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Efecte dels components bioactius del cacau sobre la microbiota i el sistema immunitari intestinal de rata

Malen Massot Cladera



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UNIVERSITAT^{DE}
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Facultat de Farmàcia
Departament de Fisiologia

**EFFECTE DELS COMPONENTS BIOACTIUS DEL CACAU
SOBRE LA MICROBIOTA I EL SISTEMA IMMUNITARI
INTESTINAL DE RATA**

MALEN MASSOT CLADERA

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EFFECTE DELS COMPONENTS BIOACTIUS DEL CACAU SOBRE LA MICROBIOTA I EL SISTEMA IMMUNITARI INTESTINAL DE RATA

Memòria presentada per **Malen Massot Cladera** per optar al títol de doctor
amb menció internacional per la Universitat de Barcelona

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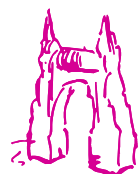
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INFORMEN

Que la memòria titulada ***Efecte dels components bioactius del cacau sobre la microbiota i el sistema immunitari intestinal de rata*** presentada per **MALEN MASSOT CLADERA** per optar al Grau de Doctor amb Menció Internacional per la Universitat de Barcelona, ha estat realitzada sota la nostra direcció al Departament de Fisiologia, i considerant-la conclosa, autoritzem la seva presentació per ser jutjada pel tribunal corresponent.

I perquè així consti, signem el present a

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Abreviatures

Ag	Antigen
AGCC	Àcids grassos de cadena curta
AID	Citidina desaminasa induïda per activació
APC	Cèl·lula presentadora d'antigen
APRIL	Lligam inductor de la proliferació
BALT	Teixit limfoide associat als bronquis
CALT	Teixit limfoide associat a la conjuntiva
CD40L	Lligam de CD40
DAMP	Patrons moleculars associats a dany
DC	Cèl·lules dendrítiques
CYP	Citocrom P450
ECA	Enzim convertidor d'angiotensina
ERK	Cinasa regulada per senyals extracel·lulars
FDC	Cèl·lules dendrítiques fol·liculars
GALT	Teixit limfoide associat a l'intestí
GJIC	Comunicacions intercel·lulars en fenedura
GLM	Ganglis limfàtics mesentèrics
HDL	Lipoproteïnes d'alta densitat
IEL	Limfòcits intraepiteliais
Ig	Immunoglobulina
IgA-SC	Cèl·lules secretores d'IgA
IL-	Interleucina
ILF	Fol·licles limfoides aïllats
IRAK	Cinasa associada al receptor d'IL- 1
IRF3	Factor 3 regulador d'interferó
LALT	Teixit limfoide associat a la laringe
LDALT	Teixit limfoide associat a la glàndula lacrimal
LDL	Lipoproteïna de baixa densitat
LP	<i>Lamina propria</i>

LPS	Lipopolisacàrids
MALT	Teixit limfoide associat a mucoses
MAMP	Patrons molecular associat a microbi
MAPK	Cinasa activada per mitogen
MHC	Complex principal d'histocompatibilitat
NALT	Teixit limfoide associat a la nasofaringe
NET	Trampes extracel·lulars de neutròfils
NF-κB	Factor nuclear κ B
NK	Cèl·lules <i>natural killer</i>
NLR	Receptors de tipus NOD
NO	Monòxid de nitrogen
NOS	Òxid nítric sintasa
PAMP	Patrons moleculars associats a patogen
PARP	Polimerasa poliADP-ribosa
PIgR	Receptor d'immunoglobulines polimèriques
PP	Plaques de Peyer
PRRS	Receptors de reconeixement de patrons
ROS	Espècies reactives d'oxigen
SALT/DALT	Teixit limfoide associat a les glàndules salivals
S-IgA	IgA-secretora
TALT	Teixit limfoide associat a les trompes d'Eustaqi
Tc	Limfòcit T citotòxic
TCR	Receptor de limfòcits T
TGF-β1	Factor transformador de creixement β 1
TGI	Tracte gastrointestinal
Th	Limfòcit T col·laborador
TIR	Receptor <i>toll/IL-1</i>
TJ	Unions estretes
TLR	Receptors tipus toll
TNF-α	Factor de necrosis tumoral α

RESUM/ABSTRACT

In the last few years, cocoa has become one of the main subjects of study due to its high content in flavonoids. Several studies have associated the cocoa intake with health benefits. Moreover, immunomodulatory properties in rats have been also attributed to cocoa.

On this basis, the aim of the present thesis was to establish the impact of diets enriched with cocoa, cocoa flavonoids or cocoa fiber on the fecal microbiota composition and its activity as well as on the immune function in the gut.

To achieve this objective, preclinical studies were carried out in rats fed a 10% conventional cocoa-enriched diet, diets elaborated with different amounts of non-fermented cocoa extracts and cocoa fiber diet.

Regarding microbiota results, differential composition pattern was observed after all the experimental diets intake but only the cocoa fiber diet increased the *Bifidobacterium* spp. and *Lactobacillus* spp. proportion. In addition, the cocoa fiber diet was the one which caused the most pronounced changes in the short chain fatty acids (SCFA) production. Particularly, it increased the cecal and fecal concentration of acetic, propionic and butyric acids. Moreover, both the 10%-cocoa diet and cocoa fiber diets differentially modulated the TLR gene expression in the colon.

Concerning the mucosal immunoglobulin production, all cocoa polyphenol-enriched diets modulated the intestinal IgA secretion although this effect was not proportional to their flavonoid content. The cocoa fiber diet also exerted an effect on intestinal IgA secretion but in a different way depending on the compartment. Focusing on the extraintestinal compartment, although the cocoa fiber diet

showed the same down-modulatory effect on IgA and IgM secretion as the cocoa diet, its mechanisms were different. In addition, all cocoa flavonoid-enriched diets decreased IgA-coated bacteria proportion in a non-dose dependent manner whereas this percentage increased by the cocoa fiber intake.

The 10% cocoa diet was the only one that caused a slower body weight gain. This effect is correlated with the microbiota modulation. The change induced by cocoa diet on the expression of genes involved in the lipid metabolism in the colon could be also involved.

Regarding the urinary metabolites, the cocoa and the cocoa fiber diets caused differential metabolic profile that can be used as consumption marker. The metabolic fingerprint correlated well with the body weight, the metabolic hormones, the intestinal immunity and the microbiota composition. Moreover, all these variables showed also an association between them.

Therefore, the effects produced by cocoa intake are due to the differential effects caused by each one of its main bioactive compounds - polyphenols and fiber - which act in a synergistic or opposite manner depending on the variable. Other cocoa compounds are also involved in such effects.

**OBJECTIUS/
*OBJECTIVES***

In the last few years, the interest in finding out new functional natural products with beneficial properties for the host has increased considerably. In this sense, cocoa has become one subject of study due to its content in fiber and minerals but mainly because it is also considered a great source of bioactive compounds such as flavonoids which have shown to possess antioxidant properties.

Several studies have evidenced the association between the cocoa intake and the protection to develop inflammation- and oxidative stress-related diseases. Moreover, studies carried out in our group of research in the Department of Physiology at the Faculty of Pharmacy have evidenced that a 10% cocoa diet possesses immunomodulatory effects in rats. Particularly, a diet containing a 10% cocoa modulates the spleen, mesenteric lymph nodes and Peyer patches' lymphocyte functionality. Furthermore, cocoa intake modifies the synthesis and secretion of intestinal (IgA and IgM) and systemic (IgA, IgM and IgG) immunoglobulins in young and adults rats. It has also been reported a differential gene expression pattern of molecules involved in the crosstalk between the microbiota and the immune system in small intestine together with a lower body weight gain in the 10% cocoa-fed animals.

On basis on these previously described effects, the hypothesis that supports the current thesis is that **cocoa might act on the intestinal microbiota modulating its composition as well as its functionality. Consequently, these changes could affect the crosstalk between the microbiota and the immune system which may modify the intestinal immune functions. Alternatively, the compounds present in cocoa**

could directly affect the colonic epithelial cell function and the gut-associated lymphoid tissue activity.

In addition, it remains to be known which of the cocoa compounds is the main responsible for such effects. In this sense, many *in vivo* and *in vitro* studies have attributed to the cocoa flavonoids the ability to protect against pathologies. Similarly, the cocoa fiber intake has also been associated with beneficial effects in experimental models.

Therefore, considering this background, the main objective of the thesis was:

To establish the effect of diets containing either cocoa, cocoa flavonoids or cocoa fiber on the intestinal microbiota composition and activity and the immune function in the gut.

To achieve this, the following specific objectives were proposed:

1. To establish the impact of a cocoa-enriched diet on the intestinal microbiota composition and its interaction with the intestinal immune system.

The results obtained from this objective are part of the following publication:

- *Cocoa modulatory effect on rat faecal microbiota and colonic crosstalk. Arch Biochem Biophys*, 2012; 527:105-112.

2. To establish the contribution of the cocoa flavonoids in the cocoa modulatory effect on the microbiota composition and functionality and these from the immune system.

The results obtained from this objective make the following publications:

- *Cocoa flavonoid-enriched diet modulates systemic and intestinal immunoglobulin synthesis in adult Lewis rats. Nutrients*, 2013; 5: 3272-3286.
- *Impact of cocoa polyphenol extracts on the immune system and microbiota in two strains of young rats. Br J Nutr*, 2014; 112:1944-1954.

3. To establish the contribution of the cocoa fiber in the cocoa modulatory effect on the microbiota composition and functionality and these from the immune system.

The results obtained from this objective are part of the followings publications:

- *Prebiotic effects of cocoa fiber on rats. J Funct Foods*, 2015; 19: 341-352.
- *Cocoa polyphenols and fiber modify colonic gene expression in rats. Eur J Nutr*, under review.
- *Cocoa and cocoa fibre differentially modulate the IgA and IgM content at mucosal sites. Submitted.*
- *Association between cocoa diet effects on rat's body weight, microbiota and intestinal immunity and the urine metabolic profile. Submitted.*

INTRODUCCIÓ

1. SISTEMA IMMUNITARI

El tracte gastrointestinal (TGI) conté la superfície mucosal més extensa i complexa del cos humà, arribant a cobrir una àrea de 100 m². A més presenta una gran varietat topològica, amb zones de característiques microecològiques molt diverses pel que fa a temperatura, atmosfera, pH, humitat, superfícies d'adherència o concentració de nutrients. D'altra banda, l'intestí es troba contínuament en contacte amb una elevada càrrega antigènica d'origen alimentari-ambiental. Per tant, la capacitat de discriminar, per una banda, entre antígens (Ag) innocus i microorganismes autòctons i, per altra banda, entre elements nocius i patògens és fonamental per al manteniment de l'homeòstasi intestinal [1].

L'intestí disposa de mecanismes de defensa que limiten l'accés d'elements nocius dins l'organisme com són les barreres microbiològica, física i immunològica.

1.1. BARRERA MICROBIOLÒGICA O MICROBIOTA INTESTINAL

Les seves característiques microecològiques converteixen el TGI en una multiplicitat d'àrees colonitzables que constitueixen els nínxols ecològics per diferents tipus de microorganismes. El TGI es caracteritza per una gran complexitat, densitat poblacional i diversitat de microorganismes. Concretament, s'estima que els microorganismes presents a l'intestí tenen en el seu contingut més de 100 vegades el nombre de gens que en el genoma humà. Aquest conjunt dinàmic de microorganismes es coneix com a microbiota autòctona intestinal [2–

5]. Així doncs, la microbiota està constituïda pels microorganismes que presenten una associació estable amb les mucoses de l'intestí on resideixen i, a més, on desenvolupen importants funcions complementàries [6].

1.1.1. Colonització microbiana intestinal

La colonització de la microbiota intestinal es produeix, per primer cop, durant el part (sent també el tipus de part un factor determinant) [7,8] i va incrementant en diversitat i riquesa al llarg de les diferents etapes de la vida [9,10] (**Figura 1**). La idea inicial àmpliament estesa de l'esterilitat del fetus està essent actualment qüestionada [11]. El que està clar és que l'evolució de la composició de la microbiota està molt determinada pel règim alimentari de l'infant [9,12–14]. Així doncs, durant els primers anys de vida, la colonització de l'intestí es troba en un canvi continu degut a la ingesta de llet com a principal font d'aliments [15]. En aquest sentit, s'han descrit diferències importants en la composició i diversitat de la microbiota intestinal d'infants alimentats amb llet materna i dels que han rebut llet de fórmula [16]. La lactància materna confereix una microbiota amb major diversitat taxonòmica que la llet de fórmula donat que és una font important de substàncies prebiòtiques (en forma d'oligosacàrids) i probiòtics (sent l'aportació estimada entre 10^5 i 10^7 bacteris/dia). D'aquesta manera, la lactància materna promou el creixement dels lactobacils i bifidobacteris, a la vegada que inhibeix l'adhesió de bacteris patògens en el TGI de l'infant [17–20]. Als 5-6 mesos s'incorporen les farinetes de fruites que, pel seu elevat contingut en mono- i polisacàrids, promouen la colonització per part de bacteris del *phylum Firmicutes* a més de la diversificació de l'ecosistema intestinal. L'inici de l'alimentació a base de purés de verdures facilita l'arribada dels

Bacteroidetes i l'assentament definitiu dels *Firmicutes*. La colonització per *Escherichia coli* i altres membres del phylum *Proteobacteria* coincideix amb la incorporació gradual d'aliments proteics (carn, peix i ou). D'aquesta manera, al voltant dels 3-4 anys, un cop s'assoleix l'alimentació dels adults, els microorganismes responsables de mantenir l'homeòstasi intestinal ja es troben en les proporcions i diversitat típiques de l'edat adulta (**Figura 1**).

1.1.2. Diversitat i composició

La variació interindividual existent en la composició de la microbiota pot reflectir, almenys en part, les diferències en la ingesta alimentària. Ara bé, cal tenir en compte que la resposta de la microbiota als canvis dietètics, i consegüentment la seva funcionalitat i efectes, pot diferir entre individus [21]. Els factors genètics i ambientals són els principals determinats de la composició de la microbiota intestinal, tot i que s'ha vist que la dieta juga un paper important [13,22–24].

En aquest sentit, recentment s'han identificat tres enterotips característics amb rellevància metabòlica. L'enterotip resulta de la classificació dels microorganismes vius presents a l'ecosistema intestinal humà depenent de l'abundància relativa de determinats gèneres [25]. Així doncs, es parla de l'enterotip 1 el que es caracteritza per l'abundància de bacteris dels gèneres *Bacteroides* i *Parabacteroides*; l'enterotip 2 en el qual són més abundants els gèneres *Prevotella* i *Desulfovibrio* i, finalment, l'enterotip 3, que és el més freqüent i es caracteritza per un predomini de *Ruminococcus*, *Staphylococcus*, *Gordonibacter* i *Akkermansia* [26].

A diferència dels humans, la microbiota intestinal de rates presenta menys variabilitat inter-individual i s'ha descrit el predomini per part

de determinats gèneres com són els *Bacteroides*, *Clostridium*, *Bifidobacterium* i cocs grampositius anaeròbics [27].

A mesura que avancem cap a la vellesa, els hàbits alimentaris i l'estil de vida es modifiquen, a la vegada que s'experimenten alteracions (malalties inflamatòries, infeccions, etc.) que requereixen intervenció farmacològica. Tot plegat implica que la composició de la microbiota intestinal així com la seva funcionalitat continuï modificant-se [10] (Figura 1).

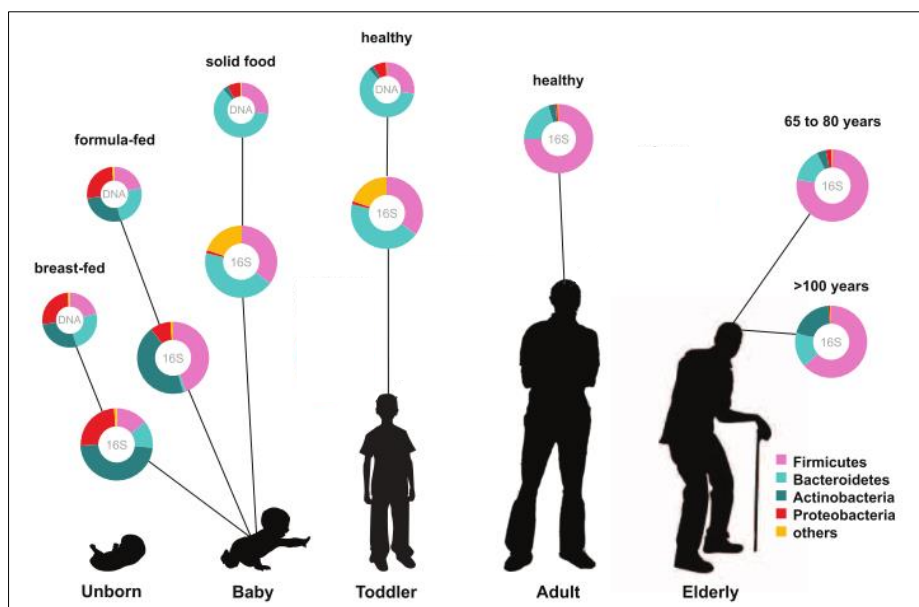


Figura 1. Evolució de la microbiota intestinal amb l'edat (adaptada de [10]).

La colonització bacteriana es produeix de forma desigual al llarg de les diferents regions del TGI (Figura 2A) [28]. En concret s'ha vist que la part superior del TGI, des de la boca fins a l'intestí prim, conté una

microbiota dispersa i poc abundant que prové de l'orofaringe i que és arrossegada al llarg del TGI amb els àpats [29]. A nivell d'intestí prim proximal, l'acidesa estomacal i les secrecions biliars i pancreàtiques poden afectar negativament els bacteris allà establerts. La concentració bacteriana va incrementant al llarg del jejú, on hi ha un predomini de *Lactobacillus*. Aquesta major representació bacteriana continua al llarg de l'ili on, a més, hi ha una major diversitat, semblant a la que es troba a nivell d'intestí gros. Així doncs, l'intestí gros es caracteritza per la seva elevadíssima densitat microbiana. Tot i que hi ha un predomini de bacteris (arribant a una concentració de 10^{12}) [30], també s'ha evidenciat la presència de *Archaea*, fongs i protozous. A més, hi ha una gran diversitat ja que s'han identificat més de 1000 espècies. Entre les més predominants destaquen els membres de les famílies *Lactobacillaceae*, *Clostridium*, *Enterococcaceae*, *Streptococcaceae*, *Bacteroidaceae*, *Ruminococcaceae*, entre d'altres [28]. A nivell fecal també es troben concentracions elevades de microorganismes (**Figura 2B**). Cal destacar que la microbiota present en les mostres fecals, tot i no reflectir exactament el contingut d'altres trams gastrointestinals [31,32], és, juntament amb el còlon, on major nombre de bacteris es poden trobar [33]. Concretament, els bacteris poden arribar a representar al voltant del 60% dels sòlids fecals [34].

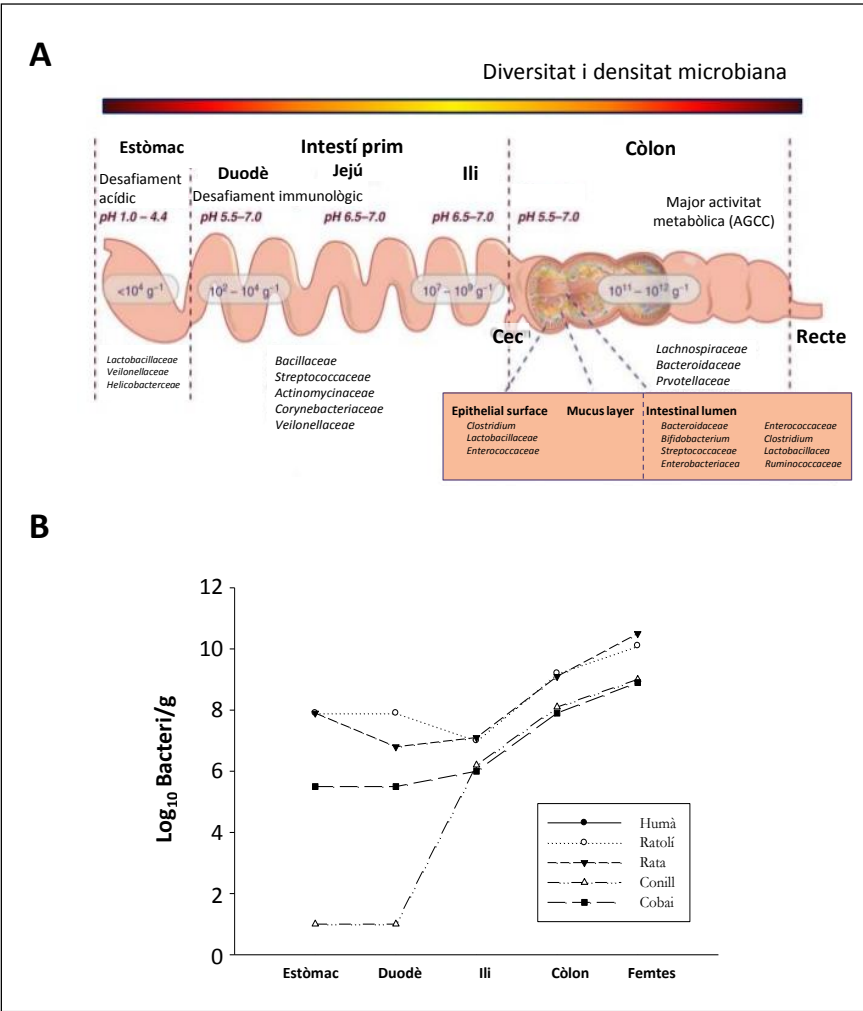


Figura 2. (A) Distribució de la microbiota intestinal en el TGI en humans. (B) Similituds en la concentració al llarg del TGI i en mostres fecals en diferents mamífers (adaptada de [28,33]).

1.1.3. Funcions de la microbiota intestinal

Com s'ha mencionat anteriorment, la microbiota estableix una relació de simbiosi amb l'organisme [30], de manera que molts dels senyals derivats dels bacteris són essencials per a la fisiologia intestinal [35,36]. Mentre l'intestí proporciona un hàbitat ric en fonts d'energia procedents dels aliments ingerits, els microorganismes que constitueixen la microbiota intestinal desenvolupen diverses funcions:

A) **Funció metabòlica:** Permeten l'aprofitament de nutrients no digeribles gràcies a l'aportació d'enzims capaços de metabolitzar els hidrats de carboni i proteïnes de la dieta amb la conseqüent producció d'àcids grassos de cadena curta (AGCC) [37]. Aquests AGCC tenen efecte tròfic sobre l'epiteli intestinal [38]. A més, la microbiota intestinal sintetitza una gran varietat de vitamines essencials (biotina, àcid fòlic, B, K, E), afavoreix l'absorció de calci, magnesi i ferro; i transforma els àcids biliars i el colesterol en una gran quantitat de metabòlits [39,40].

B) **Funció protectora:** Té un paper protector o de barrera, el qual és fonamental per a la resistència a la colonització de microorganismes exògens potencialment patògens. Pertorbacions en la microbiota habitual, el que es coneix com a disbiosi, sovint desencadenen infeccions tant a nivell intestinal com extraintestinal [3,41–50]. La microbiota protegeix del sobrecreixement endogen dels microorganismes oportunistes, fonamentalment, d'espècies estrictament anaeròbies (*E. coli* i *Estreptococs*) a través de diversos mecanismes: competint pels llocs d'adhesió i pels nutrients i/o mitjançant la secreció d'inhibidors metabòlics per part dels bacteris residents (bacteriocines) [38,51,52].

C) **Funció immunomoduladora:** A banda d'aquestes funcions directes de la microbiota protegint de la invasió de patògens, els bacteris contribueixen, alhora, al desenvolupament, maduració i manteniment del sistema immunitari mucosal i sistèmic. Aquesta funció es pot considerar des de dos punts de vista:

- Promoció del desenvolupament del sistema immunitari en la infància [21,53]. La importància de la colonització bacteriana en la maduració immunitària infantil queda evidenciada per estudis realitzats en animals criats en condicions asèptiques o *germ-free*. Aquests animals presenten un sistema immunitari immadur, amb alteracions en la morfologia intestinal, unes plaques de Peyer (PP, *Peyer patches*) de menor mida, un menor nombre i, a la vegada, major immaduresa dels limfòcits i cèl·lules dendrítiques (DC, *dendritic cells*) intestinals [54]. Concretament, les cèl·lules productores d'immunoglobulina (Ig) A es troben en menor quantitat i, per això, existeix un dèficit de les Ig presents en la llum intestinal i també sèriques.

- Contribució al manteniment funcional del sistema immunitari associat a l'intestí de l'adult. Així, per exemple, s'ha vist que els bacteris de la microbiota contribueixen a l'expansió de limfòcits en l'epiteli i a l'adquisició de la seva activitat citotòxica, potencien la funció fagocítica de l'organisme, tenen un paper en la inducció i manteniment de la tolerància oral a Ag presents en la dieta i augmenten la producció d'IgA luminal [21,53].

Així doncs, la comunitat bacteriana intestinal pot ser considerada com un òrgan metabòlic ocult degut al seu gran impacte en la salut humana. En aquest sentit, aquesta contribueix positivament al metabolisme, a la fisiologia, a la nutrició o al sistema immunitari de l'hoste [3].

1.2. BARRERA FÍSICO-QUÍMICA O EPITELI INTESTINAL

L'epiteli intestinal, a més de ser responsable de l'absorció de nutrients, representa per a la mucosa una segona línia de defensa enfront a patògens. Constitueix una barrera física ubicada estratègicament que està formada per una monocapa de cèl·lules epitelials que separen la gran diversitat d'Ag luminals de les cèl·lules immunitàries presents en la lamina propria (LP) subjacent. A més, conté nombroses modificacions físico-químiques que permeten discernir l'ambient extern del medi intern de l'individu [55]. Les cèl·lules epitelials estan unides entre si, entre d'altres, per mitjà de les unions estretes, conegudes com a tight junctions (TJ), les qual tenen un paper important en la permeabilitat de la barrera intestinal [56]. Les TJ són estructures dinàmiques força complexes que consten de proteïnes transmembrana (claudines, molècules d'unió per adhesió i occludines, entre d'altres) i proteïnes de suport estructural citoplasmàtiques, les quals estan directament connectades amb el citoesquelet intercel·lular d'actina i en contacte amb proteïnes amb activitat reguladora (proteïnes G, aPKC, etc.) [57]. Així doncs, les TJ i les microvellositats de l'extrem apical cel·lular són components estructurals fonamentals en la barrera [58].

El paper de barrera física es veu potenciat per la presència d'adaptacions bioquímiques com és la capa de glicocàlix. Aquesta es forma per la secreció per part de les cèl·lules caliciformes de diverses mucines que s'uneixen a la membrana apical de les cèl·lules epitelials formant una capa gruixada de mucus relativament impermeable (Figura 3) [59]. L'alliberament de grànuls de mucina incrementa davant la presència d'agressions físiques, químiques o infeccioses. Les

mucines interactuen amb polisacàrids de la superfície bacteriana a fi de poder expulsar-los per peristaltisme intestinal [60].

Les cèl·lules epitelials intestinals secreten pèptids antimicrobians (defensines i catelicidines, entre d'altres) a la llum intestinal, els quals promouen la formació de porus en la paret cel·lular bacteriana [58]. L'epiteli constitueix alhora una barrera dinàmica crucial pel desenvolupament o inducció de respostes immunitàries innata i adaptativa i, conseqüentment, per al control de la defensa immunitària i manteniment de l'homeòstasi [61].

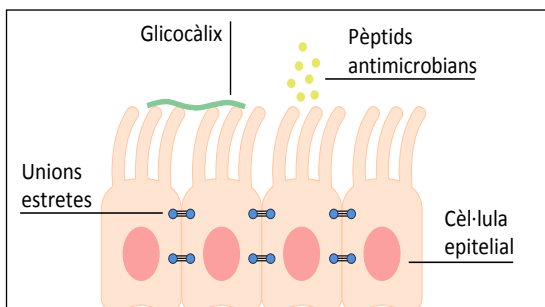


Figura 3. Barrera epitelial de l'intestí. Les cèl·lules epitelials presenten adaptacions físico-químiques per mantenir la integritat de la barrera (adaptada de [58]).

De forma general, els bacteris no penetren la barrera epitelial a no ser que expressin mucinases o bé factors d'adherència, colonització o invasió. Així doncs, l'accessibilitat a la superfície apical de l'epiteli i, la conseqüent ruptura de la barrera, són dues propietats que permeten diferenciar als microorganismes patògens dels innocus per a l'individu [61].

1.3. BARRERA IMMUNOLÒGICA O SISTEMA IMMUNITARI DE LES MUCOSES

Els mamífers han desenvolupat el teixit limfoide associat a mucoses (MALT, *mucosa-associated lymphoid tissue*), el qual constitueix el principal punt de partida per a la generació de les respostes immunitàries mucosals [62]. De la mateixa manera que en el compartiment sistèmic, a les mucoses, les respostes immunitàries innata i adaptativa constitueixen dos tipus de mecanismes de defensa essencials en l'individu i funcionen de forma coordinada i sinèrgica [63].

1.3.1. Organització anatòmica

El MALT constitueix un òrgan limfoide que pot dividir-se en subàrees connectades funcionalment. Aquestes inclouen el teixit limfoide associat a l'intestí (GALT, *gut-associated lymphoid tissue*), el teixit limfoide associat a la nasofaringe (NALT, *nasopharynx-associated lymphoid tissue*), als bronquis (BALT, *bronchus-associated lymphoid tissue*), a l'aparell genitourinari, a les glàndules salivals (SALT/DALT, *salivary gland-or duct-associated lymphoid tissue*), a la glàndula lacrimal (LDALT, *lachrymal-drainage-associated lymphoid tissue*) i a la laringe (LALT, *larynx-associated lymphoid tissue*) (**Figura 4**). Alguns autors també parlen del teixit limfoide associat a la trompa d'Eustaqui (TALT, *Eustachian tube-associated lymphoid tissue*) o a la conjuntiva (CALT, *conjunctiva-associated lymphoid tissue*) [64–68].

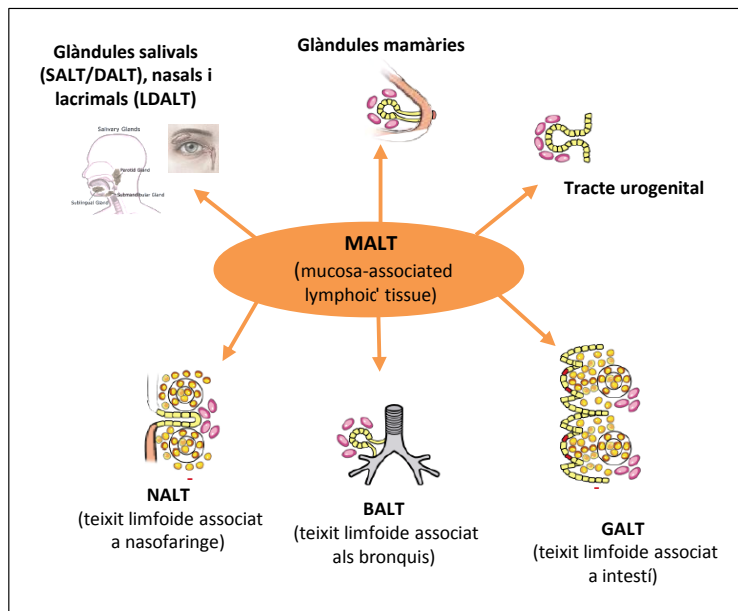


Figura 4. Principals components del teixit limfoide associat a mucoses (MALT).

El teixit limfoide més extens del cos és el GALT el qual, anatòmicament, inclou el compartiment organitzat i el difús. En el compartiment organitzats es troben les PP, els fol·licles limfoides aïllats (ILF, *isolated lymphoid follicles*) i els ganglis limfàtics mesentèrics (GLM), que són els responsables de la captació dels Ag directament de la superfície mucosal i estimulació dels limfòcits T i B verges, i indueixen la resposta immunitària. Per altra banda, el GALT difús, està format per limfòcits dispersos al llarg de l'epiteli (IEL, *intraepithelial lymphocytes*) i de la *lamina propria* (LP) de la mucosa i constitueix el lloc efector de la resposta immunitària, on les cèl·lules efectores, després de la seva extravasació i diferenciació, porten a terme la seva funció [69] (**Figura 5**). Tot i que els GLM estructuralment formen part del compartiment inductor o organitzat de la resposta

immunitària, existeix certa controvèrsia a l'hora de considerar-los, o no, part del GALT [68].

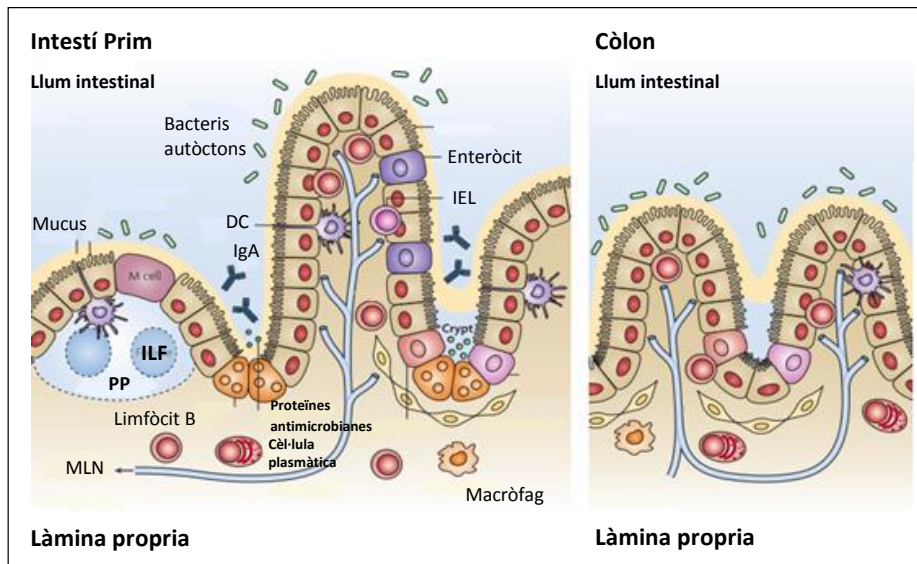


Figura 5. Representació esquemàtica del teixit limfoide associat a l'intestí (GALT) prim i gros (adaptada de [1]). **DC:** dendrític cell; **IEL:** limfòcits intraepitelials; **IgA:** immunoglobulina A; **ILF:** fol·licles limfoides aïllats; **MLN:** ganglis limfàtics mesentèrics; **PP:** Plaques de Peyer.

Com s'ha mencionat anteriorment, les glàndules salivals també es consideren part del sistema immunitari mucosal. Concretament, formen part del compartiment efector de la resposta immunitària [67,70]. Els limfòcits prèviament estimulats en els compartiments inductors del GALT i NALT arriben a les glàndules salivals promovent la producció d'anticossos específics en la saliva, majoritàriament d'isotip IgA [67,70]. Les glàndules salivals es troben ubicades dins la boca i al voltant de la boca i la gola i poden classificar-se en glàndules salivals

majors o menors. De glàndules menors n'hi ha entre 800-1000 distribuïdes al llarg de la cavitat oral, mentre que són tres les glàndules majors: la paròtide, la submaxil·lar i la sublingual [71].

1.3.1.1. Plaques de Peyer

Les PP constitueixen el principal teixit limfoide organitzat de l'intestí prim i es localitzen en la cara antimesentèrica, fonamentalment de l'ili i, amb menor freqüència, del jejú, d'humans i rosegadors. Estan formades per diversos fol·licles macroscòpics de limfòcits B [72] entre els que s'ubiquen limfòcits T CD4+ i DC [66].

En humans, les PP es comencen a formar a les 11 setmanes de gestació, i a les 19 ja s'observen les àrees de limfòcits T i B. No obstant, és just després del naixement quan, l'estimulació constant per part dels bacteris intestinals, indueix la formació dels centres germinals [73]. La quantitat i mida de les PP depèn de la presència, o no, de microbiota i també de l'edat, assolint el seu màxim entre els 15-25 anys d'edat [74,75].

En rata, no és fins als 12 dies d'edat que les PP comencen a ser visibles macroscòpicament tot i que els centres germinals es desenvolupen més tardanament [76]. Pel que fa a la composició cel·lular en rata, els limfòcits B i T suposen al voltant del 60% i 25%, del contingut cel·lular de les PP, respectivament i les cèl·lules *natural killer* (NK) constitueixen el 2,5% [77].

Les PP representen el lloc més prominent d'inducció de la resposta immunitària mucosal a Ag intestinals. Una de les seves principals funcions és la d'albergar la formació de centres germinals. És aquí on té lloc el contacte entre els limfòcits B, els Ag presentats per les DC

fol·liculars (FDC, *follicular dendritic cells*) i els limfòcits T col·laboradors o *T helper* (Th) fol·liculars que comporta la producció d'IgA. A més, és en les PP on els limfòcits B experimenten el canvi d'isotip d'IgM a IgA (*class switching*) [66]. També s'ha suggerit la implicació de les PP en la inducció de la tolerància oral, tot i que no existeixen estudis concloents [78].

1.3.1.2. Fol·licles limfoides aïllats

Els ILF són agregats més petits que les PP distribuïts al llarg de la LP de l'intestí prim, principalment a la part més distal de l'ili, i del còlon [79] [66,80]. A diferència de les PP, els ILF es desenvolupen un cop s'ha produït la colonització bacteriana de l'intestí [74]. Representen el prototip de teixit limfoide intestinal induït per la microbiota intestinal ja que és essencial en el manteniment de l'equilibri entre la microbiota intestinal i el sistema immunitari. Els ILF estan formats per un fol·licle de limfòcits B envoltat per una gran quantitat de DC i algun limfòcit T CD4+ [66]. Els ILF contribueixen significativament a la síntesi d'IgA a l'intestí. De fet les DC dels ILF són capaces d'activar els limfòcits B provocant així el canvi d'isotip d'IgM a IgA, fins i tot, en absència de limfòcits T o centres germinals [74].

1.3.1.3. Ganglis limfàtics mesentèrics

Els GLM són estructures ovoides lleugerament aplanades que es troben ubicades en el mesenterí de l'intestí prim i que constitueixen un punt de trobada per Ag, cèl·lules presentadores d'Ag (APC, *antigen presenting cells*) i limfòcits procedents de la sang [69].

En un gangli es distingeixen tres compartiments amb una composició cel·lular característica: escorça, paraescorça i medul·la. La regió més superficial és l'escorça que es caracteritza per tenir un elevat nombre de limfòcits B i de FDC. La paraescorça, ubicada just per sota l'anterior, presenta una elevada proporció de limfòcits T i de APC. Finalment, la regió més interna del gangli, la medul·la, conté acumulacions de cèl·lules plasmàtiques, limfòcits T i B, macròfags i DC [81].

Els limfòcits T verges circulants arriben als GLM guiats per quimiocines produïdes per les DC a través de vècules especialitzades [82]. Les DC residents en l'escorça capten i processen els Ag que arriben a través de la limfa i, és llavors quan les DC més madures migren cap a la paraescorça on presenten l'Ag als limfòcits verges Th o T citotòxics (Tc, *cytotoxic T cells*). Així és com es generen els limfòcits T efectors i com s'activa la resposta immunitària adaptativa [82].

1.3.1.4. Limfòcits de la lamina propria

La LP es troba immediatament per sota de l'epiteli i la membrana basal, estenent-se entre les criptes i cap a l'eix de cada vellositat intestinal. La LP conté una rica xarxa sanguínia i limfàtica cap a les quals s'absorbeixen els productes de la digestió.

A diferència dels ILF o les PP, la LP conté menys limfòcits B i, a nivell d'intestí prim, la població limfocitària de la LP en humans està bàsicament constituïda per cèl·lules plasmàtiques productores d'IgA i un nombre quasi igual de TCR $\alpha\beta$ +CD4+ i TCR $\alpha\beta$ +CD8 $\alpha\beta$ + [83]. En rata, la població limfocitària de la LP està constituïda per 50-60% de limfòcits CD4+, 10-15% de limfòcits CD8+, 3-4% de limfòcits NK i 30% de limfòcits B [84]. La composició de cèl·lules limfocitàries a la LP li confereix la característica de ser, juntament amb els IEL, un

compartiment clau en la generació de la resposta immunitària efectora.

1.3.1.5. Limfòcits intraepiteliais

Els IEL resideixen a l'espai interepitelial, per sota de les unions estretes i per sobre de la membrana basal. Donat la llargada de la superfície mucosal i la seva proporció respecte a les cèl·lules epitelials (1:4-9), els IEL representen una gran proporció de la població de cèl·lules immunitàries [85]. Tot i que són una població bastant heterogènia, la majoria d'IEL tenen un fenotip citotòxic o supressor poc habitual en els teixits limfoides i que és característic del compartiment mucosal (CD8 $\alpha\alpha$ +) [86]. S'ha demostrat que els IEL tenen el fenotip típic dels limfòcits efectors o de memòria amb una habilitat immunoreguladora capaç de produir una resposta efectora immediata i intensa. És per això que juguen un paper essencial en el procés de la tolerància oral [87].

1.3.2. Immunitat innata intestinal

Els elements que formen part de la resposta immunitària innata apareixen en edat primerenca i defensen d'agressions externes. La resposta immunitària innata a l'intestí està constituïda per elements como les barreres físiques i químiques (epiteli i substàncies antimicrobianes), la pròpia microbiota intestinal i les cèl·lules fagocítiques i citotòxiques [88].

1.3.2.1. Interacció microbiota-sistema immunitari intestinal

El sistema immunitari intestinal ha de ser capaç de discernir ràpidament entre “*el que és propi*” i “*el que és aliè*” a fi de mantenir de manera constant l’homeòstasi intestinal. La interacció entre els bacteris intestinals i l’hoste es coneix com a “*crosstalk*”, mostrant així una forma de comunicació pacífica i dinàmica entre ambdós. Aquesta interacció es produeix mitjançant molècules específiques presents a les parets bacterianes que reben el nom de PAMP o MAMP, acrònims de patró molecular associat a patògen (*pathogen-associated molecular patterns*) i patró molecular associat a microbi (*microbe-associated molecular patterns*), respectivament, els qual són reconeguts per les cèl·lules de l’organisme a través dels receptors de reconeixement de patrons (PRRS, *pattern recognition receptors*). Així doncs, els PRRS contribueixen a la primera línia de defensa de l’hoste enfront patògens i juguen un paper important tant en la resposta immunitària innata com en la resposta immunitària adaptativa donant pas al posterior procés inflamatori.

Els PRRS presenten una àmplia distribució i poden trobar-se ubicats en la membrana cel·lular o en el citosol. Entre els receptors citosòlics es troben les dues famílies de PRRS més estudiades: els receptors de tipus *NOD* (NLR) i el gens induïbles per àcid retinoic (RIG-1, *retinoic acid inducible gene 1*). Els PRRS que es troben a les membranes cel·lulars inclouen els receptors tipus *toll* (TLR, *toll-like receptors*). La seva activació pot produir-se tant a nivell de les mucoses com en el compartiment sistèmic. En condicions fisiològiques, quan els PRRS funcionen de manera correcta, petites quantitats de MAMP i Ag exògens procedents de microorganismes són neutralitzats amb normalitat, mantenint l’homeòstasi. No obstant, qualsevol desregulació en la funcionalitat d’aquests receptors pot ocasionar una inflamació greu i/o crònica, a més de desordres immunitaris [89,90].

Els TLR són glucoproteïnes transmembrana de tipus I, el domini extracel·lular de les quals consisteix en seqüències d'aminoàcids repetides riques en leucina, mentre que el component intracel·lular conté un domini TIR (*Toll/IL-1 receptor*). Fins ara s'han identificat 13 TLR diferents en mamífers, 10 dels quals són funcionals en humans (TLR1-10) i 13 en rosegadors (TLR1-9, TLR11-13 comuns en rata i ratolí i el TLR10 únicament en rata) [91–94]. Individualment, aquests sensors confereixen protecció davant un nombre reduït de PAMP, però conjuntament, reconeixen una elevada quantitat de molècules derivades de protozous, fongs, bacteris i virus [95,96] (**Taula 1**). També intervenen en el reconeixement de molècules intracel·lulars endògenes alliberades per cèl·lules activades o necròtiques i per la matriu extracel·lular, anomenades patrons moleculars associats a dany (DAMP, *damage-associated molecular patterns*), desencadenant-se així una resposta inflamatòria per tal de promoure la reparació del teixit [97].

La majoria dels TLR (TLR1, TLR2, TLR4, TLR5, TLR6 i TLR10) s'expressen extracel·lularment mentre que la família dels TLR3, TLR7, TLR8, i TLR9 són receptors intracel·lulars localitzats en els compartiments endosomals i són responsables del reconeixement de patògens intracel·lulars tals com virus i alguns bacteris [98]. Aquests receptors es poden expressar en cèl·lules epitelials intestinals, macròfags, DC, limfòcits B i T o en cèl·lules de l'estroma, entre d'altres [99].

Taula 1. Molècules reconegudes pels TLR funcionals identificats en mamífers.

TLR	Funcionalitat en mamífers	Molècules que reconeix
1	H, R	Triacil lipopèptid
2	H, R	Diacil i triacil lipopèptid, polisacàrid A
3	H, R	RNA de doble cadena
4	H, R	Lipopolisacàrid (LPS)
5	H, R	Flagelina bacteriana
6	H, R	Diacil lipopèptid
7	H, R	RNA víric de cadena simple i diversos anàlegs sintètics d'imidazoquinolina i guanosina
8	H, R	RNA víric de cadena simple i diversos anàlegs sintètics d'imidazoquinolina i guanosina
9	H, R	DNA amb motius CpG no metilats freqüentment present en genomes vírics i bacterians
10	H	n.e.
11	R	Proteïnes de protozous (p.e. profilina)
12	R	n.e.
13	R	n.e.

H: Humans; **R:** Rosegadors; n.e.: no especificat

De forma general, davant d'un reconeixement microbià específic, aquests receptors incorporen proteïnes adaptadores tals com MyD88, TRIF i TRAM. Cada TLR recluta una determinada combinació d'adaptadors a fi de promoure la resposta inflammatòria adequada. La unió d'aquests adaptadors mitjançant el seu domini TIR implica el reclutament de cinases associades al receptor d'interleucina (IL)-1 (IRAK, *IL-1R-associated kinases*) i la posterior activació de factors de transcripció tals com el factor nuclear NF- κ B, el factor 3 regulador de interferó (IRF3) i cinases activades per mitògens (MAPK). Aquesta activació indueix, a la vegada, a l'expressió de mediadors pro-inflamatoris antimicrobians, entre els que destaquen IL-6, factor de necrosis tumoral α (TNF- α , *tumoral necrosis factor*) i IL-1 β [100–102].

La senyalització mitjançant TLR a l'intestí està implicada en funcions crucials per al manteniment de la barrera epitelial com és la proliferació epitelial, la producció d'IgA, el manteniment de les unions estretes i l'expressió de pèptids antimicrobians [103]. No obstant, les diferents condicions de l'intestí prim i del còlon, ja sigui per la diferència en la densitat bacteriana, la qual arriba a ser de 10^9 a la part més distal de l'intestí prim, justifiquen la diferència d'expressió en cada regió intestinal [103]. A més a més, hi ha una gran varietat de factors que poden afectar a l'expressió dels TLR, entre els que destaquen citocines, estrès cel·lular, agents infecciosos, així com factors dietètics.

Tot i que els TLR tenen, fonamentalment, un paper de defensa en la resposta immunitària innata, la seva inapropiada activació ha estat implicada en la patogènesi de molts desordres gastrointestinals i malalties autoimmunitàries com ara la malaltia celíaca, la malaltia inflamatòria intestinal, el càncer de còlon i la colitis infecciosa [98,99]. Degut a la contínua exposició a la microbiota, l'organisme ha de desenvolupar mecanismes que redueixin el risc d'activació inapropiada dels TLR a fi de mantenir l'homeòstasi mucosal.

1.3.2.2. Immunitat cel·lular innata

Si els microorganismes superen la barrera epitelial intestinal, el sistema immunitari innat és capaç de fer front als possibles microorganismes patògens mitjançant altres mecanismes de defensa com són les cèl·lules fagocítiques i citotòxiques disperses pels teixits intestinals.

Els macròfags intestinals són cèl·lules clau en la defensa innata capaces de portar a terme respostes fagocítiques ràpides a través,

d'entre d'altres, el reconeixement de PAMP per mitjà dels TLR i NLR que expressen. Aquestes cèl·lules, exerceixen la seva funció a través de mecanismes moleculars que permeten desenvolupar fortes respostes fagocítiques i bactericides. No obstant, els macròfags intestinals presenten una baixa expressió de receptors com ara els receptors de LPS, IgA i IgG a més d'una baixa producció de citocines pro-inflamatòries (IL-1, IL-6 i IL-8) [104].

Els neutròfils són cèl·lules granulocítiques que representen aproximadament el 60% de la població leucocitària sanguínia en estat no infecció [105]. Contribueixen en la lluita contra els patògens invasors mitjançant l'alliberament de molècules antimicrobianes i espècies reactives d'oxigen (ROS; *reactive oxygen species*) així com amb el reclutament i activació de les APC. Els neutròfils també ajuden al manteniment de l'homeòstasi intestinal. Es requereixen un seguit de senyals (citocines, molècules d'adhesió i quimiocines d'elevada especificitat) per tal d'afavorir la seva migració cap a la llum intestinal [105]. Els neutròfils eliminen els microorganismes patògens a través de la fagocitosi i de la producció de "trampes" extracel·lulars o NET (*neutrophils extracellular traps*). Aquestes trampes permeten agrupar als agents antimicrobians per tal d'agafar i matar als agents patògens [106]. De forma similar que amb els TLR, l'activació descontrolada dels neutròfils pot conduir a la destrucció cel·lular i a un seguit d'esdeveniments que agreugen la patologia, com es el cas de la malaltia inflamatòria intestinal [105].

Les cèl·lules NK són un tipus de limfòcits citotòxics granulars de gran mida que constitueixen entre el 5-20% dels limfòcits en sang [107]. En humans i rosegadors adults, el percentatge del limfòcits NK+ intraepiteliais i de LP intestinal és molt baix i, fins i tot, indetectables [108,109]. En canvi, en rates de poca edat, el percentatge és relativament elevat, sent la majoria CD8+ en ambdós compartiments.

Aquest fet evidencia la importància d'aquesta població limfocitària en la resposta immunitària inespecífica durant el primers anys de vida, moments en els que les subpoblacions limfocitàries involucrades en la resposta immunitària adaptativa no estan ben desenvolupades [86,109]. Quan els senyals dels receptors d'activació superen als dels receptors d'inhibició, els limfòcits NK inicien les seves funcions efectores entre les que s'inclouen la secreció de citocines tipus Th1 com INF- γ , i la funció citotòxica directa [110].

1.3.3. Immunitat adaptativa intestinal

Si els agents patògens aconsegueixen evadir la resposta immunitària innata, els vertebrats disposen de mecanismes més evolucionats, dirigits principalment per limfòcits, els quals constitueixen l'anomenada resposta immunitària específica o adaptativa. Aquest tipus de resposta es desenvolupa, bàsicament, mitjançant dos mecanismes fonamentals: la resposta immunitària cel·lular portada a terme per cèl·lules, entre les que destaquen els limfòcits T; i la resposta immunitària humoral que, a nivell d'intestí, és exercida principalment per limfòcits B implicats en la secreció d'IgA o IgM.

Les respostes immunitàries adaptatives permeten prevenir l'entrada de virus a les cèl·lules, neutralitzar els efectes d'exotoxines, opsonitzar amb anticossos els patògens a fi de potenciar la seva eliminació mitjançant el sistema del complement i la fagocitosi [111]. D'altra banda, la microbiota suposa un repte pel sistema immunitari adaptatiu degut a l'elevada càrrega antigènica que ha de ser suportada sense induir resposta defensiva.

La resposta immunitària adaptativa intestinal inclou les següents fases:

- **Captació de l'Ag:**

Els Ag luminals poden travessar la barrera mucosal i arribar al GALT mitjançant diverses vies (**Figura 6**). L'entrada es produeix, principalment, a través de les cèl·lules M presents a l'epiteli associat al fol·licle de les PP i dels ILF. Aquestes cèl·lules estan especialitzades en la captació i transport a través de l'epiteli d'Ag procedents de macromolècules, virus, bacteris i paràsits [61,112]. En la seva superfície apical, en lloc de microvellositats com la resta d'enteròcits, presenten microplecs que les fan més accessibles al material luminal afavorint d'aquesta manera la transcitosi [61]. Les cèl·lules M expressen receptors d'IgA que els permet capturar complexos IgA-bacterians i transportar-los a través de l'epiteli [113]. A més, expressen PRRS, com els TLR, en la seva membrana apical el que afavoreix el reconeixement dels agents patògens [114]. La membrana basolateral de les cèl·lules M presenta una profunda invaginació que alberga subpoblacions específiques de limfòcits B i T verges i de memòria, i DC que seran les encarregades de processar els Ag per a la posterior presentació antigènica [115].

Ara bé, les cèl·lules M no són l'única via d'accés dels Ag ja que també poden ser captats per mecanismes transcel·lulars i paracel·lulars (mitjançant les unions estretes entre les cèl·lules de l'epiteli intestinal). Els enteròcits també poden captar els Ag solubles a través de la membrana apical tot i superant la dificultat d'accedir als agregats macromoleculars i microorganismes degut a la presència del glicocàlix [116]. A més, les DC especialitzades de la LP de l'intestí prim expressen les mateixes proteïnes presents en les TJ podent així estendre les seves dendrites entre les TJ de les cèl·lules epitelials per tal de captar directament l'Ag a nivell luminal [117].

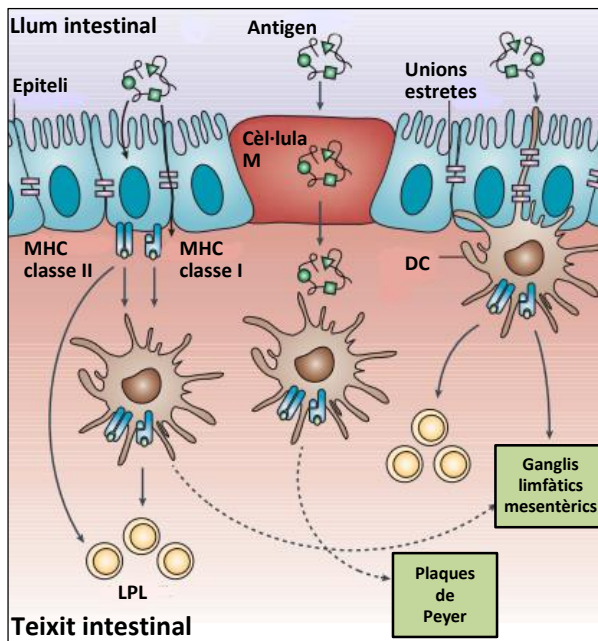


Figura 6. Mecanismes de captació antigènica a l'intestí (adaptada de [320]). **DC:** cèl·lula dendrítica; **LPL:** limfòcits de lamina pròpia; **MHC:** complex principal d'histocompatibilitat

○ Presentació de l'Ag i migració cel·lular:

Les cèl·lules M entreguen l'Ag intacte a les APC professionals ubicades en la cúpula subepitelial de les PP. Tot seguit, les APC amb l'Ag es dirigeixen a les àrees de limfòcits T i/o fol·licles de limfòcits B on interactuen amb els limfòcits verges [69]. Les APC interioritzen i processen l'Ag, fragments del qual passaran a expressar-se en la membrana plasmàtica associats a molècules del complex principal d'histocompatibilitat (MHC, *major histocompatibility complex*) per tal de ser reconeguts pel receptor de limfòcits T (TCR). Les APC activades poden interaccionar amb limfòcits B i T de les PP o ILF, migrant posteriorment als GLM mitjançant els vasos limfàtics (**Figura 7**). Un cop activats, els limfòcits T o B poden portar a terme les seves funcions efectores.

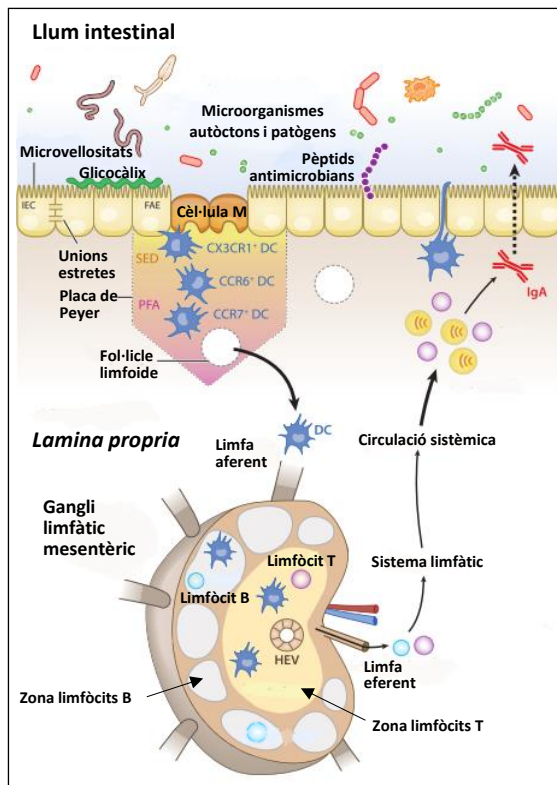


Figura 7. Mecanismes de captació i presentació antigènica, migració cel·lular i funció efectora a l'intestí. Les cèl·lules M capten els Ag de la llum intestinal i els transfereixen a les DC que els transporten fins als GLM a través del sistema de vasos limfàtics aferents. Les DC promouen la diferenciació dels limfòcits T efectors i regulen el canvi d'isotip dels limfòcits B. Els limfòcits activats es dirigeixen cap a la sang i tornen a la LP per realitzar les seves funcions. En la LP, els limfòcits B IgA+ es diferencien a cèl·lules plasmàtiques productores de dímers d'IgA, que són transportats a la llum intestinal [321].

La generació d'una resposta immunitària efectiva depèn de la migració o *homing* organitzada de les diverses subpoblacions de limfòcits B i T als diferents teixits. La migració de cèl·lules activades en les PP o GLM cap a la LP de l'intestí prim, on té lloc la resposta efectora, depèn de l'expressió de receptors de quimiocines i integrines [118,119]. Aquest patró d'expressió és diferent als que expressen els limfòcits T activats en altres òrgans limfoides.

- **Síntesi d'immunoglobulina A**

La producció d'IgA a l'intestí representa una estratègia clau per conferir protecció immunitària no inflamatòria [62]. La IgA és l'isotip més abundant dels anticossos produïts a les secrecions mucosals [55,120] i s'estima que al voltant del 80-90% es troba en la mucosa intestinal [121]. La IgA és capaç d'interaccionar amb el receptor d'immunoglobulines polimèriques (pIgR), transportador d'anticossos que s'expressa en la superfície basolateral de les cèl·lules epitelials. Un cop s'ha unit al pIgR a través de ponts disulfur mitjançant una glicoproteïna coneguda com a cadena J de la IgA, els dímers d'IgA són transportats cap a la superfície mucosal mitjançant un procés de transcitosi epitelial. Durant aquest procés, en quedar unit el component secretor del pIgR a la IgA, es forma la IgA-secretora (S-IgA). El component secretor confereix protecció a la IgA front les proteases presents a la llum intestinal [52].

La S-IgA és capaç de neutralitzar toxines i patògens sense generar inflamació donat a la seva incapacitat d'activar el sistema del complement [62,122]. A més, fixa bacteris al mucus, i bloqueja la seva entrada a la mucosa intestinal, facilita el creixement dels bacteris de la microbiota i inhibeix alhora el dels patògens, promou la relació microbiota-hoste disminuint l'expressió d'epítops proinflamatoris per part de la microbiota, a la vegada que neutralitza compostos amb activitat proinflamatòria [30]. Així doncs, la S-IgA es considera un element clau en el manteniment de l'homeòstasi entre la tríada formada per la microbiota, les cèl·lules epitelials ubicades al llarg del TGI i el sistema immunitari mucosal subjacent [120].

La síntesi d'IgA pot produir-se per dues vies:

- ***Síntesi d'IgA dependent de limfòcits T***

La majoria d'Ag provoquen una resposta mucosal de tipus IgA a través de mecanismes dependent de limfòcits T que tenen lloc a les PP i, en menor proporció, als GLM [55,62,66]. Les PP presenten tres característiques que promouen la síntesi d'IgA. En primer lloc els centres germinals de les PP afavoreixen la interacció entre limfòcits B i T a més d'expressar l'enzim citidina desaminasa induïda per activació (AID), específica de limfòcits B i necessària pel canvi de classe. D'altra banda, la proporció de limfòcits B respecte als limfòcits T és molt superior comparat amb els ganglis limfàtics perifèrics i a més expressen una gran quantitat de citocines inductores de la síntesi d'IgA (com IL-5, IL-6, IL-21 i, especialment, el TGF- β 1 [30].

Per a la generació de limfòcits B IgA+ específics en les PP, el TGF- β 1 s'uneix al lligam CD40 (CD40L), expressat en els limfòcits T CD4+. A la vegada, IL-6, IL-10 i IL-21 faciliten l'expansió de les limfòcits B IgA+ i la seva diferenciació a cèl·lules plasmàtiques secretores d'IgA (IgA-SC, *IgA secreting cells*). Les DC de les PP juguen un paper essencial ja que són les úniques productores d'IL-6 i TGF- β 1 [123]. Un cop madures, les cèl·lules IgA+ activades circulen per sang fins a la LP on tindrà lloc la diferenciació a IgA-SC [62]. En aquest procés de migració cap a l'intestí, l'expressió de la integrina $\alpha_4\beta_7$ i el seu receptor CCR9 en els limfòcits B IgA+ [119] així com l'expressió del receptor CCR10 i el seu lligam CCL28 expressat a l'intestí prim i còlon [124] juguen un paper important.

A la LP, els limfòcits B IgA+ es diferencien en cèl·lules plasmàtiques productores d'IgA, capaces de sintetitzar la cadena d'unió J que

permetrà la formació d'oligòmers estables d'IgA amb major afinitat per l'Ag [30].

- ***Síntesi d'IgA no dependent de limfòcits T***

Per tal de compensar la limitació de la via anterior, la mucosa intestinal ha desenvolupat una via més ràpida de generar IgA sense necessitat de limfòcits T en resposta a Ag ben conservats de bacteris i patògens mitjançant els TLR. Arrel d'aquesta nova via, s'ha qüestionat la necessitat dels limfòcits T en la producció d'IgA. Aquesta generació té lloc als espais fol·liculars i extrafol·liculars, incloent la LP [112].

Els TLR poden activar directament les limfòcits B o bé induir l'alliberament del factor d'activació de limfòcits B denominat BAFF i el seu homòleg (APRIL, *a proliferation-inducing ligand*) per part de les DC, monòcits i cèl·lules epitelials entre d'altres. Aquests estimulen la recombinació i canvi d'IgG a IgA en humans i ratolins al unir-se als limfòcits B a través del receptor TACI (*transmembrane activator and calcium modulator and cyclophilin ligand interactor*) [62].

2. CACAU

El cacau s'obté de les llavors de l'arbre anomenat *Theobroma cacao* per Carl Linnaeus al 1753, terme que significa l'"aliment dels déus" [125] (**Figura 8**).

Estudis recents mostren que el cacau i el seu derivat, la xocolata, presenten efectes beneficiosos per a la salut. Ara bé, els beneficis del cacau ja eren aplicats per les civilitzacions mesoamericanes, les quals des de l'any 700 a.C. preparaven begudes a partir de les llavors de cacau per usar-les en la pràctica mèdica [126,127].



Figura 8. Fruits de l'arbre *Theobroma cacao*

Al 1502, els europeus van descobrir el cacau durant el viatge de Cristòfor Colom i la seva tripulació al "Nou Món". Poc temps després, al 1528, Hernan Cortés, conscient del seu valor, va introduir les llavors de cacau a la Península Ibèrica. De forma gradual, l'hàbit de consumir la beguda preparada a partir de l'anomenat "*or marró*" es va difondre per Europa i es va convertir en la beguda de moda [127,128]. El cacau begut però, era exclusiu de les classes socials altes donat l'elevat preu de les llavors a causa de la seva escassetat. El consum de begudes amb cacau es va estendre ràpidament per l'aristocràcia europea. No obstant, no va ser fins al segle XIX quan es va elaborar el primer cacau sòlid passant a ser considerat una llaminadura [129].

2.1. PROCÉS D'ELABORACIÓ DEL CACAU

El procés habitual d'elaboració del cacau inclou processos com fermentació, assecat i un posterior torrat de les llavors del cacau. Seguidament s'espel·lofen i s'obtenen els *nibs*, que a continuació són sotmesos al procés de mòlta, el que permet aconseguir la pasta o licor de cacau, un producte líquid a temperatura ambient. El cacau en pols és el resultat de l'eliminació parcial del greix present en el licor de cacau, mentre que la xocolata resulta de la mescla del licor de cacau amb sucre. Segons la proporció de licor de cacau i sucre s'obtindrà un determinat tipus de cobertura (xocolata dolça, amarga o semi amarga) [130] amb unes característiques organolèptiques i una composició nutricional determinades.

Les característiques del producte final depenen, a més, de la varietat de cacau usada. N'existeixen tres: la cultivada més àmpliament, sobretot a Àfrica occidental i Brasil, és la varietat Forastero, el Criollo és sembrat a Veneçuela, Carib i Madagascar, entre altres, i és considerat un "cacau fi i d'aroma", i el Trinitario, la tercera varietat, és un híbrid de les dues anteriors i es cultiva principalment a les Antilles i Trinitat [131].

2.2. COMPOSICIÓ DEL CACAU

El cacau en pols aporta proteïnes (15-20%), carbohidrats (~15%), lípids (10–24%, majoritàriament 10-12%) i fibra (26-40%). També és considerat bona font de minerals (fòsfor, magnesi, calci, ferro, zinc, potassi, clor i coure), vitamines (A, del grup B, C i E) [131] (**Taula 2**) i polifenols [132]. A més, conté metilxantines, com cafeïna (0,2%) i, sobretot, teobromina (~2,1%).

Taula 2. Cacao en pols: informació nutricional per 100 g (adaptada de [131]).

Nutrients	Contingut per 100 g
Macronutrients	
Energia	
kcal	201
kJ	841
Proteïnes (g)	22
Hidrats de carboni (g)	12,5
Midó	11,5
Sucre	1
Lípids (g)	11
Saturats	6,9
Monoinsaturats	3,8
Poliinsaturats	0,3
Fibra (g)	32
Metilxantines (g)	2,3
Teobromina	2,1
Cafeïna	0,2
Micronutrients	
Na (mg)	30
K (mg)	3000
Ca (mg)	150
Mg (mg)	550
P (mg)	700
Cl (mg)	10
Fe (mg)	35
Zn (mg)	7
Cu (mg)	4
Vitamina A (mg) (retinol)	<0,02
Vitamina B1 (mg)	0,1
Vitamina B2 (mg)	0,3
Vitamina B3 (mg)	2
Vitamina B5 (mg)	1,5
Vitamina C (mg)	<0,1
Vitamina E (mg) (tocoferol)	2,3

2.2.1. Fibra

El cacau és una excel·lent font de fibra dietètica. De fet, la quantitat de fibra en el cacau és superior a la quantitat present en aliments habitualment considerats com a “rics en fibra” tals com les fruites fresques (0,1-3,3%), llegums (9,0-18,7%), verdures i hortalisses (1,0-3,5%), cereals i derivats (0,6-2,7%) i, fins i tot, les algues Nori i Wakame (~ 34%) [133,134].

Un 83,32% de la fibra de cacau és de tipus insoluble i el 16,67% restant és fibra soluble (**Taula 3**). Al voltant d'un terç de la fibra insoluble correspon a substàncies no amilàcies (18,1% de la matèria seca) i a més, conté glucosa, el que suggereix que la cel·lulosa és el principal polisacàrid no digerible de la fibra de cacau (al voltant del 10% de la matèria seca). La fibra de cacau insoluble també conté petites quantitats de monosacàrids com arabinosa, xilosa, manosa, o galactosa juntament amb un contingut significatiu d'àcids urònics, que són indicatius de la presència d'hemicel·luloses i substàncies pèctiques, ambdues fermentables. En quant a la fracció soluble de fibra, s'han trobat quantitats apreciables de galactosa i manosa que podrien ser indicatius de la presència de galactomanans o formar part de les substàncies pèctiques [135].

Per altra banda, la fibra de cacau conté 9% de greix i un 18% de proteïnes referit a pes sec [136]. A més conté un petit percentatge de polifenols (normalment 1,15% del pes sec), el qual és lleugerament superior al descrit per a determinats aliments d'origen vegetal com fruites seques, fruites fresques i verdures [137].

Per tant, la fibra de cacau, juntament amb els polifenols amb capacitat antioxidant podrien tenir un paper actiu en el manteniment de la salut humana i en la prevenció de patologies amb elevada incidència com són les malalties cardiovasculars o el càncer [136].

Taula 3. Fibra de cacau: contingut i composició de les fraccions soluble i insoluble (adaptada de [135]).

	% matèria seca
Fibra dietètica soluble	10,09 ± 0,38
Ramnosa	0,29 ± 0,04
Fucosa	n.d.
Arabinosa	0,29 ± 0,01
Xilosa	0,09 ± 0,01
Manosa	0,51 ± 0,01
Galactosa	1,36 ± 0,02
Glucosa	0,41 ± 0,03
Sucres neutres	2,96 ± 0,10
Àcids urònics	7,13 ± 0,29
Fibra dietètica insoluble	50,42 ± 0,29
Ramnosa	0,15 ± 0,01
Fucosa	0,06 ± 0,02
Arabinosa	0,94 ± 0,01
Xilosa	0,97 ± 0,01
Manosa	0,96 ± 0,01
Galactosa	0,91 ± 0,01
Glucosa	10,53 ± 0,15
Sucres neutres	14,53 ± 0,16
Àcids urònics	3,48 ± 0,13
Lignans Klason	32,41 ± 0,40
Fibra dietètica total	60,51 ± 0,32

2.2.2. Flavonoides

Un altre dels components bioactius presents en el cacau en pols que ha guanyat interès en els darrers anys són els polifenols, particularment els flavonoides. Aquests productes són metabòlits secundaris de les plantes i contribueixen a la pigmentació blava, vermellosa i ataronjada present en flors, fruites i vegetals [131,138,139]. Els flavonoides també estan presents en llavors, grans, espècies i begudes derivades com el vi, el te o la cervesa [138,140].

Els principals flavonoides presents en el cacau són els flavanols, com la (+)-epicatequina, la més abundant (21–43 mg/g) i la (-)-catequina, que constitueixen un 37% del contingut polifenòlic. A més, conté oligòmers derivats de les catequines anomenats procianidines (també coneguts com a tanins condensats) [141,142].

El contingut de flavonoides en el producte final pot ser molt variable en funció de diversos factors. En concret, factors genètics i agrícoles [143] així com els diferents processos als que és sotmesa la llavor de cacau (fermentació, torrat o alcalinització) poden modificar notablement el contingut final de polifenols [131,144,145].

Per tal d'evitar la pèrdua de flavonoides s'ha intentat desenvolupar noves metodologies per tal d'obtenir un producte amb major contingut dels components bioactius del cacau i potenciar-ne així els seus efectes beneficiosos. En aquest sentit, s'ha elaborat un producte a partir de llavors de cacau que no ha estat sotmès a fermentació ni a torrefacció, comercialitzat com a Cocoapure® (inicialment anomenat CocoanOX®), el qual manté en una gran proporció els flavonoides presents en la llavor [146,147]. Per considerar l'activitat biològica dels flavonoides cal tenir en compte també la seva biodisponibilitat, absorció i metabolisme a nivell gastrointestinal, la seva distribució

tissular i cel·lular així com les noves formes bioactives derivades del seu metabolisme [143,148].

La biodisponibilitat dels flavonoides està influenciada pel seu grau de polimerització així com per la matriu alimentària en la que es troben i les interaccions amb altres components de la dieta [149–151]. En aquest sentit, l'elevat contingut en fibra de cacau pot contribuir en el transport dels flavonoides al llarg del TGI [152]. Altres factors que també influeixen són l'edat, la genètica, la presència d'alteracions fisiològiques, el metabolisme, la composició de la microbiota o l'activitat enzimàtica colònica de l'individu [153].

Un cop ingerits, els polifenols són metabolitzats com a substàncies xenobiòtiques. Els flavanols en forma monomèrica són absorbits a nivell de l'intestí prim on són metabolitzats per enzims de fase II que donen lloc a conjugats glucuronitzats [154,155]. Seguidament poden incorporar-se a la circulació sistèmica o bé ser eliminats en la bilis. Per contra, la major part dels flavonoides presents en el cacau en forma de procianidines formades per tres o més monòmers no s'absorbeixen fàcilment a l'intestí prim i arriben al còlon on passen a ser substrat del metabolisme bacterià [156]. S'ha identificat un reduït nombre de espècies bacterianes amb capacitat de catabolitzar els flavanols en la microbiota intestinal. La majoria pertanyen als *phyla Firmicutes* (*Clostridium* spp. i *Bifidobacterium* spp.) i *Actinobacteria* (*Lactobacillus* spp.) [157–159]. En el còlon, a partir de les procianidines es generen una gran varietat d'àcids fenòlics. Entre ells destaquen l'àcid fenilpropioníc, l'àcid fenilacètic i els derivats de l'àcid benzoic [158,160–163]. Aquesta conversió és essencial per a l'absorció de les procianidines i dóna lloc a metabòlits que són més beneficiosos que les formes originals [164–166]. Així doncs, hi ha autors que promouen l'estudi dels metabòlits derivats de la microbiota així com dels

generats pel propi hoste a l'hora d'establir associacions entre la ingesta de polifenols del cacau i els seus efectes sobre la salut [167]. Tot seguit, els metabòlits també poden ser metabolitzats pels enzims de fase II i, finalment, entren a la circulació sistèmica on poden ser font de compostos potencialment bioactius [168] o bé poden ser eliminats en orina [156,166]. A la vegada, una proporció de metabòlits microbians són eliminats en femtes. L'excreció dels metabòlits generats a partir del metabolisme microbià varia notablement entre individus i, per algunes persones, en funció del substrat [156].

2.3. EFECTES BIOLÒGICS DEL CACAU

El cacau, gràcies al seu contingut en polifenols i els metabòlits derivats d'aquests, presenta efectes antioxidants ja que és capaç de neutralitzar radicals lliures, actuar com a quelant de metalls (Fe^{2+} i Cu^+) implicats en la formació de ROS; inhibir enzims relacionats amb la producció d'oxidants (xantina oxidasa) i estimular mecanismes antioxidants endògens [169]. Concretament, s'ha vist que el cacau presenta una activitat antioxidant dues vegades superior a la descrita pel vi negre, de dues a tres vegades superior a la que presenta el te verd i de quatre a cinc vegades superior a la que s'ha descrit pel te negre [145]. Tot i que la capacitat antioxidant del cacau ha estat àmpliament estudiada *in vitro* i *in vivo* [170–186], s'ha vist que els flavonoides poden actuar també com a prooxidants quan s'administren a dosis elevades o bé en presència de metalls amb activitat redox [187,188]. Per altra banda, s'ha descrit que altres components presents en el cacau com les metilxantines o la fibra també poden contribuir a la capacitat antioxidant del cacau [135,189,190].

A través de la seva capacitat antioxidant, o bé per altres mecanismes, el cacau exerceix efectes beneficiosos en l'organisme. De fet, el consum de cacau ha estat associat principalment amb efectes positius sobre el sistema cardiovascular [191], la carcinogènesi [192,193], sistema nerviós central, la pell, l'obesitat, la resistència a la insulina [132,194,195] i sobre la resposta inflamatòria i el sistema immunitari [196].

2.3.1. Efectes cardiovasculars

Les malalties cardiovasculars constitueixen una de les principals causes de mort a nivell mundial i sobretot en països industrialitzats [197]. La dieta és un dels elements clau a l'hora de controlar els factors de risc cardiovasculars (hipertensió, tabaquisme, hiperlipidèmia i diabetis mellitus) [132] i reduir-ne així la seva incidència [198]. En aquest sentit, diversos estudis demostren una correlació inversa entre la ingesta de cacau o productes derivats i les malalties cardiovasculars [167, 199–202].

L'interès per l'efecte del cacau sobre la prevenció de la hipertensió arterial s'inicià amb el descobriment d'una població aborigen originària de l'arxipèlag de San Blas (Panamà), els indis nadius de Kuna, els qual presentaven taxes clarament inferiors d'hipertensió (~2,2%) que no augmentaven amb l'edat [200,203,204]. No obstant, si els indis Kuna migraven a àrees urbanes de Panamà mostraven una major prevalença d'hipertensió i arribaven a igualar les xifres de la resta d'habitants del nucli urbà [205]. Es van analitzar els diferents factors medioambientals implicats, incloent-hi la dieta, i es va descobrir que a l'arxipèlag, els indis consumien cinc tasses al dia de cacau ric en

polifenols (900 mg polifenols/dia), dieta que no seguien a l'emigrar [204–206].

Diversos estudis *in vitro* i *in vivo* han permès la identificació de dianes cel·lulars i moleculars a través de les quals el cacau pot exercir els seus efectes sobre el sistema cardiovascular [147,207,208]. Els efectes beneficiosos del cacau poden atribuir-se a l'activació del sistema òxid nítric sintasa (NOS), a la modulació del sistema renina-angiotensina-aldosterona, a la capacitat de millorar el perfil lipídic i la sensibilitat a la insulina i al seu potencial d'inhibir l'activació i agregació plaquetària [191,209–214].

2.3.1.1. Estudis *in vitro*

Estudis *in vitro* demostren que el cacau és capaç d'inhibir l'activitat de l'enzim convertidor d'angiotensina (ECA) [211,215], enzim implicat en la formació d'angiotensina II, un potent vasoconstrictor. S'ha descrit que l'addició d'extracte de cacau (6,25-100 µg/mL) a cèl·lules endotelials de vena umbilical humana disminueix l'activitat de l'ECA de forma dosi depenent als 10 minuts i, després de 24 h, la dosi més elevada provoca un increment de la síntesi de monòxid de nitrogen (NO) [208]. Un altre estudi realitzat també amb cèl·lules de vena umbilical humana, ha mostrat l'efecte inhibidor de l'epicatequina en l'expressió gènica i l'activitat de l'arginasa 2, enzim implicat en la síntesi de NO en la paret vascular [216]. Aquests resultats es correlacionen amb un estudi realitzat en conills en el que es va veure que les procianidines polimèriques del cacau són capaces d'induir relaxació endotelial en l'aorta aïllada de conills a través de l'activació de NOS [217]. Per altra banda, els flavonoides del cacau (catequina, epicatequina i procianidines) eviten l'oxidació de les lipoproteïnes de

baixa densitat (LDL, *low-density lipoprotein*) *in vitro*, procés que està estretament relacionat amb la formació de les paques d'ateroma [218–221].

2.3.1.2. Estudis *in vivo*

També s'ha avaluat l'efecte del cacau sobre la pressió arterial, el desenvolupament de plaques d'ateroma i el perfil lipídic en models animals. En aquest sentit, s'ha demostrat l'efecte protector de l'epicatequina en models d'hipertensió espontània en rates [222] i en rates tractades amb un inhibidor potent de NOS [223]. La dieta suplementada amb epicatequina els va permetre mantenir les xifres de tensió arterial en els rangs òptims, va prevenir l'increment dels biomarcadors d'estrès oxidatiu a més de recuperar els nivells de NO [222,223]. Igualment, s'ha evidenciat que una única administració per via oral de varies dosis d'un extracte natural de cacau enriquit en flavonoides reduïa les xifres de pressió arterial en un model d'hipertensió espontània en rates [147].

El consum prolongat de cacau en conills hipercolesterolèmics és capaç d'enlentir l'oxidació de les LDL i de reduir l'àrea de lesions ateroscleròtiques en l'aorta [207]. A més a més, s'ha descrit una menor concentració de colesterol, triglicèrids i LDL en un model d'aterosclerosi en hámsters als que es va administrar cacau durant 10 setmanes [224]. Així mateix, en rates alimentades amb una dieta hipercolesterolèmica, la ingesta de cacau durant quatre setmanes és capaç de disminuir la concentració de colesterol en plasma i incrementar l'eliminació de colesterol i àcids biliars en femtes. No obstant, l'efecte no va ser evident si la dieta contenia una mescla

d'epicatequina i catequina demostrant la implicació d'altres substàncies del cacau [225].

En aquest sentit, Sánchez *et al.* [226] han demostrat que la ingesta prolongada d'un producte soluble de fibra de cacau en l'aigua de beguda és també capaç de protegir enfront la hipertensió arterial en un model d'hipertensió espontània en rata. Aquest producte va reduir la hipertensió i la concentració de malondialdehid plasmàtica. No obstant quan es va produir la retirada del producte es va observar un increment en les xifres de pressió arterial [226]. D'altra banda, s'ha evidenciat l'efecte hipocolesterolèmic i una reducció en la peroxidació lipídica en rates alimentades amb una dieta rica en greix suplementada amb fibra de cacau durant tres setmanes [136,227]. D'altra banda, Ried *et al.* [228] descarten el paper de la teobromina de cacau com a responsable d'aquesta disminució de la pressió arterial tot i la seva coneguda activitat com a vasodilatador [229].

2.3.1.3. Estudis clínics

S'ha avaluat l'impacte de la ingesta de cacau i xocolata en diferents grups de normotensos, incloent joves, adults, amb sobrepès i hipercolesterolèmia; pre-hipertensos i hipertensos amb i sense tolerància a la glucosa afectada. En general s'ha demostrat l'efecte anti-hipertensiu del cacau i la xocolata [228,230]. Estudis en individus hipertensos lleus no tractats indiquen que el consum d'una dosi de 1052 mg de flavanols del cacau/dia durant 6 setmanes és capaç de reduir les xifres de pressió arterial [231]. S'ha observat un efecte similar en individus adults amb sobrepès que van consumir 74 g de xocolata negra sòlida [232]. Estudis intervencionals en individus sans indiquen que el consum de cacau redueix l'oxidació de LDL [220] a la

vegada que incrementa el colesterol HDL (*high-density lipoprotein*) a nivell plasmàtic [233–235]. També s'ha descrit un descens dels triglicèrids i dels biomarcadors de la peroxidació lipídica plasmàtics en individus sans que han consumit xocolata o begudes de cacau [182,236,237]. De forma contrària, altres estudis indiquen que la ingesta regular de productes de cacau en individus sans no influeix en la concentració d'aquests biomarcadors [233,238] i que l'oxidació de LDL no es veu modificada per un consum diari de 48 g de xocolata o 18 g de cacau durant 6 setmanes en individus que presenten malalties coronàries [239].

D'altra banda, s'ha evidenciat una millora en la funció endotelial i plaquetària per la ingesta de xocolata negra (amb un 70% de cacau) en individus sans [240,241], amb sobrepès [232] i trasplantats del cor [242]. A més d'una millora en la funció endotelial, voluntaris sans que van consumir diàriament cacau ric en flavanols (821 mg flavanols/dia) durant 5 dies presentaren vasodilatació perifèrica [243].

Entre els possibles mecanismes mitjançant els qual el cacau exerceix els seus efectes antihipertensius destaquen la seva capacitat d'incrementar la producció de NO [238] i prostaciclina, un inhibidor de l'agregació plaquetària [244].

Donat que els estudis preclínics i clínics recolzen els efectes beneficiosos dels flavonoides presents en el cacau a nivell cardiovascular, l'*European Food Safety Authority* (EFSA) va elaborar al 2010 i 2012 les declaracions de propietats saludables dels flavonoides del cacau en relació al sistema cardiovascular [245,246].

Per altra banda, s'ha descrit que altres components presents en el cacau, com la fibra de cacau, exerceixen també un efecte protector davant les malalties cardiovasculars. En aquest sentit, la ingesta de

dues racions al dia de 15 g d'un producte de cacau enriquit en fibra de cacau reduïa les xifres de pressió arterial, la peroxidació lipídica i els nivells de glucosa en individus amb hipercolesterolèmia moderada [247].

2.3.2. Efectes anticancerígens

La inflamació crònica i l'estrès oxidatiu són factors involucrats en els processos cancerígens. La formació de ROS, espècies reactives intermediàries d'oxigen i de nitrogen (ROI i RNI, respectivament) poden lesionar el DNA i/o interferir en la seva reparació i donar lloc a mutacions que impedeixen el control de la replicació i creixement cel·lular. Així doncs, donada la capacitat antioxidant dels flavonoides del cacau, no és d'estranyar que la seva ingesta estigui també associada a una millora en els processos cancerígens.

2.3.2.1. Estudis *in vitro*

S'ha evidenciat la capacitat antiproliferativa, proapoptòtica, antimutagènica i quimioprotectora dels extractes de cacau, juntament amb la seva acció preventiva en la formació de ROS [192,248–253].

En un model d'escissió del DNA per mitomicina C, el licor de cacau - ric en catequina i procianidines del tipus B [254] - és capaç d'inhibir la lesió del DNA [248].

D'altra banda, s'ha demostrat que un extracte de flavonoides del cacau en cèl·lules epitelials de fetge de rata inhibeix diversos processos induïts per H₂O₂ i relacionats amb la carcinogènesi [252]. Concretament, el cacau inhibeix la formació de comunicacions

intercel·lulars en fenedura (GJIC, *gap junctional intercellular communication*), la fosforilació i internalització de la connexina 43, l'acumulació de ROS i l'activació de les cinases regulades per senyals extracel·lulars (ERK, *extracellular signal-regulated kinases*) [252]. Els efectes antiproliferatius de les procianidines del cacau també s'han observat a través del bloqueig de la fase G2/M del cicle cel·lular, i la disminució de l'activitat de l'ornitina descarboxilada i la S-adenosilmetionina descarboxilada, dos enzims clau en la biosíntesi de poliamines [249].

En relació als efectes antimutagènics i quimioprotectors, Ohno *et al.* [251] han observat que l'extracte de cacau inhibeix un mutagen químic, el benzo[α]pirè, que provoca danys al DNA quan el seu metabolisme s'activa pel citocrom P450 (CYP). En cèl·lules tumorals de càncer de mama, s'ha vist que l'extracte de cacau provoca la sobreexpressió del CYP que activa el metabolisme del benzo[α]pirè el que afavoreix el metabolisme i la producció de metabòlits estrogènics cap a formes no genotòxiques. Aquest fet ocasiona una davallada en els nivells estrogènics presents en els tumors mamaris i contribueix a l'activitat citotòxica del tamoxifen, un fàrmac antiestrogen amb efectes citotòxics [253].

2.3.2.2. Estudis *in vivo*

Els estudis realitzats *in vivo* sobre l'efecte antitumoral del cacau se centren, especialment, en models animals. S'ha estudiat l'efecte de l'extracte de licor de cacau sobre l'activitat enzimàtica de marcadors tumorals com la fosfatasa alcalina, la γ -glutamil transpeptidasa, el glutatió-S-transferasa i el glutatió reductasa, tant en plasma com en fetge obtinguts de rates amb hepatocarcinoma. Els resultats obtinguts

mostren que l'administració diària de l'extracte de licor de cacau durant dues setmanes, redueix l'activitat dels marcadors tumorals en els dos compartiments i demostren així el seu potencial en reduir la gravetat de l'hepatocarcinogènesi [255].

També s'ha avaluat l'efecte d'un extracte de cacau en pols (Acticoa) en la incidència de càncer de pròstata en rates Wistar. Després d'induir el càncer, els animals van ser alimentats amb Acticoa (24 o 48 mg/kg). Després de nou mesos d'aquesta dieta, el grup amb la dosi més baixa van presentar una menor incidència de tumors prostàtics i una taxa de supervivència superior, que va arribar a ser similar a la del grup control sa [256]. També s'ha descrit l'efecte protector del licor de cacau enfront el càncer de pulmó en un model de carcinogènesi múltiple en rates F344 i en fases inicials d'un model de carcinogènesi pancreàtic en rates Sprague-Dawley [257,258].

Per altra banda s'ha demostrat que una dieta rica en cacau exerceix efectes antiproliferatius en rates Wistar amb càncer de còlon a través de la reducció dels nivells d'ERK, proteïna cinasa B i ciclina D, molècules involucrades en processos de senyalització durant la divisió i supervivència cel·lular inhibint els processos apoptòtics. A més, la dieta va mostrar efectes proapoptòtics en reduir els nivells de Bcl-XL i incrementar els nivells de Bax i l'activitat de caspasa-3 [259].

Pel que fa a nivell clínic, hi ha estudis observacionals que han descrit una menor incidència de diferents tipus de càncer a conseqüència de la ingesta d'una dieta rica en flavonoides, inclosos els procedents del cacau [260]. De fet, els indis nadius de Kuna, ja presentaven una menor freqüència de mort per càncer a la que presentava la població que residia a Panamà, fet que es podria explicar, entre d'altres, per l'elevada ingesta de cacau que presentaven els primers [261].

3. CACAU I SISTEMA IMMUNITARI

La ingesta de cacau també té efectes sobre el sistema immunitari. Concretament, la dieta rica en cacau influeix en la resposta inflamatòria i en la resposta immunitària adaptativa, tant a nivell sistèmic com al compartiment intestinal. La revisió que es presenta a continuació recull les evidències científiques, publicades fins aleshores, en relació a l'efecte immunomodulador del cacau i proposa, alhora, possibles futures directrius amb l'objectiu d'esbrinar la contribució dels principals components bioactius del cacau en aquests efectes i les possibles conseqüències d'aquests efectes.



The effects of cocoa on the immune system

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Cocoa is a food relatively rich in polyphenols, which makes it a potent antioxidant. Due to its activity as an antioxidant, as well as through other mechanisms, cocoa consumption has been reported to be beneficial for cardiovascular health, brain functions, and cancer prevention. Furthermore, cocoa influences the immune system, in particular the inflammatory innate response and the systemic and intestinal adaptive immune response. Preclinical studies have demonstrated that a cocoa-enriched diet modifies T cell functions that conduce to a modulation of the synthesis of systemic and gut antibodies. In this regard, it seems that a cocoa diet in rats produces changes in the lymphocyte composition of secondary lymphoid tissues and the cytokines secreted by T cells. These results suggest that it is possible that cocoa could inhibit the function of T helper type 2 cells, and in line with this, the preventive effect of cocoa on IgE synthesis in a rat allergy model has been reported, which opens up new perspectives when considering the beneficial effects of cocoa compounds. On the other hand, cocoa intake modifies the functionality of gut-associated lymphoid tissue by means of modulating IgA secretion and intestinal microbiota. The mechanisms involved in these influences are discussed here. Further research may elucidate the cocoa compounds involved in such an effect and also the possible medical approaches to these repercussions.

Keywords: cocoa, lymphocyte, macrophage, antibody, cytokine, gut-associated lymphoid tissue, thymus, spleen

INTRODUCTION

In addition to the beneficial effects on oxidative stress, cardiovascular health, nervous system diseases, aging, and cancer prevention, cocoa has been revealed as a food with immunoregulatory properties. In the following sections, the influence of cocoa or its flavonoids on the innate and adaptive immunity are reviewed. Firstly, the anti-inflammatory properties of cocoa are briefly summarized, and secondly, the effects of cocoa on the adaptive immune system and intestinal immunity are reported.

To achieve this objective, a systematic search in SCOPUS-V.4 (Elsevier) – SciVerse was conducted for the following key terms: “cocoa” AND “lymphocyte” OR “immun*” OR “inflammation” OR “microbiota.” In order to prevent potential misclassification of relevant articles no exclusion criteria were used. The search included the period from January 1990 to March 2013.

ANTI-INFLAMMATORY POTENTIAL OF COCOA

Inflammation is the response of tissues to an aggression caused by pathogens, chemicals or wounding. Inflammation involves a complex network of reactions initially designed to protect the host from injury and to heal damaged tissue. The activation and migration of leukocytes to the site of the lesion and the release of growth factors, cytokines, reactive oxygen species (ROS), and nitric oxide (NO) are known to play a crucial role in the inflammatory response. Constant overproduction of pro-inflammatory molecules leads to chronic inflammation.

In general, flavonoids are associated with anti-inflammatory properties. In this regard, the flavanols contained in cocoa have

been the subject of both *in vitro* and *in vivo* studies (reviewed in Pérez-Cano et al., 2010). Many studies have reported cocoa's ability to reduce cytokines, chemokines, ROS, NO, etc. involved in inflammatory response. However, few studies have focused on the *in vivo* anti-inflammatory activity of cocoa.

COCOA EFFECTS ON INFLAMMATORY CELLS

Cocoa extracts or single flavonoids, both as monomers (epicatechin, catechin) or polymers (procyanidins) have demonstrated *in vitro* their anti-inflammatory potential, although there have been some controversial results.

A cocoa flavonoid-enriched extract and the monomers epicatechin and isoquercitrin were able to decrease the production of inflammatory molecules such as tumor necrosis factor (TNF)- α and monocyte chemoattractant protein (MCP)-1 by macrophages under stimulation with lipopolysaccharide (LPS; Ramiro et al., 2005a). Similarly, epicatechin in stimulated whole blood cells culture suppressed the production of interleukin (IL)-6 and IL-8 (Al-Hanbali et al., 2009). However, monomer to pentamer units and longer chain fractions of cocoa flavanols increased the secretion of TNF- α , IL-1, and IL-6 in LPS-stimulated peripheral blood mononuclear cells (PBMC; Mao et al., 2002; Kenny et al., 2007; Wisman et al., 2008).

Aside from cytokines, other inflammatory molecules can be influenced by cocoa. Epicatechin, procyanidin B₁, procyanidin B₂, and a cocoa extract reduced NO release by stimulated macrophages (Ono et al., 2003; Ramiro et al., 2005a; Hämmäläinen et al., 2007). Likewise, the *in vitro* treatment with cocoa fractions or flavonoids

alone decreased the production of ROS from several kinds of cells (Sanbongi et al., 1997; Erlejman et al., 2006; Granado-Serrano et al., 2007; Ramiro-Puig et al., 2009).

Neutrophils also play an important role during inflammation. It has been demonstrated that cocoa has the potential to positively modulate the neutrophil inflammatory activity. In this sense, certain flavanols and procyanidins isolated from cocoa moderated some signaling pathways induced by LPS on neutrophils, particularly those of oxidative bursts and activation markers, and cocoa could influence selected apoptosis mechanisms (Kenny et al., 2009).

Regarding the mechanisms of action, it has been reported that hexameric cocoa procyanidins have the capacity to modulate TNF- α -induced NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation in intestinal epithelial cells (Erlejman et al., 2008). NF- κ B is a transcription factor involved in the regulation of genes encoding cytokines (IL-1, IL-2, IL-6, IL-8, TNF- α , among others), adhesion molecules (e.g., intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and endothelial leukocyte adhesion molecule 1), acute phase proteins, inducible enzymes [inducible NO synthase (iNOS) and cyclooxygenase 2 (COX-2)], etc. (Pahl, 1999).

ANTI-INFLAMMATORY POTENTIAL OF COCOA IN HEALTHY CONDITIONS

In a more physiological approach, using cells isolated from humans or animals fed with diets containing cocoa, the *in vitro* ability to produce inflammatory mediators and the serum concentrations of inflammatory molecules has been studied.

Some studies have focused on the *in vitro* response of macrophages isolated from rats fed cocoa. It has been demonstrated that these cells produced lower amounts of TNF- α , IL-6, NO, and ROS (Ramiro-Puig et al., 2007a; Castell et al., 2009). Similarly, serum concentration of MCP-1 decreased after a cocoa diet in rats (Ramos-Romero et al., 2012a).

With regard to studies in humans, it has been reported that a supplementation with cocoa products in healthy humans did not affect inflammation markers (Mathur et al., 2002); however, a cross-sectional analysis showed that the regular intake of dark chocolate by a healthy population in Southern Italy was inversely related to serum C-reactive protein concentration (di Giuseppe et al., 2008). In addition, cocoa consumption for 4 weeks decreased some adhesion molecules involved in the recruitment of inflammatory cells (Monagas et al., 2009). More recently, leukocytes from healthy volunteers showed a decrease in the activation of NF- κ B and also in the serum concentrations of some adhesion molecules, such as intercellular adhesion molecule 1 and E-selectin, 6 h after receiving 40 g of cocoa powder (Vázquez-Agell et al., 2013).

COCOA DIET AND INFLAMMATORY DISEASES

Although at present no human intervention studies applying cocoa treatment in inflammatory conditions have been reported, some studies in animal models of diseases suggest the anti-inflammatory effect of cocoa. In this context, the oral administration of a cocoa polyphenolic fraction to mice has seen to inhibit ear edema in a dose-dependent manner (Lee et al., 2006a). Moreover, rats that received cocoa for a week (4.8 g/kg/day) developed

a lower paw edema induced by carrageenan and by bradykinin (Ramos-Romero et al., 2008; Castell et al., 2009).

The anti-inflammatory activity of cocoa has been extended to inflammatory bowel disease (IBD). Using IBD models, a number of flavonoids, such as quercitrin, rutin, diosmin, hesperidin, morin, and silymarin have demonstrated anti-inflammatory activity (reviewed in Comalada et al., 2013). However, a study using a cocoa diet in a dextran sodium sulfate (DSS) model demonstrated that cocoa intake did not improve clinical colitis, although it certainly contributed to reducing colonic oxidative activity and serum inflammatory mediator concentrations (Pérez-Berezo et al., 2012a). These results agree with those obtained with luteolin and with a lemon verbena infusion rich in polyphenolic compounds (Karrasch et al., 2007; Lenoir et al., 2011). More interestingly, it has recently been reported that a polyphenol-enriched cocoa extract was able to decrease acute DSS colitis in mice (Andújar et al., 2011), thus evidencing the need for a high polyphenol content in the cocoa to achieve anti-inflammatory activity in the IBD.

The effect of a cocoa diet on *in vivo* models of neuronal inflammation and systemic chronic inflammation such as adjuvant arthritis (AA) and collagen-induced arthritis (CIA) has also been reported. Rats fed a diet enriched with cocoa produced a decrease in the inflammatory response to an acute and chronic noxious stimulus of trigeminal ganglion neurons (Cady and Durham, 2010).

In AA, a cocoa-enriched diet was able to decrease the synthesis of antibodies against the pathology inducer, and to reduce the proportion of Th lymphocytes in blood and regional lymphoid tissues, but the cocoa diet produced only a tendency to modulate hind-paw swelling (Ramos-Romero et al., 2012b). It must be added that the oral administration of some flavonoids such as quercetin and hesperidin were only able to partially reduce AA swelling (Mamani-Matsuda et al., 2006; Li et al., 2010) or only slightly decreased this chronic inflammatory model (Rovenský et al., 2009). However, a cocoa diet was able to reduce the oxidative stress associated with AA (Ramos-Romero et al., 2012c).

Concerning the CIA model in rats, a diet enriched in cocoa beginning 2 weeks before CIA induction and given throughout the process, has been applied. Although arthritic cocoa-fed rats decreased specific autoantibody titers, the production of pro-inflammatory mediators from peritoneal macrophages, and the Th proportion in lymph nodes, they developed a similar hind-paw swelling as the reference arthritic animals (Ramos-Romero et al., 2012a). On the contrary, Miyake et al. (2008) reported that the oral administration of highly oligomeric procyanidins isolated from Jatoba (*Hymenaea courbaril*) ameliorated CIA in mice and also decreased the serum concentrations of some specific autoantibodies. Similarly, the oral consumption of an extract of green tea polyphenols reduced the incidence, the arthritis index and the autoantibody concentration of CIA in mice (Haqqi et al., 1999). Similarly, single flavonoids administered orally, such as hesperidin, resulted in preventive and therapeutic effects in mice CIA (Kawaguchi et al., 2006).

COCOA AND LYMPHOID TISSUES

Primary and secondary lymphoid tissues constitute two major categories of lymphoid organs. The formation of the primary

repertoire of lymphocytes takes place in the primary tissues such as thymus and bone marrow. Secondary lymphoid tissues are responsible for the coordination of immune responses by spatially organizing the interaction of immune effector cells (Drayton et al., 2006). By means of preclinical studies in rats, it has been evidenced that a cocoa diet can induce changes in the cell composition of both primary and secondary lymphoid organs. In particular, a cocoa diet has an influence on the proportion of B lymphocytes and T cell subsets, i.e., T cell receptor (TCR) $\alpha\beta^+$ cells, TCR $\gamma\delta^+$ cells, T helper (Th), cells and T cytotoxic (Tc) cells (Figure 1).

A cocoa diet influences antioxidant status and the cell composition of rat thymus. A diet containing 10% cocoa in rats increased the thymic content of catalase and superoxide dismutase and promoted the progression of immature thymocytes (double negative TCR $\alpha\beta^{\text{low}}$ and double positive TCR $\alpha\beta^{\text{high}}$ cells) toward more mature T cell stages (CD4 $^+$ CD8 $^-$ TCR $\alpha\beta^{\text{high}}$ cells; Ramiro-Puig et al., 2007b). Similarly, a diet with 10% cocoa was able to influence a secondary lymphoid tissue such as the rat spleen and lymph nodes (Figure 1). Young rats fed cocoa decreased the spleen percentage of Th cells while increasing that of B cells (Ramiro-Puig et al., 2007a). Additionally, adult Louvain rats fed 10% cocoa for 6 weeks reduced the proportion of TCR $\alpha\beta^+$ cells in inguinal lymph nodes (Ramos-Romero et al., 2012a). Likewise, the percentage of Th cells was reduced in mesenteric lymph nodes (MLNs) at the expense of Tc cells that increased in young Wistar rats fed 10% cocoa for 3 weeks, but not 4% cocoa (Ramiro-Puig et al., 2008; Figure 1). A high-cocoa diet also affects the lymphocyte composition of intestinal Peyer's patches (PPs). In particular, cocoa intake reduced the TCR $\alpha\beta^+$ cell percentage, mainly due to a decrease in the Th cell proportion, and increased B cell and TCR $\gamma\delta^+$ cell percentages (Ramiro-Puig et al., 2008; Figure 1). The increase in TCR $\gamma\delta^+$ cell percentages in PPs and MLNs induced by cocoa is similar to the effects of apple polyphenol intake in healthy mice (Akiyama et al., 2005) and could be especially important during childhood, when the immune system is maturing (Pérez-Cano et al., 2005), or in the prevention of food allergies.

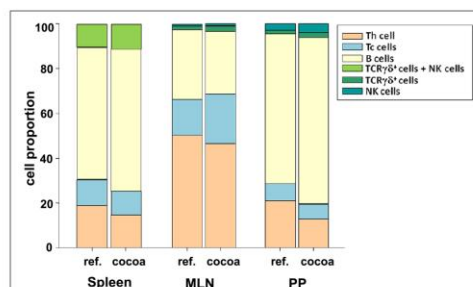


FIGURE 1 | Summary of the effects of a 10% cocoa diet in rats on lymphocyte proportion in secondary lymphoid tissues (based on Ramiro-Puig et al., 2007a, 2008). MLN, mesenteric lymph nodes; PP, Peyer's patches.

INFLUENCE OF COCOA ON ADAPTIVE IMMUNE RESPONSE

The adaptive immune response is an intricate reaction comprising a number of intracellular and intercellular events from the antigen entry until the development of effector mechanisms. Dendritic cells (DC), acting as antigen-presenting cells, take up, process and present antigen to TCR-specific Th lymphocytes. The interaction between DC and Th cells involves a lot of co-stimulatory molecules thus forming the immune synapses (Dustin and Groves, 2012). Next, Th lymphocytes proliferate and differentiate, becoming effector cells such as Th1, Th2, Th17, or regulatory T cells that produce cytokines. Some of these cytokines involve the activation of other antigen-specific cells such as Tc cells or B cells. Activated B cells differentiate into plasma cells, which synthesize antibodies that specifically bind the antigen that has triggered the adaptive immune response.

The first event in adaptive response refers to Th cell activation. Specific recognition of antigenic peptide by TCR together with co-stimulatory molecules causes production of IL-2 (Malek, 2008). IL-2 binds to a receptor consisting of the subunits α , β , and γ (CD25, CD122, and CD132, respectively) to produce cell proliferation (Boyman and Sprent, 2012). CD25 is expressed after Th cell activation. The binding of IL-2 to its receptor leads to the stimulation of complex transduction signals involving mitogen-activated protein kinase (MAPK), Janus kinase/signal transducer and activator of transcription (JAK/STAT), and phosphatidylinositol 3-kinase (PI3K)/Akt pathways that eventually mediates multiple biological processes including T cell and B cell growth and differentiation (Malek, 2008; Boyman and Sprent, 2012).

COCOA AND TH CELL ACTIVATION

IL-2 and CD25 are markers of early lymphocyte T activation. Some *in vitro* studies have reported the effect of isolated cocoa flavonoids and cocoa extracts in the synthesis of IL-2. Sanbongi et al. (1997) found that cocoa liquor polyphenols inhibited both IL-2 gene expression and IL-2 secretion in human blood T cells. Likewise, Mao et al. (1999, 2000) showed that a crude cocoa extract and pentamer, hexamer and heptamer procyanidins from cocoa also reduced IL-2 transcription in phytohemagglutinin (PHA)-stimulated human PBMC. Similarly, in a lymphoid cell line activated with phorbol 12-myristate 13-acetate (PMA) and IL-1 and cultured in the presence of epicatechin or a cocoa extract, it has been established that cocoa flavonoids were able to decrease the expression of surface CD25 and to diminish IL-2 secretion (Ramiro et al., 2005b). The ability of the cocoa extract to decrease CD25 expression was higher than that of epicatechin alone, which may be due to the effect of other cocoa flavanols (Ramiro et al., 2005b). Overall, these *in vitro* studies agree that cocoa flavonoids can decrease IL-2 production in Th cells. These results are also in line with those described with other flavonoids, such as genistein (Atluru et al., 1991) and Pycnogenol® (Cho et al., 2001). How cocoa flavonoids modulate IL-2 gene is not known but it has been demonstrated that epicatechin and dimeric procyanidins decrease NF- κ B activation on PMA-activated Jurkat cells, a lymphoid cell line (Mackenzie et al., 2004). The inhibition of NF- κ B might mediate the downregulation of both IL-2 and CD25 in a similar way to that of the decrease in pro-inflammatory mediators.

It is interesting to note the effect of cocoa procyanidins on the plasma membrane of Jurkat T cells. After the adsorption of flavonoids, the plasma membrane became more fluid, and procyanidins prevented the leakage of small molecules from vesicles (Verstraeten et al., 2004). These effects could also influence the establishment of immune synapses thus attenuating the interaction between the Th cell and the antigen-presenting cell.

In spite of the *in vitro* results, *in vivo* studies do not confirm the downregulation of IL-2 by cocoa flavonoids. Some studies carried out in rats showed the effect of a diet containing cocoa on the functionality of immune cells isolated from spleen or lymph nodes. Splenocytes from rats fed cocoa (a diet with either 4 or 10% defatted cocoa) did not decrease IL-2 production or CD25 surface expression after stimulation with PMA plus ionomycin (Ramiro-Puig et al., 2007a; Pérez-Berezo et al., 2009). Likewise, these cells showed a similar or even higher proliferative response (Ramiro-Puig et al., 2007a). In the same way, lymphocytes from cocoa-fed rat MLN produced higher or equal amounts of IL-2 (Ramiro-Puig et al., 2008; Pérez-Berezo et al., 2009; Ramos-Romero et al., 2012b).

COCOA AND EFFECTOR T CELLS

After naïve Th cell activation and proliferation, effector Th lymphocytes appear. Depending on the cytokines released to the medium, which are eventually related to the antigen that trigger the immune response, activated Th1 cells, Th2 cells, Th17 cells, or regulatory T cells result (Nakayama and Yamashita, 2008; Amsen et al., 2009; Korn et al., 2009). Th1 cells direct cell-mediated immunity against intracellular pathogens by means of the synthesis and release of interferon (IFN)- γ , TNF- α , and TNF- β , among others. These cytokines promote phagocytosis and cytotoxicity recruiting macrophages, natural killer (NK) cells, Tc cells, and also the enhancement of complement-activating antibodies synthesis. Th1 activity is usually associated with inflammation (Nakayama and Yamashita, 2008). Th2 cells are designed to fight against extracellular pathogens, activating mast cells and eosinophils, and the production of antibodies which are not able to activate the complement system. Th2 cells are involved in the humoral immunity and allergic reactions (Nakayama and Yamashita, 2008). The Th2 subset produces cytokines such as IL-4 and IL-5 that help B cells to proliferate and differentiate, and IL-10 with anti-inflammatory properties. IL-4 is mainly produced by activated Th2 cells and plays an important role in regulating Th1/Th2 balance (Nakayama and Yamashita, 2008). Recently, the effectors Th cell family expanded with the discovery of Th17 cells. These cells produce IL-17 and exhibit effector functions distinct from Th1 and Th2 cells. The primary function of Th17 cells appears to be the clearance of pathogens that are not adequately handled by Th1 or Th2 cells and they are potent inducers of tissue inflammation (Korn et al., 2009).

The effect of cocoa diets in rats on the cytokine production by Th1 and Th2 cells has been reported. The secretion of IFN- γ , the main cytokine related to Th1 activity, has been quantified in cells isolated from the spleen and lymph nodes of rats fed a cocoa diet. No changes in the secretion of this cytokine were observed in splenocytes (Ramiro-Puig et al., 2007a; Ramos-Romero et al., 2012b), although others found increased values (Pérez-Berezo

et al., 2009), and *in vitro* studies demonstrated a suppression of IFN- γ production by PHA-stimulated PBMC (Jenny et al., 2009).

More interestingly, a cocoa diet in rats produced a lower IL-4 secretion in isolated splenocytes (Ramiro-Puig et al., 2007a; Pérez-Berezo et al., 2009) and MLN cells (Ramiro-Puig et al., 2008). However, IL-10 secretion was not modified in rats fed a cocoa diet (Ramiro-Puig et al., 2007a, 2008). The results obtained in these *in vivo* experiments did not exactly fit with those obtained in *in vitro* studies with cocoa flavonoids. Thus, an increase in IL-4 secretion after epicatechin addition in a lymphoid cell line and PBMC has been reported (Mao et al., 2000; Ramiro et al., 2005b), whereas hexamer to octamer cocoa procyanidins presented an inhibitory effect on this cytokine (Mao et al., 2000).

COCOA AND HUMORAL IMMUNE RESPONSE

As stated before, an increase in the percentage of B cells in spleen was observed in rats fed cocoa (Figure 1). However, the antibody response of these cells has been found to be attenuated. Thus, the ability to produce IgG, IgM, and IgA by splenocytes from rats fed cocoa was depressed (Ramiro-Puig et al., 2007a). This effect was also reflected in serum immunoglobulin concentrations. Three-week-old rats fed with 10% cocoa for 3 weeks, but not those fed 4% cocoa, had lower serum IgG, IgM, and IgA concentrations (Ramiro-Puig et al., 2007a; Pérez-Berezo et al., 2012b). However, when the cocoa diet began later and the dose was lower, the effect was not so patent (Pérez-Berezo et al., 2011).

The influence of a 4 and 10% cocoa diet on the antibody synthesis in immunized rats has been reported. When animals were fed cocoa before and during an immunization process, the synthesis of specific antibodies and the number of IgG-secreting cells decreased, although the proliferation rate of lymph node and spleen cells was maintained (Pérez-Berezo et al., 2009). The analysis of antibodies demonstrated that the impact on humoral response did not affect all antibody isotypes equally. The most attenuated isotypes were specific IgM, IgG1, IgG2a, and IgG2c whereas anti-OVA IgG2b concentrations held steady or increased with the 10% cocoa diet. IgG isotypes can be associated with Th1 or Th2 immunity. In the rat, IgG1 and IgG2a are related to the Th2 response, while IgG2b depends on the Th1 response (Binder et al., 1995; Gracie and Bradley, 1996; Saoudi et al., 1999; Bridle et al., 2007). These results agree with others that evaluated certain food polyphenols, such as those from apple or soybean (Akiyama et al., 2005; Kogiso et al., 2006). From all these results, it has been suggested that a cocoa diet mainly downregulates the Th2 immune response, whereas it maintains Th1 immunity. This hypothesis was supported by a lower IL-4 secretion from splenocytes and a higher production of IFN- γ from lymph node cells (Pérez-Berezo et al., 2009).

Because of a cocoa diet seems to attenuate antibody synthesis, it has been tested in experimental disease models in which antibodies play a pathogenic role, such as autoimmune diseases and allergic processes.

Rheumatoid arthritis (RA) is a systemic autoimmune disease in which chronic inflammation of synovial joints results in joint destruction, pain, disability, and a reduced life expectancy (Wegner et al., 2010). The pathology of the RA is mediated by specific autoantibodies, mainly against citrullinated proteins such as

collagen type II (Wegner et al., 2010). In consequence, CIA in rats or mice is the gold standard *in vivo* model for RA studies (Asquith et al., 2009). In such rat experimental model, the influence of a cocoa diet on joint inflammation and autoantibody titers has been reported (Ramos-Romero et al., 2012a). Louvain rats fed cocoa from 2 weeks before arthritis induction, and during the disease period studied (4 weeks), reduced the synthesis of specific antibodies against type-II collagen, but this effect was not enough to mitigate the hind-paw swelling in arthritic animals during the study period (Ramos-Romero et al., 2012a).

Allergic reactions are mainly caused by IgE-mediated hypersensitivity. In allergic patients, the immune system reacts to innocuous substances by producing IgE. These antibodies bind to mast cells and, after allergen reaction, produce degranulation of mast cell mediators with a subsequent generation of allergic manifestations (Amim, 2012). The effect of cocoa in an allergy model has been preclinically studied. A diet containing 10% cocoa prevented the synthesis of antibodies involved in allergic reaction in young rats, in particular, rats fed a cocoa diet showed lower titers of specific IgG1, IgG2a and a decrease of specific IgE of about 60–70% (Abril-Gil et al., 2012). The effect of a cocoa diet on allergic manifestations has not yet been established. However, it is noteworthy that the modulation of specific IgE was also observed in allergy models after treatment with flavonoids, and in these studies the effect on IgE synthesis was associated with lower allergy signs. This is the case in treatment with luteolin (Das et al., 2003), baicalin (Won Jung et al., 2012), biochanin A (Ko et al., 2011), quercetin (Cruz et al., 2012), myricetin (Medeiros et al., 2008), and hesperidin (Rogerio et al., 2007; Joskova et al., 2011). In addition, it can be added that clinical trials applying a treatment with Pycnogenol®, an extract of *Pinus maritima* containing procyanidins, demonstrated the efficacy of such intervention in reducing some signs of allergic asthma (Hosseini et al., 2001; Lau et al., 2004; Belcaro et al., 2011). From all these studies it can be concluded that foods enriched in flavonoids, such as cocoa, open a new perspective in their use as a nutraceutical in allergic diseases.

EFFECTS OF COCOA ON INTESTINAL IMMUNITY

The digestive system is the first compartment reached by dietary compounds. Bacteria, epithelial cells, and immune cells in the intestine are the first ones to be affected by diet. Every day, the gut-associated lymphoid tissue (GALT), which constitutes the most extensive and complex part of the immune system in the body, receives a huge antigenic load and has to distinguish between invasive pathogens and innocuous antigens from food and commensal bacteria. Briefly, the intestinal immune response is initiated in the M cells from PPs which uptake luminal antigens and transport them toward DC, which interact with interfollicular T lymphocytes or migrate toward MLN (Cerutti and Rescigno, 2008). This process induces differentiation and maturation of B cells, which become IgA⁺ cells and later IgA-secreting cells (Kunisawa and Kiyono, 2005). The main resulting product of the GALT is the secretory-IgA (S-IgA; Mora and von Andrian, 2008; Brandtzaeg, 2010). This immunoglobulin constitutes the first line of non-inflammatory immune protection at mucosal surfaces by neutralizing microbial pathogens and exotoxins and

by interacting with innocuous dietary antigens and commensal microbes (Corthésy, 2007; MacPherson et al., 2008).

Few studies addressing the dietary effects of cocoa on GALT function in healthy animals or humans have been reported to date. Dietary intervention with cocoa did not morphologically affect the intestinal structure (Ramiro-Puig et al., 2008), but is capable of modifying some important aspects of the GALT composition and functionality in rats as next detailed (Ramiro-Puig et al., 2008; Pérez-Berezo et al., 2011, 2012b; Figure 2).

COCOA DIET AND MESENTERIC LYMPH NODE LYMPHOCYTE ACTIVATION

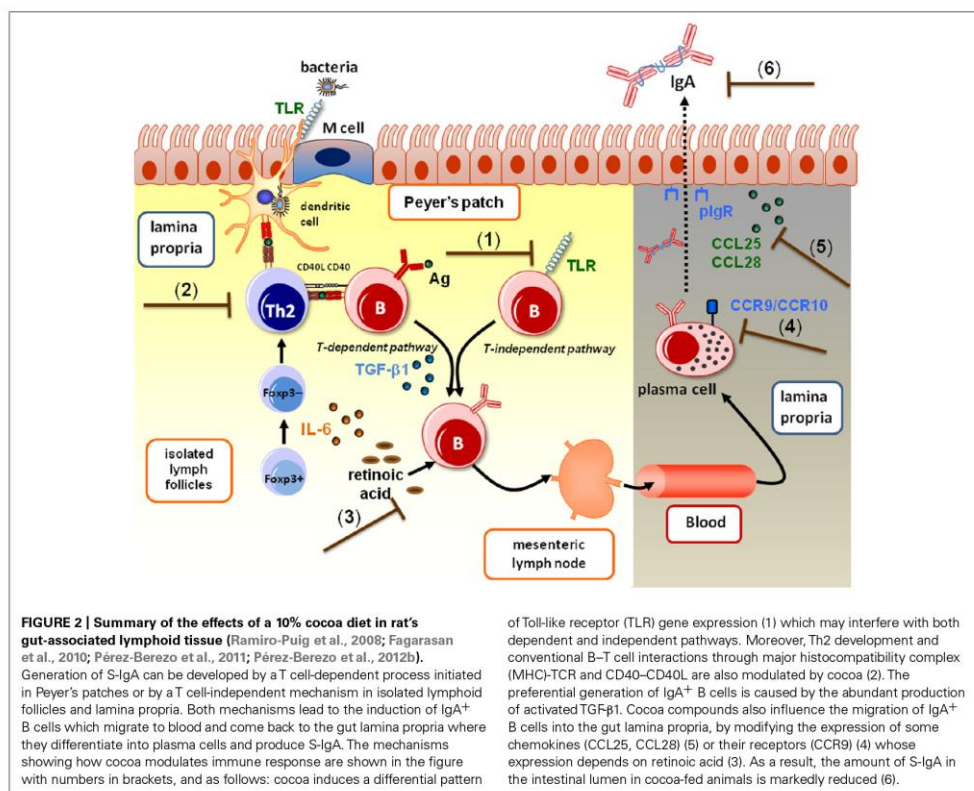
Rat interventional nutrition with a cocoa diet modulates MLN lymphocyte activation in certain conditions. Isolated MLN cells from young rats fed 10% cocoa for 3 weeks strongly enhanced IL-2 secretion; nevertheless, the proliferation rate did not increase after 48 h of cell culture (Ramiro-Puig et al., 2008). On the other hand, isolated MLN lymphocytes from rats fed a long-term cocoa diet (9 weeks) did not change IL-2 production after *in vitro* mitogen activation nor their proliferative ability after *in vitro*-specific activation (Pérez-Berezo et al., 2009).

To ascertain whether cocoa modified Th effector cell functionality in the GALT, IFN γ , IL-4, and IL-10 cytokine production was studied in stimulated MLN cells isolated from animals fed a cocoa diet (Ramiro-Puig et al., 2008). The nutritional 10% cocoa intervention for 3 weeks resulted in a lower IL-4 secretion, IL-10 secretion tended to decrease whereas that of IFN- γ tended to increase. A 4% cocoa intake for 3 weeks did not produce any significant modification (Ramiro-Puig et al., 2008). These results suggest that high-cocoa diets, similarly to the results found in the systemic compartment, downregulate Th2 responses, and therefore, may downregulate B cell differentiation and immunoglobulin production even at mucosal sites.

COCOA AND SECRETORY-IGA

Secretory-IgA plays a key role in the maintenance of gut homeostasis and oral tolerance and its function and production are tightly regulated (Cerutti and Rescigno, 2008). The relationship between a cocoa diet and S-IgA has been demonstrated in different experimental designs using rats, where the effect of varied proportions of cocoa diets (2, 4, 5, and 10%), different age at the beginning of the dietary nutritional (3 or 6 weeks of age) and length of diet (3, 6, or 9 weeks) have been analyzed. As demonstrated next, cocoa reduces S-IgA protein and gene expression which conduces a different pattern of IgA-coating bacteria. The effect of cocoa on S-IgA might be due to the influence of cocoa on genes related to Th maturation, Th-B cell interactions, and IgA⁺ B cell gut-homing, among others (Pérez-Berezo et al., 2011, 2012b).

First data showing a downmodulatory effect of cocoa on S-IgA were established in fecal samples after 2 weeks of both 4 and 10% cocoa intake in young rats. However, this effect only remained for 3 weeks in the 10% cocoa diet. The decrease in fecal IgA correlated with a lower concentration of S-IgA and S-IgM in gut washes (Ramiro-Puig et al., 2008). These results were confirmed in a study that extended the dietary intervention with a 10% cocoa diet up to 6 weeks (Pérez-Berezo et al., 2012b). If dietary intervention began later in rat age (6-week-old animals), the attenuating effect of



cocoa remained for 5 and 10% cocoa diet, showing a rapid effect even 1 week after the diet start (Pérez-Berezo et al., 2011). However, a lower cocoa proportion (2%) tended to reduce fecal IgA levels after only 3 weeks of diet. Therefore, cocoa diets, especially those with a higher cocoa proportion, decreased S-IgA concentration in the intestinal lumen of rats (Ramiro-Puig et al., 2008). This effect was associated with a lower number of PP cells with a high capacity to secrete IgA (Ramiro-Puig et al., 2008), and with a downregulation of IgA gene expression in PP cells and in the wall of the small intestine and colon (Pérez-Berezo et al., 2011, 2012b; Massot-Cladera et al., 2012).

Some commensal intestinal bacteria in humans and rodents coat S-IgA by an apparent non-random immunological phenomenon (Tsuruta et al., 2010). In fecal samples from rats collected before and after a cocoa diet, the IgA-coating bacteria were enumerated. After 6 weeks, reference animals showed an increase in the percentage of IgA-coating bacteria that was avoided with the 10% cocoa diet (Massot-Cladera et al., 2012).

In order to look further into the downregulation of S-IgA through a cocoa diet in rats, the gene expression of several

molecules involved in intestinal immune response was established using different cocoa proportions (2, 5, and 10%), supplementation periods (3 or 6 weeks) and initial age of rats (3 or 6 weeks; Pérez-Berezo et al., 2011, 2012b). A pathway for B cells to become IgA-secreting cells is a T cell-dependent process located in either PPs or MLNs, inductive sites of the intestinal immune system (Kunisawa and Kiyono, 2005; Mora and von Andrian, 2008). The maturation of mucosal Th cells depends on IL-6, among others; the interaction between activated Th cells and B cells requires the interaction of CD40 ligand with CD40 (Islam et al., 1991; Cerutti, 2008), and the differentiation of B cells into IgA⁺ B cells involves transforming growth factor-β1 (TGF-β1), IL-5, IL-6, IL-10, and IL-21 (Schoenbeck et al., 1989; Briere et al., 1994; Ramsay et al., 1994; Dullaers et al., 2009). The study of a 10% cocoa diet for 3 or 6 weeks in rats on the mechanisms of S-IgA secretion revealed that the cocoa diet did not modify TGF-β1 gene expression in PPs, MLNs, or the small intestine; however, IL-6 gene expression was reduced ~95% in MLNs after 6 weeks of a 10% cocoa diet (Pérez-Berezo et al., 2012b) but not earlier (Pérez-Berezo et al., 2011). Likewise, cocoa intake did not modify CD40 gene expression

either in PPs or in MLNs (Pérez-Berezo et al., 2012b), which is in accordance with previous studies that have shown that a cocoa diet increased the proportion of B cells in PPs (Ramiro-Puig et al., 2008); however, a 10% cocoa diet (but not lower proportions) for 6 weeks (but not in a shorter period), reduced CD40 gene expression in the small intestine (Pérez-Berezo et al., 2011, 2012b).

When IgA⁺ B cells become activated they leave PPs, go to the bloodstream and come back to the intestine or other mucosa (MacPherson et al., 2008; Brandtzaeg, 2010). The gut-homing system requires the integrin $\alpha\beta7$ and some chemokine receptors on activated gut lymphocytes (Mora and von Andrian, 2008). Chemokines produced by epithelial cells such as CCL25 and CCL28 interact with the chemokine receptors CCR9 and CCR10 respectively, to recruit IgA⁺ B cells (Hieshima et al., 2004). The CCR9 expression on IgA⁺ B cells is induced by retinoic acid (Mora et al., 2006) through its ligation to nuclear retinoic acid receptors (RAR; Ross et al., 2009). Diets containing 2, 5, or 10% cocoa for 3 weeks in 6-week-old animals did not affect the gene expression of CCR9, CCL25, RAR α , or RAR β but increased the CCL28 gene expression in the small intestine wall (Pérez-Berezo et al., 2011). The increase in the CCL28 gene expression could reflect a “rescue mechanism” to strongly attract the IgA⁺ B cells to the gut, in an attempt to compensate the S-IgA downregulation. When the cocoa diet began earlier and lasted longer (3-week-old animals fed 10% cocoa for 6 weeks), the gene expression of gut-homing molecules such as RAR, CCR9, and CCL28, but not CCL25, was downregulated in the small intestine (Pérez-Berezo et al., 2012b). Overall, these results demonstrate the longer the cocoa intake the greater the sensitivity of gut-homing mechanisms in the intestine.

Finally, when IgA⁺ B cells reach the intestine, they differentiate into IgA-secreting cells mainly releasing dimers of IgA. This immunoglobulin is actively secreted to the apical surface of epithelial cells by a polymeric immunoglobulin receptor (pIgR) expressed on the basolateral surface of epithelial cells (Cerutti and Rescigno, 2008). The gene expression of pIgR was not modified by any cocoa diet given for 3 or 6 weeks (Pérez-Berezo et al., 2011, 2012b).

In summary, a high-cocoa diet induces a lower number of IgA⁺ B cells reaching the intestinal lamina propria by down-regulating either the expression of chemokine or that of their receptors (Figure 2). However, in the gut lamina propria some other mechanisms remain working efficiently.

COCOA AND BACTERIA RECOGNITION

The GALT maintains mucosal homeostasis by inducing a state of non-responsiveness to innocuous antigens, such as commensal bacteria, or by responding actively to counteract pathogens (Fagarasan et al., 2010). In this regard, toll-like receptors (TLRs), through the recognition of conserved molecular motifs on microorganisms, are important molecules involved in the cross-talk between microorganisms and gut epithelial and immune cells (Abreu, 2010). It has been reported that the generation of IgA⁺ B cells can be independent of Th cells and involve TLR non-specific recognition (Fagarasan et al., 2010). In this context, cocoa diets in rats have shown differential TLR expression patterns for TLR2, TLR4, TLR7, and TLR9 in PPs, MLNs, the small intestine

and colon (Pérez-Berezo et al., 2011, 2012b; Massot-Cladera et al., 2012).

A high (10%) and continuous cocoa diet produced an upregulation of TLR4 and TLR9 and a downregulation of TLR2 and TLR7 in PPs and MLNs (inductor sites of intestinal immune response; Pérez-Berezo et al., 2011, 2012b). Conversely, in the small intestine and the colon, cocoa-fed animals showed lower TLR4 and TLR9 and higher TLR2 and TLR7 gene expression (Massot-Cladera et al., 2012; Pérez-Berezo et al., 2012b). TLR4 expression is positively correlated to the number of IgA-secreting cells in the lamina propria and their recruitment through CCL28 (Shang et al., 2008; Abreu, 2010). In consequence, the down-regulation of TLR4 through a cocoa diet in the intestine (as effector site) could be associated with the decrease in S-IgA in feces.

Toll-like receptors are expressed preferentially in tissues that are in constant contact with microorganisms (Cario, 2005; Abreu, 2010). Therefore, changes in the TLR expression could reflect changes in the intestinal microbiota and/or its relation to intestinal immune cells (Shibolet and Podolsky, 2007).

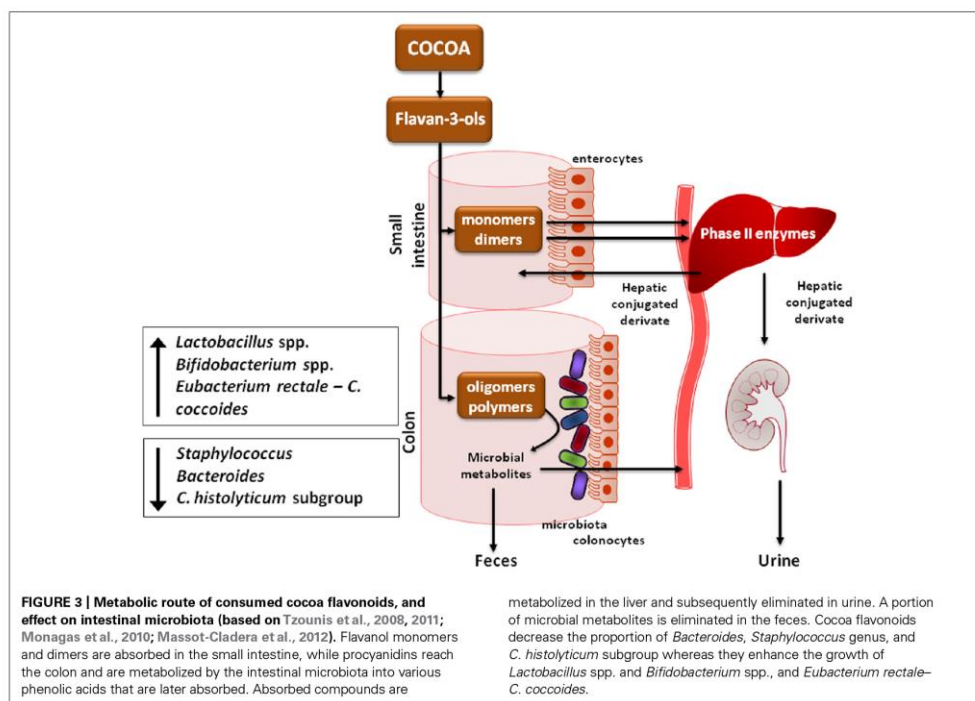
INFLUENCE OF COCOA ON GUT MICROBIOTA

The above data demonstrate that cocoa-enriched diets in rats influence the intestinal immune system either through a direct effect on intestinal immune cells and/or an indirect effect mediated by changes in microbiota which would influence the cross-talk with the host (i.e., through TLR). Therefore, it remained to be seen whether cocoa diets modulated microbiota composition and, consequently, the intestinal immune system. This is possible due that cocoa flavonoids reaching the colon can interact with intestinal microbiota through a bidirectional relationship. Thus, bacteria can be involved in the polyphenol metabolism, and flavonoids can influence microbiota growth and composition (Hayek, 2013). In this context, changes in intestinal microbiota composition may influence the immune system as well as the compounds originated by the bacterial metabolism (Figure 3).

COCOA FLAVONOIDS METABOLISM

Cocoa flavonoids have a particular bacterial metabolism due to the high degree of polymerization of its flavanols. After cocoa intake, monomers (i.e., catechin and epicatechin) are rapidly absorbed in the small intestine, while the largest proportion of dietary polyphenols (90–95%) in the form of oligomers and polymers (i.e., cocoa procyanidins) pass intact through the gastrointestinal tract, reaching the colon (Monagas et al., 2010). This fact allows them to be metabolized by the intestinal microbiota (Figure 3). Colonic bacteria is composed of more than 500 species and a bacterial load of approximately 10^{11} to 10^{12} bacteria/g of colonic contents (O'Hara and Shanahan, 2006). It is known that microbiota has the ability to metabolize polyphenols to simpler metabolites and this conversion is often essential for absorption and modulates the biological activities of these compounds which are more beneficial than the original forms found in food (Clifford, 2004; Tzounis et al., 2008; Selma et al., 2009; Monagas et al., 2010; Neilson and Ferruzzi, 2011).

Cocoa polyphenols are extensively degraded in the colon by a broad range of reactions able to generate various phenolic



acids, mainly including phenylpropionic, phenylacetic, and benzoic acid derivatives (Déprez et al., 2000; Kohri et al., 2003; Aura, 2008; Tzounis et al., 2008; Fogliano et al., 2011). Later, colon bacterial metabolites are absorbed into the bloodstream, providing another source of potentially bioactive compounds (Rios et al., 2003). Once absorbed, the microbial metabolites from flavanols are mainly metabolized in the liver by phase-II enzymes as hepatic conjugated derivatives that are subsequently eliminated in urine (Neilson and Ferruzzi, 2011; Figure 3). In particular, the presence of 5-(3',4',5'-trihydroxyphenyl)- γ -valerolactone and 5-(3',4'-dihydroxyphenyl)- γ -valerolactone in urine is considered to be a potential biomarker of flavan-3-ols consumption in humans after cocoa products intake (Urpi-Sarda et al., 2009). At the same time, a portion of microbial metabolites (non-conjugated microbial metabolites) is eliminated in the feces. The excretion of microbial metabolites varies markedly between subjects and, for some individuals, it may also vary with the substrate (Monagas et al., 2010).

Regarding the intestinal bacteria with the ability to catabolize flavanols, a limited number of bacterial species have been identified as being involved in the polyphenols catabolism. Interestingly, the majority of the bacteria characterized belong to the Clostridia group, which is a large component of the gut microbiota (Wang et al., 2001; Tzounis et al., 2008).

COCOA INFLUENCE ON GUT MICROBIOTA COMPOSITION

It is known that unabsorbed dietary phenolics and their metabolites can exert significant effects on the intestinal environment by modulation of the microbiota (Lee et al., 2006b). Although there is limited information concerning the ability of (+)-catechin and (–)-epicatechin, the main monomers present in cocoa, to promote or inhibit the growth of selected intestinal bacteria, there are some *in vitro*, preclinical and clinical studies regarding this subject (Tzounis et al., 2008, 2011; Massot-Cladera et al., 2012).

In vitro studies have shown the antimicrobial properties of some polyphenols (Puuupponen-Pimiä et al., 2005; Lee et al., 2006b). To date, Tzounis et al. (2008) showed that (+)-catechin induced an inhibitory effect in the growth of the *Clostridium histolyticum* group using the batch culture approach, at the same time that both (+)-catechin and (–)-epicatechin enhanced the growth rate of the beneficial bacteria group, *Eubacterium rectale*-*C. coccoides*. Furthermore, there were increases in both *Lactobacillus* spp. and *Bifidobacterium* spp. genus following (+)-catechin exposure, as well as a small but significant increase in the growth of *E. coli* after (+)-catechin incubation (Tzounis et al., 2008; Figure 3).

The effects of cocoa polyphenols observed in animal models are partially in line with the above results. Young rats receiving a 10% cocoa intake for 6 weeks showed a significant decrease in the proportion of *Bacteroides*, *Staphylococcus* genus, and *C. histolyticum*

subgroup (Massot-Cladera et al., 2012). The effect of this defatted cocoa powder on microbiota was also observed in rats fed for 4 weeks with diets based on cocoa polyphenols-enriched powders (Massot-Cladera et al., 2013).

With regard to human studies, evidence of the effects of cocoa or cocoa products intake on microbiota composition is scarce. A human intervention study evaluated the high-cocoa flavanol consumption effect on microbiota composition from healthy volunteers (Tzounis et al., 2011). The results showed that a 4 weeks daily ingestion of a high-cocoa flavanol beverage containing 494 mg flavanols increased the growth of *Lactobacillus* spp., and *Bifidobacterium* spp. in comparison with a control low-cocoa flavanol drink that contained only 29 mg flavanols (Tzounis et al., 2011). Although these results were not found in the preclinical interventions, it prompted the redefinition of cocoa polyphenols as prebiotics. It is important to mention that divergence between the animal and human data could be ascribed to several factors, such as the cocoa composition (fiber and flavonoids pattern), dose, and differential composition and distribution ecosystem (rats vs human gut). Overall, all these findings strengthen the evidence that cocoa polyphenols can have significant effects on the growth of select gut microbiota (Figure 3).

CONCLUDING REMARKS

A cocoa diet has been shown to influence the immune system: in the innate inflammatory response as well as in the adaptive immunity, and in both systemic and intestinal compartments.

From the results concerning the effects of cocoa on inflammatory reaction, it could be concluded that although cocoa demonstrates clear anti-inflammatory properties *in vitro*, when tested *in vivo* results are more controversial. To date, it can be concluded that if inflammation is mild and cocoa has a high polyphenol content, it could help in the resolution of inflammatory response, and, in any case, due to its antioxidant properties, cocoa can be a complementary anti-inflammatory therapy.

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4. CACAU, FLAVONOIDES I TLR

La comunicació entre les cèl·lules immunitàries de l'hoste, principalment intestinals, i la microbiota autòctona és el que es coneix com a *crosstalk*. Aquesta interacció es produeix a través del reconeixement de determinats patrons moleculars dels microorganismes pels receptors TLR. L'activació inadequada d'aquests receptors pot desencadenar processos inflamatoris crònics i desordres immunològics. En aquest sentit, s'ha suggerit que els flavonoides presents en la dieta són capaços de modular les vies de senyalització mitjançada pels TLR. La revisió que es presenta a continuació és un recull de les evidències científiques en relació a la capacitat dels flavonoides, i entre ells els del cacau, de modular l'activitat i l'expressió dels TLR, donant una visió global dels possibles mecanismes d'acció involucrats en aquests efectes.

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Review

Flavonoids Affect Host-Microbiota Crosstalk through TLR Modulation

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Abstract: Interaction between host cells and microbes is known as crosstalk. Among other mechanisms, this takes place when certain molecules of the micro-organisms are recognized by the toll-like receptors (TLRs) in the body cells, mainly in the intestinal epithelial cells and in the immune cells. TLRs belong to the pattern-recognition receptors and represent the first line of defense against pathogens, playing a pivotal role in both innate and adaptive immunity. Dysregulation in the activity of such receptors can lead to the development of chronic and severe inflammation as well as immunological disorders. Among components present in the diet, flavonoids have been suggested as antioxidant dietary factors able to modulate TLR-mediated signaling pathways. This review focuses on the molecular targets involved in the modulatory action of flavonoids on TLR-mediated signaling pathways, providing an overview of the mechanisms involved in such action. Particular flavonoids have been able to modify the composition of the microbiota, to modulate TLR gene and protein expression, and to regulate the downstream signaling molecules involved in the TLR

pathway. These synergistic mechanisms suggest the role of some flavonoids in the preventive effect on certain chronic diseases.

Keywords: TLR; polyphenols; inflammation; vegetables; intracellular signaling

1. Host-Microbiota Crosstalk and Dietary Factors

1.1. Interaction between Microbiota and the Host

The intestinal mucosa is constantly interacting with a high load of antigens, which comprises, besides those from food, those derived from microbes, both commensal microbiota and invading pathogens. For this reason, the gastrointestinal tract contains the intestinal epithelial cells (IEC) that act as a physical barrier, and the largest part of the immune system: the gut-associated lymphoid tissue (GALT), with approximately 70% of the body immune cells [1]. The main function of the IEC and immune cells located in the intestine is to discriminate precisely between the potential harmful antigens, and then mounting an effective immune response, and those which are innocuous and therefore do not require counteraction from the host, a process known as oral tolerance [2]. The key and ineludible first action in this discrimination consists of the recognition of microbial components in the intestine.

The microbial interaction with host cells (both immune and non-immune cells) is known as crosstalk. Certain microbiota components can be recognized through the pattern-recognition receptors (PRRs). These are host innate immune receptors involved in the detection of pathogens that recognize conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) as well as microbe-associated molecular patterns (MAMPs) (host-commensal interactions) and induce the production of innate effector molecules. These can be located on the cell membrane or in the cytosol joining to bacterial-surface-exposed structures or nucleic acids, respectively. The cytosolic receptors are composed of two major families: the nucleotide oligomerization domain (NOD)—like receptors (NLRs) such as NOD1, NOD2 and NLRP1, and the retinoic acid inducible gene I (RIG-1)—like receptors (RLRs) which recognizes viral RNAs. The cell membranes' PRRs include toll-like receptors (TLRs) that may reside outside the cell or in specialized vesicular compartments (endosomes) where they detect DNA and RNA derived from viruses and bacteria [3].

PRRs represent the first line of host defense against pathogens, play a pivotal role in both innate and adaptive immunity and can lead to the subsequent inflammatory process. Their activation results not only in local mucosal immunity but also in systemic immune responses. In this sense, small amounts of MAMPs and exogenous antigens from microbes are handled in a homeostatic manner when the PRRs are operating in a healthy manner, leading then to an appropriate pathway activation and balanced mediators secretion. However, dysregulation in the activity of such receptors can lead to the development of chronic and severe inflammation as well as immunological disorders, including septic shock, atherosclerosis, diabetes and cancer [4,5].

It can be of importance to study the influence of diet in such initial activation. Among components present in the diet, polyphenols, mainly flavonoids, have been suggested as dietary factors able to modulate TLR-mediated signaling pathways [5].

1.2. Toll-Like Receptors

Each host TLR recognizes distinct PAMPs derived from various microbial pathogens including viruses, bacteria, protozoa and fungi. However, besides their microbial-sensing activity, TLRs also play a role in the recognition of endogenous intracellular molecules released by activated or necrotic cells and extracellular matrix, called damage-associated molecular patterns (DAMPs), which also lead to an inflammatory response which is needed to promote tissue repair [6].

In addition, TLR activation induces the expression of many immune and inflammatory genes via the stimulation of nuclear factor κ B (NF- κ B) and mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), p38 and c-jun N-terminal kinase (JNK) [7]. Generally, NF- κ B and MAPKs, involving p38MAPK or JNK, contribute in activating some proinflammatory cytokines [8,9], whereas activation of ERK promotes cell survival [10].

There are 11 human TLRs identified so far, divided into three groups according to the target in which they interact: lipids and lipopeptides (TLR1, 2, 4 and 6), proteins (TLR5) and nucleic acids (TLR3, 7, 8 and 9). TLR1 forms heterodimers with TLR2 (TLR1/2) and recognizes triacyl lipopeptides. TLR2 in concert with TLR1 or TLR6 recognizes a wide variety of PAMPs, including peptidoglycan, lipopeptides and lipoproteins of Gram-negative bacteria, mycoplasma lipopeptides and fungal zymosan. TLR6 in association with TLR2 (TLR2/6) recognizes diacyl lipopeptides. TLR4 interacts with bacterial lipopolysaccharide (LPS). TLR5 recognizes bacterial flagellin. TLR3 recognizes double-strand RNA, and TLR7 and TLR8 interact with the single-strand RNA found during viral replication. TLR9 recognizes unmethylated deoxycytidyl-phosphate-deoxyguanosine (CpG) motifs commonly present in bacterial and viral genomes. Finally, human TLR11 has been reported to be non-functional because of the presence of a stop codon in the gene and TLR10 is able to homodimerize or heterodimerize with TLR1 and TLR2, but its ligand remains unknown [11].

Recognition of PAMPs by host TLRs triggers intracellular signaling cascades through a set of toll/interleukin-1 receptor (TIR) -domain-containing adaptors, including myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP/Mal), TIR domain-containing adaptor inducing interferon (IFN) (TRIF/TICAM1) and TRIF-related adaptor molecule (TRAM/TICAM2) [12]. Each TLR recruits a specific combination of adaptors to activate different transcription factors, giving rise to appropriate inflammatory responses. All TLRs, with the exception of TLR3, share the adaptor MyD88 which recruits IL-1 receptor-associated kinase-4 (IRAK-4) and leads to its phosphorylation. The phosphorylated IRAK-4 then induces the phosphorylation of IRAK-1 [13], which, in turn, activates the tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 induces phosphorylation of the I κ B kinase (IKK) complex, resulting in activation of the transcription factor NF- κ B [14].

TIRAP mediates the activation of the MyD88-dependent pathway downstream of TLR2 and TLR4 [15]. TRIF is another adaptor molecule for TLR3 and TLR4 that produces a MyD88-independent [16,17] signaling pathway (TRIF-dependent). This pathway activates both NF- κ B and interferon regulatory factor 3 (IRF3) to induce the activation of type I interferons (IFNs), and also causes delayed NF- κ B activation mediated through the receptor-interacting protein-1 (RIP1) [17]. TRAM selectively mediates the TRIF-dependent pathway downstream of TLR4, but not TLR3.

1.3. Flavonoids

Flavonoids are vegetal compounds that contribute to the brilliant shades of blue, scarlet and orange colors in leaves, flowers and fruits of plants. In addition, they are found in seeds, nuts, grains and spices, and in some beverages such as wine, tea and beer [18]. Chemically, flavonoids are polyphenols which are commonly found conjugated to sugars (as a glycosylated form) although some of them can exist as free aglycones [19].

The basic flavonoid structure is the flavan nucleus consisting of 15-carbon skeleton arranged in two phenyl rings bound by a three-carbon bridge commonly cyclized with oxygen, then forming three rings labeled A, B, C (Figure 1) [18]. The various classes of flavonoids differ in the level of oxidation and pattern of substitution on the C ring while individual compounds within a class differ in the pattern on substitution of the A and B rings. The major classes of flavonoids are flavonols, flavones, flavanones, isoflavones, flavanols and anthocyanidins (Figure 1).

Figure 1. Chemical structure and representative compounds from the main families of flavonoids.

Basic structure	
Chemical structure	Family name: representative compounds
	Flavanols: epicatechin, epicatechin-3-gallate, epigallocatechin-3-gallate
	Flavanones: eriodictyol, hesperetin, naringenin
	Flavones: baicalein, chrysin, eupalín, luteolin
	Isoflavones: daidzein, equol, genistein
	Flavonols: quercetin, morin, kaempferol, myricetin
	Anthocyanidins: cyanidin, delphinidin, malvidin

Flavonols are ubiquitous in plants and are found mainly in fruit skin, being the yellow and red onions one of the richest sources of them [20]. The flavonol class includes kaempferol, quercetin, and myricetin typically found as glycosides. The flavones, such as apigenin, luteolin, and baicalin, are less widely distributed, although substantial amounts of apigenin and luteolin have been detected in cereal grain and some aromatic herbs (parsley, rosemary, thyme) [18,20]. The most abundant *flavanone* is naringenin, followed by hesperidin, eriodictyol and hesperetin. They are present in especially high amounts in citrus fruits and usually occur as rutinosides and neohesperidosides, which differ in taste [19]. Isoflavones are found most often in leguminous plants with substantial quantities of daidzein and genistein occurring in soybean [20]. *Flavanols* (flavan-3-ols) include monomeric and polymeric forms called procyanidins (condensed tannins). Monomeric forms are catechins and gallic acid esters of catechins and epicatechins [19]. They are widely distributed in plants and fruits and also in tea leaves [18]. *Anthocyanidins* have a fully aromatized C ring and as a result they are positively charged. The most common of these natural water-soluble compounds are pelargonidin, cyaniding, delphinidin, and malvidin, which form conjugates with sugars and organic acids to generate a multitude of anthocyanins that confer a wide range of colors, ranging from orange and red to blue and purple, on fruits (specially in berries like strawberries, blueberries, blackberries and blackcurrants), vegetables and plants [21,22].

2. Objective

There is increasing scientific evidence of a relationship between diets rich in natural antioxidants and the prevention of several chronic diseases. The inverse association between dietary antioxidants and inflammatory-like disorders is supported by several epidemiological and interventional studies [23]. Polyphenols, including flavonoids, are the most commonly consumed dietary antioxidants and therefore they may be one of the major responsible for the healthy effect of fruit-and-vegetable-enriched diets. As the activation of the TLR pathway constitutes the first step in the inflammatory cascade activation, and its deregulation can lead to severe chronic inflammation and immune disorders, it is plausible to hypothesize a downmodulatory action of flavonoids in the TLR-induced pathways.

This review focuses on the molecular targets and mechanisms involved in the modulatory action of flavonoids on TLR-mediated signaling pathways. In this context, it aims to provide an overview of the three major levels involved in such action: by varying the composition of the microbiota, by modulating the expression and activation of TLRs and, finally, by modifying the downstream signaling pathways involved. The synergy of all these mechanisms may explain the high potential of flavonoids for preventing certain types of disorders (Figure 2). The suppression of TLR activation by flavonoids offers new potential interventions for ameliorating inflammatory diseases and therefore constitutes a new alternative to current approaches.

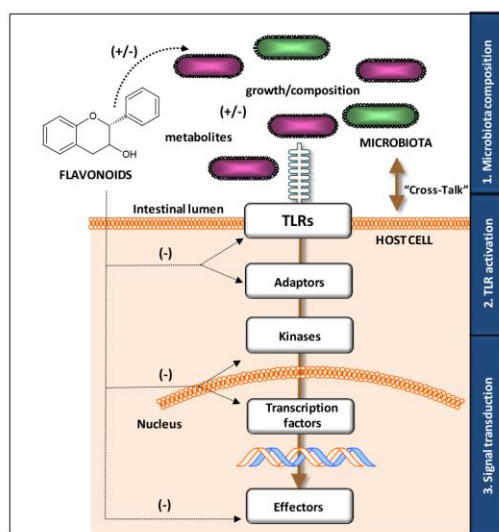
3. Mechanisms of Modifying “Cross-Talk” by Flavonoids

3.1. Influence on Growth and Composition of Microbiota

Bacterial composition (*i.e.*, types of MAMP and PAMP) is one of the major factors influencing TLR activation. In turn, dietary habits are known to modulate the composition of the gut microbiota [24]. For this reason, the earliest level involved in modifying the host-bacteria crosstalk by dietary flavonoids

involves the modification of the microbial inhabitants in the gut (Figure 2). There is evidence showing that several kinds of flavonoids, including flavanones, flavonols, flavanols, and isoflavones, modify the microbiota composition. This has been recently reviewed by Etxeberria *et al.* [22] who extensively compile the latest investigations regarding the impact of phenolic compounds, as pure chemicals or in the food context, on microbial communities in both *in vitro* and *in vivo* studies (in animal and human interventions).

Figure 2. Overview of the mechanisms involved in the regulation of microbiota-host crosstalk by flavonoids. They can act at three different levels by modulating: (1) microbiota composition, by means of directly (flavonoid) or indirectly (metabolite) affecting the growth; (2) Toll-like receptor (TLR) activation, by means of acting on the receptor and its adaptor proteins; (3) signal transduction, by means of interfering with upstream and downstream kinases as well as the transcription factors involved in the inflammatory and immune response activation.

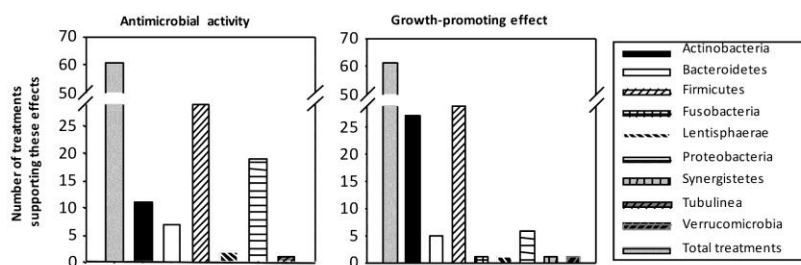


With regard to pure flavonoids, an *in vitro* study tested the impact of particular flavonols (rutin, quercetin), flavanols (catechin) and flavanones (naringin, naringenin, hesperidin and hesperetin) on the growth of pure cultures of six bacteria species [25]. The author showed the dose-dependent inhibitory effect of the aglycones naringenin and quercetin on the growth of all bacteria species studied, which was stronger than that of their glycosides [25]. On the other hand, both quercetin (aglycone) and rutin (glycoside) significantly increased the growth of the major intestinal *Bacteroidetes* and *Firmicutes* phyla, with a greater increase in the former than in the latter *in vitro* [26]. However hesperetin and catechin (both aglycones) showed no or weak antibacterial activity against most of the analysed bacteria [25], which was later corroborated by an *in vitro* study evaluating the antimicrobial activity of

five aglycones on the growth of the probiotic *Bifidobacterium adolescentis* [27]. Similarly, the repressing and/or stimulating effect induced by *flavanols*, such as catechin and epicatechin, on the growth of certain bacteria has been reported in *in vitro* and batch cultures studies [28,29].

Besides those studies of particular flavonoids on bacteria *in vitro*, there are a number of studies showing the changes induced by flavonoid-rich foods on microbiota composition. In order to synthesize the available data, and based on the recompilation performed by Etxeberria *et al.* [22], the Figure 3 below classifies the number of studies or treatments with polyphenols, mainly flavonoids, into the antimicrobial activity or the growth-promoting effect on the more prevalent bacterial groups.

Figure 3. Summary of the number of treatments using beverages (cocoa, tea and wine), fruits or vegetables (soy, pomegranate, grapes, berries and apples) rich in flavonoids and their impact on bacterial growth. There is a high number of studies with flavonoids (*in vitro* and *in vivo*) showing both antimicrobial activity (**left**) and growth-promoting effects (**right**) on the more prevalent bacterial groups.



Globally, more than 100 experiments have shown that bacterial groups in gut microbiota are affected by beverages (cocoa, tea and wine) and fruits/vegetables rich in flavonoids, either showing antimicrobial activity or, on the contrary, promoting the growth of certain bacteria. These effects have been shown in all types of flavonoids.

Concerning the effect of flavonoid-rich beverages, the intake of cocoa, which mainly contains epicatechin and catechin, and their polymeric forms, was able to inhibit the growth of *Clostridium histolyticum*/C. *perfringens*, *Staphylococcus* and *Streptococcus* genera in preclinical and clinical studies [28,30]. In the same way, tea flavonoids exerted an antimicrobial activity against selective bacterial species [31], whereas bifidogenic properties have also been attributed to them [32,33]. Differences in the proportion of the four major bacteria phyla in humans (*Proteobacteria*, *Fusobacteria*, *Firmicutes*, and *Bacteroidetes*) have been observed after a nutritional intervention with red wine which contains a complex mixture of polyphenols, mainly including flavanols and anthocyanins [34]. Similar effects have been described in rats treated for 16 weeks with wine polyphenol, which showed a predominant proportion of *Lactobacillus* and *Bifidobacterium* spp. [35], while controversial results were obtained in an *in vitro* batch culture [33,36]. Nevertheless, these effects were not so pronounced or even disappeared in humans when consuming dealcoholized red wine [34].

Changes have also been observed in the *Firmicutes*, *Actinobacteria* and/or *Bacteroidetes* proportions induced by soy products, which mainly contain isoflavones, in *in vitro*, preclinical and clinical studies [37–40]. In addition, the potential modulation of gut microbiota composition by fruits has been thoroughly reviewed by Etcheberria *et al.* [22]. To date, a large amount of evidence suggests that fruits and their derivatives are able to significantly boost the growth of colonic-friendly bacteria, such as *Bifidobacterium* and *Lactobacillus* [41–44].

Overall, it can be summarized that the intake of flavonoids is able to modulate the composition of the gut microbiota, potentiating the growth of specific beneficial bacteria strains (*Lactobacillus* and *Bifidobacterium* in >70% of the studies), and also inhibiting the growth of certain pathogenic bacteria (*i.e.*, *Clostridium*). It must be added that, despite the evidence described above, natural flavonoids are not the only responsible for this effect on gut microbiota but their metabolites are also involved. Flavonoids and related compounds can reach the colon where they become substrate for the microbial metabolism [45,46]. Indeed, intestinal bacteria have the ability to metabolize flavonoids, more or less efficiently, depending on their chemical structure, to simpler and more bioavailable metabolites [20,47]. This transformation modulates the biological activities of these compounds, which, in general, are more beneficial after colonic metabolism than in their original forms [45,46,48–50]. Besides the known biological activity of most small phenolic metabolites, several microbial catabolites from epicatechin, epigallocatechin or naringenin have been described possessing important antimicrobial activity, especially against Gram-negative species [51].

3.2. Modulatory Action on TLR Gene and Protein Expression

TLR pathways can be modulated by flavonoids at different levels (Figure 2). Both TLR gene expression and cell membrane expression, which are directly related with TLR functionality, can be affected by flavonoids. Under homeostatic conditions IEC have low expression of TLR2 and TLR4, and therefore in a healthy context, TLR activation is low; however, in an inflammatory scenario, TLR expression on IEC increases and then TLR signaling is triggered [52].

Both *in vitro* studies (Table 1), using primary cells or cell lines, and *in vivo* studies (Table 2) have evidenced the influence of different classes of flavonoids on TLR gene and protein expression. The best results regarding the down-regulatory effect of flavonoids have been raised in the presence of TLR activators (*i.e.*, LPS as an *in vitro* TLR4 activator) whereas lack of effect is found under non stimulatory conditions (*i.e.*, in healthy animals/subjects).

LPS-stimulated J774 macrophages treated with the flavonols engeletin and astilbin showed inhibition of TLR4 gene expression [53]. In addition, kaempferol-3-*O*-sophoroside down-regulated the cell surface expression of TLR2 and TLR4 in endothelial cells [54] as did quercetin in PBMC after challenge [55]. Flavanones as naringenin and hesperidin have shown similar effects. In particular, naringenin down-regulates TLR2 and TLR4 protein expression and TLR2 mRNA levels in PBMC after being challenged with *Chlamydia trachomatis* [56]. Other assays performed *in vitro* in 3T3-L1 cells during adipocyte differentiation and *in vivo* in C57Bl/6j mice after a high fat diet (HFD) agree with the inhibitory effect of naringenin on TLR2 gene expression, although no effect was found for TLR4 [57].

Table 1. Flavonoids on TLR gene and protein expression in *in vitro* studies.

Flavonoid	Dose	Duration ¹	TLR ²	Studied Target	Cell (Challenge)	Reference
FLAVONOLS						
Engletin	10/50 µM	2 h	↓ TLR4	mRNA	J774 macrophages (LPS)	[53]
Astilbin	–	6 h	↓ TLR4	mRNA		
Kaempferol-3-O-sophoroside	–	6 h	↓ TLR2 ↓ TLR4	Protein	Endothelial cells	[54]
Quercetin	25 µM	1 h	↓ TLR2 ↓ TLR4	Protein	PBMC	[55]
FLAVANONES						
Naringenin	1 µg/mL	48 h	↓ TLR2	Protein/mRNA	J774 macrophages (<i>C. trachomatis</i>)	[56]
Naringenin	100 µM	–	↓ TLR4	mRNA		
FLAVANOLS						
Epigallocatechin-3-gallate	1 µM	24 h	↓ TLR2	mRNA	3T3L1 cells (adipocyte differentiation)	[57]
Epigallocatechin-3-gallate	10 µM	24 h	↓ TLR4	Protein/mRNA	RAW 264.7 macrophages (LPS)	[58]
FLAVONES						
Baicalin	40/80 µM	24 + 3 h	↔ TLR4 ↔ TLR1-9	mRNA	Murine bone marrow-derived DCs (unstimulated) Human oral keratinocytes (LPS)	[59] [60]
Baicalin	5/10 µg/mL	0.5–6 h	↓ TLR2 ↓ TLR4	Protein/mRNA Protein/mRNA	PC12 and primary rat neurons (oxygen glucose deprivation)	[61]

Notes: ¹ Treatment duration is showed when detailed in the article, if it is not provided a (–) is showed; ² Changes in gene expression are summarized by means of an increase (↑), decrease (↓) or not affected (↔) by the intervention.

Flavones have been shown to also modulate TLR gene expression in both *in vitro* and *in vivo* studies. Isoliquiritigenin decreased, among other targets, the gene expression of TLR4 in RAW 264.7 macrophage line [68], and luteolin administrated at different doses to rats was able to suppress the overexpression of TLR4 and TLR5 induced by ischemia in the cerebral cortex at both protein and gene level [62]. Baicalin added to human oral keratinocytes culture was able to down-regulate *P. gingivalis* LPS-induced expression of downstream genes associated with TLR signaling, but almost no changes were found in the gene expression of TLR1-9 determined by the PCR array [60]. However, in another study, baicalin down-regulated protein and gene expression of TLR2 and TLR4 both *in vitro*—PC12 and primary neurons under oxygen glucose deprivation conditions—and *in vivo*—hippocampus cells from mice after carotid arteries ligation [61].

With regard to flavanols, gene and cell surface expression and total protein level of TLR4 were markedly reduced by the addition of epigallocatechin gallate (EGCG) to LPS-stimulated RAW 264.7 macrophages [58]. However, it did not affect TLR4 gene expression when murine bone marrow-derived DCs were in resting conditions [58], which is in agreement with other studies where the flavonoid is not able to modify TLRs in the absence of stimulatory conditions. On the contrary, a semi-synthetic flavonoid called flavopiridol did not alter the TLR4 cell surface expression in LPS-stimulated RAW 264.7 macrophages [69].

Some extracts rich in flavonoids have also shown an effect on TLR gene expression, mainly in *in vivo* studies. Thus, the oral treatment of Wistar rats with a hydroalcoholic extract of *Achyrocline satureioides*, a rich source of quercetin and luteolin, decreased TLR4 expression on neutrophils [63]. Wistar rats fed cocoa, a rich source of procyanidins among other flavonoids, showed changes in the expression of several TLR in different tissues of the intestinal immune system [64]: a cocoa diet induced an up-regulation of TLR4 and TLR9 gene expression and a down-regulation of TLR2 and TLR7 gene expression in Peyer's patches (PPs) and mesenteric lymph nodes (MLNs). Conversely, in small intestine (SI) tissue, cocoa-fed animals showed lower values for TLR4 and TLR9 mRNA and a higher value for TLR2 and TLR7 [65]. However, a similar design did not show a clear effect on colonic tissue, where microbiota is mainly found [30]. It should be taken into account that the effects induced by these extracts cannot be exclusively attributed to flavonoids because other components may be acting as well (*i.e.*, theobromine and fiber in cocoa).

In humans, two interventional studies using orange juice have evaluated the increase in TLR2 and TLR4 mRNA and protein expression in PBMC, induced by a high-fat high-carbohydrate meal, in volunteers [66,67].

In addition to flavonoids, the effect on TLR genes or surface expression by other polyphenols has also been reported. Thus, curcumin decreases TLR2 gene and surface expression in immune cell lines (monocytic THP-1 cells and dHL-60 promyelocytic leukaemia cells) and in primary cells (peripheral blood polymorphonuclear neutrophils, PMN) [70], and a complement containing resveratrol prevented the increase in TLR4 gene expression in PBMC induced by a high-fat, high-carbohydrate meal in healthy subjects [71].

Finally, it has recently been described that TLR gene expression is subject to circadian rhythms [72,73] which might be related to the presence of flavonoids and other exogenous regulators of TLR in the diet.

It should be also mentioned that certain flavonoids *in vivo* may not be the only direct responsible of the induced effects because indirect effects, through one or more of its microbial metabolites, might be also considered. However, in the *in vitro* studies the TLR modulation should be due to direct effects because the microbial metabolism is not present in such approach. Further studies with this aim should be developed.

3.3. Modulatory Action in TLR Activation

The activation of TLR intracellular signaling activation requires further processes that can also be targeted by flavonoids. These processes include the accumulation of lipid rafts, microdomains rich in cholesterol and sphingolipids that act as platforms for downstream molecules and seem to be a key factor in the activation of immune cells, TLR dimerization/oligomerization [74], its glycosilation and the participation of adaptor proteins.

Thus, quercetin and luteolin could interact with lipid rafts [75], whereas isoliquiritigenin was able to suppress LPS-induced dimerization of TLR4 in a dose-dependent manner in RAW 264.7 macrophage cell line [68]. Polyphenols such as curcumin and resveratrol have also shown this effect [74]. With regard to the influence on TLR glycosilation, *Helicobacter pylori* infection seems to initiate the inflammatory cascade in gastric epithelial cells by TLR4 glycosilation, fact that has been reported suppressed by EGCG [47].

There are at least five adaptor proteins of TLR and each of these has a TIR domain (*i.e.*, TRIF or MyD88), which directly or indirectly binds to the TIR domain of a TLR [76]. Flavonoids seem to modulate the pathways involving such adaptors, however from the literature it is difficult to ascertain which is the specific target affected and mainly focus on downstream molecules (Figure 4). As examples, isoliquiritigenin in RAW 264.7 macrophages was able to suppress the MyD88-dependent pathway (*i.e.*, IRAK1 degradation) [68] whereas other *in vitro* studies performed in RAW 264.7 cells and also in 293T (human embryonic kidney cell line) showed that EGCG treatment was able to modulate both MyD88- and TRIF-dependent signaling pathways [77].

On the other hand, it has to be taken into account that TLR gene expression can be self-regulated by downstream molecules participating in its signaling (*i.e.*, NF- κ B) [78], which potentiates the down-modulatory effect of those flavonoids able to act on both targets.

3.4. Effect on Signal Transduction Molecules

A large number of articles can be found regarding the effect of flavonoids on the activity of different protein kinases that regulate various intracellular signaling cascades, such as MAPK [79,80]. For this reason flavonoids may also modify the downstream kinases activated after TLR triggering. Furthermore, oxidative stress accompanying inflammation, which also leads to MAPK activation, seems to be controlled by the direct action of flavonoids (Figure 4).

The activation of MAPK engages ERK 1 and 2, JNK and p38 MAPK linked to stress stimuli, such as ROS overproduction or inflammation, and among other actions, this stimulation induces the phosphorylation of inhibitor proteins such as κ B inhibitor proteins (I κ Bs), involved in the NF- κ B pathway. The modulation of the activity of NF- κ B, a pro-inflammatory transcription factor, is one of the clearest effects of certain flavonoids [81,82]. NF- κ B triggers the expression of over 100 genes, many of

them involved in the inflammatory response [83]. NF- κ B is found in the cytoplasm of non-stimulated cells bound to I κ Bs which are modulated by MAPK action.

To date in the TLR context, the flavanols epicatechin gallate (ECG) and EGCG in human dental pulp fibroblast were able to block the ligand-stimulated TLR2 cell signaling pathways throughout the down-regulation of downstream mechanisms such as p38MAPK, SAP/JNK, ERK1/2 and phosphorylation of I κ Ba and p65 subunit of NF- κ B [84]. Likewise, in DC, EGCG inhibited LPS-induced MAPK and NF- κ B activation [59].

Flavones, flavonols and flavanones also modulate these kinases. Luteolin inhibits p38 MAPK [62] and I κ B kinase activity [85] both indicating a role for this flavonoid in MyD88-dependent signaling pathway. Baicalin may inhibit *P. gingivalis* LPS-induced activation of NF- κ B, p38 MAPK and JNK [60], and same inflammatory pathway when the study was performed in ischemic neurons [86]. The flavonol flavopiridol—quercetin analogue—was also able to regulate the downstream TLR2 and TLR3 pathways through the MyD88-dependent pathway but not the independent pathway, as was demonstrated by *in vitro* studies showing inhibition of the activation of NF- κ B and MAPKs but not TRAF6 [69]. Flavanones such as naringenin inhibited TLR2 pathways by the modulation of I κ B degradation and JNK phosphorylation [57], and also down-regulated the induced increase of phosphorylation of p38 MAPK in macrophages infected with *Chlamydia trachomatis* [56].

Foods rich in flavonoids, such as cocoa, have also shown this potential *in vitro*, either by their scavenging action or by direct interaction with the downstream kinases [87,88]. In this context, epicatechin, catechin and dimeric B procyanidins seem to be accumulated in the cytosol and act at the early stages of NF- κ B activation, regulating oxidant levels and reducing I κ Bs phosphorylation, whereas cocoa dimeric procyanidins seem to be able to enter the nuclei and selectively avoid NF- κ B binding to DNA [89].

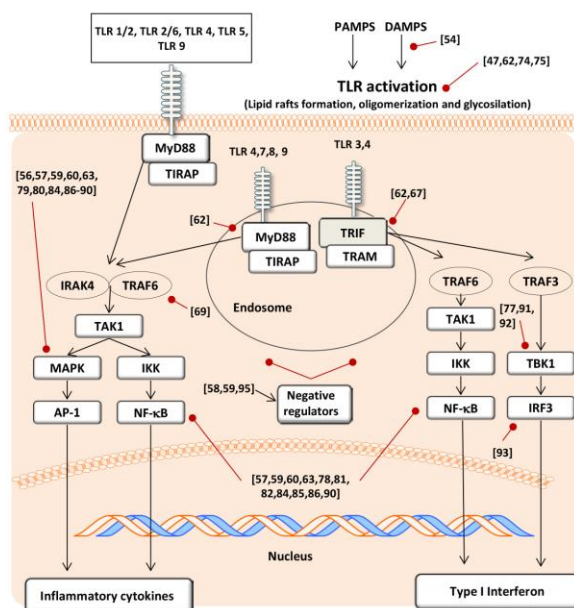
Overall, it is plausible that the different structures of flavonoids and their regiospecific modifications in their side chains lead to different bioactivities. In this sense, Lim *et al.* [90] studied the effect of five classes of plant-derived flavonoids (flavonols, flavones, flavanones, catechins and cyanidins) on TLR2 pathway *in vitro* and found differential effects on the targets studied, such as p38 MAPK and NF- κ B.

TBK1, a representative kinase found in TRIF-dependent TLR activation, participates in IRF3 activation and type I IFNs production (Figure 4). Some flavonoids, such as EGCG, luteolin, quercetin, chrysin and eriodictyol, showed inhibitory effects on this downstream kinase whereas others, such as naringenin and hesperitin, had no effect [77,91,92]. Moreover, isoliquiritigenin was able to suppress TLR3 and TLR4 agonist-induced IRF3 activations in RAW 264.7 macrophage cell line [93]. These results demonstrated the down-regulatory activity of certain flavonoids on the TLR3 and TLR4 TRIF-dependent pathways (Figure 4).

Another mechanism can be proposed as an alternative to the direct actions of flavonoids on specific targets of the TLR pathway. In recent years, the importance of the intracellular negative regulators of TLRs such as SOCS1, TOLLIP, IRAK or SIGIRR has been described [94]. In this regard, procyanidin dimer B2-treated macrophages up-regulate IRAK-M protein and therefore also suppress TLR4 signaling and phosphorylation of MAPKs such as ERK1/2, p38 and JNK [95]. Moreover, EGCG has been shown to up-regulate TOLLIP protein expression through a 67-kDa Laminin receptor (67LR) in macrophages [58] and in DCs [59].

Furthermore, as previously described, DAMPs can also activate TLR pathways and it seems that flavonoids can target these molecules as well. For example, a DAMP known as high mobility group box protein 1 (HMGB1) acts as an endogen ligand of TLR2 and TLR4 when it is released to the extracellular compartment [6] and this release by endothelial cells is modulated by kaempferol-3-*O*-sophoroside [54].

Figure 4. Signal transduction molecules affected by the flavonoids modulatory action on TLR activation. Both, the MyD88 dependent (**left**) and independent (**right**) pathways are modulated by the effect of several flavonoids on targets from different upstream (TLR expression and activation; adaptors modulation) and downstream levels (kinases and transcription factors). Black arrows indicate stimulation of the pathway and red arrows indicate inhibition of the pathway. In brackets there are the references cited in the text.



Overall, it can be suggested that changes in microbiota composition and TLR modulation by flavonoids may have also an impact on the intestinal immune activation and oral tolerance. In this sense, flavonoids by modulating intestinal microbiota (Section 3.1) may polarize the immune response towards the T-cell mediated tolerogenic pathway. At the same time the repression of the TLR signaling, which is associated to innate immune tolerance [96], can be also produced by flavonoids (Section 3.2). As certain flavonoids have been described to present immunomodulator activity by inducing changes on the GALT [97] and on systemic immune cells [98], as well as on regulatory T cells [99,100], we may suggest the possible implication of TLR signaling modulation herein described by flavonoids in such immunoregulatory effects.

4. Conclusions

Flavonoids are able to act as exogenous regulators of TLR and therefore modulate the host-microbiota crosstalk. This is possible due to their ability to modulate the TLR signaling pathways at different levels. On one hand, flavonoids can modify the composition of the microbiota, either by direct action or through their microbial catabolites, acting as antimicrobial agents on certain bacterial groups or as growth promoters for other bacteria. On the other hand, flavonoids can modulate the gene expression, the initial activation process of the receptor, and the upstream and downstream signaling molecules involved in the TLR pathway. These synergistic mechanisms make some flavonoids interesting modulatory agents on TLR-mediated inflammatory responses and therefore it is plausible to think that their preventive effect on certain chronic diseases, associated with exacerbated TLR activation, may be at least in part result from TLR regulation.

Based on these effects, we suggest that more preclinical, *in vitro* and animal model, studies, as well as human clinical trials, should be carried out with flavonoids regarding this aspect. Taking into account the lack of toxic effects and the high availability from natural sources, they should be considered as tools to counteract the increase in inflammatory-based diseases in our society.

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Author Contributions

Francisco J. Pérez-Cano and Margarida Castell were mainly responsible for designing the study; Francisco J. Pérez-Cano, Maria J. Rodríguez-Lagunas, Malen Massot-Cladera and Margarida Castell carried out the scientific searches and drafted the manuscript; all authors contributed to and approved the final version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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RESULTATS

Article 1

“Cocoa modulatory effect on rat faecal microbiota and colonic crosstalk”

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Els resultats del present article han permès la presentació de les comunicacions:

- *Effects of a cocoa-enriched diet on rat intestinal immune system by changes in microbiota and toll-like receptors.* III Workshop Probióticos, Prebióticos y Salud. Evidencia Científica, Castelldefels (Espanya), Desembre 2011. Comunicació oral.
- *Cocoa polyphenols intake in rats modulates the colonic crosstalk between intestinal microbiota and immune system.* 5th International Conference on Polyphenols and Health, Sitges (Espanya), Octubre 2011.
- *Rat microbiota modulation by a long term cocoa-enriched diet.* 15th International Congress of Mucosal Immunology Paris (França), Juliol 2011.

RESUM

Objectiu: Establir l'efecte d'una dieta enriquida amb cacau sobre la composició de la microbiota, l'expressió gènica dels receptors *toll-like* (TLR) i la secreció d'IgA en intestí de rata.

Material i mètodes: L'estudi es va realitzar en rates de 4 setmanes d'edat de la soca Wistar, les quals van ser alimentades amb una dieta amb un 10% de cacau o una dieta estàndard durant 6 setmanes. Abans i després de la intervenció nutricional es va caracteritzar la microbiota intestinal per la tècnica de FISH acoblada a citometria de flux (FISH-FCM) així com determinar el percentatge de bacteris units a IgA. Al final de l'estudi es va avaluar l'expressió gènica de TLR i d'IgA en mostres de còlon per la tècnica de PCR a temps real i la secreció d'IgA intestinal per la tècnica d'ELISA.

Resultats: La intervenció dietètica amb cacau durant 6 setmanes va disminuir la concentració d'IgA intestinal. A més, els animals alimentats amb la dieta de cacau van presentar canvis en la composició de la microbiota amb una menor proporció dels gèneres *Bacteroides* spp., *Clostridium* spp. i *Staphylococcus* spp., efectes que van anar acompanyats d'un menor percentatge de bacteris units a IgA. La dieta de cacau va induir canvis en el patró d'expressió dels receptors TLR en el còlon.

Conclusió: La ingesta d'una dieta de cacau al 10% modifica el creixement de determinats gèneres bacterians de la microbiota intestinal de rata que s'associen a canvis en l'expressió gènica dels TLR.



Cocoa modulatory effect on rat faecal microbiota and colonic crosstalk

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ABSTRACT

Previous studies have reported the effect of a cocoa-enriched diet on the intestinal immune system in rats. Cocoa contains fibre and polyphenols that can directly influence the intestinal ecosystem and its relationship with the immune system. The aim of this study was to evaluate the effects of a cocoa-enriched diet on gut microbiota, toll-like receptor (TLR) expression and immunoglobulin (Ig) A (IgA) intestinal secretion in rats. Four-week-old Wistar rats were fed a standard or cocoa diet for 6 weeks. Faecal samples were collected before the beginning of the diet and at the end of the study. After the nutritional intervention, colon samples were obtained to quantify TLR and IgA gene expression and IgA protein. Microbiota composition was characterized by fluorescent in situ hybridization (FISH) coupled to flow cytometry (FCM) analysis using specific probes directed to 16S rRNA of the main bacteria genus present in rat intestine. The cocoa dietary intervention resulted in a differential TLR pattern and a decrease in the intestinal IgA secretion and IgA-coating bacteria. Moreover there was a significant decrease in the proportion of *Bacteroides*, *Clostridium* and *Staphylococcus* genera in the faeces of cocoa-fed animals. In conclusion, cocoa intake affects the growth of certain species of gut microbiota in rats and is associated with changes in the TLR pattern which could be responsible for the changes observed in the intestinal immune system.

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Introduction

Extensive research has demonstrated that diet is a crucial aspect of maintaining an optimal immune function [1]. In this context, there has been an increasing interest in focusing on the identification of natural biologically active nutrients with the potential to modulate the activity of the immune system [2,3]. As the intestine is the first compartment reached by dietary immunomodulatory compounds, the cells from the gut-associated lymphoid tissue (GALT)¹ are the first ones to be affected. The intestine

is constantly exposed to dietary and environmental compounds and harbours a complex community of trillions of bacteria [4]. As a consequence of this continuous antigenic load, the intestinal immune system undergoes a dynamic remodelling process and maintains mucosal homeostasis by inducing a state of non-responsiveness to food antigens and commensal bacteria and counteracting them when it is exposed to pathogens [5]. In this regard, toll-like receptors (TLR) are important receptors involved in the recognition of conserved molecular motifs on micro-organisms and constitute key mediators of immunity during the cross-talk between the intestinal microbiota and gut cells [6]. Structurally, GALT is divided into organised lymphoid structures and diffusely distributed cell populations, which are considered primary mucosal immune inductive and effector sites, respectively. As a consequence of effector immune response, the intestinal mucosa contains secretory immunoglobulin A (S-IgA), the main antibody in the intestine.

Over the last decade, cocoa has become the subject of increasing interest because of its high content of polyphenols, mainly (–)-epicatechin, (+)-catechin and various polymers derived from these monomers called procyanidins [7–9]. These compounds, among others present in cocoa, have been shown to modify the composition and functionality of diverse lymphoid tissues in rats, including the GALT [10–13]. In this regard, a high and continuous cocoa intake interfered with some of the mechanisms involved in intestinal

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¹ Abbreviations used: BMI, body mass index; cDNA, complementary DNA; CO, colon; COCOA, cocoa group; Cy3, indocarbocyanine fluorochrome; Cy5, indodicarbocyanine fluorochrome; ELISA, Enzyme-Linked Immunosorbent Assay; FBS, foetal bovine serum; FCS, forward scatter signal; FCM, flow cytometry; FISH, fluorescent in situ hybridization; FISH-FCM, fluorescent in situ hybridization coupled to flow cytometry; FITC, Fluorescein isothiocyanate; GALT, gut associated lymphoid tissue; HB, hybridization buffer; Ig, immunoglobulin; IgA-SCs, IgA-secreting cells; MLNs, mesenteric lymph node; NaCl, sodium chloride; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PFA, paraformaldehyde; PI, Propidium iodide; PPS, Peyer's patches; REF, reference group; RNA, ribonucleic acid; rRNA, ribosomal RNA; SDS, dodecylsulfate sodium; SI, small intestine; SSC, side scatter signal; S-IgA, secretory immunoglobulin A; TE, tris-EDTA buffer; TLR, toll-like receptors.

IgA production, leading to a lower IgA secretion [14]. Moreover, cocoa diets induced differential TLR expression patterns for TLR2, TLR4, TLR7 and TLR9 in Peyer's patches (PPs), mesenteric lymph nodes (MLNs) and small intestine (SI) tissue [13,14]. This effect might be reflecting changes in the intestinal microbiota composition, as previous studies show that cocoa procyanidins and fibre reach the colon intact after a cocoa intake and may act as bacterial growth substrate [15].

In order to characterize the bacterial population, a wide variety of techniques have been described. In recent years, fluorescent *in situ* hybridization (FISH) technique with specific anti-16S rRNA oligonucleotide probes has become widely used for the detection of specific bacterial groups in multi-species bacterial samples such as faeces [16–19]. This method is based on the hybridization of synthetic oligodeoxynucleotide probes to specific regions within the bacterial ribosome without the need to cultivate the bacteria. In addition, FISH technique allows for the analysis of unlabeled bacteria, which is not possible with other emerging techniques such as quantitative PCR-based methods [20]. Specific oligonucleotide probes have been designed for many bacterial genera and species which are known to be present in the intestinal tract [21]. Traditionally, labelled bacteria are counted under a fluorescence microscope by the investigator, which results in a laborious, slow and poorly reproducible work [22,23]. For this reason, over the past five years, FISH has been combined with other detection techniques in order to improve efficiency and specificity. In this context, FISH technique, combined with flow cytometry (FCM-FISH), has been proposed as a complex but automated and objective methodology which may be considered as an alternative to microscopy.

The objective of this study was to ascertain the effects of a high and continuous cocoa intake on faecal microbiota composition, immunoglobulin-coating bacteria and their cross-talk with the immune system in order to establish a possible correlation among a cocoa diet and gut microbiota, colon TLR gene expression and mechanisms of bacterial exclusion through S-IgA.

Material and methods

Diets and animals

Three-week-old female Wistar rats were obtained from Harlan (Barcelona, Spain) and housed in cages under conditions of controlled temperature and humidity in a 12:12 light–dark cycle. After 6 days of acclimatization, the rats were randomly assigned to two dietary groups ($n = 7$ each group): the reference group (REF) which was fed with a standard diet AIN-93M (Harlan, Barcelona, Spain), and the cocoa group (COCOA) which received chow containing 10% (w/w) cocoa for 6 weeks. Natural Forastero cocoa (Nutrexpa S.A, Barcelona) containing 10.62 mg/g polyphenols was used for this study. The cocoa diet was obtained from modified AIN-93M containing 100 g cocoa/kg as previously described [13]. The resulting cocoa-enriched chow was isoenergetic and had the same proportion of macronutrients (carbohydrates, lipids and proteins) and also insoluble fibre as the standard diet, but was supplemented with particular compounds of cocoa such as soluble fibre and polyphenols. Diet compositions are detailed in Table 1.

Animals were given free access to water and chow *ad libitum*. Body weight and food intake were monitored throughout the experiment. 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.

Sample collection and processing

After 6 weeks of dietary intervention, the rats were anaesthetized intramuscularly with ketamine (90 mg/kg) and xylazine (10 mg/kg). The colon (CO) and the small intestine (SI) were excised. The CO was immediately immersed in RNAlater® (Ambion, Life technologies, Austin, TX, USA), incubated at 4 °C overnight and stored at –20 °C until PCR analysis. The SI was divided into two halves and carefully rinsed with cold sterile 0.9% sodium chloride (NaCl) solution in order to remove faecal content. The distal half was opened lengthwise and was used to obtain the gut wash for IgA quantification by enzyme-linked immunosorbent assay (ELISA), as previously described [11].

Faecal samples were collected before and after six weeks of the diet beginning and stored at –20 °C until processing. In this processing, faeces were weighed, diluted 1:20 (w/v) in phosphate-buffered saline (PBS, pH 7.2) and homogenized using a Polytron® (Kinematica, Lucerne, Switzerland). The homogenates obtained were then centrifuged (300g, 1 min, 4 °C) for bacterial characterization following procedures described by Nadal et al. [24] with some modifications. Faecal homogenates were used for IgA-coating bacteria and microbiota analysis. In the later case, part of the supernatant was fixed overnight by adding 4% paraformaldehyde (PFA) (Merck, Madrid, Spain) solution in PBS. After fixation, bacteria were washed twice with PBS (12000g, 5 min, 4 °C). Finally, cell pellets were suspended in a 1:1 PBS/ethanol mixture, stored at –20 °C for at least 1 h and then stored at –80 °C until analysis.

Microbiota analysis by fluorescence *in situ* hybridization coupled to flow cytometry (FISH-FCM)

The bacterial groups contained in faeces were characterized using FISH technique with group- or genus-specific fluorochrome-conjugated probes which target the bacterial 16S rRNA (Sigma-Aldrich, Madrid, Spain). The specific probes used in this study include the EUB338 probe (5'-GCTGCTCCCGTAGGAGT), which targets all eubacteria and was also used to determine total counts, the *Lactobacillus-Enterococcus* group probe (Lab158, 5'-GGTATTAGCAYCTGTTTCCA), the *Bacteroides-Prevotella* group probe (Bac303, 5'-CCAATGTGGGGACCTT), the *Bifidobacterium* genus probe (Bif164, 5'-CATCCGGCATTACACCC), the *Staphylococcus* genus probe (Staphy, 5'-TCTCTCCATATCTCTGCGC), the *Streptococcus* genus probe (Strep, 5'-CACTCTCCCTTCTGCAC) and the *Clostridium* group probe (Chis150, 5'-TTATGCGGTATTAATCT(C/T)CCTTT) [25,26]. The group-specific probes were labelled at the 5'-end with indodicarbocyanine fluorochrome (Cy5) while the EUB338 probe was labelled with indodicarbocyanine fluorochrome (Cy3). The NON-EUB338-Cy5 probe (5'-ACTCTACGGGAGGAGCAGC) was applied as a negative control in order to eliminate background fluorescence. Specific cell enumeration was performed by combining each of the group-specific Cy5 probes with the EUB 338-Cy3 probe.

Fixed cell suspensions were incubated in the presence of each fluorescent probe (50 ng/μL) in preheated hybridization buffer (HB, 10 mmol/L Tris-HCl pH 8, 0.9 mol/L NaCl, 0.1% sodium dodecyl sulfate (SDS)) [24,27] in a 0.2 mL tube in a thermocycler at 48 °C for 2 h in the dark [28]. In the case of *Lactobacillus*, samples were washed in Tris-EDTA (TE) buffer and permeabilized with 0.25 mg/mL lysozyme (Serva, Heidelberg, Germany) for 10 min at room temperature prior to the hybridization process with the same HB but adding 30% formamide [29,30]. Hybridized cell suspensions were then washed in HB without SDS for 30 min at 48 °C in order to eliminate non-specific binding of the probes. The hybridized cells were collected by centrifugation (12 000g, 5 min), washed twice in PBS [31] and resuspended in 200 μL of PBS solution for flow cytometry analysis. Samples were kept in the dark at 4 °C

Table 1
Composition of experimental diets (g/kg diet).

Components	Standard diet (AIN-93n0031M; g/kg)	10% Cocoa diet (g/kg)
Casein	140	118
L-Cystine	1.8	1.8
Corn starch	465.69	449.69
Maltodextrin	155.0	155.0
Sucrose	100.0	100
Soybean oil	40.0	29
Cellulose	50.0	24.5
Mineral mix (TD94049)	35.0	35.0
Vitamin mix (TD94047)	10.0	10.0
Choline bitartrate	2.5	2.5
tert-Butylhydroquinone	0.008	0.008
Natural cocoa powder		100
22% Protein		22
16% Carbohydrate		16
11% Lipid		11
34% Fiber		34
Insoluble (75%)		25.5
Soluble (25%)		8.5
1.062% Polyphenols		1.062
Epicatechin		83 mg
Catechin		14 mg
Procyanidin B2		65 mg
Quercetin		1.5 mg
Isouercetin		4 mg
Quercetin-3-arabinose		2 mg
Quercetin-3-glucuronide		0.6 mg
kcal/g diet	3.6	3.6

until the FCM assay to avoid loss of the signal intensity of the hybridized cells.

FISH technique and cytometric analysis were optimized using *Bifidobacterium breve*, *Lactobacillus paracasei*, *Lactobacillus plantarum*, *Escherichia coli* MC 400 and *E. coli* bl 21 strains to evaluate the specificity and efficiency of the probes and hybridization/washing conditions. Moreover, this approach allowed us to establish the acquisition gate on the basis of bacterial morphology (forward and side scatter; FSC/SSC).

Immunoglobulin-coating bacterial analysis

A volume of 35 µL of the faecal homogenates was diluted in 500 µL 1% (v/v) foetal bovine serum (FBS)/PBS and after high-speed centrifugation (8000g, 5 min, 4 °C) the resulting pellet was resuspended in 50 µL 1% (v/v) FBS/PBS containing fluorescein isothiocyanate (FITC)-anti-rat IgM (BD Biosciences; Heidelberg, Germany), phycoerythrin (PE)-anti-rat IgA (Open Biosystems, Huntsville, AL, USA) or FITC-anti-rat Ig antibodies (Abcam; Cambridge, UK). Each mixture was incubated for 30 min in the dark at room temperature. A non-stained mixture of each sample was used as control. Thereafter, suspensions were washed twice with PBS (8000g, 5 min) and resuspended in 200 µL PBS until analysis. The labelled samples were mixed with 10 µL propidium iodine (PI; 1 mg/mL, Sigma-Aldrich, Madrid, Spain) 15 min prior to FCM analysis in order to label total bacteria.

Flow cytometry

A FacsAria SORP sorter (BD, San José, CA, USA) was used. Bacteria morphology was selected according to their FCS/SSC signal. From here on, bacteria hybridized with group- or genus-specific probes conjugated to Cy5 or Cy3 were detected combining FSC signal with Cy5 fluorescence detectors (670/14 nm filter) or Cy3 fluorescence detectors (575/25 nm filter). The FCM parameters

were adjusted for bacterial counts, and the bacterial concentrations of the samples were adjusted to obtain particle counts of 100–1000 events per second and the events were recorded for 60 s in list mode files. Commercial Flow Check™ Fluorospheres (Beckman Coulter, Inc. FL, USA) were used to determine total counts combined with the EUB338-Cy3 probe. Analysis was performed using the Summit v4.3 software (Beckman Coulter, Inc. FL, USA).

FITC-anti-rat Ig and IgM antibodies were detected with the FITC fluorescence detectors (530/30 nm), and PE-anti-rat IgA using the PE fluorescence detectors (575/25 nm), separately. Finally, total bacteria was detected by means of PI fluorescence detectors (610/20 nm).

Microbiota composition results were expressed as the log faecal cells/g of faeces in each sample. Ig-coating bacteria results were expressed as a percentage of bacterial cells labelled with anti-rat Ig with respect to the total cell population labelled with PI.

Assessment of RNA gene expression by Real Time PCR

The RNA was isolated from tissue samples in RNeasy® 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 [14].

Four micro grams of total RNA was converted to cDNA and a final volume of 1 µL was used to confirm the reaction of each sample by conventional β-actin PCR as previously described [32].

Specific PCR TaqMan® primers and probes (Applied Biosystems, AB, Weiterstadt, Germany) were used to measure *Tlr2* (Rn02133647_s1, 1), *Tlr4* (Rn00569848_m1, 1), *Tlr7* (Rn01771083_s1, 1), *Tlr9* (Rn01640054_m1, 1) and *Iga* (331943, made to order). Quantitative PCR assays were performed in duplicate for each sample using an ABI PRISM®7700 Sequence Detection System (AB). Quantification of the genes being studied was normalized to the housekeeping gene *Gusb* (Rn00566655_m1, 1). The amount of target mRNA relative to the endogenous control expression was calculated for the COCOA group relative to values from the REF group which represents 100% gene expression, using the $2^{-\Delta\Delta Ct}$ method, as previously described [33]. Results are expressed as the mean ± SEM of the percentage of these values.

Statistical analysis

The software package SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Levene's and Kolmogorov-Smirnov's tests were applied to the equality of variance and normal distribution assays of the studied groups, respectively. Non-parametric Mann-Whitney *U* and Wilcoxon tests were used in order to assess significance for independent and related samples, respectively. The Pearson's correlation test was used to calculate the correlations between bacterial count changes and either IgA or TLRs gene expression changes as a result of the dietary intervention. Significant differences were established at *P* values less than 0.05.

Results

Effect of cocoa diet on body weight

Body weight and chow intake were monitored throughout the study. The initial body weight (71.42 ± 0.01 g) increased differentially in both dietary groups and at the end of the study the REF animals reached 193.84 ± 0.25 g of body weight whereas the COCOA rats had significantly lower growth (167.36 ± 0.40 g,

$p < 0.01$). This effect was not associated with a lower food intake because it was similar throughout the study between both groups (data not shown).

FISH-FCM methodology for faecal bacteria enumeration

In order to optimize the methodology of coupling FCM to FISH, non-mixed bacterial populations were used as controls (Fig. 1). Critical settings were related to probe hybridization specificity and efficiency. Conditions set up in such pure bacteria include, among others, hybridization and washing temperatures and incubation periods (from 1 to 24 h), requirement of lysozyme treatment for *Lactobacillus*, and composition of the permeabilization buffer (SDS concentration ranging from 0.001% to 10%). Final conditions are described in the Material and methods and FCM gating is shown in Fig 1.

Effects of cocoa diet on microbiota composition

The faecal microbiota composition was characterized by FCM-FISH technique before the start of the diet and at the end of the

study (Table 2). The *Lactobacillus* proportion had a tendency to be reduced by age when samples from both groups were considered together. With regards to the nutritional intervention, the high and continuous cocoa diet led to significant differences in microbiota composition. Although the total counts of bacteria were not affected by the diet, the cocoa-fed animals had a significant decrease in the proportion of *Bacteroides*, *Staphylococcus* and *Clostridium* genera compared to the REF group at the end of the study and with respect to their initial values ($p < 0.05$).

Effects of cocoa diet on colon TLR gene expression

Gene expression of TLR2, TLR4, TLR7 and TLR9 was assessed in the colon at the end of the study (Fig 2). When samples from both groups were considered together the number of the *Lactobacillus* and *Staphylococcus* genera showed a correlation with the gene expression of TLR7 and TLR9 at the end of the study ($r = 0.849$, $p = 0.016$ and $r = 0.850$, $p = 0.015$; respectively).

Colon TLR4 gene expression was not modified in the cocoa-fed animals whereas TLR2 and TLR7 expression had a tendency to be

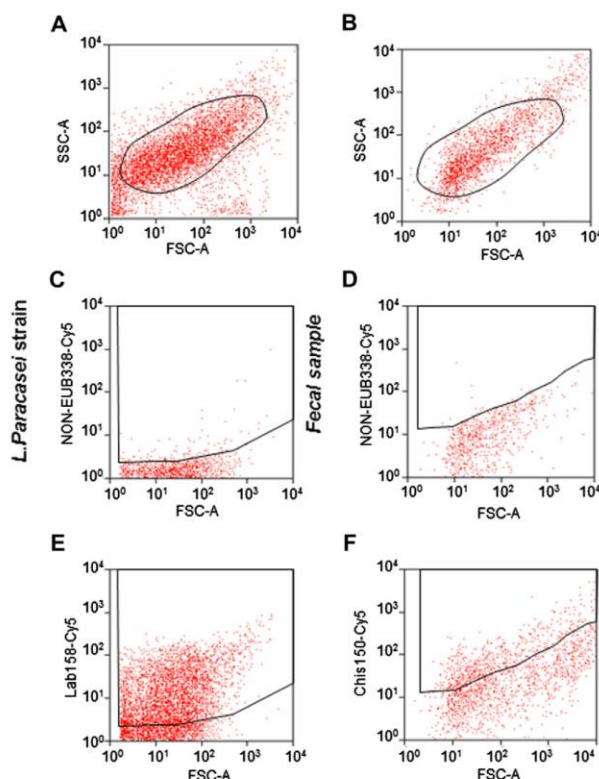


Fig. 1. Representative cytograms of the optimized FISH-FCM methodology using the *L. paracasei* strain (A, C, E) and a representative faecal sample (B, D, F). A and B cytograms show the initial acquisition gates that select the bacterial population. C and D cytograms correspond to negative controls combining morphological parameters of the bacterial population (FSC) and fluorescence for NON-EUB-Cy5 probe in the *L. paracasei* strain (C) or in the faecal sample (D) which allows the threshold for the positive region for *Lactobacillus* or *Clostridium* detection to be selected. E and F cytograms correspond to positive bacteria for Lab158-Cy5 in the *L. paracasei* strain (E) and Chis150-Cy5 in a faecal sample (F) after FISH technique.

Table 2
Bacterial counts of each genus relative to total bacteria counts determined by FISH-FCM in faeces.

	REF group				COCOA group			
	Before intervention		After intervention		Before intervention		After intervention	
	Cells/g ^a	S.M.E	Cells/g ^a	S.M.E	Cells/g ^a	S.M.E	Cells/g ^a	S.M.E
All Bacteria	9.47	0.13	9.34	0.12	9.26	0.08	9.32	0.10
<i>Lactobacillus</i>	8.30	0.16	7.81	0.28	7.86	0.18	7.35	0.15
<i>Bacteroides</i>	8.19	0.24	7.90	0.22	8.09	0.25	7.06 ^{*,†}	0.23
<i>Bifidobacterium</i>	8.31	0.22	8.13	0.11	8.19	0.17	7.68	0.21
<i>Staphylococcus</i>	8.61	0.27	8.51	0.15	8.71	0.14	7.81 ^{*,†}	0.22
<i>Streptococcus</i>	8.90	0.19	8.73	0.17	8.68	0.16	8.36	0.13
<i>Clostridium</i>	8.53	0.13	8.25	0.19	8.39	0.15	7.59 ^{*,†}	0.23

* Significant differences: $p < 0.05$ vs REF group after intervention.

† Significant differences: $p < 0.05$ vs COCOA group before intervention.

^a log faecal cells/g of faeces.

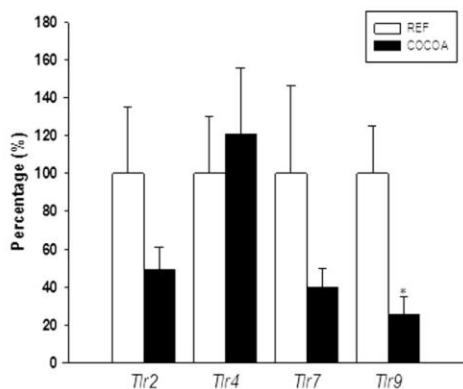


Fig. 2. TLR gene expressions in the colon after nutritional intervention with cocoa. Expression values are normalized using the expression of *Gusb* as the endogenous housekeeping gene. Each bar represents the mean $\pm$ SEM of the percentage of the COCOA group (black bars) with respect to the REF group (white bars), which represents 100% gene expression. Significant differences: * $p < 0.05$ vs REF.

reduced. Cocoa-fed animals showed 4-fold downregulation in the TLR9 expression in comparison to the REF group ($p < 0.05$).

TLR2 and TLR7 decreases correlated with values of *Lactobacillus*, *Bacteroides* and *Bifidobacterium* in the COCOA group ($p < 0.01$, in all cases).

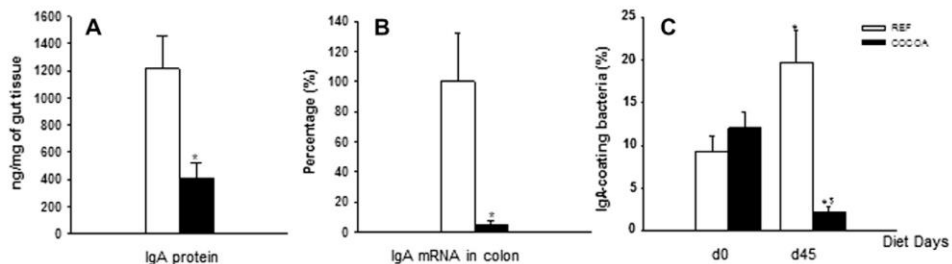


Fig. 3. Effects of a cocoa-enriched diet on intestinal IgA. Black bars correspond to the COCOA group and white bars correspond to the standard diet (REF group). Each bar represents the mean $\pm$ SEM of each variable for the cocoa group in comparison with the reference group. (A) Concentration of S-IgA in small intestine washes, (B) percentage of *Iga* gene expression in the colon compared to the reference group which represents 100% gene expression, and (C) percentage of faecal bacteria coated with IgA before and after 6 weeks of nutritional intervention. Statistical significance: * $p < 0.05$ vs REF group after the study period; † $p < 0.05$ vs their initial values.

Effects of cocoa diet on intestinal IgA

Different approaches were used to study the effect of cocoa on intestinal IgA production (Fig 3). After 6 weeks of the dietary intervention, the S-IgA concentration in the small intestine washes from the COCOA animals was significantly reduced when compared to that of the REF group ($p < 0.05$) (Fig. 3A) and significantly correlated with the decrease in the proportion of the *Clostridium* and *Streptococcus* groups ($p < 0.05$). Moreover, the colon *Iga* gene expression in those animals receiving cocoa for 6 weeks followed the same down-modulatory pattern ($p < 0.05$) (Fig. 3B). This decrease also had significant correlations with the content of *Bacteroides*, *Bifidobacterium*, *Streptococcus* and *Clostridium* in the COCOA group ($p < 0.01$, in all cases).

Faecal samples collected before the start of the diet and at the end of the study were used to determine IgA- and IgM-coating bacteria by flow cytometry (Figs. 3C and 4). Although IgM-coating bacteria were not detected in faecal samples (data not shown), both groups showed a similar IgA-coating bacteria pattern at baseline. Moreover, the proportion of the IgA-coating bacteria in both dietary groups was significantly associated with the number of faecal *Clostridium* and *Staphylococcus* ($r = 0.769$, $p = 0.026$ and $r = 0.680$, $p = 0.044$, respectively). At the end of the study the REF group showed an age-related increase in the percentage of IgA-coating bacteria which was avoided by the cocoa diet ($p < 0.05$) fig 4.

Discussion

Previous studies showed that cocoa-enriched diets in rats influence intestinal immune system [11,13,14] what is attributed either

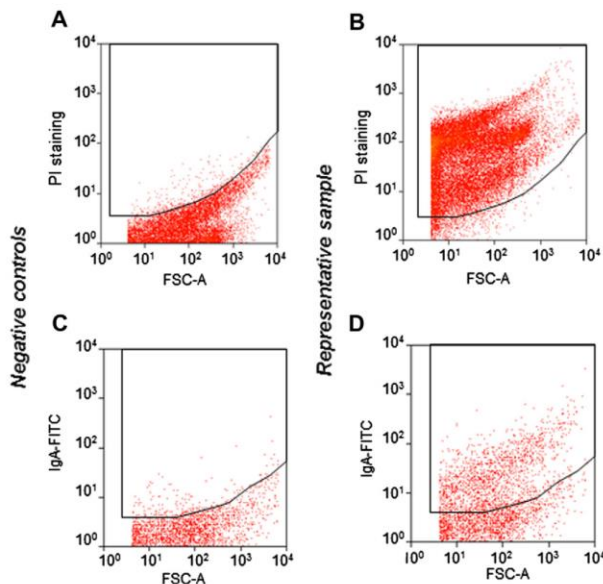


Fig. 4. Representative biparametric cytograms combining morphological parameters, total bacteria labelling and IgA-coating bacteria percentages. (A) A non-stained faecal homogenate was used as a negative control to establish the positive region for total bacteria PI labelling. (B) Population of total bacteria stained with PI in a faecal homogenate. (C) A non-stained faecal homogenate was used as a negative control to establish the positive region for IgA-coating bacteria labelled with FITC. (D) Percentage of IgA-coating bacteria in a faecal homogenate. C and D are gated on the basis of an established window on B (PI⁺ cells, bacterial population).

to a direct effect on intestinal immune cells and/or to an effect, mediated by changes in microbiota which would influence the cross-talk with the host (i.e. through TLR).

After ingestion of cocoa, its procyanidins as well as its fibre reach intact the colon [15,34]. Commensal bacteria can metabolise polyphenols [34] and then intact procyanidins and their metabolites may influence both on the immune cells and the own microbiota. Here we demonstrate that a 10% cocoa diet for 6 weeks decreased the amount of the *Bacteroides*, *Staphylococcus* and *Clostridium* groups, intestinal IgA production and TLR9 gene expression.

Although the antimicrobial activity of polyphenols has been demonstrated *in vitro*, there are few *in vivo* studies. Tea phenols and some metabolites show *in vitro* inhibitory effects on intestinal human bacteria such as *Clostridium*, *Bacteroides* and *Staphylococcus* while commensal anaerobes such as *Lactobacillus* and *Bifidobacterium*, among others, are less affected [35]. These results are in line with those found here, probably due to the fact that tea and cocoa share a similar but non-identical pattern of flavanols. Similarly, (+)-catechin shows an inhibitory effect on the growth of the *Clostridium histolyticum* using the batch culture approach [36], and berries and its phenols also inhibited *in vitro* the *Staphylococcus* growth [37]. Our results regarding the decreasing proportion of some bacteria genera are also in line with the effects of dietary tannins in several animal models [38,39]. However, other *in vivo* studies have shown the ability of certain flavonoids to promote the growth of selected gut microbiota. Thus, using the batch culture approach, pomegranate polyphenols have been reported to specifically enhance the growth of *Bifidobacterium* spp. and the *Lactobacillus-Enterococcus* group [40]. The *Bacteroides*, *Lactobacillus* and *Bifidobacterium* intestinal proportions increased in rats fed with

wine polyphenols [41], and pigs receiving tea polyphenols showed an increase in their *Lactobacillus* proportions [42].

Interventional studies evaluating the consumption of cocoa products in humans are very limited, but in line with some of our results, a recent study using high cocoa flavanol consumption for 4 weeks found a significant decrease in the *C. histolyticum* group [43]. However, the high cocoa beverage also caused a promoting effect on the growth of *Lactobacillus* spp. which does not reflect our findings. These differential results could be attributed to several factors such as the cocoa composition (fibre and flavonoids pattern) dose, and differential composition and distribution ecosystem (rats vs human gut) [44]. In this sense, the predominant microorganisms in rats are *Peptostreptococcus* and *Fusobacterium* genus [44], whereas three enterotypes (different species and functional composition) in the human gut microbiota called, due to its predominance, *Bacteroides*, *Prevotella* and *Ruminococcus* [45] has been recently demonstrated. Therefore, there are certain limitations to this kind of interventional studies in humans due to its high interpersonal variability. This could be a key factor in studies evaluating the potential health effects of polyphenols; as these effects may be dependent on the volunteers' microbiota composition the results can be very diverse. This important variability factor may be better controlled by using experimental animals in which the microbiota from the same colonies is similar.

This study also shows the effect of a cocoa diet on body weight. As shown in previous studies, a high cocoa intake produces lower weight increase [11,46]. These results are in line with some studies reporting weight loss and reduced fat deposition after polyphenol intake [47–49]. Some explanations have been given in this regard relating to hypolipidemic and cellular energy stimulating the properties of flavonoids [50,51]. However, as a reduction in some

bacterial groups such as *Clostridium* is significantly correlated with weight loss and body mass index (BMI) z-score reduction in a total population [24], the effect of cocoa on reducing the *Clostridium* group in the gut microbiota, may also explain partially the effects of flavonoids on the body weight.

Besides the changes in microbiota composition after the cocoa diet, some TLRs, which are key molecules for the recognition of micro-organisms [52,53], were also studied in the colon. The results showed a significant downregulation of TLR9 and a tendency to decrease TLR2 and TLR7 in the colon of cocoa-fed animals. Previous studies using a similar experimental design have found a pattern of changes in TLR expression in inductive GALT and a different pattern in the effector GALT in the small intestine [13,14]. In our current study, the changes in TLR2, TLR4 and TLR7 gene expression in the colon after cocoa intervention seems to demonstrate a similar behaviour to the results obtained in the inductor sites. Conversely, the colon TLR9 expression in cocoa-fed animals adopts the same tendency to that which was previously observed in the effector sites. These changes in TLR expression could reflect the changes found in the intestinal microbiota and/or its direct relation with intestinal epithelial and immune cells. In addition, these changes may modulate the recruitment of IgA-secreting cells to the intestinal lamina propria [14].

We found that high cocoa diet had a down-modulatory effect on intestinal IgA at protein level, in line with previous studies [11], and also at gene level, which concurs with the effect found in small intestine and Peyer's patches from rats fed a cocoa diet [14]. The mucosal, but also systemic, Ig down-modulation may be important in diseases involving dysregulation of intestinal antibody responses such as celiac disease or food allergies. Here we have also investigated the possible effects of cocoa on IgA-coating bacteria. Regardless of the lowering effect of cocoa on IgA-coating bacteria, the proportion of the IgA-coating bacteria in both dietary groups is particularly closely associated with the number of faecal *Clostridium* and *Staphylococcus*, which agrees in part with the findings of others [24]. These results may suggest that these bacteria have the biggest influence on the IgA-coating effect.

All these findings increase the evidence that cocoa can modulate intestinal microbiota composition. This effect may be due to its highly polymerized polyphenols which are associated to dietary fibre repressing the growth of certain bacteria. Moreover, microbiota changes could be partly responsible for the lower differentiation in IgA + B cells and/or lower IgA synthesis capacity in this compartment, probably due to the changes produced in TLR gene expression. In summary, a high and continuous cocoa intake in rats is capable of modifying some functional aspects at intestinal level including microbiota composition and immune response.

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Article 2

“Cocoa flavonoid-enriched diet modulates systemic and intestinal immunoglobulin synthesis in adult Lewis rats”

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Els resultats del present article han permès la presentació de la comunicació:

- *Influence of several sources of cocoa flavonoids on humoral immune system in young rats.* 6th International Immunonutrition Workshop, Palma de Mallorca (Espanya), Octubre 2012.

RESUM

Objectiu: Avaluar si els efectes causats per la dieta rica en cacau en la secreció d'IgA intestinal en rata depenen del seu contingut en flavonoides.

Material i mètodes: L'estudi es va realitzar en rates de 8 setmanes d'edat de la soca Lewis les quals van ser alimentades durant dues setmanes amb una dieta estàndard o bé amb dietes amb un contingut creixent de flavonoides del cacau (0,2% a partir d'un extracte de cacau convencional fermentat; 0,4% o un 0,8% a partir d'un extracte de cacau que no ha estat sotmès als processos de fermentació i de torrefacció conservant així l'elevat contingut en polifenols). Al llarg de l'estudi es va monitoritzar el consum de pinso i el pes corporal i es va mesurar el pH fecal. Abans i després d'una i dues setmanes d'intervenció nutricional es va quantificar, per tècniques d'ELISA, la IgA fecal i la concentració sèrica d'IgM i IgG. En mostres fecals també es va establir el percentatge total de bacteris i la proporció de bacteris que expressen la IgA en la seva superfície.

Resultats: La dieta amb el menor contingut de flavonoides del cacau (0,2%) va ser la única en provocar menor guany de pes corporal al final de l'estudi. Aquest efecte, no va ser degut a una menor ingesta de pinso. Totes les dietes enriquides amb flavonoides del cacau van atenuar l'increment associat a l'edat en la concentració fecal d'IgA, el percentatge de bacteris i la proporció de bacteris units a IgA en mostres fecals. Aquests efectes però, no es van correlacionar amb la quantitat de flavonoides de la dieta. Cap de les dietes va modificar la concentració d'IgG mentre que, únicament la dieta de 0.4% de flavonoides va reduir la concentració sèrica d'IgM.

Conclusió: La influència de la dieta rica en cacau sobre la secreció d'IgA intestinal i el percentatge de bacteris en femtes no depèn exclusivament del seu contingut en flavonoides.

Article

Cocoa Flavonoid-Enriched Diet Modulates Systemic and Intestinal Immunoglobulin Synthesis in Adult Lewis Rats

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Abstract: Previous studies have reported that a diet containing 10% cocoa, a rich source of flavonoids, has immunomodulatory effects on rats and, among others effects, is able to attenuate the immunoglobulin (Ig) synthesis in both systemic and intestinal compartments. The purpose of the present study was focused on investigating whether these effects were attributed exclusively to the flavonoid content or to other compounds present in cocoa. To this end, eight-week-old Lewis rats were fed, for two weeks, either a standard diet or three isoenergetic diets containing increasing proportions of cocoa flavonoids from different sources: one with 0.2% polyphenols from conventional defatted cocoa, and two others with 0.4% and 0.8% polyphenols, respectively, from non-fermented cocoa. Diet intake and body weight were monitored and fecal samples were obtained throughout the study to determine fecal pH, IgA, bacteria proportions, and IgA-coated bacteria. Moreover, IgG and IgM concentrations in serum samples collected during the study were quantified. At the end of the dietary intervention no clear changes of serum IgG or IgM concentrations were quantified, showing few effects of cocoa polyphenol diets at the systemic level. However, in the intestine, all cocoa polyphenol-enriched diets attenuated the age-related increase of both fecal IgA and IgA-coated bacteria, as well as the proportion of bacteria in feces. As these effects were not dependent on the dose of polyphenol present in the diets, other compounds and/or the precise polyphenol composition present in cocoa raw material used for the diets could be key factors in this effect.

Keywords: cocoa polyphenols; immune system; immunoglobulins

1. Introduction

Cocoa, obtained from dried and fermented seeds derived from *Theobroma cacao*, was consumed as early as 1100 B.C. by ancient Mesoamerican civilizations [1]. In fact, the Olmecs, and eventually the Mayans and Aztecs, consumed food and beverages produced from cocoa, which was considered a divine beverage with medicinal properties [2]. Although health benefits of cocoa were already known by these ancient civilizations, it was not until recently that cocoa gained recognition because of its high content of fiber and polyphenols, particularly flavonoids. Cocoa powder mainly contains flavanols such as (–)-epicatechin and (+)-catechin (5%–10% of total polyphenols) as well as procyanidins (≥90%), the oligomers derived from these monomers [3–11]. Other minor polyphenols have been identified in cocoa, e.g., quercetin, isoquercitrin (quercetin 3-*O*-glucoside), quercetin 3-*O*-arabinose, hyperoside (quercetin 3-*O*-galactoside), naringenin, luteolin, apigenin, and others [12].

In addition to the antioxidant capacity of cocoa, its interaction with the immune system *in vitro* [13–16] and *in vivo* has been reported. Regarding *in vivo* effects, previous studies have demonstrated that a dietary intervention with cocoa was capable of modulating the serum IgA, IgM and IgG concentrations as well as the intestinal IgA and IgM contents in young rats [17–19]. In addition, a 10% cocoa diet was able to modify the composition and functionality of several lymphoid tissues [17,20–22] including the gut-associated lymphoid tissue (GALT) [20,21]. Cocoa intake modified, at the same time, the fecal microbiota composition and its crosstalk with the immune system by means of the modulation of the toll-like receptors' (TLR) gene expression in the colon [19].

As occurs in other plant-derived foods, the phenolic content of cocoa-derived products is largely dependent upon the cultivar, origin, agricultural and postharvest practices, and the processing [11,23,24]. Until the cocoa liquor is obtained, the cocoa seed has to be subjected to different procedures (specifically fermentation, alkalization or *Dutching* and roasting processes) which lead to a considerable loss of cocoa polyphenols [9,11,24]. On account of the beneficial effects and the poor bioavailability of polyphenols, developing a new flavonoid-enriched cocoa powder has become a subject of great interest. In order to achieve this, control of the processing steps as well as some modifications of its habitual processing procedure have been proposed to keep the maximum amount of cocoa polyphenols [24]. To this end, Naturex S.A. developed CocoaPure, a new flavonoid-enriched cocoa powder from unfermented, blanch-treated, and non-roasted cocoa beans [25].

In this regard, two types of cocoa extracts with a different proportion and composition of polyphenols have been used to ascertain whether the cocoa modulatory effects on the systemic and intestinal immunoglobulin synthesis observed in previous studies can be attributed exclusively to its flavonoid content.

2. Material and Methods

2.1. Animals and Diets

Twenty-four male Lewis rats (8 weeks old) were obtained from Harlan (Barcelona, Spain) and housed in cages under conditions of controlled temperature and humidity in a 12:12 light-dark cycle. After one week of acclimatization, the rats were randomly assigned to four dietary groups ($n = 6$ /each): the reference group (REF) which was fed with the standard diet AIN-93M (Harlan, Barcelona, Spain); the cocoa group (PC0.2), which received chow containing 0.2% of polyphenols from conventional cocoa powder; and the PC0.4 and PC0.8 groups, receiving 0.4% and 0.8% of polyphenols, respectively, from non-fermented cocoa (CocoaPure). The diet lasted two weeks. Conventional natural Forastero cocoa containing 21.20 mg/g of polyphenols was provided by Nutrexpa S.L. CocoaPure containing 510 mg/g of polyphenols was provided by Naturex Spain S.L. The three cocoa diets were prepared from a basal mix diet and particular components, both supplied by Teklad Global Diets (Harlan, Indianapolis, IN, USA). The addition of 100 g/kg of conventional cocoa, 8.7 g/kg or 17.8 g/kg CocoaPure was established in order to obtain a final diet with 0.2%, 0.4% and 0.8% of polyphenols, respectively, and the same proportion of macronutrients as AIN-93M formula. (Table 1). The polyphenol characterization of PC0.2, PC0.4 and PC0.8 diets is also summarized in Table 1.

Table 1. Composition of experimental diets (g/kg diet). The three experimental diets provided similar amounts of proteins, lipids and carbohydrates to the reference diet (AIN-93M).

Components	REF (AIN-93M; g/kg)	PC0.2 (g/kg)	PC0.4 (g/kg)	PC0.8 (g/kg)
Cocoa powder	-	100	8.7	17.4
Nutrients provided by the basal mix:				
Casein	121.8	97.1	120.7	119.5
L-Cystine	1.8	1.4	1.8	1.8
Corn Starch	419.1	421.0	418.3	417.5
Maltodextrin	147.3	116.8	147.3	147.3
Sucrose	117.0	108.7	117.0	117.0
Soybean oil	40.0	26.2	39.4	38.9
Cellulose	50.0	24.5	48.3	46.5
Minerals	27.0	27.8	27.0	27.0
Vitamins	2.0	7.2	2.0	2.0
Choline bitartrate	2.5	2.0	2.5	2.5
tert-Butylhydroquinone	0.008	0.006	0.008	0.008
Nutrients provided by cocoa powder:				
Protein	-	22	1.13	2.26
Carbohydrate	-	16	0.82	1.64
Lipid	-	11	0.56	1.12
Fiber (insoluble/soluble)	-	34 (25.5/8.5)	1.75 (1.31/0.44)	3.5 (2.63/0.88)
Total polyphenols *	-	2.12	4.44	8.88

Table 1. Cont.

Polyphenols provided by cocoa powder **:				
Catechin	-	0.073	0.048	0.097
Epicatechin	-	0.204	0.689	1.379
Isoquercetin	-	0.0053	n.d.	n.d.
Quercetin	-	0.0029	n.d.	n.d.
Procyanidin B ₁	-	n.d.	0.127	0.254
Procyanidin B ₂	-	0.167	0.356	0.713
Total procyanidins	-	n.d.	3.897	7.795

* The analytical method employed to estimate the total polyphenol compounds was the spectrophotometric method of Folin-Ciocalteu [26]. ** Polyphenol profile was identified and quantified using a reversed-phase high performance liquid chromatography (RP-HPLC) coupled to a diode array detector (DAD) (RP-HPLC-DAD) [27], and procyanidin B1 and B2 were quantified by normal-phase HPLC coupled to mass spectrometry (MS) [9] with fluorescence detection (n.d. means non determined).

Animals were given free access to water and chow. Body weight and food intake were monitored twice per week throughout the experiment. 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.

2.2. Sample Collection and Processing

From the beginning of the diet until the end of the study, blood was collected through the saphenous vein and, after centrifugation, serum was separated and kept at -20°C until ELISA quantification. Fecal samples were collected at the same time points and kept at -20°C until analysis. Fecal homogenates for bacteria proportion and immunoglobulin-coating bacterial analysis (5% w/v) and ELISA IgA quantification (2% w/v), were obtained as previously described [19,20] and frozen at -20°C until analysis.

2.3. Fecal pH Determination

Feces collected at the beginning and at the end of the nutritional intervention were used to determine fecal pH using a surface electrode (Crison Instruments, S.A., Barcelona, Spain).

2.4. Immunoglobulin Quantification in Serum and Feces (ELISA)

Fecal IgA, serum IgM and IgG concentrations were quantified by ELISA as previously described [28]. In brief, 96-well polystyrene plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with anti-rat IgA, IgM or IgG mAbs (BD Biosciences, Heidelberg, Germany) (2 mg/mL in phosphate-buffered saline, PBS) and, after blocking with 1% bovine serum albumin (BSA, Sigma-Aldrich, Madrid, Spain) in PBS (PBS-BSA, 1 h, room temperature), appropriate diluted samples and standard dilutions (purified IgA, IgM or IgG, BD Biosciences) were added. After washing, biotin-conjugated anti-rat IgA, IgM or IgG mAbs (BD Biosciences) and subsequently, peroxidase-conjugated ExtrAvidin (Sigma-Aldrich) was added. An *o*-phenylenediamine

dihydrochloride-H₂O₂ solution (Sigma-Aldrich) was used for the detection of bound peroxidase. 3 M H₂SO₄ was added to stop the reaction. Optical density (OD) was measured on a microtiter microplate photometer (LabSystems Multiskan, Helsinki, Finland) at 492 nm. Data were interpolated by means of Ascent v.2.6 software (Thermo Fisher Scientific, S.I.U., Barcelona, Spain) into the standard curves, and expressed as µg/mL in serum and as ng/mL in faecal samples.

2.5. Fecal Bacteria and IgA-Coating Bacterial Analysis

A volume of 35 µL of the fecal homogenates was diluted in 500 µL 1% (v/v) fetal bovine serum (FBS)/PBS and, after centrifugation (8000 g, 5 min, 4 °C), the resulting pellet was resuspended in 50 µL 1% (v/v) FBS/PBS containing fluorescein isothiocyanate (FITC)-anti-rat Ig antibodies (Abcam, Cambridge, UK). Each mixture was incubated for 30 min in the dark at room temperature. A non-stained mixture of each sample was used as control. Thereafter, suspensions were washed twice with PBS (8000 g, 5 min) and resuspended in 200 µL PBS until analysis. The labeled samples were mixed with 10 µL propidium iodide (PI, Sigma-Aldrich) (1 mg/mL) 15 min prior to flow cytometry (FCM) analysis in order to label total bacteria [19]. A FACS Aria SORP sorter (BD, San José, CA, USA) was used. The FCM parameters were adjusted to obtain particle counts of 100–1000 events per second and the events were recorded for 60 s in list mode files. Data analysis was performed using the FlowJo v7.6.5 software (Tree Star, Inc., Ashland, OR, USA).

For bacteria proportion evaluation, a gate according to forward- and side-scattered light (FSC/SSC) was established on the basis of bacteria morphology from previous studies by using specific rRNA probes. The percentage of events stained with PI (by means of 610/20 nm fluorescence detectors) inside this gate is indicative of the proportion of bacteria present in the fecal sample. For IgA-coating bacteria, FITC-anti-rat Ig bound to bacteria were detected with the FITC fluorescence detectors (530/30 nm) and the IgA-coating bacteria proportion was expressed as positive FITC counts within the PI positive bacteria present in the above FSC/SSC morphology gate. Previously, we had demonstrated that Ig coated to bacteria was exclusively IgA [19].

2.6. Statistical Analysis

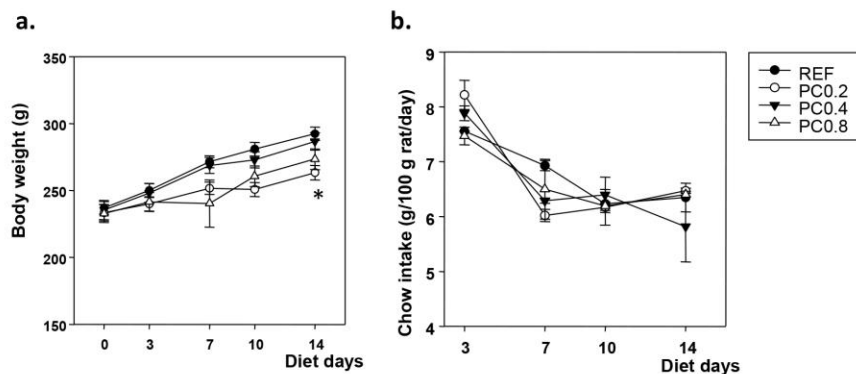
The software package SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Levene's and Kolmogorov-Smirnov's tests were applied to evaluate the equality of variance and normal distribution assays of the studied groups, respectively. The two-way analysis of variance followed by Bonferroni's *post hoc* significance test was used when the assumptions of normality and equal variance were found in variables along time (*i.e.*, weight, chow intake, IgG). In the opposite case, non-parametric tests (Kruskal-Wallis and Mann-Whitney U rank-sum test) were used to assess significance (*i.e.*, IgM and IgA). Moreover, the non-parametric Wilcoxon test was used to compare matched groups at unique time point (*i.e.*, total bacteria at the end of the period). Significant differences were established at *P* values less than 0.05 when comparing two variables, and less than 0.0167 when comparing four variables (*i.e.*, four experimental diets).

3. Results

3.1. Body Weight and Chow Intake

During the study, the body weight slope was similar between the groups with the exception of animals, which received the diet with a lower polyphenol content (PC0.2 group) who showed a lower increase than that of the reference rats, being statistically significant at the end of the study (Figure 1a). This effect was not related to a lower chow intake, as chow intake in all polyphenol-enriched groups and in the reference group ranged from 5 to 9 g/100 g/day, with no statistical differences between them (Figure 1b).

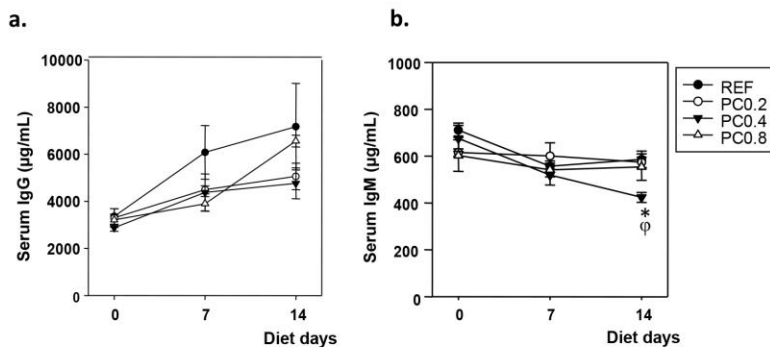
Figure 1. (a) Body weight and (b) chow intake (g/100 g rat/day) monitored during the period of dietary intervention with the cocoa polyphenol-enriched diets. Values are expressed as mean $\pm$ SEM ($n = 6$). Statistical differences: * $p < 0.0167$ vs. reference group (REF).



3.2. Serum IgG and IgM Concentrations

Serum concentrations of IgG and IgM from all experimental groups throughout the study are summarized in Figure 2. Although none of the cocoa polyphenol-enriched diets was able to significantly modify the serum IgG concentration, all dietary interventions had a tendency to lower the age-increasing pattern (Figure 2a). With regards to serum IgM antibodies, the animals fed with PC0.4 diet showed a significant decrease of IgM concentration two weeks after cocoa polyphenol intake began (Figure 2b) compared to those of the REF and PC0.2 groups ($p < 0.0167$).

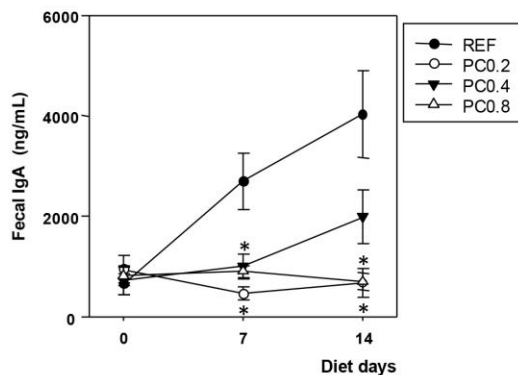
Figure 2. Effects of cocoa polyphenol-enriched diets on serum IgG (a) and IgM (b) concentrations at the beginning, and after one and two weeks of diet. Values are expressed as mean $\pm$ SEM ($n = 6$). Statistical differences: * $p < 0.0167$ vs. REF and ϕ $p < 0.0167$ vs. cocoa group (PC)0.2.



3.3. Fecal IgA Concentration

Intestinal IgA production was quantified in feces. The fecal IgA concentration increased about four-fold over the study period in REF rats. Overall, this age-dependent increase was avoided by all cocoa polyphenol-enriched diets. IgA concentrations were already lower than in the REF group one week after nutritional intervention in the PC0.2 and PC0.8 groups ($p < 0.0167$) and remained significant until the end of the study (Figure 3).

Figure 3. Effect of cocoa polyphenol-enriched diets on fecal IgA concentration at the beginning of the study and after one and two weeks of dietary intervention. Values are expressed as mean $\pm$ SEM ($n = 6$). Statistical differences: * $p < 0.0167$ vs. REF.

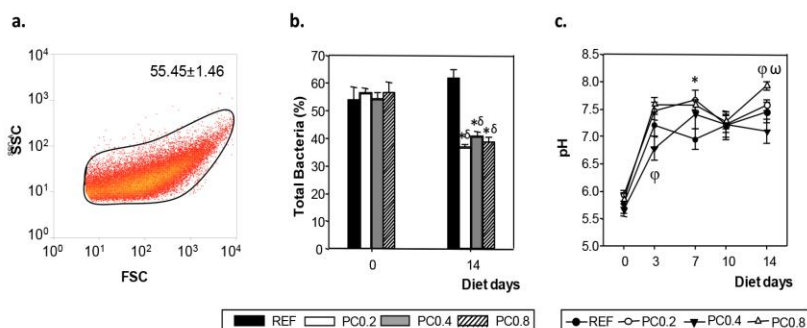


3.4. Total Bacteria Determination

Fecal samples collected at the beginning of the diet and two weeks after the nutritional intervention allowed us to determine the percentage of total bacteria. For that, bacteria morphology was selected according to their FSC/SSC signal and by means of PI fluorescence detectors (Figure 4a). There was no difference in the percentage of total bacteria in any of the groups at the baseline (55.45% $\pm$ 1.46%). The REF group showed a significant age-related increase in this percentage, from ~55% at the beginning of the diet to ~62% two weeks later ($p < 0.05$). This pattern was avoided by all cocoa polyphenols-enriched diets regardless of dose ($p < 0.0167$) (Figure 4b).

Fecal samples collected weekly allowed fecal pH to be determined (Figure 4c). Although there were some punctual differences at Day 7 for the PC0.2 diet and at the end of the study for PC0.8, no significant changes due to the nutritional interventions of cocoa were observed and a similar pattern was seen for pH.

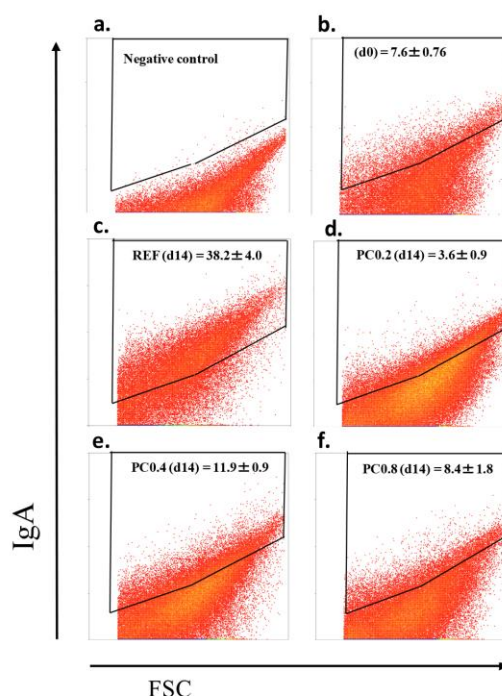
Figure 4. Representative biparametric cytogram showing the initial acquisition gate to select the bacterial population according to its forward- and side-scattered light (FSC/SSC) signal with the mean $\pm$ SEM of all groups at the baseline (a). Effect of cocoa polyphenol-enriched diets on total bacteria (b) and fecal pH (c) after two weeks of nutritional intervention. Results are expressed as mean $\pm$ SEM ($n = 6$). Statistical differences: δ $p < 0.05$ vs. initial values, * $p < 0.0167$ vs. REF, ϕ $p < 0.0167$ vs. PC0.2 and ω $p < 0.0167$ vs. PC0.4 in same day of study.



3.5. IgA-Coating Bacteria Analysis

IgA-coating bacteria were determined by flow cytometry using fecal samples collected before the start of the diet and at the end of the study (Figure 5). A non-stained sample was used as a negative control (Figure 5a). At the beginning, all groups showed a similar IgA-coating pattern (Figure 5b). The REF group showed an age-related increase in the percentage of IgA-coating bacteria from up to ~38% two weeks later (Figure 5c). This pattern was avoided by all cocoa polyphenol-enriched diets ($p < 0.0167$) (Figure 5d–f) with the down modulatory effect in animals that received the diet with a lower polyphenol content (PC0.2 group, $p < 0.0167$) (Figure 5d) being significantly higher.

Figure 5. Effects of cocoa polyphenol-enriched diets on percentage of fecal bacteria coated with IgA before and after 2 weeks of nutritional intervention. Biparametric cytogram corresponding to a negative fecal control (a). It combines morphological parameters of the bacterial population (FSC/SSC) and fluorescence for IgA-fluorescein isothiocyanate (FITC) which allow the threshold for the positive region to be established. Cytogram, which represents the percentage of IgA-coating bacteria at baseline for all groups (b). Cytograms for IgA-coating bacteria detection for REF (c), PC0.2 (d), PC0.4 (e) and PC0.8 (f) groups at the end of the study. Results are expressed as mean $\pm$ SEM ($n = 6$).



4. Discussion

In previous studies, cocoa-enriched diets have demonstrated their immunomodulatory role on rats' systemic and intestinal compartments. It is believed that this effect is mainly due to the polyphenols present in cocoa. The purpose of the present work was focused on investigating whether these effects are attributed exclusively to its polyphenol content or to other compounds present in cocoa. For that, Lewis rats were fed three cocoa diets formulated with two distinct cocoa raw materials differing in polyphenol dose and composition. The study lasted two weeks, because in previous studies, this period was enough to down-regulate intestinal IgA [20]. From the present results it emerges that all dietary

interventions with cocoa attenuated the humoral intestinal immune response, demonstrating for the first time the immunomodulatory effect of non-fermented-polyphenolic-enriched cocoa. Although all dietary interventions with cocoa attenuated the humoral intestinal immune response, it was achieved in a polyphenol-dose-independent manner suggesting that other compounds present in cocoa powder also play a role.

Previous reports concerning the effect of cocoa on the immune system *in vivo* have been developed using 2%–10% cocoa-enriched diets formulated with defatted cocoa containing 20–30 mg/g of polyphenols [19–21]. In the present study we used a conventional cocoa powder with 2% of polyphenols to elaborate the PC0.2 diet. We added 100 g of cocoa powder to 900 g of basal mix and the resulting diet contained 0.2% polyphenols in addition to the proteins (2.2%), carbohydrates (1.6%), lipids (1.1%) and fiber (3.5%) provided by cocoa. The present work includes the study of the effect of CocoaPure, which is a cocoa powder obtained by a modification of conventional cocoa powder processing, thus avoiding the loss and keeping the high amount of polyphenols present in the cocoa seed [26]. In the elaboration of the CocoaPure diets (PC0.4 and PC0.8), a small amount of cocoa powder was used to double or to quadruple the polyphenol content of the conventional 10% cocoa diet (PC0.2). As about 50% of CocoaPure corresponded to polyphenols, the PC0.8 diet contained only 0.23% cocoa proteins, 0.16% cocoa carbohydrates, 0.11% cocoa lipids and 0.35% cocoa fiber, whereas the PC0.4 diet contained half proportions of these nutrients. Therefore, in the present study, three cocoa diets with increasing proportions of polyphenols (0.2%–0.8%) and decreasing content of other cocoa compounds, such as fiber, were designed to dissect the effects of cocoa polyphenols on the immune system.

During the study, all the rats fed the cocoa polyphenol-enriched diets had similar chow intakes to the reference animals. However, the animals in the PC0.2 group had lower body weight increase than the reference group during the study. These results are similar to those previously shown in high-dose cocoa-fed rats [17,21]. In this regard, flavonoids seem to stimulate cellular energy [29,30], cocoa intake is capable of inhibiting the lipid digestion and absorption [31–33] or of reducing fat deposition [33–36]. However, in the present study, the conventional cocoa diet (PC0.2), which provided the lowest amount of polyphenols, was the diet with the strongest effect on body weight. This result suggests that other compounds present in cocoa, such as fiber might also contribute to the inhibitory potency of the polyphenols [37]. Additionally the kind of flavonoids (monomeric, oligomeric or polymeric flavanols) contained in cocoa materials could also play a role in this effect on body weight increase. Another possible mechanism involved in the cocoa's ability to reduce body weight and in lipid metabolism may be the polyphenols' effect on microbiota composition. Differential effects of gut bacteria on the efficiency of energy extraction from the diet and changes in the host metabolism of absorbed calories have been described [38,39] and, for example, a high *Firmicutes*-to-*Bacteroidetes* ratio might have an important role in obesity [39–41]. In this sense, previous studies have demonstrated that cocoa reduces the *Clostridium* and *Staphylococcus* genera belonging to the *Firmicutes* phylum in the gut microbiota [19]. Although in the present study changes in microbiota composition were not evaluated, a reduction in the proportion of bacteria in feces was found in all the animals fed with cocoa polyphenol diets whereas no significant change in fecal pH was observed. This effect could be attributed to the antimicrobial activity of polyphenols, which has been reported both *in vitro* [42–44] and in experimental animals [45,46]. Focusing on cocoa polyphenols,

our findings regarding the decreasing percentage of total bacteria seem to be in accordance with a previous study showing that polyphenols present in cocoa can produce an inhibitory effect on the growth of certain gut microbiota in rats [19]. Nevertheless, other studies have shown the ability of certain flavonoids present in cocoa to promote the growth of selected bacteria genera [47,48]. In any case, further studies should be carried out to look in detail at the effects of cocoa polyphenols on the microbiota composition and metabolism.

Regarding the systemic humoral response, previous studies have shown that immunoglobulin production is attenuated by experimental cocoa-enriched diets. Thus, splenocytes from young rats fed cocoa had a lower capacity to produce IgG, IgM and IgA and cocoa-fed rats had decreased serum IgG, IgM and IgA concentrations [17,18]. However, in the present study carried out in older rats for two weeks, all cocoa polyphenol diets tested had a tendency to lower the age-increasing pattern observed in the reference group but without significant differences and lacking a polyphenol dose-dependent effect. Therefore, it can be concluded that when the cocoa diet begins later in life and it lasts just two weeks, the immunomodulating effect is not as clear as in early age. We cannot discard the idea that a longer exposure to cocoa diet might have a more significant effect. A lack of or low effect has also been described when the cocoa proportion included in the diet was lower [28].

Contrary to what happened in the systemic compartment, the suppressing effect of cocoa polyphenols on antibody concentration was higher in the intestinal compartment. Our results from a defatted conventional cocoa diet (PC0.2) confirmed the down-modulatory effect on intestinal IgA content shown in previous studies, including varied proportions of cocoa diets (2%–10%), different ages at the beginning of the dietary intervention (3–6 weeks old) and length of diet (3–9 weeks) [17,18,28]. In the same way, the PC0.4 and PC0.8 diets, with a higher polyphenol content than the above-mentioned diet, also drastically decreased the IgA production but without a polyphenol dose-dependent effect. The effect of cocoa on intestinal IgA could be due, as has been described, to the influence of cocoa on genes related to Th maturation, Th-B cell interactions, and IgA+ B cell gut homing, among others [18,28]. Regardless of the cocoa-induced mechanisms, antibody-attenuating effects have also been previously described for different dietary polyphenols such as genistein [49], luteolin [50] and apigenin [51] in healthy or hypersensitivity animal models.

With regards to IgA-coating bacteria, similar results to the intestinal IgA content were found. The lowering effect of a defatted cocoa polyphenol diet (PC0.2) on IgA-coating bacteria in old animals agrees with previous studies performed on younger animals [19]. Again, there was a lack of correlation between the total polyphenol content in the diet and IgA-modulating effects, which means that cocoa diets based on higher flavonoids/lower cocoa fiber (CocoaPure) displayed similar effects. Therefore, the intestinal IgA down-modulatory effects described here could be partially attributed to cocoa polyphenols, because all diets, even with a very low cocoa fiber content (PC0.4 and PC0.8), were able to modulate the intestinal IgA production. However, a particular effect according to the kind of flavanols found on conventional or unfermented cocoa matter cannot be discarded.

In any case, a high whole cocoa or cocoa polyphenol intake might play an immunoregulatory role that could be beneficial on the hypersensitivity status. The immunomodulatory ability of cocoa can be especially significant in diseases involving a dysregulation of intestinal antibody responses such as celiac disease or food allergies.

5. Conclusions

The immunomodulatory effects of diets containing high cocoa could be partially attributed to its polyphenol content. However, other cocoa compounds and the particular polyphenol composition could also be key factors in this effect. Further studies evaluating dissected components of cocoa, such as individual polyphenols, fiber, theobromine, *etc.* should be carried out in order to evaluate whether it is possible to reproduce, at least partially, the previously and herein described cocoa effects on the immune system.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Article 3

“Impact of cocoa polyphenol extracts on the immune system and microbiota in two strains of young rats”

Malen Massot-Cladera, Mar Abril-Gil, Sandra Torres,
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Els resultats del present article han permès la presentació de la comunicació:

- *Influence of different sources of cocoa polyphenols on intestinal microbiota and IgA of Wistar rats*. IV Workshop Probióticos, Prebióticos y Salud. Evidencia Científica, Madrid (Espanya), Gener 2013. Comunicació oral. Publicada en *Nutrición Hospitalaria* (2013), 28 (Suppl 1), S26.

RESUM

Objectiu: Avaluar si els efectes causats per la dieta rica en cacau sobre la microbiota i el teixit limfoide intestinal en rata depenen del seu contingut en flavonoides i de la soca de rata utilitzada.

Material i mètodes: L'estudi es va realitzar en rates de 3 setmanes d'edat de les soques Wistar i Brown Norway, les quals van ser alimentades durant quatre setmanes amb una dieta estàndard o bé amb dietes amb un contingut creixent de flavonoides de cacau (0,2% a partir d'un extracte de cacau convencional fermentat; 0,4% partint d'un extracte de cacau elaborat amb llavors de cacau blanquejades sense fermentar ni torrar o un 0,8% pel qual es va usar un extracte d'etanol procedent de les llavors de cacau blanquejades sense fermentar ni torrar). Abans i després de la intervenció nutricional es va caracteritzar la composició de la microbiota intestinal per la tècnica de FISH-FCM així com determinar el percentatge de bacteris units a IgA. Per ELISA es va quantificar setmanalment la concentració sèrica d'IgG1, IgG2a, IgG2b i IgA, la concentració fecal d'IgA i al final de l'estudi la concentració d'IgA en rentat intestinal. També es va realitzar la caracterització limfocitària en ganglis limfàtics mesentèrics i plaques de Peyer.

Resultats: En alguns aspectes, les rates de la soca Wistar van ser més sensibles als efectes de les dietes enriquides amb cacau que la soca Brown Norway. Totes les dietes experimentals van disminuir la concentració sèrica d'IgG1 i IgG2b, independentment de la quantitat de flavonoides de la dieta. A més, les dietes amb un contingut de 0,2% i 0,8% de polifenols van inhibir el creixement dels gèneres

Streptococcus spp. i *Lactobacillus* spp., el percentatge de bacteris fecals units a IgA així com la secreció d'IgA intestinal. La dieta amb el menor contingut de flavonoides (0,2%) va incrementar la proporció de limfòcits NK+ en plaques de Peyer.

Conclusió: Alguns dels efectes immunomoduladors associats a la ingesta de dietes enriquides amb cacau, els quals depenen de la soca de rata utilitzada, són parcialment atribuïts al seu contingut en flavonoides. No obstant, altres components del cacau contribueixen també a aquests efectes.



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Impact of cocoa polyphenol extracts on the immune system and microbiota in two strains of young rats

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Abstract

A diet containing 10% cocoa, a rich source of polyphenols and fibre, is able to modify intestinal immune status as well as microbiota composition. The present study was aimed at investigating whether cocoa flavonoid content is uniquely responsible for these modulatory effects of cocoa, and to establish whether these effects depend on the rat strain. To this end, 3-week-old Wistar and Brown Norway rats were fed, for 4 weeks, either a standard diet or the following three isoenergetic diets containing increasing proportions of cocoa flavonoids from different sources: one with 0.2% polyphenols (from conventional defatted cocoa), and two others with 0.4 and 0.8% polyphenols (from non-fermented cocoa, very rich in polyphenols). Serum Ig concentrations, faecal IgA levels, microbiota composition and IgA-coating bacterial proportion were evaluated at the beginning and at the end of the study. After the nutritional intervention, the composition of lymphocytes in Peyer's patches and mesenteric lymph nodes was evaluated. In some respects, the Wistar strain was more sensitive to the impact of the cocoa diets than the Brown Norway strain. After 4 weeks of dietary intervention, similar modulatory effects of the diets containing 0.2 and 0.8% polyphenols on mucosal IgA levels and microbiota composition were found, although the 0.2% diet, with a higher proportion of theobromine and fibre, had more impact, suggesting that polyphenols are not the only components involved in such effects.

Key words: Cocoa: Immune system: Intestinal immune system: Immunoglobulins: Microbiota

Cocoa powder is a product derived from the beans of the *Theobroma cacao* plant that is considered a rich source of fibre (26–40%), proteins (15–20%), carbohydrates (about 15%) and lipids (10–24%). It also provides minerals and vitamins⁽¹⁾. It has become a subject of increasing interest due to its high content of flavonoids, compounds with antioxidant activity. Cocoa mainly contains the monomers (–)-epicatechin and catechin as well as various polymers derived from both of these compounds, known as procyanidins. Methylxanthines have also been identified in cocoa powder⁽²⁾.

To date, there have not been many studies addressing the dietary effects of polyphenols on gut-associated lymphoid tissue (GALT)⁽³⁾, and there have been even fewer studies about the effects of cocoa polyphenols on the functionality or composition of GALT in animals⁽⁴⁾. GALT constitutes the most extensive and complex part of the immune system in the body. Structurally, it is composed of diffused cells, considered to be the effector site of the immune response

(intraepithelial and lamina propria lymphocytes), and organised lymphoid structures considered as the inductive site of the intestinal immune response (Peyer's patches (PP), isolated follicles and mesenteric lymph nodes (MLN)). Previous studies⁽⁵⁾ have reported that the ratios of T:B lymphocytes and Th:Tc cell subsets in PP and MLN are modified after a high intake of cocoa polyphenols in young rats.

IgA is the most abundant Ig isotype produced in the body, and it is estimated that about 80% of all IgA antibody-secreting cells reside in the gut mucosa⁽⁶⁾. This antibody constitutes the first line of non-inflammatory immune protection at mucosal surfaces by neutralising microbial pathogens and exotoxins and by interacting with innocuous dietary antigens and commensal microbes^(7,8). The capacity to modulate secretory IgA (sIgA) levels by different polyphenols has been described previously^(9,10). Focusing on cocoa polyphenols, their effects on sIgA levels have been studied in different experimental designs using rats that have confirmed that polyphenols

Abbreviations: FBS, fetal bovine serum; FCM, flow cytometry; FSC, forward-scatter characteristics; GALT, gut-associated lymphoid tissue; mAb, monoclonal antibodies; MLN, mesenteric lymph nodes; NK, natural killer; PC0.2, diet containing 0.2% polyphenol from conventional cocoa; PC0.4, diet containing 0.4% polyphenol from non-fermented cocoa rich in polyphenols; PC0.8, diet containing 0.8% polyphenol from non-fermented cocoa rich in polyphenols; PP, Peyer's patches; REF, reference; RPMI, Roswell Park Memorial Institute; sIgA, secretory IgA; SSC, side-scatter characteristics; TCR, T-cell receptor.

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reduce sIgA protein and sIgA gene expression, which leads to a different pattern of IgA-coating bacteria^(5,11,12).

The crosstalk between GALT and the microbiota has been shown to be critical for mucosal tissue homeostasis, maintenance of mucosal barrier function and protection against infectious and inflammatory diseases occurring at mucosal sites. The interaction is a complex and tightly regulated process that distinguishes invasive pathogens and innocuous antigens from food and commensal bacteria⁽¹³⁾. During the metabolism of food and xenobiotics such as polyphenols, the host and its gut microbiota co-produce a large number of small molecules, many of which play critical roles in this crosstalk⁽¹³⁾. Some of these signals from commensal intestinal bacteria continuously support the maintenance of mucosal lymphoid tissue⁽¹⁴⁾.

It has also been also observed that polyphenol-rich dietary sources have an influence on the gut microbiota^(15,16). In relation to this, it has recently been demonstrated that high intakes of polyphenols from cocoa are able to produce changes in microbiota composition in animals⁽¹²⁾ and in humans⁽¹⁷⁾. These changes in the microbiota could be partly responsible for the changes observed in GALT. The aim of the present study was to ascertain whether cocoa flavonoid content is uniquely responsible for the modulatory effects of cocoa on the immune system and microbiota as reported in previous studies, and at the same time, to evaluate whether these changes are rat strain dependent. To achieve this objective, two rat strains with a different immune response pattern were fed diets containing the following three different proportions of polyphenols from different sources of cocoa: one diet contained a conventional cocoa powder and the other two diets contained flavonoid-enriched cocoa powders obtained from non-fermented, blanched and non-roasted cocoa beans.

Materials and methods

Diets

A conventional natural Forastero cocoa powder (Nutrexpa S.L.) containing 21–20 mg/g of polyphenols was used to elaborate a diet with 0.2% polyphenol (PC0.2). A cocoa polyphenol-enriched extract obtained from non-fermented, blanched and non-roasted cocoa beans and containing 160.3 mg/g was used to elaborate a diet with 0.4% polyphenol (PC0.4). Moreover, a 0.8% polyphenol diet (PC0.8) was elaborated using an ethanol extract from the same non-fermented, blanched and non-roasted cocoa beans and containing 510 mg/g of polyphenols. The polyphenol characterisation of the three cocoa powders is summarised in Table 1.

The three cocoa diets were elaborated from a standard AIN-93M formula by subtracting the amount of carbohydrates, proteins, lipids and insoluble fibre provided by each cocoa source. The resulting cocoa polyphenol-rich chow diets were isoenergetic and had the same proportion of macronutrients (carbohydrates, proteins and lipids) and insoluble fibre as the standard diet, and provided the corresponding quantity of particular compounds such as cocoa soluble and insoluble fibre and cocoa polyphenols (Table 1).

Table 1. Composition of nutrients and polyphenols provided by the three cocoa powders in the experimental diets (g/kg diet)

Components	REF (AIN-93M, g/kg)	PC0.2 (g/kg)	PC0.4 (g/kg)	PC0.8 (g/kg)
Casein	121.8	97.1	115.4	119.5
L-Cystine	1.8	1.4	1.8	1.8
Maize starch	419.1	421.0	415.7	417.5
Maltodextrin	147.3	116.8	147.3	147.3
Sucrose	117.0	108.7	117.0	117.0
Soyabean oil	40.0	26.2	37.7	38.9
Cellulose	50.0	24.5	42.9	46.5
Minerals	27.0	27.8	27.0	27.0
Vitamins	2.0	7.2	2.0	2.0
Choline bitartrate	2.5	2.0	2.5	2.5
tert-Butylhydroquinone	0.008	0.006	0.008	0.008
Cocoa powder	–	100	25	17.4
Protein	–	22	4.61	2.26
Carbohydrate	–	16	3.35	1.64
Lipid	–	11	2.30	1.12
Fibre	–	34	7.12	3.5
Insoluble	–	25.5	5.34	2.63
Soluble	–	8.5	1.78	0.88
Total polyphenols*	–	2.12	4	8.88
Catechin	–	0.073	0.080	0.097
Epicatechin	–	0.204	0.500	1.379
Isoquercetin	–	0.0053	ND	ND
Quercetin	–	0.0029	ND	ND
Procyanidin B1	–	ND	0.045	0.254
Procyanidin B2	–	0.167	0.310	0.713
Total procyanidins	–	ND	3.200	7.795

REF, reference diet; PC0.2, diet containing 0.2% polyphenol from conventional cocoa; PC0.4, diet containing 0.4% polyphenol from non-fermented cocoa rich in polyphenols; PC0.8, diet containing 0.8% polyphenol from non-fermented cocoa rich in polyphenols; ND, not determined.

*Total polyphenol compounds were estimated by the Folin–Ciocalteu spectrophotometric method. The polyphenol profile was identified and quantified using a reversed-phase HPLC coupled to a diode array detector, and the procyanidin profile (from monomers to >decamers) by normal-phase HPLC coupled to MS with fluorescence detection.

Animals

Female Wistar and Brown Norway rats (3 weeks old) were obtained from Harlan and housed in cages under conditions of controlled temperature and humidity in a 12 h light–12 h dark cycle. After an acclimatisation period of 1 week, the rats were randomly distributed into four dietary groups (*n* 6 per group) for each strain. The reference (REF) groups were fed with a standard AIN-93M diet (Harlan), and the cocoa groups received the PC0.2, PC0.4 or PC0.8 diets. The diets lasted for 4 weeks.

The rats were given free access to water and chow *ad libitum*. Body weight and food intake were monitored throughout the experiment. The 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 (reference no. 358/12).

Sample collection and processing

Blood samples were collected weekly from the beginning of the diet until the end of the study, and after centrifugation, serum was separated and kept at –20°C until Ig quantification.



by ELISA. Faecal samples were collected at the same time points and were processed for Ig quantification and bacterial characterisation. For this purpose, faecal homogenates were obtained, as described previously^(5,12), and frozen at -20°C until analysis.

After 4 weeks of dietary intervention, the rats were anaesthetised intramuscularly with ketamine (90 mg/kg; Merial Laboratories, S.A., Barcelona) and xylazine (10 mg/kg; Bayer). The small intestine and MLN were collected. The small intestine was divided into two halves and carefully rinsed with cold 0.9% NaCl solution in order to remove faecal content. The distal half was opened lengthwise and visible PP were excised. The intestinal tissue without PP was used to obtain the gut wash for IgA quantification by ELISA, as established previously in our laboratory⁽⁵⁾, while the proximal half was rejected. MLN and PP were used to isolate lymphocytes and to perform their phenotypic characterisation.

Lymphocyte isolation from Peyer's patches and mesenteric lymph nodes

The excised PP were incubated in a shaker with Roswell Park Memorial Institute (RPMI) medium containing 1 mM-dithiothreitol (Sigma-Aldrich) for 5 min at 37°C . Thereafter, PP were washed with RPMI medium and passed through a $40\text{ }\mu\text{m}$ mesh (BD Biosciences). The filtered cell suspension was resuspended in RPMI medium containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and 0.05 mM-2- β -mercaptoethanol (Merck). Cell counting and viability were determined by double staining with acridine orange and ethidium bromide (Sigma-Aldrich), followed by fluorescence light microscopy analysis using a Zeiss microscope (Axioskop MC80; Zeiss).

MLN cell suspension was obtained by passing the tissue through a sterile $40\text{ }\mu\text{m}$ mesh and incubating on ice to remove tissue debris by sedimentation for 10 min. Then, the suspensions were centrifuged (500 g, 5 min, 4°C), and the cells were resuspended in RPMI medium containing 10% FBS and 0.05 mM-2- β -mercaptoethanol. Cell counting and viability were also determined.

Lymphocyte immunofluorescence staining and flow cytometry analysis

PP and MLN lymphocytes were stained with anti-rat monoclonal antibodies (mAb) followed by flow cytometry (FCM) analysis. Mouse anti-rat mAb conjugated to fluorescein isothiocyanate, phycoerythrin or peridinin chlorophyll protein used herein included the following: anti-CD4 (OX-35); anti-T-cell receptor (TCR)- $\alpha\beta$ (R73); anti-CD8 α (OX-8); anti-CD25 (OX-39); anti-CD-161a (10/78); anti-CD45RA (OX-33) (BD Biosciences); anti-IgA (Thermo Fisher Scientific). Cells (2×10^5) were labelled with saturating concentrations of conjugated mAb in PBS (pH 7.2) containing 1% FBS and 0.09% sodium azide (Merck) for 30 min at 4°C in the dark. Negative control staining using isotype-matched mAb was included for each sample. After washing with PBS, the cells were fixed with 0.5% *p*-formaldehyde (Merck) and stored at 4°C in the dark. Analyses were performed

using an FC 500 Series Flow Cytometer (Beckman Coulter, Inc.), and data were assessed by the FlowJo version 7.6.5 software (Tree Star, Inc.). Lymphocyte populations were defined as follows: Th (TCR $\alpha\beta^+$ CD4 $^+$) cells; Tc (TCR $\alpha\beta^+$ CD8 $^+$) cells; natural killer (NK) T (TCR $\alpha\beta^+$ NK $^+$) cells; NK (TCR $\alpha\beta^-$ NK $^+$) cells; B (CD45RA $^+$ CD4 $^-$) cells. Results are expressed as percentages of positive cells in the lymphocyte population selected according to their forward-scatter characteristics (FSC) and side-scatter characteristics (SSC), as reported previously⁽⁵⁾.

Ig quantification in serum, faeces and gut washes by ELISA

The amounts of sIgA in gut washes and faeces, and serum IgA, IgG1, IgG2a, IgG2b concentrations were quantified by ELISA, as described previously⁽¹¹⁾. In brief, ninety-six-well polystyrene plates (Nunc MaxiSorp) were coated with anti-rat IgA, IgG1, IgG2a or IgG2b (2 mg/ml in PBS; BD Biosciences) and incubated overnight. After blocking with PBS containing 1% bovine serum albumin (1 h), the plates were washed and appropriate diluted samples and standard dilutions were added (3 h). After washing, biotin-conjugated anti-rat IgA, IgG1, IgG2a or IgG2b mAb were added (1 mg/ml; BD Biosciences) and after 2 h, peroxidase-conjugated ExtrAvidin (4 mg/ml; Sigma-Aldrich) was incubated for 30 min. Finally, the enzyme-substrate reaction was developed and stopped by adding 3M-H $_2$ SO $_4$ (Merck). Absorbance was measured in a microplate photometer (LabSystems Multiskan) at 492 nm. Data were interpolated using ASCENT version 2.6 software (Thermo Fisher Scientific) into the standard curves, and expressed as $\mu\text{g/ml}$ for IgG1, IgG2a and IgG2b and as ng/ml for IgA results.

Fluorescence in situ hybridisation of gut microbiota

The bacterial groups present in the faeces were characterised using the fluorescence *in situ* hybridisation technique with group- or genus-specific fluorochrome-conjugated probes (Sigma-Aldrich), as established previously in our laboratory⁽¹²⁾ with some modifications. The specific probes and controls used in the present study, as well as the hybridisation conditions, are summarised in Table 2. The EUB338 probe labelled at the 5'-end with indocarbocyanine fluorochrome (Cy3) was used to label total bacteria, whereas the NON-EUB338-indocarbocyanine fluorochrome (Cy5) probe was applied as a negative control in order to eliminate background fluorescence. Briefly, fixed cell suspensions were incubated in the presence of each fluorescent probe (50 ng/ μl) in pre-heated hybridisation buffer (10 mM-Tris-HCl, pH 8, 0.9 M-NaCl and 0.1% SDS) in a 0.2 ml tube in a thermocycler at appropriate temperature (Table 2) for 2 h in the dark. In the case of *Lactobacillus*, samples were washed with Tris-EDTA buffer and permeabilised with 0.25 mg/ml of lysozyme (Serva) for 1 h at room temperature before the hybridisation process with the same hybridisation buffer but adding 30% formamide. Hybridised cell suspensions were then washed with hybridisation buffer with 0.001% SDS for 30 min at the same temperature as hybridisation was done in order to eliminate non-specific binding of the probes. The hybridised cells

Table 2. Oligonucleotide probes and hybridisation conditions used in the analysis of intestinal bacteria by fluorescence *in situ* hybridisation–flow cytometry analysis

Target bacterial groups	Probe	Sequence (5'–3')	Hybridisation conditions (°C)	References
Domain bacteria	EUB338-I	GCTGCCTCCCGTAGGAGT	46	Daims <i>et al.</i> ⁽³⁶⁾
	EUB338-II	GCAGCCACCCGTAGGTGT	46	
	EUB338-III	GCTGCCACCCGTAGGTGT	46	
<i>Bacteroides/Prevotella</i>	Bac303	CCAATGTGGGGGACCTT	46	Manz <i>et al.</i> ⁽³⁷⁾
<i>Bifidobacterium</i>	Bif164	CATCCGGCATTACACCC	50	Langendijk <i>et al.</i> ⁽³⁸⁾
<i>Lactobacillus/Enterococcus</i>	Lab158	GGTATTAGCAYCTGTTTCCA	50 (lysozyme)	Harmsen <i>et al.</i> ⁽³⁹⁾
<i>Escherichia coli</i>	Ec1531	CACCGTAGTGCCTCGTCATCA	37	Poulsen <i>et al.</i> ⁽⁴⁰⁾
<i>Clostridium histolyticum/C. Perfringens</i>	CHis150	TTATGCGGTATTAATCTYCCTT	50	Franks <i>et al.</i> ⁽⁴¹⁾
<i>Staphylococcus</i> spp.	Staphy	TCCTCCATATCTCTGCGC	46	Trebesius <i>et al.</i> ⁽⁴²⁾
<i>Streptococcus</i> spp.	Strept	CACCTCTCCCTTCTGCAC	46	Trebesius <i>et al.</i> ⁽⁴²⁾
Negative control	NON-EUB	ACTCCTACGGGAGGAGCAGC	46	Wallner <i>et al.</i> ⁽⁴³⁾

were collected by centrifugation (12 000 *g*, 5 min), washed twice in PBS, resuspended for FCM analysis and kept in the dark at 4°C until analysis.

Ig-coating bacterial staining

Faecal homogenates were diluted in 1% (v/v) FBS/PBS and centrifuged (8000 *g*, 5 min, 4°C). The resulting pellet was resuspended in 1% (v/v) FBS/PBS containing fluorescein isothiocyanate–anti-rat IgA antibodies (Abcam). Each mixture was incubated for 30 min in the dark, washed twice with PBS (8000 *g*, 5 min) and resuspended in PBS until analysis. A non-stained mixture of each sample was used as the control. To label total bacteria, the samples were mixed with propidium iodide (Sigma-Aldrich) before FCM analysis.

Flow cytometry bacterial analysis

For bacterial characterisation and IgA-coated bacterial determination, FCM analysis was performed using a FACSAria SORP sorter (BD Biosciences), as described previously⁽¹²⁾. Bacterial morphology was selected according to their FSC/SSC signal. Subsequently, bacteria hybridised with group- or genus-specific probes conjugated to Cy5 or Cy3 were detected by combining the FSC signal with fluorescence detectors. FCM parameters were adjusted for bacterial counts, and bacterial concentrations of the samples were adjusted to obtain particle counts of 100–1000 events/s, and the events were recorded for 60 s in list mode files. Commercial Flow-Check™ Fluorospheres (Beckman Coulter, Inc.) were used to determine total counts combined with the EUB338-Cy3 probe. The proportion of IgA-coating bacteria was expressed as positive fluorescein isothiocyanate counts within the propidium iodide-positive bacteria present in the above FSC/SSC morphology gate. Both analyses were performed using the FlowJo version 7.6.5 software (Tree Star, Inc.).

The results of microbiota composition are expressed as the percentage of each genus with respect to the total bacterial population hybridised with EUB probes in each sample. Similarly, the results of IgA-coating bacteria are expressed as the percentage of bacterial cells labelled with anti-rat IgA with respect to the total cell population labelled with propidium iodide.

Statistical analysis

Statistical analysis was performed using the software package SPSS 18.0 (SPSS, Inc.). Levene's and Kolmogorov–Smirnov tests were applied to assess variance equality and normal distribution, respectively. Conventional one-way ANOVA was performed when normal distribution and equality of variance existed. The Bonferroni test was applied when specific cocoa intake had a significant effect on the dependent variable. Non-parametric Mann–Whitney *U* and Wilcoxon tests were used in order to assess significance for independent and related samples, respectively. Significant differences were established at $P < 0.05$.

Results

Effects of cocoa polyphenols on body weight and chow intake

During the study, although the body weight of Brown Norway rats was lower than that of Wistar rats, its slope was similar among the dietary groups in every rat strain with the exception of those that received the diet with lower polyphenol content (PC0.2 group). In Wistar rats, the PC0.2 diet produced a statistically significant lower increase than that in the REF groups during the study ($P < 0.05$; Fig. 1(a)), whereas in Brown Norway rats, the significance appeared only at the end of the study ($P < 0.05$; Fig. 1(b)). This effect was not related to lower chow intake since it was similar throughout the study among all the experimental groups in both rat strains (data not shown).

Effects of cocoa polyphenols on serum IgG1, IgG2a and IgG2b concentrations

Serum concentrations of IgG1, IgG2a and IgG2b from all the experimental groups throughout the study in both strains are summarised in Fig. 2. Wistar rats showed an age-increasing pattern in the second half of the study in the three IgG isotype concentrations (Fig. 2(a)–(c)). With regard to the nutritional intervention, the diets containing cocoa polyphenols partially reduced this increase in comparison with the REF diet, being statistically significant ($P < 0.05$) for IgG1 at day 21 (Fig. 2(a)) and for IgG2b at day 14 in the PC0.2 group and at the end

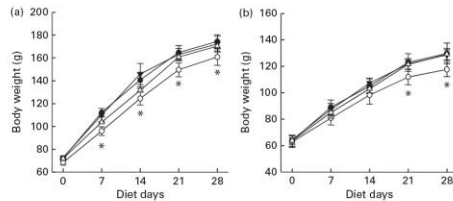


Fig. 1. Effects of cocoa polyphenol-enriched diets on body weight in (a) Wistar rats and (b) Brown Norway rats. Values are means (n 6), with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference group ($\bullet$); ($P < 0.05$) on the same day of the study. $\circ$, Diet containing 0.2% polyphenol from conventional cocoa; $\blacktriangle$, diet containing 0.4% polyphenol from non-fermented cocoa rich in polyphenols; $\triangle$, diet containing 0.8% polyphenol from non-fermented cocoa rich in polyphenols.

of the study after the intake of the three diets (Fig. 2(c)). No significant effects were observed for IgG2a.

Brown Norway rats showed higher IgG concentrations than Wistar rats, and at the end of the study, their values were 9-fold the IgG1 concentration (Fig. 2(d)), 3-fold the IgG2a concentration (Fig. 2(e)) and 2-fold the IgG2b concentration (Fig. 2(f)) compared with those found in Wistar rats. No significant effects were observed after the nutritional interventions with any of the cocoa polyphenol-enriched diets in this rat strain with the exception of a punctual increase in IgG1 concentration by the PC0-2 diet.

Effects of cocoa polyphenols on serum, faecal and gut wash IgA concentration

Serum obtained during the period of the study was also used to quantify IgA. There were no effects on IgA concentration in Wistar rats after any nutritional interventions (Fig. 3(a)). On the contrary, Brown Norway rats fed with cocoa polyphenols decreased serum IgA concentration after 3 weeks of diets, the concentration being significantly lower than the REF groups ($P < 0.05$), though at the end of the study, the levels were similar among all the dietary groups (Fig. 3(b)).

Intestinal IgA concentration was also quantified in the faeces and gut washes. Faecal IgA concentration quickly increased over the study period in REF Wistar rats. This age-dependent increase was partially but significantly avoided by all cocoa polyphenol-enriched diets 14 d after the nutritional intervention ($P < 0.05$). Nevertheless, the inhibitory effect on faecal IgA remained significant only in rats that were fed with the PC0-2 and PC0-8 diets at the end of the study (Fig. 3(c)). The age-dependent increase in faecal IgA concentration observed in the Wistar strain was also observed in REF Brown Norway rats for the first 3 weeks of the study. The three polyphenol-enriched diets partially avoided such an increase ($P < 0.05$). After 1 week, faecal IgA concentration was similar among the dietary groups (Fig. 3(d)).

The results obtained for gut wash IgA at the end of the study were similar in both strains (Fig. 3(e) and (f)). All the three experimental diets reduced their concentrations, but they

were only statistically significant in rats fed with the PC0-2 and PC0-8 diets ($P < 0.05$), which showed up to four to five times lower IgA concentrations in gut washes.

Effects of cocoa polyphenols on lymphocyte composition in mesenteric lymph nodes and Peyer's patches

The percentages of B, Th, Tc, NK and NKT cells in MLN and PP for both strains and each cocoa polyphenol-enriched diet are summarised in Fig. 4.

Although no significant effects were observed in any of the studied MLN cell populations from Wistar and Brown Norway rats after cocoa polyphenol-enriched diets, the PC0-8 diet tended to reduce Th proportion and at the same time tended to increase B cells (Fig. 4(a) and (b)).

With regard to the PP lymphocyte population, all three polyphenol-enriched diets have a tendency to up-regulate the NK proportion in both studied strains, but only the PC0-2 diet significantly increased its proportion in Wistar rats ($P < 0.05$; Fig. 4(c) and (d)). Moreover, all diets tended to reduce the proportion of both Th and Tc cells with an increase in the proportion of B cells in Wistar rats. On the contrary, in Brown Norway rats, a tendency to increase the proportion of B cells while decreasing that of Th cells was only observed for the PC0-4 diet (Fig. 4(d)).

Effects of cocoa polyphenols on gut microbiota composition

As the Wistar strain showed a higher impact of the cocoa diets than the Brown Norway strain on faecal IgA concentration at the end of the study (Fig. 3(c) and (d)), the following determinations were only performed in Wistar rats.

The composition of faecal microbiota in Wistar rats was characterised by the FCM-fluorescence *in situ* hybridisation technique before the start of the diet and at the end of the study in all dietary groups (Table 3). All groups showed a similar microbiota composition pattern at baseline.

The proportion of *Bacteroides*, *Bifidobacterium*, *Streptococcus* and *Staphylococcus* increased by age in the REF groups ($P < 0.05$). With regard to the nutritional intervention, all the three cocoa polyphenol-enriched diets led to significant differences in microbiota composition. After 4 weeks of nutritional intervention, the rats fed the PC0-2 diet showed a significant decrease in the total counts of bacteria compared with their baseline values ($P < 0.05$) and with the REF diet ($P < 0.05$), and a lower proportion of *Lactobacillus* with respect to their baseline values ($P < 0.05$). This effect on *Lactobacillus* was also observed in the PC0-4 and PC0-8 groups. The age-dependent increase in *Staphylococcus* genera was significantly lower after the intake of the PC0-2 diet than after the intake of the REF diet ($P < 0.05$). In contrast, the increase in the proportion of *Streptococcus* by age was inhibited by the PC0-2 and PC0-8 diets ($P < 0.05$).

Effects of cocoa polyphenols on IgA-coating bacteria

The percentage of IgA-coating bacteria was determined by FCM according to their FSC/SSC signal using faecal samples collected

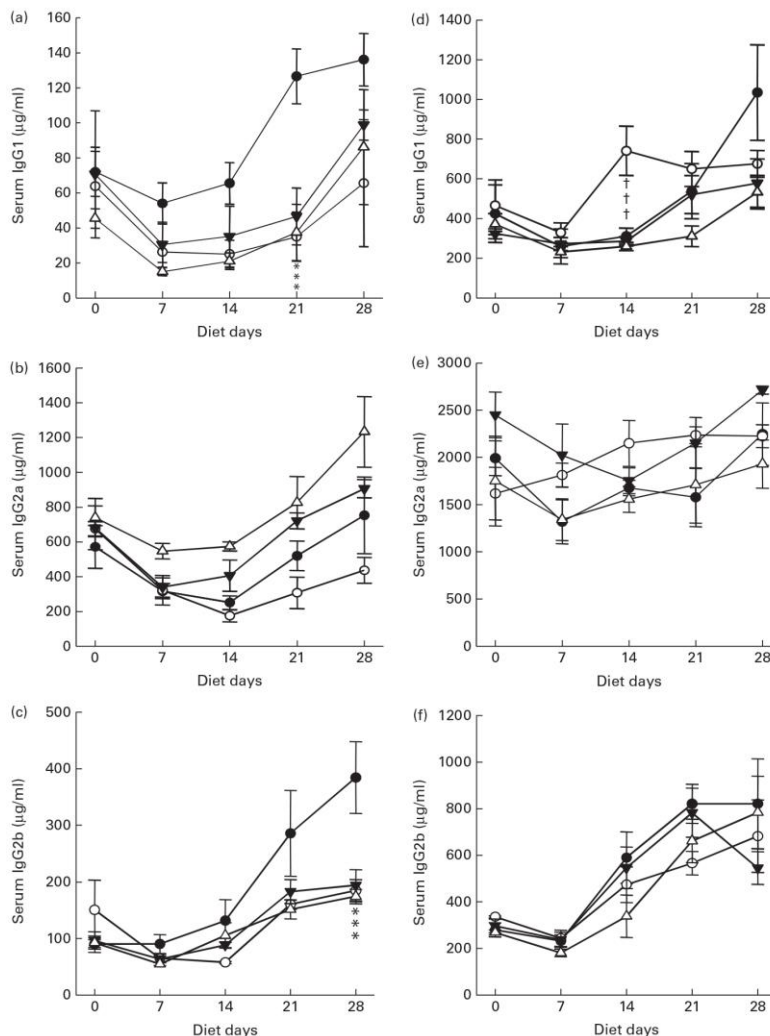


Fig. 2. Effects of cocoa polyphenol-enriched diets on serum IgG1 (a, d), IgG2a (b, e) and IgG2b isotypes (c, f) in (a–c) Wistar rats and (d–f) Brown Norway rats. Values are means (n 6), with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference group (REF; $P < 0.05$) on the same day of the study. †Mean value was significantly different from that of the group consuming the diet containing 0.2% polyphenol from conventional cocoa (CON; $P < 0.05$) on the same day of the study. —●—, Diet containing 0.4% polyphenol from non-fermented cocoa rich in polyphenols; —○—, diet containing 0.8% polyphenol from non-fermented cocoa rich in polyphenols.

before the start of the diet and at the end of the study (Fig. 5(a)). At the beginning of the study, all groups showed similar IgA-coating patterns (Fig. 5(b)). This proportion decreased by age up to 10% in the REF groups at day 28. The groups that received

the PC0-2 and PC0-8 diets also had a decreased percentage of IgA-coating bacteria with respect to those found at baseline ($P < 0.05$), with the reduction by the PC0-2 diet being stronger than that by the REF diet ($P < 0.05$; Fig. 5(c)).



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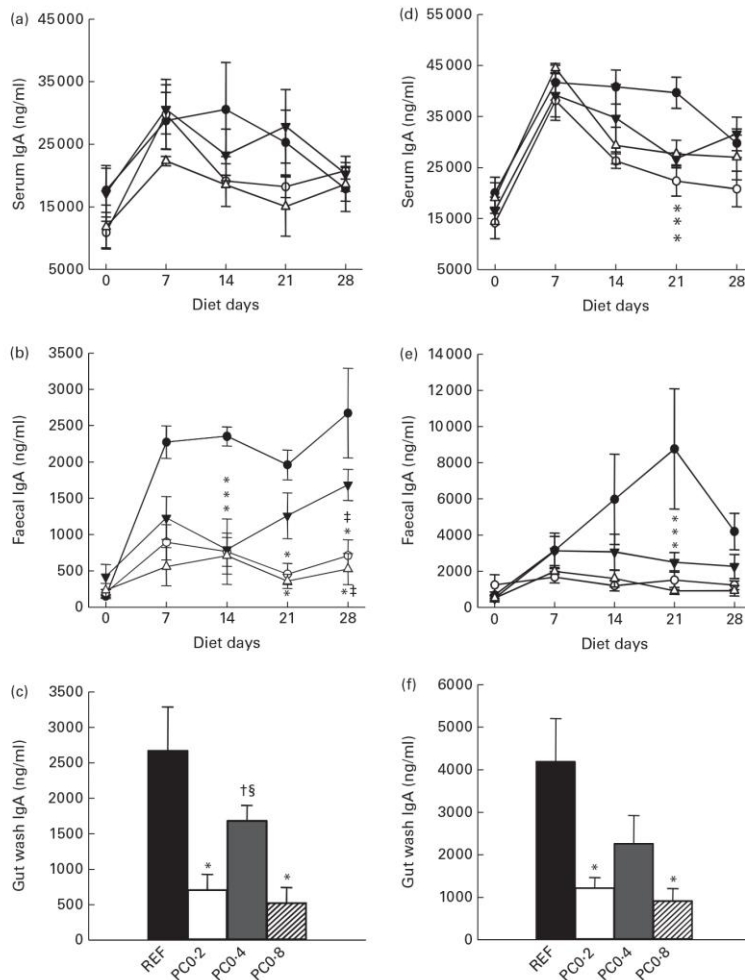


Fig. 3. Effects of cocoa polyphenol-enriched diets on systemic and intestinal IgA concentrations. Systemic IgA concentration was measured in serum samples from (a) Wistar rats and (d) Brown Norway rats. Intestinal IgA concentration was quantified in faecal samples (b, e) and in small-intestinal washes (c, f) from Wistar rats (b, c) and Brown Norway rats (e, f) at day 28. Values are means (n 6), with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference (REF) groups (●; $P < 0.05$) on the same day of the study. †Mean value was significantly different from that of the group consuming the diet containing 0.2% polyphenol from conventional cocoa (PC0.2, ○; $P < 0.05$) on the same day of the study. ‡Mean value was significantly different from that of the group consuming the diet containing 0.4% polyphenol from non-fermented cocoa rich in polyphenols (PC0.4, ▲; $P < 0.05$) on the same day of the study. §Mean value was significantly different from that of the group consuming the diet containing 0.8% polyphenol from non-fermented cocoa rich in polyphenols (PC0.8, ▨; $P < 0.05$) on the same day of the study.

Discussion

In previous studies, cocoa-enriched diets have demonstrated their immunomodulatory role in the systemic and intestinal compartments of rats⁽⁴⁾. It is believed that this effect is

mainly due to the polyphenols present in cocoa. The present study focused on investigating whether these effects are attributed exclusively to them or should be attributed to other compounds present in cocoa, and, in addition, on evaluating

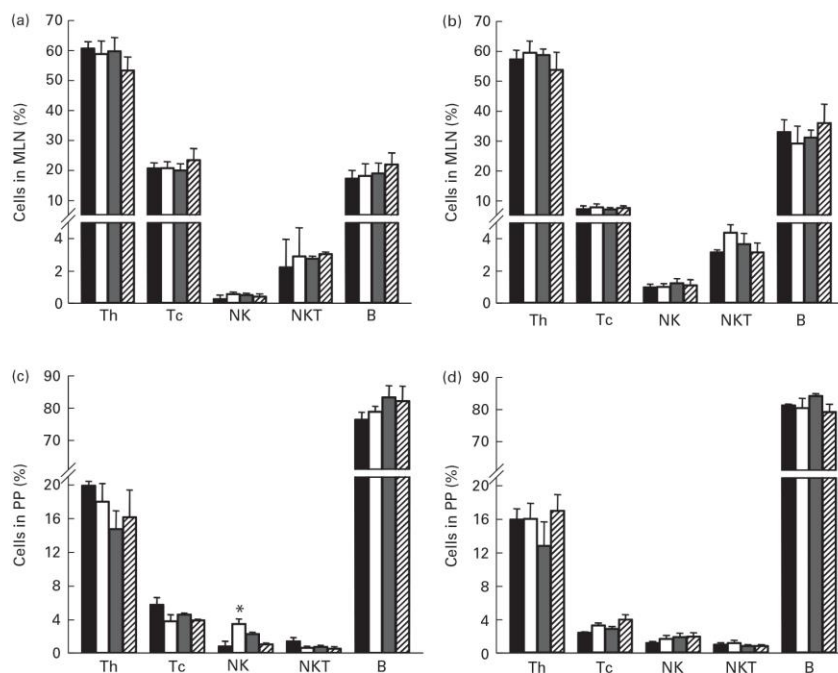


Fig. 4. Effects of cocoa polyphenol-enriched diets on (a, b) mesenteric lymph nodes (MLN) and (c, d) Peyer's patches (PP) from (a, c) Wistar rats and (b, d) Brown Norway rats. Values are means (n 6), with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference group ($P < 0.05$). NK, natural killer. □, Diet containing 0.2% polyphenol from conventional cocoa; ■, diet containing 0.4% polyphenol from non-fermented cocoa rich in polyphenols; ▨, diet containing 0.8% polyphenol from non-fermented cocoa rich in polyphenols.

whether these changes are rat strain dependent. To achieve this, two different rat strains were fed three cocoa diets formulated with three distinct cocoa raw materials differing in the proportion of polyphenols and other cocoa compounds. From the results, it emerges that Wistar rats are, in some aspects, more sensitive than Brown Norway rats to the effects of a cocoa diet on the immune system. In addition, the influence of cocoa on intestinal IgA and microbiota depends on polyphenols; however, as these effects are not dose dependent, other compounds must be involved in this influence.

Previous reports concerning the effect of cocoa on the immune system *in vivo* were developed using 2–10% cocoa-enriched diets formulated with conventional cocoa containing, among other compounds, 20–30 mg/g of polyphenols^(5,12,18). The present study includes the effect of two cocoa powders very rich in polyphenols (about 160 and 500 mg/g, respectively), because they were obtained without fermentation, allowing them to keep the polyphenols contained in cocoa seeds⁽¹⁹⁾. In the elaboration of the PC0.4 and PC0.8 diets, small amounts of cocoa powders were used to double or to quadruple the polyphenol content of the conventional 10% cocoa diet (PC0.2). The amount of conventional cocoa

powder used to elaborate the PC0.2 diet provided about 34 mg/g of fibre, including both soluble and insoluble fibre, and about 2 mg/g of theobromine. In summary, in the present study, three cocoa diets with increasing proportions of polyphenols (0.2–0.8%) and decreasing content of other cocoa compounds, such as fibre and theobromine, were designed to dissect the effects of cocoa on the immune system.

First, the influence of cocoa polyphenol intake on the increase in body weight was evaluated during the study period. Although during this period, there was no difference regarding food intake among the experimental groups, those fed with the PC0.2 diet in both rat strains had a lower increase in body weight, a result that is in line with previous studies carried out using conventional cocoa^(12,18,20,21). However, it is worth noting that diets containing non-fermented cocoa with a higher amount of polyphenols did not produce such an effect, suggesting, therefore, that the influence of cocoa on body weight does not depend on cocoa polyphenol content. In this sense, we can suggest that methylxanthines found in higher concentrations in the PC0.2 diet than in the PC0.4 and PC0.8 diets could play an important role⁽²²⁾.

Table 3. Bacterial percentage of each genus with respect to total bacteria determined by fluorescence *in situ* hybridisation–flow cytometry analysis in the faeces
(Mean values with their standard errors)

Group...	Before intervention								After intervention							
	REF		PC0-2		PC0-4		PC0-8		REF		PC0-2		PC0-4		PC0-8	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total bacteria	80.24	2.67	85.60	5.25	81.17	4.93	80.04	7.10	81.19	5.19	62.30*†	3.58	78.29	5.33	84.26‡	2.03
<i>Bacteroides</i>	16.82	1.66	18.87	3.50	13.87	3.41	11.43	1.44	32.81*	4.53	22.24	0.23	26.94	4.57	23.55	5.26
<i>Bifidobacterium</i>	1.84	0.45	1.86	0.76	1.99	0.22	3.85	0.56	8.64*	1.87	4.79	1.45	3.85	1.18	3.27	0.09
<i>Lactobacillus</i>	11.00	3.33	18.50	1.32	19.25	0.75	11.25	0.75	5.75	1.83	4.00*	2.02	2.83*	0.83	4.83*	0.60
<i>Escherichia coli</i>	3.01	0.04	3.40	0.31	3.79	0.45	5.38	1.73	3.73	0.97	1.70	0.84	1.79	0.13	1.33	0.36
<i>Clostridium histolyticum</i> / <i>C. perfringens</i>	7.00	1.74	6.50	0.76	3.50	1.00	4.00	0.50	14.67	3.17	6.17	0.83	15.00§	2.65	4.17	1.09
<i>Streptococcus</i>	25.16	5.20	34.42	5.77	27.35	5.06	33.36	5.49	57.05*	7.26	23.95†	5.73	34.57	5.14	25.27†	7.30
<i>Staphylococcus</i>	3.96	1.16	5.58	0.90	11.81	4.94	14.01	4.52	54.60*	3.65	25.32†	3.55	44.63†	1.50	43.60*‡	4.41

REF, reference diet; PC0-2, diet containing 0.2 % polyphenol from conventional cocoa; PC0-4, diet containing 0.4 % polyphenol from non-fermented cocoa rich in polyphenols; PC0-8, diet containing 0.8 % polyphenol from non-fermented cocoa rich in polyphenols.

* Mean value was significantly different from that before the intervention ($P < 0.05$).

† Mean value was significantly different from that of the REF group at the end of the study ($P < 0.05$).

‡ Mean value was significantly different from that of the PC0-2 group at the end of the study ($P < 0.05$).

§ Mean value was significantly different from that of the PC0-8 group at the end of the study ($P < 0.05$).

Further studies must delve into the cocoa compounds and the mechanisms involved in this effect.

One of the immune aspects considered in the present study is the influence of cocoa polyphenols on serum Ig concentrations. It has been reported that food polyphenols have the ability to modulate systemic Ig production^(23,24). In the present study, we observed that the three cocoa diets attenuated the age-increasing pattern observed for serum IgG1 and IgG2b concentrations in Wistar rats but not in Brown Norway rats. This effect did not depend on the polyphenol dose alone, thus suggesting that other compounds in the formulation of cocoa must have also contributed and even potentiated the effect when lower amounts of polyphenols are present. In contrast, serum IgG concentrations in Wistar rats are much lower than those in Brown Norway rats and, perhaps as a consequence, the first strain is more sensitive to cocoa intake than the latter one. The attenuation of serum IgG concentration produced by cocoa is in line with previous studies also carried out in Wistar rats⁽¹¹⁾.

With regard to intestinal IgA, the present results from a conventional cocoa diet (PC0-2) confirmed the down-modulatory effect on intestinal (faecal and gut wash) IgA content shown in previous studies, including several proportions of cocoa in diets (2–10 %), different ages at the beginning of the dietary intervention (3–6 weeks old) and different lengths of the diet (3–9 weeks)^(5,12,25). The attenuating effect on intestinal IgA produced by the PC0-2 diet was also produced by non-fermented cocoa when used at 0.8 % concentration in both Wistar and Brown Norway rats, in a similar way to what was observed in older Lewis rats fed with similar PC0-2 and PC0-8 diets for 2 weeks⁽²¹⁾. However, when considering IgA-coating bacteria, its percentage decreased after the intake of the PC0-2 diet but not after the PC0-8 diet, thereby partially reflecting the attenuating effect on intestinal IgA. The lowering effect of a conventional cocoa polyphenol diet (PC0-2) on bacteria coated with IgA observed herein is in line with previous studies conducted with a longer intervention in

Wistar rats⁽¹²⁾ and also in older Lewis rats⁽²¹⁾. Overall, these results suggest that polyphenols can be responsible for the decrease in intestinal IgA concentrations, but some other compounds present in conventional cocoa must have also contributed or potentiated the effect of polyphenols.

The study of lymphocyte composition in PP and MLN can shed light on the effect of cocoa on intestinal IgA. Some studies have reported the ability of polyphenols to modulate the proportion of some mucosal lymphocyte populations⁽²⁶⁾, and also previous results have shown the effect of conventional cocoa on these compartments⁽⁵⁾. However, in the present study, the diets containing conventional cocoa did not produce profound changes with the exception of the increase of the NK population in PP lymphocytes. Similarly, diets containing a higher amount of polyphenols did not produce any effect on lymphocyte composition in MLN and PP. Since no significant changes were observed in the percentage of Th and B cells, the proportion of these cells in MLN or PP cannot explain the attenuating effect of cocoa polyphenols on intestinal IgA concentration, and this fact suggests that intracellular mechanisms, released immune mediators or the transcytosis of IgA across the intestinal wall could be responsible for the effect on intestinal IgA concentration⁽¹¹⁾. In contrast, the present results support the up-modulatory effect of conventional cocoa on the proportion of NK cells in the PP of Wistar rats, and agree with the enhancing effect of cocoa on innate immunity, as described previously⁽⁹⁾.

With regard to the gut microbiota, a decrease in total bacteria counts and some composition changes were produced by 4 weeks of cocoa diets, this being more pronounced again with the lowest concentration of polyphenols provided by the conventional cocoa. In general, cocoa diets inhibited the growth of *Staphylococcus*, *Streptococcus* and *Clostridium histolyticum*/*C. perfringens* (belonging to the Firmicutes phylum) produced by age. The inhibitory effect on the growth of *Staphylococcus* and *Streptococcus* genera induced by the PC0-2 diet is partially in line with previous findings in young

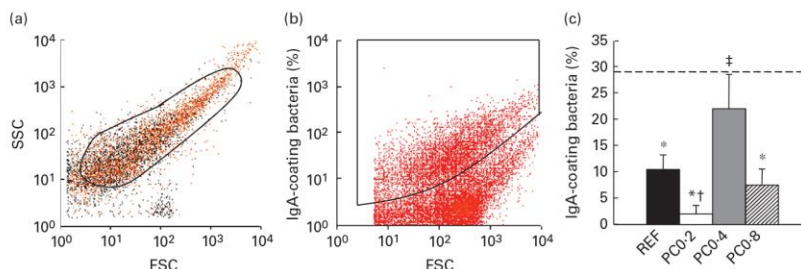


Fig. 5. Representative biparametric cytogram showing the (a) initial acquisition gate to select the bacterial population according to its forward-scatter characteristic (FSC)/side-scatter characteristic (SSC) signals. (b) Cytogram representing the mean of the percentage of IgA-coating bacteria, with their standard errors at baseline for all groups at day 0 (29.1 (SEM 3.3)). (c) IgA-coating bacteria at the end of the 28 d of nutritional intervention in all the groups. ---, Basal value. Values are means (n 6), with their standard errors represented by vertical bars. *Mean value was significantly different from that before the intervention ($P < 0.05$). †Mean value was significantly different from that of the reference (REF) groups ($P < 0.05$) on the same day of the study. ‡Mean value was significantly different from that of the group consuming the diet containing 0.2% polyphenol from conventional cocoa (□; PC0-2, $P < 0.05$) on the same day of the study. ■, PC0-4, diet containing 0.4% polyphenol from non-fermented cocoa rich in polyphenols; ▨, PC0-8, diet containing 0.8% polyphenol from non-fermented cocoa rich in polyphenols. (A colour version of this figure can be found online at <http://www.journals.cambridge.org/bjn>)

Wistar rats after 6 weeks of nutritional intervention⁽¹²⁾. In this context, polyphenols have been associated with an inhibitory effect on the growth of *C. bistoliticum*/*C. perfringens* genera in *in vitro*, preclinical and clinical studies^(12,17,27–30). In contrast, the rats fed with the three cocoa polyphenol-enriched diets showed a decrease in the proportion of *Bifidobacterium*, which is not in line with other authors who attributed a bifidogenic effect to the polyphenols^(17,29,31,32). These differential results could be attributed to several factors such as the source of the polyphenols (pure flavonoids or polyphenol-rich dietary sources), dose, methodology (*in vitro*, preclinical or clinical studies) and differential composition and distribution ecosystems (rats *v.* human gut). In addition, it has been reported that polyphenols have diverse selective prebiotic effects and antimicrobial activities against certain genera of the gut microbiota⁽³³⁾, Gram-negative bacteria being more resistant than Gram-positive bacteria⁽³⁴⁾, and excessive amounts of polyphenols may inhibit the growth of beneficial colonic microbiota⁽³⁵⁾. In any case, from these data and unpublished results from our ongoing research, it could be suggested that cocoa polyphenols influence microbiota composition, but some other compounds, such as fibre and/or methylxanthines, must enhance this effect.

In summary, some immunomodulatory effects of diets containing high cocoa depend on the rat strain and could be partially attributed to their polyphenol content. Other cocoa compounds could also be key factors in this effect. Further studies evaluating dissected components of cocoa, such as fibre and theobromine, should be carried out in order to evaluate whether they are able to reproduce, at least partially, the previously and herein described cocoa effects on the intestinal immune system.

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The authors' contributions were as follows: M. M.-C., M. A.-G., À. F., M. C. and F. J. P.-C. conceived and designed the research; M. M.-C., S. T. and M. A.-G. carried out the experiments; M. M.-C., M. A.-G. and F. J. P.-C. carried out the data analysis; M. M.-C., M. C. and F. J. P.-C. were involved in the interpretation of the data; M. M.-C. contributed to the drafting of the manuscript; M. M.-C., M. A.-G., À. F., M. C. and F. J. P.-C. contributed to the critical revision of the manuscript; F. J. P.-C. had primary responsibility for the final content. All authors read and approved the final version of the manuscript.

None of the authors has any conflict of interest to declare.

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Article 4

“Prebiotic effects of cocoa fibre on rats”

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Els resultats del present article han permès la presentació de les comunicacions:

- *Cocoa fiber influences the proportion of short chain fatty acids in the intestine and sera.* V Workshop Probióticos, Prebióticos y Salud. Evidencia Científica, València (Espanya), Gener 2014.
- *Changes in fecal microbiota composition and prebiotic effect of cocoa fiber in Wistar rats.* VI Workshop Probióticos, Prebióticos y Salud. Evidencia Científica, Oviedo (Espanya), Febrer 2015.
- *Cocoa fiber as a new prebiotic.* International Scientific Association for Probiotics and Prebiotics (ISAPP) Conference, Washington DC (Estats Units), Maig 2015.

RESUM

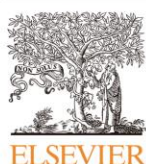
Objectiu: Avaluar si els efectes causats per la dieta rica en cacau sobre la composició i funcionalitat de la microbiota i la seva interacció amb el sistema immunitari a través dels TLR són deguts al seu contingut en fibra de cacau.

Material i mètodes: L'estudi es va realitzar en rates de 3 setmanes d'edat de la soca Wistar. Es van alimentar durant tres setmanes amb una dieta estàndard, una dieta enriquida amb un 10% de cacau (que conté polifenols i fibra de cacau), amb una dieta a base de fibra de cacau amb baix contingut de polifenols (<0,2%) o bé amb una dieta elaborada amb inulina. Es va registrar setmanalment el pes corporal i la ingesta de pinso. La composició de la microbiota intestinal es va caracteritzar per la tècnica de FISH-FCM juntament amb el percentatge de bacteris fecals units a IgA després de tres setmanes d'intervenció nutricional. La concentració fecal, cecal i sèrica d'àcids grassos de cadena curta es va determinar per cromatografia de gasos. Després de la intervenció nutricional es va avaluar l'expressió gènica de TLR i ocludina en mostres de còlon per tècniques de PCR a temps real.

Resultats: La dieta de cacau al 10%, a diferència de la dieta de fibra de cacau, va provocar un menor guany corporal al llarg de tot l'estudi. La dieta elaborada amb fibra de cacau va incrementar el nombre de *Lactobacillus* spp. i *Bifidobacterium* spp., efectes que van anar acompanyats d'una major proporció de bacteris units a IgA, una major concentració d'àcids grassos de cadena curta en femtes, contingut cecal i sèrum. A més, la dieta de fibra de cacau va induir canvis en el

patró d'expressió dels TLR2, TLR5, TLR7 i ocludina diferents als derivats de la dieta amb cacau.

Conclusió: La dieta de fibra de cacau, a diferència de la dieta amb un 10% de cacau, presenta efectes prebiòtics ja que promou el creixement de bacteris beneficiosos (*Bifidobacterium* spp. i *Lactobacillus* spp.), incrementa la producció d'àcids grassos de cadena curta i el percentatge de bacteris units a IgA, modifica el *crosstalk* entre la microbiota i el sistema immunitari i reforça la funció de barrera de l'epiteli intestinal.



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Prebiotic effects of cocoa fibre on rats



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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 in Wistar rats 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 were 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.

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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, Pérez-Berezo, Franch, Castell, & Pérez-Cano, 2012; Pérez-Berezo et al., 2011; Pérez-Cano, Massot-Cladera, Franch, Castellote, & Castell, 2013), but understanding of the mechanisms and specific components within cocoa which exert these effects is limited. Cocoa contains

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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, foetal 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 iodide; 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

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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 (Etcheberria 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, Espín, & Tomás-Barberán, 2009; Tzounis et al., 2008). Both the dietary polyphenols and their microbially-derived phenolic metabolites modulate the gut microbiota composition (Etcheberria et al., 2013; Lee, Jenner, Low, & Lee, 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 (CO₂, CH₄ and H₂) and heat (Jakobsdottir et al., 2013; Puertollano, Kolida, & Yaqoob, 2014; Wong & Jenkins, 2007). Acetate, propionate 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, de Souza, Kendall, Emam, & Jenkins, 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, Li, Green, Holzman, & Lin, 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 (Tourinho, Fuguet, Vinardelu, Cascante, & Torres, 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. Materials 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 3 weeks. Natural Forastero cocoa and cocoa fibre (mainly rich in cellulose, hemicellulose and pectic substances) powders (Nutrexpia 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.

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 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-Butylhydroquinone	0.008	0.006	0.006	0.006
Water	72.4	63	71.1	72
Cocoa powder	–	100	–	–
Cocoa fibre powder	–	–	52.3	–
Inulin powder	–	–	–	8.5

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

Components	Cocoa powder (g/kg)	Cocoa fibre powder (g/kg)	Inulin powder (g/kg)
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 ^a	4	0.02	–
Water	7	–	–

^a Total polyphenol compounds were quantified by Folin-Ciocalteu method.

2.2. Sample collection and processing

At the beginning and end of the study, blood samples were collected and serum was kept at -20°C 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°C overnight and stored at -20°C 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°C) and kept at -20°C 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, Vogt, & Wolever, 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 (14,000 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 Nukol™ Capillary Column (30 m $\times$ 0.53 mm $\times$ 1.0 μm , SUPELCO™ Analytical, Gilligham, UK) and flame ionization 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; $+20^{\circ}\text{C}/\text{min}$ to 145°C ; $+4^{\circ}\text{C}/\text{min}$ 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, isobutyric, 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 was centrifuged (18,000 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 3 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 $\pm$ SEM ($\mu\text{g}/\text{mL}$).

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 iodide (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 °C).

2.7. Flow cytometry bacterial analysis

FCM analysis for bacterial characterization was performed using a FACS Aria SORP sorter (BD, San José, CA, USA) as previously described (Massot-Cladera et al., 2012). Commercial Flow Check™ 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 RNeasy® 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. Two micrograms 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 *Ocln* (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 $2^{-\Delta\Delta C_T}$ method, as previously described (Pérez-Cano et al., 2009). Results are expressed as the mean $\pm$ 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, SCFA 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 ($p < 0.01$ days 7, 14, 21) (Fig. 1A). This effect was not

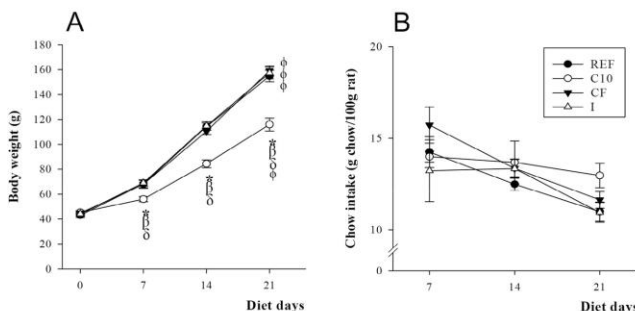


Fig. 1 – Body weight (A) and chow intake (g/100 g rat/day) (B) monitored throughout the nutritional intervention. Values are expressed as mean $\pm$ SEM (n = 10). Reference diet (REF); cocoa diet (C10); cocoa fibre diet (CF); inulin diet (I). Statistical differences: * $p < 0.05$ vs REF; α $p < 0.05$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

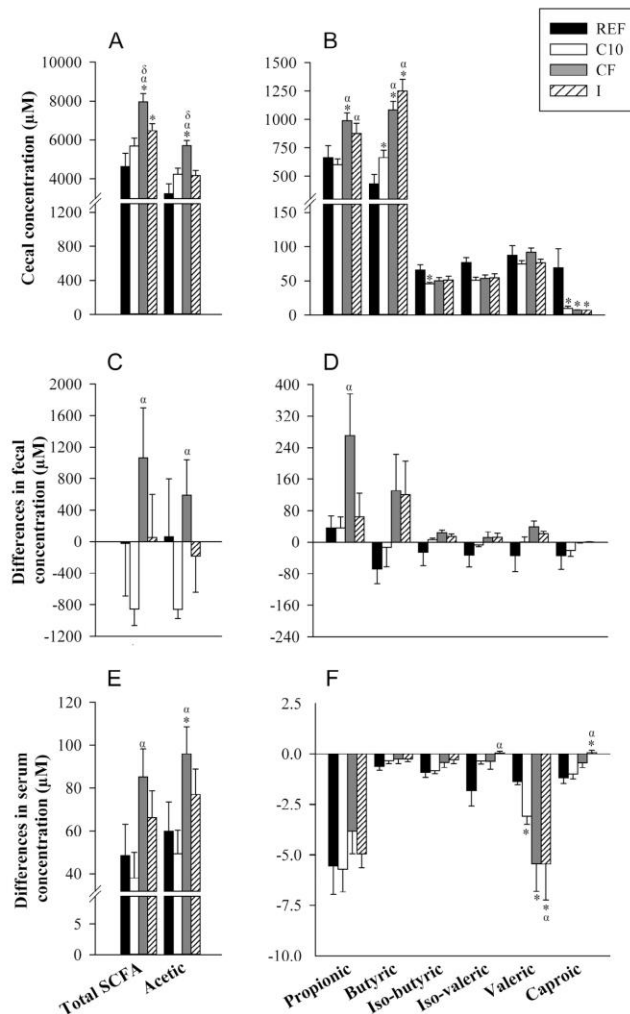


Fig. 2 – Effects of cocoa fibre on SCFA production. Caecal SCFA concentration at the end of the study (A, B); and difference in faecal (C,D) and serum (E,F) SCFA concentration compared to the initial values after 3 weeks of nutritional intervention. Values are expressed as mean $\pm$ SEM (n = 10). Reference 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.

related to lower chow intake, which was similar throughout the study among all experimental groups (Fig. 1B).

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 $\pm$ 305.2 μM), which was approximately twofold higher than that quantified in faecal samples (3053 $\pm$ 1451.4 μM). The lowest concentration of SCFA was detected in serum samples (216.9 $\pm$ 6.2 μM).

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

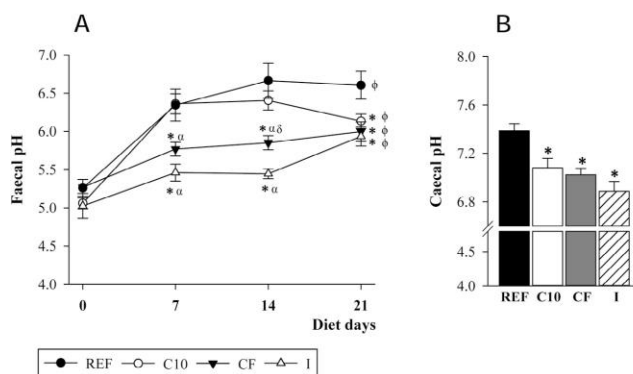


Fig. 3 – Effects of cocoa fibre on faecal (A) and caecal pH (B) after 3 weeks of nutritional intervention. Results are expressed as mean $\pm$ SEM ($n = 10$). Reference diet (REF); cocoa diet (C10); cocoa fibre diet (CF); inulin diet (I). Statistical differences: * $p < 0.05$ vs REF; α $p < 0.05$ vs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

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 $\mu\text{g/mL}$).

Concerning faecal SCFA, all groups showed a similar pattern at baseline (data not shown). However, after 3 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 acid 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 $\mu\text{g/mL}$) and also after 3 weeks of nutritional intervention (4.38 ± 0.96 $\mu\text{g/mL}$) 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 3 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 3 weeks later all groups showed a similar concentration (0.75 ± 0.17 $\mu\text{g/mL}$).

3.3. Faecal and caecal pH

All groups showed a similar faecal pH at baseline (~ 5.5) which significantly increased up to ~ 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 2 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).

3.4. Microbiota characterization by FCM-FISH

After 3 weeks of nutritional intervention, there were significant differences in the gut microbiota composition between groups (Table 3).

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 $\pm$ SEM of bacterial cells $\times 10^7$ /g of faeces. Firmicutes to Bacteroidetes ratio is expressed for each experimental group as the mean $\pm$ 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; $^a p < 0.05$ vs C10; $^b p < 0.05$ vs

Groups	After intervention							
	REF		C10		CF		I	
	Mean ($\times 10^7$ cells/g)	SEM	Mean ($\times 10^7$ cells/g)	SEM	Mean ($\times 10^7$ cells/g)	SEM	Mean ($\times 10^7$ 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 ^{ab}	2.69	16.54 ^{ab}	5.09
Lactobacillus (F)	4.21	0.94	7.25	2.65	12.20*	2.77	24.38 ^{ab}	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 ^a	3.68	4.99 ^a	1.06
Streptococcus (F)	5.94	1.83	1.27*	0.21	18.30 ^{ab}	5.20	6.55 ^a	0.74
Clostridium histolyticum/C. perfringens (F)	3.40	1.22	6.53	1.69	11.69*	2.94	12.40 ^{ab}	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 ^a	1.83	9.11 ^a	2.82

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 ($p = 0.021$). *C. histolyticum/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 *C. coccoides/E. rectale* groups compared to the REF group ($p = 0.016$).

There was an inverse correlation between the faecal pH and the number of *C. 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 ($p = 0.023$).

3.5. IgA-coated bacteria

The proportion of faecal IgA-coated bacteria after the nutritional intervention was $\sim 10\%$ 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).

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*).

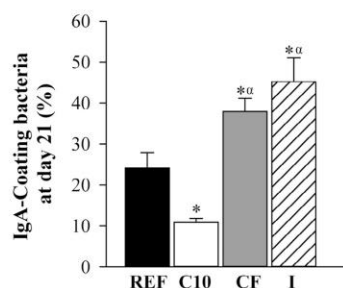


Fig. 4 – Effects of cocoa fibre on percentage of faecal bacteria coated with IgA after 3 weeks of nutritional intervention. Results are expressed as mean percentage $\pm$ SEM (n = 10). Reference diet (REF); cocoa diet (C10); cocoa fibre diet (CF); inulin diet (I). Statistical differences: * $p < 0.05$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

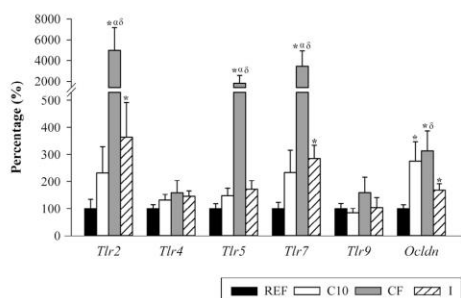


Fig. 5 – TLR gene expressions in the colon after nutritional intervention with cocoa fibre diet. Expression values are normalized using the expression of *Gusb* as the endogenous housekeeping gene. Each bar represents the mean $\pm$ SEM of the percentage of the C10 group (white bars), CF group (grey 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.

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 approximately 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).

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 3 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 fer-

mentation 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, not only 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, Franch, Castellote, Castell, & Pérez-Cano, 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, Meyer, & Gibson, 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, Tuohy, & Gibson, 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 (Etcheberry 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 *E. 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 defence 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, 2010; Abreu, Fukata, & Arditi, 2005). 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, Massot-Cladera, Rodríguez-Lagunas, & Castell, 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, Hurst, Stuart, & Lambert, 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, Rastmanesh, & Agostonic, 2013). Specifically, it has been reported that *Bacteroidetes* possess fewer genes for enzymes involved in lipid and carbohydrate metabolism than *Firmicutes* (Jumper 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 defences 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.

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Conflict of interest

The authors declare no competing financial interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.09.021.

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Article 5

“Cocoa polyphenols and fiber modify colonic gene expression in rats”

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Sotmés a publicació

Els resultats del present article han permès la presentació de les comunicacions:

- *Expression of colonic genes related to immune function in Wistar rats fed a cocoa-enriched diet.* VIII Societat Catalana d’Immunologia (SCI), Barcelona (Espanya), Novembre 2014.
- *Changes in intestinal gene expression induced by diets enriched in cocoa fiber and polyphenols in young Wistar rats.* 7th International Immunonutrition Workshop, Carovigno (Itàlia), Maig 2014.

RESUM

Objectiu: Establir el mecanisme mitjançant el qual la ingesta de cacau modula el metabolisme lipídic i la funció immunitària intestinal a través de l'estudi de canvis en l'expressió gènica. A més, esbrinar la contribució de la fibra de cacau a aquests efectes.

Material i mètodes: L'estudi es va realitzar en rates de 3 setmanes d'edat de la soca Wistar alimentades durant tres setmanes amb una dieta estàndard, una dieta enriquida amb un 10% de cacau (que conté polifenols i fibra de cacau), amb una dieta a base de fibra de cacau amb baix contingut de polifenols (<0,2%) o bé amb una dieta elaborada amb inulina. Es va estudiar l'expressió gènica en còlon per tècniques de microarray i validats per tècniques de PCR a temps real.

Resultats: El grup alimentat amb la dieta de cacau al 10% va ser el que major nombre de canvis en l'expressió gènica va presentar. La dieta de cacau va incrementar l'expressió gènica dels gens *Scgb1a1* i *Scnn1g* mentre que va disminuir la dels *Tac4*, *Mcpt2*, *Fcer1a* i *Fabp1*, la majoria d'ells relacionats amb el metabolisme lipídic i la funció immunitària. Les dietes a base de fibra van disminuir l'expressió dels gens *Serpina10* i *Apoa4*. Es van obtenir patrons similars d'expressió per tècniques de PCR a temps real.

Conclusió: S'estableix la modulació de l'expressió gènica com a un dels mecanismes mitjançant el qual el cacau exerceix els seus efectes. Particularment, la dieta de cacau, a diferència de la dieta de fibra de cacau, disminueixen l'expressió d'un elevat nombre de gens involucrats en les vies metabòliques lipídiques i en la funcionalitat del sistema immunitari en mostres de còlon.

Cocoa polyphenols and fiber modify colonic gene expression in rats

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Abstract

Purpose: Cocoa intake has been associated with health benefits, improving cardiovascular function and metabolism, as well as modulating intestinal immune function. The aim of this study was to take an in-depth look into the mechanisms affected by the cocoa intake by evaluating the colonic gene expression after nutritional intervention, and to ascertain the role of the fiber of cocoa in these effects.

Method: To achieve this, Wistar rats were fed for 3-weeks with either a reference diet, a diet containing 10% cocoa (C10), a diet based on cocoa fiber (CF) or a diet containing inulin (I). At the end of the study, colon was excised to obtain the RNA to evaluate the differential gene expression by microarray. Results were validated by RT-PCR.

Results: The C10 group was the group with most changes in colonic gene expression, most of them under-represented but a few in common with the CF diet. The C10 diet significantly up-regulated the expression of *Scgb1a1* and *Scnn1g* and down-regulated *Tac4*, *Mcpt2*, *Fcer1a* and *Fabp1* by twofold, most of them related to lipid metabolism and immune function. The CF and I diets down-regulated the expression of *Serpina10* and *Apoa4* by twofold. Similar patterns of expression were found by PCR.

Conclusion: Cocoa consumption down-regulated genes related to the immune system (B cell and mast cell functionality), lipid metabolism in the colon tissue. Most of the beneficial effects attributed to cocoa were due not only to its fiber content, but also to the possible contribution of polyphenols and other compounds.

Keywords: cocoa · colonic gene expression · dietary fiber · immune function · lipid metabolism

Introduction

The increasing interest in finding food-based strategies with health-improving effects has turned cocoa, the fruit of the cocoa tree (*Theobroma cacao* L.), into one of the subject matters of study. The health benefits provided by cocoa consumption in the prevention of diseases and physiological disorders have been described. Thus, cocoa consumption has long been demonstrated to improve cardiovascular status, decreasing blood pressure and reducing platelet aggregation [1, 2]. In addition, cocoa intake has been shown to increase HDL cholesterol, to protect against LDL oxidation, and to improve hyperlipidemia, insulin sensitivity and hyperglycemia [3, 4], and there is emerging evidence of the beneficial role of chocolate in reducing body weight/body fat [5, 6]. In addition, studies concerning the cocoa protector effect against carcinogenesis [7], neurodegenerative diseases and cognitive function [8] have also been published.

Preclinical studies have demonstrated that cocoa exerts immune modulation in both systemic and intestinal compartments [9]. In this context, immunoglobulin production is modulated after cocoa intake and, in consequence, cocoa has been tested as a possible adjuvant treatment in allergic reactions [10]. In addition, the intake of cocoa affects the intestinal compartment by modulating the microbiota, as assessed *in vivo* and clinical studies [11] and also the toll-like receptor (TLR) expression, key molecules involved in the recognition of microorganisms and inducers of inflammatory response [12] both in the inductive and effectors intestinal immune tissues, where antigens are sampled lymphocytes are activated and; where the effector cells contribute to the formation of S-IgA antibodies, respectively [13–15].

Whatever the cocoa effect, most of these beneficial actions of cocoa have been attributed to its high content of polyphenols, mainly flavonoids such as (–)-epicatechin, (+)-catechin and their polymeric forms called procyanidins [16]. Thus, flavonoids have been related to cocoa-induced cardiovascular effects [1, 2], protection against carcinogenesis [7], and cognitive function [8]. Nevertheless, it must be added that cocoa, besides being a strong source of flavonoids, also has a high content of dietary fiber (26 – 40%, mostly insoluble fiber) which could strengthen or be in part responsible for the effects described above. In general, dietary fiber appears to be particularly beneficial against various Western diseases, including metabolic syndrome [17] and, in particular, cocoa fiber can prevent obesity by reducing body weight and improving glucose levels as well as controlling the blood lipid profile and lipid peroxidation in animals and human interventions [18].

One of the several possible mechanisms involved in the body weight control might be through the cocoa fiber's capacity to increase fat elimination in feces [19, 20] by binding bile acids and dietary

fats, and interfering with lipid absorption in the small intestine [21, 22]. In addition, the effects of cocoa fiber assisting in the control of arterial blood pressure in hypercholesterolemic subjects has been reviewed [20]. Protection against disorders related to the gastrointestinal tract (i.e. constipation, irritable bowel syndrome) or colorectal and other types of cancer has also been attributed to cocoa fiber fermentation by the colonic microbiota which leads to the formation of short chain fatty acids (SCFA) [23].

Taking the above evidences together, the influence of cocoa consumption on the systemic and intestinal compartments is clear. These effects can be attributed to both its polyphenols and fiber content which can reach the colon, but also to their microbial metabolites produced after cocoa consumption. Therefore, we hypothesize that important events after cocoa intake are produced in the colon environment, where bioactive compounds of cocoa would interfere then in the gene expression of colon cells. The objective of the present work was to study in-depth the mechanisms involved in the beneficial effects of cocoa by evaluating changes in colonic gene expression after a preclinical nutritional intervention, and to ascertain whether cocoa fiber is responsible for these effects. For this purpose, the technique chosen is microarray analysis due to the fact that it is a powerful tool that allows global analysis of the expression of thousands of genes to be performed in a single assay [24].

Material and Methods

Animals and diets

Female Wistar rats (3-week-old) were obtained from Janvier (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) was fed with a diet containing 0.4% of polyphenols, 0.85% soluble fiber and 2.55% of insoluble fiber provided by 10% cocoa; the cocoa fiber group (CF) received a diet with the same cocoa soluble and insoluble fiber proportion as the C10 group but with a very low amount of polyphenols (<0.02%); and a fourth group (I) received the same amount of soluble fiber as the C10 group (0.85%) but in the form of inulin in order to distinguish the particular effect of cocoa fiber (Table 1). Natural Forastero cocoa and cocoa fiber powders (Idilia Foods SL (formerly Nutrexpa SL), 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 fiber. The three experimental diets were elaborated from AIN-93M

formula by subtracting the amount of carbohydrates, proteins, lipids and insoluble fibers provided by the corresponding cocoa, cocoa fiber extracts. The resulting chows were isoenergetic and had the same proportion of macronutrients (carbohydrates, proteins and lipids) and insoluble fiber as the reference diet (Table 1).

Animals were given free access to water and chow. The diets lasted for three weeks. 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 3-week nutritional intervention, the rats were anesthetized intramuscularly with ketamine (90 mg/kg) (Merial Laboratorios, S.A., Barcelona, Spain) and xylazine (10 mg/kg) (Bayer A.G, Leverkusen, Germany). A 5 mm piece of the middle of the colon was collected from all animals in aseptic conditions in RNeasy Lysis Buffer and kept at 4 °C overnight before storing at -20 °C for further gene expression analysis by microarray

RNA extraction

For RNA isolation, tissue samples were transferred into lysing matrix tubes (MP Biomedicals, Illkirch, France) containing an appropriate buffer and were homogenized in a FastPrep® instrument (MP Biomedicals) for 30 s. Lysates were centrifuged for 3 min at 12,000 *g* to eliminate excess tissue debris. The RNA was isolated by the RNeasy Mini Kit (Qiagen, Madrid, Spain) following the manufacturer's recommendations and then quantified with a NanoDrop spectrophotometer and NanoDrop IVD-1000 v.3.1.2 software (NanoDrop Technologies, Wilmington, DE, USA). The Agilent 2100 Bioanalyzer with the RNA 6000 LabChip kit (Agilent Technologies, Madrid, Spain) was used to provide an RNA integrity number for each sample. All samples used for further experiments showed an RNA integrity number (RIN) ≥ 9 and purity between 1.885 and 2.042 using the A_{260}/A_{280} ratio.

Table 1 Composition of nutrients and polyphenols provided by the three experimental diets (g/kg diet).

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
<i>tert</i> -Butylhydroquinone	0.008	0.006	0.006	0.006
Water	72.4	63	71.1	72
Cocoa powder	-	100	-	-
Protein	-	22	-	-
Carbohydrate	-	16	-	-
Lipid	-	11	-	-
Fiber (insoluble/soluble)	-	34 (25.5/8.5)	-	-
Minerals	-	6	-	-
Total Polyphenols ¹	-	4	-	-
Water	-	7	-	-
Cocoa fiber powder	-	-	52.3	-
Protein	-	-	8	-
Carbohydrate	-	-	0.5	-
Lipid	-	-	4.8	-
Fiber (insoluble/soluble)	-	-	31.9 (23.4/8.5)	-
Minerals	-	-	7	-
Total Polyphenols ¹	-	-	0.2	-
Water	-	-	-	-
Inulin powder	-	-	-	8.5
Protein	-	-	-	-
Carbohydrate	-	-	-	-
Lipid	-	-	-	-
Fiber (insoluble/soluble)	-	-	-	8.5 (-/8.5)
Minerals	-	-	-	-
Total Polyphenols ¹	-	-	-	-
Water	-	-	-	-

Microarray procedure

The study of the differential expression profiling was carried out with a SurePrint-G3 Rat GE 8x60K microarray kit (ID 028279, Agilent Technologies, Madrid, Spain), following a *loop* experimental design in which a pairwise comparison was done. Quadruplicate Samples for each experimental condition were employed, and a dye swaps (Cy3 and Cy5) was performed to the RNA amplified from each sample. RNA quality was assessed using a TapeStation (Agilent Technologies). RNA concentration and dye incorporation was measured using a UV-VIS spectrophotometer (Nanodrop 1000, Agilent Technologies, Wilmington, DE, USA). Labeling and hybridization to microarray was conducted following the manufacturer's two-color protocol (*Two-Color Microarray-Based Gene Expression Analysis v. 6.5, Agilent Technologies*), using *LowInput QuickAmp Labeling Kit* and *Agilent Microarray Hybridization Chamber Kit* for labeling and hybridization, respectively. Microarray chips were then washed and immediately scanned using a DNA Microarray Scanner (Model G2505C, Agilent Technologies) by the Genetic Diagnostic Bioarray facilities (Bioarray, Alicante, Spain).

Microarray data analysis

Data extraction was performed with Agilent Feature Extraction Software v.10.7 (Agilent Technologies). Bioinformatic analysis was performed with Bioconductor software under R environment, using the following packages: *limma* (v.3.16.1) for background correction and normalization, *Marray* and *pcaMethods* for quality control plots, *RankProd* for differential expression, and finally *GOSTats* (v.2.26.0.) and *GSEABase* for Gene Ontology functional analysis. Latest gene annotations available were used. Raw feature intensities were background corrected using *normexp* background correction algorithm. Within-array normalization was done using spatial and intensity-dependent *loess*. *Aquantile* normalization was used to normalize between arrays. The expression of each gene is reported as the base 2 logarithm of ratio of the value obtained of each condition relative to control condition (REF group). A gene is considered differentially expressed if it displays a PFP (Percentage of false prediction, equivalent of false discovery rate, FDR) less than 0.05 by rank product non-parametric method (RankProd). Significantly altered genes were plotted in a heatmap using R statistical software (v.3.0.2.). Venn Diagrams in GX allowed finding differently expressed genes that followed the same pattern (e.g. up regulated or down regulated) in common among the experimental conditions. Finally, over and under expressed genes were analyzed in terms of gene ontology by using a hypergeometric analysis (GOSTats). The output of this analysis was then filtered using two different criteria. On the one hand, data were filtered by fold expression, generating lists of differentially expressed genes by at least twofold, whereas on the

other hand, data were filtered taking into account the statistical significance of the enrichment analysis for the over- and under-represented GO terms belonging to the biological process (BP), cellular component (CC) and molecular function (MF) domains. The microarray data have been lodged in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) as accession number: GSE7095.

Validation of gene expression by Real-Time PCR

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: *Scgb1a1* (Rn00564903_m1, inventoried (I)), *Scnn1g* (Rn00566891_m1, I), *Fabp1* (Rn00664587_m1, I), *Serpina10* (Rn00592428_m1, I), *Mcpt2* (Rn00756479_g1, I), *Fcer1a* (Rn00562369_m1, I), *Apoa4* (Rn00562482_m1, I) and *Tac4* (Rn00597278_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 genes being studied was normalized to the housekeeping genes *Gusb* (Rn00566655_m1, I). The SDS v2.4 software (AB) was used to analyze the expression data. Results are expressed as the fold change of the amount of target mRNA relative to the endogenous control expression calculated using the standard $2^{-\Delta\Delta Ct}$ method for the three experimental groups relative to values from the REF group, which represents a onefold change in gene expression.

Statistics

Statistical analysis of PCR results was performed using the software package SPSS 22.0 (SPSS, Inc.). Levene's and Kolmogorov-Smirnov tests were applied to assess variance equality and normal distribution, respectively. Non-parametric tests were performed when normal distribution and equality of variance did not exist. Specifically, Kruskal-Wallis and Mann-Whitney *U* tests were used in order to assess significance for independent samples. Significant differences were established at $P < 0.05$.

Results

Body weight and chow intake

Body weight and chow intake were monitored throughout the study. The initial body weight was similar among the groups (44.4 ± 0.7 g) increasing during the study up to 154.8 g in the REF group. In animals fed 10% cocoa diet, body weight increase was lower than that in the other groups, the difference already being significant at day 7 and, at the end, the body weight was ~ 35 g less than

that in the rest of the groups ($P < 0.05$). This effect, also observed in previous studies [13, 14, 25], was not related to a lower chow intake since this was similar in all the experimental groups (data not shown) throughout the study as well as in reported studies [13, 14, 25]. No changes in body weight were found due to the CF or I diets.

Colon gene expression profiles and commonly modulated genes

Three weeks of diet containing either 10% cocoa, the equivalent amount of cocoa fiber or the equivalent amount of inulin as soluble fiber, produced changes in the gene expression of colon. The C10 group produced more changes than the CF and I groups, with most of these changes being down-regulated and only a few of them in common with the CF diet. Likewise, very few similarities were detected between the CF and I diets, the I group being the one with the fewest changes.

In order to study in-depth the number of genes affected in common, a Venn Diagram representation was drawn comparing separately the list of significantly up- and down- modified genes ($P < 0.05$) obtained for the three experimental diet groups of animals compared to the REF group (Fig. 1). The C10 and CF groups shared a total of 79 modulated genes, 31 of which were up-regulated and 48 down-regulated. The CF and I groups were found to have a total of 28 modulated genes in common, of which 8 were up-regulated and 20 down-regulated. Finally, a total of 18 modulated genes -3 up-regulated and 15 down-regulated- were shared between the C10 and I groups. The expression of 14 genes was found to have been modified in common by the three experimental diets (3 up- and 11 down-regulated).

Focusing on the uncommon modulated genes (Fig. 1), the cocoa-fed animals showed the highest modulation for both up- and down-regulations (64 and 175, respectively), whereas both the cocoa fiber and inulin diets modulated a similar number of different genes, with the number of up- and down-regulated genes also being similar.

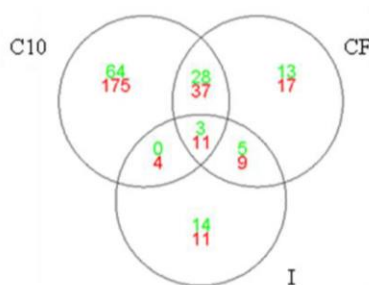


Fig. 1 Differentially expressed genes among all three experimental groups vs REF group are visualized by means of a Venn diagram ($n=4/\text{group}$). The diagram shows the number of genes that belongs to each of the individual lists, the genes in common between each pair of lists and the genes in common among all three lists (in the center of the representation) for each type of regulation (Green: up-regulation and Red: down-regulation) compared to the REF group.

Gene ontology (GO) analysis

We have considered the most statistically significant enriched for the over-represented and -under-represented GO terms belonging to the biological process (BP), cellular component (CC) and molecular function (MF) domains, i.e. including both the *P*-value together with the counts and the expected counts for each GO term in the C10 and the CF groups in comparison with the REF group. When those GO terms were also commonly enriched by the I diet, the number of the *P*-value together with the counts and the expected were also included (Tables 2 and 3). Regarding the most significantly enriched over-represented GO terms by C10 diet were those related to the chemical homeostasis (belonging to BP domain), the hemoglobin complex (CC domain) and the oxygen binding (MF domain) (Table 2). The C10 and CF groups resulted in the enrichment of several GO terms in common, showing in most cases equal statistical significance. Only two of the above common enriched GO terms was also found in the I group (extracellular region in the CC domain and CCR10 chemokine receptor binding in the MF domain).

With regard to the most significantly enriched under-represented ones (Table 3), digestive system process (BP domain), extracellular region (CC domain) and serine-type endopeptidase activity (MF domain) were identified as the GO terms most significantly enriched by the C10 diet. In this case, the three experimental diet groups had more significantly enriched under-represented GO terms in common, with the CF group being the one with the most significance for most of them.

Impact of cocoa diet on gene expression

The complete list of modified genes can be found in the Supplementary file.

The $\geq$ twofold up- and under-represented genes expression after cocoa diet intake (C10 group) compared to the REF group is shown in Tables 4 and 5, respectively. Likewise, the fold change expression of the same genes by the CF and I diets is included. The two genes with the highest fold up-regulation after C10 diet were *Scgb1a1* and *Scnn1g* (3.948 and 3.119 log fold change, respectively) (Table 4), the first encoding one a small epithelium-derived protein related to anti-inflammation, and the second one the gamma subunit of a membrane-bound ion-channel which plays an essential role in electrolyte (basically sodium transport in kidney, colon, lung and sweat glands) and blood pressure homeostasis. If we consider GO terms, the most up-regulated genes in the C10 group (*Scgb1a1*, *Cyp2f4*, *Dpysl4*, *Fmo2*, *Hoxd10*, *Spink3*, *Angptl4*, *Padi3*, *Pde7a*, *Cregl* and *Alas2*, among others) (Table 4) belong to the most represented term in the BP and in the MF domains which are the metabolic process (including total, primary and cellular processes,

GO:0008152, GO:0044238 and GO:0044237, respectively) and the binding pathways (GO:0005488 in MF domain) terms, respectively. Moreover, the most significantly enriched GO terms are also represented here. Particularly, *Mt2A*, *Sftpd*, *Atp12a*, *Trpv1*, *Angptl4*, *Guca2b*, *Alox15* and *Alas2* belong to the response to inorganic substance term (GO:0010035 in BP domain) whereas most of the significantly enriched GO terms (GO:0019825, GO:0020037, GO:0046906, GO:0005506, GO:0022892) in the MF domain are represented by the *Cyp2f4*, *LOC287167*, *LOC689064*, *Alox15*, *Alas2*, *Scnn1g*, *Fxyd4*, among others.

On the other hand, the most up-regulated genes after CF and I diet intake were *Fxyd4* (a protein-coding gene member of a family of small membrane proteins) and *Vom2r3* (a protein-coding gene involved in G protein-coupled receptor signaling pathway), respectively. The last gene did not undergo a twofold changed by 10% cocoa diet (See supplementary file).

The gene with the highest down-regulation after the C10 group was *Tac4* (Tachykinin 4), a member of the tachykinin family of neurotransmitter peptides involved in the immune system regulation, which decreased by 2.766 (Table 5). This gene and other genes down-regulated by 10% cocoa diet are related to the immune system. Thus, for *Fcer1a* ($\log_2$ FC -1.497) encoding the expression of the high-affinity receptor I for IgE Fc fragment for alpha polypeptides, and *Ms4a2* ($\log_2$ FC -1.144) encoding the same receptor for beta polypeptides. Both genes, together with *Tac4* are implicated in pathways related to the mast cell-mediated immunity (GO:0002448), its activation (GO:0045576; GO:0002279, GO:0033008, GO:0033005, GO:0033006, GO:0033003) and its degranulation (GO:0043303, GO:0043304, GO:0043306) in the BP domain. *Fcer1a* and *Ms4a2*, acting together with *Fcgr3a* ($\log_2$ FC -1.230), are also involved in the immunoglobulin binding pathway (GO:0019865 in the MF domain) and, together with *Adipoq* ($\log_2$ FC -1.826), are involved in cytokine production (GO:0001816 in the BP domain).

In addition, in C10 group a great number of down-regulated genes, such as *Adipoq*, *Fabp1*, *Fabp4*, *Fcer1a*, *Ptgis*, *Cyp4f1*, *Npc1l1* among others, affect the lipid and fatty acid metabolic process pathways (GO:0006629 and GO:0006631 in the BP domain, respectively). Many other genes down-regulated by 10% cocoa diet are involved in the proteolysis pathway such as *Mcpt1*, *Mcpt2*, *Mcpt4*, *Mcpt8*, *Mcpt10*, *Pga5*, etc. (GO:0006508 in the BP domain); response to stress such as *F2*, *Cd55*, *Nox1*, *Fcer1a*, *Tph1*, *Tff1*, etc (GO:0006950 in the BP domain), plasma membrane such as *Tmprss9*, *Tnfrsf12a*, *Casq1*, *Smpx*, *Fcgr3a*, etc. (GO:0005886 in the CC domain), and protein binding such as *Retnlg*, *Fabp4*, *Npw*, *Myl2*, *Bhlha15*, etc. (GO:0005515 in the MF domain).

Most of these genes highly modified by the cocoa diet were not or were only slightly modified in

the CF and I groups with the exception of *Ros1* (an orphan receptor tyrosine kinase that plays a role in cell differentiation) and *Tac4*, which were also modified in the CF-fed animals, and *Atf3* (activating transcription factor 3) and *Retnlb* (a cysteine-rich cytokine expressed in the colon and implicated in immunity) in the I-fed animals.

The CF and I diets down-regulated more than twofold the expression of *Serpina10* ($\log_2$ FC -2.729 and -2.987, respectively), a gene codifying a serpin family member predominantly expressed in the liver and involved in the regulation of the coagulation factors' activity, and *Apoa4* ($\log_2$ FC -1.955 and -1.273, respectively), a protein-coding gene that plays a role in lipoprotein metabolism (See supplementary file). On the contrary, these genes were not so affected by 10% cocoa diet ($\log_2$ FC 0.475 and -0.645, respectively) (See supplementary file).

Validation of microarray results by Real-Time PCR analysis

Eight genes representative of the affected pathways and with differential expression were selected for further analysis and validation of microarray results using quantitative RT-PCR. The studied genes included *Scgb1a1*, *Scnn1g* (highly up-regulated in the C10 group), *Tac4*, *Mcpt2*, *Fcer1a* and *Fabp1* (highly down-regulated in the C10 group). In addition, *Serpina10* (highly down-regulated in both the CF and I groups) and *Apoa4* (highly-down-regulated in the CF group) were also studied.

Results are shown in Fig 2, where the fold change expression determined both in the microarray and by PCR for each gene in each group are summarized. The quantitative PCR results are consistent with that of microarray. Although not all changes by PCR achieved statistical significance, a similar pattern of expression was determined using both techniques (Fig. 2). The statistical analysis of the Real-Time PCR results showed that only the expression of *Mcpt2* ($P=0.01$) and *Fcer1a* ($P=0.01$) was significantly down-regulated in the C10 group and that the cocoa fiber diet produced a significant down-regulation of *Serpina10* expression ($P=0.04$).

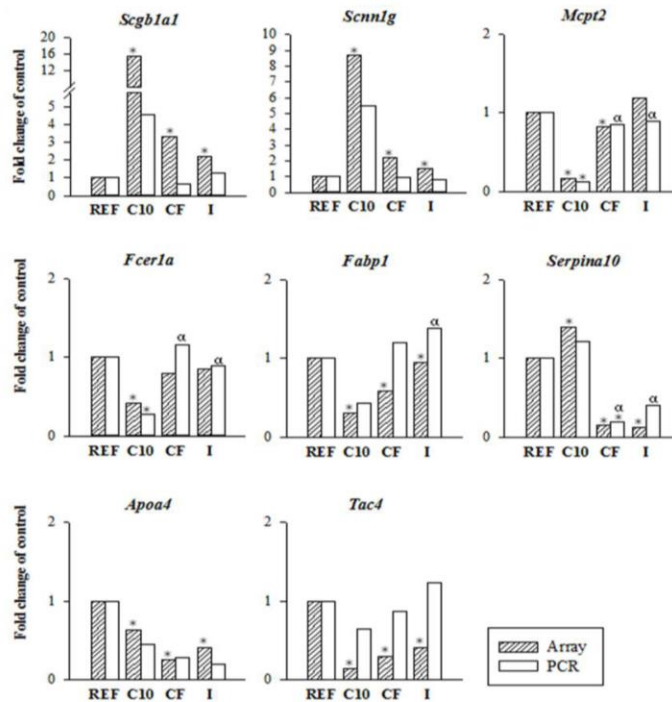


Fig. 2 Expression of selected genes by Real-Time PCR. The values are presented as fold change in comparison with the REF group that was considered 1 (n=10/group). Filled bars represent the expression for each specific gene as determined in the microarray experiments and empty bars represent the expression for each specific gene as determined by PCR normalized by *Gusb*. Statistical significance: * $P < 0.05$ vs REF group; α $P < 0.05$ vs C10 group.

Discussion

We have previously reported that the intake of a 10% cocoa diet in young rats induces changes in body weight and in the intestinal environment modulating the gut immune system and microbiota composition [13]. The involvement of cocoa fiber in such effects as well as its precise mechanism of action are unknown to date. In the present study, a microarray of the colon tissue RNA allowed the in depth study of the modifications of gene expression induced in rats by either a 10% cocoa diet, or two other diets with the same proportion of soluble and insoluble fibers: one based on cocoa fiber and the other containing inulin. Although the three experimental diets produced the up- or

down-regulation of a large number of genes, the cocoa intake caused the more pronounced and numerous changes (322 by the C10 diet vs 123 by the CF and 57 by the I diet), with most of them being down-regulations (227 down vs 95 up). From the 123 modifications induced by cocoa fiber intake, 79 were in common with those produced by the cocoa diet and 28 with those produced by inulin intake, with only 14 genes being modified in common by the three experimental diets.

A group of genes down-regulated by cocoa intake allowed us to study in-depth the mechanisms of this diet and its involvement in the immune function. In fact, the most down-regulated gene in the C10 group was that of tachykinin 4 (*Tac4*), which has been described as the promoter of B lineage cells [26]. As B lymphocytes are the immune cells responsible for antibody synthesis, these results could explain previous studies showing the cocoa-induced attenuation of immunoglobulin concentration in rats in both the systemic and intestinal compartments [15, 25, 27, 28]. This gene modification could also be responsible, at least partially, for the decrease of IgE synthesis in allergic animals induced by the 10% cocoa diet [10] and even for the same effect reported by other polyphenols [29]. Focusing on allergy, it seems that cocoa intake could also be beneficial in aspects other than IgE synthesis because, as shown here, it down-regulates the expression of genes, such as *Fcer1a*, *Ms4a2* and *Tac4*, involved in several GO terms related to mast cell-mediated immunity, its activation and its degranulation; key events in allergic response. When considering the expression of these immune-related genes in the cocoa fiber and inulin groups, the effects were not so marked, and therefore this may suggest that polyphenols or other cocoa compounds, but not cocoa fiber, are mainly responsible for the immunomodulatory properties of cocoa by interfering in the B-cell ability to produce antibodies and also in the mast cell functionality.

A cocoa diet might also exert its immunomodulatory effects through the down-modulation of the expression of genes involved in cytokine production (*Fcer1a*, *Ms4a2* and *Adipoq*). In addition, cocoa diet in rats has demonstrated certain protective effects against chronic inflammation [30, 31] that could also be related to the down-regulation of the cytokine gene expression and also to the up-regulation of the expression of *Scgb1a1*, the gene that codifies a protein related to anti-inflammation [32]. In this context, both fiber diets - of cocoa and inulin - also increased the *Scgb1a1* gene expression, although in a lower intensity compared to that observed in the C10 group, suggesting that fiber is partially responsible for the anti-inflammatory effect of cocoa but that other cocoa compounds such as flavonoids must also be involved [33]. However, contrary to this suggestion, a significant down-regulation of *Scgb1a1* gene expression has been found in a lung tumor mice model treated for 20 weeks with green tea [34].

Another group of genes down-regulated by the 10% cocoa diet was related to the lipid metabolism. In particular, the expression of *Adipoq*, an adipocyte-derived hormone involved in glucose and lipid metabolism, and *Fabp1*, an acid-binding protein found in liver, intestine and kidney involved in fatty acids uptake, transport and metabolism, were decreased by 1.826 and 1.682 FC logarithms. Moreover, the expression of *Angptl4*, a circulating lipoprotein lipase inhibitor, was up-regulated in the C10 group. Since these genes were less affected by the CF and I diets, we can suggest that the cocoa diet modifies lipid metabolism and this effect cannot be totally attributed to its fiber content and cocoa flavonoids are probably involved. The results agree with the reported hypolipidemic capacity of a cocoa diet [35–38] as well as in line with the results obtained, here and also in previous studies, on body weight [13, 25, 28, 39, 40]. In fact, the down-regulation of genes related to lipid metabolism has also been described by flavonoids, such as quercetin. The administration of quercetin in mice fed with a high-fat diet reduces the expression of hepatic genes related to lipid and fatty acid production, such as *Aldh1b1*, *Abcg5*, *Acaca*, *Fasn*, *Gpam* and *Apoa4* [36]. Although some other studies report body weight loss and a reduced fat deposition after polyphenol intake [36, 41, 42], previous studies carried out in our laboratory using three distinct cocoa materials with different proportions of polyphenols suggest that those effects on body weight cannot be exclusively attributed to cocoa polyphenols but also to other compounds present in cocoa [27]. As in the present study, we evidenced the lack of effect of the CF diet on body weight and on the same genes related to lipid metabolism as the entire cocoa diet, other bioactive compounds of cocoa, such as methylxanthines, could be proposed as being responsible for these effects on lipid metabolism gene expression proteins and body weight. In this context, methylxanthines are effective in stimulating resting energy expenditure per se [43] as well as regulating the lipid profile in obese-diabetic rats [44]. In addition, the synergistic interactions between methylxanthines and flavonoids in the thermogenesis have been suggested which could explain their effect on body weight [43].

Regarding cocoa fiber, in spite of the fact that it did not show the effects on the same lipid metabolism genes as cocoa, it is worth noting that the CF diet down-regulated the expression of another gene related to lipoprotein metabolism, the *Apoa4*. As also validated by RT-PCR, all three experimental diets down-regulated this gene but the highest effect was observed after CF intake. These results could justify the hypolipidemic effect reported after consumption of either insoluble or soluble CF. In this context, the modulation of serum and liver lipid profile has been reported both in rats fed insoluble CF [18] and those fed soluble CF [19, 20]. *Apoa4* can also be associated with the response to superoxide and to oxygen radical pathways (GO:0000303 and GO:0000305, respectively), therefore its down-regulation could elucidate the mechanism by which CF as well as cocoa flavanols are able to diminish the levels of certain lipid peroxidation biomarkers [37, 45].

From our results we can also observe the cocoa effects on other genes associated with the antioxidant activity [37]. Thus, a significant increase in the expression of *Hba-a2* and *Hbb* genes, encoding alpha and beta betaglobin subunits, respectively, was found in both the C10 group and the CF group, although the influence was lower than a twofold change.

Another beneficial effect of cocoa intake concerns blood pressure. Cocoa flavanols have been described to possess antihypertensive properties [46], which have made them a subject matter of health claims by the European Food Safety Authority [47]. Possible feasible mechanisms whereby they decrease blood pressure could either be maintaining the endothelium-dependent vasodilatation [48], or inhibiting the angiotensin-converting enzyme (ACE) activity [49]. Partially in line with those mechanisms, in our study, a significant decrease on the expression of *Mcpt1* and *Mcpt4* - both genes involved in the regulation of systemic arterial blood pressure by renin-angiotensin (GO:0003081) - have been observed in 10% cocoa-fed animals but not in the CF group. Nevertheless, the expression of the *Scnn1g* gene, involved in blood pressure homeostasis, was up-regulated in the C10 group and also in the CF animals, although with a < twofold-change. In addition, both the CF diet and the inulin diet down-regulated by more than twofold the expression of *Serpina10*, a gene involved in the regulation of the coagulation factors' activity, whose deficiency has been related to the risk of thrombosis [50]. Overall, our results show that cocoa intake modifies some colonic genes related to blood pressure regulation, and these effects cannot be attributed to its fiber content.

It remains to be elucidated which component from cocoa, but not cocoa fiber, is the main responsible of these changes in gene expression: both flavonoids and theobromine, present in high proportion in cocoa, are good candidates for such effects.

In summary, this study sheds light on the mechanisms involved in the reported effects of cocoa intake. As shown here, cocoa consumption down-regulates some genes related to the immune system (B cell and mast cell functionality), to lipid metabolism and also to blood pressure in the colon tissue. In addition, after comparing the effects of cocoa in its entirety and those produced by only cocoa fiber, it can be concluded that most of the beneficial effects attributed to cocoa are not only due to its fiber content but that polyphenols and other compounds could also be key factors.

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Table 2 Summary of the most enriched up-represented GO terms belonging to biological process (BP), cellular component (CC) and molecular function (MF), in the C10 and FC groups in comparison with the REF group, adding those shared with the I diet. The number of the expected genes, the counts as well as the *P*-value for each GO term are included (n=4/group).

GO TERM		C10 vs REF			CF vs REF			I vs REF		
		Exp Counts	Counts	P-Value	Exp Counts	Counts	P-Value	Exp Counts	Counts	P-Value
Biological Process	chemical homeostasis (GO:0048878)	3	12	1.57E-05	1	6	1.04E-03			
	response to inorganic substance (GO:0010035)	2	9	2.68E-05	1	7	3.62E-06			
	detoxification of copper ion (GO:0010273)	0	2	3.60E-05						
	ion homeostasis (GO:0050801)	2	10	5.03E-05	1	5	2.17E-03			
	oxygen transport (GO:0015671)	0	2	7.19E-05	0	2	1.34E-05			
	homeostatic process (GO:0042592)	4	13	9.04E-05	2	7	1.02E-03			
	cellular response to zinc ion (GO:0071294)	0	2	1.20E-04						
	prostate glandular acinus morphogenesis (GO:0060526)	0	2	2.50E-04	0	1	1.07E-02			
	prostate epithelial cord arborization involved in prostate glandular acinus morphogenesis (GO:0060527)	0	2	2.50E-04	0	1	1.07E-02			
	regulation of cell-cell adhesion mediated by integrin (GO:0033632)	0	2	3.32E-04	0	1	1.22E-02			
	gas transport (GO:0015669)	0	2	5.32E-04	0	3	1.00E-04			
	response to stress (GO:0006950)	8	15	5.66E-03	0	11	1.14E-04			
	hydrogen peroxide catabolic process (GO:0042744)	0	2	2.20E-03	0	2	4.19E-04			
	protein heterooligomerization (GO:0051291)	0	3	5.75E-03	0	3	5.13E-04			
	response to hypoxia (GO:0001666)	1	5	3.40E-03	0	4	8.99E-04			
	response to decreased oxygen levels (GO:0036293)	1	5	3.55E-03	0	4	9.34E-04			
Cellular Component	hemoglobin complex (GO:0005833)	0	5	2.07E-10	0	4	2.94E-09			
	haptoglobin-hemoglobin complex (GO:0031838)	0	2	7.31E-05	0	2	1.94E-05			
	extracellular region (GO:0005576)	5	15	7.50E-05	3	10	1.20E-04	1	4	2.01E-03
	cytosolic part (GO:0044445)	1	5	4.58E-04	0	5	1.83E-05			
	rough endoplasmic reticulum (GO:0005791)	0	3	1.18E-03						
	4-aminobutyrate transaminase complex (GO:0032144)	0	1	3.53E-03	0	1	1.83E-03			
	subapical complex (GO:0035003)	0	1	3.53E-03						
	apical part of cell (GO:0045177)	1	5	4.41E-03	1	3	1.80E-02			
	intracellular canaliculus (GO:0046691)	0	1	7.05E-03						
	invadopodium membrane (GO:0071438)	0	1	7.05E-03						
	phosphopyruvate hydratase complex (GO:0000015)				0	1	7.31E-03			
	apical plasma membrane (GO:0016324)	1	4	8.77E-03	0	3	8.34E-03			
	sodium channel complex (GO:0034706)				0	1	2.89E-02			
	Aggresome (GO:0016235)				0	1	3.25E-02			
Molecular Function	oxygen binding (GO:0019825)	0	5	1.09E-08	0	4	6.70E-08			
	oxygen transporter activity (GO:0005344)	0	4	5.31E-08	0	3	1.08E-06			
	heme binding (GO:0020037)	1	8	8.44E-08	0	6	4.22E-07			
	tetrapyrrole binding (GO:0046906)	1	8	1.14E-07	0	6	5.29E-07			
	iron ion binding (GO:0005506)	1	9	2.39E-07	0	6	6.30E-06			

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haptoglobin binding (GO:0031720)	0	2	3.98E-05	0	2	1.09E-05			
hemoglobin alpha binding (GO:0031721)	0	2	3.98E-05	0	2	1.09E-05			
CCR10 chemokine receptor binding (GO:0031735)	0	2	3.98E-05	0	2	1.09E-05	0	1	1.67E-03
substrate-specific transporter activity (GO:0022892)	3	12	6.57E-05	2	5	1.96E-04			
hemoglobin binding (GO:0030492)	0	2	2.76E-04	0	2	7.62E-05			

Table 3 Summary of the most enriched under-represented GO terms belonging to biological process (BP), cellular component (CC) and molecular function (MF) in the C10 and FC groups in comparison with the REF group, adding those shared with the I diet. The number of the expected genes, the counts as well as the *P*-value for each GO term are included (n=4/group).

	GO TERM	C10 vs REF			CF vs REF			I vs REF		
		Exp Counts	Counts	P-Value	Exp Counts	Counts	P-Value	Exp Counts	Counts	P-Value
Biological Process	digestive system process (GO:0022600)	1	8	5.43E-08	0	6	8.69E-09	0	3	1.10E-04
	cellular cation homeostasis (GO:0030003)	3	15	3.81E-07						
	digestion (GO:0007586)	1	8	6.67E-07	0	6	6.00E-08	0	3	2.83E-04
	chemical homeostasis (GO:0048878)	7	23	8.08E-07						
	regulation of biological quality (GO:0065008)	19	40	1.85E-06	6	12	5.75E-03			
	response to organic substance (GO:0010033)	16	35	2.10E-06				3	8	3.74E-03
	cation homeostasis (GO:0055080)	4	15	2.38E-06						
	regulation of calcium ion transport into cytosol (GO:0010522)	0	6	2.98E-06						
	cellular metal ion homeostasis (GO:0006875)	3	13	3.22E-06						
	cytosolic calcium ion transport (GO:0060401)	1	7	3.81E-06						
	intestinal cholesterol absorption (GO:0030299)	0	2	2.81E-03	0	3	1.39E-06	0	2	8.81E-05
	lipid digestion (GO:0044241)	0	2	4.24E-03	0	3	2.71E-06	0	2	1.34E-04
	intestinal absorption (GO:0050892)				0	3	1.33E-05	0	2	3.71E-04
	steroid metabolic process (GO:0008202)	2	9	2.13E-04	1	6	2.44E-05			
	regulation of digestive system process (GO:0044058)				0	3	4.16E-05	0	3	9.14E-06
	positive regulation of cholesterol esterification (GO:0010873)				0	2	1.00E-04	0	2	3.68E-05
	regulation of intestinal cholesterol absorption (GO:0030300)				0	2	1.00E-04	0	2	3.68E-05
	cellular hormone metabolic process (GO:0034754)				0	4	1.17E-04			
Cellular Component	extracellular region (GO:0005576)	12	44	5.78E-15	4	16	1.22E-07	2	12	1.86E-07
	extracellular space (GO:0005615)	6	26	2.03E-10	2	9	5.58E-05	1	8	5.31E-06
	extracellular region part (GO:0044421)	8	27	5.42E-09	2	10	5.59E-05	1	9	2.79E-06
	sarcoplasmic reticulum (GO:0016529)	0	5	2.72E-05						
	sarcoplasm (GO:0016528)	0	5	4.74E-05						
	sarcoplasmic reticulum lumen (GO:0033018)	0	2	4.26E-04						
	endoplasmic reticulum part (GO:0044432)	5	13	1.14E-03						
	myofibril (GO:0030016)	1	6	1.39E-03						
	contractile fiber (GO:0043292)	1	6	1.90E-03						
	extracellular matrix (GO:0031012)	3	9	3.03E-03				1	3	1.50E-02
	very-low-density lipoprotein particle (GO:0034361)	0	2	8.98E-03	0	2	8.42E-04	0	2	2.90E-04
	triglyceride-rich lipoprotein particle (GO:0034385)	0	2	8.98E-03	0	2	8.42E-04	0	2	2.90E-04
	high-density lipoprotein particle (GO:0034364)				0	2	1.06E-03	0	2	3.65E-04
	plasma lipoprotein particle (GO:0034358)	0	2	2.19E-02	0	2	2.14E-03	0	2	7.43E-04
	protein-lipid complex (GO:0032994)	0	2	2.35E-02	0	2	2.30E-03	0	2	7.99E-04
	endocytic vesicle (GO:0030139)				0	2	1.34E-02			

Resultats – Article 5

Molecular Function	meiotic cohesin complex (GO:0030893)	0	1	5.00E-02	0	1	1.52E-02			
	serine-type endopeptidase activity (GO:0004252)	1	9	1.07E-05						
	enzyme inhibitor activity (GO:0004857)	2	11	2.28E-05	1	5	1.03E-03	0	5	4.87E-05
	serine-type peptidase activity (GO:0008236)	2	9	2.71E-05						
	serine hydrolase activity (GO:0017171)	2	9	3.08E-05						
	peptidase inhibitor activity (GO:0030414)	1	8	8.85E-05	0	4	9.38E-04	0	4	1.06E-04
	carbonate dehydratase activity (GO:0004089)	0	3	9.56E-05						
	endopeptidase activity (GO:0004175)	3	11	1.85E-04						
	immunoglobulin binding (GO:0019865)	0	3	2.07E-04						
	IgE receptor activity (GO:0019767)	0	2	2.16E-04						
	ATPase inhibitor activity (GO:0042030)	0	2	2.16E-04						
	steroid dehydrogenase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor (GO:0033764)				0	3	4.73E-05			
	phosphatidylcholine-sterol O-acyltransferase activator activity (GO:0060228)				0	2	4.73E-05	0	2	1.34E-05
	steroid dehydrogenase activity (GO:0016229)				0	3	9.13E-05			
	3-beta-hydroxy-delta5-steroid dehydrogenase activity (GO:0003854)	0	2	1.48E-03	0	2	1.65E-04			
	sterol transporter activity (GO:0015248)				0	2	4.28E-04	0	2	1.22E-04
	cholesterol transporter activity (GO:0017127)				0	2	4.28E-04	0	2	1.22E-04
	endopeptidase inhibitor activity (GO:0004866)				0	4	9.38E-04	0	4	7.94E-05
	endopeptidase regulator activity (GO:0061135)	1	6	2.59E-03	0	4	1.11E-03	0	4	9.43E-05

	C10 vs REF	CF vs REF	I vs REF
Symbol	log2 FC	log2 FC	log2 FC
<i>Scgb1a1</i>	3.948	1.716	1.139
<i>Scnn1g</i>	3.119	1.133	0.573
<i>Fxyd4</i>	2.583	2.089	0.718
<i>Slc15a1</i>	2.332	1.388	0.475
<i>Gbp1</i>	2.207	1.662	0.432
<i>Cyp2f4</i>	2.185	1.436	0.367
<i>Dpysl4</i>	2.069	1.387	0.242
<i>Fmo2</i>	2.040	1.007	0.426
<i>Pdyn</i>	1.873	0.194	0.175
<i>Mt2A</i>	1.656	0.644	-0.147
<i>RGD1561239</i>	1.597	1.787	0.742
<i>Sftpd</i>	1.583	0.163	0.351
<i>Mme</i>	1.580	0.636	0.353
<i>Hoxd13</i>	1.575	0.615	0.100
<i>Chst5</i>	1.533	0.591	0.082
<i>Atp12a</i>	1.457	1.630	0.500
<i>Thbs4</i>	1.450	0.908	0.302
<i>Hoxd10</i>	1.403	0.668	0.164
<i>Trpv1</i>	1.390	0.749	0.058
<i>Hoxb13</i>	1.358	0.786	0.101
<i>Spink3</i>	1.338	1.162	0.463
<i>LOC687303</i>	1.327	0.734	0.755
<i>Abat</i>	1.319	1.272	0.477
<i>Angptl4</i>	1.316	0.914	0.670
<i>Aox4</i>	1.311	0.054	0.320
<i>B3gnt7</i>	1.265	0.403	0.019
<i>Pcsk9</i>	1.253	0.048	0.063
<i>RGD1560608</i>	1.225	0.759	0.709
<i>Guca2b</i>	1.211	0.811	0.287
<i>Padi3</i>	1.190	0.832	0.462
<i>Scnn1b</i>	1.181	0.499	0.057
<i>LOC287167</i>	1.178	0.607	0.296
<i>RGD1308274</i>	1.142	0.073	0.321
<i>Sme1b</i>	1.133	0.968	0.031
<i>Pde7a</i>	1.129	0.708	0.405
<i>LOC100363350</i>	1.126	0.621	0.446
<i>Gsta2</i>	1.111	0.537	0.157
<i>Slc20a1</i>	1.110	0.624	0.056
<i>RGD1310110</i>	1.097	0.410	0.231
<i>LOC689064</i>	1.095	1.024	0.501
<i>Rnase1</i>	1.094	1.063	1.218
<i>Dnase1</i>	1.088	1.087	0.124
<i>siat7D</i>	1.058	0.363	0.096
<i>Serpinc1</i>	1.053	0.873	0.469
<i>LOC494499</i>	1.029	0.576	0.276
<i>Fbxl13</i>	1.028	1.009	0.067
<i>Alox15</i>	1.028	0.387	0.191
<i>Creg1</i>	1.021	0.406	-0.008
<i>Rnase1l2</i>	1.010	0.857	1.012
<i>Alas2</i>	1.007	0.997	0.452

Table 4 List of up-regulated genes differentially expressed by $\geq$ twofold with a $P < 0.05$ obtained after the 10 % cocoa diet intake (C10 group) compared to the REF group (n=4/group).. This includes the gene symbol for all the up-regulated genes. Values are expressed as a $\log_2$ of fold change and shown when ≥ 1 . Values for the two other experimental diets were also included, although independently of their fold change and/or their significance. Values in bold and cursive represent the non-statistically significant changes.

Table 5 List of down-regulated genes differentially expressed by ≥ 2 -fold with a $P < 0.05$ obtained after the 10 % cocoa diet intake (C10 group) compared to the REF group (n=4/group). This includes the gene symbol for all the down-regulated genes. Values are expressed as a $\log_2$ of fold change and shown when ≤ -1 . Values for the two others experimental diets were also included, although independently of their fold change and/or their significance. Values in bold and cursive represent the non-statistically significant changes.

	C10 vs REF	CF vs REF	I vs REF		C10 vs REF	CF vs REF	I vs REF
Symbol	$\log_2$ FC	$\log_2$ FC	$\log_2$ FC	Symbol	$\log_2$ FC	$\log_2$ FC	$\log_2$ FC
<i>Syce2</i>	-1.000	-1.117	0.028	<i>Olfm4</i>	-1.291	-0.826	0.002
<i>Chga</i>	-1.011	-0.328	-0.133	<i>Mt3</i>	-1.298	-0.631	-0.372
<i>Smpx</i>	-1.016	-0.349	-0.381	<i>Casq2</i>	-1.298	-0.643	-0.218
<i>Myl2</i>	-1.019	-0.940	-0.823	<i>Hsd3b5</i>	-1.302	-0.648	-0.340
<i>Ptgis</i>	-1.026	-0.370	-0.173	<i>Gsta5</i>	-1.308	-0.560	-0.158
<i>Cfd</i>	-1.037	-0.087	0.009	<i>Pln</i>	-1.309	-0.384	-0.349
<i>Mshn</i>	-1.041	0.382	0.377	<i>Nox1</i>	-1.313	-0.256	0.207
<i>LOC360228</i>	-1.041	0.559	0.231	<i>Me1</i>	-1.324	-0.443	-0.264
<i>Kcnj5</i>	-1.043	-1.062	-0.766	<i>Cac4</i>	-1.334	-0.688	-0.715
<i>Car3</i>	-1.045	0.278	-0.263	<i>LOC682360</i>	-1.335	-0.459	-0.314
<i>Slpi2</i>	-1.050	0.102	0.425	<i>Sln</i>	-1.360	-0.649	-0.410
<i>Ca2</i>	-1.055	-0.551	-0.087	<i>RGD1563231</i>	-1.370	-0.170	-0.580
<i>Rgs2</i>	-1.059	-0.856	-0.760	<i>Slpi</i>	-1.378	0.188	0.552
<i>Mfsd2a</i>	-1.059	-0.455	-0.262	<i>RGD1562127</i>	-1.382	-0.687	-0.360
<i>Tnfrsf12a</i>	-1.059	-0.600	-1.353	<i>Sgcg</i>	-1.386	-0.884	-0.543
<i>Car8</i>	-1.060	-0.525	-0.339	<i>LOC685106</i>	-1.390	0.215	-0.613
<i>Mybpc2</i>	-1.064	-0.459	-0.353	<i>Hdc</i>	-1.393	-0.281	-0.076
<i>Gent3</i>	-1.071	-0.549	-0.183	<i>Retnlb</i>	-1.420	1.223	1.939
<i>Mrap</i>	-1.072	-0.045	-0.363	<i>RGD1565374</i>	-1.430	-0.901	-0.378
<i>Tmprss9</i>	-1.077	-1.024	-0.582	<i>Apobec2</i>	-1.436	-0.613	-0.243
<i>Gldn</i>	-1.085	-0.522	-0.161	<i>Sypl2</i>	-1.457	-0.801	-0.867
<i>Hsd3b6</i>	-1.086	-0.518	-0.228	<i>Fcer1a</i>	-1.497	-0.248	-0.283
<i>Casq1</i>	-1.086	-0.436	-0.282	<i>Hsd17b6</i>	-1.503	-0.988	-0.325
<i>RGD1560314</i>	-1.089	-0.959	-0.119	<i>RGD1565970</i>	-1.509	-0.198	0.413
<i>RGD1309651</i>	-1.094	-0.426	-0.103	<i>Tph1</i>	-1.518	-0.642	-0.305
<i>Ly49i2</i>	-1.102	-0.604	-1.177	<i>Cfb</i>	-1.551	0.167	0.560
<i>LOC363060</i>	-1.108	-0.829	-0.616	<i>Slfn3</i>	-1.558	0.166	0.249
<i>LOC688635</i>	-1.113	-0.189	-0.038	<i>Retnlg</i>	-1.560	-0.018	0.509
<i>Kiflc</i>	-1.125	-0.943	-0.701	<i>LOC679045</i>	-1.562	-0.216	-0.234
<i>LOC687842</i>	-1.126	-0.830	-0.489	<i>Cma1</i>	-1.583	-0.288	0.256
<i>Scx</i>	-1.131	-0.368	-0.413	<i>Cpa3</i>	-1.609	-0.258	-0.115
<i>Slc13a1</i>	-1.134	-0.585	-0.301	<i>Retnla</i>	-1.627	0.878	1.162
<i>Ccr10</i>	-1.137	0.036	0.419	<i>Cyp4f1</i>	-1.634	-0.582	0.169
<i>Mzb1</i>	-1.140	0.064	0.772	<i>Npc1l1</i>	-1.662	-1.294	-0.707
<i>Ms4a2</i>	-1.144	-0.127	0.012	<i>Tff1</i>	-1.663	-0.786	-0.310
<i>Rup2</i>	-1.145	-1.201	-0.220	<i>Aif3</i>	-1.681	-1.015	-1.715
<i>Rrad</i>	-1.150	-0.320	-0.193	<i>Fabp1</i>	-1.682	-0.780	-0.074
<i>Fbln7</i>	-1.156	-0.540	-0.398	<i>Tnfrsf17</i>	-1.690	-0.139	0.572
<i>Bpifb6</i>	-1.156	-0.825	-0.560	<i>Spink4</i>	-1.714	-2.376	-1.237
<i>Cd55</i>	-1.162	-0.438	-0.237	<i>LOC100360169</i>	-1.718	0.232	1.101
<i>F2</i>	-1.165	-0.640	-0.315	<i>Ros1</i>	-1.733	-1.914	-1.672
<i>Fabp4</i>	-1.170	-0.233	-0.334	<i>Thrsp</i>	-1.747	-0.067	-0.380
<i>Myom2</i>	-1.171	-0.772	-0.257	<i>RatNP-3b</i>	-1.761	-2.005	-0.576
<i>RGD1305928</i>	-1.180	-0.851	-0.388	<i>RGD1564563</i>	-1.793	-0.383	-0.092
<i>Slc18a1</i>	-1.184	-0.791	-0.599	<i>Mcpt8</i>	-1.824	-0.324	0.315
<i>Rgs1</i>	-1.184	-0.675	-0.604	<i>Mcpt8l3</i>	-1.824	-0.462	0.151
<i>Dapl1</i>	-1.196	-0.753	-0.375	<i>Adipoq</i>	-1.826	0.053	-0.265

<i>Tmem22</i>	-1.199	-0.583	-0.419	<i>Mcpt9</i>	-1.834	-0.384	0.240
<i>Ccl12</i>	-1.212	0.206	0.243	<i>Mcpt4</i>	-1.952	0.233	0.296
<i>Siglec1</i>	-1.213	0.130	0.102	<i>Mcpt1</i>	-1.966	-0.014	0.285
<i>Fcgr3a</i>	-1.230	-0.225	0.136	<i>Mcpt10</i>	-1.985	-0.523	0.192
<i>LOC686141</i>	-1.230	-0.200	0.140	<i>LOC100359793</i>	-1.987	-0.141	1.137
<i>LOC683753</i>	-1.232	-0.795	-0.121	<i>Scd1</i>	-1.998	0.138	-0.400
<i>Bhlha15</i>	-1.233	-0.496	-0.067	<i>Mcpt8l2</i>	-2.003	-0.532	0.196
<i>LOC688507</i>	-1.238	-0.184	0.110	<i>Pga5</i>	-2.086	-1.394	-0.775
<i>H19</i>	-1.242	-0.227	-0.317	<i>Gkn2</i>	-2.099	-1.000	-1.584
<i>Cxcl11</i>	-1.260	-0.859	-0.267	<i>Mcpt114</i>	-2.170	-0.112	0.239
<i>Rgs4</i>	-1.262	-0.570	-0.720	<i>RGD1562035</i>	-2.179	-0.248	0.176
<i>Ces2c</i>	-1.269	-0.944	-0.445	<i>Pzp</i>	-2.223	-1.221	-1.117
<i>Fut9</i>	-1.270	-0.997	-0.334	<i>Fcrls</i>	-2.342	-0.529	0.208
<i>Srpk3</i>	-1.272	-0.242	-0.042	<i>Bpifb2</i>	-2.508	-1.532	-1.300
<i>RGD1560949</i>	-1.273	-1.471	-1.135	<i>Mcpt2</i>	-2.545	-0.290	0.255
<i>Npw</i>	-1.276	-0.777	-0.406	<i>Tac4</i>	-2.766	-1.731	-1.279
<i>Rbp4</i>	-1.283	-0.026	-0.205				

Article 6

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

Malen Massot-Cladera, Àngels Franch,
Francisco J Pérez-Cano, Margarida Castell

Sotmés a publicació

Els resultats del present article han permès la presentació de la comunicació:

- *Intestinal IgA and IgM concentrations are modified in rats by a diet rich in polyphenols and fibre.* 3rd International Lymphoid Tissue Meeting (LT3), Rotterdam (Països Baixos), Setembre 2013.

RESUM

Objectiu: Avaluar els efectes de la ingesta de cacau sobre la producció d'IgA i IgM als compartiments mucosals intestinal i extraintestinal. A més, esbrinar la contribució de la fibra de cacau a aquests efectes.

Material i mètodes: L'estudi es va realitzar en rates de 3 setmanes d'edat de la soca Wistar alimentades durant tres setmanes amb una dieta estàndard, una dieta enriquida amb un 10% de cacau (que conté polifenols i fibra de cacau), amb una dieta a base de fibra de cacau amb baix contingut de polifenols (<0,2%) o bé amb una dieta elaborada amb inulina. La concentració d'IgA i IgM es va quantificar al final de l'estudi en teixits mucosals intestinals (plaques de Peyer, ganglis limfàtics mesentèrics i rentat intestinal) i extraintestinals (glàndules salivals: submaxil·lar i paròtide) i en mostres sèriques per ELISA. Després de la intervenció nutricional es va avaluar l'expressió gènica per tècniques de PCR a temps real de molècules implicades en la producció d'IgA i reclutament mucosal en la glàndula submaxil·lar.

Resultats: La dieta de cacau va disminuir la concentració d'IgA i IgM a nivell intestinal. La dieta de fibra de cacau va inhibir la producció i secreció d'IgA de forma menys potent que la dieta de cacau en alguns compartiments intestinals (rentats intestinals) mentre que, en altres, va exercir un efecte contrari i va incrementar la concentració d'IgA (plaques de Peyer i ganglis limfàtics mesentèrics). Pel que fa a nivell extraintestinal, la dieta de cacau va disminuir la concentració d'IgA i IgM mentre que la dieta de fibra de cacau únicament va reduir la concentració d'IgA. La dieta de fibra de cacau, a diferència de la dieta de cacau al 10%, incrementa la concentració sèrica d'IgA. D'altra banda, la dieta de cacau modula l'expressió gènica de molècules

involucrades en la síntesi d'IgA a nivell extraintestinal mentre que la dieta de fibra de cacau ho fa sobre les implicades en el reclutament mucosal i transcitosi a través de la glàndula salival.

Conclusió: La fibra de cacau no és la principal responsable de l'efecte inhibidor del cacau sobre la concentració d'IgA i IgM observada a nivell intestinal i extraintestinal. De fet, la dieta de fibra de cacau exerceix, en alguns compartiments, l'efecte contrari i augmenta la concentració d'IgA.

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

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Running title: Changes in mucosal Ig production by cocoa

Keywords: cocoa fibre; cocoa polyphenols; immunoglobulin; MALT

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, Wistar rats were fed either a standard diet, a diet containing 10% 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 both 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 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 β 1 (TGF- β 1), 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.

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 (main study). In addition, taking into account the obtained results, a similar design but with just one week of diet duration was executed (confirmatory study).

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).

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-Butylhydroquinone	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.

Sample collection and preparation

At the end of the third week (main study) or one week (confirmatory study) of 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 RNeasy® (Qiagen, Crawley, UK), incubated at 4 °C overnight and stored at -20 °C 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 A₂₆₀/A₂₈₀ 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* (Rn00566655_m1, I). 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 $\pm$ 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).

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.

In MLN, the C10 intake resulted only in a significant IgM reduction in comparison with those in the REF, CF and I groups ($P<0.05$) (Fig. 1(c) and (d)). 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 ($P<0.05$). The I diet did not significantly modify the IgA and IgM concentration in MLN.

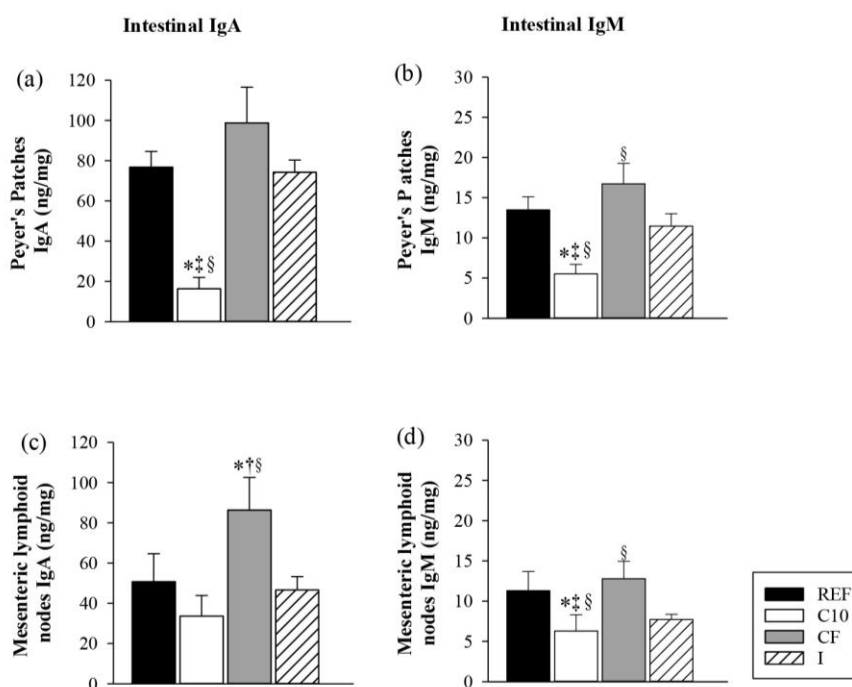


Fig. 1. Effects of cocoa diet on IgA and IgM content in the inducible intestinal compartments after three weeks of diets. IgA (a) and IgM (b) concentration in Peyer's patches, and IgA (c) and IgM (d) concentration in mesenteric lymph nodes. Values are mean (n 10) with their standard errors represented 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) ($P<0.05$).

As the I diet did not significantly modify either IgA or IgM in any of the studied compartments at the end of the main study (Fig. 1), the confirmatory study was restricted to comparing the C10 and CF diets with that of REF. In this context, the same changes on PP IgA and IgM concentrations were already observable after just one week of nutritional intervention with the C10 diet ($P<0.05$) (Table 2), whereas no statistical differences on MLN IgM or IgA were observed in the short-term study. The CF diet already increased MLN IgA and IgM after one week of diet when compared to the REF-fed animals ($P<0.05$).

After 1-week intervention							
		REF		C10		CF	
		IgA	IgM	IgA	IgM	IgA	IgM
Intestinal immune compartments							
<i>Inductor sites:</i>	PP	16.18 ± 1.88	38.59 ± 6.20	7.46 ± 1.66* [‡]	16.68 ± 4.83* [‡]	19.71 ± 2.00	41.98 ± 3.35
	MLN	14.54 ± 2.70	29.55 ± 4.47	21.65 ± 6.29	34.79 ± 10.39	25.33 ± 2.95*	40.42 ± 2.88*
<i>Effector site:</i>	GW	4.32 ± 0.35	1.54 ± 0.23	1.40 ± 0.06* [‡]	1.33 ± 0.10	4.21 ± 0.75	1.40 ± 0.10
Extra-intestinal immune compartments							
<i>Effector sites:</i>	SMG	12.78 ± 1.10	15.19 ± 4.11	2.18 ± 0.27* [‡]	2.46 ± 0.95*	6.70 ± 0.37*	6.11 ± 1.70
	PSG	17.13 ± 2.84	19.77 ± 0.51	4.64 ± 0.33* [‡]	10.62 ± 2.12*	7.97 ± 1.21*	16.25 ± 3.90

Table 2. Effects of cocoa diet on IgA and IgM content in the inductor (Peyer's patches (PP) and mesenteric lymph nodes (MLN)) and effector (gut wash (GW)) intestinal and extraintestinal (submaxillary salivary gland (SMG) and parotid salivary gland (PSG)) compartments after one week of diets. Values are mean (n 5) 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) ($P<0.05$).

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)). 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.

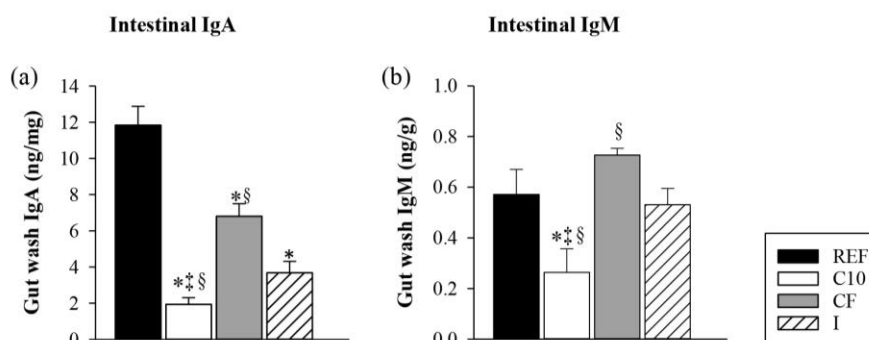


Fig. 2. Effects of cocoa diet on IgA and IgM content in the effector intestinal compartments after three weeks of diets. IgA (a) and IgM (b) concentration in gut washes. Values are mean (n 10) with their standard errors represented by vertical bars. *Mean value was significantly different from that of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

IgA and IgM concentrations in the small intestine were also determined after only one week of the REF, C10 and CF diets. Only the C10-fed animals already showed lower IgA content in GW after one week of nutritional intervention ($P<0.05$) (Table 2).

Immunoglobulin content in the effector extra-intestinal mucosal compartments

Regarding the salivary glands, a similar pattern was observed in the SMG and PSG for both Ig (Fig. 3). The C10 diet significantly reduced the IgA and IgM content in both salivary glands compared to the rest of the diets ($P<0.05$). The CF diet also reduced IgA concentration in both salivary glands in a more modest way in comparison to the REF diet (Fig. 3(a) and (c)), without affecting the IgM concentration (Fig. 3(b) and (d)). No changes in IgA and IgM were observed in the salivary glands after I diet intake.

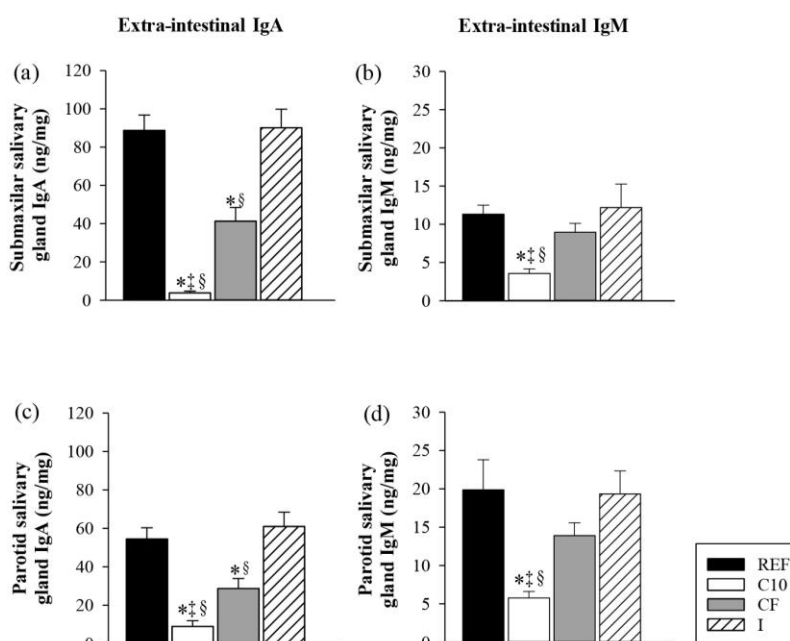


Fig. 3. Effects of cocoa diet on IgA and IgM content in the effector extra-intestinal mucosal compartments after three weeks of diets. IgA (a) and IgM (b) concentration in the submaxillary gland and IgA (c) and IgM (d) concentration in the parotid gland. Values are mean (n 10) with their standard errors 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) ($P<0.05$).

IgA and IgM were also determined in both salivary glands in animals fed with the REF, C10 and CF diets for 7 days. This period was enough time for both diets to down-regulate IgA in SMG and PSG compared to the REF group ($P<0.05$) (Table 2). The C10-fed animals also showed lower concentrations of IgM in both SMG and PSG glands in comparison with the REF group ($P<0.05$) (Table 2).

Systemic immunoglobulins

Serum concentrations of IgA, IgM and IgG from all experimental groups at the end of the main study 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)).

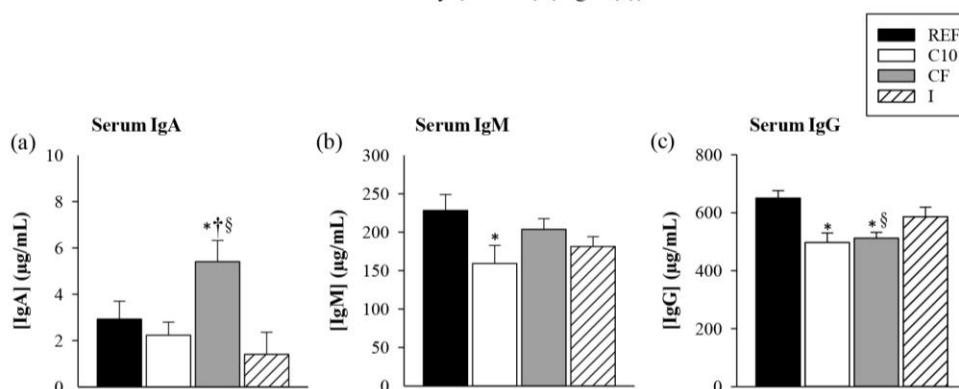


Fig. 4. Effects of cocoa diet on serum IgA (a), IgM (b) and IgG (c) concentrations after three weeks of diets. Values are mean (n 10) with their standard errors represented by vertical bars. *Mean value was significantly different from that of the reference (REF) group ($P<0.05$); †Mean value was significantly 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- β 1, RAR α , RAR β , 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- β 1 gene expression has been observed in the C10 diet. On the other hand, the CF diet fed animals showed significantly lower expression of TGF- β 1 and RAR α 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.

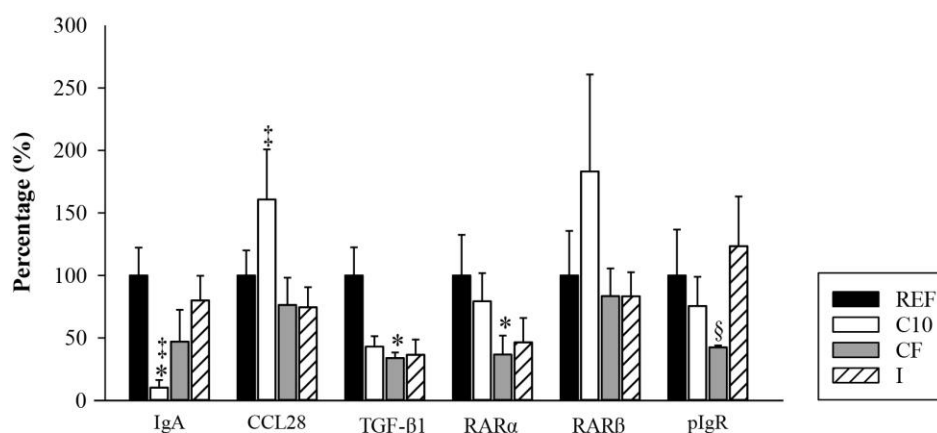


Fig. 5. Expression of genes associated with IgA synthesis, secretion, switching, intestinal homing in submaxillary salivary gland after three weeks of diets. Expression levels were normalized using the expression of *Gusb* as the endogenous housekeeping gene. Values are mean (n 5-6) with their standard 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 (main study) were already significant after just one week of the diet (confirmatory study). 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 at the end of the main study, 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- β 1. Although IL-6 mRNA was not detected in the SMG, the TGF- β 1 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 RAR α and RAR β in the salivary gland we found that the C10 intake for three weeks did not modify either the expression of RAR α or RAR β 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 RAR α but not RAR β , 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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The authors' contributions were as follows: M.M.-C., À.F., F.J.P.-C and M.C. conceived and designed the research; M.M.-C., F.J.P.-C. and M.C. carried out the experiments, the data analysis and were involved in the interpretation of the data; M.M.-C. contributed to the initial draft of the manuscript; À.F., F.J.P.-C and M.C. contributed to the critical revision of the manuscript; M.C. has primary responsibility for the final content. All authors have read and approved the final version of the manuscript for publication.

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Article 7

“Association between cocoa diet effects on rats’ body weight, microbiota and intestinal immunity and the urine metabolic profile”

Malen Massot-Cladera, Jordi Mayneris-Perxachs, Adele Costabile, Jonathan R. Swann, Àngels Franch, Francisco J Pérez-Cano, Margarida Castell

Sotmés a publicació

Els resultats del present article han permès la presentació de la comunicació:

- *Metabolic impact of cocoa diet on urinary metabolic profiles of rats.* 2nd International Congress on chocolate and Cocoa in Medicine (ISCHOM), Barcelona (Espanya), Setembre 2015.
- *Relationship between the urine metabolic profile and the effects of cocoa polyphenols and fiber on microbiota and immunity.* VII Workshop Probióticos, Prebióticos y Salud. Evidencia Científica, Sevilla (Espanya), Gener 2016.

RESUM

Objectiu: Es pretén establir, en animals que han rebut una dieta rica en cacau, la relació entre el perfil metabòlic en orina, els efectes del cacau sobre el pes corporal, les hormones metabòliques, la resposta immunitària intestinal i la composició de la microbiota. A més, esbrinar la contribució de la fibra de cacau a aquests efectes.

Material i mètodes: L'estudi es va realitzar en rates de 3 setmanes d'edat de la soca Wistar alimentades amb una dieta enriquida amb un 10% de cacau (que conté polifenols i fibra de cacau), amb una dieta a base de fibra de cacau amb baix contingut de polifenols (<0,2%) o bé amb una dieta elaborada amb inulina. A les dues setmanes de la intervenció nutricional, es va recollir orina de 24 h per determinar el perfil metabòlic mitjançant espectrometria de ressonància magnètica nuclear. En mostres obtingudes a les tres setmanes de la intervenció nutricional es va quantificar la concentració d'IgA en femtes per ELISA i la concentració plasmàtica d'hormones metabòliques per immunodetecció de tipus Luminex. Es va realitzar un estudi de correlació entre el pes corporal, les hormones metabòliques, la IgA fecal, la composició de la microbiota i els metabòlits detectats en orina.

Resultats: El consum de cacau i de fibra de cacau va modificar de forma diferencial el perfil metabòlic en orina pel que fa a metabòlits del cacau i el metabolisme energètic i d'aminoàcids. La dieta amb cacau va disminuir el contingut fecal d'IgA i la concentració plasmàtica de glucagó i GLP-1. Pel contrari, la dieta de fibra de cacau va incrementar la concentració plasmàtica de leptina. Tot i que les dues dietes van augmentar la concentració de grelina, la dieta de cacau va

provocar un increment més pronunciat. La concentració de grelina es correlaciona inversament amb la presència de *Bifidobacterium* spp., el grup *Clostridium histolyticum*/*C. perfringens*, la proporció de *Firmicutes*/*Bacteroidetes* i la concentració d'IgA en femtes. Existeix una correlació inversa entre el pes corporal i la concentració de grelina, mentre que apareix una correlació directa entre el pes corporal i les concentracions plasmàtiques de glucagó i leptina, la presència de *Bifidobacterium* spp. i *Streptococcus* spp., la relació *Firmicutes*/*Bacteroidetes* i la concentració d'IgA en femtes. La concentració d'IgA en femtes es correlaciona positivament amb la proporció de *Streptococcus* spp. D'altra banda, hi ha una correlació entre pes corporal, hormones metabòliques, IgA fecal i composició de la microbiota amb alguns dels metabòlits derivats del cacau detectats en orina derivats tant del metabolisme bacterià com de l'hoste.

Conclusió: La ingesta de cacau modifica el metabolisme energètic i d'aminoàcids tant bacterià com del propi hoste donant lloc a un perfil metabòlic diferencial, el qual pot ser usat com a marcador d'ingesta. A més, es pot establir una relació entre aquest perfil i les variables de pes corporal, hormones metabòliques i contingut fecal d'IgA.



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La **Dra Margarida Castell Escuer** i el **Dr. Francisco J Pérez Cano**, directors de la Tesi Doctoral elaborada per **Malen Massot Cladera**, amb el títol "**Efecte dels components bioactius del cacau sobre la microbiota i el sistema immunitari intestinal en rata**"

INFORMEN que l'article titulat "**Association between cocoa diet effects on rat's body weight, microbiota and immunity and their urine metabolomic profile**" el qual forma part de la Tesi Doctoral anteriorment esmentada, ha estat elaborat en coautoria amb el Dr. Jordi Mayneris-Perxachs del Imperial College London (UK). La participació d'ambdós autors ha estat la següent:

- Malen Massot Cladera s'ha encarregat, juntament amb els directors de la Tesi, del disseny experimental, elaboració de les dietes, seguiment dels animals, registres durant l'estudi i obtenció de mostres. A més, s'ha encarregat dels experiments i de la interpretació de les dades obtingudes a través de l'anàlisi de l'expressió gènica així com l'anàlisi de les variables immunològiques i metabòliques.
- Ambdós autors han realitzat els experiments relacionats amb l'estudi metabolòmic
- El Dr. Jordi Mayneris Perxachs ha estat l'encarregat de l'anàlisi de les dades obtingudes de l'estudi metabolòmic i d'elaborar les correlacions estudiades.
- Ambdós autors han contribuït de igual manera a l'elaboració del primer esborrany del manuscrit.

A més, els directors **INFORMEN** que l'article "**Association between cocoa diet effects on rat's body weight, microbiota and immunity and their urine metabolomic profile**" no constitueix part de cap altre Tesi Doctoral.

Barcelona, a 19 d'octubre de 2015

Signat:

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Association between cocoa diet effects on rats' body weight, microbiota and intestinal immunity and the urine metabolic profile

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Abbreviation list

α KMV, α -keto- β -methyl-*n*-valerate; **α KIC**, α -ketoisocaproate; **1-MX**, 1-methylxanthine; **2-HIB**, 2-hydroxyisobutyrate; **2-OG**, 2-oxoglutarate; **3-HIB**, 3-hydroxyisobutyrate; **3-IS**, 3-indoxyl-sulfate; **3-MX**, 3-methylxanthine; **4-GB**, 4-guanidinobutanoic acid; **-CS**, 4-cresol sulfate; **4-CG**, 4-cresol glucuronide; **HMB**, β -hydroxy- β -methylbutyrate; **4-HPA**, 4-hydroxypropionic acid; **7-MX**, 7-methylxanthine; **BCAA**, branched-chain amino acids; **C10**, 10% cocoa diet; **CF**, cocoa fiber diet; **DF**, dietary fiber; **DMA**, dimethylamine; **DMG**, dimethylglycine; **DMU**, dimethyluric acid; **GLP-1**, glucagon-like peptide-1; **IAA**, indole-3-acetic acid; **NAG**, *N*-acetylglycoprotein; **NMN**, nicotine mononucleotide; **NMNA**, *N*-methyl-nicotinic acid; **NMND**, *N*-methyl-nicotinamide; **OPLS-DA**, Orthogonal projection to latent structures-discriminant analysis; **PAG**, phenylacetyl-glycine; **REF**, Reference diet; **RD**, recycle delay; **SCFA**, short chain fatty acids; **TMAO**, trimethylamine *N*-oxide **TSP**, 3-trimethylsilyl-1-[2,2,3,3-²H₄] propionate.

Keywords: Cocoa / Hormones / IgA / Metabonomic / Microbiota

Abstract

Scope: The aim of this study was to elucidate the relationship between the urine metabolic fingerprint and the effects of cocoa and cocoa fiber on body weight, hormone metabolism, intestinal immunity and microbiota composition.

Methods and results: Wistar rats were fed, for three weeks, a diet containing 10% cocoa (C10), or two other diets with same proportion of soluble fiber: one based on cocoa fiber (CF) and another containing inulin as a reference diet (REF). Twenty-four hour urine samples were analyzed by an untargeted ^1H -NMR metabonomic approach. Concentration of fecal IgA and plasma metabolic hormones were also quantified. The C10 diet decreased the intestinal IgA, plasma GLP-1 and glucagon concentrations and increased ghrelin levels compared to the REF group. Clear differences were observed between the urines from the C10 group and those from the CF group ($Q^2Y=0.89; p=0.001$). Some urine metabolites correlated with the cocoa effects on body weight, immunity and microbiota.

Conclusions: These results indicate that mainly whole cocoa intake alters the host and bacterial metabolism concerning energy and amino acid pathways leading to a specific metabolic fingerprint that can be used as consumption markers. This metabolic profile correlates with body weight, metabolic hormones, intestinal immunity and microbiota composition.

1 Introduction

Cocoa is considered a great source of bioactive compounds such as polyphenols and dietary fiber (DF), which have been attributed consistent positive health effects [1–5]. Cocoa contains monomeric flavonoids such as the flavanols (+)-catechin and (-)-epicatechin, and mainly its oligomers and polymers known as procyanidins. These polymeric compounds are able to pass intact through the small intestine and to reach the colon [6], where they are metabolized by the intestinal microbiota. This conversion is crucial for their absorption and also for the generation of new compounds which are biologically more active than the original ones [6–8]. Thus, there is a growing body of evidence on the reciprocal relationship between bacteria and polyphenols that may help understand the documented benefits of polyphenols consumption [9]. In fact, it has been extensively reported that both dietary polyphenols, including those from cocoa, and the corresponding microbially-derived phenolic metabolites modulate the gut microbiota composition in *in vitro*, *in vivo* and clinical studies [8,10–13].

Similar ability to modulate the gut microbiota composition has been attributed to the DF, which has been described to indirectly modulates the short chain fatty acids (SCFA) production [14–18]. Recently, cocoa fiber-enriched intake in rats has been associated with the modulation of the gut microbiota and the SCFA production [17]. The DF fraction in cocoa is mainly rich in cellulose, followed by highly fermented pectic substances and hemicellulose, which is less fermentable than the former [19].

After microbial transformation, the cocoa metabolites either from flavonoids or cocoa fiber (i.e. SCFA) are absorbed into the bloodstream, providing another source of potentially bioactive compounds [20,21]. The microbial metabolites from flavanols, are mainly metabolized by liver phase-II enzymes to conjugated derivatives that are subsequently eliminated in urine [7].

Previous preclinical studies carried out in our laboratory have evidenced that cocoa, cocoa flavanols and cocoa fiber modify some aspects of the intestinal and systemic immune response [2,13,17,22]. On the other hand, a diet with cocoa, but not with its flavonoids or fiber, is able to reduce the body weight gain [13,17,23]. Given that this effect on weight is not associated with a lower chow intake, it is necessary to go deep into the mechanism involved in such effect.

Therefore, the aim of this study was to elucidate the relationship between the urine metabolic fingerprint by ^1H NMR spectroscopy and the effects of cocoa on body weight, hormone metabolism, intestinal immunity and microbiota composition. The particular involvement of cocoa fiber in such effects has also been studied.

2 Materials and methods

2.1 Animals and diets

Female Wistar rats (3-week-old) were obtained from Janvier (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 three dietary groups: cocoa (C10), cocoa fiber (CF) and reference (REF) groups ($n=10$ /each). The C10 group received chow containing 10% cocoa that finally provided a 0.4% of polyphenols, 0.85% soluble fiber and 2.55% of insoluble fiber; the CF group received a diet with the same cocoa soluble and insoluble fiber proportions as the C10 group but with a very low amount of polyphenols ($<0.02\%$); and the REF group received the same amount of fiber as the C10 group, being the soluble portion (0.85%) as inulin in order to distinguish the particular effect of cocoa fiber. Natural Forastero cocoa and cocoa fiber 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 fiber. The three experimental diets were elaborated on basis of the AIN-93M formula by subtracting the amount of carbohydrates, proteins, lipids and insoluble fiber provided by the corresponding supplement. The three resulting diets were isoenergetic and had the same proportion of macronutrients (carbohydrates, proteins and lipids) and fiber as has been previously reported [17]. Animals were given free access to water and food. The diets lasted for three weeks.

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).

2.2 Sample collection and processing

The 24 h urine samples were collected, by means of metabolic cages, at 15 days after beginning the nutritional intervention. Centrifuged urines were kept at $-80\text{ }^{\circ}\text{C}$ until analysis. Moreover, blood samples were collected after three weeks and plasma was kept at $-80\text{ }^{\circ}\text{C}$ prior to metabolic hormones determination. Fecal samples were also collected at the third week of diet and the homogenates were obtained as previously described [17] and frozen at $-20\text{ }^{\circ}\text{C}$ until analysis.

2.3 Sample preparation for ^1H NMR analysis

Urine samples were defrosted and prepared for ^1H NMR spectroscopy by combining 400 μL of sample with 200 μL of phosphate buffer (pH 7.4; 100% D_2O) containing 1 mM of 3-trimethylsilyl-1-[2,2,3,3- $^2\text{H}_4$] propionate (TSP) as an external standard and 2 mM sodium azide as a bactericide. Samples were vortexed and particles were removed by centrifugation (13000 g for 10 min) prior to transferring 550 μL to a 5 mm NMR tube. Standard one-dimensional ^1H NMR spectra of the urine samples were acquired on a 500 MHz Bruker NMR spectrometer using a standard noisy experiment incorporating a pre-saturation pulse to attenuate the water signal. This experiment consisted of [recycle delay (RD)-90 $^\circ$ - t_1 -90 $^\circ$ - t_m -90 $^\circ$ -acquire free induction decay]. The water signal was suppressed by irradiation during the RD of 2 s, with a mixing time (t_m) of 10 μs . The acquisition time was set to 2.91 s and the 90 $^\circ$ pulse length was 15.87 μs . For each sample, 8 dummy scans were followed by 128 scans and collected in 64K data points using a spectral width of 16 ppm. Prior to data analysis, NMR spectra were phased, corrected for baseline distortions and calibrated using the reference standard TSP. ^1H NMR spectra (δ 0.2-10.0) were digitized into consecutive integrated spectral regions (~20,000) of equal width (0.00055 ppm) using Matlab (Mathworks). The regions containing signals from urea (δ 5.5 – 6.0) and the residual water (δ 4-7 – 5.2) were removed to minimize baseline effects arising from imperfect water suppression. Chemical shift variation was minimized across the dataset by applying a recursive segment-wise peak alignment (RSPA) algorithm to each spectrum. Each spectrum was normalized to unit area to account for variation in sample concentration.

2.4 Quantification of metabolic hormones in plasma

Plasma concentrations of ghrelin, glucagon, glucagon-like peptide (GLP)-I and leptin were determined in plasma using the Bio-Plex ProTM Diabetes Assay (Bio-Rad, Madrid, Spain) according to the manufacturer's instructions. Analysis was carried out with the Bio-Plex[®] MAGPIXTM Multiplex Reader and the Bio-Plex Data ProTM software (BioRad) as in previous studies [24]. The limits of quantification can be found as Supporting information.

2.5 Fecal IgA quantification

The concentration of IgA in feces was quantified by ELISA following the manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, TX, USA). Absorbance was measured in a microplate photometer (LabSystems Multiskan) and data were interpolated using ASCENT version 2.6 software (Thermo Fisher Scientific, Barcelona, Spain) into the standard curves.

2.6 Statistical analysis

Statistical analysis for body weight, chow intake, fecal IgA and metabolic hormones was performed using the software package IBM SPSS Statistics 22.0 (SPSS, Inc. USA). Levene's and Kolmogorov–Smirnov tests were applied to assess variance equality and normal distribution, respectively. Conventional one-way ANOVA was performed when normal distribution and equality of variance existed. The Bonferroni test was applied when specific cocoa intake had a significant effect on the dependent variable. Non-parametric Mann–Whitney U and Wilcoxon tests were used in order to assess significance for independent and related samples, respectively.

Multivariate modeling was performed in Matlab using in-house scripts. This included principal components analysis using pareto scaled data and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) using a unit variance scaling approach. Pairwise OPLS-DA models were constructed to aid model interpretation and identify discriminatory metabolites between the study groups. Here, ^1H NMR spectroscopic profiles served as the descriptor matrix (X) and the experimental groups (REF, C10, CF) were used pairwise as the response variable (Y). Orthogonal signal correction filters were used to remove variation in the descriptor matrix unrelated to the response variable to assist model interpretation. Loading coefficient plots were generated by back-scaling transformation where covariance is plotted between the Y-response matrix and the signal intensity of the metabolites in the NMR data (X). These plots are colored based on the correlation coefficient (r^2) between each metabolite and the Y-response variable, with red indicating strong significance and blue indicating weak significance. The predictive performance (Q^2Y) of the model was calculated using a seven-fold cross validation approach and model validity was established by permutation testing (1.000 permutations).

Clustering analysis. Unsupervised hierarchical clustering analysis (HCA) was performed to identify general patterns of metabonomic variation between samples. To do so, we used the normalized levels of metabolites identified to contribute to class separation through the OPLS-DA models. For comparative analysis across different metabolites, data were standardized as z-scores across samples for each metabolite before clustering, so that the mean is 0 and the standard deviation is 1. This standardized matrix was subsequently used in unsupervised HCA for samples and metabolites using Euclidean distance and average linkage, by means of the `pdist` and `linkage` functions in the MATLAB bioinformatics toolbox. Heatmaps and dendrograms following HCA were generated with MATLAB `imagesc` and `dendrogram` functions, respectively. In the heatmaps, a red-blue color scale is used so that shades of red and blue represent higher and lower values, respectively, compared

with the mean. Different diet groups are color-coded and shown under the dendrogram for each sample.

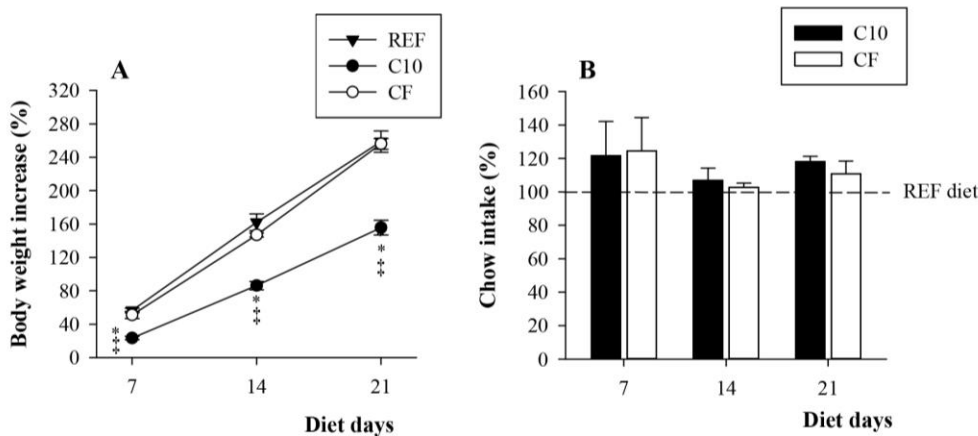
Correlation analysis. To explore the functional correlation between the changes on body weight, metabolism and intestinal immunity,metabonome perturbations and gut microbiome., Spearman's correlation analyses were performed on all the samples grouped together. The Benjamini-Hochberg method was used to adjust *p*-values for multiple testing considering a 5% false discovery rate (FDR).

3 Results

3.1 Body weight and food intake

Body weight and food intake were monitored weekly throughout the study (**Fig. 1**). Initially, no differences in body weight were observed between groups (mean (g) $\pm$ SEM, REF: 43.7 $\pm$ 1.1; C10: 45.4 $\pm$ 1.4; CF: 44.8 $\pm$ 1.1). However, from day 7, a lower body weight gain was observed in C10 animals compared to the other groups ($p < 0.05$) (**Fig. 1A**). This effect was not related to lower food intake, which was similar throughout the study among all experimental groups (**Fig. 1B**). No changes in body weight gain were found as a result of CF diet intake.

Figure 1. Body weight increase (%) compared to the baseline (A) and chow intake (%) compared to the REF diet which represents 100% (B) monitored throughout the nutritional intervention. Values are expressed as mean $\pm$ SEM (n=10). * $p < 0.05$ vs REF diet; and $^{\dagger}p < 0.05$ vs CF diet.



3.2 Metabolic hormones

The metabolic hormones quantified in plasma after three-week dietary intervention for all groups are summarized in **Table 1**. Both the C10 and CF diets increased the concentration of ghrelin compared to the REF group ($p < 0.05$). This increase was higher in the C10 group compared to the CF group ($p < 0.05$). Both diets also resulted in a lower plasma GLP-1 concentration in comparison with the REF group ($p < 0.05$). Moreover, the C10 diet reduced the glucagon concentration compared to the REF and CF diets ($p < 0.05$). The leptin concentration was not affected after the C10 diet but it was up-regulated as a result of the CF diet intake compared to the rest of the groups ($p < 0.05$) (**Table 1**).

Table 1. Metabolic hormones in plasma after three weeks of nutritional intervention. Results are expressed as mean $\pm$ SEM (n=7). * $p < 0.05$ vs REF diet; † $p < 0.05$ vs C10 diet and ‡ $p < 0.05$ vs CF diet.

	REF	C10	CF
Ghrelin (ng/mL)	30.57 $\pm$ 3.86	98.63 $\pm$ 18.73*‡	43.59 $\pm$ 4.27*
GLP-1 (pg/mL)	29.4 $\pm$ 16.4	7.4 $\pm$ 3.1*	4.2 $\pm$ 0.2*
Glucagon (pg/mL)	180.1 $\pm$ 25.3	100.4 $\pm$ 3.1*‡	166.2 $\pm$ 26.9
Leptin (pg/mL)	647.7 $\pm$ 135.5	335.4 $\pm$ 127.5	968.7 $\pm$ 177.6*†

3.3 Fecal IgA

The C10 diet intake resulted in a decrease in the IgA concentration compared to the others groups ($p < 0.05$) (**Fig. 2**). The CF diet did not produce any change in the IgA concentration, which was similar to that quantified in the REF group.

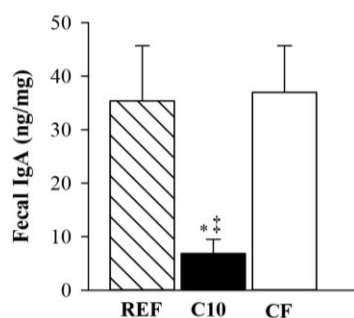


Figure 2. Fecal IgA concentration determined after three weeks of nutritional intervention. Results are expressed as mean $\pm$ SEM (n=9-10). * $p < 0.05$ vs REF diet; † $p < 0.05$ vs CF diet.

3.4 Urinary metabolic profile

An OPLS-DA model with strong predictive ability ($Q^2Y=0.93$; $p = 0.001$) was returned comparing the metabolic profiles from rats receiving the C10 diet and the REF diet (**Fig. 3A and 4**). Rats fed the C10 diet excreted **cocoa-derived metabolites** such as *N*-methylnicotinic acid (NMNA) and nicotine mononucleotide (NMN), catechin derivatives as well as methylxanthine metabolites, i.e. theobromine, xanthine, 1-methylxanthine (1-MX), 3-methylxanthine (3-MX), 7-methylxanthine (7-MX), imidazole, dimethyluric acid (DMU). In addition, urines from C10 group contained cocoa microbial-derived metabolites such as hippurate and 2-hydroxyisobutyrate (2-HIB), and 4-hydroxypropionic acid (4-HPA), phenylacetylglutamine (PAG) and indole-3-acetic acid (IAA) that could be derived from the monoamines contained in cocoa. Moreover, the urine from these rats contained taurine derived from the amino acid metabolism.

On the other hand, those animals receiving the C10 diet excreted lower amount of energy metabolism-related metabolites -acetone, citrate, 2-oxoglutarate (2-OG) and *N*-methylnicotinamide (NMND)- compared to the REF group. Other metabolites excreted in lower amounts were those related to amino acid metabolism by endogenous pathway such as α -keto-isocaproate (α KIC), α -keto-methylvalerate (α KMV), hydroxymethylbutyrate (HMB), 3-hydroxyisobutyrate (3-HIB) and glycine. Likewise, lower excretion of microbial-derived amino acid metabolites-3-indoxyl-sulfate (3-IS), 4-cresol sulfate (4-CS) and 4-cresol glucuronide (4-CG)- were found in C10 urines. In addition, metabolites related to choline metabolism -dimethylamine (DMA), dimethylglycine (DMG) and choline-, and metabolites related to dietary metabolism (sucrose, glucose, tartrate) were also reduced in C10 group. The C10 diet also resulted in a lower excretion of others metabolites such as sebacate, 4-guanidinobutanoate, creatinine, allantoin, and pseudouridine compared to the REF diet.

Regarding CF diet, a clear metabolic variation was observed in the urine from rats fed this diet compared to those fed the REF diet (**Fig. 3B and 4**; OPLS-DA model $Q^2Y=0.65$; $p = 0.001$). Cocoa-derived metabolites such as NMNA, NMN, theobromine, xanthine, 1-MX, 3-MX, 7-MX, and DMU were found in the urine of rats receiving the CF diet but not those receiving the REF diet. Moreover, rats following the CF diet excreted 2-HIB, IAA, citrate, acetone, NMND, sucrose, glucose, acetate and tartrate, which are related to microbial, energetic or dietary metabolism. Lower amounts of 4-HPA, hippurate, 3-IS, PAG, α KI, DMG, sebacate, 4-guanidinobutanoate, ethanol, creatinine, allantoin and pseudouridine were found in the CF group when compared to REF group.

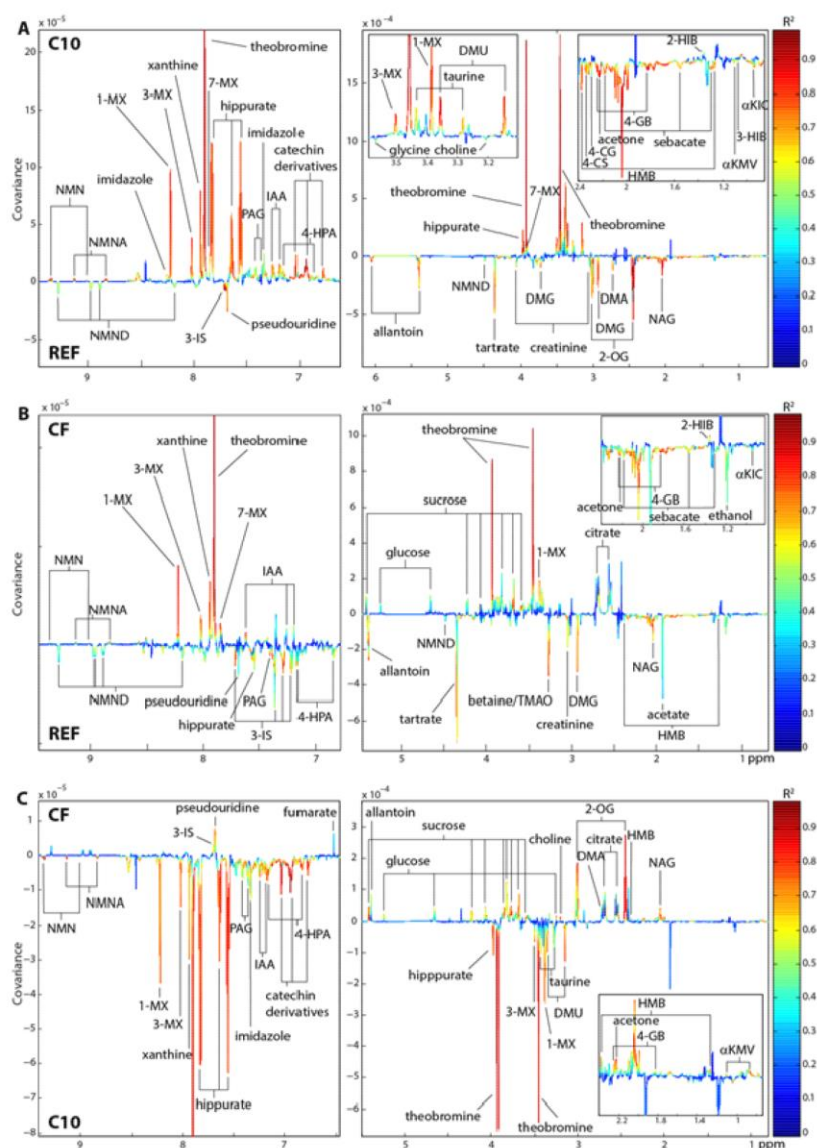


Figure 3. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) comparing the urinary metabolic profiles of rats receiving different dietary regimens. Coefficient plots extracted from the OPLS-DA models comparing rats receiving REF diet with C10 diet (A); REF diet with CF diet (B); and C10 diet with CF diet (C). α KMV, α -keto- β -methyl-*n*-valerate; α KIC, α -ketoisocaproate; 1-MX, 1-methylxanthine; 2-HIB, 2-hydroxyisobutyrate; 2-OG, 2-oxoglutarate; 3-HIB, 3-hydroxyisobutyrate; 3-IS, 3-indoxyl-sulfate; 3-MX, 3-methylxanthine; 4-GB, 4-guanidinobutanoic acid; -CS, 4-cresol sulfate; 4-CG, 4-cresol glucuronide; HMB, β -

hydroxy- β -methylbutyrate; **4-HPA**, 4-hydroxypropionic acid; **7-MX**, 7-methylxanthine; **C10**, 10% cocoa diet; **CF**, cocoa fiber diet; **DMA**, dimethylamine; **DMG**, dimethylglycine; **DMU**, dimethyluric acid; **IAA**, indole-3-acetic acid; **NAG**, *N*-acetylglucoprotein; **NMN**, nicotine mononucleotide; **NMNA**, *N*-methyl-nicotinic acid; **NMND**, *N*-methyl-nicotinamide; **PAG**, phenylacetylglutamine; **REF**, Reference diet; **TMAO**, trimethylamine *N*-oxide.

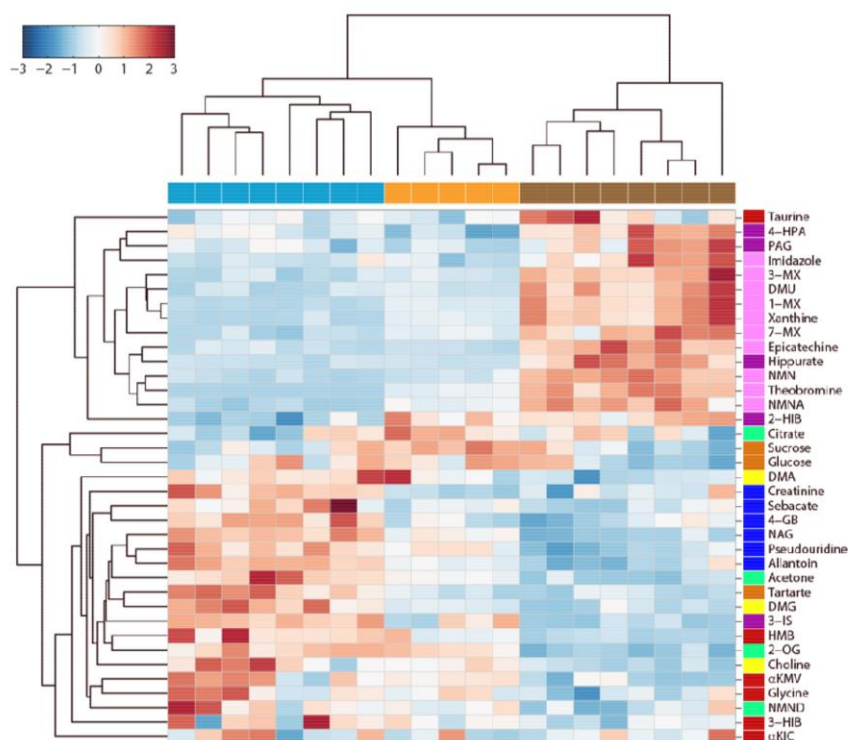
Finally, the OPLS-DA model contrasting the urinary metabolic phenotypes from rats receiving the C10 diet and those fed the CF diet (**Fig. 3C and 4**; $Q^2Y=0.89$; $p = 0.001$) also showed some clear differences. As expected, rats consuming the C10 diet excreted higher levels of cocoa- (NMNA, NMN), catechin- and methylxanthine- (theobromine, xanthine, 1-MX and 3-MX, imidazole, DMU) derivatives compared to those fed the CF diet. The C10 diet fed animals also excreted higher amounts of 4-HPA, hippurate, PAG, IAA and taurine. However, compared to CF group, the C10 diet fed animals eliminated lower amounts of 3-IS, acetone, citrate, 2-OG, NMND, α KMV, HMB, DMA, choline, sucrose, glucose, sebacate, 4-guanidinobutanoate, allantoin, pseudouridine and fumarate.

3.5 Correlations between urine metabolites and studied variables

The correlation analysis of the urine metabolic fingerprint with the effects of cocoa on metabolic hormones (ghrelin, GLP-1, glucagon, leptin), body weight, intestinal immunity (by means of intestinal IgA levels) and microbiota composition (reported previously [17,25]) was also studied (**Fig. 5**). Globally, whereas most of the studied variables were inversely correlated with cocoa metabolites, only the ghrelin concentration was positively correlated with the amount of these metabolites. In fact, the strongest correlations were found between ghrelin and NMNA, 1-MX, xanthine and theobromine. On the other hand, plasma glucagon concentration correlated inversely with these metabolites derived from cocoa, whereas it has a positive correlation with choline and other metabolites.

The body weight showed an inverse correlation with cocoa metabolites and a positive correlation with those from the amino acid metabolism. Similarly, fecal IgA concentration showed an inverse correlation with cocoa-derived metabolites whereas it was positively correlated with choline metabolites and allantoin.

Figure 4. Dendrogram and heatmap representation of unsupervised hierarchical clustering (HCA) of the metabonome for all rats. Each column corresponds to a single rat and each row corresponds to a specific metabolite. Metabolites identified to contribute to the separation between diets through OPLS-DA models were used for sample clustering. Metabolite z-score transformation was performed on the levels of each metabolite across samples, with blue denoting a lower and red a higher level compared to the mean. Metabolites and samples are clustered using correlation distance and average linkage and color coded by diet (*Brown*, cocoa diet; *Orange*, cocoa fiber; *Blue*, REF) or pathway (*Red*: aminoacid metabolism; *Purple*: microbial metabolism; *Orange*: dietary; *Green*: energy metabolism; *Yellow*: choline metabolism; *Blue*: miscellaneous; *Pink*: cocoa metabolites), respectively. HCA grouped the urinary metabolic profiles from the C10-fed animals together and distinct from the other studied animals. Profiles from animals receiving the CF diet clustered together and were separate from the REF diet.



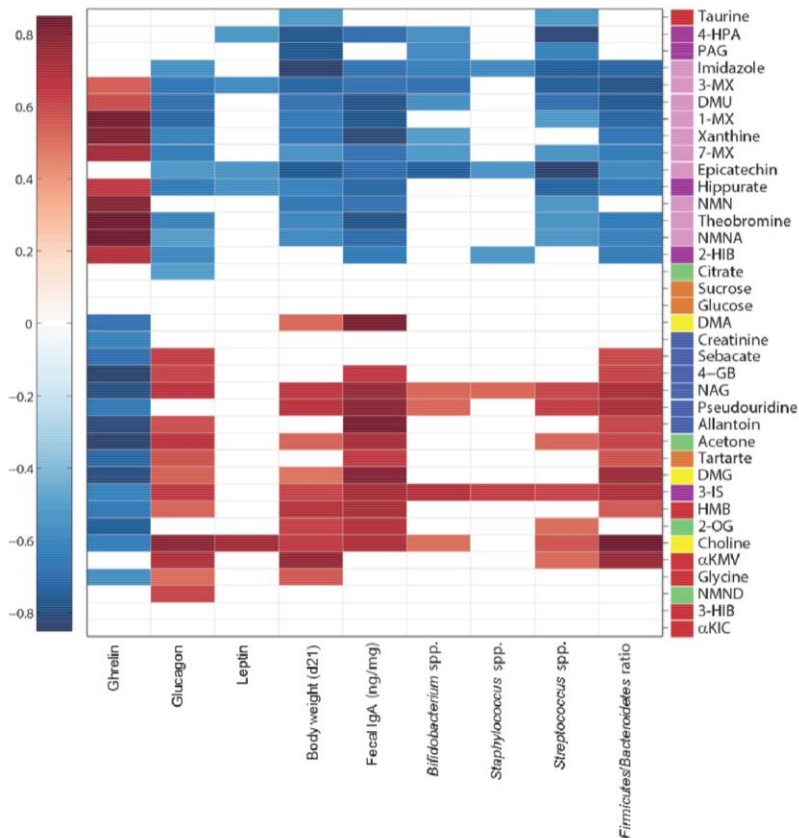
Urinary metabolites also correlated with the microbiota composition after both the C10 and CF diets intake that has been previously characterized [17]. Thus, the proportion of *Streptococcus* genus presented an inverse correlation with the amount of epicatechin (cocoa metabolite) and 4-

HPA (from the amino acid metabolism) in urine. In addition, when the *Firmicutes/Bacteroidetes* ratio was considered, a strong positive correlation was observed between its values and those from the choline-related metabolites, whereas it inversely correlated with metabolites derived from cocoa.

3.6 Other correlations between studied variables

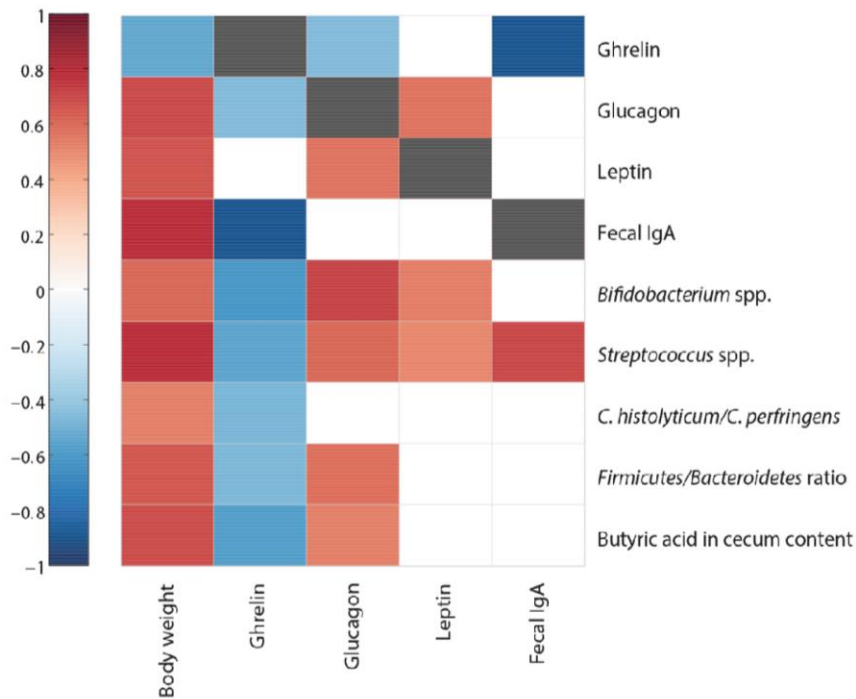
The correlation analysis between the effects of cocoa on the metabolic hormones, body weight, intestinal immunity and microbiota composition and functionality [17] has been also studied (Fig. 6).

Figure 5. Correlations between metabolites and responses. The intensity of the colors represents the degree of correlation, with red and blue indicating positive and negative correlations, respectively. Metabolites identified to contribute to the separation between diets through OPLS-DA models were to obtain the correlations. The order of metabolites is the same obtained from the unsupervised hierarchical clustering in Figure 4. Only significant correlations after applying a Benjamini and Hochberg procedure for controlling for a false discovery rate of 5% are shown. Correlation coefficients were based on Spearman’s correlation.



When samples from all the groups were considered together, the body weight correlated positively with both plasma concentrations of glucagon and leptin, but negatively with plasma ghrelin concentration. In addition, body weight showed a positive correlation with the fecal IgA content and the *Streptococcus* genus proportion, among others.

Figure 6. Correlations between body weight, metabolic hormones, intestinal immunity and microbiota composition and functionality. The intensity of the colors represents the degree of correlation, with red and blue indicating positive and negative correlations, respectively. Only significant correlations after applying a Benjamini and Hochberg procedure for controlling for a false discovery rate of 5% are shown. Correlation coefficients were based on Spearman's correlation



The metabolic hormones showed significant associations with the microbiota composition and functionality (**Fig. 6**). Particularly, the glucagon concentration was positively correlated with the fecal counts of *Bifidobacterium* spp., whereas the ghrelin concentration correlated inversely with *Bifidobacterium* spp. proportion and the cecal butyric acid concentration. Finally, fecal IgA was

positively associated with the fecal *Streptococcus* spp. counts and inversely with ghrelin concentration.

4 Discussion

We have previously reported that a 10% cocoa diet in rats produces a lower body weight increase, attenuates intestinal IgA secretion and modifies gut microbiota composition [13,17,25–28]. In the present study, we demonstrate that the cocoa intake produces some changes in metabolic hormones and it also results in a distinct urinary metabolic pattern. Moreover, correlations between urinary metabolites and those variables in which cocoa has an effect (i.e. body weight, metabolic hormones and intestinal immunity) were established, as well as among these modified variables. In addition, we have also correlated them with the cocoa effects on microbiota composition and functionality from a previous study [17]. The effects on the metabolic hormones and urinary metabolic pattern differed after the whole cocoa or the cocoa fiber intake, showing the relevance of other cocoa compounds in these effects.

As expected, the urinary metabolic profiles in the animals from the C10 group showed the excretion of cocoa-derived metabolites which is in line with previous controlled cocoa dietary intervention studies [29–31]. These metabolites include both the flavonoid and methylxanthine derivatives, some of them produced by the gut microbiota, reinforcing the fact that some of these cocoa compounds reach intact the colon, where are metabolized by intestinal bacteria [6,8]. However, these metabolites were found in a lower proportion in the urine of animals fed the cocoa fiber, confirming thus the lower concentrations of polyphenols and methylxanthines in the fiber powder used in the present study.

Microbial cocoa metabolism also led to a high excretion of 4-HPA, IAA, hippurate and PAG in the C10 group which could come from different cocoa compounds. On the one hand, cocoa is rich in monamines, such as tyramine, tryptamine and 2-phenylethylamine, which can be degraded by gut bacteria to 4-HPA, IAA, and PAG, respectively. On the other hand, microbiota degradation of cocoa polyphenols can produce quinic acid and benzoic acid which are then oxidized to hippurate and other phenol-related metabolites such as 4-HPA and PAG. The gut microbiota also produce potentially toxic substances derived from protein metabolism [32], such as the uremic toxins 4-CS and 3-IS, derived from tyrosine and tryptophan, respectively. These metabolites were lowered excreted after the C10 diet demonstrating a decrease in the amino acid degradation by gut microbiota in those animals. This lower degradation was also demonstrated with the reduced excretion of α KMV and α KIC in the C10 group, both derived from the branched-chain amino acids

(BCAA) endogenous catabolism. These changes, together with the lower urinary excretion of acetone, suggest an alteration in energy metabolism through the modulation of the ketogenesis and the BCAA metabolism in the C10 group. Similarly, in comparison with the CF diet, the amount of glucose, sucrose and tricarboxylic acid (TCA) cycle intermediates -including citrate, 2-OG, and fumarate- was lower in the C10 group, indicating that the TCA cycle was also down-regulated after the cocoa intake. Overall, this shift in the energy consumption pattern could contribute to the reduction of the body weight in C10-fed rats, reported here and in previous studies [23,25,27]. To date, in hamsters fed a high-fat/high-cholesterol diet TCA cycle was suppressed and intermediate levels were decreased, which indicated that the energy metabolism was shifted to fatty acid oxidation in response to hyperlipidemia [33].

Urine metabolic profiles obtained after nutritional interventions can be associated with physiological changes. Regarding the low body weight gain induced by the C10 diet, there was an inverse correlation between cocoa metabolites and body weight, the highest correlation being with imidazole, one of the smallest metabolites derived from methylxanthines. Therefore, it can be suggested the contribution of theobromine, a methylxanthine present in cocoa in a high concentration, and its metabolites to the effect of cocoa on body weight. An inverse association was also found between the body weight and the presence of metabolites derived from the microbial metabolism, especially with PAG and 4-HPA. Thus, regardless of others reported mechanisms [34–36], the contribution of microbiota composition might be considered in the cocoa regulatory effect on body weight. In fact, it has been showed that the C10 diet intake decreased the *Staphylococcus* and *Streptococcus* genera (from the *Firmicutes* phylum) and increased the *Bacteroides* spp. (*Bacteroidetes* phylum) leading to a lesser ratio of *Firmicutes* to *Bacteroidetes* [17,25]. This decreased ratio has been associated with lower body weight [37]. Agreeing with that, an inverse correlation was also found between the counts of *Streptococcus* spp. and the excreted amounts of 4-HPA and epicatechin in urine.

On the other hand, the relationship between urinary metabolites and intestinal immunity has been established. Whereas the CF did not affect the IgA content, the C10 diet, agreeing with previous studies [2,13,26], resulted in a significantly lower fecal IgA concentration. In this sense, inverse correlations between the fecal IgA concentration and the amounts of cocoa epicatechin and methylxanthine derivatives and those from microbial metabolism were obtained. Overall, these results suggest that cocoa metabolism may lead to the formation of bioactive products that interact with the mechanisms involved in IgA secretion and/or its synthesis. Indeed, not only the microbiota-generated metabolites would be important, but also the microbiota composition. In this

sense, the lower counts of *Streptococcus* genus are positively correlated with the lessening of the fecal IgA concentration.

Besides urinary metabolites and fecal IgA, metabolic hormones were also determined after the C10 and CF intake. In line with previous studies [24], the GLP-1 and glucagon, peptide hormones involved in the glucose metabolism, were reduced by the C10 diet, but only the former was also modified after the CF intake. However, only glucagon concentration correlated inversely with the cocoa metabolites.

Regarding hormones involved in regulating food intake, whereas leptin helps to control the appetite and to maintain a stable body weight [38], ghrelin stimulates the appetite, increases fat mass deposition and weight gain and influences glucose and lipid metabolism [39]. Although the C10 diet increased ghrelin concentration, but did not significantly affect that of leptin, it did not provoked changes in food intake. Therefore it is unlikely that these regulating appetite hormones contribute to cocoa influence on body weight. In fact, the ghrelin concentration was inversely correlated associated with the body weight. On the other hand, it has been suggested a relationship between leptin and ghrelin and gut microbiota [38,40]. In this sense, here we have observed that the proportions of *Bifidobacterium* and *Streptococcus* genera showed a positive correlation with the leptin concentration and an inverse correlation with ghrelin, which are partially in line with reported studies [38]. Thus, it can be postulated that the hormones would affect microbiota composition and/or the changes on microbiota would affect the hormone production. In this sense, it has been described that leptin stimulates the mucin production in the intestine which could affect the composition of the microbiota [41]. However, it has also been suggested that the microbiota may modulate these hormones through the physiological regulation of the levels and type of autoantibodies produced against them [42]. Moreover, it has been described that two of the main SCFA (butyrate and propionate) regulate gut hormone release [43]. In this regard, our study demonstrates that the ghrelin concentration was inversely correlated with the butyric acid concentration in cecum content. Therefore, although the hormone effects on microbiota can not be discarded, it can be suggested that the cocoa modulatory effect on the microbiota composition and functionality is partially responsible for the changes observed in both hormone concentrations.

In summary, through a metabonomic approach, we have described the urinary metabolites derived from cocoa and cocoa fiber intake that can be used as consumption markers. Moreover, the lower excretion of certain host and microbial metabolites reflected a shift in the dietary energy and amino acid metabolism due to cocoa, but not to cocoa fiber diet. These metabolic profiles are associated with the body weight, intestinal IgA, the metabolic hormone profile and microbiota composition.

Results from diet containing only cocoa fiber evidence the contribution of other cocoa compounds on such effects. Further studies should be carried out to evaluate the precise contribution of cocoa methylxanthines.

Author contributions

The authors' contributions were as follows: M. M. -C., À. F., F. J. P. -C and M. C. conceived and designed the research; M. M.-C. and J. M.-P. carried out the metabolomics experiments whereas M. M.-C. carried out the rest of the experiments; J.M.-P. and J.R.S. and A.D. carried out the metabolomics data analysis and were involved in the interpretation of these data whereas M. M. -C., F. J. P.-C. and M. C. carried out the luminex and IgA data analysis and interpretation of the data; M. M. -C. and J. M.-P. contributed equally to the initial draft of the manuscript; A.C., J.R.S., 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.

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Supplementary data

Lower (LLOQ) and upper limits of quantification (ULOQ) for metabolic hormones according to the Bioplex-Pro assay applied.

Metabolic hormones (pg/mL)		
	LLOQ	ULOQ
Ghrelin	159.58	155595.25
Glucagon	23.43	1316.37
GLP-1	8.44	1999.39
Leptin	123.73	130750.77

DISCUSSIÓ

Estudis previs portats a terme pel nostre grup de recerca han demostrat que una dieta enriquida amb cacau en rates és capaç de modificar el sistema immunitari intestinal, tal com es recull en el treball de revisió que forma part de la introducció [196]. Entre d'altres efectes, la intervenció nutricional amb cacau en rata és capaç de modificar la composició limfocitària intestinal i atenuar la producció d'IgA mitjançant la regulació de l'expressió gènica de diverses molècules implicades en la seva síntesi i en el reclutament intestinal de les cèl·lules productores [77,262,263]. D'altra banda, està establert que components de la dieta com els flavonoides interactuen i modifiquen la microbiota intestinal, tal i com es recullen en el segon treball de revisió que forma part de la introducció [96]. En base a aquests resultats preliminars, la hipòtesi que sustenta aquesta tesi ha estat que els efectes produïts pel cacau sobre el sistema immunitari no serien deguts exclusivament a la interacció directa entre compostos del cacau i les cèl·lules immunitàries, sinó que també estarien mediat per la modificació de la composició i/o funcionalitat de la microbiota i de la seva interacció amb l'hoste. En aquest sentit, el **primer objectiu** de la tesi es va encaminar a establir l'efecte d'una dieta rica en cacau sobre la composició de la microbiota intestinal i la seva interacció amb el sistema immunitari intestinal. Els resultats obtinguts a partir del desenvolupament d'aquest objectiu es recullen a la **publicació 1**. A continuació, donat que se sabia poc sobre quins eren els components del cacau responsables dels seus efectes immunomoduladors, l'estudi es va encaminar a esbrinar la participació de dos components bioactius presents en el cacau: els flavonoides i la fibra. Així, el **segon objectiu** va consistir en establir la participació dels flavonoides en l'activitat moduladora del cacau sobre la composició i funció de la microbiota i el sistema immunitari intestinal; objectiu que es va

desenvolupar en les **publicacions 2 i 3**. A més a més, es va plantejar el **tercer objectiu** centrat en establir la participació de la fibra de cacau en l'activitat moduladora de la composició i funció de la microbiota i el sistema immunitari intestinal, el que ha permès l'obtenció dels resultats inclosos en les **publicacions 4, 5, 6 i 7**.

Com a resultat del primer objectiu, s'ha caracteritzat la **microbiota intestinal** en rates Wistar abans i després de sis setmanes d'intervenció nutricional amb una dieta amb un 10% de cacau; resultats que s'han comparat amb la microbiota d'animals alimentats amb una dieta estàndard (**publicació 1**). Aquest estudi ha demostrat que una dieta amb un 10% de cacau modifica de forma clara la composició de la microbiota intestinal tot disminuint la proporció dels gèneres bacterians *Bacteroides*, *Staphylococcus* i *Clostridium*.

Aquests efectes de la dieta rica en cacau sobre la microbiota es podrien atribuir al seu contingut en polifenols en base a diverses dades bibliogràfiques d'estudis tant *in vitro* com *in vivo*. Així, *in vitro*, s'ha descrit, per exemple, la capacitat inhibidora del creixement de certes espècies bacterianes per part de polifenols del te [264–266], del creixement de *Staphylococcus* spp. per part de fruites del bosc [267], i del creixement de *C. histolyticum* per efecte de la catequina [158]. Recentment s'ha descrit que flavanols del raïm disminueixen el creixement de *C. histolyticum* *in vitro* mentre que promouen el creixement del grup *Lactobacillus-Enterococcus* en la microbiota humana [268]. En el mateix sentit, s'ha observat *in vivo* que tanins de la dieta disminueixen alguns gèneres bacterians de la microbiota [269,270], mentre que d'altres flavonoides, com els que es troben en algunes formes de te, inhibeixen el creixement de bacteris dels gèneres *Clostridium* i *Bacteroides* i augmenten el de *Bifidobacterium*

spp. i *Lactobacillus* spp. [271]. Tanmateix flavonoides presents a la magrana promouen el creixement de *Bifidobacterium* spp. i del grup *Lactobacillus-Enterococcus* [272], i extractes de poma augmenten els recomptes de *Bifidobacterium* spp. i *Lactobacillus* spp. [273]. Per altra banda, s'ha descrit que els polifenols del vi poden augmentar la proporció de *Bacteroides* spp., *Lactobacillus* spp. i *Bifidobacterium* spp. en rata [274], mentre que els polifenols del te incrementen la presència de *Lactobacillus* spp. en porc [275].

Pel que fa a estudis d'intervenció nutricional amb cacau en humans, s'ha vist que el consum d'una beguda a base de cacau amb un elevat contingut de flavanols durant quatre setmanes redueix significativament la proporció de *C. histolyticum* i promou el creixement de *Lactobacillus* spp. [276].

Per tant, en base a aquests antecedents que mostren la interacció dels flavonoides sobre la microbiota intestinal, s'ha intentat establir el paper dels polifenols del cacau en les modificacions en la microbiota causades per aquesta dieta. Per això s'ha comparat l'efecte del cacau convencional amb el de dietes amb Cocoapure® (antigament anomenat CocoanOX®, de Naturex). El Cocoapure® és un producte del cacau que no ha estat sotmès a fermentació ni torrefacció i presenta una riquesa de 160-500 mg/g de polifenols [277]. L'addició de petites quantitats de Cocoapure® en el pinso de rata permet proporcionar un elevat contingut de flavonoides del cacau juntament amb una menor quantitat d'altres components. Concretament s'ha duplicat i quadruplicat l'aportació de flavonoides del cacau convencional i s'ha comparat amb l'efecte produït per aquesta dieta (**publicacions 2 i 3**). S'ha pogut constatar que, en rates Lewis adultes, les tres dietes enriquides amb diferents proporcions de polifenols són capaces de reduir la proporció de bacteris fecals, encara que no ho fan de forma dosi-dependen (**publicació 2**). Així mateix, rates Wistar joves

alimentades durant quatre setmanes amb les diferents fonts i quantitats de flavonoides del cacau modifiquen la composició de la microbiota intestinal, inhibint l'augment de la proporció de *Staphylococcus* spp., *Streptococcus* spp. i del grup *C. histolyticum/C. perfringens* que es produeix amb l'edat (**publicació 3**). Per tant, es constata que part de l'efecte del cacau sobre aquests gèneres i grups bacterians són deguts a l'acció dels flavonoides del cacau. Ara bé, donat que els efectes més pronunciats corresponen a la dieta amb la menor quantitat de flavonoides (la procedent del cacau convencional), sembla ser que, encara que els flavonoides del cacau modulen el creixement bacterià, altres components presents en el cacau convencional amb més proporció, com pot ser la fibra i/o les metilxantines, entre d'altres, poden exercir un efecte addicional o sinèrgic sobre la microbiota intestinal.

A partir d'aquests resultats, l'interès es va centrar en la fibra de cacau, ja que aquest component, a l'igual que les formes polimèriques dels flavonoides, pot arribar al còlon i actuar sobre la microbiota intestinal [156,165]. Per això es va disposar d'un grup d'animals que van rebre una dieta amb un contingut de fibra de cacau equivalent a l'aportat per la dieta amb un 10% de cacau però sense pràcticament flavonoides (<0,02%), i els resultats es van comparar amb els obtinguts després de la ingesta d'un 10% de cacau i també després d'una dieta amb un contingut d'inulina equivalent a la quantitat de fibra soluble que aportava la dieta rica en cacau (**publicació 4**). S'ha pogut constatar que la dieta amb fibra de cacau, de forma similar a la dieta rica en inulina, és capaç d'augmentar el recompte de bacteris *Bifidobacterium* spp. i *Lactobacillus* spp. en la microbiota intestinal després de tres setmanes de dieta, el que permet suggerir el seu paper com a prebiòtic. Pel contrari, amb la dieta amb cacau no només no s'indueix aquest efecte sinó que fins i tot es redueix la proporció del gènere

Bifidobacterium spp., el que suggereix que altres compostos del cacau inhibeixen l'efecte prebiòtic de la seva fibra (**publicació 4**).

Per altra banda, més enllà de la composició microbiana, també s'ha estudiat la seva funcionalitat. Per això, s'han quantificat els **àcids grassos de cadena curta** (AGCC) a nivell cecal, fecal i sèric després d'una dieta rica en cacau, fibra de cacau o inulina. Aquests AGCC es produeixen a partir de carbohidrats complexos no digeribles de l'aliment, que són metabolitzats i fermentats per la microbiota intestinal i exerceixen tot una sèrie d'efectes beneficiosos per l'hoste a nivell nutricional, tròfic i immunomodulador, entre d'altres [278,279]. La ingesta de fibra de cacau va comportar canvis més pronunciats pel que fa a la producció d'AGCC en contingut cecal i femtes que la dieta rica en cacau i la dieta amb inulina (**publicació 4**). Particularment, la fibra de cacau va augmentar la síntesi dels principals AGCC com són els àcids acètic, propiònic i butíric, mentre que el cacau només va incrementar la producció d'àcid butíric en el contingut cecal. Aquests resultats permeten suggerir que altres components del cacau podrien inhibir el metabolisme colònic de la fibra o fer la fibra menys accessible als bacteris intestinals i, d'aquesta manera, tal com s'ha esmentat anteriorment, inhibir l'efecte prebiòtic de la fibra de cacau.

Cal remarcar que, malgrat la diferent composició d'AGCC obtinguda en femtes i contingut cecal després de les dietes a base de cacau, fibra de cacau o inulina, les tres dietes són capaces de reduir el pH fecal i cecal (**publicació 4**). Donat que el cacau no modifica àmpliament la composició d'AGCC, cal pensar que potser el seu contingut en polifenols i/o metilxantines podria acidificar el contingut intestinal. A més, cal destacar que les dietes amb cacau i fibra de cacau no només modifiquen la funcionalitat de la microbiota intestinal a nivell de la

producció d'AGCC sinó que també influeixen en la producció microbiana de diversos metabòlits (**publicació 7**). En aquest sentit, una aproximació metabolòmica no dirigida ha permès identificar metabòlits microbians derivats dels flavonoides i de les metilxantines del cacau en l'orina d'animals que han rebut una dieta amb 10% de cacau, coincidint amb estudis previs [280–282]. Pel contrari, els metabòlits microbians d'aquests compostos no es troben en la dieta de fibra de cacau, fet que confirma l'absència de flavonoides i metilxantines en la fibra de cacau. Així, queda pendent establir si els efectes adscrits a la dieta amb cacau són deguts als propis flavonoides o a algun/s dels seus metabòlits.

D'altra banda, tot i que l'epicatequina és el metabòlit que més es correlaciona de forma inversa amb l'abundància dels gèneres estreptococs, estafilococs i bifidobacteris, l'anàlisi metabonòmic posa de manifest que els metabòlits microbians principalment derivats de les metilxantines també presenten aquesta associació inversa amb l'abundància d'aquests gèneres bacterians (**publicació 7**). Aquest fet permet postular que les metilxantines del cacau exerceixen també una acció inhibidora sobre el creixement d'aquests bacteris. D'aquesta manera, quedaria justificat que l'efecte prebiòtic de la fibra de cacau (increment de *Bifidobacterium* spp.) no s'observi en la dieta amb cacau, amb elevada proporció d'epicatequina i metilxantines com la teobromina, que estarien contrarestant aquest efecte de la fibra.

Independentment dels canvis en la microbiota intestinal per efecte de determinats components de la dieta, cal tenir en compte que aquests compostos poden influir en la interacció entre la microbiota i l'hoste (*crosstalk*). El reconeixement de patrons bacterians es produeix, de forma inespecífica, gràcies a la presència, entre d'altres molècules,

dels receptors TLR [99,283]. En aquest sentit, s'ha vist que la dieta amb un 10% de cacau en rates modifica **l'expressió gènica de TLR en el còlon**, concretament, redueix significativament l'expressió gènica de TLR9 i tendeix a disminuir la de TLR2 i TLR7 (**publicació 1**). Estudis previs havien demostrat canvis similars pel que fa a TLR9 a l'intestí prim però, en canvi, les modificacions observades en TLR2, TLR4 i TLR7 són similars a les descrites en estudis previs en els teixits inductors de la resposta immunitària intestinal com són els ganglis limfàtics mesentèrics i les plaques de Peyer [262,263].

La reducció en l'expressió de TLR9 en el còlon per la dieta amb un 10% de cacau, juntament amb un augment de l'expressió de TLR4, s'ha confirmat en posteriors estudis (**publicació 4**), en els quals també s'ha intentat establir el paper de la fibra de cacau en el patró de TLR en el còlon. Pel contrari, s'ha pogut observar que la dieta a base de fibra de cacau augmenta l'expressió gènica de TLR2, TLR5 i TLR7, juntament amb l'expressió gènica d'occludina (**publicació 4**). Se sap que el principal paper de les molècules TLR és la defensa contra bacteris oportunistes mitjançant la inducció de senyals que promouen la secreció d'IgA i de pèptids antimicrobians, el reforç de la barrera i la proliferació de les cèl·lules epitelials [1,284]. Després de la ingesta de fibra de cacau s'incrementa l'expressió gènica de diversos TLR els quals es troben reduïts en el cas d'una dieta rica en cacau. Per tant, de nou, l'efecte de la fibra de cacau queda contrarestat per l'efecte d'altres compostos presents en el cacau com poden ser els flavonoides [96]. La modulació del patró de TLR per part de la fibra podria ser un reflex dels canvis produïts en la microbiota intestinal i/o d'un efecte directe sobre l'epiteli i les cèl·lules immunocompetents intestinals (**publicació 4**).

Tal com s'havia vist en estudis previs [77], la dieta amb un 10% de cacau és capaç d'atenuar la **secreció d'IgA intestinal** i, a més, la seva

expressió gènica en el còlon (**publicació 1**), de forma similar a com s'havia observat en plaques de Peyer [263]. Donat que s'ha descrit l'acció inhibidora de la síntesi d'anticossos per a alguns flavonoides de la dieta com genisteïna, luteolina i apigenina [285–287], s'ha intentat relacionar l'efecte de la dieta rica en cacau sobre la síntesi d'IgA amb el seu contingut en polifenols. Per això s'ha comparat l'efecte de la dieta amb un 10% de cacau que contenia 0,2% de polifenols, amb el produïts després de la ingesta de 0,4 i 0,8% de polifenols en forma de **Cocoapure®**, obtingut a partir de cacau no fermentat, tant en rates Lewis adultes (**publicació 2**) com en rates joves de les soques Wistar i Brown Norway (**publicació 3**). Encara que les tres dietes riques en polifenols tenen un efecte modulador de la secreció d'IgA intestinal, no es produeix un efecte proporcional al contingut en flavonoides, el que permet suggerir que d'altres components també tenen un paper en la modulació de la resposta immunitària intestinal.

Per això, el treball corresponent a les **publicacions 6 i 7** s'ha basat, en part, en l'establiment de l'efecte de la fibra de cacau sobre la secreció d'IgA intestinal. Mentre que la **publicació 7** s'ha centrat en l'efecte de la dieta de cacau i fibra de cacau sobre el contingut d'IgA en femtes i la seva relació amb metabòlits presents en orina, l'objectiu de la **publicació 6** va ser el d'analitzar l'efecte de la dieta rica en cacau, fibra de cacau o inulina sobre el contingut d'IgA i d'IgM dels teixits limfoides intestinals inductors (plaques de Peyer i ganglis limfàtics mesentèrics) i efectors tant intestinals com d'altres mucoses (rentat intestinal i glàndules salivals, respectivament). Els resultats demostren que la dieta amb un 10% de cacau exerceix els efectes més pronunciats en la síntesi d'immunoglobulines tant en els compartiments intestinals com extraintestinals (**publicacions 6 i 7**). La ingesta de fibra de cacau provoca una reducció menys acusada d'IgA en alguns compartiments (rentat intestinal i glàndules salivals), o bé no té efecte (**publicació 7**),

o bé, en alguns casos, ha demostrat tenir un efecte contrari al de la ingesta de cacau i augmentar la secreció d'IgA, com és en el cas dels ganglis limfàtics mesentèrics (**publicació 6**). Considerant que la ingesta de fibra de cacau, però no la d'inulina, augmenta la secreció d'immunoglobulines en plaques de Peyer i ganglis limfàtics mesentèrics, es pot considerar que la fibra de cacau conté elements estructurals no compartits amb la inulina que contribueixen a potenciar la síntesi d'IgA, potser relacionats amb el seu efecte prebiòtic (**publicació 4**). Malgrat tot, la dieta amb fibra de cacau i la dieta amb inulina causen una reducció de la concentració d'IgA en el rentat intestinal, si bé no tan acusada com la de la dieta rica en cacau (**publicació 6**). Això demostra que els efectes de la fibra en els teixits limfoides inductors no es reproduïen en els teixits efectors i que, degut a què l'efecte de la fibra no és tant pronunciat com el del cacau, altres compostos del cacau han de potenciar l'efecte de la fibra.

Tal i com s'ha mencionat, també s'ha establert l'efecte de la ingesta rica en cacau i en fibra de cacau sobre el contingut d'IgA en les **glàndules salivals**. Encara que s'han emprat flavonoides en cas de disfunció de les glàndules salivals [288–290], no es coneixia la influència de la dieta rica en cacau en aquests compartiments extraintestinals. La dieta rica en cacau atenua la concentració d'IgA i IgM en les glàndules salivals, efecte que en part sembla ser degut al seu contingut en fibra (**publicació 6**). Aquests resultats no coincideixen amb els observats en animals alimentats amb altres tipus de fibra [291], i permet suggerir que no només la fibra, sinó d'altres compostos del cacau poden interferir amb la producció d'immunoglobulines en les glàndules salivals. S'ha intentat aprofundir en els mecanismes implicats en la reducció de la concentració d'IgA en les glàndules salivals determinant l'expressió de diversos gens relacionats amb la síntesi i secreció d'IgA (**publicació 6**). S'ha observat que la dieta a base

de cacau, però no la de fibra de cacau, comporta una reducció del mRNA que codifica per IgA en aquells teixits. Per altra banda es produeix una reducció en l'expressió gènica de la citocina TGF- β 1, implicada en la síntesi d'IgA tant amb la dieta rica en cacau com en fibra, el que coincideix amb efectes observats en l'intestí prim d'animals alimentats amb cacau [262,263]. Tanmateix s'ha observat que la dieta amb un 10% de cacau augmenta l'expressió de la quimiocina CCL28, involucrada en el reclutament de limfòcits B IgA+ [292,293], el que pot suggerir un mecanisme compensatori de la reducció d'IgA. Per altra banda, en glàndules salivals, la dieta amb fibra de cacau, però no la dieta amb 10% de cacau, disminueix l'expressió gènica de RAR α , molècula de la família dels receptors de l'àcid retinoic implicada en el reclutament de cèl·lules en les mucoses [294] i de pIgR, receptor que possibilita la transcitosi d'IgA [295], resultats que poden explicar parcialment la disminució d'IgA en les glàndules salivals per la fibra de cacau.

Retornant al compartiment intestinal, s'ha observat que la dieta amb un 10% de cacau redueix la proporció de **bacteris intestinals units a IgA (publicació 1)**, fet que es pot correlacionar amb la reducció en la proporció dels gèneres *Clostridium* i *Staphylococcus* [296]. En estudiar el paper dels flavonoides del cacau en aquest efecte, s'ha observat que les tres concentracions de polifenols del cacau considerades provoquen una reducció dels bacteris fecals units a IgA (**publicació 2**) i, en el cas de rates Wistar, només la dieta amb cacau convencional i la que porta més proporció de polifenols tenen aquesta acció (**publicació 3**). En qualsevol cas, aquests resultats demostren el paper dels flavonoides en la modulació dels bacteris units a IgA, però com que no hi ha un efecte proporcional a la quantitat de flavonoides de la dieta, altres components del cacau han d'estar implicats. Per això, la

proporció de bacteris intestinals units a IgA en rata també s'ha establert de forma comparativa després d'una dieta amb un 10% de cacau, amb una dieta amb la quantitat equivalent de fibra de cacau i amb una dieta amb la quantitat equivalent de fibra soluble en forma d'inulina (**publicació 4**). Els resultats obtinguts permeten descartar a la fibra de cacau com a responsable de la reducció de bacteris intestinals units a IgA provocada per la ingesta de cacau, ja que pel contrari, la fibra indueix un increment d'aquesta proporció. Aquest fet demostra una altra vegada que altres components del cacau contraresten els efectes prebiòtics de la fibra de cacau. En aquest sentit, existeixen metabòlits en orina derivats principalment de les metilxantines, tant del metabolisme microbià com del propi metabolisme de l'hoste, que es correlacionen amb aquest efecte sobre la IgA unida a bacteris (**publicació 7**).

Per tal d'intentar aclarir algun aspecte més de l'acció del cacau sobre la síntesi d'IgA intestinal, s'ha quantificat la **composició limfocitària en plaques de Peyer i en ganglis limfàtics mesentèrics** d'animals alimentats amb dietes riques en cacau i flavonoides del cacau (**publicació 3**). No obstant, no s'han observat canvis significatius referents a les principals poblacions limfocitàries amb l'excepció d'un increment de la proporció de cèl·lules NK en les plaques de Peyer després de la intervenció amb la dieta de cacau convencional. Aquests resultats coincideixen parcialment amb els observats anteriorment pel grup de recerca [77]. Donat que la proporció de limfòcits Th i B no canvia en els òrgans estudiats en el present estudi, la modulació de la secreció d'IgA pel cacau ha d'implicar mecanismes intracel·lulars, mecanismes relacionats amb mediadors, o bé la transcitosi de la IgA a través de la paret intestinal.

En aquest sentit, l'estudi portat a terme en la **publicació 5**, que estableix les modificacions de l'expressió gènica en còlon per tècniques de *microarray*, pot ajudar a comprendre els canvis induïts en el còlon després de la dieta rica en cacau. S'ha observat que aquesta dieta modula negativament gens relacionats amb la funció immunitària. En aquest sentit, un dels gens més regulats negativament és la taquicinina (*Tac4*), que és una molècula promotora dels limfòcits B [297], cèl·lules responsables de la síntesi d'anticossos. En comparació amb la dieta rica en cacau, la dieta rica en fibra de cacau o inulina no redueixen tant aquest gen (**publicació 5**). Per altra banda l'efecte immunomodulador sobre la IgA intestinal també es podria relacionar amb una menor expressió de gens implicats en la producció de citocines, com *Fcer1a*, *Ms4a2* i *Adipoq* (**publicació 5**). La regulació d'aquests gens, juntament amb la sobreexpressió de *Scgb1a1*, gen que codifica per una proteïna amb activitat antiinflamatòria, i també sobreexpressada després de la dieta rica en cacau i en inulina, pot explicar les accions antiinflamatòries atribuïdes a la dieta rica en cacau descrites en altres estudis [298,299].

L'impacte que té el cacau i els seus components sobre la resposta immunitària humoral a nivell intestinal també pot tenir repercussió a nivell sistèmic. Per aquesta raó, s'ha determinat l'efecte dels flavonoides del cacau sobre les **immunoglobulines sèriques** en rates adultes després de dues setmanes de dieta (**publicació 2**) i no s'han observat efectes significatius i indicatius de l'efecte dels flavonoides de la dieta en la secreció d'anticossos sistèmics tal com s'havia observat en estudis previs per efecte del cacau [262,300]. Aquest estudi permet suggerir, per tant, que la immunitat intestinal, i en edats més temperanes, és la més afectada per la dieta rica en cacau. En canvi, en rates Wistar joves s'ha vist que les dietes enriquides amb

flavonoides atenuen l'increment associat a l'edat de les concentracions sèriques d'IgG1 i IgG2b, efectes que no estan associats a la concentració de flavonoides (**publicació 3**). Per tant, es pot suggerir de nou que, els efectes del cacau sobre la funcionalitat del sistema immunitari sistèmic no depenen només del seu contingut de polifenols, sinó que d'altres components han de contribuir i, fins i tot, potenciar l'efecte dels flavonoides del cacau. En l'estudi de l'efecte de la fibra de cacau sobre la concentració d'immunoglobulines sèriques (**publicació 6**) s'ha constatat que el cacau i la fibra de cacau exerceixen el mateix efecte atenuant sobre la concentració d'IgG, mentre que només la dieta rica en cacau redueix la concentració d'IgM. Pel que fa a la concentració d'IgA sèrica, aquesta no es modifica per la dieta rica en cacau però incrementa per efecte de la fibra de cacau, possiblement com un reflex de l'augment de la producció en ganglis limfàtics mesentèrics observat (**publicació 6**). En resum, la fibra de cacau no només no potencia els efectes dels flavonoides del cacau en la resposta humoral sistèmica sinó que, fins i tot, pot exercir un efecte contrari (per exemple, incrementant la IgA sèrica).

Un dels efectes col·laterals produïts per la ingesta d'un 10% de cacau és la seva influència sobre el **creixement ponderal**. La dieta amb un 10% de cacau comporta un alentiment en la corba ponderal dels animals que no es pot atribuir a una menor ingesta de menjar. Aquests resultats s'han reproduït en tots els estudis de la present tesi (**publicacions 1-7**) i també en treballs previs del grup de recerca [77,300,301]. Aquest efecte sobre el pes aniria en línia amb els descrits per a alguns polifenols, entre ells els presents en el cacau, en els que es demostra una reducció en l'absorció i el dipòsit de greix [212,302–307]. Així mateix, s'han descrit efectes hipolipidèmics i estimuladors d'energia cel·lular per als flavonoides [308–310]. Per altra banda,

donat que la dieta rica en cacau redueix la proporció de clostridis, gènere bacterià que es correlaciona amb pèrdua de pes (**publicació 7**) [296], l'efecte del cacau sobre el pes podria estar mediat per aquest canvi a la microbiota. Els bacteris de la microbiota poden tenir diferents eficiències en l'extracció d'energia de la dieta comportant canvis en el metabolisme de l'hoste [311,312]. En aquest sentit, s'ha observat una correlació positiva entre el pes corporal i la relació *Firmicutes/Bacteroidetes* (**publicació 7**), la qual s'ha relacionat estretament amb l'obesitat [312–314]. Particularment, els *Bacteroidetes* presenten un menor nombre de gens per a enzims implicats en el metabolisme de lípids i carbohidrats que els *Firmicutes* [315,316]. Per tant, l'efecte de la dieta rica en cacau sobre el pes corporal podria atribuir-se parcialment a la reducció dels gèneres *Clostridium* i *Staphylococcus* que pertanyen al fílum *Firmicutes*, i a l'increment de *Bacteroides* (inclòs en el fílum *Bacteroidetes*) que donarien lloc a una reducció de la relació *Firmicutes/Bacteroidetes* (**publicacions 1 i 4**).

A partir dels resultats de les **publicacions 2 i 3** s'ha pogut observar que l'efecte sobre la corba ponderal de la dieta amb un 10% no és degut al seu contingut en flavonoides ja que, en administrar Cocoapure®, que subministra més flavonoides del cacau i més purs, no s'observa l'efecte que té el cacau sobre el creixement ponderal. Per tant, altres components del cacau que no són flavonoides, com per exemple la fibra o les metilxantines han de contribuir a l'efecte del cacau sobre el pes corpora l[227]. En aquest sentit, s'han identificat tota una sèrie de metabòlits en orina que es correlacionen amb el pes corporal dels animals. Concretament, metabòlits derivats de l'activitat microbiana sobre flavonoides i metilxantines es correlacionen inversament amb el pes corporal, mentre que metabòlits derivats de l'activitat de l'hoste es correlacionen de forma positiva amb aquesta variable (**publicació**

7). Així mateix cal destacar que la ingesta de cacau, a diferència de la dieta amb fibra de cacau, dóna lloc a una reducció dels metabòlits procedents d'aminoàcids i, en general, del metabolisme energètic, el que podria indicar un canvi en el patró de consum d'energia i que podria incidir sobre la corba ponderal. A més, en estudiar l'efecte de la ingesta de la fibra de cacau sobre la corba ponderal (**publicació 4**), s'ha observat que la fibra no és la responsable de la regulació de la corba ponderal. Aleshores cal subratllar de nou el paper que poden tenir les metilxantines del cacau (que no estan presents en la fibra del cacau). En aquest sentit, Dulloo *et al.* [277] han demostrat el sinergisme produït per la interacció entre les metilxantines i els flavonoides en alguns processos fisiològics.

És important assenyalar la influència de la dieta rica en cacau sobre gens relacionats amb el metabolisme lipídic de còlon, tal com s'ha observat en la **publicació 5**. La dieta amb un 10% de cacau regula negativament l'expressió de *Adipoq*, una hormona derivada d'adipòcits implicada en el metabolisme de glucosa i lípids, i de *Fabp1*, proteïna implicada en la captació, transport i metabolisme d'àcids grassos. Al mateix temps aquesta dieta augmenta l'expressió de *Angptl4*, un inhibidor de la lipoproteïnlipasa (**publicació 5**). Aquests efectes poden estar induint un pitjor aprofitament dels greixos de la dieta i per tant tenir repercussió en la corba ponderal de l'animal. Aquestes modificacions en l'expressió gènica del metabolisme lipídic no són tan pronunciades després de la ingesta de fibra de cacau i per tant les induïdes pel cacau no es poden atribuir al seu contingut en fibra. Tot i que s'ha vist que el flavonoide quercetina redueix l'expressió de gens hepàtics relacionats amb la producció de lípids i àcids grassos [317], els nostres resultats amb dietes amb diferents concentracions de polifenols de cacau no permeten suggerir el paper d'aquests compostos bioactius en l'efecte del cacau sobre la corba

ponderal. En aquest sentit, les metilxantines es presenten de nou com a compostos bioactius que poden estar clarament implicats en aquests efectes.

Seguint amb el metabolisme, s'ha avaluat l'efecte de les dietes de cacau i fibra de cacau sobre la concentració plasmàtica d'algunes **hormones metabòliques (publicació 7)**. En aquest sentit s'ha observat que, tot i que ambdues dietes augmenten la concentració de grelina, la dieta de cacau és la que provoca un major increment. La grelina es correlaciona inversament amb el pes corporal, amb la proporció dels bacteris *Bifidobacterium* spp., *Streptococcus* spp., el grup *C. histolyticum*/*C. perfringens* així com amb la relació *Firmicutes*/*Bacteroidetes*. En concordança amb estudis previs [318], s'ha observat una correlació negativa entre la concentració en plasma de grelina i la d'àcid butíric en contingut cecal. També s'ha quantificat la concentració plasmàtica de leptina i glucagó després de la intervenció nutricional. La dieta de cacau va disminuir la concentració de glucagó, mentre que la dieta de fibra de cacau va incrementar la concentració de leptina. Aquest fet reforça el fet que la quantitat de pinso consumit, relacionat amb la sacietat i la leptina, no és un dels mecanismes implicats en l'efecte del cacau sobre el pes. Ara bé, ambdues hormones mostren una correlació directa amb el pes corporal i amb la proporció dels bacteris *Bifidobacterium* spp. i *Streptococcus* spp, resultats que coincideixen parcialment amb estudis previs [319].

Per últim, el desenvolupament d'aquesta tesi ha comprés la utilització de diverses soques de rata. Encara que en un principi el projecte es plantejava en rates Wistar, s'han fet estudis també en rates de les soques Lewis i Brown Norway que s'utilitzaven per a altres estudis del

grup de recerca. Mitjançant **la publicació 3** s'ha pogut evidenciar que, en alguns aspectes relacionats amb efectes de la dieta sobre el sistema immunitari intestinal, existeixen diferències importants en funció de la soca utilitzada.

En resum, i de forma global es pot destacar que el consum d'una dieta rica en cacau indueix canvis en el pes corporal, en la composició i funcionalitat de la microbiota intestinal, i en la seva interacció amb l'hoste; i també en la resposta immunitària intestinal i sistèmica. Els flavonoides del cacau semblen ser responsables parcials de la majoria d'aquests canvis, a excepció de l'efecte sobre el pes. Pel contrari, la fibra de cacau sembla modificar aquestes variables en alguns casos fins i tot al contrari del que s'observa de forma global amb el cacau, com és el cas de la seva activitat prebiòtica, la qual queda emmascarada quan la fibra no s'ingereix de forma aïllada. Diverses evidències posen de manifest el paper que les metilxantines del cacau poden estar exercint en els efectes globals del cacau i, per tant, en un futur, caldrà establir el paper de la teobromina en les variables estudiades en la present tesi.

**CONCLUSIONS/
*CONCLUSIONS***

The results obtained from this study have enabled us to draw the following conclusions:

- A 10% cocoa diet modulates the **microbiota composition** in rats, by decreasing the proportions of *Bacteroides*, *Staphylococcus* and *Clostridium* genera. This effect can be attributed, at least in part, to its flavonoid content. Otherwise, the cocoa fiber does not contribute to these effects because it even presents a prebiotic activity.
- The **microbiota** composition is well correlated with the presence of several methylxanthines-derived **metabolites** in urine.
- Whereas the cocoa-enriched diet practically does not modify the **short chain fatty acids** production by the intestinal microbiota, the cocoa fiber increases the intestinal concentration of acetic, propionic and butyric acids.
- The cocoa fiber intake modulates the immunological interaction between the host and the microbiota by strengthening the **TLR gene expression** in colon.
- The lower intestinal **IgA** production induced by the 10% cocoa diet is due to its content in both flavonoids and fiber. The modulatory effect of both the cocoa and cocoa fiber is also observed in others extraintestinal mucosal tissues such as the salivary glands. In these glands, the cocoa fiber also reduces the gene expression of molecules involved in the IgA secretion. On the contrary, the IgA synthesis is increased by the cocoa fiber diet in the systemic compartments.

- The cocoa-enriched diet intake reduces the proportion of **IgA-coated bacteria** in feces, which is produced, at least in part, by its content in flavonoids. This effect is also associated with methylxanthines-derived metabolites resulted from the bacterial metabolism. On the contrary, the cocoa fiber based diet increases the proportion of fecal bacteria-coated with IgA.
- The 10% cocoa diet intake implies changes in the **expression** of several **genes** involved in the immune response and lipid metabolism. Most of these effects are not observed when diet only contains cocoa fiber.
- The diet containing cocoa slows down the **body weight gain**, effects that are independent of its content in flavonoids and cocoa fiber. This result is associated with, among others, methylxanthines-derived metabolites resulted from the bacterial metabolism, a lower energetic metabolism, and with the microbiota composition modulation. However, this effect is not due to a modulation of the metabolic hormones such as leptin.

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