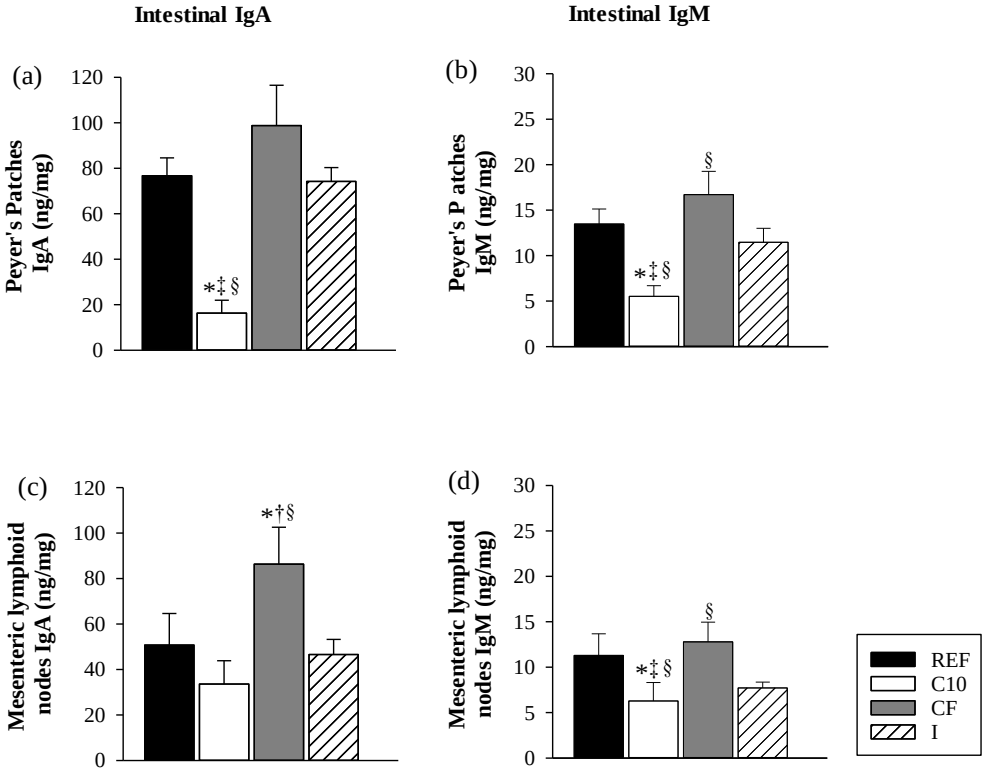
Throughout the study no changes in the increasing body weight pattern were found as a result of CF and I diet intake. However, the cocoa-fed animals showed a slower body weight increase, this being significantly lower in comparison to that of the rest of the groups throughout the study (up to 31.7% lower body weight increase in the C10 group compared to the rest of the groups at day 7, 72.2% at day 14 and 103.2% at day 21). This effect was not related to lower chow intake, which was similar throughout the study in all experimental groups (data not shown). Similar results on body weight by 10% cocoa diet have been already reported[13,15,18].

Immunoglobulin content in the inductor intestinal compartments

After three weeks of nutritional intervention, changes in IgA and IgM concentrations in both studied inductor compartments in the intestine (PP and MLN) were observed (Fig. 1). In PP, the C10 diet significantly reduced the IgA and IgM concentrations (Fig. 1(a) and (b)) compared to those observed in the rest of the groups ($P<0.05$), whereas the CF increased the IgM concentration with respect to the I-fed animals ($P<0.05$). The I diet did not significantly modify the IgA and IgM

concentration in PP. The same changes on PP IgA and IgM concentrations were already observable after just one week of nutritional intervention with the C10 diet (P<0.05) (online Supplementary Table S1).

Fig. 1. Effects of cocoa diet on IgA and IgM content in the inductor intestinal compartments after three weeks of diets. IgA (a) and IgM (b) concentration in Peyer's patches, and IgA (c) and IgM (d) concentration in mesenteric lymph nodes. Values are mean (n 10) with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference (REF) group (P<0.05); †Mean value was significantly different from that of the group consuming the cocoa diet (C10) (P<0.05); ‡Mean value was significantly different from that of the group consuming the cocoa fibre diet (CF) (P<0.05); §Mean value was significantly different from that of the group consuming the inulin diet (I) (P<0.05).



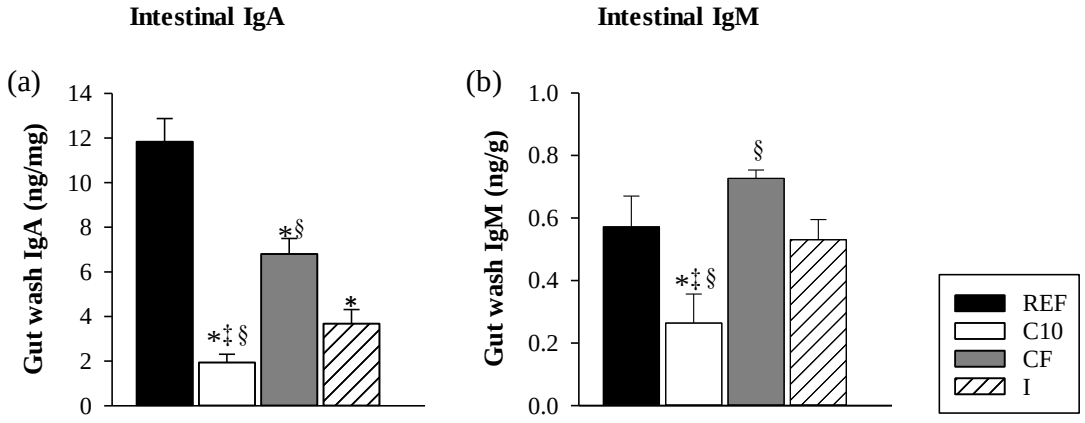
In MLN, the C10 intake did not significantly modify IgA concentration (Fig. 1(c)) but resulted in a significant IgM reduction in comparison with those in the REF, CF and I groups (P<0.05) (Fig. 1(d)). No statistical differences on MLN IgM or IgA were observed after just one week of C10 diet (online Supplementary Table S1). Moreover, in the same tissue the CF diet increased the IgA concentration compared to the other groups (P<0.05) and also the IgM concentration compared to

the I group (P<0.05) (Fig. 1(c) and (d)). This increase in MLN IgA and IgM was already observed after one week of diet (online Supplementary Table S1). The I diet did not significantly modify the IgA and IgM concentration in MLN (Fig. 1(c) and (d)).

Immunoglobulin content in the effector intestinal compartments

IgA and IgM concentrations were also determined in GW as representative of the production of Ig in the effector tissues (Fig. 2). Although all three experimental diets significantly reduced the GW IgA concentration compared to the REF group (P<0.05), it was the C10 diet that produced the most profound reduction in comparison with the CF and I diets (P<0.05) (Fig. 2(a)) that was already observed after one week of nutritional intervention (P<0.05) (online Supplementary Table S1). The decrease in IgA after the I diet intake was also more pronounced than that observed in the CF group (P<0.05). With regards to the IgM in GW, the C10 diet reduced its concentration compared to the rest of the groups (P<0.05) (Fig. 2(b)). The CF diet increased the IgM concentration compared to that produced by the I diet (P<0.05), but not when compared to the REF diet.

Fig. 2. Effects of cocoa diet on IgA and IgM content in the effector intestinal compartments after three weeks of diets. IgA (a) and IgM (b) concentration in gut washes. Values are mean (n 10) with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference (REF) group (P<0.05); ‡Mean value was significantly different from that of the group consuming the cocoa fibre diet (CF) (P<0.05); §Mean value was significantly different from that of the group consuming the inulin diet (I) (P<0.05).

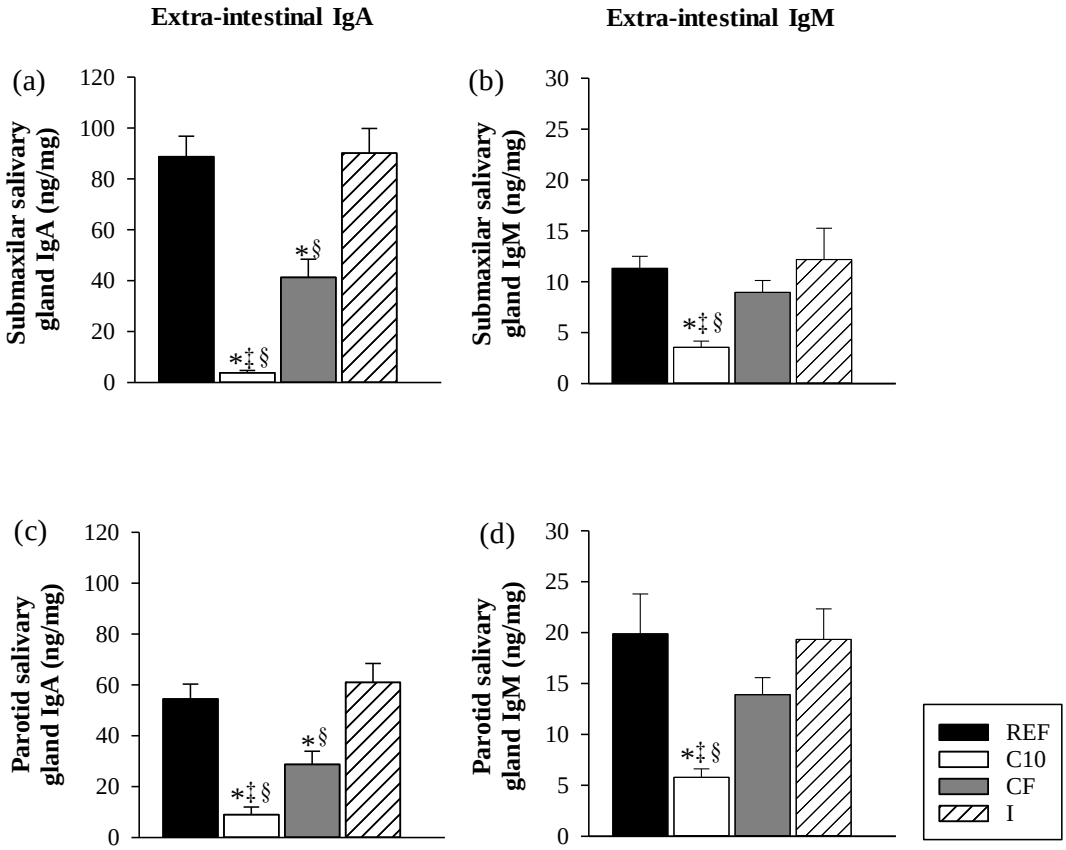


Immunoglobulin content in the effector extra-intestinal mucosal compartments

201 Regarding the salivary glands, a similar pattern was observed in the SMG and PSG for both Ig
202 (Fig. 3). The C10 diet significantly reduced the IgA and IgM content in both salivary glands
203 compared to the rest of the diets (P<0.05). The CF diet also reduced IgA concentration in both
204 salivary glands in a more modest way in comparison to the REF diet (Fig. 3(a) and (c)), without
205 affecting the IgM concentration (Fig. 3(b) and (d)). The same C10 and CF effects were already
206 observed after one week (online Supplementary Table S1). No changes in IgA and IgM were
207 observed in the salivary glands after I diet intake.

208

Fig. 3. Effects of cocoa diet on IgA and IgM content in the effector extra-intestinal mucosal compartments after three weeks of diets. IgA (a) and IgM (b) concentration in the submaxillary gland and IgA (c) and IgM (d) concentration in the parotid gland. Values are mean (n 10) with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference (REF) group (P<0.05); ‡Mean value was significantly different from that of the group consuming the cocoa fibre diet (CF) (P<0.05); §Mean value was significantly different from that of the group consuming the inulin diet (I) (P<0.05).



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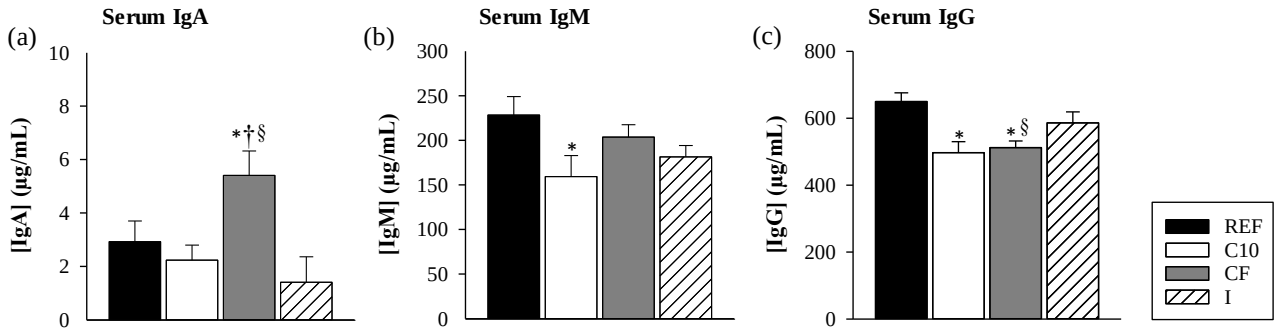
Systemic immunoglobulins

Serum concentrations of IgA, IgM and IgG from all experimental groups after three weeks of diet are summarized in Fig. 4. With regards to the IgA concentration, the CF diet intake resulted in higher levels of IgA compared to the rest of the diets (P<0.05) (Fig. 4(a)). IgM concentration was reduced by the C10 diet when compared to the REF diet after 3 weeks of intervention (P<0.05) (Fig. 4(b)). Serum IgG concentration was diminished by both the C10 and CF diets compared to the REF diet at the end of the third week of study (P<0.05) (Fig. 4(c)).

Fig. 4. Effects of cocoa diet on serum IgA (a), IgM (b) and IgG (c) concentrations after three weeks of diets. Values are mean (n 10) with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference (REF) group (P<0.05); ‡Mean value was significantly different from that of the group consuming the cocoa fibre diet (CF) (P<0.05); §Mean value was significantly different from that of the group consuming the inulin diet (I) (P<0.05).

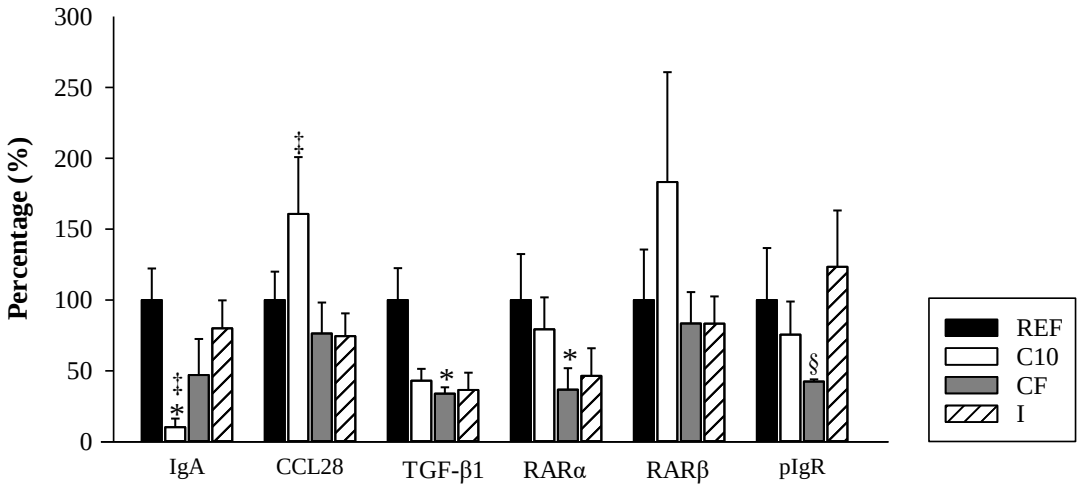


Submaxillary gland gene expression

Gene expression of IgA, CCL28, TGF-β1, RARα, RARβ, pIgR and IL-6 was assessed in the SMG at the end of the study to examine in depth the mechanism involved in the Ig modulation activity of cocoa products (Fig. 5). The IgA expression was significantly down-regulated only in the C10 group compared to the REF and CF diets, whereas CCL28 was significantly up-regulated by this diet compared to the CF diet (P<0.05). In addition, a tendency to down-regulate the TGF-β1 gene expression has been observed in the C10 diet. On the other hand, the CF diet fed animals showed significantly lower expression of TGF-β1 and RARα after three weeks of nutritional intervention compared to the REF animals (P<0.05). Moreover, the expression of pIgR in those animals fed with CF was also significantly lower than those fed with I diet (P<0.05). The I diet did not modify the

expression of any of the studied genes. The *Il6* expression was not detected in the SMG from any of the studied groups.

Fig. 5. Expression of genes associated with IgA synthesis, secretion, switching, intestinal homing in submaxillary salivary gland after three weeks of diets. Expression levels were normalized using the expression of *Gusb* as the endogenous housekeeping gene. Values are mean (n 5-6) with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference (REF) group, which represents 100% gene expression ($P<0.05$); ‡Mean value was significantly different from that of the group consuming the cocoa fibre diet (CF) ($P<0.05$); §Mean value was significantly different from that of the group consuming the inulin diet (I) ($P<0.05$).



Discussion

In previous studies, we reported that the intake of a 10% cocoa diet down-regulates the Ig content at systemic and intestinal levels in young rats[13–16]. However, the impact of cocoa at the extra-intestinal mucosal level, the mechanism by which it may act and the involvement of cocoa fibre in such effects are unknown to date. This study compares the effect of the whole cocoa intake with that produced by cocoa fibre extract (with a lower amount of polyphenols) on Ig production and that produced by a soluble fibre such as inulin. The results showed that the diet containing whole cocoa (C10 diet) induced the most pronounced effects on the synthesis of Ig in intestinal and extra-intestinal mucosal compartments. In addition, we found that most of the cocoa effects observed at the end of the third week were already significant after just one week of the diet. The

impact of the cocoa fibre on Ig production was lower than that produced by the whole cocoa in some tissues (GW and salivary glands for IgA) whereas in other compartments it had no effect (PP for both Ig and GW, MLN and serum for IgM) or it even exerted the opposite effect (MLN and serum for IgA). Finally, the soluble fibre-enriched diet only modified the GW IgA levels, decreasing its concentration, similar to those effects resulting from CF intake. The consequence and meaning of these results in each compartment are discussed next.

At the GALT inductor sites (PP and MLN), cocoa intake attenuated the production of the IgM synthesis in MLN and that of IgA and IgM in PP after three weeks of diet, the latter effects already being observed after one week of the diet. On the contrary, the CF diet (containing a very low amount of polyphenols) exerted the opposite effect of the C10 diet by increasing the IgA content in both PP and MLN (only significant in the latter). These results are in line with those previously reporting a higher proportion of IgA-coated bacteria in faeces from the CF-fed animals[19]. To our knowledge, this is the first report about the immune-potentiating effect of cocoa fibre. In line with these results, a rise in the IgA concentration has also been described for well-known substances with prebiotic effects[21]. Contrary to that, the prebiotic substance used in the present study (inulin) did not modulate Ig production, as reported in other studies[22], which 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[23]. Likewise, the CF diet increased the IgM content in PP and MLN with respect to the I group, suggesting that cocoa fibre has particular compounds and/or composition that are not in common with inulin, which could contribute to these effects. In addition, it is possible that the prebiotic effects attributed to the cocoa fibre[19] may be involved in the mechanism by which cocoa fibre increased the IgA concentration in this compartment. This enhancement is also reflected in serum from the CF group, in which IgA concentration is also increased, behaviour that is not always present after prebiotic intake[24,25]. Moreover, this means that the fibre present in cocoa can be discarded as being the main factor responsible for the down-regulation caused by the whole cocoa, and this allows us to suggest that other compounds present in cocoa may be masking and counteracting the immune-potentiating effect of the cocoa fibre.

Regarding the effector intestinal compartment (GW), previous studies carried out in our laboratory reported a reduction in IgA and IgM concentration after a six-week cocoa diet[15]. In line with those results, in the present study we have observed that this reductive effect of cocoa is already evidenced after three weeks and one week of diet, thus showing the early down-regulatory effects of cocoa. The cocoa fibre- or inulin-fed animals also presented lower IgA concentration in this

compartment. Therefore, the CF effect on inductor sites is not reproduced at effector level. However, as the CF influence was not as pronounced as that produced by the whole cocoa diet, cocoa fibre might only be partially contributing to the down-regulatory effects of cocoa and other cocoa compounds, such as flavonoids, might be potentiating the effect of fibre, or even be the key factor responsible for this effect. Nonetheless, studies using three different polyphenol-enriched cocoa extracts evidenced that although they were also able to modulate the Ig production, they were not the main compound in charge of such an effect[18,26]. Therefore, further studies should be carried out in order to fully elucidate the contribution of other cocoa components, such as methylxanthines on these intestinal effects.

A third compartment included in the current study was the salivary glands. Despite the existing evidence about the protective effect of flavonoid consumption from medication and/or age-related dysfunction of salivary glands[27–29], there is no information about the impact of polyphenols on the salivary glands' Ig production. In fact, to our knowledge, this is the first report describing the impact of whole cocoa (containing polyphenols and fibre) on Ig production in the extra-intestinal mucosal compartments. We observed that the C10 diet diminished the IgA and IgM concentrations in the salivary glands at the end of just one week of dietary intervention. Similarly, there is scarce information about the influence of indigestible carbohydrates on IgA response in the salivary glands, this being the first time the impact of fibre from cocoa or as inulin on the Ig production in these tissues has been described. Here we have reported that the CF-fed animals, although in a more moderate way than the C10 group, also presented lower concentrations of IgA in this compartment at the same time points. These results are not in line with those observed in animals fed with fructooligosaccharide (FOS) and a mixture of polydextrose and lactitol which induced higher submandibular IgA concentration[25]. Unlike the prebiotic substances and the results mentioned above, the cocoa fibre did not exert immune-potentiating effects in the salivary glands, thus suggesting that not only the fibre but also other compounds present in cocoa might be interfering with the Ig production in the salivary glands.

In order to obtain insights into the underlying mechanism associated with the reduction in IgA concentration in the salivary glands due to the diets, the gene expression of some key molecules involved in IgA synthesis and regulation, as well as IgA-SCs mucosal homing has been evaluated in this compartment. Here, we have observed that the IgA gene expression was drastically down-regulated by the C10 group but not by the CF and I groups. This result agrees with the most pronounced effects of the C10 diet on SMG IgA content, which could be due to the presence of a

lower number of IgA-SCs cells in this tissue and/or a lower ability to secrete it. Some of the molecules involved in the maturation and differentiation of B cells in becoming IgA-SCs are IL-6 and TGF-β1. Although IL-6 mRNA was not detected in the SMG, the TGF-β1 gene expression tended to be lessened by all three experimental diets, only being statistically significant in the CF diet. These results suggest the attenuating effect of soluble fibre in the differentiation of B cells into the salivary glands, and could partially explain the decreased IgA content found after the C10 and CF diets. Similar results have been found in the small intestine from animals fed with a cocoa diet for three and seven weeks[15,30].

Once the IgA-secreting B cells are activated, they migrate to the blood and later return to the mucosal effector compartments[5]. Mucosal homing is mainly regulated by adhesion molecules and chemokine-mediated interaction. To date, CCL28 chemokine and its corresponding receptor are crucial in the recruitment of IgA+ B cells to the extra-intestinal as well as intestinal mucosa[31,32]. Here, similarly to what has been reported in the small intestine[30], the C10 intake, but not the CF, up-regulated the expression of CCL28. These results may point to a compensatory mechanism to strongly attract the IgA+ B cells to avoid the down-modulation on IgA concentration. Other molecules involved in the intestinal cell homing mechanism are those that constitute the retinoic acid nuclear receptor family (RAR)[33]. After evaluating the expression of both RARα and RARβ in the salivary gland we found that the C10 intake for three weeks did not modify either the expression of RARα or RARβ at this level, which agrees with those results obtained in the small intestine and MLN after three weeks of diet[15]. However, the CF diet significantly reduced the expression of RARα but not RARβ, which could contribute to the IgA-decreasing effect of the CF on salivary glands. Finally, in order to reach the mucosal lumen, the IgA requires the association with the transmembrane epithelial protein pIgR[4]. We found that pIgR gene expression was not modified by the C10 diet at the extra-intestinal level or in the intestine[15], thus indicating that the reduction of IgA concentration associated with the cocoa intake is not due to a lower transport across the epithelium. Little is known about the impact of fibre on the pIgR gene expression. To date, an increase in its expression after the intake of FOS and a mixture of polydextrose and lactitol in rodents has been reported[25,34]. However, in the present study the CF intake resulted in a lower pIgR gene expression, suggesting that the reduction on IgA content in the salivary glands as a result of cocoa fibre intake also takes place by means of IgA transport.

In summary, we have demonstrated that a cocoa diet led to a lower IgA and IgM secretion in both inductor and effector intestinal compartments as well as in the extra-intestinal mucosal

compartment in young Wistar rats. Most of these effects could be already observed after one week of nutritional intervention. Soluble fibre seems to be partially responsible for the lower IgA secretion into the intestinal lumen. In spite of the results produced by the cocoa diet, it is worth noting that the enhancement by the CF diet of the IgA synthesis in the inductor intestinal compartment was also detectable at the systemic level, where higher IgA concentration was quantified. Both the C10 and CF diets produced a down-regulatory effect on the IgA secretion in the extra-intestinal tissues. The C10 diet interacted with the mechanisms involved in the IgA synthesis in a similar way to that observed in the small intestine, whereas the cocoa fibre showed particular effects on homing and transcytosis of IgA across the epithelial gland.

Overall, cocoa fibre has IgA immune-potentiating effects that are counteracted when it is present in the whole cocoa product. This fact suggests that other cocoa components are involved in such an effect. This immunomodulation is not found in the inulin diet. Further studies must be considered to ascertain the mechanism by which cocoa fibre is able to act in up-regulating the IgA production at the intestinal inductor compartments whereas it down-regulates its production in those of the mucosal effectors.

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