UNIVERSITAT DE BARCELONA

Influència d'una dieta rica en cacau sobre el sistema immunitari intestinal de rates sensibilitzades

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INFORMA

Que la memòria titulada ***Influència d'una dieta rica en cacau sobre el sistema immunitari intestinal de rates sensibilitzades*** presentada per **MARIONA CAMPS i BOSSACOMA** per optar al Títol de Doctora per la Universitat de Barcelona, ha estat realitzada sota la meva direcció a la Secció de Fisiologia, i considerant-la conclosa, autoritzo la seva presentació per ser jutjada pel tribunal corresponent.

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'The important thing is not to stop questioning'

Albert Einstein

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ABREVIATURES

APC	Cèl·lules presentadores d'antigen
BALT	*Bronchus-associated lymphoid tissue*
BN	Brown Norway
CALT	*Conjuntiva-associated lymphoid tissue*
CD	*Cluster of differentiation*
COX	Ciclooxigenasa
DALT	*Salivary duct-associated lymphoid tissue*
DC	Cèl·lula dendrítica
DN	*Double negative*
GALT	*Gut-associated lymphoid tissue*
GF	*Germ-free*
GLM	Ganglis limfàtics mesentèrics
GM-CSF	*Granulocyte macrophage colony-stimulatig factor*
i.p.	Intraperitoneal
IDO	Idoleamina 2,3-dioxigenasa
IEL	Limfòcits intraepitelials
IFN	Interferó
Ig	Immunoglobulina
IL	Interleucina
ILC	*Innate lymphoid cells*
LALT	*Larynx-associated lymphoid tissue*
LDALT	*Lacrimal duct-associated lymphoid tissue*
LOX	Lipooxigenasa
LPL	Limfòcits de la làmina pròpia
MALT	*Mucosa-associated lymphoid tissue*
MHC	*Major histocompatibility complex*
NALT	*Nasopharynx-associated lymphoid tissue*
NF-κB	Factor de transcripció nuclear kappa B
NK	*Natural killer*
NO	Òxid nitric

PAMPS *Pathogen-associated molecular patterns*

PARP Poli ADP (difosfat d'adenosina)-ribosa polimerasa

PDGF *Platelet-derived growth factor*

PP Plaques de Peyer

PRR *Pattern-recognition receptor*

RA Àcid retinoic

RALDH Retinal deshidrogenasa

SCFA *Short-chainfatty acids*

SP *Single positive*

TCR *T cell receptor*

TGF *Transformin growth factor*

Th Limfòcit *T helper*

TLR Receptor *toll-like*

TNF Factor de necrosis tumoral

Treg Cèl·lula T reguladora

VEGF *Vascular endothelial growth factor*

ABSTRACT

Due to the rising prevalence of food allergy, the development of animal models of oral food sensitization is of interest in studying the mechanisms involved in this sensitization and its possible treatments.

Previous studies have reported that cocoa influences the intestinal and systemic immune systems, modifying lymphoid tissue composition and functionality and the synthesis of intestinal and systemic antibodies.

On these bases, the aim of the current thesis was to study in depth the immunomodulatory properties of cocoa, in particular to ascertain the effect and possible mechanisms induced by a cocoa diet on a rat oral sensitization model and also to determine which compound/s is/are responsible for such effect.

To achieve the initial part of the goal, a rat oral sensitization model was developed using ovalbumin as allergen and cholera toxin as adjuvant. Afterwards, three-week-old female Lewis rats from the successful model developed were fed for four weeks with a 10% cocoa diet. With regard to the results, the 10% cocoa diet was able to prevent the synthesis of the anti-ovalbumin antibodies. Moreover, the cocoa intake decreased intestinal IgA concentration due to fewer IgA cells reaching the lamina propia. In terms of the gut-associated lymphoid tissues (GALT), cocoa diet increased the proportion of TCR$\gamma\delta$+ cells and NK cells in mesenteric lymph nodes, Peyer's patches and epithelium together with some modifications in gene expression of various molecules and cytokines in the mesenteric lymph nodes and small intestine. Cocoa intake also influenced gut microbiota in this rat oral sensitization model.

To attain the second part of the goal, the determination of the compounds responsible for the immunoregulatory effect of cocoa, two different approaches were made. Firstly, approximations were carried out in order to determine the effect of hesperidin, a flavonoid not found in cocoa, on rat immune response. However, with the dosages and the immunization process used, we were unable to detect changes on specific antibody production, although some modifications were evident in intestinal lymphocyte composition and functionality.

On the other hand, in order to determine whether theobromine, the main methylxanthine in cocoa, was responsible for the cocoa's effects on microbiota, antibody immune response and on lymphocyte composition, the effects of the cocoa diet were compared with those obtained from rats fed a 0.25% theobromine diet (the same amount of theobromine provided by the cocoa diet). Theobromine affected microbiota composition but not in the same manner as cocoa intake, suggesting that other cocoa compounds, such as polyphenols and fibre, may have an effect on the intestinal bacteria. Nevertheless, theobromine was responsible for the short-chain fatty acid increase and the lower proportion of IgA-coated bacteria. Moreover, theobromine seems to be the main

component responsible for the decrease in cocoa antibodies together with almost all modifications to the lymphoid tissues.

To sum up, cocoa, due to its theobromine content, is able to prevent the development of a rat oral sensitization model, contributing towards oral tolerance. These results suggest the role of cocoa as a potential nutraceutical for avoiding or preventing food allergies.

Introducció

1. IMMUNITAT SISTÈMICA I INTESTINAL

1.1 RESPOSTA IMMUNITÀRIA

La funció fisiològica del sistema immunitari consisteix en la defensa conjunta i coordinada enfront qualsevol substància perjudicial (agents infecciosos o toxines) que es trobi en contacte amb l'organisme. Aquesta defensa té lloc mitjançant mecanismes defensius genèrics que constitueixen la resposta immunitària innata i mecanismes defensius específics, que formen la resposta adaptativa [1]. Una altra funció del sistema immunitari és evitar la resposta contra el que és propi i contra les substàncies innòcues que entren en contacte amb l'organisme, procés que s'anomena tolerància [1].

La immunitat innata, també anomenada immunitat natural o espontània, és la primera línia de protecció enfront a agents estranys. Consisteix en mecanismes de defensa cel·lulars i bioquímics que reconeixen l'antigen de forma inespecífica i donen una resposta ràpida i similar davant les diferents substàncies alienes. Les cèl·lules que majoritàriament hi intervenen són els macròfags, els neutròfils i les cèl·lules natural killer (NK) [2].

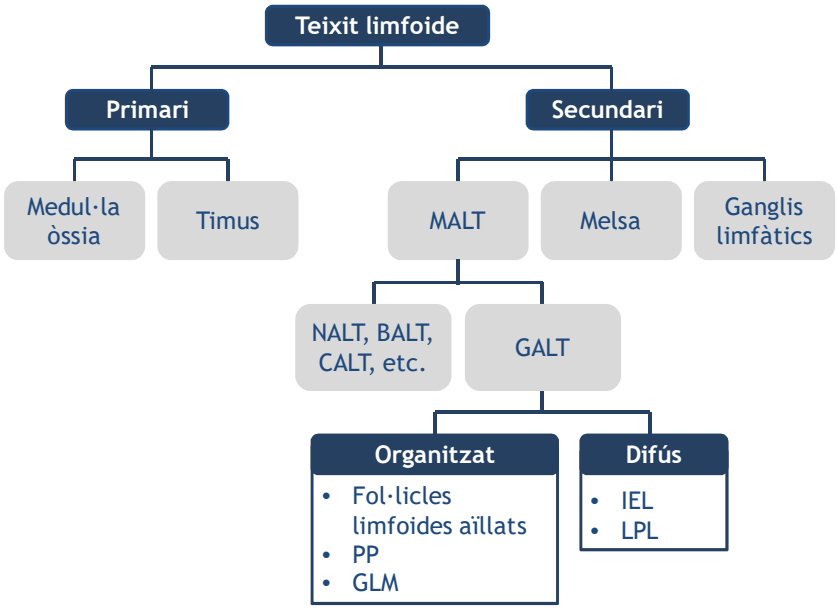
Per altra banda, la immunitat adaptativa, també anomenada específica, tal com aquest darrer nom indica, elabora una resposta concreta davant de cada agent infecciós. Les cèl·lules que majoritàriament hi intervenen són els limfòcits [2].

1.2 TEIXITS LIMFOIDES

Els limfòcits es formen i acumulen en teixits o òrgans limfoides, compartiments de gran importància no només per al desenvolupament i regulació dels limfòcits, sinó també per a la iniciació de les respostes immunitàries [3]. Des del punt de vista funcional es classifiquen en primaris i secundaris (**Figura 1**), encara que, darrerament, s'ha descrit una nova categoria, els terciaris [4]. Els teixits limfoides terciaris són acumulacions ectòpiques de cèl·lules limfoides que sorgeixen durant una inflamació crònica [4]. Concretament, es formen durant infeccions cròniques, malalties autoimmunitàries i en localitzacions amb càncer, entre altres [5].

Figura 1. Classificació del teixit limfoide

BALT (*bronchus-associated lymphoid tissue*); CALT (*conjuntiva-associated lymphoid tissue*); GALT (*gut-associated lymphoid tissue*); GLM (ganglis limfàtics mesentèrics); IEL (limfòcits intraepitelials); LPL (limfòcits de la làmina pròpia); MALT (*mucosa-associated lymphoid tissues*); NALT (*nasopharynx-associated lymphod tissue*); PP (plaques de Peyer)



1.2.1 Teixits limfoides primaris

Els teixits limfoides primaris o centrals són els encarregats de la formació i maduració dels limfòcits a partir de les cèl·lules mare hematopoètiques, és a dir, de la limfopoesi. En aquesta categoria s'inclouen la medul·la òssia i el timus (**Figura 1**).

- **Medul·la òssia**

A la medul·la òssia s'hi generen els precursors hematopoètics, és on té lloc la formació d'eritròcits, monòcits, granulòcits, limfòcits i plaquetes [6]. A més, a la medul·la òssia es produeix la maduració dels limfòcits B. En canvi, els precursors dels limfòcits T abandonen la medul·la per acabar de madurar al timus.

La medul·la òssia està molt vascularitzada per vasos sanguinis, no per limfàtics, i constitueix una part important de la xarxa de recirculació dels limfòcits, per on passen milers de milions de limfòcits al dia [7].

- **Timus**

El timus és un òrgan bilobulat situat al tòrax, a la regió anterior del mediastí, darrera de l'estèrnum i per sobre del cor. Cada lòbul està dividit en múltiples lobels per envans fibrosos, que consten d'una escorça externa i una medul·la interna [2]. Els precursors dels

limfòcits T arriben a l'escorça a través de la sang [8] i, a mesura que maduren, avancen cap a la medul·la (**Figura 2**). Quan les cèl·lules progenitores entren al timus no presenten el receptor antigènic de limfòcits T (TCR), ni els coreceptors CD4 o CD8, i s'anomenen cèl·lules doble negatives (DN, CD4-CD8-). Els limfòcits DN es poden subdividir en quatre estats de diferenciació en funció de l'expressió de les molècules CD44 i CD25 (DN1-4). A excepció de les DN1 (CD44+CD25-), els limfòcits en aquest estat de maduració expressen el pre-receptor TCR. A continuació, les cèl·lules passen a ser doble positives (DP, CD4+CD8+) i expressen correctament el receptor TCR [8]. A partir d'aquí, les cèl·lules DP interaccionen amb les cèl·lules epitelials de l'escorça que expressen el complex principal d'histocompatibilitat (MHC) de classe I o II associat a antígens propis, i en funció del MHC amb el que interaccionin i del grau de reconeixement del pèptid presentat, les cèl·lules pateixen la mort per negligència, la selecció positiva o la selecció negativa. Quan els limfòcits DP interaccionen dèbilment amb cèl·lules epitelials de l'escorça, es produeix apoptosi, l'anomenada mort per negligència. Per altra banda, s'evidencia una selecció positiva quan les cèl·lules DP expressen un TCR que reconeix adequadament el pèptid propi presentat pel MHC de cèl·lules epitelials o dendrítiques de la medul·la. Quan les cèl·lules T han reconegut el MHC de classe I mantenen l'expressió de CD8 (*single positive*, SP, CD4-CD8+), mentre que quan el limfòcit T reconeix el MHC de classe II, aquests preserven l'expressió de CD4+ (SP, CD4+CD8-) [2,8] (**Figura 2**). En canvi, els limfòcits DP experimenten una selecció negativa quan reconeixen fortament el complex MHC i el pèptid propi, i dóna lloc a la seva apoptosi per tal d'evitar la circulació de limfòcits autoreactius [2,8] (**Figura 2**).

Un cop els limfòcits T han madurat inicien la migració cap als òrgans limfoides secundaris via sanguínia o limfàtica (**Figura 3**).

Figura 2. Maduració dels limfòcits T al timus
Basada en [8]

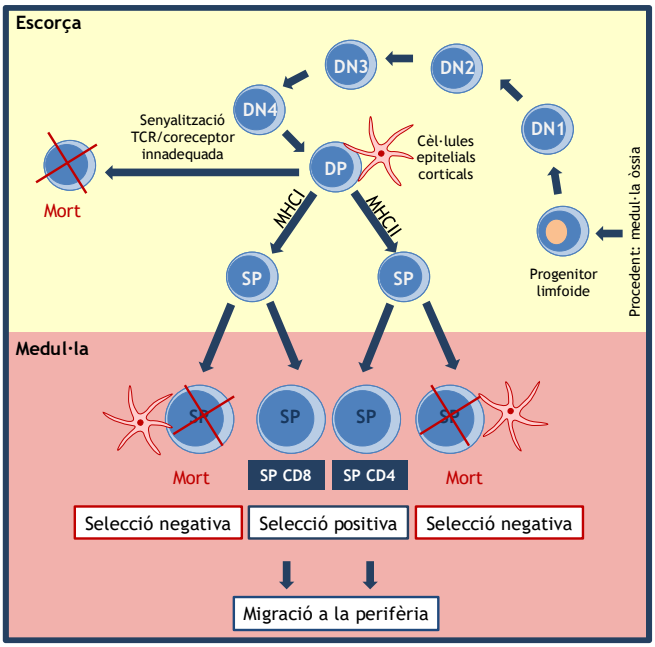
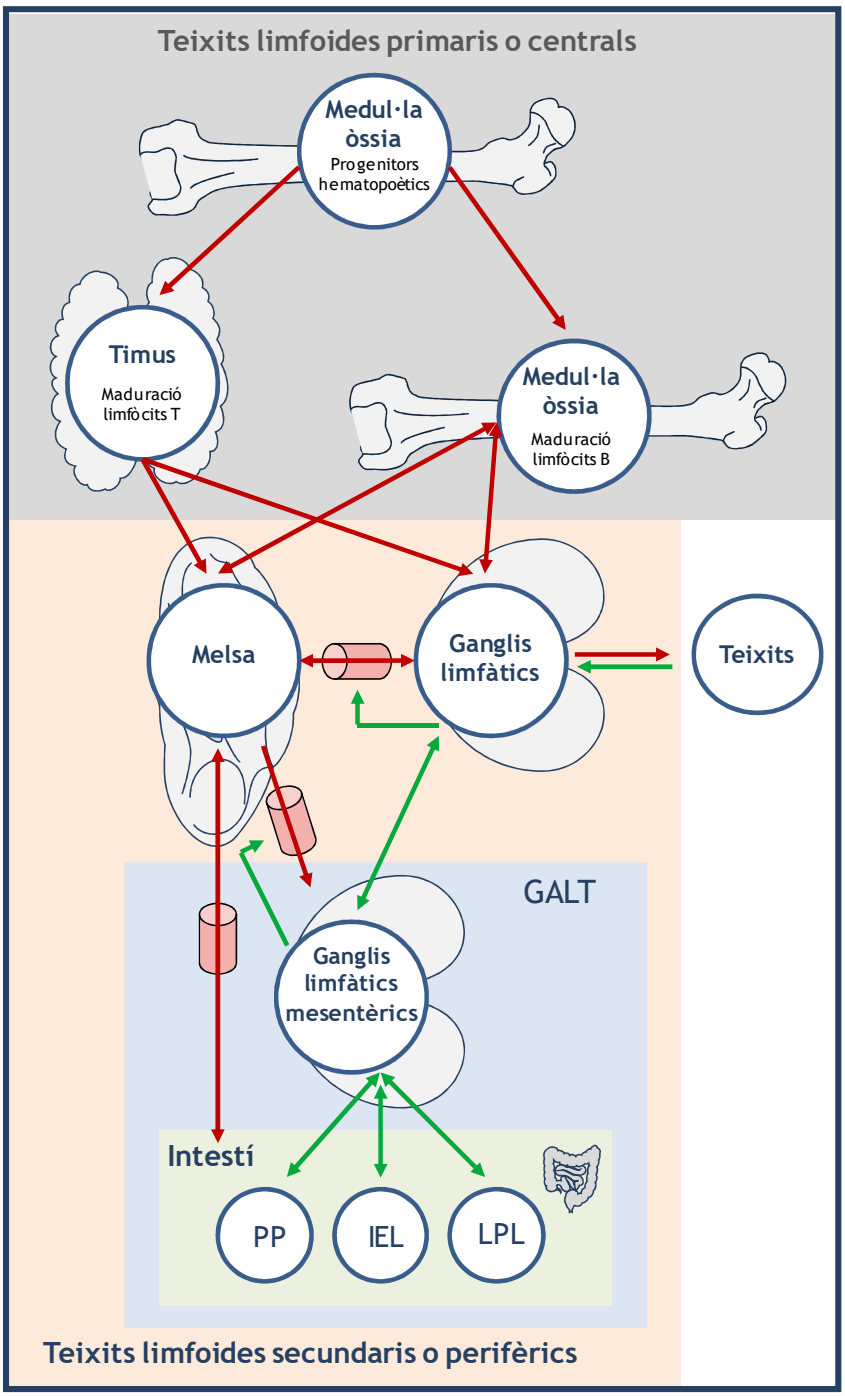


Figura 3. Connexions entre el teixit limfoide

Fletxes vermelles: transport per sang. Fletxes verdes: transport per limfa. GALT (*gut-associated lymphoid tissue*); IEL (limfòcits intraepitelials); LPL (limfòcits de la làmina pròpia); PP (plaques de Peyer)



1.2.2 Teixits limfoides secundaris

Els teixits limfoides secundaris o perifèrics constitueixen el lloc d'estimulació dels limfòcits madurs per tal de respondre a patògens. En aquesta categoria s'inclouen la melsa, els ganglis limfàtics i el teixit limfoide associat a mucoses (MALT) (**Figura 1**).

- **La melsa**

La melsa és un gran òrgan limfoide que es troba ubicat a la part superior esquerra de l'abdomen, just per sota el diafragma, amb un pes aproximat d'uns 150 g en una persona adulta [9]. Està formada per dos compartiments funcionalment i morfològicament diferents: la polpa blanca i la polpa vermella. La polpa blanca està plena de limfòcits i està organitzada en dues zones: la de limfòcits T i la de limfòcits B. La seva funció consisteix en afavorir la resposta immunitària adaptativa enfront a antígens transportats per la sang. Per altra banda, a la polpa vermella s'hi filtra la sang i s'elimina material estrany i eritròcits vells o danyats [9,10].

- **Ganglis limfàtics**

Els ganglis limfàtics són petits òrgans en forma de ronyó amb un pes aproximat d'un gram que es troben dispersos per tot el cos i connectats entre ells mitjançant vasos limfàtics. La limfa arriba als ganglis a través de diferents vasos limfàtics aferents, es filtra a través de la substància del gangli i drena per un vas limfàtic eferent. Els ganglis limfàtics presenten una escorça externa i una medul·la interna i es troben envoltats per una càpsula fibrosa. En concret, els ganglis limfàtics proporcionen una interfase entre la sang i el sistema limfàtic i permeten que les cèl·lules presentadores d'antigen (APC), residents als ganglis limfàtics o bé que han emigrat des de teixits perifèrics, presentin l'antigen als limfòcits T i inicïïn la resposta immunitària [11]. Els limfòcits verges passen des de la sang cap els ganglis limfàtics, i a través de vasos limfàtics tornen a la circulació sanguínia diverses vegades (**Figura 3**), fins que reconeixen un antigen a l'interior d'un gangli. Una vegada han reaccionat amb un antigen es diferencien a cèl·lules efectores i memòria i emigren cap al focus infecciós via sanguínia (**Figura 3**).

- **Teixit limfoide associat a mucoses (MALT)**

Les superfícies mucoses del sistema digestiu i de les vies respiratòries, amb una extensió superior a 300 m^2 en humans, juntament amb altres mucoses, es troben colonitzades per limfòcits, formant el teixit limfoide associat a mucoses (MALT). El MALT participa en respostes immunitàries contra antígens ingerits o inhalats [12] i, en concret, inclou el teixit limfoide associat a l'intestí (*gut-associated lymphoid tissue*, GALT), a la nasofaringe (*nasopharynx-associated lymphoid tissue*, NALT), als bronquis (*bronchus-associated lymphoid tissue*, BALT), a la conjuntiva (*conjuntiva-associated lymphoid tissue*, CALT), al conducte lacrimal (*lacrimal duct-associated lymphoid tissue*, LDALT), a la laringe (*larynx-associated lymphoid tissue*, LALT) i al conducte salival (*salivary duct-associated lymphoid tissue*, DALT) [10]. Les principals funcions del MALT són la producció i la secreció d'IgA específica a través de la mucosa.

1.2.3 Teixit limfoide associat a l'intestí (GALT)

El GALT constitueix el 70% de la totalitat del sistema immunitari, essent la principal ruta de contacte amb l'exterior i, per tant, amb patògens (bactèries, protozous, fongs, virus) o substàncies tòxiques [13]. Conté 10^{12} cèl·lules limfoides per metre d'intestí i més cèl·lules productores d'immunoglobulines que la resta del cos [14]. Anatòmicament el GALT es divideix en dos compartiments, el GALT organitzat i el GALT difús (**Figura 1**).

El GALT organitzat és l'inductor de la resposta immunitària intestinal i inclou els fol·licles limfoides aïllats, les plaques de Peyer (PP) i els ganglis limfàtics mesentèrics (GLM) (**Figura 1**).

Les PP són fol·licles limfoides localitzats a la cara antimesentèrica de la mucosa intestinal [15]. La seva mida i densitat incrementa del jejú a l'ili, trobant-se amb més freqüència a la part distal de l'ili [16]. Les PP es troben separades de la llum intestinal per un epiteli especialitzat, anomenat epiteli associat al fol·licle, on es troben les cèl·lules M. A les PP s'hi troben moltes cèl·lules B rodejades de petites àrees de limfòcits T [16]. Les PP tenen un paper molt important en la resposta immunitària mucosal enfront antígens o bactèries i en la inducció de la tolerància oral [17,18].

Els GLM es troben en el mesenteri de l'intestí prim i és on té lloc la resposta immunitària específica, sent el principal compartiment d'inducció de la tolerància oral [19].

El GALT difús és el teixit efector de la resposta immunitària. Aquest teixit inclou els limfòcits intraepitelials (*intraepithelial lymphocytes*, IEL) i els limfòcits de la làmina pròpia (*lamina propia lymphocytes*, LPL) (**Figura 1**). Els IELs contenen molts limfòcits T localitzats entremig de les cèl·lules epitelials, i interactuen amb aquestes per tal de mantenir l'homeòstasi mucosal [20,21]. En concret, els IELs es troben amb una freqüència de 10-15 per 100 cèl·lules epitelials [22] i inclouen limfòcits T amb diferent estructura de receptor antigènic: les cèl·lules TCR$\alpha\beta$ i les cèl·lules TCR$\gamma\delta$, que també es poden classificar en funció de l'expressió del coreceptor CD8. La majoria dels IEL TCR$\gamma\delta$ expressen el coreceptor CD8$\alpha\alpha$ [23]. Els IELs estant destinats a proporcionar una protecció immediata i evitar l'entrada i propagació d'agents infecciosos que entren per via intestinal [20].

Els LPLs inclouen limfòcits T (amb proporció similar de CD4+ i CD8+), cèl·lules plasmàtiques, cèl·lules dendrítiques, mastòcits i macròfags [15,16] i es troben localitzats a la làmina pròpia de l'intestí, just per sota de l'epiteli. En aquest compartiment se sintetitzen anticossos (sobretot IgA) i hi té lloc la presentació antigènica local.

1.3 RESPOSTA IMMUNITÀRIA INTESTINAL

Malgrat l'existència de les barreres físiques, químiques i biològiques que protegeixen el tracte gastrointestinal, de vegades, els antígens poden penetrar la mucosa intestinal i arribar al GALT [15].

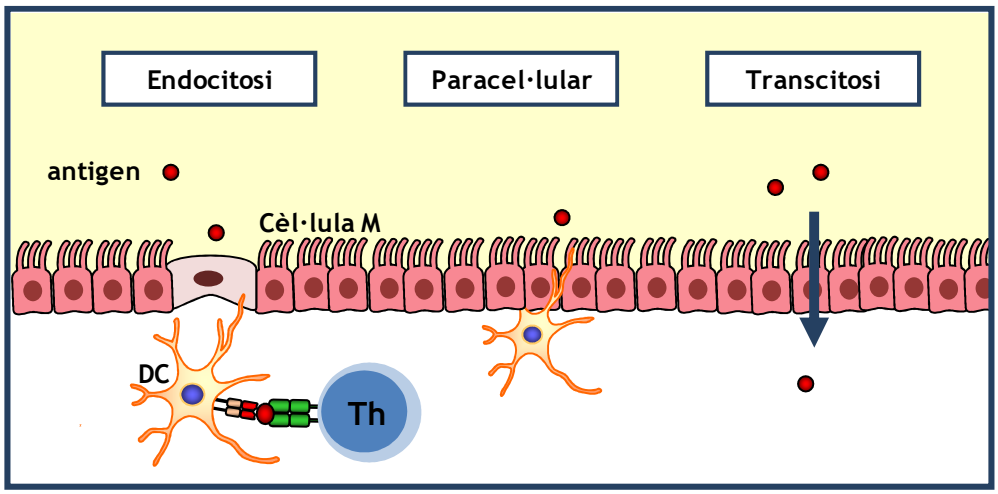
Les possibles vies d'entrada dels antígens són (**Figura 4**):

- **Endocitosi:** les cèl·lules M –localitzades a les PP- o les cèl·lules caliciformes, permeten l'adhesió, captació i internalització d'antígens. Mitjançant mecanismes d'endocitosi, els antígens són transportats a través de vesícules a la membrana basolateral, on són alliberats a l'espai extracel·lular [24] i es troben amb les APC, fonamentalment les cèl·lules dendrítiques (DC).

- **Difusió paracel·lular**: entre els enteròcits, les cèl·lules dendrítiques poden projectar les seves dendrites mitjançant proteïnes associades a les unions estretes i captar l'antigen directament de la llum intestinal.

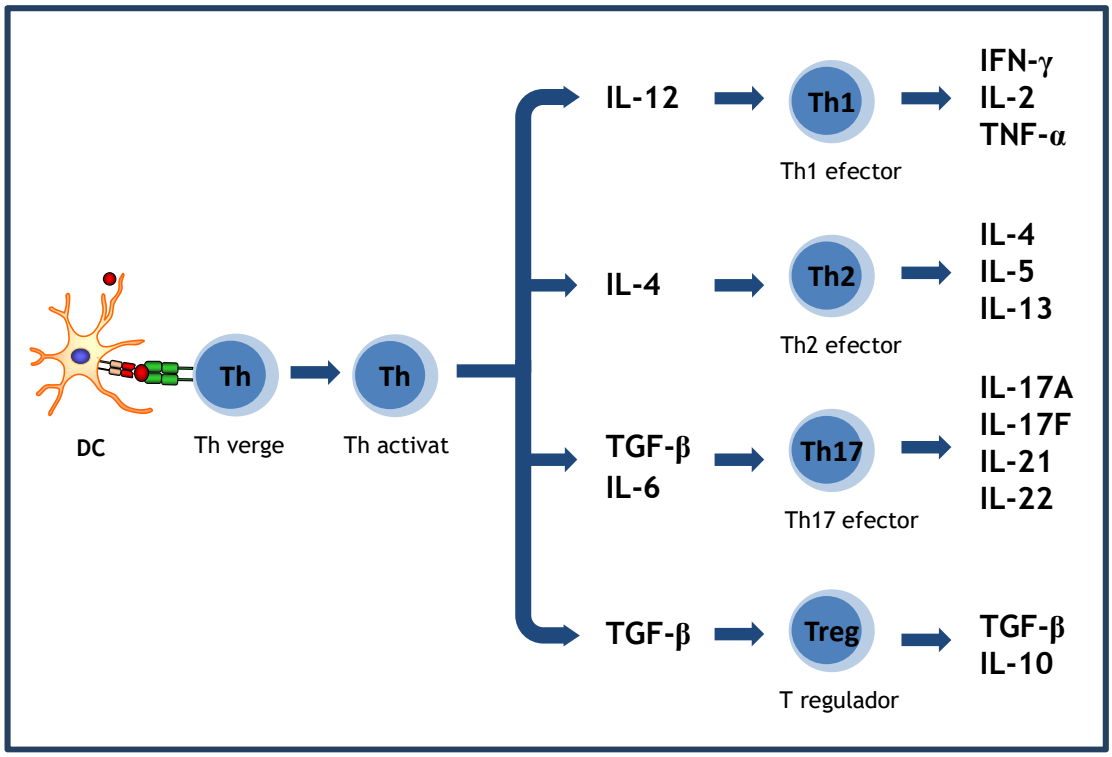
- **Transcitosi:** certs antígens poden ser captats pels enteròcits i alliberats a la làmina pròpia on trobaran les APC. Aquesta via és més restrictiva a causa del recobriment extern d'aquestes cèl·lules amb glicocàlix, fet que impedeix l'entrada d'agregats macromoleculars i de microorganismes.

Figura 4. Vies d'entrada dels antígens intestinals
DC (cèl·lula dendrítica); Th (limfòcit T helper)



Les DC del GALT actuen d'APC de forma similar al sistema immunitari sistèmic. Les DC interioritzen i processen l'antigen per tal d'expressar els pèptids antigènics a la seva membrana associats al MHC de classe II i presentar-los als limfòcits T específics que els reconeixeran a través del seu receptor (TCR). Aquesta interacció entre DC i limfòcits T pot tenir lloc a la pròpia PP o bé als GLM, després de la migració de la DC a través de vasos limfàtics aferents. Amb la presentació antigènica, els limfòcits s'activen i proliferen, i es diferencien, en funció de les citocines que secreten, en les subpoblacions efectores Th1, Th2 o Th17 o en les cèl·lules T reguladores (Treg) (**Figura 5**).

Figura 5. Resum de l'activació de cèl·lules T i els tipus cel·lulars resultants
DC (cèl·lula dendrítica); IFN (interferó); IL (interleucina); TGF (*transforming growth factor*); Th (limfòcit T *helper*); TNF (factor de necrosis tumoral)



Els limfòcits Th1 es caracteritzen per la secreció d'interferó γ (IFN-γ), interleucina (IL)-2, i del factor de necrosis tumoral (TNF)-α, entre d'altres. La seva principal funció és la defensa, mitjançant fagocitosi i activació de la citotoxicitat contra infeccions, especialment de microorganismes intracel·lulars (virus, bacteris i alguns protozous). Per altra banda, els limfòcits Th2 produeixen, sobretot, IL-4, IL-5 i IL-13 i actuen com a principals mediadors de reaccions al·lèrgiques i en defensa d'infeccions extracel·lulars i paràsits (d'helmints i artròpodes). La subpoblació efectora Th17 es caracteritza per la secreció d'IL-17A, IL-17F, IL-21 i IL-22 [25]. El factor de creixement transformant (TGF)-β1 i la IL-6 promouen la diferenciació dels limfòcits Th17, però una vegada aquests estant diferenciats, la IL-23 és la citocina que indueix la seva proliferació (**Figura 5**). Els tres tipus cel·lulars efectors s'inactiven mútuament. Així doncs els limfòcits efectors Th1, mitjançant IFN-γ inhibeixen l'activitat dels Th2 i dels Th17; les cèl·lules efectores Th2 mitjançant la IL-4 inhibeixen la proliferació dels limfòcits Th1 i Th17, i, per últim, les cèl·lules efectores Th17 inhibeixen l'activació dels limfòcits Th1 i Th2.

A més d'aquestes tres subpoblacions limfocítiques efectores, s'ha descrit l'existència de limfòcits Treg, associats a la presència del TGF-β. Aquestes cèl·lules produeixen IL-10 i

TGF-β i intervenen en la prevenció de malalties autoimmunitàries, la supressió d'al·lèrgia i d'asma i en el desenvolupament de la tolerància oral [26].

En funció de quin tipus cel·lular s'activi i proliferi en resposta a un antigen, es desenvoluparà la tolerància oral a antígens o la sensibilització oral (**Apartats 1.4 i 1.6.2**).

1.4 TOLERÀNCIA ORAL

EL GALT de tots els individus es troba permanentment en un entorn amb una gran quantitat i diversitat d'antígens, concretament, amb el pas de més de 30 kg de proteïnes alimentàries cada any, el que representa més de 100 g de proteïna al dia [27,28]. Generalment aquestes substàncies no indueixen una resposta defensiva però, en determinades persones i ambients, es pot activar el sistema immunitari. La tolerància oral consisteix en la supressió específica de la resposta immunitària enfront a un antigen que entra per via digestiva [29]. Mantenir la tolerància requereix complexes interaccions entre les cèl·lules no immunitàries i les cèl·lules immunitàries del GALT [28]. Aquests complexos mecanismes depenen de diversos factors, tals com la dosi de l'antigen, la seva estructura, el temps i ruta d'exposició, la susceptibilitat genètica i la composició i activitat metabòlica de la microbiota [30].

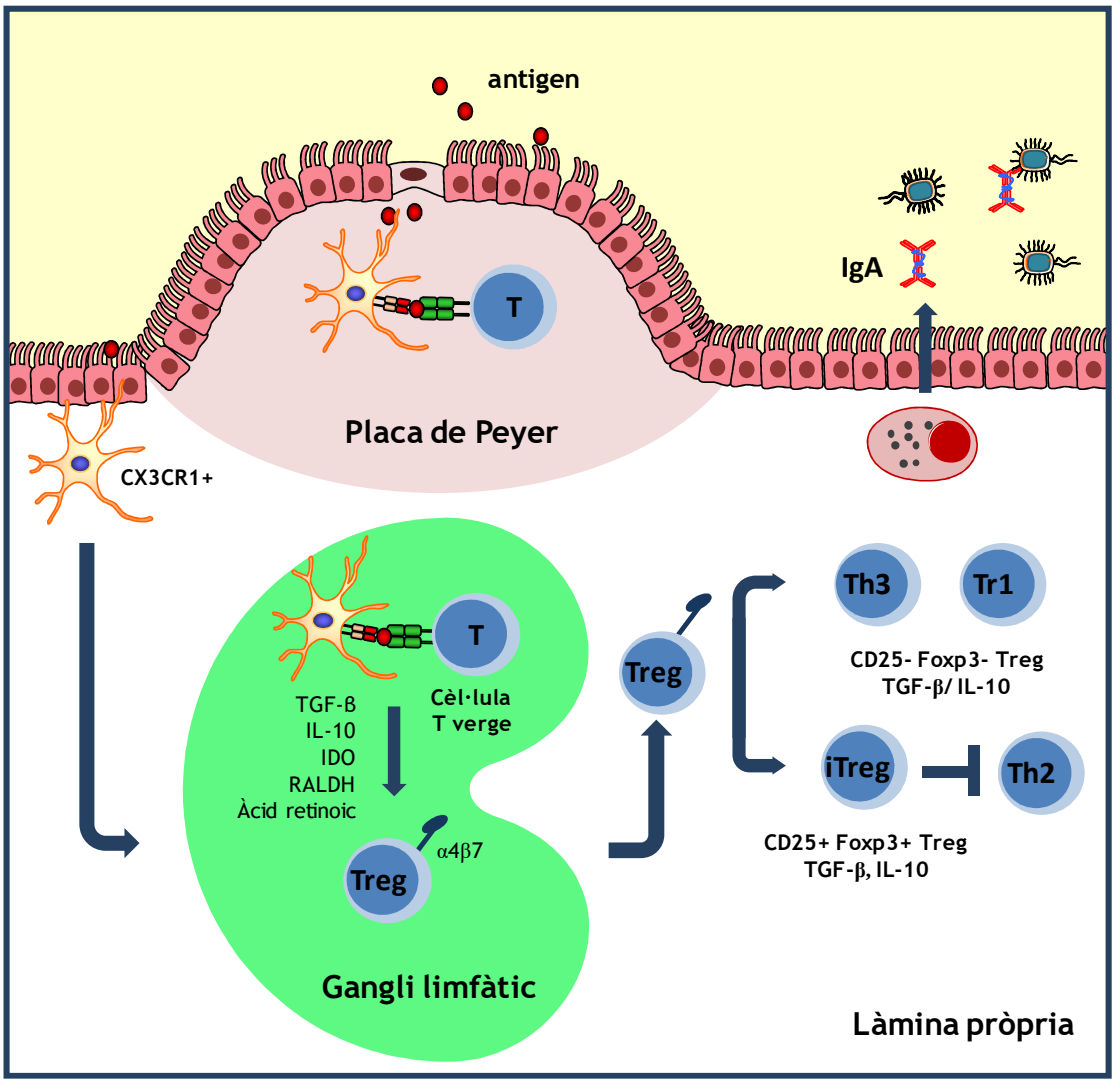
En el desenvolupament de la tolerància, les DC hi tenen un paper important. Un cop la DC ha capturat l'antigen, la DC (CD103+) migra cap als GLM on es produeixen diferents citocines (TGF-β, IL-10), enzims (retinal deshidrogenasa -RALDH- i idoleamina 2,3-diogixenasa -IDO-) i metabòlits (àcid retinoic -RA-) que indueixen la diferenciació dels limfòcits T verges a limfòcits Treg. Aquestes cèl·lules expressen a la seva superfície marcadors que activen la migració intestinal (integrina α4β7 i el receptor CCR9), on desenvolupen els seus efectes tolerogènics (**Figura 6**).

Les cèl·lules Treg que es formen poden ser principalment de tres tipus (**Figura 6**):

- **Cèl·lules Th3:** limfòcits que no expressen Foxp3 ni CD25. Produeixen TGF-β i poden promoure el desenvolupament de les cèl·lules Treg induïdes (iTreg) amb la producció de TGF-β.
- **Cèl·lules Tr1:** limfòcits que no expressen Foxp3 ni CD25 i participen a la tolerància oral amb la producció d'IL-10.
- **Cèl·lules iTreg:** cèl·lules Treg CD4+CD25+Foxp3+ involucrades en la inhibició de respostes Th2, ja que promouen la formació d'IgG4 anti-al·lergogen i generen DC tolerogèniques.

Quan algun d'aquests mecanismes de tolerància es desregula pot comportar el desenvolupament d'una reacció d'hipersensibilitat [31], tal com es detalla a l'**apartat 1.6.2**.

Figura 6. Esquema del desenvolupament de la tolerància a antígens alimentaris
IDO (idoleamina 2,3-dioxigenasa); IL (interleucina); iTreg (limfòcits T reguladors induïts); RALDH (retinal deshidrogenasa); T (limfòcit T); TGF (*transforming growth factor)*; Th (limfòcit T helper); Treg (limfòcit T regulador); Tr1 (limfòcts T reguladors de tipus 1)



1.5 MICROBIOTA INTESTINAL

El tracte gastrointestinal conté un complex i dinàmic ecosistema que inclou una gran varietat de microorganismes. En concret, el cos humà conté com a mínim 10^{14} bactèries [32], suposant una massa total d'uns 2 kg [33], i essent el tracte gastrointestinal el compartiment més densament colonitzat [34]. El genoma de la microbiota intestinal, anomenat microbioma, inclou, almenys, 150 vegades més gens que el genoma humà [35].

La microbiota intestinal no és igual per a totes les persones i dins de la mateixa persona depèn de la seva edat i de diversos factors ambientals. El desenvolupament de la microbiota intestinal es troba influït per les condicions del naixement, la genètica, l'epigenètica, la dieta, l'edat, l'entorn, l'ús de medicaments, entre d'altres, i es va modificant al llarg del temps [34,36–38]. Es considera que el fetus és estèril fins el moment del part, encara que s'ha descrit la composició bacteriana en el meconi del nounat, i s'ha suggerit una possible colonització microbiana a l'úter [39,40]. Tanmateix, es considera que, en el moment del part, el nadó és ràpidament colonitzat, principalment, pel contacte vaginal i, a partir d'aquest moment, s'origina una transmissió vertical de mare a fill juntament amb altres factors externs tant de la mare com del nen, i es va modificant la microbiota del nadó. Durant el primer any de vida la microbiota de l'infant conté menys nombre i diversitat de bactèries que la de l'adult. La composició bacteriana en aquest període es caracteritza per un predomini d'*Actinobacteria* i *Proteobacteria*. En condicions normals, quan el nen assoleix els tres anys de vida presenta una composició bacteriana que, en l'estat de salut, es pot mantenir estable la resta de vida [41]. La microbiota de l'adult inclou més de 1000 espècies bacterianes diferents [12] i es caracteritza per tenir principalment un 70-75% de bacteries dels dos següents fílums [42]:

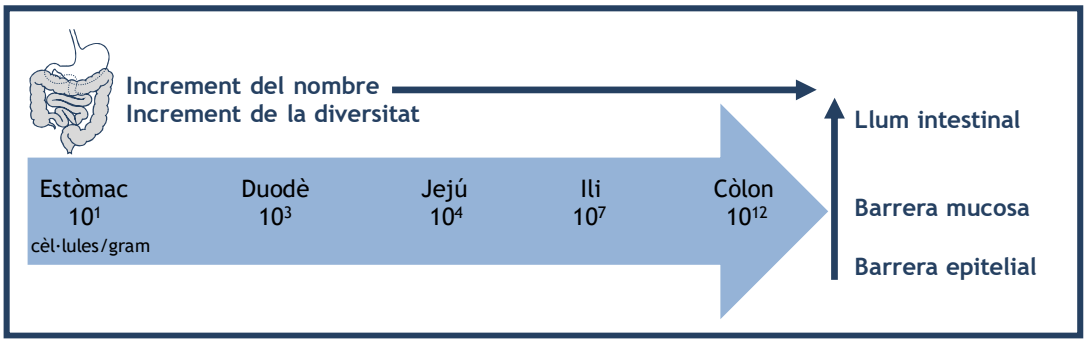
- *Firmicutes*: principalment dels gèneres *Clostridium*, *Faecalibacterium*, *Blautia*, *Ruminococcus* i *Lactobacillus*.
- *Bacteroidetes*: principalment dels gèneres *Bacteroides* i *Prevotella*.

En petites quantitats, també es troben bacteris dels fílums *Actinobacteria* (*Bifidobacteria*), *Proteobacteria* (*Enterobacteriaceae*), *Verrucomicrobia*, *Fusobacteria* i *Cyanobacteria* [41–43].

La composició de la microbiota intestinal varia de persona a persona i, en funció d'aquesta, la població humana s'ha categoritzat en 3 enterotips [44]: l'enterotip 1 (anomenat *Bacteroides*), l'enterotip 2 (anomenant *Prevotella*) i l'enterotip 3 (anomenat *Ruminococcus*). Aquests enterotips s'han relacionat amb la dieta que l'individu consumeix: l'enterotip 1 està associat amb el consum d'elevades proporcions de proteïnes i grasses, l'enterotip 2 amb elevades quantitats de carbohidrats i sucres simples i l'enterotip 3 amb grans quantitats de grasses poliinsaturades [45].

La microbiota intestinal no es troba distribuïda homogèniament al llarg del tracte gastrointestinal, sinó que la densitat microbiana incrementa al llarg d'aquest. L'estómac és l'òrgan menys colonitzat (10^1-10^4 cèl·lules per gram de contingut), i el còlon el que més (10^{10}-10^{12} cèl·lules/g de femta) (**Figura 7**). Es considera que només en el còlon hi ha un 70% del total de bactèries presents al cos humà [43].

Figura 7. Variacions en la quantitat de microbiota al llarg del tracte gastrointestinal
Basada en [43]



Durant la darrera dècada, l'estudi de la microbiota intestinal humana ha experimentat un gran creixement i ha permès aprofundir en la seva composició i funcions. Aquest coneixement deriva, fonamentalment, de dos grans estudis. Als Estats Units, el projecte NIH HMP (*Human Microbiome Project,* 2007) intenta descriure els gens bacterians humans, agrupar-los en estat de salut i predisposició de malalties [46]. A Europa, el projecte MetaHIT (*Metagenomics of the Human Intestinal Tract,* 2008) ha permès descriure, agrupar i caracteritzar 3,3 milions de gens bacterians trobats en mostres fecals de persones europees [35] i s'ha centrat en el paper de la microbiota en la malaltia inflamatòria intestinal i en l'obesitat [47].

1.5.1 Funcions de la microbiota

La microbiota intestinal participa en l'homeòstasi de l'organisme a través de funcions metabòliques i nutricionals, fisiològiques i immunològiques.

- **Funció metabòlica i nutricional:** la microbiota exerceix una funció metabòlica important ja que algunes bactèries contenen enzims capaços de digerir certs carbohidrats que no han sigut processats (fibra insoluble o el midó resistent de blat de moro). Aquestes activitats metabòliques comporten la producció de nutrients com àcids grassos de cadena curta (SCFA), vitamines (vitamina K, vitamina B_{12} i àcid fòlic) i aminoàcids [34], essent els SCFA una font important d'energia i de metabolisme lipídic.

- **Funció fisiològica i immunològica:** la microbiota participa en la defensa contra patògens amb la producció de components antimicrobians. També està implicada en el desenvolupament, maduració i manteniment de les funcions gastrointestinals de la barrera intestinal i del sistema immunitari mucosal.

En condicions de salut, la microbiota intestinal i l'hoste estableixen simbiosi. Tot i així, en certs moments de la vida, es pot presentar una alteració de la composició microbiana o

disbiosi, tal com s'ha associat amb diverses malalties (metabòliques, neurològiques, inflamatòries o al·lèrgiques) [34,41,48].

1.5.2 Microbiota i sistema immunitari

La microbiota té un paper molt important en el desenvolupament del sistema immunitari intestinal i sistèmic. Estudis en animals nascuts i mantinguts en condicions estèrils (*germ-free*, GF) han demostrat que aquests animals presenten un menor nombre de cèl·lules secretores d'IgA i una reducció en la secreció d'aquesta immunoglobulina, variacions en la producció de citocines, en la generació de la tolerància oral, i alteracions en la formació de la melsa, dels ganglis limfàtics i de les PP [49–53]. Així doncs, l'adquisició de la microbiota en el nounat és rellevant per al correcte desenvolupament de la immunitat innata i l'adaptativa.

1.5.2.1 Microbiota i immunitat innata

Les cèl·lules del sistema immunitari innat es troben localitzades a la interfase entre l'hoste i la microbiota, fet que permet que interactuïn davant bacteris i els seus productes metabòlics i, així, s'indueixin diferents respostes fisiològiques. A aquest nivell, s'han descrit diverses formes d'interacció entre la microbiota i l'hoste, ja sigui amb les cèl·lules epitelials, les cèl·lules mieloides o les cèl·lules limfoides innates (*innate lymhoid cells*, ILC) [54].

- **Cèl·lules epitelials:** les cèl·lules epitelials intestinals presenten a la seva superfície receptors associats a la immunitat innata. Entre aquests, disposen dels receptors de reconeixement de patrons (*pattern-recognition receptors,* PRR) que reconeixen estructures moleculars conservades dels patògens (*pathogen-associated molecular patterns,* PAMPs) [36,55]. Els més coneguts són els *toll-like receptors* (TLR), els quals, a través del factor de transcripció nuclear kappa B (NF-κB) [36], estan relacionats amb la proliferació de les cèl·lules epitelials, la síntesi d'IgA, el manteniment de les unions estretes i l'expressió de pèptids antimicrobians, funcions crítiques per al manteniment de la barrera epitelial [56] (**Figura 8**).

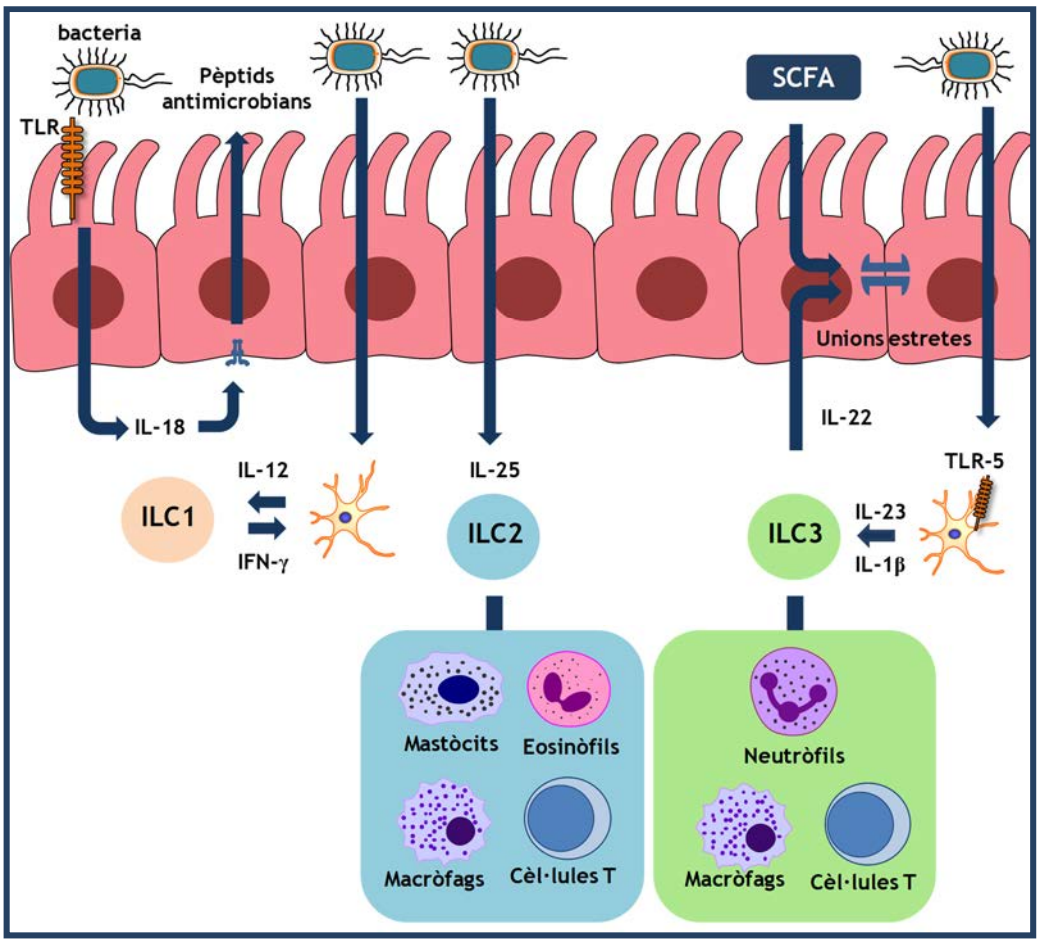
- **Cèl·lules mieloides:** la microbiota i els seus metabòlits influeixen en el desenvolupament, maduració i funció de les cèl·lules mieloides circulants (neutròfils i basòfils) i de teixits (macròfags) mitjançant l'activació de PRR [54].

- **Cèl·lules limfoides innates (ILC):** les ILC són cèl·lules del sistema immunitari innat recentment caracteritzades i classificades en categories [57]. Les ILC inclouen cèl·lules citotòxiques (NK) i no citotòxiques (ILC1, ILC2 i ILC3). Les ILC1 sintetitzen IFN-γ i TNF, les ILC2 produeixen citocines Th2 (IL-4, IL-5, IL-9, IL-13) i les ILC3 secreten IL-17A, IL-17F, IL-22, entre d'altres [58]. Encara que el seu desenvolupament no depèn de la microbiota, la seva funcionalitat està

condicionada per aquesta. La comunicació de les ILC amb la microbiota es porta a terme mitjançant citocines, PRR o pèptids antimicrobians. En la majoria dels casos, les cèl·lules epitelials o mieloides comuniquen les ILC amb la microbiota. Les ILC1 s'activen per la producció d'IL-12 per part d'una DC en resposta a un bacteri. Les ILC2 s'activen per citocines produïdes per les cèl·lules epitelials depenent de la microbiota. Seguidament les ILC2 interactuen amb mastòcits, eosinòfils, basòfils i macròfags. Les ILC3 s'activen, entre d'altres, per la IL-23 produïda per activació del TLR5 en resposta a la flagel·lina i provoquen la producció d'IL-22 responsable d'accions antimicrobianes i de la fortificació de la barrera intestinal [54]. Alhora, les ILC3 interaccionen amb altres cèl·lules immunitàries (**Figura 8**).

Figura 8. Efecte de la microbiota sobre la immunitat innata

Basada en [54]. IFN (interferó); IL (interleucina); ILC (*innate lymphoid cells*); SCFA (*short-chain fatty acids*); TLR (receptor *toll-like*)



1.5.2.2 Microbiota i immunitat adaptativa

La microbiota intestinal també influeix en el desenvolupament de la immunitat adaptativa. Concretament, s'ha relacionat amb un increment de les cèl·lules que expressen IgA, i en la quantitat i diversitat d'aquesta immunoglobulina a la llum intestinal. A més, la colonització bacteriana s'ha associat amb la maduració i manteniment de les cèl·lules Th17 i Treg [54,59,60].

1.5.2.3 Microbiota i al·lèrgia

El 1989, l'investigador Strachan va introduir la "hipòtesi de la higiene" en la que suggereix que la manca d'exposició bacteriana durant la infantesa com a resultat de procedir de famílies amb un nombre reduït de membres i d'un increment en les condicions d'higiene, comporta una elevada prevalença d'al·lèrgia [36,61,62]. Seguint aquesta premissa, una gran diversitat d'estudis han descrit la relació entre la microbiota intestinal i el desenvolupament d'al·lèrgia.

S'ha trobat una menor prevalença d'asma i d'al·lèrgies en persones que viuen en el medi rural, concretament en granges [63,64]. Per altra banda, s'han associat animals GF amb una elevada síntesi d'IgE [65] i una alta resposta inflamatòria [66]. En aquest mateix sentit, la reducció de la microbiota intestinal per causa de l'ús d'antibiòtics produeix un increment de la IgE sèrica i una exagerada inflamació al·lèrgica [67]. Els animals GF o tractats amb antibiòtics presenten menys cèl·lules Treg (CD4+Foxp3+) i més predisposició a desenvolupar una sensibilització al·lèrgica [68]. Aquest nombre reduït de cèl·lules reguladores s'incrementa quan, en aquests ratolins GF, se'ls hi administra bacteris (*Clostridia* i *Bacteroides flagilis*) o SCFA i, en conseqüència, es redueix la sensibilització al·lèrgica [69,70]. Així doncs, a més de la relació directa entre la microbiota i l'al·lèrgia, diversos articles han associat l'increment de SCFA amb la millora de la tolerància oral. En concret, s'ha vist que l'elevada proporció d'acetat i butíric protegeix del desenvolupament d'al·lèrgia [71].

Per tant, la bona colonització i el manteniment de la microbiota intestinal redueix la probabilitat de desenvolupar al·lèrgia i afavoreix la tolerància oral.

1.6 AL·LÈRGIA ALIMENTÀRIA

L'al·lèrgia alimentària es defineix com un efecte advers per a la salut com a resultat d'una resposta immunitària específica i reproduïble a l'exposició d'un determinat aliment [72]. Actualment, l'al·lèrgia alimentària és un problema de salut pública creixent que afecta tant a la població infantil com a l'adulta, produint una reducció de la qualitat de vida del pacient i del seu entorn familiar i social [73]. S'estima que afecta de mitjana a un 4-8% d'infants i al voltant d'un 5% d'adults [74].

La predisposició a patir una al·lèrgia alimentària depèn de molts factors tals com el sexe, la raça, la genètica, l'atòpia, el dèficit de vitamina D, l'obesitat, la higiene i la ruta d'exposició de l'aliment [39,74].

Es considera que l'al·lèrgia alimentària pot ser [75,76]:

- **Al·lèrgia mitjançada per IgE:** caracteritzada per la formació d'anticossos IgE i per l'aparició de símptomes abans de les dues hores després de la ingesta o exposició a l'al·lergen [76]. Generalment, afecta a la pell, el tracte gastrointestinal o el tracte respiratori.

- **Al·lèrgia no mitjançada per IgE:** provocada per mecanismes immunitaris no depenents de la IgE, sinó que, per altres isotips d'anticossos i cèl·lules immunitàries. En general afecta el tracte gastrointestinal. Concretament, es manifesta en forma d'enterocolitis, proctocolitis (inflamació de recte i còlon) i enteropatia [77].

- **Mixta:** aquest tipus d'al·lèrgia alimentària està mitjançada per mecanismes dependents d'IgE i per d'altres mecanismes. Generalment, la reacció d'hipersensibilitat retardada, que pot que aparèixer amb posterioritat a la ingesta, és el resultat d'una barreja d'aquestes dues formes d'al·lèrgia [78].

1.6.1 Al·lergògens

Els al·lergògens o al·lèrgens alimentaris són components dels aliments reconeguts per cèl·lules del sistema immunitari que provoquen reaccions immunitàries específiques. Generalment són proteïnes (de 15 kDa a 40 kDa) o glicoproteïnes (de 10 kDa a 70 kDa) [79]. Els al·lèrgens alimentaris donen lloc a reaccions tant si s'ingereixen crus com cuits, si bé n'hi ha que només es comporten com al·lergògens en alguna d'aquestes dues formes [77]. Segons aquestes propietats, els al·lèrgens es divideixen en:

- **Al·lèrgens de classe 1:** són glicoproteïnes estables al calor i a la digestió enzimàtica (resistents a àcids i proteases) [80]. En conseqüència, originen la sensibilització per via intestinal.

- **Al·lèrgens de classe 2:** solen ser làbils a la calor i a la digestió, probablement perquè contenen epítops conformacionals. Per tant, la via de sensibilització no és la digestiva, sinó que es produeix per una reacció encreuada amb pneumoal·lèrgens o al·lèrgens de contacte [81]. La base immunològica de la reacció que generen aquests al·lèrgens es basa en la presència d'IgE específica enfront a dues molècules que comparteixen una estructura semblant.

Un individu pot presentar una resposta immunitària a varies proteïnes d'un mateix aliment i envers a diversos epítops d'una proteïna concreta [82]. Actualment, ja s'han descrit més de 400 al·lergògens alimentaris [79] i el nombre continua creixent a causa de l'aparició de nous antígens alimentaris i, a més, és probable que amb l'augment dels aliments transgènics apareguin al·lèrgens fins ara desconeguts [83].

Actualment, els aliments implicats amb més freqüència en reaccions al·lèrgiques a nivell europeu són: la llet de vaca, el blat i l'ou (6%, 3,6% i 2,5%, respectivament) [84]. Pel que fa a l'estat espanyol, les al·lèrgies alimentàries més comunes són:el llagostí, el préssec, el tomàquet, el blat, el blat de moro i les nous (4,9%, 3,8%, 3,5%, 3,4%, 3,2% i 3,1%, respectivament) [85].

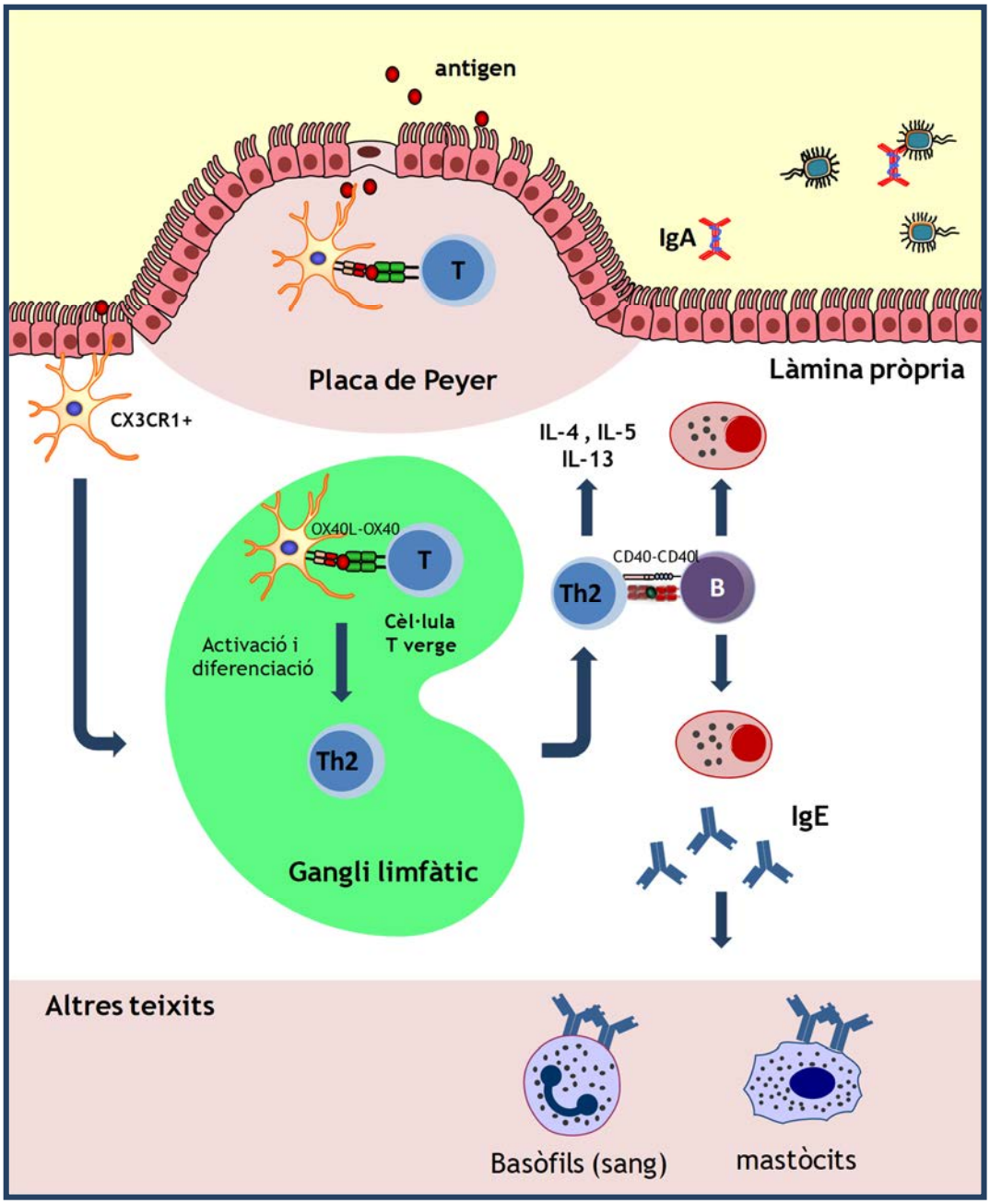
1.6.2 Al·lèrgia mitjançada per IgE: mecanismes implicats

L'al·lèrgia mitjançada per IgE, es considera que és una resposta immunològica inapropiada, amb un increment de limfòcits Th2 i una elevada producció d'IgE. En concret, en un primer contacte amb l'antigen, s'estimula la diferenciació de les cèl·lules Th verges a limfòcits Th2 efectors, que produeixen les citocines IL-4, IL-5 i IL-13 i potencien la producció d'IgE per part dels limfòcits B. Seguidament l'anticòs IgE s'uneix a la superfície de mastòcits i basòfils (**Figura 9**). Generalment la resposta immunitària de tipus Th2 protegeix contra infeccions parasitàries, encara que, quan es desregula, aquestes respostes contribueixen al desenvolupament d'al·lèrgia. Amb una nova exposició a l'al·lergen, aquest s'uneix a les molècules d'IgE fixades a la superfície dels tipus cel·lulars detallats i s'activen els basòfils, els mastòcits i, de forma més tardana, els eosinòfils (**Taula 1**). Així s'inicia una cascada de senyals que comporten l'exocitosi dels gränuls, la síntesi i secreció de mediadors lipídics, i la síntesi i secreció de citocines (**Taula 2**). L'alliberació d'aquests mediadors origina les manifestacions clíniques del procés al·lèrgic (**Taula 3**).

A més del paper que tenen les cèl·lules Th2 en el desenvolupament de l'al·lèrgia, recentment, s'ha postulat la participació d'altres tipus cel·lulars tals com els limfòcits Th9, Th17 i Th22 [30]. La diferenciació de les cèl·lules Th9 és deguda a la presència de TFG-β i IL-4, i dóna lloc a IL-9. Per altra banda, en presència d'IL-6, TGF-β i IL-23, es produeix la diferenciació de les cèl·lules Th17, productores d'IL-17A, d'IL17F i d'IL-22, tal com prèviament s'ha detallat. Per últim, s'ha descrit que les cèl·lules Th22, productores d'IL-22, també poden contribuir en el desenvolupament d'al·lèrgia [30].

No es coneix el mecanisme exacte per a la inducció d'una resposta Th2 en individus al·lèrgics, encara que tal com s'ha comentat anteriorment, la genètica i diferents factors ambientals hi tenen un paper important. Alteracions genètiques en els gens d'IL-4, la cadena α d'IL4R, IL-9, IL-13, entre altres, en són possibles candidats [86]. A més, per molt que anteriorment es creia que estava causada per un desequilibri entre les respostes Th1/Th2, actualment, també s'associa a un defecte en l'activitat supressora de les cèl·lules Treg. Així mateix, s'ha relacionat la presència d'IL-33 amb el desenvolupament de la sensibilització, el que produeix un increment de la permeabilitat intestinal i promou la diferenciació de limfòcits Th2 per part de les DC [87]. La diferenciació de cèl·lules Th2 també està condicionada per la presentació antigènica, en concret, per les unions de OX40-OX40L, TIM4-TIM1 i *jagged-notch* entre les DC i les cèl·lules T verges [87]. A més, s'ha descrit que diferents alteracions qualitatives i quantitatives de la població de cèl·lules NK hi poden intervenir, suggerint així un paper regulador en aquest tipus cel·lular [86,88].

Figura 9. Esquema del desenvolupament de la sensibilització al·lèrgica
B (limfòcit B); T (limfòcit T); Th (limfòcit T helper); IL (interleucina)



Taula 1. Cèl·lules implicades en l'al·lèrgia [2]

Mastòcits	Són cèl·lules que s'originen a la medul·la òssia i es troben àmpliament distribuïdes pels teixits, sobretot prop dels vasos sanguinis i dels nervis i en localitzacions subepitelials. Presenten receptors per a la porció Fc de la IgE a la seva superfície (FcεRI) i gràsnuls citoplasmàtics rics en histamina. Junt amb els basòfils, produeixen citocines importants en la inflamació i responsables de la resposta inflamatòria tardana tot afavorint la resposta al·lèrgica i la síntesi d'IgE, el desenvolupament dels limfòcits Th2 i el reclutament d'eosinòfils.
Basòfils	Es troben circulant per sang. Els basòfils comparteixen amb els mastòcits la presència de receptors FcεRI per a la IgE i de gràsnuls rics en histamina.
Eosinòfils	Tenen un paper important en la reacció tardana. Alliberen un ventall de mediadors tan gran com els mastòcits i, entre ells, produeixen la proteïna bàsica major i la proteïna catiònica d'eosinòfils. També, activen directament els mastòcits i així s'allarga l'alliberament de mediadors. D'aquesta manera, s'aconsegueix reclutar, amplificar i mantenir la resposta inflamatòria sense necessitat de que es produeixi una nova exposició a l'antigen.

Taula 2. Mediadors alliberats en l'al·lèrgia [2]

Amines biògenes	Histamina
Proteases neutres i proteoglicans	Triptasa, carboxipeptidasa A, catepsina G, hidrolases àcides, condroitin sulfat, heparina
Mediadors lipídics	Derivats de la via de la ciclooxigenasa (COX): prostaglandines Derivats de la via de la lipooxigenasa (LOX): leucotriens
Citocines	IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-15, IL-16, TNF-α, factor estimulant de colònies de granulòcits i macròfags (GM-CSF), factor de creixement endotelial vascular (VEGF), factor de creixement derivat de plaquetes (PDGF), TGF-β

Taula 3. Manifestacions clíniques de l'al·lèrgia mitjançada per IgE [76,89]

Manifestacions cutànies Més d'un 80% de les reaccions a aliments pateixen manifestacions cutànies	Immediates: Urticària, angioedema i pruïja
	Cròniques: Dermatitis atòpica
Manifestacions respiratòries	Del tracte respiratori superior: Esternuts, rinorrea, congestió nasal i pruïja
	Del tracte respiratori inferior: Dispnea, sibilàncies, tos i laringoespasme
Manifestacions oculars	Eritema conjuntival, pruïja i llagrimeig
Manifestacions gastrointestinals	Pruïja i/o formigueig dels llavis, la llengua, el paladar i la gola amb o sense inflor, nàusees, dolor o rampes abdominals, vòmits i/o diarrea
Anafilaxi	Reacció sistèmica greu que afecta a més de dos òrgans o sistemes. És de risc vital i amenaça la vida del pacient

1.7 MODELS ANIMALS D'AL·LÈRGIA ALIMENTÀRIA

La creixent prevalença d'al·lèrgia alimentària comporta que la recerca en aquest àmbit se centri en l'estudi dels factors de risc, la fisiopatologia, les proves diagnòstiques i el tractament d'aquestes reaccions [90]. És per això que, actualment, l'obtenció de models animals que mimetitzin un procés d'al·lèrgia alimentària és un dels principals objectius en aquesta àrea de recerca.

S'han estudiat models d'al·lèrgia alimentària utilitzant diferents espècies, soques, sexes, edats, adjuvants (toxina colèrica, toxina de *Bordetella pertussis*, carragenina), al·lergògens, pautes d'administració (dosi i freqüència) i rutes de sensibilització [91,92]. En concret, s'han intentat desenvolupar models en porcs, gossos i, sobretot, en rosegadors [92]. L'avantatge principal que presenten aquests últims és la seva elevada disponibilitat i el gran nombre de soques existents. A l'hora, la seva petita mida i baix cost de manteniment fa possible l'estudi a partir d'un gran nombre d'animals [31]. S'han obtingut models d'al·lèrgia principalment en ratolins, sobretot de la soca Balb/c [93–95]. També, en rates, majoritàriament, en les soques Wistar [96] i Brown Norway (BN) [83,97]. S'ha

vist que les rates BN solen ser especialment indicades per estudiar protocols de sensibilització ja que, aquesta soca presenta una alta capacitat de generar anticossos IgE específics d'antigen en resposta a certs al·lèrgens i, per tant, desenvolupa un patró semblant a un individu amb predisposició al·lèrgica [31], encara que presenten un elevat cost. A més, s'ha demostrat que en rosegadors, les femelles produeixen una resposta superior en anticossos específics que els mascles [98], per molt que en humans, l'al·lèrgia alimentaria en edat infantil és més freqüent en nens i, en adults, en dones [99].

Dins el grup de recerca d'Autoimmunitat i Tolerància s'ha dut a terme el desenvolupament d'un model d'al·lèrgia alimentària en rates de la soca BN amb una immunització prèvia per via intraperitoneal (i.p.) [97]. Tot i així, en els models d'al·lèrgia alimentària es valora que la sensibilització es realitzi únicament per via oral, ja que aquesta s'inicia degut a la ingesta oral de l'al·lergen però, apareix una complicació important a superar: la tolerància oral [91].

2. FLAVONOIDES I SISTEMA IMMUNITARI

Els flavonoides són productes naturals presents en els vegetals que s'ingereixen a través de la dieta. Una gran varietat d'estudis mostren la influència de la dieta rica en flavonoides sobre el sistema immunitari.

En aquest apartat s'incorpora la revisió realitzada per elaborar un capítol del llibre "INMUNONUTRICIÓN Y ESTILO DE VIDA", coordinat per la Dra. Ascensión Marcos del Departament de Metabolisme i Nutrició de l'Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN, CSIC). En aquest capítol es defineixen els flavonoides i es fa un resum dels estudis *in vitro,* preclínics i clínics dels efectes dels flavonoides sobre el sistema immunitari i sobre les reaccions al·lèrgiques. L'extensió del capítol (màxim 10000 paraules) i el nombre de referències bibliogràfiques (màxim 60) estan condicionades per l'editorial.

CAPÍTULO

10

Influencia de los flavonoides sobre el sistema inmunitario

Mariona Camps-Bossacoma, Àngels Franch, Francisco J. Pérez-Cano y Margarida Castell

Resumen

Los flavonoides, productos del metabolismo secundario de las plantas, se encuentran habitual de la dieta rica en frutas, verduras, té, cacao y vino. Además de los conocidos efectos antioxidantes y antiinflamatorios, los flavonoides poseen una importante acción inmunorreguladora en la respuesta inmunitaria adquirida. En este capítulo se resumen los efectos de estos componentes *in vitro* e *in vivo*. Se hace especial énfasis en las acciones de los flavonoides en las diferentes fases de la respuesta inmunitaria adquirida, desde la presentación antigénica hasta la formación de los linfocitos efectores y la secreción de citocinas. Asimismo, dado que los flavonoides forman parte de la dieta, se resume su acción sobre el sistema inmunitario intestinal y la microbiota. Además de estas propiedades inmunorreguladoras, se detallan algunos ejemplos del efecto protector de varios flavonoides particulares y de extractos vegetales ricos en flavonoides en modelos animales de alergia, en los cuales han mostrado su efecto modulador de la síntesis de IgE, de la activación de mastocitos, basófilos y eosinófilos y de la liberación de los mediadores responsables de la sintomatología de la alergia. Por último se incluyen algunas evidencias clínicas de sus efectos inmunomoduladores a través de estudios epidemiológicos y de estudios de intervención nutricional. Sin embargo, las evidencias de los efectos inmunomoduladores de los flavonoides en humanos son escasos y por ello se necesitan más estudios observacionales en poblaciones de riesgo así como estudios de intervención nutricional.

Palabras clave: activación linfocitaria, alergia, citocinas, inmunidad intestinal, flavonas, flavonoles, microbiota

INTRODUCCIÓN

Los flavonoides constituyen un grupo de moléculas orgánicas ubicuas en las plantas vasculares como producto de su metabolismo secundario. Numerosos estudios demuestran los efectos saludables de su consumo y, concretamente y de forma genérica, se ha establecido el papel protector de los flavonoides en los procesos cancerígenos y en las reacciones inflamatorias implicadas en la patogenia de enfermedades como la obesidad, la aterosclerosis o la enfermedad de Alzheimer. En este capítulo se resumirán las acciones de los flavonoides sobre el sistema inmunitario, tanto sobre la respuesta inmunitaria innata como en la respuesta inmunitaria adquirida, considerando de forma más concreta, su acción sobre los procesos alérgicos. No se incluyen las evidencias de los efectos antiinflamatorios de los flavonoides, objeto de excelentes revisiones (1,2) e incluso de libros enteros (3). Sin embargo, sí que se incluye una síntesis de la influencia de los flavonoides de la dieta sobre el sistema inmunitario intestinal, que es el que de forma más precoz y directa está en contacto con estos componentes dietéticos. El capítulo resume efectos *in vitro* (procedentes de la adición de flavonoides sobre líneas celulares o cultivos primarios), preclínicos (obtenidos en animales de experimentación tras la administración oral de flavonoides) y finalmente, se recogen las pocas evidencias obtenidas a partir de estudios epidemiológicos y estudios clínicos.

CLASIFICACIÓN DE LOS FLAVONOIDES

Químicamente los flavonoides presentan una estructura básica formada por un esqueleto C6-C3-C6 (2-fenilbenzopirano), y se clasifican en diversas familias según el grado de oxidación y saturación presente en el anillo C. Las familias principales de los flavonoides incluyen flavonoles, dihidroflavonoles, flavonas, flavanonas, flavanoles (flavan-3-ol, flavan-4-ol y flavan-3,4-diol) y flavanos (4). Entre los flavonoides también se incluyen los isoflavonoides, los cuales poseen un esqueleto de 3-fenilcromona, y que incluyen una familia importante desde el punto de vista fisiológico, las isoflavonas. Los flavonoides pueden ser modificados químicamente (hidroxilación, metoxilación, *O*-glicosilación, etc.), lo que contribuye a que hoy en día se conozcan más de 8000 compuestos distintos (4,5).

Las diferentes familias de flavonoides se encuentran ampliamente distribuidas en alimentos y bebidas de origen vegetal, como frutas, verduras, té, cacao y vino. Los flavonoles se pueden encontrar en cebollas, puerros, manzanas, frutos en bayas, vino tinto y té, siendo la quercetina el flavonol más común. La apigenina y la luteolina son las flavonas más abundantes y se encuentran en la piel de la fruta, el perejil y el apio. Las flavanonas (naringenina, hesperidina, etc.) son exclusivas de frutas cítricas. Los flavanoles incluyen formas monoméricas (epicatequina, catequina, epigalocatequina, galato de epigalocatequina – EGCG-, etc.) y poliméricas (proantocianidinas o taninos condensados como procianidinas, prodelfinidinas y propelargonidinas) y se encuentran mayoritariamente en el cacao y en el té. Las isoflavonas (genisteína, daidzeína, glicitina, etc.) están presentes exclusivamente en plantas leguminosas, sobre todo en la soja.

FLAVONOIDES E INMUNIDAD INNATA

EFECTO DE LOS FLAVONOIDES SOBRE CÉLULAS DE LA INMUNIDAD INNATA

Estudios *in vitro* han demostrado las propiedades moduladoras de diversos flavonoides sobre la inmunidad innata. En estos estudios, los flavonoides se adicionan a líneas celulares de monocitos, macrófagos o bien se incuban sobre cultivos primarios de células obtenidas de sangre o tejidos linfoides.

Los flavonoides han mostrado actividad sobre monocitos, macrófagos y neutrófilos, modulando su funcionalidad. Esta acción se puede atribuir a su capacidad antioxidante, necesaria para el correcto funcionamiento de las células inmunitarias, y también a su acción reguladora de la actividad enzimática y de la producción o liberación de mediadores, principalmente de tipo inflamatorio.

La estructura polifenólica de los flavonoides es la responsable de su elevada actividad antioxidante. Los flavonoides son capaces de secuestrar radicales libres y también protegen

contra oxidantes como especies reactivas de oxígeno (ROS) y especies reactivas de nitrógeno. Los flavonoides son agentes quelantes de metales y modulan el estrés oxidativo generado durante la inflamación (6). Por ejemplo, flavonoles y flavonas reaccionan frente al oxígeno singlete; isoflavonas, como quercetina y luteolina, entre otros, reaccionan con el ácido hipocloroso, mientras que taxifolina y quercetina neutralizan a peroxinitrito (revisado en (6,7)). Además, algunos flavonoles como miricetina, quercetina o kampferol, entre otros, presentan capacidad secuestradora de metales, evitando así que se perpetúe la reacción en cadena de la oxidación radicalaria.

Por otra parte, los flavonoides ejercen su acción sobre el sistema inmunitario innato a través de la regulación de un gran número de enzimas. Se ha descrito que estos compuestos son capaces de modular más de 50 enzimas implicados en el vías metabólicas de los mamíferos, como las involucradas en la proliferación celular, la supervivencia de las células, la expresión génica y la señalización celular (6). Por ejemplo, los flavonoles quercetina y fisetina son capaces de inhibir las proteína tirosincinasas (PTK), la proteína cinasa C (PKC) y las mapcinasas (MAPK). Quercetina y las flavonas baicaleína, luteolina, apigenina, y nobitelina también pueden actuar sobre la fosfolipasa A_2, las ATPasas, las lipoxigenasas (LOX) o las ciclooxigenasas (COX), lo que contribuye a su efecto antiinflamatorio (6–8). La inhibición enzimática se traduce en la modulación de diversos mecanismos de señalización celular como receptores, citocinas, quimiocinas, moléculas de adhesión, factores de transcripción como el factor nuclear κB (NF-κB), etc. (revisado en (6–8)). En general, las agliconas son más activas que los flavonoides glicosilados, siendo la isoflavona genisteína uno de los compuestos más activos (6).

De forma global, numerosos estudios en células de tipo macrófago o monocito muestran la capacidad inhibidora de los flavonoides, y entre ellos flavonoles, flavanonas e isoflavonas, disminuyendo la producción de citocinas pro-inflamatorias (factor de necrosis tumoral α o TNF-α, interleucina 1 o IL-1, etc.), así como de eicosanoides derivados del ácido araquidónico (prostaglandinas y leucotrienos), también con importante acción pro-inflamatoria. Estos efectos parecen ser debidos a la regulación de su transcripción, en el caso de citocinas, o de las enzimas implicadas en su formación (a través de la inhibición de COX), en el caso de los eicosanoides.

Los efectos antiinflamatorios de los flavonoides también se han observado en mastocitos y basófilos, por su potencial inhibitorio de su desgranulación y liberación de histamina, tal como se detalla más adelante.

FLAVONOIDES E INMUNIDAD ADQUIRIDA

EFECTO DE LOS FLAVONOIDES SOBRE CÉLULAS DE LA INMUNIDAD ADQUIRIDA

La respuesta inmunitaria adquirida se inicia con la presentación antigénica, proceso por el cual las células dendríticas captan el antígeno, asumen su papel de células presentadoras de antígeno (APC), e interaccionan con los linfocitos Th (CD4+) vírgenes específicos. Esta interacción conducirá a la activación de los linfocitos Th, los cuales proliferarán y se diferenciarán a linfocitos efectores, y con ello se desarrollará la respuesta inmunitaria adquirida. Las células dendríticas actúan como centinelas del sistema inmunitario y se encuentran distribuidas en los tejidos periféricos mostrando un fenotipo inmaduro, caracterizado por una baja expresión de moléculas del complejo principal de histocompatibilidad de clase II (MHC-II) y moléculas coestimuladoras (CD11c, CD40, CD80, CD83 y CD86). Cuando las células dendríticas captan un antígeno, se produce su maduración y migración hacia los órganos linfoides secundarios para encontrarse con los linfocitos Th específicos. La interacción entre la célula dendrítica y el linfocito Th tiene lugar a través de la llamada sinapsis inmunitaria, en la que intervienen, las moléculas MHC-II de la célula dendrítica y las moléculas coestimuladoras de ambos tipos celulares.

Numerosos flavonoides han mostrado su influencia en el proceso de diferenciación y presentación antigénica por parte de células dendríticas (9) (**Figura 1**). Así, por ejemplo, flavanoles como galato de epigalocatequina (EGCG) son capaces de disminuir la expresión de moléculas clave en la sinapsis inmunitaria como son CD83, CD80, CD11c y MHC-II (10). De forma similar, la quercetina es capaz de

inhibir el proceso de captación antigénica, modulando la endocitosis, y la migración de las células dendríticas, sin modificar la expresión del receptor CCR7 (11). La flavona crisina también ha mostrado su capacidad inhibitoria sobre la diferenciación, maduración y función de las células dendríticas (12). Por otra parte, EGCG, curcumina, quercetina, apigenina, fisetina y silibinina son capaces de inhibir la maduración *in vitro* de células dendríticas inducida por agonistas de TLR4, como es el lipopolisacárido (LPS), concretamente se inhibe la expresión de moléculas coestimuladoras, de MHC-II y la producción de IL-12, relacionada con la activación de células dendríticas (revisado en (9,13)). De esta manera, las células dendríticas incubadas en presencia de flavonoides modulan la activación y proliferación de linfocitos Th y con ello el desarrollo de la respuesta inmunitaria adquirida. Los mecanismos responsables de estos efectos parecen ser debidos a la interacción con las vías de transcripción NF-κB y MAPK (9,11).

La siguiente fase de la respuesta adquirida consiste en la activación y proliferación clonal del linfocito Th específico, proceso que depende, entre otros factores, de la síntesis, de la secreción y del efecto autocrino de la citocina IL-2 (**Figura 1**). A este nivel, numerosos flavonoides han demostrado *in vitro* su capacidad de controlar la proliferación linfocitaria y modular la síntesis de IL-2. Este efecto se ha manifestado por ejemplo para isoflavonas, como la genisteína, capaz de disminuir la secreción de IL-2 y la proliferación linfocitaria en células mononucleares de sangre periférica (PBMCs) de voluntarios sanos (14). Asimismo, flavanoles como EGCG inhiben la proliferación linfocitaria y la producción de IL-2 cuando se añaden *in vitro* a células mononucleares humanas o a linfocitos ganglionares (15). Flavonoides de la misma familia, como epicatequina, son capaces de inhibir la síntesis de IL-2 y su receptor (CD25) en una línea celular de ratón (16). Sin embargo las procianidinas son capaces de inhibir la proliferación de cultivos de linfocitos esplénicos sin modificar la síntesis de IL-2 (17). Por otra parte, el flavonol quercetina es capaz de reducir la síntesis de IL-2, junto con una menor expresión de su receptor (IL-2Rα o CD25) en linfocitos Th activados de ratones (18). En conclusión, los flavonoides son capaces de inhibir la síntesis de IL-2 que conlleva la inhibición de la proliferación de los linfocitos Th.

Además de todos estos efectos en estudios *in vitro,* distintos ensayos realizados *in vivo* pueden complementar estos resultados. Así, por ejemplo, la flavanona diosmina, abundante en los frutos cítricos, administrada a ratones Balb/c es capaz de atenuar la llegada de linfocitos T al foco inflamatorio inducido con LPS, efecto asociado a una reducción de la producción de IL-2 (19).

El efecto regulador de la proliferación linfocitaria y/o de la secreción de IL-2 se ha evidenciado también en estudios con diferentes extractos vegetales ricos en flavonoides como por ejemplo, extractos de cacao, ricos en flavanoles, flavonoides aislados de *Vitex agnus-castus* (*Verbenaceae*) y de *Artemisia princes* (*Asteraceae*) (revisado en (13)). Asimismo, recientemente, se ha descrito el papel modulador de la activación linfocitaria de extractos de la leguminosa *Campylotropis hirtella* (Franch.) Schindl., ricos en isoflavonas, isoflavononas y flavonoles (20) y de *Eriosema campestre* var. *macrophylum* (21). Sin embargo, flavonoides aislados de pieles de cítricos son capaces de potenciar la proliferación de esplenocitos de ratón de forma dosis independiente (22).

La tercera fase de la respuesta inmunitaria adquirida incluye la diferenciación de los linfocitos efectores que secretarán un perfil de citocinas particular de cada respuesta inmunitaria (Th1, Th2, Th17) (**Figura 1**). En esta fase, las evidencias descritas son más contradictorias (revisado en (13)). Muchos estudios muestran la capacidad moduladora de la síntesis de interferón γ (IFN-γ), citocina típica de la respuesta Th1, o de IL-4, representativa de la respuesta Th2, tras la adición *in vitro* de flavonoides. Entre los compuestos capaces de atenuar la secreción de IFN-γ se encuentran flavonoles (quercetina y kamferol) (18,23), isoflavonas (genisteína) (14) y flavanoles (EGCG, procianidinas y los presentes en el cacao) (15,17). Algunos estudios han profundizado en el mecanismo modulador de estos compuestos y así se ha demostrado que quercetina, uno de los más estudiados, puede modular la expresión de T-bet, factor de transcripción que se une directamente en el promotor de IFN-γ e incrementa y activa su transcripción (18).

Por otra parte, en cuanto a la producción de IL-4 existen resultados contradictorios (inhibición o potenciación) sobre la influencia de isoflavonas (genisteína), flavanoles (epicatequina y extracto de cacao) (16) si bien elevadas concentraciones de flavonas (luteolina y apigenina) y flavonoles (fisetina) son capaces de reducir la síntesis de esta citocina (revisado en (24)). Asimismo, se ha descrito que las procianidinas de manzana modulan la síntesis de IL-4 en un cultivo primario de esplenocitos (17).

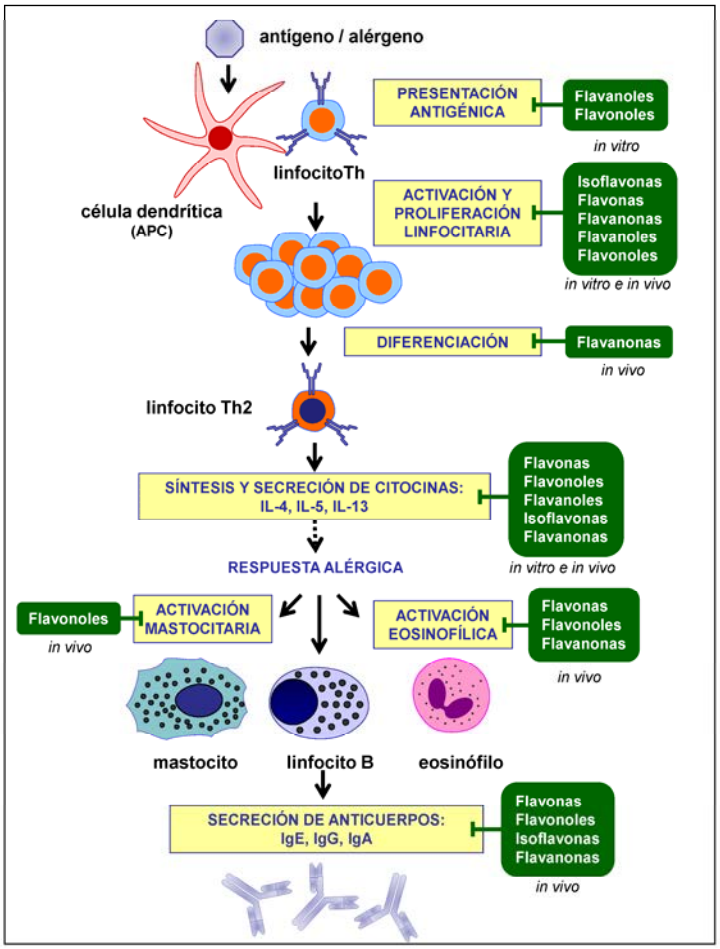


Figura 1: Secuencia de activación de la respuesta inmunitaria adquirida y puntos en los que se han descrito efectos de los flavonoides. APC: célula presentadora de antígeno, IL: interleucina; Th: T helper. Imágenes individuales obtenidas de Motifolio.

EFECTO DE LOS FLAVONOIDES SOBRE LAS CÉLULAS EFECTORAS DE LAS REACCIONES ALÉRGICAS

Además de las acciones sobre las células dendríticas y los linfocitos, los flavonoides también ejercen una acción moduladora sobre basófilos y mastocitos, principales células efectoras de las reacciones alérgicas. Los mastocitos y los basófilos se pueden activar mediante la unión de la IgE a su superficie ya que poseen receptores de alta afinidad para esta inmunoglobulina (FcεRI). Cuando se sintetiza IgE contra un alérgeno, este anticuerpo se une al receptor FcεRI de mastocitos y basófilos. Con un nuevo contacto con el alérgeno, este se

une a la IgE fijada a la superficie de estos tipos celulares causando su desgranulación, con liberación de histamina y proteasas, y la síntesis de nuevos mediadores como citocinas y derivados del ácido araquidónico. El conjunto de estos mediadores son los responsables de la sintomatología alérgica.

Ensayos in vitro sobre diversas líneas celulares de mastocitos y basófilos (*rat basophilic leukemia cells* o RBL-2H3, *human basophilic cell line* o KU-812 y *human leukemic mast cell line* o HMC-1) o sobre células aisladas de lavados peritoneales, médula ósea o sangre han permitido establecer el papel de los flavonoides en esta reacción de hipersensibilidad (revisado en (24,25)).

Los flavonoides son capaces de inhibir la desgranulación de mastocitos y basófilos y reducir la liberación de histamina, proteasas (β-hexosaminidasa), y citocinas como IL-4, IL-6, IL-8 e IL-13 (revisado en (6)). En general, los flavanoles y las flavanonas son poco activos. Algunas isoflavonas, como daidzeína y genisteína son poco activas, mientras que otras, como la tectorigenina, ha mostrado su capacidad inhibidora de la secreción de proteasas. Flavonoides de tipo flavonol o flavona son los que muestran una mayor actividad (24). Concretamente, el flavonol quercetina ha mostrado su capacidad de inhibir la desgranulación y la síntesis y liberación de histamina de mastocitos (26). Esta capacidad moduladora también se ha observado para otros flavonoles como fisetina, kaemferol, miricetina y morina. La fisetina destaca por su capacidad moduladora de la liberación de histamina.

Las flavonas constituyen los flavonoides con una mayor actividad inhibitoria de la liberación de histamina de mastocitos (revisado en (24)). Algunas de las flavonas que han mostrado esta acción son la diosmetina, la luteolina y la apigenina, siendo las dos primeras las más activas (24). La luteolina, también es inhibidora de la 5-LOX, enzima importante para la síntesis de leucotrienos (LT) a partir de mastocitos, mediadores importantes del proceso de broncoconstricción en las reacciones alérgicas (26). Además, distintas flavonas (apigenina, luteolina) y flavonoles (kampferol y quercetina) han mostrado su papel inhibidor de la producción de citocinas proinflamatorias como son TNF-α e IL-4 en la línea celular RBL-2H3 (27).

Recientemente también se ha descrito que una homoisoflavona aislada de *Cremastra appendiculata* Makino, inhibe la producción de citocinas proinflamatorias (IL-6 y TNF-α) y reduce la síntesis de la prostaglandina 2 y los leucotrienos LTB_4 y LTC_4 de mastocitos estimulados. Estas acciones se han relacionado con una inactivación de la señalización de la tirosin quinasa del bazo (*Syk*) y de la supresión de la fosfolipasa A_2 citosólica (cPLA2) (25). Asimismo, el flavanol morina inhibe la desgranulación y la síntesis de citocinas proinflamatorias (IL-4 y TNF-α) en células RBL-2H3 y BMMCs estimuladas debido a una inhibición en la activación de las quinasas *Fyn* y *Syk* (25).

Otro tipo celular presente en las reacciones alérgicas es el eosinófilo. Se ha observado eosinofilia en las reacciones de hipersensibilidad tipo I y también en otras situaciones como la esofagitis eosinofílica, jugando un papel importante en la patogenia de enfermedades en las que también intervienen los linfocitos Th2, los mastocitos y los linfocitos B (26). Por ello, la atenuación de la actividad de los eosinófilos ha ganado importancia en los últimos años. Entre los moduladores naturales de la actividad eosinofílica, destacan las hierbas medicinales, como *Ephedra sinica* (*ma huang*), muy utilizada como antialérgica en la medicina china, si bien su efecto se atribuye más bien a los pseudoalcaloides que contiene, más que a sus flavonoides (26). Por otra parte, se ha descrito que el flavonol quercetina es capaz de modular las condiciones eosinofílicas, mediante la inhibición de la sobreexpresión del receptor de la histamina, de LOX, de COX, de una excesiva actividad de células dendríticas y de una excesiva producción mucosa (26).

EFECTO DE LOS FLAVONOIDES EN MODELOS ANIMALES DE ALERGIA

Además de los estudios realizados *in vitro*, algunos flavonoides han demostrado su influencia sobre el sistema inmunitario *in vivo*, sobre todo cuando se ha procedido a la estimulación de la respuesta inmunitaria mediante la inducción de un proceso de hipersensibilidad inmunitaria, como es la alergia. Como se ha comentado, los procesos alérgicos se caracterizan por la estimulación de la respuesta inmunitaria de tipo Th2 que da

lugar a la activación de mastocitos, basófilos y eosinófilos, y asimismo a la formación de anticuerpos de tipo IgE, responsables de las reacciones anafilácticas, y de determinados isotipos de anticuerpos de la clase IgG. A nivel preclínico, se ha establecido la influencia de dietas ricas en flavonoides sobre modelos de alergia utilizando como biomarcadores la concentración de anticuerpos o los niveles de citocinas sintetizadas, además de indicadores clínicos de la respuesta alérgica. También se ha estudiado el efecto de la administración de flavonoides en modelos de asma y de dermatitis atópica, y los resultados son, en general, satisfactorios (revisado en (5,13,24,28,29)). A modo de resumen, se citan a continuación algunos ejemplos.

Considerando las flavonas, dietas ricas en crisina, baicaleina, apigenina, luteolina y diosmetina han demostrado su capacidad para reducir la concentración sérica de IgE y también modular la expresión génica de citocinas típicas de la respuesta alérgica como son la IL-2, IL-4, IL-10 e IL-13. En un modelo murino de asma, crisina evita la infiltración de células inflamatorias, especialmente eosinófilos, en el fluido broncoalveolar, la producción de citocinas Th2 (IL-4 y IL-13) y el incremento de la IgE sérica total (30). Otra flavona, la eupatilina, es capaz de inhibir la expresión de eotaxina en la línea celular epitelial bronquial humana, que constituye el principal agente quimiotáctico para eosinófilos (31). Análogamente, en un modelo de asma, apigenina disminuye la concentración sérica de IgE, la acumulación de eosinófilos, la concentración de las citocinas IL-6 e IL-17A y la hiperreactividad bronquial (32). Asimismo, en un modelo de asma en cobayas, las flavonas han demostrado su actividad antiasmática, siendo la actividad de la luteolina y de la apigenina superior a la de baicaleína y crisina. Concretamente, las flavonas disminuyen la resistencia de las vías aéreas y, en el fluido broncoalveolar reducen la infiltración de leucocitos, la liberación de histamina, la actividad fosfolipasa A2 y la liberación de mediadores de eosinófilos (33). Por otro lado, en modelos de alergia alimentaria, de rinitis alérgica y de asma alérgica, baicaleína ha resultado ser eficiente en la prevención de síntomas alérgicos y en la atenuación de la producción de IgE y de citocinas proinflamatorias, efectos relacionados con un incremento de la actividad de las células T reguladoras (31). Cabe destacar también el papel de la baicaleína en un modelo de alergia alimentaria (34). Esta flavona es capaz de reducir las manifestaciones alérgicas, efecto asociado a una disminución de la síntesis de IgE y de la reactividad de los linfocitos T. Asimismo, baicaleína tiene un efecto sobre el intestino, aumentando la función barrera intestinal y la diferenciación de linfocitos T reguladores (34). Por otra parte, también se ha descrito el papel protector de las flavonas, concretamente de apigenina y baicaleína en modelos de dermatitis atópica (28).

Los flavonoles (como los que se encuentran en las cebollas, brócoli, manzanas, uva, etc.) también son eficaces en la modulación de la hipersensibilidad alérgica. Entre los flavonoles, la quercetina presenta una actividad antialérgica demostrada por la inhibición de la liberación de histamina, la disminución de determinadas citocinas (IL-4, por ejemplo) y leucotrienos, y la inhibición de la formación de IgE (revisado en (35,36)). Así, por ejemplo, quercetina es capaz de modular la síntesis de citocinas Th2 y la liberación de la histamina de mastocitos, inhibir la activación de eosinófilos, suprimir la dermatitis de contacto en ratón y controlar un modelo de asma alérgica (37). Asimismo, la administración de quercetina atenúa la reacción anafiláctica en ratas sensibilizadas con cacahuete (38). Concretamente, este flavonol reduce la producción de histamina, la aparición de síntomas alérgicos y la inhibición en la síntesis de IgE. De forma similar, fisetina es capaz de atenuar manifestaciones de dermatitis atópica en ratón, reducir la concentración de IgE sérica y la infiltración de mastocitos, eosinófilos y linfocitos (39). Un extracto rico en kamferol es responsable de mitigar los síntomas de shock anafiláctico en un modelo de alergia alimentaria, efecto asociado con una reducción de la respuesta Th2 (31).

Por otro lado, las isoflavonas también se han mostrado activas en modelos animales de alergia. Así, una dieta que con daidzeína y genisteína es capaz de inhibir la producción sérica de IgE, reducir los síntomas anafilácticos y la desgranulación mastocitaria en un modelo de sensibilización alérgica de cacahuete (40).

En cuanto a las flavanonas, se ha demostrado que, en un modelo de asma alérgica, la

administración de naringenina no modifica la concentración sérica de IgE pero es capaz de reducir la producción de citocinas Th2 (IL-4, IL-5 e IL-13) por parte de células esplénicas sin modificar su proliferación. Además, naringenina inhibe la infiltración de eosinófilos en el fluido broncoalveolar (41). En un reciente estudio, la naringenina es capaz de modular un modelo de asma inducido en ratón mediante la reducción de la infiltración eosinofílica y de las citocinas IL-4 y IFN-γ (42).

Por otra parte, también se ha establecido el efecto de extractos con mezclas de flavonoides sobre modelos animales de alergia. Así, por ejemplo, un extracto de *Kalanchoe pinnata* (*Crassulaceae*), rico en derivados de quercetina, quercitrina y kampferol, es capaz de atenuar tanto la producción de IgE como la de citocinas Th2 (43). De forma similar, se ha observado la eficacia de un extracto de hojas de *Citrus Tachibana* (ricas en rutina, apigenina, kampferol y quercetina) en un modelo de alergia alimentaria. Concretamente, este extracto vegetal, principalmente por la acción del flavanol kampferol, atenúa la reacción anafiláctica en un modelo de alergia alimentaria inducido en ratón, efecto que se acompaña de una menor producción de citocinas Th2 (IL-4, L-5 e IL-13) a partir de linfocitos de ganglios linfáticos mesentéricos (44). Asimismo, una mezcla de extractos de *Phellodendron amurense* y *Sanguisorba officinalis* muestran un efecto sinérgico en la prevención de la dermatitis atópica en un modelo en ratón, efecto asociado a una disminución de IgE, IgG1 y de la expresión de citocinas Th1 y Th2 (45).

Además, recientemente se ha demostrado que un extracto de cacao, rico en flavanoles, es capaz de atenuar los síntomas alérgicos en un modelo de dermatitis atópica, incluyendo una disminución de la síntesis de IgE, una disminución de la expresión génica de citocinas Th2 y regulación de la infiltración de eosinofilos y mastocitos en las lesiones de la piel (46). En modelos de alergia, ya sea en alergia alimentaria o en una sensibilización oral, la ingesta de cacao es capaz de atenuar la producción de IgE, IgG1 e IgG2a, junto con un incremento de la proporción de linfocitos TCRγδ en tejidos linfoides intestinales como son el epitelio intestinal, los ganglios linfáticos mesentéricos y las placas de Peyer (47–49).

Por lo tanto, en resumen, la mayoría de los estudios sugieren que los flavonoides producen un efecto inmunosupresor que puede ser de utilidad en reacciones alérgicas mediadas por la IgE.

FLAVONOIDES E INMUNIDAD INTESTINAL

El sistema inmunitario intestinal incluye estructuras organizadas (placas de Peyer y ganglios linfáticos mesentéricos) y células dispersas en el epitelio (linfocitos intraepiteliales o IEL) o en la lámina propia (linfocitos de lámina propia o LPL). Estas células actúan de forma coordinada con el epitelio intestinal e incluso con los microorganismos de la microbiota. Aunque no existen muchos estudios que evalúen el efecto directo de los flavonoides sobre la inmunidad intestinal, hay suficientes evidencias, sobre todo a nivel preclínico, que demuestran que los flavonoides pueden modificar su funcionalidad a través de los mecanismos expuestos anteriormente sobre la inmunidad innata (actividad antioxidante, moduladora de liberación y producción de mediadores inflamatorios, reguladora de mecanismos de señalización, etc.) y/o sobre la inmunidad adaptativa (acción antiproliferativa y reguladora de la respuesta Th2 y por tanto asociada a la producción de ciertos anticuerpos, con especial relevancia sobre la IgA). La **Figura 2** muestra los principales efectos y mecanismos descritos para algunos flavonoides en relación a la inmunidad y microbiota intestinal.

EFECTO DE LOS FLAVONOIDES SOBRE LA MICROBIOTA INTESTINAL

La microbiota, formada por los microorganismos que se encuentran colonizando las mucosas del cuerpo humano (sobre todo a nivel intestinal), ejerce diferentes funciones beneficiosas para la defensa del organismo: tanto una acción anti-infecciosa frente a patógenos al interferir en su colonización, como contribuyendo al desarrollo, maduración y mantenimiento del sistema inmunitario intestinal. Uno de los mecanismos por los cuales los flavonoides pueden modular la respuesta inmunitaria intestinal, puede ser, de

forma indirecta, a través de la modificación de la composición y funcionalidad de la microbiota, así como de la acción sobre los sistemas de interacción de ésta con el hospedador (**Figura 2**). En este sentido, se ha observado el efecto modulador de la microbiota mediante muchos tipos diferentes de flavonoides, los de tipo flavanona, flavonol, flavanol e isoflavona (revisado en (50)).

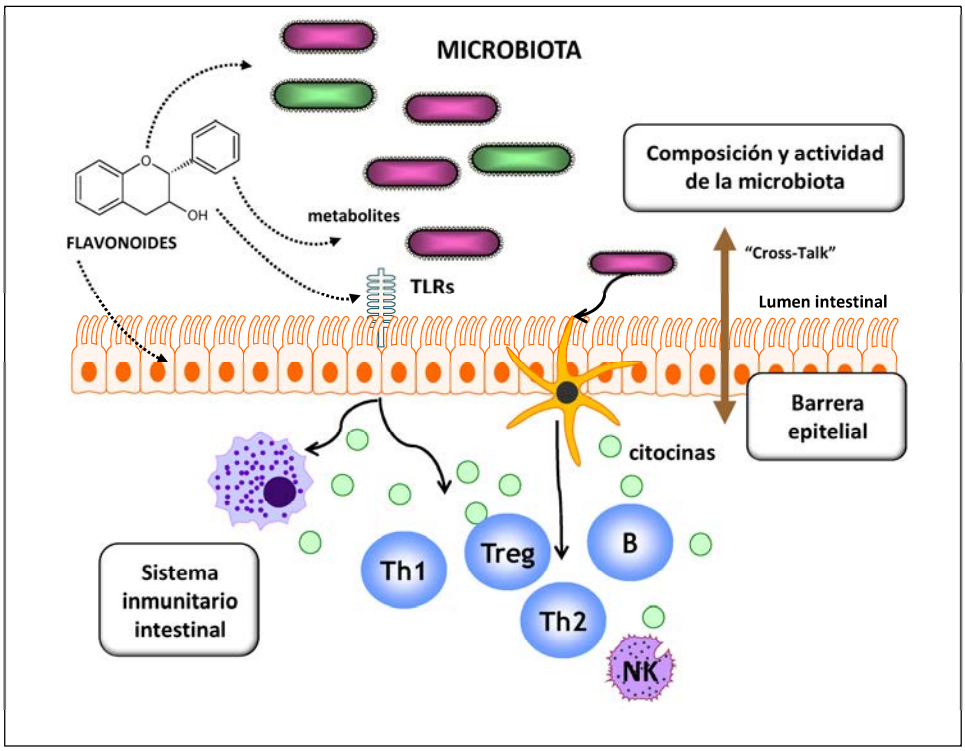


Figura 2: Efectos de los flavonoides sobre la inmunidad intestinal. Imágenes individuales obtenidas de Motifolio.

De hecho, numerosos estudios demuestran la capacidad de diferentes polifenoles, y entre ellos los flavonoides, para limitar el sobrecrecimiento de algunos grupos o especies bacterianas, acción relacionada con el conocido efecto "antibiótico" de este tipo de compuestos sobre ciertos patógenos (siendo de especial relevancia los catabolitos microbianos de la epicatequina, la epigalocatequina o la naringenina) (51). Además, ciertos flavonoides también pueden promover el crecimiento selectivo de otros grupos bacterianos, entre los que destaca la acción prebiótica de algunos flavonoides, como por ejemplo los del cacao, sobre las bifidobacterias (52).

Las características de los flavonoides es un aspecto esencial en esta función, ya que dependerá no sólo de la subclase a la que pertenezcan sino también a la posición de sus radicales, de si se encuentra en forma de aglicona libre o formando un glucósido, de su grado de polimerización (los flavonoides que forman polímeros como las procianidinas o taninos parecen tener una mayor influencia), y finalmente, de si éstos están retenidos o no por otros componentes alimentarios como la fibra, hecho que permite que alcancen el colon con más facilidad y por lo tanto que puedan ejercer su función de manera más evidente. Los cambios en la microbiota inducidos por los flavonoides pueden implicar una diferente actividad metabólica (por ejemplo, diferente producción de ácidos grasos de cadena corta), pero, al mismo tiempo, una diferente interacción con los receptores del hospedador

dirigidos a su reconocimiento como los receptores de tipo *toll* (TLR), entre otros. Además, existen estudios, principalmente *in vitro*, que demuestran que flavonoides de tipo flavonol (quercetina), flavanona (naringenina), flavanoles (epigalocatequin-3-galato) o flavonas (baicalina) pueden modificar la expresión génica, proteica e incluso la activación de estos receptores. Así, mediante este mecanismo también se afecta la interacción entre la microbiota y el sistema inmunitario. De forma general, los flavonoides disminuyen su expresión, por lo que se pueden asociar con un menor estímulo inflamatorio para el organismo (revisado en (50)).

ESTUDIOS CLÍNICOS CON FLAVONOIDES

Los estudios *in vitro* y preclínicos han permitido esbozar los efectos de los flavonoides sobre el sistema inmunitario, los mecanismos implicados y también el efecto protector en las reacciones alérgicas. Sin embargo, existen pocos ensayos clínicos que permitan corroborar los efectos inmunomoduladores de los flavonoides. A continuación, se resumen algunas evidencias que muestran la influencia de los flavonoides en el sistema inmunitario humano y también los estudios preclínicos en la enfermedad alérgica obtenidos en los últimos años.

En primer lugar cabe destacar una reciente revisión que recoge datos del estudio Moli-sani y analiza el contenido de polifenoles de la dieta (flavonoles, flavonas, flavanonas, flavanoles, antocianinas, isoflavonas y lignanos) de una cohorte de 5948 mujeres y 5965 hombres del área mediterránea, con edad igual o superior a 35 años, y lo asocia a biomarcadores de bajo grado de inflamación (53). Este estudio muestra la relación inversa entre estos biomarcadores y la cantidad de flavonoides de la dieta ingeridos en forma de frutas, verduras, frutos secos, semillas, mermeladas, te, vino tinto y cerveza.

Por otra parte, se han realizado estudios clínicos con frutas, verduras, té, cacao, vino, derivados de la soja, y con suplementos de quercetina. En general, los resultados obtenidos no permiten extraer conclusiones claras y reproducibles sobre el efecto de los flavonoides sobre la funcionalidad inmunitaria, si bien los resultados obtenidos en situaciones de estrés inflamatorio son más congruentes en el efecto regulador de los flavonoides de la dieta (revisado en (54)). Así, por ejemplo, el consumo de té negro o té verde (con un rango de 318-928 mg/día de catequinas y durante 3-30 semanas) mostró poca influencia sobre la concentración de citocinas circulantes como IL-1β, IL-6, TNF-α (54). En el mismo sentido, el consumo de cacao (40 g de cacao con cerca de 500 mg de polifenoles, durante cuatro semanas), con unos flavonoides parecidos a los del té, en pacientes de riesgo cardiovascular no modificó los niveles de IL-6 circulante si bien moduló la expresión de marcadores inflamatorios en monocitos (55). Sin embargo, flavonoides procedentes del vino o de extractos de uva (incluyendo un contenido de flavonoides de 95-2490 mg/día durante 4 semanas hasta un año) fueron capaces de reducir las concentraciones plasmáticas de las citocinas proinflamatorias TNF-α, IL-1α, IL-6 e IL-18 (54). Por otra parte, estudios realizados con isoflavonas de soja (70-112 mg/día durante periodos que oscilan entre 4 semanas y 2 años) muestran acciones contradictorios sobre el efecto modulador de estos flavonoides en las concentraciones plasmáticas de TNFα o IL-6 (54). Asimismo, con la evaluación del consumo de zumos de frutas o vegetales no se obtienen resultados concluyentes en la disminución de citocinas proinflamatorias (54). En este mismo sentido, diferentes ensayos clínicos han evaluado las concentraciones plasmáticas de IL-6, IL-8 y TNFα tras el suplemento dietético con quercetina (50-1400 mg/día, durante 2-12 semanas) y los resultados son igualmente contradictorios si bien dominan los estudios que demuestran una cierta disminución de los mediadores inflamatorios (54). Por otra parte cabe destacar los efectos inmunomoduladores descritos en ancianos delicados de salud que tomaron suplementos ricos en epigalocatechina (Leucoselect® Phytosome®). En este estudio se observó cómo los flavonoides aumentan las respuestas Th1 (IFN-γ) en estas personas de riesgo, lo que permite postular su posible efecto protector contra enfermedades alérgicas crónicas (56).

En cuanto a trabajos concretos que muestran el posible efecto protector de los flavonoides en las enfermedades alérgicas humanas, estudios epidemiológicos realizados hace dos décadas sugieren el efecto protector de los flavonoides contra enfermedades crónicas, incluyendo el

asma (57). Un estudio finlandés, con una cohorte de más de 10.000 voluntarios adultos, demuestra la asociación inversa entre ingesta de quercetina y hesperidina con la incidencia de asma (58). Asimismo, un estudio longitudinal, llevado a cabo en madres gestantes, ha relacionado la ingesta de frutas y verduras con una menor incidencia de alergia en los descendientes (58).

Por otra parte se han realizado diversos ensayos clínicos mediante la administración de determinados flavonoides o extractos ricos en flavonoides que indican efectos beneficiosos en la rinitis alérgica (revisado en (59)).

En el caso de asma, se han llevado a cabo pocos ensayos clínicos, si bien se ha observado la efectividad de Pycnogenol, extracto rico en flavonoides (proantocianidinas). Este producto es capaz de disminuir los leucotrienos presentes en sangre, mostrando una mejoría en la función pulmonar y en la sintomatología, lo que conllevaba a una reducción del uso de inhaladores (revisado en (59)). Sin embargo, un metanálisis sobre las intervenciones con derivados vegetales en asma, concluye que no hay suficientes evidencias objetivas para realizar recomendaciones con estos productos ya que la mayoría de los estudios incluyen un número pequeño de pacientes, son de corta duración, y utilizan una metodología simple (60).

CONCLUSIONES FINALES

Los flavonoides procedentes de la dieta pueden tener un papel inmunorregulador sobre el organismo. Esta influencia se ha evidenciado a partir de numerosos estudios desarrollados *in vitro* que muestran el poder antiinflamatorio y antioxidante de flavonoles, flavonas, flavanonas e isoflavonas, mediante la inhibición de la formación de mediadores inflamatorios, como son algunas citocinas, la actividad de diversas enzimas y la formación de especies oxidantes. Asimismo, los flavonoides interactúan con células implicadas en la respuesta inmunitaria adquirida, interfiriendo en diversas fases de esta respuesta, como la presentación antigénica, la proliferación linfocítica y la secreción de citocinas características de las respuestas Th1 y Th2. Estudios llevados a cabo en modelos de alergia han puesto en evidencia el poder regulador de los flavonoides en la formación de anticuerpos, sobre todo IgE, en la activación y atracción de mastocitos y eosinófilos, y en las consecuencias clínicas producidas. Por último, como componentes de la dieta, los flavonoides interactúan con el sistema inmunitario intestinal y también con la microbiota de este compartimento. Concretamente, se ha demostrado que algunos flavonoides promueven el crecimiento de determinadas especies bacterianas y, por otra parte, modifican moléculas implicadas en el reconocimiento de bacterias por parte del hospedador. A pesar de que los estudios preclínicos demuestran que la dieta rica en determinados flavonoides influye sobre la inmunidad intestinal y la inmunidad sistémica y que algunos flavonoides tienen el potencial de atenuar los procesos alérgicos, las evidencias clínicas aún son escasas y, por ello, se necesitan más estudios observacionales en poblaciones de riesgo así como estudios de intervención nutricional.

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3. CACAU I SISTEMA IMMUNITARI

3.1 ORIGEN I OBTENCIÓ DEL CACAU

El cacau és un producte que s'obté de la llavor del cacauer (arbre de la família de les Esterculiàcies), anomenat *Theobroma cacao* per Carl Linnaeus el 1753. Aquest nom prové de les paraules gregues *theos* i *broma* que signifiquen "menjar de déu" [100], ja que en els seus inicis es considerava un aliment diví. El cacauer és un arbre natiu de les regions humides tropicals de la part nord de Sud-Amèrica [101], encara que actualment també es conrea a l'Àfrica occidental, a l'Amèrica central i a l'Extrem Orient [102]. Es cultiven tres grans varietats: Foraster, Criolll i Trinitari [103].

L'origen del cacau se situa en les cultures Olmeques, Maies i Asteques a Mesomèrica abans de l'any 2000 a.C [104]. En els seus inicis, el cacau era consumit en la seva forma líquida, anomenada *xocoatl,* i era usat com a moneda de canvi. El primer contacte d'Europa amb el cacau va ser a Amèrica, l'any 1502, quan Cristòfor Colom i la seva tripulació varen capturar una canoa a Guanaja (Hondures) que contenia cacau [103]. En aquell moment, però, els europeus desconeixien la importància que tenia a Mesomèrica. Posteriorment, el 1528, Hernando Cortés en arribar a terres mexicanes va ser obsequiat amb cacau líquid i, en el seu viatge de retorn, el va portar a Espanya per donar a conèixer els seus magnífics efectes [100,105]. A continuació es va difondre i usar ràpidament a tot Europa, primer com a producte exclusiu per a l'alta societat i posteriorment es va estendre a la població en general. Al segle XIX van aparèixer els primers preparats sòlids derivats del cacau, tals com les rajoles de xocolata i els bombons.

L'obtenció de productes derivats del cacau requereix una sèrie de passos (**Figura 10**):

1. **Recol·lecció:** el fruit del cacauer creix durant tot l'any. Solen fer-se dues collites a l'any.
2. **Desgrana: e**ls fruits del cacauer s'obren longitudinalment i s'extreuen les llavors amb la polpa que les envolta.
3. **Fermentació:** les llavors del cacau s'apilen i es cobreixen amb fulles de plàtan o bé es col·loquen en caixes de fusta per produir la fermentació durant uns 5-6 dies, en funció de la varietat del cacau. Durant aquest procés es facilita la desaparició

de la polpa viscosa que envolta les llavors i s'inicia l'adquisició del color i gust característic tot reduint la seva amargor i astringència. L'èxit de la fermentació requereix una sèrie d'activitats microbianes, tant de llevats, bacteris productors d'àcid làctic i bacteris productors d'àcid acètic [106–108].

4. **Assecatge:** després de la fermentació, les llavors requereixen el procés d'assecatge que, en funció de la zona, pot ser natural, al sol, o provocat de forma artificial. En aquest procés es redueix el contingut d'aigua que conté el cacau del 50% al 6-7% i així s'evita el creixement de fongs.

5. **Torrefacció:** les llavors de cacau se sotmeten a torrefacció durant un temps variable de 5 a 120 min i a una temperatura de 120 a 150 ºC per originar l'aroma i el gust típic de la xocolata [107]. En aquest procés es produeix l'esterilització microbiològica de les llavors. Les llavors torrades es trenquen, s'esclofollen i s'obtenen les *nibs* (amb un contingut de mantega de cacau del 50-56%) [108].

6. **Mòlta:** durant la mòlta (50-70 ºC) es provoca el trencament de l'estructura cel·lular i l'alliberament de la mantega de cacau, i es produeix així l'anomenat licor del cacau, que és la base de tots els seus derivats [109]. Tot seguit, el licor de cacau s'introdueix en una premsa hidràulica on s'obté la torta de cacau i la mantega de cacau. A partir de la torta de cacau, amb un altre pas de mòlta, s'aconsegueix el cacau en pols mentre que la xocolata s'obté combinant el licor de cacau amb mantega de cacau i sucre [110]. El cacau en pols constitueix el que es coneix com a cacau natural.

7. **Alcalinització:** per tal de produir el cacau alcalinitzat, sobre la pols, la torta o les *nibs* es realitza l'alcalinització mitjançant l'addició d'una solució alcalina, majoritàriament de carbonat potàssic. Durant aquesta etapa tenen lloc canvis en el color (que passa de vermellós a marró o fins i tot negre), en el pH, en el gust (més suau, menys amargant, menys àcid i menys astringent) i en la solubilitat (més soluble).

Figura 10. Procés per obtenir el licor de cacau
Imatges extretes de diferents webs [111–113]

3.2 COMPOSICIÓ DEL CACAU

El cacau en pols conté més de 500 compostos, en concret, presenta un contingut del 26-40% de fibra, del 15-20% de proteïnes, del 15% de carbohidrats, del 10-24% de lípids, i també conté minerals (magnesi, coure, potassi, calci i ferro), vitamines (A, E, B, àcid fòlic), polifenols (majoritàriament flavanols) i metilxantines (teobromina i cafeïna) [110,114].

3.2.1 Polifenols: flavonoides

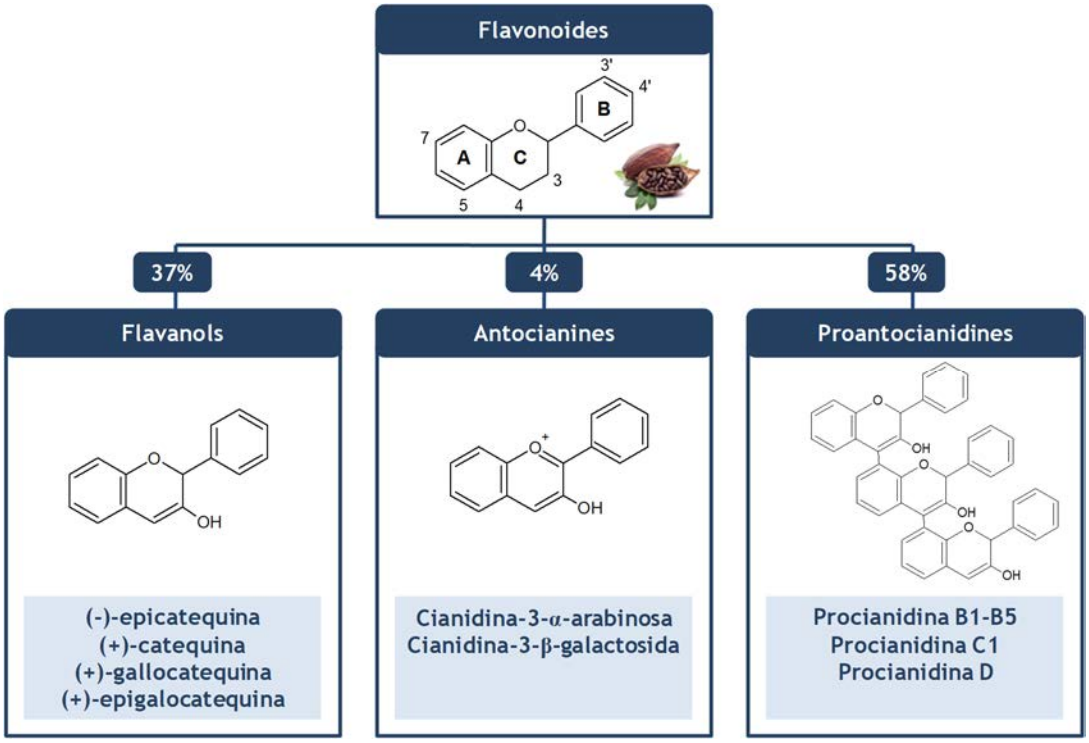
Els polifenols són productes del metabolisme secundari de les plantes que es troben en una gran varietat de fruites, vegetals, llavors, flors, begudes i diferents productes confeccionats [115]. Els polifenols comprenen diferents famílies i, entre elles, la més important és la dels flavonoides [115]. Els flavonoides, en funció de la seva estructura química, es classifiquen en vuit subclasses: flavans, flavanones, isoflavanones, flavones, isoflavones, antocianines, xalcones i flavonolignans. S'han identificat més de 8000

flavonoides diferents i generalment, de forma natural, es troben conjugats a sucres formant glicòsids [116].

El cacau conté un elevat contingut de polifenols per pes sec (12-18%) [117], superior al de diversos aliments, incloent el te i el vi [104]. Els polifenols de les llavors del cacau es troben dins les cèl·lules pigmentàries dels cotiledons [115] i la seva quantitat depèn de molts factors tals com la varietat de la planta, el clima, les característiques del sòl, el maneig post-collita, la fermentació, l'assecatge i la torrefacció [104,118].

Els polifenols més abundants del cacau són flavonoides, dels quals 37% són flavanols, 4% antocianines i 58% proantocianidines (**Figura 11**) [115]. El principal flavanol present al cacau és la (-)-epicatequina, que arriba a constituir el 35% de tot el contingut de polifenols presents a la llavor del cacau [103]. En menys quantitat, el cacau conté (+)-catequina i traces de (+)-gallocatequina i (+)-epicatequina. Les antocianines presents al cacau consisteixen, principalment, en la cianidina-3-α-L-arabinosa i la cianidina-3-β-D-galactosida mentre que les procianidines que conté són majoritàriament dímers, trímers o oligòmers de flavan-3,4-diols [117]. Concretament, les principals procianidines presents són la B1, B2, B3, B4, B5, C1 i D [119].

Figura 11. Els flavonoides del cacau

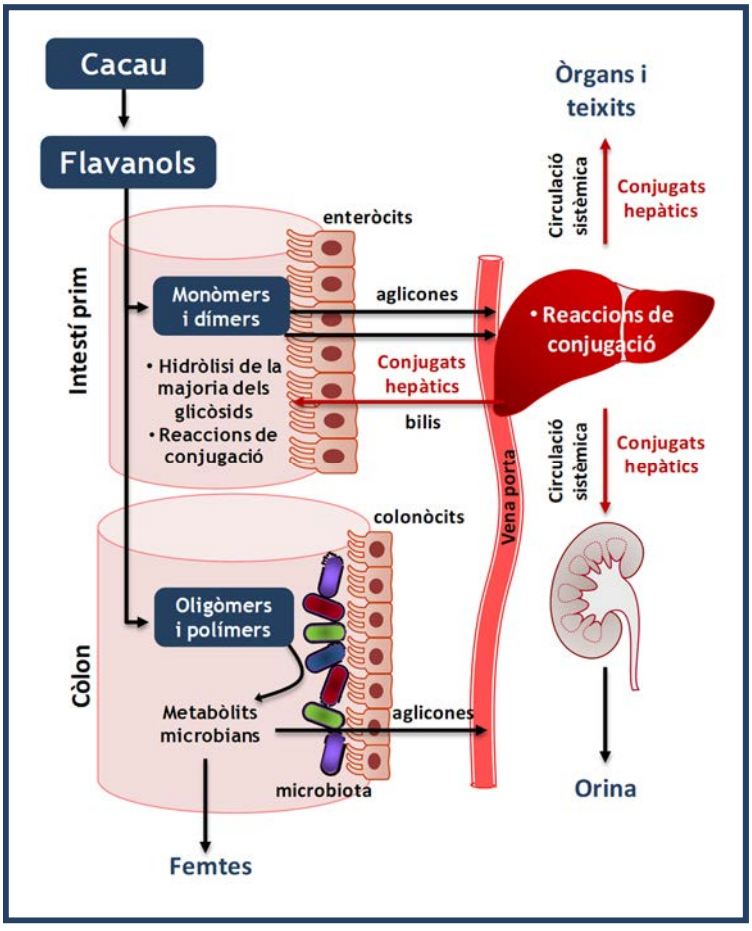


L'estructura química dels flavonoides influeix en la seva biodisponibilitat. Generalment els polifenols que es troben en el menjar són glicòsids, però només les aglicones i alguns glucòsids poden ser absorbits per la mucosa intestinal. És per això, que diferents enzims humans o microbians són importants per assolir la forma adient per a la seva absorció.

Els flavonoides del cacau són estables en el seu pas per l'estómac i la seva absorció intestinal depèn de la seva pròpia estructura química [120], encara que existeixen moltes interaccions amb altres aliments de la dieta [121]. Els flavonoides monomèrics i els dimèrics s'absorbeixen a l'intestí prim, mentre que els oligomèrics i els polimèrics són difícilment absorbits a l'intestí prim, però, presenten una funció local important neutralitzant oxidants i compostos carcinogènics [114,122]. Un cop al còlon, són biotransformats per la microbiota intestinal i, seguidament, absorbits [114,123].

Els flavonoides són metabolitzats per enzims de fase II a l'enteròcit (metilació, sulfatació, o glucurinació), passen a sang, arriben al fetge on poden ser novament metabolitzats i retornen a la circulació sanguínia per ser finalment secretats per l'orina. Alguns conjugats hepàtics són excretats per via biliar i els metabòlits no absorbits s'eliminen per les femtes [122–124] (**Figura 12**).

Figura 12. Metabolisme dels flavonoides del cacau
Basada en [122–124]

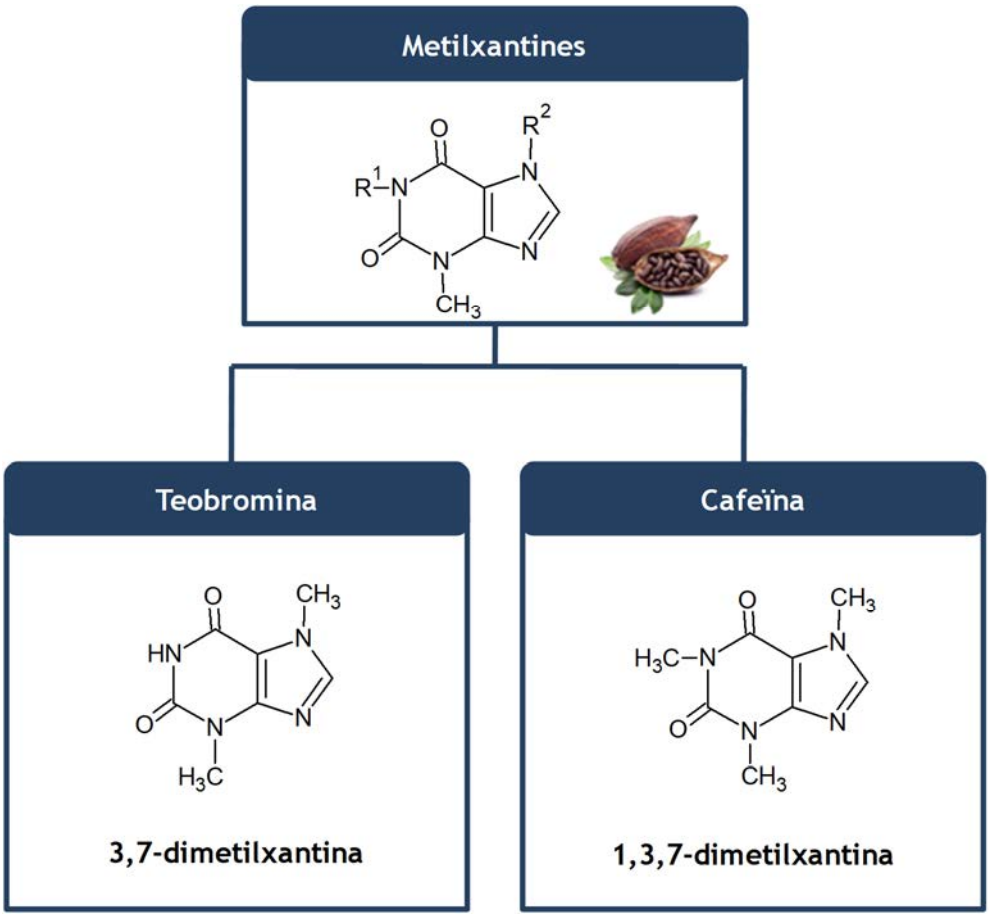


3.2.2 Metilxantines

Les metilxantines són productes del metabolisme secundari de les plantes sintetitzades per a protegir-se de patògens. Són produïdes en quantitats considerables per un nombre limitat d'espècies botàniques [125]. Les tres principals fonts de metilxantines són el cafè (*Coffea* sp.), el te (*Camellia sinensis*) i el cacau (*Theobroma cacao*), i són la cafeïna, la teofil·lina i la teobromina, respectivament, les seves principals metilxantines [125].

El cacau conté cafeïna i teobromina (**Figura 13**), essent la teobromina la més abundant [126]. Els cotiledons de les llavors madures contenen entre un 2,2-2,7% de teobromina per pes sec [127]. El contingut de teobromina varia en funció de la varietat del cacau i del seu procés d'elaboració [126].

Figura 13. Les metilxantines del cacau



La cafeïna i la teobromina s'absorbeixen fàcilment en el tracte gastrointestinal i es distribueixen àmpliament per tot el cos. Es metabolitzen al fetge i són sotmeses a un reciclatge enterohepàtic fins a excretar-se per l'orina, ja sigui com a metabòlits o bé en la seva forma original [126].

Les propietats de la cafeïna són ben conegudes. És un potent estimulant del sistema nerviós central i provoca un increment de la consciència, claredat de pensament i disminució de la fatiga [125]. A més, se li han atribuït propietats cardiovasculars, diürètiques, natriurètiques, i anticancerígenes, entre altres [125].

Pel que fa a la teobromina, recentment ha crescut l'interès pels seus efectes sobre la salut. Per molt que aquesta metilxantina és tòxica per alguns mamífers, com els gossos, la toxicitat en humans és molt baixa [128]. Algun dels seus beneficis descrits són la millora de la salut oral [129], la prevenció de la tos [130] i la inhibició de la cristal·lització de l'àcid úric [131].

3.3 EFECTES DEL CACAU SOBRE LA SALUT

L'evidència més primerenca de l'ús del cacau per finalitats mèdiques o curatives es troba en les civilitzacions de Mesomèrica, els maies i els asteques, uns 600 anys a.C [100,105]. Malgrat que actualment el seu ús generalitzat com aliment ha superat la seva utilitat mèdica, aquests darrers anys s'ha incrementat novament l'interès i recerca del paper del cacau sobre la salut. S'han dut a terme estudis *in vitro*, *in vivo* i clínics que confirmen el potencial efecte del cacau en malalties cardiovasculars, en el sistema nerviós central, a la pell, en càncer i en el sistema immunitari, entre altres [110,119,132,133].

Pel que fa als efectes del cacau en la salut cardiovascular, s'ha vist que, entre altres beneficis, presenta efectes en la prevenció de l'aterosclerosi, en la regulació de la pressió arterial i en la inhibició de l'activació plaquetària [134–136]. La producció d'aquests efectes es deu, en part, a l'elevada capacitat antioxidant que presenta el cacau pel seu elevat contingut fenòlic, el que contribueix en la inhibició de l'oxidació de lípids [134,137]. A més, s'ha vist que el cacau incrementa la producció de l'òxid nítric (NO) [138], redueix l'activitat de la xantina oxidasa, inhibeix l'enzim convertidor d'angiotensina i regula l'expressió de NF-κB [137,139].

També existeixen evidències que relacionen el cacau amb el sistema nerviós central. En concret s'ha descrit que el cacau protegeix de la neurodegeneració (a les malalties d'Alzheimer i Parkinson), modula la funció neuronal i evita la depressió i l'estrès [110,132,140]. Molts d'aquests beneficis també s'han relacionat amb l'increment de NO que, a més de regular la vascularització perifèrica, incrementa la perfusió cerebral. A més a més, s'ha descrit que el cacau redueix la formació d'espècies reactives d'oxigen i la neurotoxicitat produïda per les proteïnes β amiloides [141]. La prevenció de la depressió s'ha associat amb el paper que tenen els polifenols del cacau en el bloqueig de la captació de diferents neurotransmissors i en la conversió del triptòfan present en el cacau a serotonina, entre d'altres [142].

Per altra banda, el cacau presenta propietats endocrines importants com antidiabètic i antiobesitat [143]. Aquests efectes són deguts al paper que tenen els polifenols del cacau en la millora de la funció endotelial i antioxidant, tot modulant el metabolisme lipídic [132,144].

Pel que fa a la salut dental, el cacau actua protegint les càries dentals i evita malalties periodontals. Aquests efectes són deguts a la reducció en la formació del biofilm i en la inhibició de la producció d'àcid [145].

El cacau també mostra un efecte beneficiós a la pell. Diferents estudis han relacionat el paper dels flavanols del cacau en la fotoprotecció, millora de la circulació dèrmica i en les qualitats i hidratació de la pell [110,146].

Per altra banda, diversos estudis *in vitro* i *in vivo* suggereixen l'utilitat del cacau en la inhibició del creixement de les cèl·lules cancerígenes [147]. En concret, s'ha vist que el cacau pot reduir el càncer de mama [148], el càncer pancreàtic [149], el càncer de pulmó [150] i el càncer de còlon [151] en rosegadors o en línies cel·lulars representatives. Aquests efectes són deguts a la seva activitat antioxidant i a la regulació de diferents senyals de transducció que modulen la inflamació, la proliferació cel·lular, la diferenciació i l'apoptosi [151].

Per últim, el cacau presenta diferents efectes en el sistema immunitari (**apartat 3.4**) que podrien contribuir en un efecte beneficiós en les reaccions d'hipersensibilitat i autoimmunitàries, encara que cal desenvolupar estudis clínics en aquest sentit.

3.4 CACAU I RESPOSTA IMMUNITÀRIA

Diferents estudis *in vivo* i *in vitro* demostren la influència de la ingesta de cacau en el sistema immunitari innat i adquirit (sistèmic i intestinal). Tots aquests efectes es troben detallats en la revisió que hi ha a continuació, on també es descriu el paper del cacau en la prevenció de diferents alteracions del sistema immunitari. Com que aquesta revisió, en premsa a la revista *Frontiers in Nutrition* (section Nutritional Immunology), s'ha escrit a la fase final de la tesi s'hi inclouen resultats que formen part dels articles presents a la secció de Resultats.

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REVIEW
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Cocoa Diet and Antibody Immune Response in Preclinical Studies

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The ability of cocoa to interact with the immune system *in vitro* and *in vivo* has been described. In the latter context, a cocoa-enriched diet in healthy rats was able to modify the immune system's functionality. This fact could be observed in the composition and functionality of lymphoid tissues, such as the thymus, spleen, and lymph nodes. Consequently, immune effector mechanisms, such as antibody synthesis, were modified. A cocoa-enriched diet in young rats was able to attenuate the serum levels of immunoglobulin G (IgG), IgM, and IgA and also the intestinal IgM and IgA secretion. Moreover, in immunized rats, the intake of cocoa decreased specific IgG1, IgG2a, IgG2c, and IgM concentrations in serum. This immune-regulator potential was then tested in disease models in which antibodies play a pathogenic role. A cocoa-enriched diet was able to partially prevent the synthesis of autoantibodies in a model of autoimmune arthritis in rats and was also able to protect against IgE and T helper 2-related antibody synthesis in two rat models of allergy. Likewise, a cocoa-enriched diet prevented an oral sensitization process in young rats. In this review, we will focus on the influence of cocoa on the acquired branch of the immune function. Therefore, we will focus on how a cocoa diet influences lymphocyte function both in the systemic and intestinal immune system. Likewise, its potential role in preventing some antibody-induced immune diseases is also included. Although further studies must characterize the particular cocoa components responsible for such effects and nutritional studies in humans need to be carried out, cocoa has potential as a nutraceutical agent in some hypersensitivity status.

Keywords: gut-associated lymphoid tissue, IgA, immunoglobulin G, IgM, immunoregulator, lymph nodes, spleen, tolerance

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INTRODUCTION

Antibody response is a kind of acquired immune response produced by complex interactions between several types of immune cells after the entry of an antigen into the body. In brief, when dendritic cells come into contact with an antigen in the skin or the mucosa, they become antigen-presenting cells and will be in charge of finding specific helper (Th) cells in order to trigger an acquired immune response (1). Activated specific Th cells will differentiate into effector T cells that, by means of different patterns of cytokines, will enhance the function of cells, such as B lymphocytes, macrophages, natural killer (NK) cells, cytotoxic T (Tc) lymphocytes, mast cells, or eosinophils. The activation of B cells, mainly related to Th2-immune response, will produce the formation of plasma cells that will eventually synthesize antibodies against the triggering antigen (1). In addition, inside the germinal centers of the secondary lymphoid tissues, another kind of

antigen presentation occurs. Follicular dendritic cells (FDC) retain the native antigen that could prime B cells to synthesized specific antibodies (2, 3). The FDC can form immune complex-coated bodies known as iccosomes that could also affect B cell activation, maturation, and maintenance (4). Whereas antibodies will neutralize or facilitate antigen destruction, sometimes, such as in hypersensitivity and autoimmune reactions, they could have a harmful effect on the body.

Although the earliest evidence for the medical use of cocoa was found in Mesoamerican civilizations (5), nowadays, the healthy properties of cocoa and its derivatives are re-emerging. In addition to the effects of cocoa on cardiovascular health (6, 7), the nervous system (8), and cancer (9–11), cocoa also has an effect on the immune system. The immunomodulatory properties of cocoa include its potential anti-inflammatory role, demonstrated in both *in vitro* and *in vivo* studies (12–14). However, only few clinical studies with this aim have been carried out, and recently it was suggested that there is scarce evidence of the anti-inflammatory effects of cocoa consumption in humans (15). Nevertheless, researchers in this field have joined on several occasions to discuss in depth the effects of chocolate and cocoa on medicine and have demonstrated the increasing emergence of cocoa as a diet compound able to prevent some diseases, or even being a coadjuvant in some therapies (16, 17). In this review, we will focus on the influence of cocoa on the acquired branch of the immune function. Therefore, we will focus on how a cocoa diet influences lymphocyte function both in the systemic and intestinal immune system. Likewise its potential role in preventing some antibody-induced immune diseases is also included.

COCOA INFLUENCES SYSTEMIC ANTIBODY SYNTHESIS

Preclinical studies performed 10 years ago showed for the first time the *in vivo* influence of a cocoa diet on the immune system (14, 18). These studies were carried out in young rats that were fed a diet containing 10% cocoa or in rats that were orally administered with a dose equivalent to 4% cocoa in food intake for 3 weeks. Results showed that the 10% cocoa-enriched diet, but not the 4% dose, was able to decrease serum immunoglobulin G (IgG), IgM, and IgA concentrations (18) (**Table 1**). A further analysis of IgG isotypes showed that 3-week-old rats fed a 10% cocoa diet for 3 weeks resulted in attenuated levels of IgG2b antibodies but increased levels of IgG2a (19) (**Table 1**). However, in a study in which the cocoa diet was given later, at 6 weeks of age, the 10% cocoa-enriched diet was associated with lower values of serum IgG2a but higher serum IgG2c concentrations than those present in animals fed the standard diet (20) (**Table 1**). Moreover, it was observed that the minimum dose to achieve such an effect was 5% cocoa in the diet (20) and, at any rat age, a 5 or 10% cocoa diet attenuated the serum levels of IgM and IgA (19, 20), the effects being clearer when animals were younger and the diet lasted longer. Therefore, these studies in rats showed that a cocoa diet influences systemic immunoglobulin production but the effect depends on

TABLE 1 | Summary of the effects of cocoa diet in serum immunoglobulins and specific antibodies in healthy rats.

Strain	Initial age (weeks)	Cocoa dose	Length of the study (weeks)	Results	Reference
Wistar rats	3	4% by oral gavage	3	=IgG =IgM =IgA	(18)
		10% in the food		↓IgG ↓IgM ↓IgA	
Wistar rats	6	2% in the food	3	=IgG1, IgG2a, IgG2b, IgG2c =IgM =IgA	(20)
		5% in the food	3	=IgG1, IgG2b, ↓IgG2a, ↑IgG2c =IgM =IgA	
		10% in the food	3	=IgG1, IgG2b, IgG2c, ↓IgG2a ↓IgM =IgA	
Wistar rats	4	10% in the food	7	=IgG1, IgG2a, IgG2c, ↓IgG2b ↓IgM ↓IgA	(19)
Wistar rats	3	4% in the food	9	=Specific IgG2a, IgG2b ↓Specific IgG1, IgG2c ↓Specific IgM	(21)
		10% in the food	9	↓Specific IgG1, IgG2a, IgG2c ↑Specific IgG2b ↓Specific IgM	

Arrows indicate increases or decreases, equals sign means no changes.
Ig, immunoglobulins.

the antibody isotype, the age of the animal, and the length of the cocoa intervention.

Apart from the cocoa's influence on basal serum immunoglobulin levels, it was interesting to shed some light on the antibody response in rats after a specific challenge, i.e., in immunized rats. In these conditions, animals were fed cocoa before and during an immunization process, and the overall synthesis of specific antibodies was also lowered (21) (**Table 1**). Specifically, the accurate analysis of antibodies revealed that the most attenuated isotypes were specific IgM, IgG1, IgG2a, and IgG2c antibodies, whereas specific IgG2b concentrations held steady or even increased with the 10% cocoa diet. As IgG rat isotypes can be associated with Th1 (IgG2b) or Th2 (IgG1 and IgG2a) immune response (22), these results may suggest a regulatory effect of cocoa in Th2-immune responses. This lowering effect on specific IgG1 and IgG2a, and therefore on Th2-related response, could be associated with cocoa polyphenols given that other polyphenols, such as genistein, chrysin, and apigenin (23, 24), and those from apple or soybean caused similar results (23, 25).

After establishing cocoa's influence on immunoglobulin synthesis, the reason why this diet produced such an effect remained to be studied.

COCOA INFLUENCES COMPOSITION AND FUNCTIONALITY OF PRIMARY AND SECONDARY LYMPHOID TISSUES

To ascertain the mechanisms induced by cocoa on the antibody immune response, lymphoid tissue composition and lymphocyte functionality were then determined. In addition, as cocoa intake can interact with gut-associated lymphoid tissue (GALT), several investigations were carried out to ascertain the influence of cocoa in this particular compartment of the immune system. Preclinical studies carried out in rats demonstrated that a cocoa diet modifies lymphoid tissue composition and function (13). Lymphoid tissues are considered as primary or secondary depending on whether they are devoted either to the formation of the lymphocyte repertoire or to the development of the immune response, respectively (26). Thymus is a primary lymphoid tissue where T-cell maturation takes place, whereas lymph nodes, spleen, and mucosal lymphoid tissue belong to the secondary lymphoid tissue category (27).

Cocoa and Systemic Lymphoid Tissue Composition

It was demonstrated that Wistar rats receiving a 10% cocoa diet for 3 weeks accumulate cocoa polyphenol metabolites in immune tissues, such as the thymus, lymph nodes, and spleen (28). In particular, the highest accumulation was in the thymus, where phenotypic changes were found due to the diet. In particular, cocoa intake resulted in an enhancement of the progression of immature thymocytes (those with low expression of the αβ T-cell receptor—TCR $\alpha\beta^{-}$, and expressing or non-expressing the clusters of differentiation CD4 and CD8, i.e., $TCR\alpha\beta^{low}CD4^{-}CD8^{-}$ or $TCR\alpha\beta^{low}CD4^{+}CD8^{+}$) toward more mature stages ($TCR\alpha\beta^{high}CD4^{+}CD8^{-}$) (29) (**Table 2**). In spite of this increase in $CD4^{+}$ (Th) cells in the thymus, the analysis of a secondary lymphoid tissue, such as the spleen, revealed that a 10% cocoa diet in young rats for 3 weeks increased the proportion of spleen B cells and decreased that of Th lymphocytes (18) (**Table 2**).

Lymph nodes were also affected by a cocoa diet. In particular, in mesenteric lymph nodes, a cocoa-enriched diet for 3 or 4 weeks in rats increased the proportion of innate cytotoxic lymphocytes, such as cells expressing γδ T-cell receptor ($TCR\gamma\delta^{+}$) and NK cells, and also that of the Tc lymphocytes and B cells, whereas the proportion of Th cells decreased (30, 31) (**Table 2**). These effects were only produced by a 10% cocoa diet whereas a 4% cocoa dose was insufficient to influence the phenotype of mesenteric lymph nodes (30). Similarly, the intake of a 10% cocoa-enriched diet given to rats for 6 weeks decreased the proportion of $TCR\alpha\beta^{+}$ cells but did not modify that of regulatory T cells (Treg) in inguinal lymph nodes in rats (32) (**Table 2**).

A more in-depth analysis of lymphocytes in mesenteric lymph nodes revealed that the increase of $TCR\gamma\delta^{+}$ cells was attributed to the presence of a higher amount of $CD8\alpha\alpha^{+}$ cells, a typical intestinal phenotype, which could be due to the migration of this cellular type from the intestine (34). The increase of Tc cells in mesenteric lymph nodes was accompanied by a higher proportion of activated cells ($CD25^{+}CD8^{+}$ cells) and cells expressing the αE-integrin ($CD103^{+}CD8^{+}$ cells) and a lower proportion of cells bearing L-selectin ($CD62L^{+}CD8^{+}$ cells) (31) (**Table 2**). CD103 is a subunit of the αE-integrin that can mediate cell adhesion and migration to the gut (35), whereas L-selectin is involved in lymphocyte rolling on the endothelium and the homing to secondary lymphoid tissues (36). These results could mean that the cocoa diet decreased the arrival of blood lymphocytes to mesenteric lymph nodes whereas it may favor intestinal cells entering. As cocoa compounds can reach the small intestine and even the colon (37, 38), they can affect the intestinal lymphocytes and promote their migration to mesenteric lymph nodes.

Overall, the increased proportion of $CD8\alpha\alpha^{+}TCR\gamma\delta^{+}$ cells, NK cells, and $CD103^{+}$ Tc cells in mesenteric lymph nodes could be involved in cocoa's influence on antibody immune response. $TCR\gamma\delta^{+}$ cells have been associated with an attenuating effect on the synthesis of antibodies (39), and NK cells could also contribute to the regulation of antibody synthesis (40). Moreover, $CD103^{+}$ cells have been associated with a regulatory function given that their proportion increased after treatment with immunosuppressive agents (41).

After feeding a cocoa-enriched diet, cocoa flavonoid metabolites are stored in the lymphoid tissues (thymus, lymph nodes, and spleen) as well as in the liver. In fact, epicatechin metabolites have been reported to be accumulated in concentrations twofold higher in the thymus, testes, and liver than in lymph nodes and spleen (28). With regard to the liver, the 10% cocoa intake in rats enhanced hepatic antioxidant capacity, without modifying hepatic superoxide dismutase and catalase activities (29).

Cocoa and Lymphocyte Function

The development of the acquired immune response implies the involvement of complex interactions between immune cells

TABLE 2 | Summary of the effects of cocoa diet in lymphocyte composition of lymphoid tissues.

Lymphoid tissue	Cocoa dose	Length of the diet (weeks)	Results (% cells)	Reference
Thymus	10% in the food	3	↓TCRαβ^{low}CD4$^-$CD8$^-$ ↓TCRαβ^{low}CD4$^+$CD8$^+$ ↑TCRαβ^{high}CD4$^+$CD8$^-$	(29)
Spleen	4% by oral gavage 10% in the food	3	No changes ↑B ↓Th	(18)
Lymph nodes	4% by oral gavage 10% in the food	3	No changes ↑TCRγδ$^+$ ↓Th ↑Tc	(30)
	10% in the food	4	↑NK ↑B ↓TCRαβ$^+$ ↑TCRγδ$^+$ (↑CD8αα$^+$) ↓Th (↓CD62L$^+$) ↑Tc (↑CD25$^+$, ↑CD103$^+$, ↓CD62L$^+$)	(31)
	10% in the food	6	↓TCRαβ$^+$ ↓Th ↑Tc =Treg	(32)
Peyer's patches	4% by oral gavage 10% in the food	3	No changes ↑B ↓TCRαβ$^+$ ↑TCRγδ$^+$ ↓Th	(30)
	10% in the food	4	↑TCRγδ$^+$ ↑NKT ↓Th (↑ CD25$^+$, ↑CD103$^+$, ↓CD62L$^+$) =Tc (↑ CD103$^+$)	(33)
Intestinal intraepithelium	10% in the food	4	↑TCRγδ$^+$ ↑NK ↓TLR4$^+$ ↑CD4$^+$CD103$^+$	(33)
Intestinal lamina propria	10% in the food	4	↓NKT ↓IgA$^+$	(33)

Arrows indicate increases or decreases, equals sign means no changes.
CD, cluster of differentiation; TCRαβ^{low}, cells with low expression of αβ T-cell receptor; Th, T helper lymphocytes; Tc, T cytotoxic lymphocytes; TCRγδ$^+$, cells with γδ T-cell receptor; NK, natural killer cells; NKT, natural killer T cells; Treg, T regulatory cells; TLR, toll-like receptor; Ig, immunoglobulin.

by means of particular surface molecules and the secretion of cytokines. The gene or protein expression of those molecules involved in the immune synapses, as well as cytokines and other molecules secreted by immune cells, can be evaluated.

In vitro studies carried out in lymphoid cell lines showed the ability of cocoa to reduce the synthesis of interleukin 2 (IL-2) involved in early T lymphocyte proliferation (42, 43). This cytokine is mainly produced by Th cells after antigen activation (44) and plays a crucial role in immune response, enhancing Tc cell, NK cell cytotoxic activities, T cell differentiation, and stimulating the proliferation and the antibody synthesis (45). These effects could be responsible for the cocoa downregulation of the antibody synthesis. However, the results obtained *in vivo* on IL-2 secretion or lymphocyte proliferation could not confirm such a mechanism (18, 21, 30) (**Figure 1A**). In particular, IL-2 secretion was not modified in spleen cells from rats fed 10% cocoa for 3 weeks, even though lymphocyte proliferation increased (18). On the other hand, higher or unmodified amounts of IL-2 secretion were detected after the stimulation of lymph node cells of rats fed a 10% cocoa diet for 3 or 9 weeks (21, 30). Therefore, the interaction of a cocoa diet in the initial phases of immune activation seems not to explain the attenuating effect on antibody synthesis. However, a recent study on the gene expression of mesenteric lymph node cells shows that certain molecules present on antigen-presenting cells (dendritic cells) were modified by this diet. In particular, a cocoa diet increased the gene expression of CD11c and OX40L (31) (**Figure 1A**). It has been suggested that, in a model of oral sensitization, a subset of dendritic cells (CD11c$^+$, CD103$^+$, and CD8$^+$) that migrates and activates in the mesenteric lymph nodes seems responsible for the Th2 polarization in this model (46). OX40L–OX40 interaction has been related to follicular Th cells and promotes the generation of Th2 response during antigen presentation (47, 48), and it was increased in an oral sensitization process (31). Despite these results, the cocoa

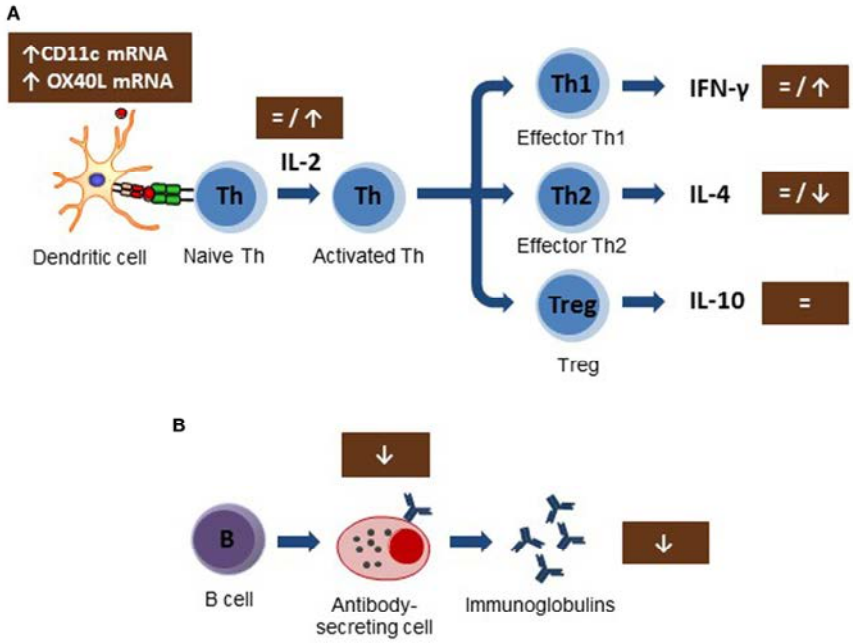

FIGURE 1 | Summary of the mechanisms involved by cocoa diet on lymphocyte function: **(A)** Cocoa effect on the induction of acquired immune response, involving from antigen presentation until the development of effector T cells. **(B)** Cocoa effect on the B-cell activation and antibody production. Arrows indicate increases or decreases, equals sign means no changes. Th, T helper cells; Treg, T regulatory cells; IL, interleukin; IFN, interferon; CD, cluster of differentiation.

diet attenuated the antibody synthesis and, therefore, this diet must interact with downstream pathways of the Th2-immune responses that would eventually inhibit antibody synthesis.

In general, the cytokine pattern secreted by activated lymphocytes reveals the stimulation of Th1, Th2, Th17, or Treg cells (49). Interferon γ (IFN-γ) is the most representative cytokine in Th1 activation (50). No changes were detected either in serum levels of IFN-γ in rats fed cocoa for 4 weeks (32) or in the secretion of IFN-γ by activated splenocytes or lymph node cells from rats fed a cocoa diet for 3 weeks (18) or 4 weeks (31). Nevertheless, an increase of IFN-γ was observed in lymph node lymphocytes from rats fed a cocoa diet for 8 weeks (21) (**Figure 1A**). Therefore, it seems that a cocoa intake over longer periods promotes Th1 immune response.

More interesting results were found in IL-4, the most representative Th2 cytokine (51). A reduction of IL-4 was found in activated lymph node cells from rats fed cocoa for 3 weeks (30) and in splenocytes from rats fed cocoa for 3 or 8 weeks (18, 21). However, no changes in IL-4 were found after 4 weeks of nutritional intervention (31). On the other hand, this downregulation on IL-4 secretion did not match with previous data *in vitro* (42, 43, 52) but it partially explains the down-modulatory role of the cocoa diet on antibody synthesis (**Figure 1A**). IL-4 promotes IgE upregulation and increases intestinal permeability (53, 54); therefore, the decrease in IL-4, along with the TCRγδ$^+$ cell increase induced by the cocoa diet, may be beneficial in reducing certain stages of hypersensitivity, such as food allergy. However, some reports focused on IL-10, a regulatory cytokine (55), did not detect any modification by the 10% cocoa diet (30, 31).

The effects of cocoa lowering IL-4 secretion in some lymphocytes populations agree with those found when the specific antibody-secreting cells after an immunization were enumerated. A significant decrease in the specific IgG-secreting cell numbers was reported by 5 and 10% cocoa diets, either in spleen or lymph node tissues, although no changes were observed in specific IgM-secreting cells (21) (**Figure 1B**). In summary, a cocoa-enriched diet plays an immune-regulatory role in the antibody immune response to an antigen that involves a lower number of specific antibody-secreting cells and, therefore, a decrease in antibody synthesis.

COCOA INTAKE INFLUENCES INTESTINAL IMMUNE SYSTEM

Cocoa Intake and Intestinal Immunoglobulins

Several years ago, Ramiro-Puig et al. first demonstrated that a cocoa-enriched diet influences the GALT by means of the

modulation of the intestinal secretory IgA (S-IgA) (30). Feeding just a 4% cocoa-enriched diet caused a decrease in the fecal S-IgA levels in the second week of the diet, but they were restored at the end of the third one. The 10% cocoa intervention caused lower fecal S-IgA secretion throughout the study (30), and this effect remained when the diet was maintained for 7 weeks (19). However, when a dose–effect study was performed with diets containing 2, 5, or 10% cocoa, the 2% diet was not enough to modify intestinal immunoglobulins (20). With regards to the gut wash—a typical sample used to evaluate intestinal immunoglobulins that consists of incubating the intestine with saline buffer at 37°C in a shaker for a few minutes to allow the release of the mucosa-linked antibodies—a lower secretion of S-IgA and S-IgM was detected (19, 20, 30, 56). These results evidence a lack of the S-IgM compensatory mechanism in certain states of S-IgA deficiency (57), probably because cocoa is also acting on S-IgM. Other studies have confirmed the previous attenuating effect of a 10% cocoa diet on S-IgA levels both in fecal samples and in gut washes (31, 58). Moreover, the immunoglobulin content has also been determined in intestinal tissues, such as Peyer's patches (PP) and mesenteric lymph nodes; and, in both tissues, the 10% cocoa diet for 3 weeks was able to decrease the levels of IgA and IgM (56).

The downregulation of intestinal immunoglobulins produced by a cocoa diet may be due to the influence of some cocoa compounds on the complex immune response developed in the GALT. This immune compartment includes inductive sites (PP and mesenteric lymph nodes) and effector sites [lamina propria (LPL) and intraepithelial lymphocytes (IEL)] (59). As explained, cocoa intake induced some changes in mesenteric lymph nodes, but the cocoa effect is not only restricted to that particular compartment. Therefore, further studies were then focused on looking in more depth the effect of a cocoa diet on PP as well as LPL and IEL.

Cocoa Intake and Lymphocyte Composition in Small Intestine and Colon

The attenuation of serum or intestinal immunoglobulin synthesis may be the result of multitude pathways, but the reduction of mucosal IgA observed after cocoa dietary intervention may possibly involve specific mechanisms located at the intestinal site.

The rat intake of 10% cocoa for 4 weeks modified the composition of lymphocytes in the PP and in the intraepithelial compartment whereas no modifications were seen in LPL (33) (**Table 2**). With regard to PP, cocoa-enriched diets were able to reduce the proportion of TCRαβ$^+$ T cells and to increase the proportion of B lymphocytes and TCRγδ$^+$ cells (30, 33), results that agree with changes detected in the mesenteric lymph nodes (30, 31). Analyzing in depth TCRαβ$^+$ cells in the intestine, the cocoa diet decreased the proportion of Th cells and increased that of natural killer T cells (NKT). In addition, after cocoa intake, PP also had higher proportions of CD4$^+$CD25$^+$ cells, CD4$^+$CD103$^+$ cells, CD8$^+$CD103$^+$ cells, and CD4$^+$CD62L$^+$ cells. Apart from the influence of cocoa intake on PP composition, the intraepithelial compartment was also affected by this diet. In IEL from the small intestine of rats fed cocoa, there was a higher percentage of TCRγδ$^+$ cells (both CD8αα$^+$ and CD8αβ$^+$) and NK cells (33).

In summary, in the GALT, the lower production of intestinal antibodies was accompanied by a relative increase in B cell numbers and a relative decrease in TCRαβ$^+$ or Th cell numbers in the inductive sites (mesenteric lymph nodes and PP). These results suggest that the antibody synthesis in B cells might be depleted by a lower stimulation from Th cells and/or a higher regulatory effect induced by cells, such as TCRγδ$^+$, NK, NKT, CD4$^+$CD25$^+$, CD4$^+$CD103$^+$, CD8$^+$CD103$^+$, and CD4$^+$CD62L$^+$, which is in agreement with the role of some of these cells in the regulation of the antibody synthesis (25, 40, 60). In whatever way the activation and differentiation of intestinal B cells was attenuated, a depletion of the high-capacity IgA-secretory cells was produced as reported when they were counted by Enzyme-Linked ImmunoSpot in PP (30) or by an immunofluorescence analysis in the small intestine lamina propria (33). These results agree with a lower IgA gene expression in PP and small intestine seen after 4 and 7 weeks of cocoa intake (19, 33).

Effects of Cocoa Diet on T Cell-Dependent Intestinal Immune Function

The gene expression of molecules involved in the intestinal immune response can shed some light on the mechanisms induced by cocoa on the regulation of the intestinal immune system. In this context, the mRNA levels of IgA, transforming growth factor (TGF) β1, IL-6, CD40, C–C chemokine receptor (CCR) 9, retinoic acid receptor (RAR) α, and RARβ have been reported in GALT tissues, such as mesenteric lymph nodes, PP, and small intestine after 3 or 7 weeks of a cocoa diet (19, 20).

CD40 is involved in the interaction between B and Th cells to begin the antibody immune response (61), and cocoa intake did not modify the expression of this molecule in any of the tissues considered (19, 20) suggesting that cocoa had no influence in this phase of the antibody synthesis. The main pathway that brings differentiation of B cells into IgA-secreting cells takes place in PP or mesenteric lymph nodes (62) and depends on cytokines, such as TGF-β1 and IL-6, among others (63). The 10% cocoa diet significantly decreased the TGF-β1 expression in the small intestine after 3 and 4 weeks (20, 33), although no changes were found after 7 weeks of nutritional intervention (19) (**Figure 2**). On the contrary, the longest nutritional intervention, but not the shortest one, was able to downregulate the IL-6 synthesis in mesenteric lymph nodes (19, 20). Therefore, the effect on these two cytokines, TGF-β1 and IL-6, involved in the S-IgA secretion at different periods (64), might be partly responsible for the downregulatory effect of cocoa. Neither TGF-β1 nor IgA gene expressions were downregulated by the 5% cocoa diet (20), which also caused a reduction in intestinal S-IgA, indicating that additional mechanisms may be interfering in the intestinal S-IgA content.

The next stage occurs when the activated B cells leave the inductor sites (PP and mesenteric lymph nodes) and home to the effector sites (i.e., lamina propria), where the differentiation

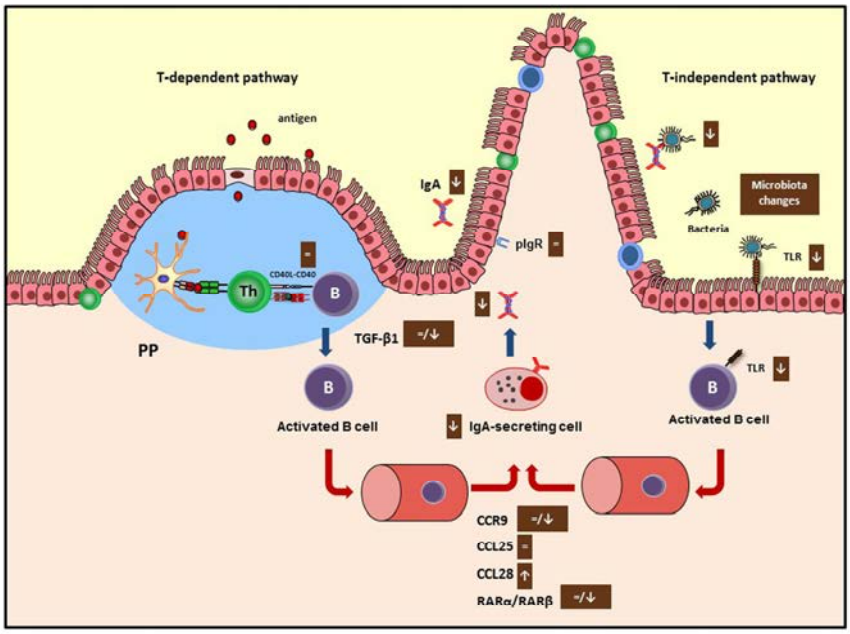

FIGURE 2 | Summary of the mechanisms involved by cocoa diet on intestinal immune function in both T-dependent and T-independent pathways. Arrows indicate increases or decreases, equals sign means no changes. Ig, immunoglobulin; PP, Peyer's patches; pIgR, polymeric immunoglobulin receptor; TLR, toll-like receptor; CCR, C–C chemokine receptor.

into IgA plasma cells takes place (65). Intestinal homing is regulated, among others, by chemokine-mediated interactions including the chemokine receptor CCR9, which binds to CCL25 and the CCL28 chemokines in the intestine (66). While 3 weeks of diet did not modify the expression of the CCR9 receptor nor the CCL25 but did increase the expression of CCL28 in the small intestine (20), 7 weeks of cocoa diet resulted in a downregulation of CCR9 and CCL28 gene expression in the same compartment (19) (**Figure 2**). In addition, retinoic acid produced by intestinal dendritic cells also plays a key role in gut homing (66) through the interaction with nuclear RAR (67). The gene expression of both RARα and RARβ was not modified in the intestinal tissue of rats fed cocoa for 3 weeks (20) but both decreased after 7 weeks (19) (**Figure 2**). Overall, these results could indicate that after being fed a cocoa diet over a long time there was an impairment of the arrival of IgA-secreting cells to the intestine because of the lack of gut-homing receptors observed. However, they do not explain the early decrease in S-IgA that was observed.

Finally, delivering IgA into the intestinal lumen depends on the transmembrane epithelial protein polymeric immunoglobulin receptor (pIgR) (68). This receptor was not modified by the cocoa intake (20), thus indicating that cocoa-induced S-IgA reduction did not occur as a consequence of a decreased transport across the epithelium.

Effects of Cocoa Diet on T Cell-Independent Intestinal Immune Function

Apart from these IgA-secreting mechanisms that depend on T-cell activation, IgA $^{+}$ B cells can be alternatively generated in a T-cell-independent manner involving toll-like receptor (TLR) signaling.

The gene and protein expression of other TLR has been modified both in the inductive sites of GALT (PP and mesenteric lymph nodes) and the effector sites (intestinal wall) after cocoa intake (19, 20, 33). In this context, among other components present in the cocoa, flavonoids have been suggested as dietary factors able to modulate TLR-mediated signaling pathways (69). TLR pathways can be modulated by flavonoids at different levels, and there are evidences of several flavonoids interfering at gene/protein expression level, in subsequent activation pathways (i.e., MyD88, TRIF), and even downstream-associated signal transduction cascades (i.e., MAPK) (69). In this sense, alternative mechanisms in TLR regulation by cocoa flavonoids have been also suggested such as the direct modulation of their intracellular negative regulators (i.e., IRAK, TOLLIP, etc.). To date, *in vitro* studies demonstrate the upregulation of IRAK-M by procyanidin dimer B2 (70), similarly to the effect described by other flavonoids like epigallocatechin-galate in TOLLIP expression (70). Anyway, the synergistic action of cocoa on all these TLR-activating signaling could also contribute to the attenuation of S-IgA synthesis.

Cocoa intake for 4 weeks reduced the proportion of $TLR4^+$ cells in the IEL compartment (33) which agrees with a decrease of TLR4 mRNA in small intestine observed in previous studies (**Figure 2**). Nonetheless, higher TLR4 gene expression was found in PP (19). The TLR4 is the receptor of bacterial endotoxin lipopolysaccharide, and its signaling has implications for IgA production (71), becoming another pathway to attenuate intestinal S-IgA synthesis.

Toll-like receptors are expressed preferentially in tissues that are in constant contact with microorganisms (72, 73), and changes in the TLR expression induced by flavonoids could reflect changes in the intestinal microbiota and/or in its relation with intestinal immune cells (69, 74). Accordingly, several studies have shown that cocoa (58, 75, 76), cocoa flavonoids (58, 77), or cocoa fiber (78) induce changes in gut microbiota composition. Moreover, a lower proportion of IgA-coated bacteria have been observed after cocoa intake (79).

In summary, it could not be discarded that the influence of cocoa on GALT was partially mediated by its effect on the intestinal microbiota, which can lead to differential TLR activation and, therefore, may also influence the lowering IgA effect of cocoa.

Recently, an analysis of an untargeted ^{1}H NMR spectroscopy-based metabolomic approach in 24-h urine samples have been carried out in order to correlate urine cocoa metabolites with cocoa effects on immunity and the gut microbiota (80). The results of this analysis demonstrate that cocoa intake, besides affecting microbiota composition, also alters the host and bacterial metabolism concerning energy and amino acid pathways leading to a particular metabolic signature that correlates with the S-IgA lowering effect of cocoa. Accordingly, a different pattern of intestinal and serum short-chain fatty acids, with increasing amounts of butyric acid, has been reported (78).

Finally, and in order to have a broader view of the molecules involved in the intestinal immune response modulated by cocoa, the changes in colonic gene expression by a microarray analysis after a cocoa nutritional intervention has been carried out (81). This study shows that a cocoa diet downregulated an extensive number of genes, many of them involved in the biological processes related to the immune system and inflammation. Specifically, the most downregulated gene after cocoa intake was tachykinin 4 (81), described as the promoter of B lineage cells (82), which could explain the attenuating effect of cocoa on antibody synthesis, despite the fact that the proportion of B cells did not decrease but, on the contrary, increased in some lymphoid tissues. Moreover, other genes involved in pathways related to the mast cell-mediated immunity, its activation, and its degranulation were downregulated (81), pointing out the possible role of cocoa in inducing tolerance in allergic processes as observed in some studies next reported.

Cocoa Intake Also Influences Another Mucosal Lymphoid Tissue

The mucosal immune system is interconnected (59). Due to cocoa's influence on the intestinal immune system, it became of interest to know whether this effect was also extended to other mucosal compartments, such as the salivary glands. The IgA and IgM content in the salivary glands (submaxillary and parotid salivary glands) was quantified after a 10% cocoa intake in rats for 3 weeks. The cocoa diet induced a decrease in the IgA and IgM content in both glands (56). This attenuating effect was associated with a drastic reduction in the IgA gene expression together with a lower expression of some molecules involved in the maturation and differentiation of B cells, such as IL-6 and TGF-β1 (56), as previously observed in the small intestinal samples (19, 20). However, in agreement with what was detected at intestinal level, no changes were detected in pIgR gene expression in the salivary glands. Therefore, in conclusion, this study shows that cocoa intake not only has an influence on the gut intestinal compartment and the systemic immunity but also on other mucosal sites in rats.

EFFECT OF COCOA DIET ON ANTIBODY-MEDIATED DISEASES

Due to the attenuating properties of cocoa on immunoglobulin levels after cocoa intake in rats, it was of interest to test its impact on diseases in which antibodies play a harmful effect. Therefore, this nutritional intervention was tested on animal models of arthritis and allergy.

Effect of Cocoa Diet on Experimental Arthritis

Rheumatoid arthritis is a symmetric, polyarticular, systemic, and autoimmune inflammatory disease in which multiple factors, including genetic, immune, and environmental ones are involved (83). Diet components such as n-3 fatty acids, vitamins D and K, and antioxidants are protective compounds against rheumatoid arthritis (84). In this context, diets containing 5 or 10% cocoa were tested on adjuvant arthritis, a model of rheumatoid arthritis widely used for the screening of anti-inflammatory drugs (85). In this animal model, cocoa diet decreased the synthesis of antibodies against the pathology inducer (**Table 3**) and was also able to decrease the proportion of Th cells in both blood and regional lymphoid tissues (86). This latter effect is important because, as anti-CD4 therapy has been shown to prevent or ameliorate adjuvant arthritis (87, 88), the cocoa-induced decrease in Th cells could be beneficial to the arthritic process. Moreover, a 10% cocoa diet avoided the Th/Tc imbalance and the reduction of the proportion of NKT cells produced by the disease (86). However, the effect of cocoa on hind-paw inflammation was very poor (86), which did not agree with the protective effect of other flavonoids in a similar inflammatory model when given by oral (quercetin) or by intraperitoneal routes (quercetin, rutin, hesperidin, and morin) (89–91). Nevertheless, a cocoa extract inhibited mice ear edema (92) and acute paw edema in rat (93, 94). Moreover, cocoa flavonoids such as epicatechin, catechin, and procyanidin B2, among others, are able to attenuate the synthesis of inflammatory mediators, such as tumor necrosis factor (TNF)-α, monocyte chemoattractant protein-1, IL-6, and IL-8 (95–100).

The influence of a 10% cocoa diet was also analyzed in collagen-induced arthritis, another model of arthritis. This inflammatory model requires T- and B-cell responses to autologous

TABLE 3 | Summary of the effects of cocoa diet in specific antibodies in rat models of arthritis and allergy.

Model	Strain	Cocoa dose	Results	Reference
Adjuvant arthritis	Wistar	5% in the food	↓Specific antibodies	(86)
		10% in the food	↓Specific antibodies	
Collagen-induced arthritis	Louvain	5–10% in the food	=Specific IgG1 ↓Specific IgG2a, IgG2b, IgG2c	(32)
Allergy induced by intraperitoneal route	Brown Norway	10% in the food	↓Specific IgG1, IgG2a =Specific IgG2b ↓Specific IgE	(22)
Food allergy induced by intraperitoneal and oral routes	Brown Norway	10% in the food	↓ specific IgG1, IgG2a =Specific IgG2b ↓Specific IgE	(101)
Oral sensitization	Lewis	10% in the food	↓Specific IgG1, IgG2b ↓Specific IgM	(31)

Ig, immunoglobulin.
Arrows indicate increases or decreases, equals sign means no changes.

collagen (102). B cells from animals with collagen-induced arthritis produce a strong specific immune response against triple helical epitopes of collagen type II (103). Anti-collagen autoantibodies bind to the joint cartilage, activate the complement cascade, and mediate the inflammatory attack on the joints, thus contributing to the disease development (104). Susceptible Louvain rats were fed with a 10% cocoa diet for 2 weeks before arthritis induction and during the latency period (2 weeks after induction), and thereafter with a 5% cocoa diet until the end of the study (an additional 2 weeks). In this case, the cocoa-enriched diet was able to reduce the synthesis of specific antibodies against type II collagen, differentially according to their isotype (**Table 3**), decrease the Th lymphocyte proportion in regional lymph nodes, and reduce the release of inflammatory mediators from peritoneal macrophages. However, these immunomodulatory effects were not enough to reduce the hind-paw swelling in arthritic animals (32). It must be taken into account that the decrease in anti-collagen antibody concentration in that rat strain was only observed at the end of the study, and it was in a lesser extent and more slowly than that expected and observed in healthy rats as shown before. In a similar context, other authors reported the beneficial effect of isolated flavonoids in improving the paw swelling in animals in long-term studies (105–107). Otherwise a nutritional intervention with the flavonoid genistein had no success (108).

Effect of Cocoa Diet on Hypersensitivity Animal Models

Cocoa on Allergy Models

The effect of the consumption of a 10% cocoa diet over 4 weeks was studied in a model of allergy induced by an intraperitoneal (i.p.) injection of ovalbumin (OVA) and toxin of *Bordetella pertussis* in alum in young Brown Norway rats (22). The cocoa diet reduced the levels of anti-OVA IgG1 and IgG2a antibodies (**Table 3**), i.e., immunoglobulins related to Th2-immune response in rats, as previously mentioned. In addition, cocoa consumption decreased the serum concentrations of total and specific IgE (**Table 3**), which is the main immunoglobulin involved in allergic reactions. These results agree with studies performed in animal models of allergy treated with polyphenols, such as baicalein (109), quercetin (110), silibinin (111), sesamin (112), or an extract of *Kalanchoe pinnata* (*Crassulaceae*) containing several flavonoids such as quercetin (113).

To analyze the mechanisms involved in such action, cytokine secretion was quantified in mesenteric lymph nodes. Contrary to what was expected, cocoa diet increased the release of IL-4, a Th2 cytokine, and decreased that of IL-10, a cytokine related to immune-regulatory responses (22). In addition, cocoa intake induced a lower secretion of TNF-α, which has been described as a contributor to the development of Th2-mediated allergic inflammation by means of promoting the homing of Th2 cells to the site of allergic inflammation. These effects of IL-10 and TNF-α agree with those reported by other flavonoids in allergic conditions (113–115).

The influence of cocoa on the GALT makes it particularly interesting to test the effect of this nutritional intervention on a food allergy process. A model of food allergy using OVA as allergen was carried out in Brown Norway rats, combining an i.p. and oral administration of the allergen. The quantification of serum anti-OVA IgG1, IgG2a, and IgE antibodies revealed that the synthesis of these antibodies was completely prevented by the cocoa diet (101) (**Table 3**). In this study, a product that was richer in cocoa flavonoids was included, but it was not able to totally reproduce the same effects as the conventional cocoa-enriched diet. Therefore, it seems that cocoa flavonoids are only partially responsible for cocoa's anti-allergy properties.

In addition, after anaphylactic shock, the increase of the serum mast cell protease II was partially prevented in the allergic group fed a cocoa diet (101). Nevertheless, other markers of anaphylaxis were not modified by the cocoa intake (body temperature and motor activity), suggesting that its modifications were not enough to prevent the food allergy reaction induced (101).

In order to shed light on cocoa's anti-allergy properties, the expression of some small intestinal genes were quantified (101). The food allergy induction increased the IgA gene expression, an effect that was prevented by a cocoa diet. Moreover, the allergic animals fed a cocoa diet also had lower mRNA levels of high-affinity IgE receptors (FcεRI), mast cell protease-II, and TGF-β1 than reference animals, molecules which could be involved in the protective effect of cocoa on food allergy. Accordingly, the inhibitory effects of flavonoids on the FcεRI surface molecule or gene expression *in vitro* were described (116, 117), and the genetic analysis of colon from rats fed cocoa assessed by microarray analysis showed the downregulation of genes involved in pathways related to mast cell activation and degranulation (81). The cytokine production of food-allergic animals was also determined in mesenteric lymph nodes and spleen (101). In these tissues, the food allergy induction increased the secretion of Th2-cytokines, such as IL-4, IL-5, and IL-13. However, the cocoa diet prevented an increase in IL-5 and IL-13 in lymph node cells and that of IL-4 and IL-13 in splenocytes.

In conclusion, in models of Th-2 immune response stimulation, the intake of cocoa prevents the secretion of typical Th2-cytokines, the synthesis of IgE involved in mast cell degranulation, and also downregulates the IgE receptors in mast cells and intestinal mast cell activation, which are the cells responsible for the most allergy symptoms. However, such effects were not able to totally prevent anaphylactic shock.

Cocoa on an Oral Sensitization Model

Although cocoa intake prevented the allergic sensitization in a model of food allergy induced by i.p. and oral allergen administration (101), it remained to find out what happened when the sensitization with the allergen was produced using only the oral route. Therefore, a 10% cocoa-enriched diet was given to 3-week-old Lewis rats submitted to an oral sensitization model induced by the oral administration of OVA together with the cholera toxin (CT) as adjuvant (118). The oral administration of OVA/CT, three times per week and for 3 weeks, was able to break down oral tolerance and induce the synthesis of specific antibodies after 4 weeks from the beginning of the sensitization protocol. Although this model did not induce detectable specific IgE synthesis, Th2-immune response related antibodies were produced (118) (**Table 3**). Feeding 10% cocoa from the beginning of the study and throughout 4 weeks attenuated the development of specific antibodies in sensitized rats fed the cocoa diet (31). In particular, the 10% cocoa diet prevented the production of anti-OVA IgG1, IgG2b, and IgM in agreement with the effect of cocoa in a food allergy model in Brown Norway rats (101).

In addition, although the IgA concentrations were not increased in this rat oral sensitization model, in contrast to other models using the same adjuvant (63, 119), the cocoa diet decreased the total IgA in both serum and intestinal compartments. As stated in previous sections, a cocoa diet influences the proliferation, differentiation, and gut homing of IgA $^{+}$ B cells (19, 31, 33), thus inducing a lower presence of these cells in the intestinal lamina propia (33) and, consequently, reducing the intestinal IgA development in line with what was reported in many studies (20, 79, 101). Additionally, the changes produced by the cocoa diet in both inductive and effector lymphoid tissues (see Cocoa Intake and Lymphocyte Composition in Small Intestine and Colon) might be responsible for the prevention of the oral sensitization. It is worth noting that the cocoa diet increased the proportion of TCRγδ^{+} and NK cells in three intestinal compartments (mesenteric lymph nodes, PP, and IEL), suggesting their role in the tolerogenic process. In line with this, unripe apple polyphenols induced an increase in the proportion of TCRγδ^{+} IEL in association with the inhibition of the development of an oral sensitization model (25), and it was also reported that the reduction of TCRγδ^{+} cells by the anti-TCRγβ antibody favors an oral sensitization in mice (120). Furthermore, NK cells could have regulatory functions contributing to the avoidance of sensitization in line with the reported prevention of allergic disease (121, 122).

Other changes induced by a cocoa diet could contribute to its tolerogenic effect (31, 33). Such changes include a reduced proportion of Th cells in mesenteric lymph nodes, PP in IEL, an increase in the percentage of CD103^{+} cells, a reduction of CD62L^{+} cells, and an increase in the percentage of CD25^{+} cells in PP. Cocoa intake also modulated the gene expression of several molecules both in mesenteric lymph nodes and in the small intestine (31, 33). In particular, cocoa consumption was associated with an increase in the gene expression of CD11c—a dendritic cell marker (123)—in mesenteric lymph nodes, whereas the mRNA levels of CD11c and CD11b were reduced in small intestinal samples; cocoa also upregulated the expression of OX40L in mesenteric lymph nodes (31)—mainly expressed on antigen-presenting cells (124). In this sense, the interaction of OX40–OX40L regulates cytokine production from T cells, antigen-presenting cells, NK cells, NKT cells, and cytokine receptor signaling (125). Additionally, cocoa decreased the gene expression of IL-1β—a potent pro-inflammatory cytokine (126)—in mesenteric lymph nodes, although no modifications were seen in the production of Th1 (IFN-γ and TNF-α), Th2 (IL-4), or Treg (IL-10) cytokines.

Overall, a cocoa intake, by means of its influence on the intestinal immune system, is able to avoid the sensitization to oral allergens, thus contributing to the downregulation of this hypersensitivity reaction.

Cocoa on an Atopic Dermatitis Model

Recently the role of a cocoa extract on atopic dermatitis has been published (127). The cocoa extract decreased the IgE levels induced by a *Dermatophagoides farinae* extract together with a reduction of atopic dermatitis symptoms. Particularly, the cocoa decreased the severity of the skin lesions, the loss of skin hydration and suppressed the infiltration of eosinophils and mast cells into the skin lesions. Moreover, an extract containing 0.25% cocoa downregulated IL-4 mRNA levels on the skin tissues, whereas an extract containing 1% cocoa decreased IL-5 gene expression at this level.

CONCLUSION

In this review, we summarize the effect of a cocoa diet on the immune system of rats, particularly in the antibody response, both in systemic and mucosal (intestinal and extraintestinal) compartments. The analyses of cells involved in such responses, as well as molecules, such as cytokines and receptors, demonstrate that the effects of a cocoa diet are exerted at multiple sites: in the antigenic presentation, in the cytokines produced by effector Th cells, and in the intestinal homing of activated cells. Eventually, these actions will reduce the synthesis of most antibody isotypes, in particular Th2-associated antibodies as IgE. The relative decrease of Th lymphocytes associated with an increase in TCRγδ^{+} cells and NK cells detected in most lymphoid tissues studied suggest the involvement of these cells in the regulatory role of cocoa. The regulatory potential of cocoa can be very beneficial in those diseases that involve hypersensitivity, such as allergy and autoimmune diseases. Nevertheless, although no signs of immunodeficiency were observed in the described studies, it must be considered that the attenuation of antibodies can be harmful when antibodies are needed to counteract a pathogenic antigen, such as infections, and to induce

antibody-dependent cytotoxicity, phagocytosis, and complement activation. Although further research must characterize the particular cocoa components responsible for such effects, and nutritional studies in humans need to be carried out, cocoa has potential as a nutraceutical agent in some hypersensitivity status.

AUTHOR CONTRIBUTIONS

MC-B, MM-C, MA-G, and AF were responsible for the manuscript preparation. MM-C and MA-G contributed to the manuscript draft. MC-B mainly wrote the manuscript. FP-C and MC contributed to its critical revision.

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Objectives

Over the recent decades, the prevalence of food allergy has increased and new potential allergens have appeared. This has given rise to the importance of having an optimal animal model of oral food sensitization for studying food allergy's pathophysiology, treatment and prevention.

Nowadays, the relationship between food and health is acknowledged. In this context, all those foods having an extra benefit apart from their nutritional role are referred to as 'functional foods'. Otherwise, when these dietary compounds are formulated and taken with a specific dosage they are termed 'nutraceutical', a combination of the words nutrition and pharmaceutical. Specifically in our research area, the study of the influence of natural entities on the immune system has gained attention and this has originated the science of Immunonutrition.

As shown in the last part of the Introduction, previous studies developed in my research group have shown the impact of cocoa on the intestinal and systemic immune system. In particular, a 10% cocoa diet is able to modify the composition and functionality of lymphoid tissues, and the synthesis of intestinal and systemic antibodies in rats. These actions prompted us to consider the potential of cocoa on those immune system alterations associated with hypersensitivity. On the other hand, in order to determine the role of cocoa polyphenols and cocoa fibre in cocoa's immunoregulatory action, several studies were carried out, and they led to conclude that these compounds were only partially responsible for such effects. Therefore, other cocoa compounds may contribute to cocoa's effects on the immune system and this also aroused our interest.

Based on this background, the hypothesis that supports the current thesis is that cocoa intake could impact the intestinal immune response as well as the intestinal microbiota composition thus contributing to the induction of oral tolerance. In addition, we hypothesized that theobromine, the main methylxanthine found in cocoa, could play a role in the cocoa's immune effects.

In consequence, the main goal of the current thesis was **to study in depth the immunomodulatory properties of cocoa, in particular to ascertain the effect and possible mechanisms induced by a cocoa diet on a rat oral sensitization model and also to determine which compound/s is/are responsible for such effect.**

The specific objectives that would together achieve this main goal were as follows:

1. **To develop an oral sensitization model in rats.**

The results obtained from the first objective are published in the following article:

Article 1: Camps-Bossacoma M, Abril-Gil M, Franch À, Pérez-Cano FJ, Castell M. Induction of an oral sensitization model in rats. *Clinical Immunology, Endocrine & Metabolic Drugs*. 2014;1(2):89–101.

2. **To establish the effect and possible mechanisms induced by a 10% cocoa diet on the rat oral sensitization model.**

The results obtained from the second objective are published in the following articles:

Article 2: Camps-Bossacoma M, Abril-Gil M, Saldaña-Ruiz S, Franch À, Pérez-Cano FJ, Castell M. Cocoa diet prevents antibody synthesis and modifies lymph node composition and functionality in a rat oral sensitization model. *Nutrients*. 2016;8(4):242.

Article 3: Camps-Bossacoma M, Pérez-Cano FJ, Franch À, Castell M. Effect of a cocoa diet on the small intestine and gut-associated lymphoid tissue composition in a rat oral sensitization model. *The Journal of Nutritional Biochemistry*. 2017;42:182–193.

Article 4: Camps-Bossacoma M, Pérez-Cano FJ, Franch À, Castell M. Gut microbiota in a rat oral sensitization model: effect of a cocoa-enriched diet. *Oxidative Medicine and Cellular Longevity*. 2017;2017:7417505.

3. **To identify the cocoa bioactive compounds responsible for cocoa's immunomodulatory effect and to ascertain the role of a non-cocoa flavonoid on the immune system.**

Although previous studies have suggested that cocoa flavonoids were not the main constituents responsible for its effects on the immune system, other reported studies showed the immunoregulatory properties of some flavonoids. For this reason, the effect of hesperidin, a non-cocoa flavonoid, on the immune response was established. This resulted in the following published paper:

Article 5: Camps-Bossacoma M, Pérez-Cano FJ, Franch À, Castell M. Influence of hesperidin on the systemic and intestinal rat immune response. *Nutrients*. 2017;9(6):580.

Finally, considering that cocoa is a rich source of theobromine, the involvement of this methylxanthine on cocoa's immune effects was investigated, and this study led to the following papers:

Article 6: Martín-Peláez S, Camps-Bossacoma M, Massot-Cladera M, Rigo-Adrover M, Franch À, Pérez-Cano FJ, Castell M. Effect of cocoa's theobromine on intestinal microbiota of rats. *Molecular Nutrition & Food research. In press.*

Article 7: Camps-Bossacoma M, Pérez-Cano FJ, Franch À, Castell M. Theobromine is responsible for the effects of a cocoa diet on the antibody immune response in rats. *Submitted.*

Resultats

ARTICLE 1

"Induction of an oral sensitization model in rats"

Mariona Camps-Bossacoma, Mar Abril-Gil, Àngels Franch, Francisco J. Pérez-Cano, Margarida Castell

Clinical Immunology, Endocrine & Metabolic Drug

2014, volum 1(2), pàgines 89-101

Indexat a: Chemical Abstracts Service (CAS), J-Gate, CNKI Scholar, Suweco CZ i EBSCO.

Els resultats del present article han estat presentats als congressos següents:

- VII Congrés de la Societat Catalana d'Immunologia, Barcelona, novembre de 2013. Camps-Bossacoma M, Abril-Gil M, Vicente F, Massot-Cladera M, Comalada, M, Pérez-Cano FJ, Franch A, Castell M. "Inducció d'un model d'immunització oral amb ovoalbúmina en rates Lewis".

- 7th International Immunonutrition Workshop, Brindisi, Itàlia, maig de 2014. Camps-Bossacoma M, Abril-Gil M, Vicente F, Massot-Cladera M, Comalada M, Pérez-Cano FJ, Franch A, Castell M. "Development of a food sensitization model in female Lewis rats".

- VIII Congrés de la Societat Catalana d'Immunologia, Barcelona, novembre de 2014. Camps-Bossacoma M, Abril-Gil M, Rigo-Adrover M, Franch A, Pérez-Cano FJ, Castell M. "Changes in intestinal gene expression induced by oral sensitization with ovalbumin in Lewis rats".

Resum ARTICLE 1

Objectiu: L'objectiu d'aquest primer article va consistir en desenvolupar un model de sensibilització oral en rata.

Material i mètodes: Rates de les soques Brown Norway o Lewis de diferents edats (de 3 a 8 setmanes) i gènere, varen rebre diverses dosis d'ovoalbúmina (OVA) per via oral (1-100 mg/animal), amb o sense l'adjuvant toxina colèrica (TC) (30 µg/animal), seguint diverses freqüències d'administració (1-7 vegades per setmana) i durant diferents setmanes (3 o 6 setmanes). L'últim dia d'estudi es va intentar induir un xoc anafilàctic administrant elevades quantitats d'al·lergen. Com a indicador de la sensibilització es va determinar la concentració d'anticossos anti-OVA mitjançant la tècnica d'ELISA.

Resultats: L'administració d'OVA en rates Brown Norway durant 6 setmanes va comportar la sensibilització del 27% dels animals. L'administració de l'antigen junt amb TC en rates d'aquesta mateixa soca de 3 setmanes d'edat, va aconseguir el desenvolupament d'anticossos anti-OVA, però, també, va ser letal per alguns animals (25%). Pel que fa a la soca Lewis, l'administració de 100 mg d'OVA i TC en rates de 8 setmanes d'edat va provocar la sensibilització del 55% dels animals. Finalment, l'administració de diferents dosis, amb o sense adjuvant, seguint diferents freqüències d'administració (1-3) en rates mascles i femelles Lewis de 3 setmanes d'edat va permetre concloure que l'administració de 50 mg d'OVA i 30 µg de TC en rates femelles, 3 vegades la setmana i durant 3 setmanes comporta la sensibilització del 100% dels animals. Aquesta sensibilització va provocar la síntesi d'IgG1, IgG2a i IgG2c específiques però no d'IgE anti-OVA.

Conclusions: El desenvolupament d'una sensibilització oral en rata depèn de l'ús de la TC com adjuvant i, en rates Lewis, de la dosi i freqüència d'administració de l'al·lergen i de l'edat i el gènere dels animals. En concret, l'administració de 50 mg d'OVA junt amb 30 µg de TC, 3 vegades per setmana i durant 3 setmanes a rates femelles Lewis permet evitar l'aparició de tolerància oral i indueix el desenvolupament d'anticossos específics.

Clinical Immunology, Endocrine & Metabolic Drugs, 2014, *1*, 89-101

Induction of An Oral Sensitization Model in Rats

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Abstract: Animal models of food allergy can provide advances in the aetiology, pathogene-sis and therapeutic treatment of this disease. The aim of the present study was to set up a model of oral sensitization in rats by testing seven different procedures and using ovalbumin (OVA) as the antigen. Brown Norway and Lewis rats aged between three and eight weeks old received, orally, different doses of the OVA together with or without cholera toxin (CT) under several dosages. Isotypes of anti-OVA antibodies were determined weekly in serum and also in intesti-nal samples (faecal or gut lavage). In the most successful procedure, mesenteric lymph nodes (MLN) and in-testine samples were excised to determine cytokines and gene expression related to allergic response, respec-tively. In both Brown Norway and Lewis rats, the quantification of specific antibodies revealed that CT was required to increase oral sensitization. In addition, OVA dosage, age and sex of animals are important in the oral sensitization of Lewis rats. Serum anti-OVA antibodies mainly belonged to IgG1 and IgG2a isotypes, and intestinal anti-OVA immune response was almost undetectable. Specific IgE was not found in the serum of any of the studied procedures, and cytokines from MLN did not clearly demonstrate a Th2 immune response. The gene expression study in small intestine samples of sensitized rats suggested changes in IL-10 and TLR-5 mRNA expression. In summary, oral sensitization in rat could be achieved in both young Brown Norway and young female Lewis rats.

Keywords: Adjuvant, allergy, animal model, Brown Norway, cholera toxin, Lewis, oral tolerance, ovalbumin.

INTRODUCTION

Allergic disease is an inappropriate immune response or hypersensitivity reaction to an innocuous antigen – mainly found in the air or in food – called allergen, which principally occurs in people with a genetic predisposition [1]. Food allergy is a Western problem, higher in children than in adults and with increasing prevalence [2]. Most food allergies described in Europe are to cow's milk, egg, nuts and seafood [3].

Physiologically, an immune response develops when the subject comes into contact with a specific antigen and their antigen-presenting cells (APC) present antigenic epitopes to specific T helper (Th) lymphocytes. Consequently, these cells become activated, proliferate, and then differentiate into effector cells. In consequence, the subject becomes sensitized. Effector cells can mainly be Th1 and Th2 lymphocytes according to the cytokine pattern they secrete. In general, Th1 cells produce gamma-interferon (IFN-γ), tumour necrosis factor-beta (TNF-β) and interleukin (IL) 2, and they are highly effective against intracellular pathogens. In response to extracellular pathogens, such as in allergic immune response, Th2 cells produce IL-4, IL-5, IL-6 and IL-10, among others, and promote the production of immunoglobulin (Ig) E, the isotype that principally mediates the allergic reaction [4]. IgE produced by the activation of this response binds to mast cells and, with further expositions to

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the same allergen, granule content and cytokines, among other mediators, are secreted from activated mast cells. Released molecules are responsible for the clinical symptoms of the allergic disease involving integumentary, respiratory, gastrointestinal or cardiovascular system disorders. The most common symptoms usually appear between minutes and 2 h after contacting with the allergen [5].

In healthy conditions, active immune tolerance is produced in response to antigens encountered via the gastrointestinal tract. In such circumstances, oral exposure results in the generation of food antigen-specific T lymphocytes with regulatory activity (Treg) [6]. These cells suppress the generation of antigen-specific effector lymphocyte responses, including pro-allergic Th2 responses and IgE production. However, in some conditions, oral tolerance is broken and allergy to food components appears [7]. Food allergies are hypersensitivity reactions after food ingestion, contact or inhalation, consisting of the specific suppression of Th immune tolerance to a food protein [6]. According to Sampson [8], food allergies are classified, depending on the route of sensitization of the allergen, as class 1 or class 2. In class 1, food allergy individuals are sensitized through the gastrointestinal tract, whereas in class 2, food allergy appears through a cross-reaction with inhaled antigens. Most of class 1 allergens are water-soluble glycoproteins with 10-70 kDa in size, stable to heat and enzymatic digestion. However, class 2 allergens often are labile to heat and digestion [8].

Nowadays, the prevalence of food allergy is increasing in Western countries [2] and new allergens are appearing due to new food biotechnology [7]. For this reason, animal models of food allergy can provide advances in understanding the aetiology and pathogenesis of such disease, as well as developing therapeutic strategies such as anti-allergic drugs or hyposensitization protocols [9]. Several rodent models of food allergy exist but some of them apply sensitization routes other than the oral route [10-12]. However, the best animal model should be achieved by using oral ingestion of the allergen, which supposes the breakdown of oral tolerance mechanisms [13]. Although some models in mice have been developed in this regard [14, 15], there are few rat models available, and some of them need a long sensitization period and also expensive rat strains such as the Brown Norway [16]. In this study, we aimed to set up a model of oral sensitization in rat, which allowed the screening of new treatments in food allergy. Here we introduce seven different options to induce food allergy and we examine the most successful one in more depth. Ovalbumin (OVA) was used as the oral allergen because it is generally accepted as a class 1 allergen [17].

MATERIALS AND METHODS

Chemicals

Ovalbumin (OVA, grade V), cholera toxin (CT), gelatine, peroxidase-conjugated extravidin, o-phenylenediamine (OPD), 30% hydrogen peroxide (H_2O_2), RPMI-1640 medium, foetal bovine serum (FBS), penicillin-streptomicin, glutamine and 3-amino-9-ethyl-carbazole (AEC) were provided by Sigma-Aldrich (Madrid, Spain). Biotin-conjugated anti-rat IgM, IgA, IgG, IgG1, IgG2a, IgG2b and IgG2c monoclonal antibodies (mAb) and purified anti-rat IgE were purchased from BD Biosciences (Madrid, Spain). Peroxidase-conjugated and unconjugated goat anti-rat IgA and the corresponding standard were from Bethyl Laboratories (Montgomery, TX, USA). Peroxidase-conjugated anti-rat Ig was provided by DakoCytomation (Glostrup, Denmark). 2-β-mercaptoethanol was provided from Merck (Darmstadt, Germany) and RNAlater® was purchased from Ambion (Applied Biosystems, Austin, TX). Ketamine was provided by Merial Laboratories S.A. (Barcelona, Spain) and xylazine by Bayer A.G. (Leverkusen, Germany).

Induction of Oral Sensitization Models

Brown Norway (BN) and Lewis rats aged between three and eight weeks old and obtained from Janvier (Saint Berthevin Cedex, France) were used. They were maintained in the Faculty of Pharmacy animals' facilities under conditions of controlled temperature and humidity in a 12:12 h light:dark cycle. Experimental procedures were reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref. 5988).

After six days of acclimatization, one of the procedures of oral sensitization summarized in Table **1** was carried out. In each experiment, a group of rats matched in sex and age and receiving the same volume of vehicle as those OVA-treated

Table 1. Procedures of oral sensitization applied.

Procedure	Rat strain	Age at the beginning (week old)	Sex	OVA (mg) /rat	CT (μg) /rat	Dosage	
						Frequency (times/week)	Duration (weeks)
p1	BN	5	Female	1	-	7	6
p2	BN	3	Female	30	30	2	3
			Male				
p3	BN	3	Female	100	30	2	3
			Male				
p4	Lewis	8-9	Female	100	30	2	3
p5	Lewis	3-4	Female	50	30	3	3
			Male				
p6	Lewis	3-4	Female	100	30	3	3
			Male				
p7	Lewis	3-4	Female	100	30	1	3
			Male				

BN: Brown Norway; CT: cholera toxin; OVA: ovalbumin.

animals was included. In addition, in those protocols using CT we added a group of animals that received OVA with the same dosage without any adjuvant. To quantify anti-OVA antibodies, faecal and sera samples were collected weekly from the beginning of the study. In some procedures, mesenteric lymph node (MLN) and intestine samples were obtained at the end of the study.

Induction of An Anaphylactic Shock

A few days after finishing the different OVA sensitization procedures in Table **1**, animals were deprived of food overnight and received oral OVA (100 mg/mL for BN rats and 200 mg/mL for Lewis rats) to induce an anaphylactic shock. To assess this response, animal behaviour was observed and rectal temperature was determined (digital thermometer, Oakton®, Vernon Hills, USA).

Quantification of Serum anti-OVA Antibodies

Indirect enzyme-linked immunosorbent assay (ELISA) was performed to determine specific serum anti-OVA antibody concentrations as in previous studies [18]. Briefly, to quantify specific total antibodies and those belonging to the IgG1, IgG2a, IgG2b, IgG2c, IgA and IgM isotypes, ELISA plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with an OVA solution (10 μg/mL), and blocked with gelatin (0.5%). After washing, appropriately diluted serum samples were incubated.

To determine total antibodies, peroxidase-conjugated anti-rat Ig and an OPD-H_2O_2 solution were added. To detect specific anti-OVA Ig isotypes, biotin-conjugated anti-rat IgG1, IgG2a, IgG2b, IgG2c, IgA or IgM mAb were used. Thereafter, peroxidase-conjugated extravidin and an OPD-H_2O_2 solution were added. A pool of sera from rats intraperitoneally immunized with OVA plus alum was used as positive control [18].

To determine OVA-specific IgE, an ELISA was applied as used in previous studies [19]. Briefly, anti-rat IgE antibody was coated to the solid phase. Later, specific anti-OVA IgE were detected with biotin-coated OVA. For OVA biotinylation, a Roche (Mannheim, Germany) kit was used according to the manufacturer's instructions. In this ELISA, a pool of sera from BN rats intraperitoneally immunized with OVA plus alum and *Bordetella pertussis* toxin was used as positive control [19].

Absorbance was measured in a microplate photometer (Labsystems Multiskan, Helsinki, Finland) at 492 nm. A positive response in antibodies was

considered when values of absorbance were higher than the mean of those obtained from non-immunized rats plus two standard deviations. In some positive groups, the antibody isotype concentration was calculated by comparison with the arbitrary units (A.U.) assigned to a pool of OVA-immunized rat sera, according to adequate dilution of the tested samples.

Quantification of Intestinal IgA and Intestinal anti-OVA Antibodies

To quantify intestinal IgA or intestinal anti-OVA antibodies, lavages from small intestine or faecal homogenates were used.

In procedure 1, intestinal anti-OVA IgA was determined in gut lavage obtained from the small intestine as performed in previous studies [20]. Briefly, the distal part of the small intestine was opened lengthwise, and cut into 5 mm pieces, weighed and incubated in a shaker at 37°C. The suspension obtained was centrifuged, and supernatants (gut lavage) were stored at −20°C until analysis.

In procedures 5-7, faecal homogenates were used as in previous studies [21]. Briefly, faecal samples were weighed, added to phosphate buffered saline (PBS, 20 mg/mL) and homogenized using a Polytron® (Kinematica, Lucerne, Switzerland). Homogenates were then centrifuged and supernatants were frozen at −20°C until analysis.

Total IgA was quantified using a sandwich ELISA as described in previous studies [22]. Specific anti-OVA antibodies were quantified using the same ELISA as for serum samples.

Mesenteric Lymph Node Lymphocyte Isolation

In procedures 1 and 5, at the end of the study, rats were anaesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). MLN were collected and lymphocytes were isolated as in previous studies [20] by passing the tissue through a sterile mesh (40 µm, Fisher Scientific). Then cells were centrifuged (600 *g*, 5 min, 4°C) and resuspended in RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL streptomycin-penicillin, 2 mM L-glutamine and 0.05 mM 2-β-mercaptoethanol. Cell counting and viability were determined with a Countess™ Automated Cell Counter (Invitrogen, Life Technologies).

Quantification of Antibody-secreting Cells in MLN by ELISPOT

In rats from procedure 1 and the corresponding reference group, anti-OVA IgA- and IgG-secreting cells (SC) and total IgA-SC were enumerated using the ELISPOT technique as in previous studies [18]. Briefly, a 96-well nitrocellulose plate (Multiscreen MAHAN 4510, Millipore, Eschborn, Germany) was coated with OVA or anti-IgA diluted in PBS (20 µg/mL) in sterile conditions (overnight, 4°C). After blocking, freshly isolated MLN cells were plated at serial dilutions ($1x10^5$, $0.5x10^5$, $0.25x10^5$ and $0.125x10^5$ cells/well) and were incubated. Biotin-conjugated anti-rat IgA or IgG mAb were incubated. After washing, extravidin-peroxidase conjugate was added. Spots (each one corresponding to one SC) were visualized by the addition of the substrate solution (AEC plus H_2O_2 in 0.1 M acetate solution). The reaction was stopped by rinsing with tap water. Spots were counted automatically by the computer-assisted ELISPOT image analysis (ELISPOT reader system, AID, Strassberg, Germany).

Quantification of Cytokine Production from MLN

From animals sensitized by procedure 5 and the corresponding reference group, MLN cells were cultured at $3x10^6$ cells/mL and stimulated with OVA (10 µg/mL) for 96 h. Supernatants were then collected to assess cytokines production. Concentrations of IL-1β, IL-4, IL-6, IL-10, IFN-γ and TNF-α were quantified using the Bioplex sandwich immunoassay (Bio-Rad, Madrid, Spain). Specific capture beads coded with distinct colours were bound to the analyte of interest. Then it was possible to reveal the specific concentration through different detection antibodies conjugated with phycoerythrin and analysed by the Luminex MAGPIX Analyzer (Bio-Rad). The quantitative determinations were performed with the following limits of detection: 8.26 pg/mL for IL-1β, 0.84 pg/mL for IL-4; 35.36 pg/mL, for IL-6; 32.31 pg/mL for IL-10; 8.51 pg/mL for IFN-γ; and 1.55 pg/mL for TNF-α.

Assessment of RNA Gene Expression by Real Time PCR

From animals sensitized by procedure 5 and the corresponding reference group, the small intestine

was carefully rinsed with cold saline solution in order to remove faecal content. A middle piece from the tissue samples was kept in RNA later® until analysis.

To isolate the RNA, the portion of small intestine in RNA later® was transferred into lysing matrix tubes (MP Biomedicals, Illkirch, France) and homogenized in a FastPrep®-24 (MP Biomedicals) for 30 s. RNA was isolated by the RNAeasy® mini kit (Qiagen, Madrid, Spain) following manufacturer's recommendations. RNA was quantified with a NanoDrop spectrophotometer and NanoDrop IVD-1000 v.3.1.2 software (NanoDrop Technologies, Wilmington, DE). The Agilent 2100 Bioanalyzer with the RNA 6000 LabChip 1 kit (Agilent Technologies, Madrid, Spain) was used to provide an RNA integrity number (RIN) for each sample.

Two µg of total RNA were reverse-transcribed in a thermal cycler PTC-100 using random hexamers and TaqMan® Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany) [23].

Specific PCR TaqMan® primers and probes (AB) were used to measure *Iga* (331943, made to order), *Tgfb1* (Rn00572010_m1, Inventoried (I)), *Cldn2* (Rn02063575, I), *Muc2* (Rn01498195_m1, I), *Foxp3* (Rn01525092_m1, I), *Il4* (Rn01456866_m1, I), *Tlr4* (Rn00569848_m1, I), *Tlr5* (Rn04219239_s1, I), *Il10* (Rn00563409_m1, I), *Il6* (Rn01410330_m1, I), *IL17a* (Rn01757168_m1, I), *Ocldn* (Rn00580064_m1, I). Quantitative PCR assays were performed in duplicate for each sample using the ABI Prism 7900 HT quantitative real-time PCR system (AB). Quantification of the genes of interest was normalized to the housekeeping gene *Gusb* (Rn00566655_m1, I). The amount of target mRNA relative to the endogenous control expression and relative to values from the reference group was calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described [24], where Ct is the cycle number at which the fluorescence signal of the PCR product crosses an arbitrary threshold set within the exponential phase of the PCR and $\Delta\Delta Ct = [(Ct_{\text{target (unknown sample)}} - Ct_{\text{endogenous control (unknown sample)}})] - [(Ct_{\text{target (reference sample)}} - Ct_{\text{endogenous control (reference sample)}})]$. Results are expressed as the percentage of these values for each experimental group compared with its reference group, which represents 100% gene expression.

Statistical Analysis

The software package SPSS 18.0 (PASW Statistics, SPSS, Chicago, IL) was used for statistical analysis. The parametric t-Student test was used to assess significance. Differences were considered statistically significant for p-values < 0.05.

RESULTS

Rat Survival, Body Weight and Food Intake

Neither the sensitization procedure nor the anaphylactic shock induction produced mortality in animals belonging to procedures 1, 4, 5, 6 and 7. In the case of the youngest BN animals (belonging to procedures 2 and 3), 25% rat death was registered.

Body weight and food intake were not modified by oral OVA administration in any of the tested procedures in comparison with the corresponding reference group (data not shown).

Serum Total anti-OVA Antibodies

The results for detectable serum total anti-OVA antibodies are summarized in Table **2**. The seropositive percentage of oral sensitized animals varied according to the procedure. Procedure 1, consisting of giving orally OVA without any adjuvant every day to female BN rats, was successful in 27% of the animals. Procedures 2 and 3, also both in BN rats, induced an anti-OVA immune response in all the surviving animals independently of the dose and rat sex. Regarding the Lewis strain, about one half of the adult rats (procedure 4) developed anti-OVA antibodies, whereas the immune response of younger rats varied according to the dosage used and the rat sex. The lowest dose of OVA tested produced the most successful response in female Lewis rats (procedure 5). The dosage used in procedures 6 and 7 caused a fewer number of sensitized animals with a low anti-OVA antibody titre (Fig. **1**).

Sensitized rats in procedure 1 showed levels of specific antibodies 35 days after the beginning of oral OVA administration which were not increased one week later (Fig. **1**). In the remaining procedures, *i.e.*, in protocols 2-7, OVA antibodies' presence was then investigated earlier and the results showed that they had already been produced 14 days after the beginning of oral OVA administration but

Table 2. Incidence of development of serum anti-OVA antibodies according to sensitization procedure.

Rat strain	Procedure	Sex	Incidence of sensitization Number of anti-OVA seropositive rats/number of total rat (percentage of incidence)
BN	p1	Female	4/15 (27%)
	p2	Female	3/3 (100%)
		Male	3/3 (100%)
	p3	Female	3/3 (100%)
		Male	3/3 (100%)
Lewis	p4	Female	5/9 (55%)
	p5	Female	4/4 (100%)
		Male	0/4 (0%)
	p6	Female	1/4 (25%)
		Male	0/4 (0%)
	p7	Female	0/4 (0%)
		Male	1/4 (25%)

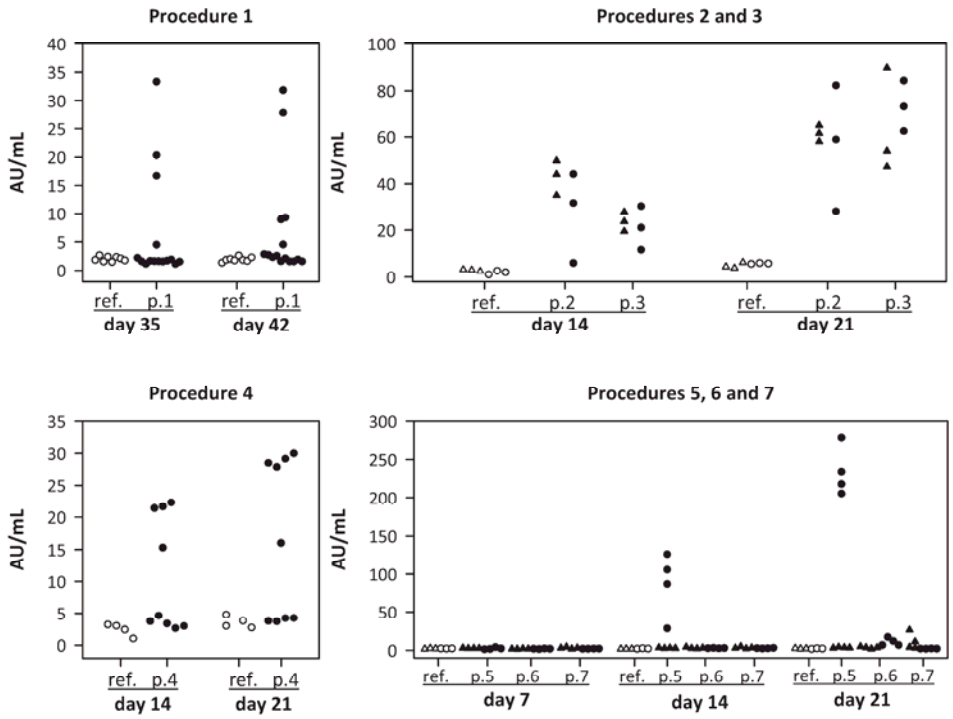

Fig. (1). Time course of total anti-OVA antibodies appearance in every procedure tested. Each dot represents an animal; reference animals are summarized in white dots whereas those in black correspond to animals receiving OVA sensitization. Circles represent females and triangles represent males.

not before (procedure 5-7). Moreover, specific anti-OVA Ig levels were increased the following week (Fig. **1**).

Serum anti-OVA IgE Antibodies and Changes after Anaphylactic Shock

There were no positive results regarding specific IgE production in any of the assessed procedures. Even in those rats in which anti-OVA antibodies were developed, specific IgE could not be detected. After the oral challenge had been performed to induce an anaphylactic shock, no changes in body temperature were observed (data not shown), keeping the rectal temperature around 38°C for up to 30 min post-oral challenge.

Serum anti-OVA Antibodies Belonging to IgG Subclasses, IgM and IgA

Anti-OVA antibody isotypes from seropositive rats were studied in depth at day 21 in the procedures with the highest incidence of sensitization both for BN (procedures 2 and 3) and Lewis (procedures 4 and 5) strains (Fig. **2**). In BN rats, higher amounts of anti-OVA IgG1 and IgG2a than specific IgG2b were detected (Figs. **2A** and **2B**). In the Lewis strain, rats from procedure 4 had similar levels of anti-OVA IgG1 and IgG2a (Fig. **2C**) whereas, in procedure 5, most of the anti-OVA antibodies belonged to the IgG2a isotype (Fig. **2D**). In this last group, specific IgM and IgA were also determined, and the results showed positive levels for both isotypes, although specific IgA titres were very low (data not shown).

IgA- and anti-OVA-secreting-cells from MLN

Total IgA-SC, anti-OVA IgA-SC and IgG-SC in MLN were enumerated at the end of the study in procedure 1, performed in BN strain (Table **3**). Regarding the number of total IgA-SC, variations were not observed with respect to the corresponding reference group. More interestingly, the number of anti-OVA IgA-SC increased significantly in

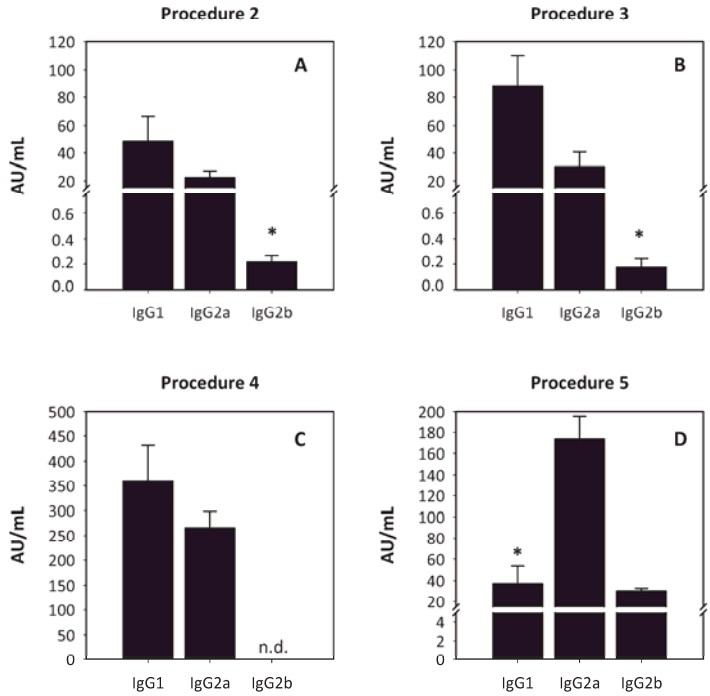

Fig. (2). Isotypes of anti-OVA antibodies in BN and Lewis seropositive animals at day 21. **A**: procedure 2; **B**: procedure 3; **C**: procedure 4; **D**: procedure 5. Results are expressed as mean + S.E.M; N= 3-6, depending on the sensitization procedure. *n.d.* means not determined. * $p<0.05$ with respect to the highest isotype.

the sensitized animals. No significant proportion of anti-OVA IgG-SC with respect to reference animals was found.

Table 3. Number of total IgA-SC, anti-OVA IgA-SC and anti-OVA-IgG SC in MLN from rats of procedure 1.

	Reference animals (SC/10^6 cells)	Procedure 1 (SC/10^6 cells)
Total IgA-SC	1009.78±127.49 (6)	1004.27±110.64 (15)
anti-OVA IgA-SC	42.22±6.73 (6)	78.57*±10.16 (15)
anti-OVA IgG-SC	28.00±3.94 (6)	34.93±5.28 (15)

Results are expressed as mean ± S.E.M, number of animals in brackets. * p<0.01 with respect to the reference animals.

Intestinal IgA and Intestinal anti-OVA Antibodies

Intestinal IgA was determined in faecal homogenates in Lewis rats from procedures 5, 6 and 7 and the corresponding reference group during the study. In this last group, the concentration of IgA was about 3.16±0.8 µg/mL on day 21. In rats from procedures 5-7 there were no significant changes on that same day in the values of faecal IgA, which were about 4.5±1.0 µg/mL in procedure 5, about 4.6±0.7 µg/mL in procedure 6, and about 2.7±0.7 µg/mL in procedure 7.

In addition, intestinal anti-OVA antibodies were tested in gut lavages from rats of procedure 1 and in faecal homogenates belonging to animals from procedures 5-7. In all samples, values were undetectable.

Cytokines Produced by Mesenteric Lymph Node Lymphocytes

In rats from the best sensitization procedure (procedure 5), isolated MLN lymphocytes were incubated with OVA for 96 h and then released cytokines were determined (Fig. **3**). Although no statistically significant differences were achieved, the production of IL-4, IL-6 and IL-10 tended to be increased in the sensitized animals, whereas lower or similar levels of IFN-γ, IL-1 and TNF-α compared to those of the reference group were found.

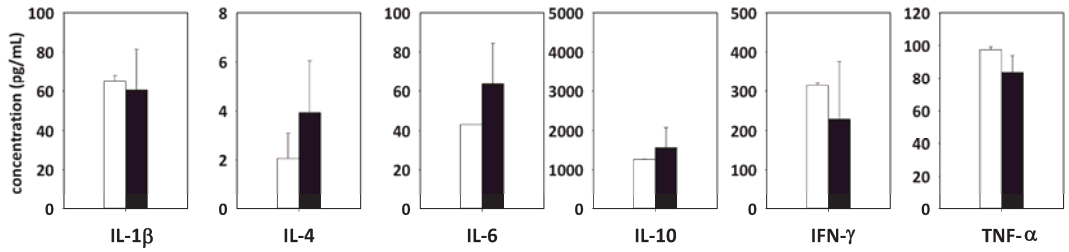

Fig. (3). Cytokines released from MLN of reference rats and those animals sensitized by procedure 5. Results are expressed as mean + S.E.M; N=4.

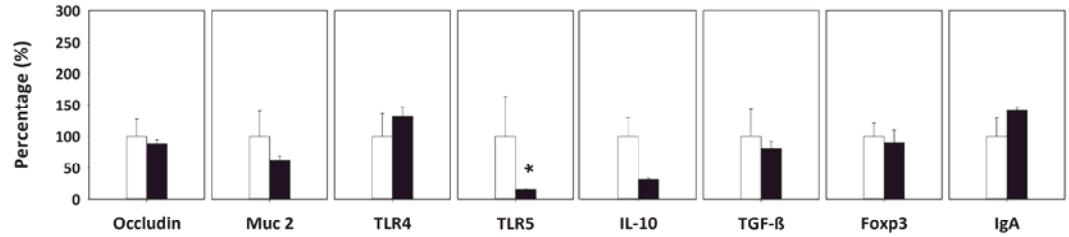

Fig. (4). Relative percentage of mRNA gene expression in small intestine samples from reference rats (white bars) and those animals sensitized by procedure 5 (black bars). Results are expressed as mean + S.E.M; N=4.

Gene Expression in Small Intestine

In sensitized animals from the procedure with the best results in the above variables (procedure 5) and its corresponding reference group, the gene expression of several molecules involved in the gastrointestinal barrier (claudin 2, occludin and mucin 2), in the molecules cross-talk (TLR-4 and TLR-5), in an inflammatory response (IL-6 and IL-17α), as well as in the regulation of immune response (TGF-β, IL-10 and Foxp3) and in the proper immune response (IgA, IL-4) was determined (Fig. **4**). No significant amounts of mRNA of claudin 2, IL-6, IL-17α and IL-4 were expressed in the small intestine wall from either the reference or sensitized animals. Neither occludin nor mucin 2 mRNA expressions varied significantly in orally sensitized animals. TLR-4 showed little change but TLR-5 underwent a down-regulation in sensitized rats. With regard to regulatory molecules, the group of orally sensitized rats tended to down-regulate IL-10 mRNA levels whereas no changes were present in TGF-β and Foxp3 mRNA, or in IgA mRNA.

DISCUSSION

In the present study we tried to set up a rat model of oral sensitization based on models described previously and including some modifications. First of all, we tested a model consisting of given orally ovalbumin (OVA) without any adjuvant every day for six weeks in an allergic-prone rat strain such as BN rats. This long procedure only provided us 27% of sensitized animals and no anti-OVA IgE was detected in any animal, which does not agree with previous results of this model [16]. In addition, intestinal specific antibodies could not be detected although significant numbers of anti-OVA IgA-SC cells were present in MLN. After obtaining these results, we then used cholera toxin (CT) as an adjuvant, which has been described as a molecule capable of breaking oral tolerance [25]. Then we tested the effect in BN rats of two different doses of OVA (30 mg or 100 mg) in addition to CT, twice a week for three weeks, following a model described in mice [14]. In this case, we started in younger BN rats, only 21 days old. Both doses caused the death of some animals regardless of the amount of OVA, probably because they were too young to be administered with such an amount of protein. However, those animals that survived produced significant amounts of anti-OVA antibodies, belonging to IgG1 ≅ Ig2a >>> Ig2b isotypes. No specific IgE or anaphylactic shock changes were similarly detected.

As IgE response expected in the BN rats was not found, and looking for an optimal animal model, we moved to the Lewis strain which is also a good immune responder strain and cheaper than BN rats. We first tested female adult rats (aged 8-9 weeks), which received the 100 mg of OVA in addition to CT. In these rats, 55% of animals produced anti-OVA IgG, belonging to IgG1 and IgG2a isotypes. As in BN rats, specific IgE was undetectable, and no changes were observed after anaphylactic shock induction. For that reason, we finally moved to younger Lewis rats (3-4 weeks old), testing then both male and female genders and also two different doses of OVA (50 mg and 100 mg) plus CT. In this case, the dosage consisted of three times per week, following reports in mice showing that two administrations of allergen with CT per week produced an earlier sensitization than once per week [14]. In these new tested procedures, because the rats were some days older than the BN rats and also because the Lewis strain has a higher body weight than that of age-matched BN rats [26], 100% of rats survived the sensitization protocol. Testing the development of antibodies, we observed that only female rats that received the lowest amount of OVA, belonging to procedure 5, produced anti-OVA antibodies. These results agree with those found in BN rats, although in this last strain there was an immune response even at a higher dose of OVA and in both sexes. In sensitized rats, specific antibodies mainly belonged to the IgG2a isotype, a few anti-OVA antibodies were IgA and none was IgE. In young Lewis rats receiving CT, no changes in total intestinal IgA were observed.

Overall, the first conclusion that can be drawn from the results presented here is that CT must be used as an adjuvant to break oral tolerance to OVA. The effect of such an adjuvant in immune response seems to be the up-regulation of proteins involved in the interaction between APC and naïve Th cell, skewing the immune response to a Th2 profile, with elevated production of IL-4 and increased synthesis of antigen-specific IgE and IgG2a in mice [27]. In addition, CT seems to induce the activation of particular dendritic cells, promoting the subsets involved in Th2 responses [9, 28]. A second conclusion from the current re-

sults is the difficulty of achieving a specific IgE response and then an anaphylactic shock in rats. In our experience, a significant IgE response could be obtained when an intraperitoneal immunization was carried out including *Bordetella pertussis* toxin [19]. However, when this toxin was omitted and only gastrointestinal sensitization was carried out the oral sensitization did not easily produce specific IgE. These results agree with those reported by Dearman and co-workers [29], although other authors achieved an IgE response only using oral sensitization [30]. In any case, specific IgG isotypes can shed light on the Th1 and Th2 polarization of immune response and show a positive oral sensitization. In rat, the correspondence of IgG isotypes with Th1/Th2 responses does not follow the same pattern as in mice. In rat, responses in IgG1 and IgG2a are attributed to Th2 activation whereas IgG2b and IgG2c levels correspond to Th1 response [30-32]. Therefore, the results obtained in animals from the successful procedures 2, 3 and 5 suggest that the immune response against OVA induced by gastrointestinal sensitization was mainly from the Th2 pattern. In sensitized animals from procedure 5, we looked for a Th2 immune response by quantifying the cytokines released from MLN cells. However, the results did not show a clear Th2 skewed response which does not agree with other studies reporting an increase in IL-4 [33]. Nevertheless, an increase in Th1 and Th2 cytokines after immunization with CT has been reported [34]. Further studies using a higher number of animals and different incubation times of MLN cells with the stimulus will confirm the current results. In addition, the isolation of lymphocytes from other compartments of gut-associated lymphoid tissue such as Peyer's patches would allow a more suitable indicator of intestinal immune response to be obtained [35]. Moreover, the study of the splenic response should be considered quite important in assessing how the oral sensitization affects at the systemic level [35].

A third conclusion from the present study can be drawn considering the anti-OVA antibody time course and the type of sample studied. Anti-OVA antibodies were detected 14 days after the beginning of oral sensitization and increased thereafter, as observed in both BN (procedures 2-3) and Lewis (procedures 4-5) rats. In addition, intestinal anti-OVA antibodies were not found in either of these strains. In animals from the procedures 5-7, CT administration did not produce changes in the levels of total intestinal IgA. These results do not agree with others that report the effect of CT potentiating the intestinal IgA synthesis [27, 36]. However, the lack of an increase in intestinal IgA could be beneficial to avoid the antigen (OVA) exclusion in the intestinal epithelia, thus favouring the immune presentation and allergy development. In this sense, intestinal IgA has been shown to have a role in oral allergy treatment [12] and mice lacking intestinal IgA develop a more severe oral hypersensitivity [37].

On the other hand, a fourth conclusion to be drawn from the current results is that both the age of the animal at the beginning of the procedure and the amount of the allergen are important to achieve an oral immune sensitization. Breaking oral tolerance is a difficult issue in which both the age of the animal [6] and the amount of protein administered play a delicate role. Our results agree with those showing that the lowest dose applied produced the highest response [38], however it should be taken into account that repeated exposure to lower doses can induce oral tolerance [39]. With regard to the age of the animal, it has been demonstrated that oral OVA administration in suckling rats prevents allergic sensitization [40] and the immune response in aged mice is different from that of younger ones [41], although rats have a more pronounced tolerogenic response than mice.

The fifth conclusion from the results obtained comparing procedures 2-3 with 5-7 is that the BN strain is better than the Lewis strain for obtaining an allergic reaction. Both male and female BN rats developed antibodies whereas only female Lewis rats succeeded in doing so. These results are in line with the higher serum immunoglobulins levels in BN rats than in Wistar serum [42] and with the different immune response observed between BN and Wistar rats after food allergy induction [32]. Regarding the gender of rats, it has been evidenced that sex differences exist in clinical and preclinical immune disturbances, although the reasons for this are not well understood [43]. In general, the severity of immune diseases such as allergy, is higher in female than male rodents, although it depends on the strain and immunization route [43, 44].

Finally, the gene expression of some molecules in the small intestine of sensitized Lewis rats by procedure 5 should shed some light about the

pathogenesis of oral sensitization in these rats but the low number of sensitized animals prevented clear results being obtained. The gene expression of both occludin, a protein in the tight junctions between epithelial cells, and mucin 2, a major component in the intestinal mucus, suggested no defects in the gastrointestinal barrier. On the other hand, the mRNA expression of TLR-4, the lipopolysaccharide receptor increasing in an intestinal inflammation [45] was only slightly increased, and mRNA of IL-6 and IL-17α, both mediators of intestinal inflammation [46], were undetectable, indicating the lack of an inflammatory response at least at the end of our study. Gene expression of TLR-5, an agonist flagellin associated with immunomodulatory functions, was down-regulated. This result suggests a change in the TLR expression on the epithelial cells and also in the dendritic cells present in the intestinal lamina propria, and agrees with the role of TLR-5 in the prevention of murine intestinal allergy [47]. In the case of IL-10 gene expression, regulatory cytokine produced by Treg cells to keep oral tolerance [6, 48], was partially down-regulated in orally sensitized animals, suggesting the breakdown of oral tolerance. These results agree with those from Sun *et al.* [49] in a model of soybean allergy in mice. However, TGF-β, another tolerogenic cytokine [6], did not change and neither did Foxp3, the forkhead box protein 3 in Treg cells, suggesting that the number of these cells could be kept even in these conditions. IL-4 mRNA was not detected, which could be due to the fact that mRNA was obtained from the entire intestine wall and not from intestinal lymphocytes, in consequence its low expression being diluted by epithelial and connective cells. Therefore, the isolation of lymphocytes from the intestinal lamina propria could allow to obtain more significant results, like those reported [33].

CONCLUSION

In conclusion, oral sensitization in rat could be achieved in both Brown Norway and Lewis strains, although an IgE immune response cannot be achieved at least in the current conditions. The development of oral sensitization depends on the use of cholera toxin as the adjuvant and, in Lewis rats, it also depends on the dosage, age and sex of the animals. Female Lewis rats and a specific dosage of the allergen are required to reach oral sensitization.

LIST OF ABBREVIATIONS

APC	=	Antigen-presenting cells
A.U	=	Arbitrary units
BN	=	Brown Norway
BSA	=	Albumin from bovine serum
CT	=	Cholera toxin
ELISA	=	Enzyme-linked immunoabsorbent assay
FBS	=	Foetal bovine serum
IFN-γ	=	Gamma-interferon
Ig	=	Immunoglobuline
MLN	=	Mesenteric lymph nodes
OPD	=	O-phenylenediamine
OVA	=	Ovalbumin
PBS	=	Phosphate-buffered saline
PCR	=	Polimerase chain reaction
TGF-β	=	Transforming growth factor-beta
Th	=	T helper cells
TNF-β	=	Tumour necrosis factor-beta
TLR	=	Toll Like Receptors
Treg	=	T regulatory cells

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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ARTICLE 2

"Cocoa diet prevents antibody synthesis and modifies lymph node composition and functionality in a rat oral sensitization model"

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Els resultats del present article han estat presentats al congrés següent:

- Food Allergy Summit & Hypersensivity 2016, Londres, juliol de 2016. Camps-Bossacoma M, Franch A, Pérez-Cano FJ, Castell M. "Cocoa as a nutraceutical to avoid preclinical oral sensitization".

Resum ARTICLE 2

Objectiu: Una vegada establert un model de sensibilització oral, el següent objectiu va consistir en determinar l'efecte d'una dieta amb un 10% de cacau sobre la producció d'anticossos i en la composició i funcionalitat de ganglis limfàtics mesentèrics (GLM) en aquest model de sensibilització.

Material i mètodes: Rates Lewis de 3 setmanes d'edat es varen distribuir en quatre grups en funció de la dieta (estàndard o amb cacau) i de si varen rebre la sensibilització oral o no. Els dos grups als que se'ls hi va induir la sensibilització, varen rebre, per via oral, l'al·lergen ovoalbúmina (OVA) i l'adjuvant toxina colèrica, 3 vegades per setmana i durant 3 setmanes. Les rates varen ser alimentades amb una dieta amb un 10% cacau o una dieta estàndard durant les 4 setmanes d'estudi. En mostres obtingudes al llarg de l'estudi es va avaluar la producció d'anticossos anti-OVA per tècniques d'ELISA. Al final de l'estudi, es va quantificar el desenvolupament d'anticossos específics i la concentració sèrica i intestinal d'IgA. A més, es va avaluar la composició de limfòcits dels GLM (citometria de flux), l'expressió gènica de diferents molècules en aquest teixit limfoide (PCR a temps real) i la producció de citocines a l'intestí i en els GLM (Multiplexed Bead-Based Immunoassays, BD Cytometric Bead Array).

Resultats: La sensibilització oral va provocar la síntesi d'anticossos específics anti-OVA dels isotips IgG1, IgG2a, IgG2b i IgM. La dieta rica en cacau va atenuar la producció d'aquests anticossos, principalment d'IgG1, IgG2b i IgM específiques. Aquesta dieta, també va disminuir la concentració sèrica i intestinal d'IgA total. La sensibilització oral no va modificar la composició dels limfòcits de GLM, encara que, la dieta rica en cacau, independentment de la sensibilització, va augmentar la proporció de limfòcits B, de limfòcits TCR$\gamma\delta$+ (per un increment de cèl·lules CD8$\alpha\alpha$+) i de cèl·lules NK i va disminuir el percentatge de limfòcits TCR$\alpha\beta$+ (per una disminució de la proporció de cèl·lules Th). A més, en aquest mateix teixit, el cacau va incrementar la proporció de cèl·lules CD8+CD25+ i CD8+CD103+ i va disminuir la de cèl·lules CD4+CD62L+ i CD8+CD62L+. La sensibilització oral va produir un increment de l'expressió gènica d'OX40L, i la dieta rica en cacau va augmentar l'expressió d'OX40L, CD11c, IL-1β i va reduir la d'IL-17α. A més, en el rentat intestinal, es va detectar un increment d'IL-10 a conseqüència de la sensibilització oral i, també, de la dieta rica en cacau en rates no sensibilitzades.

Conclusions: La dieta amb cacau indueix tolerància en un model de sensibilització oral en rates, tal com es reflecteix en l'atenuació de la síntesi d'anticossos específics. Aquest efecte ve acompanyat de diferents canvis cel·lulars en els ganglis limfàtics mesentèrics, tals com un increment en la proporció de limfòcits TCR$\gamma\delta$+ i de cèl·lules CD8+CD103+, i una disminució del percentatge de cèl·lules CD4+CD62L+ i CD8+CD62L+. Aquests canvis, juntament amb la regulació de diferents gens, podrien contribuir a l'efecte tolerogènic del cacau.

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Cocoa Diet Prevents Antibody Synthesis and Modifies Lymph Node Composition and Functionality in a Rat Oral Sensitization Model

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Abstract: Cocoa powder, a rich source of polyphenols, has shown immunomodulatory properties in both the intestinal and systemic immune compartments of rats. The aim of the current study was to establish the effect of a cocoa diet in a rat oral sensitization model and also to gain insight into the mesenteric lymph nodes (MLN) activities induced by this diet. To achieve this, three-week-old Lewis rats were fed either a standard diet or a diet with 10% cocoa and were orally sensitized with ovalbumin (OVA) and with cholera toxin as a mucosal adjuvant. Specific antibodies were quantified, and lymphocyte composition, gene expression, and cytokine release were established in MLN. The development of anti-OVA antibodies was almost totally prevented in cocoa-fed rats. In addition, this diet increased the proportion of TCRγδ+ and CD103+CD8+ cells and decreased the proportion of CD62L+CD4+ and CD62L+CD8+ cells in MLN, whereas it upregulated the gene expression of OX40L, CD11c, and IL-1β and downregulated the gene expression of IL-17α. In conclusion, the cocoa diet induced tolerance in an oral sensitization model accompanied by changes in MLN that could contribute to this effect, suggesting its potential implication in the prevention of food allergies.

Keywords: cholera toxin; flavonoids; intestinal sensitization; nutraceutic; oral tolerance; ovalbumin; specific antibodies; Tγδ+ cells

1. Introduction

Cocoa powder, derived from *Theobroma cacao* tree seeds, has a mixed composition of over 500 different compounds [1]. It contains macronutrients (carbohydrates, proteins, and lipids, both monounsaturated and saturated fatty acids), fiber (soluble and insoluble), minerals (calcium, cooper, magnesium, potassium), polyphenols (in particular it is rich in flavonoids such as epicatechin, catechin, and procyanidins), and methylxanthines (caffeine and theobromine) [2].

Today, cocoa powder and cocoa products are consumed worldwide [3] and different health benefits have been associated with their consumption [3–5]. Cocoa is a rich source of polyphenols, greater than that of tea and wine [3,6], with a potent antioxidant capacity [2,7] due to its phenolic hydroxyl groups [8]. Most of cocoa's health properties have been attributed to its polyphenol content [3,7] and, in this context, modulation of allergic reactions by several flavonoids has been described [8,9].

Focusing on cocoa and the immune system, previous studies have demonstrated that a 10% cocoa diet has an immunomodulatory effect in the intestinal and systemic immune compartments in rats. Changes in the percentage of B lymphocytes and T cells, including T cell receptor (TCR)

αβ+ cells, TCRγδ+ cells, T helper (Th), and T cytotoxic (Tc) cells in mesenteric lymph nodes (MLN), have been described [10,11]. In addition, cocoa diet influences immune functions by modulating cytokine synthesis in MLN cells [12] and attenuating the development of specific IgE, IgG1, IgG2a, IgG2c, and IgM antibodies after intraperitoneal immunization with ovalbumin [12,13].

Food allergies are abnormal immunological reactions to food proteins that generate a wide variety of immune changes and consequently different clinical symptoms and signs [14,15]. The main site of sensitization to food proteins is the gut-associated lymphoid tissue (GALT) [16], which can be classified into inductive sites (Peyer's patches, isolated lymph nodes, and MLN) and effector sites (lymphocytes in the lamina propria and intestinal epithelium). With regard to unresponsiveness to food antigens, MLN are the primary site for the induction of oral tolerance [17].

Currently, food allergy is becoming a worldwide problem [18]. In particular, its prevalence is increasing in Westernized countries [19]. In this context, oral-sensitized animal models are of interest in order to assess its mechanisms and to evaluate therapeutic and nutritional interventions. Previously, we set up a model of oral sensitization consisting of the oral co-administration of the food allergen (ovalbumin; OVA) plus cholera toxin (CT) [20]. CT is an effective mucosal adjuvant that breaks down oral tolerance to co-administered protein antigens [21], altering some regulatory mechanisms of the intestinal mucosa [22,23], although the exact pathways involved are still unclear.

Different approaches are used to treat or prevent oral sensitizations [24,25] and, in this sense, nutraceutics could have a potential role. Based on this background, the purpose of the present study was to establish the effect of cocoa consumption, with its recognized immunomodulatory activities, in a rat oral sensitization model. Likewise, in an attempt to gain insight into the mechanisms induced by a cocoa diet, the composition and functionality of cells in MLN were assessed. For these purposes, rats were fed with a 10% cocoa diet for four weeks and for the first three weeks were orally sensitized with OVA and CT. Immune responses were established by specific antibody response during the study as well as by MLN characterization at the end of the study.

2. Materials and Methods

2.1. Reagents

Albumin from bovine serum (BSA), albumin from chicken egg white (OVA; grade V), CT, gelatine, peroxidase-conjugated extravidin, *o*-phenylenediamine (OPD), 30% hydrogen peroxide (H_2O_2), fetal bovine serum (FBS), penicillin-streptomycin, glutamine, Folin-Ciocalteu phenol reagent, gallic acid monohydrate, L-asparagine monohydrate, L-arginine, folic acid, HEPES, and nystatin were purchased from Sigma-Aldrich (Madrid, Spain). Biotin-conjugated anti-rat IgG1, IgG2a, IgG2b, IgG2c, IgM, and IgA monoclonal antibodies were obtained from BD Biosciences (Madrid, Spain). Goat anti-rat IgA, its peroxidase-conjugated antibody, and rat IgA standard were provided by Bethyl Laboratories (Montgomery, TX, USA). Peroxidase-conjugated anti-rat Ig was from Dako Cytomation (Glostrup, Denmark). 2-β-mercaptoethanol, Na_3N, and paraformaldehyde were purchased from Merck (Darmstadt, Germany). Anti-rat monoclonal antibodies conjugated to a fluorochrome were provided from BD Biosciences (San Diego, CA, USA). Ketamine was obtained from Merial Laboratories S.A. (Barcelona, Spain) and xylazine from Bayer A.G. (Leverkusen, Germany). Dulbecco's Modified Eagle Medium (DMEM)-GlutaMAX media and gentamicin were obtained from Gibco™ and RNAlater® from Ambion (Thermo Fisher Scientific, Barcelona, Spain). Natural Forastero cocoa was provided by Idilia Foods S.L. (formerly Nutrexpa S.L., Barcelona, Spain) and AIN-93M diet and basal mix by Harlan Teklad (Madison, WI, USA).

2.2. Animals and Diets

Thirty-six female Lewis rats were purchased from Janvier Labs (Saint Berthevin, France) and maintained in polycarbonate pathogen-free cages (three rats per cage) with controlled conditions of temperature and humidity and in a 12:12 h light:dark cycle in the Faculty of Pharmacy's animal facility.

All experimental procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref.5988).

After one week of acclimatization, three-week-old rats were randomly assigned into the following four groups: reference group (RF/R), reference cocoa group (RF/C), sensitized group (OVA/R), and sensitized cocoa group (OVA/C), as detailed in Table 1.

Table 1. Experimental design.

Group	Oral Administration Days 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21	Diet
RF/R (n = 9)	Vehicle (1 mL/rat)	AIN-93M
RF/C (n = 9)	Vehicle (1 mL/rat)	10% cocoa
OVA/R (n = 9)	50 mg OVA + 30 µg CT/rat	AIN-93M
OVA/C (n = 9)	50 mg OVA + 30 µg CT/rat	10% cocoa

The oral sensitization was performed as previously described [20]. Briefly, rats received orally 50 mg of OVA with 30 µg of CT as adjuvant in 1 mL of distilled water, three times per week (Monday, Wednesday, and Friday) for three weeks. RF/R and RF/C groups received 1 mL of vehicle on the same days. During the 28 days of the study, animals were given free access to water and food. AIN-93M formula was used as the standard diet and a cocoa-enriched diet was produced with the addition of 100 g of defatted cocoa powder to 900 g of a basal mix, the resulting composition finally providing an isoenergetic chow. The two experimental diets provided similar amounts of proteins, lipids, and carbohydrates (Table 2).

Table 2. Composition of the diets.

Components	Reference Diet	10% Cocoa Diet	
	AIN-93M (g/kg Diet)	Basal Mix (g/kg Diet)	Cocoa Powder (g/kg Diet)
Carbohydrates	721.9	692.5	16.8
Proteins	140.8	118.2	23.1
Lipids	38.7	27	11.5
Fiber	50	24.5	35.6
Micronutrients	48.6	37.8	6.3
Flavonoids [1]	0	0	4.02
Theobromine	0	0	2.5
Total	1000	1000	

[1] total polyphenol content was determined according to the Folin–Ciocalteu method. The cocoa used in this study contained 40.18 mg/g of total polyphenols (expressed as catechin). Reversed-phase high performance liquid chromatography coupled to a diode array detector revealed that cocoa contained 2.34 mg/g epicatechin and 0.4 mg/g catechin.

2.3. Sample Collection and Processing

Blood samples were collected weekly from the beginning of the study. After centrifugation, serum was obtained and frozen at −20 °C until antibody quantification.

One week after the last oral administration, rats were anaesthetized with ketamine/xylazine (90 mg/kg/10 mg/kg) and exsanguinated. Urine was collected directly from the urinary bladder with the help of a syringe, and the small intestine and MLN were carefully dissected.

In sterile conditions, MLN were passed through a sterile mesh cell strainer (40 µm, Thermo Fisher Scientific) and the resulting cell suspension was centrifuged (538 g, 5 min, 4 °C) and resuspended with RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 IU/mL streptomycin-penicillin, 2 mM L-glutamine, and 0.05 mM 2-β-mercaptoethanol. Cell counting and viability were assessed by Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific). Some isolated lymphocytes from MLN were stained to be analyzed by flow cytometry (explained

below). Other MLN cells were stimulated *in vitro* to promote cytokine release and the remaining cells were kept in RNAlater® until gene expression analysis. MLN cells were stimulated *in vitro* by culturing 3×10^6 cells/mL in DMEM supplemented with 10% heat-inactivated FBS, 36 mg/L L-asparagine monohydrate, 116 mg/L L-arginine, 10 mg/L folic acid, 500 mg/L HEPES, 10 mg/mL gentamicin, 10,000 U/mL nystatin, 100 U/mL streptomycin-penicillin, and 0.05 mM 2-β-mercaptoethanol. At the same time, a specific stimulus was added (OVA, 10 μg/mL) and, after 72 h, supernatants were collected to assess cytokine production.

The proximal part of the small intestine was opened lengthwise, cut into small pieces, weighed, and incubated in a shaker at 37 °C. After centrifugation, supernatants were collected, aliquoted, and stored at −80 °C until cytokine and IgA quantification.

2.4. Determination of Total Polyphenol Content

Total phenolic content was determined according to Folin–Ciocalteu's method. Briefly, 250 μL of Folin–Ciocalteau's reagent and 1.25 mL of 20% Na_2CO_3 solution were added to 500 μL of diluted urine. After 2 h at room temperature, the absorbance was measured at 765 nm. A standard curve prepared with gallic acid was used.

2.5. IgA and Specific Anti-OVA Antibodies

Total serum and intestinal IgA from intestinal lavage were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA), as previously described [26].

Specific anti-OVA antibody (total anti-OVA antibodies and anti-OVA IgG1, IgG2a, IgG2b, IgG2c, IgM, and IgA isotypes) levels were measured by an indirect ELISA. In brief, 96-well polystyrene plates (Nunc Maxisorp®, Wiesbaden, Germany) were coated overnight at room temperature with 10 μg/mL of an OVA solution in carbonate buffer (pH 9.6). The plates were washed and blocked with 0.5% gelatin. Afterwards, appropriately diluted samples and standards were added for 3 h. In order to assess total anti-OVA antibodies, peroxidase-conjugated anti-rat Ig and OPD-H_2O_2 solution were added. To quantify specific anti-OVA Ig isotypes, biotin-conjugated anti-rat IgG1, IgG2a, IgG2b, IgG2c, IgM, or IgA monoclonal antibodies were used and, thereafter, peroxidase-conjugated extrAvidin and an OPD-H_2O_2 solution were added.

Absorbance was measured in a microplate photometer (LabsystemsMultiskan, Helsinki, Finland) at 492 nm and data was interpolated by Ascent v.2.6 software (Thermo Fisher Scientific). The relative anti-OVA antibody concentration was calculated giving the value of 1 to the mean value obtained from samples from the RF/R group tested in the same conditions and, therefore, all values were expressed as an increase of the mean value of RF/R group.

2.6. Immunofluorescence Staining and Flow Cytometry Analysis

Lymphocytes from MLN (5×10^5 cells) were stained using mouse anti-rat monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridininchlorophylla protein (PerCP), or allophycocyanin (APC). The antibodies used were anti-CD4, anti-CD8α, anti-CD8β, anti-TCRαβ, anti-TCRγδ, anti-NKR-P1A, anti-CD62L, anti-CD25, and anti-CD103. Cells were incubated with a mixture of saturating concentrations of antibodies in PBS containing 2% FBS and 0.1% Na_3N, at 4 °C in darkness for 20 min. After washing, cells were fixed with 0.5% p-formaldehyde and stored at 4 °C in darkness until analysis by flow cytometry. A negative control staining using an isotype-matched monoclonal antibody was included in each cell sample. Analyses were performed with a Gallios™ Cytometer (Beckman Coulter, Miami, FL, USA) in the Scientific and Technological Centers of the University of Barcelona (CCiTUB).

2.7. Gene Expression from MLN Lymphocytes

Lymphocytes from MLN were kept in RNAlater® until analysis. On the day of RNA extraction, lavages with PBS were performed to remove RNAlater®. Immediately, cells were homogenized in a

vortex for 2 min. Total RNA was extracted by RNeasy® mini kit (Qiagen, Madrid, Spain) in accordance with the manufacturer's instructions. RNA quantification was performed with a NanoPhotometer (BioNova Scientific, CA, USA) and reverse-transcribed with TaqMan® Reverse Transcription Reagents (Applied Biosystems, Thermo Fisher Scientific) [27]. Real-time PCR assays (ABI Prism 7900 HT, AB) were performed using specific PCR TaqMan® primers (Applied Biosystems): OX40L (Rn00585582_m1, Inventoried (I)), NF-κB (Rn01399572_m1, I), CD11c (Rn01511082_m1), IL-1β (Rn00580432_m1), IL-12 (Rn00584538_m1), IL-17α (Rn01757168_m1, I), and IL-33 (Rn01759835_m1). The expression of HPRT1 (Rn01527840_m1) was used to normalize the quantification of the studied genes. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described [20]. The relative mRNA level was calculated giving the value of 1 to the mean value obtained from samples from the RF/R group tested in the same conditions.

2.8. Cytokine Quantification

Interleukin (IL) 4, IL-10, interferon (IFN) γ, and tumor necrosis factor (TNF) α were quantified by BD™Cytometric Bead Assay Rat Soluble Protein Flex Set (BD Biosciences, Madrid, Spain) as detailed in previous studies [13].

2.9. Statistical Analysis

Data are expressed as means ± standard error. All statistical analyses were performed with IBM Social Sciences Software Program (SPSS, version 22.0, Chicago, IL, USA).

Levene's test was performed to assess variance equality, followed by Kolmogorov–Smirnow to determine its distribution. When the results demonstrated equality of variance and normal distribution, a two-way ANOVA test was performed (oral sensitization and diet). When the interaction between oral sensitization and diet was statistically significant, Bonferroni's *post hoc* test was performed between groups.

Otherwise, when the results had high variance and/or non-normal distribution (food and water intake, anti-OVA antibody concentration, cytokine concentration in MLN cell supernatants), non-parametric tests, such as Kruskal–Wallis and Mann–Whitney U tests were performed. When $p < 0.05$, statistical difference was considered significant.

3. Results

3.1. Food and Water Intake, Flavonoid Absorption and Body Weight

Food and water intake were monitored throughout the study (Table 3). No differences were found among groups (established by Kruskal–Wallis and Mann–Whitney U tests).

Total polyphenol concentration was quantified in urine samples at the end of the study. Rats fed standard diet showed values ranging between 3.16 and 18.6 μg/mL (mean ± standard error, 10.12 ± 1.63). Cocoa-fed animals had concentrations significantly higher ranging between 26.1 and 61.8 μg/mL (35.86 ± 3.24) (diet effect $p = 0.000$ by two-way ANOVA; no significant effect of oral sensitization, $p = 0.079$, or interaction, $p = 0.960$).

Body weight (Table 3) increased progressively during the study (time effect $p = 0.000$ by two-way ANOVA) and oral sensitization did not affect it ($p = 0.873$ by two-way ANOVA). However, the cocoa diet produced a slower growth ($p = 0.000$ by two-way ANOVA). No significant interactions were found between oral sensitization and diet or time, between diet and time, or between oral sensitization, diet, and time.

Table 3. Body weight (g) and food and water intake (g/100 g rat/day) from the four groups over the study. Data represent mean ± standard error (n = 9 for body weight, n = 3 for water and food intake established in each cage). Statistical difference: * means statistical significant difference induced by the diet by two-way ANOVA ($p < 0.001$).

Day	Variable	Group [1]			
		RF/R	RF/C	OVA/R	OVA/C
0	Body weight	59.9 ± 4.27	59.78 ± 4.58	60.86 ± 4.24	58.62 ± 4.16
0–7	Food intake	10.38 ± 2.35	13.46 ± 1.12	9.72 ± 2.50	13.43 ± 0.82
0–7	Water intake	12.06 ± 0.17	23.85 ± 4.03	12.06 ± 1.81	23.04 ± 3.04
7	Body weight	92.7 ± 6.05	82.28 ± 5.30 *	94.00 ± 5.88	80.90 ± 4.80 *
7–14	Food intake	11.65 ± 0.97	13.05 ± 0.51	11.72 ± 1.04	12.77 ± 0.33
7–14	Water intake	9.68 ± 0.90	21.08 ± 3.11	10.68 ± 0.65	22.64 ± 2.10
14	Body weight	129.04 ± 5.96	107.82 ± 5.84 *	129.03 ± 6.06	109.35 ± 4.53 *
14–21	Food intake	9.63 ± 1.03	11.12 ± 0.95	9.07 ± 0.92	11.24 ± 0.12
14–21	Water intake	9.12 ± 0.26	17.26 ± 0.09	9.13 ± 0.38	21.39 ± 0.99
21	Body weight	154.29 ± 3.72	133.80 ± 5.24 *	152.73 ± 5.67	135.55 ± 4.63 *
21–28	Food intake	6.79 ± 1.86	9.74 ± 0.51	6.82 ± 1.99	8.8 ± 1.36
21–28	Water intake	9.11 ± 0.10	14.99 ± 0.53	10.24 ± 0.96	25.45 ± 4.04
28	Body weight	174.13 ± 3.23	153.32 ± 5.67 *	171.61 ± 4.35	150.53 ± 2.57 *

[1] Groups: RF/R (reference group: vehicle and AIN-93M diet); RF/C (reference cocoa group: vehicle and 10% cocoa diet); OVA/R (sensitized group: OVA plus CT and AIN-93M diet); and OVA/C (sensitized cocoa group: OVA plus CT and 10% cocoa diet).

3.2. Immune Response to OVA: Serum Anti-OVA Antibodies

As shown in Figure 1a, specific total anti-OVA antibodies appeared progressively with the oral sensitization process in the OVA/R group, there being a 7.4-, 82.5-, 75.5-, and 424.5-fold increase with respect to the RF/R group at days 7, 14, 21, and 28, respectively. At the end of the study, total anti-OVA antibodies concentration in the OVA/R group was significantly higher than that in the RF/R group (p = 0.038 by Mann–Whitney U test). A total of 78% of animals of the OVA/R group developed antibodies established as mean value of RF/R group plus 2 × SD. On the contrary, levels in the OVA/C group throughout the study were quite similar to those found in the RF/R and RF/C groups (ranging between 1.5- and 3.4-fold increase with respect to the RF/R group) and were significantly lower than those found in the OVA/R group (p = 0.035 by Mann–Whitney U test on day 28).

Isotypes of serum anti-OVA antibodies were determined at the end of the study when results could be analyzed with higher sensitivity (Figure 1b). Although no detectable levels of specific IgG2c and IgA were found, the oral sensitization procedure led to the production of anti-OVA IgG1, IgG2a, IgG2b and IgM in such a way that levels were IgG1 (477.8-fold increase of RF/R group) > IgG2a (292.6-fold increase of RF/R group) >> IgG2b (13.9-fold increase of RF/R group) > IgM (2.0-fold increase of RF/R group). These concentrations were significantly higher than those found in the RF/R group (p = 0.000, p = 0.002, p = 0.000, p = 0.041 for IgG1, IgG2a, IgG2b, and IgM, respectively, according to Mann–Whitney U test).

In comparison with the OVA/R group, the cocoa-enriched diet significantly attenuated the increase of anti-OVA IgG1, IgG2b, and IgM (p = 0.016, p = 0.000, p = 0.000 by Mann–Whitney U test, respectively) in such a way that concentrations ranged between a 0.5-fold increase for IgM and a 17.9-fold increase for IgG1 of the RF/R group. With regard to IgG2a, although the cocoa diet values were more than 10 times lower than those in the OVA/R group, no statistically significant difference was found with respect to this group (p = 0.164 by Mann–Whitney U test).

Anti-OVA antibodies were also analyzed in intestinal lavage but these results were under the limit of detection.

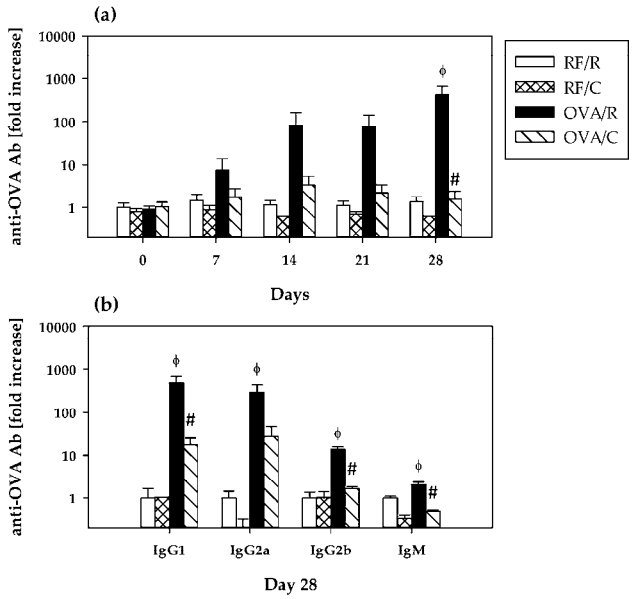

Figure 1. Serum anti-OVA antibodies. (**a**) Total anti-OVA antibody levels throughout the study; (**b**) anti-OVA IgG1, IgG2a, IgG2b, IgM at the end of the study. Values are expressed as mean ± standard error (n = 9). Statistical differences: $^{\Phi}$ $p < 0.05$ compared with RF/R group, and # $p < 0.05$ compared with OVA/R group by Mann–Whitney U test. Groups: RF/R = reference group; RF/C = reference group fed cocoa diet; OVA/R = sensitized group; OVA/C = sensitized group fed cocoa diet.

3.3. Total IgA Antibodies: Serum and Intestinal Concentrations

To assess the influence of oral sensitization on the main intestinal immunoglobulin, serum and intestinal IgA concentrations were quantified at the end of the study (Figure 2). In both cases, oral sensitization did not significantly modify the IgA concentration (p = 0.564 and p = 0.830 for serum and intestinal values, respectively, by two-way ANOVA). However, the 10% cocoa diet produced a significant decrease in the IgA levels (p = 0.000 in both cases by two-way ANOVA), that was more marked in the intestinal compartment. No significant interaction was found between oral sensitization and diet (p = 0.074 and p = 0.525 for serum and intestinal values, respectively, by two-way ANOVA).

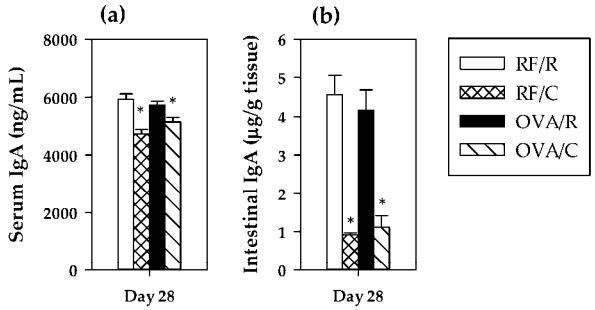

Figure 2. Serum (**a**) and intestinal (**b**) IgA concentrations at the end of the study. Values are expressed as mean ± standard error (n = 9). Statistical difference: * means statistical significant difference induced by the diet by two-way ANOVA ($p < 0.001$). Groups: RF/R = reference group; RF/C = reference group fed cocoa diet; OVA/R = sensitized group; OVA/C = sensitized group fed cocoa diet.

3.4. Lymphocyte Composition of MLN

The proportion of the main lymphocytes subsets in MLN was established at the end of the study (Figure 3). The oral sensitization did not modify significantly the percentage of B, TCRαβ+, TCRγδ+ and NK cells ($p = 0.054$, $p = 0.055$, $p = 0.662$, and $p = 0.866$, respectively, by two-way ANOVA) in this compartment (Figure 3a). The cocoa diet significantly increased the proportion of B, TCRγδ+, and NK cells ($p = 0.000$, $p = 0.000$, and $p = 0.007$, respectively, by two-way ANOVA) whereas it decreased that of TCRαβ+ cells ($p = 0.000$ by two-way ANOVA) (Figure 3a), producing a lower T/B ratio ($p = 0.005$ by two-way ANOVA) (Figure 3d). The increase of TCRγδ+ cell percentage in animals fed a cocoa diet was due to a higher proportion of CD8αα ($p = 0.000$ according to two-way ANOVA) (Figure 3b,e).

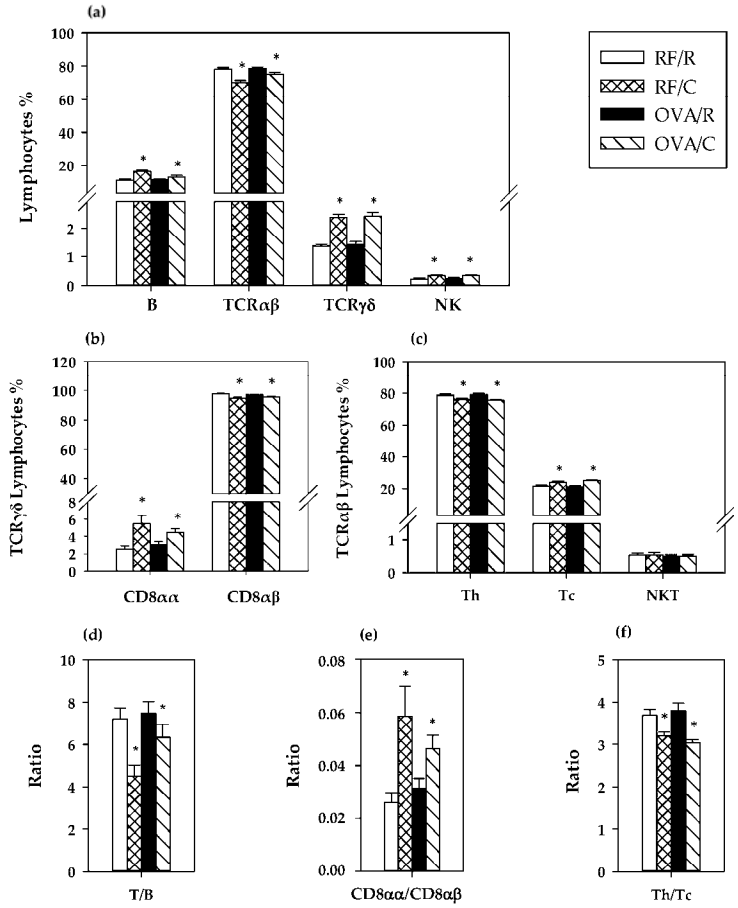

Figure 3. MLN lymphocyte composition. (**a**) Main lymphocyte subsets; (**b**) main TCRγδ+ lymphocyte subsets; (**c**) main TCRαβ+ cell subsets; (**d**) TCRαβ+/B lymphocytes ratio; (**e**) CD8αα/CD8αβ ratio in TCRγδ+ cells; (**f**)Th/Tc ratio in TCRαβ+ cells. Values are expressed as mean ± standard error ($n = 9$). Statistical difference: * means statistical significant difference induced by the diet by two-way ANOVA analysis ($p < 0.001$). Groups: RF/R = reference group; RF/C = reference group fed cocoa diet; OVA/R = sensitized group; OVA/C = sensitized group fed cocoa diet.

Further analysis of TCRαβ+ cell subsets showed that the reduction in the total TCRαβ+ cell percentage by the cocoa diet was accompanied by an increase in the proportion of Tc cells together

with a decrease in that of Th cells ($p = 0.000$ in both cases according to two-way ANOVA) (Figure 3c), which involved a lower Th/Tc ratio ($p = 0.000$ by two-way ANOVA) (Figure 3f). This means that the reduction in TCRαβ+ cell percentage was mainly due to Th cells. No effect on the low percentage of NKT cells was observed by either oral sensitization or cocoa diet ($p = 0.654$ and $p = 0.930$, respectively, by two-way ANOVA).

To analyze the Th and Tc subsets in depth, the proportion of activated cells, of cells expressing the L-selectin adhesion molecule and of cells bearing the integrin αE, was determined by means of expression of the clusters of differentiation CD25, CD62L, and CD103, respectively (Figure 4). Regarding activated cells (CD25+ cells), no differences were detected in Th lymphocytes ($p = 0.912$ and $p = 0.266$ by oral sensitization and diet, respectively, according to two-way ANOVA) (Figure 4a). Nevertheless, when considering the percentage of CD25+ cells in Tc lymphocytes, a significant interaction between oral sensitization and cocoa diet was found ($p = 0.022$ by two-way ANOVA), whereas neither condition significantly modified the proportion of CD25+ in Tc cells ($p = 0.425$ and $p = 0.360$ by oral sensitization and diet, respectively, according to two-way ANOVA). Further analysis revealed that CD25+ cell proportion in Tc lymphocytes increased in RF/C animals with respect to the RF/R group ($p = 0.030$ according to the Bonferroni test) but decreased in oral sensitized animals ($p = 0.031$ according to the Bonferroni test).

With regard to the expression of the L-selectin (CD62L+ cells) in Th and Tc cells, a decrease in the percentage of CD62L+ was observed in both subsets as a consequence of the diet ($p = 0.018$ and $p = 0.013$, respectively, by two-way ANOVA) (Figure 4b).

Finally, the proportion of Th and Tc cells bearing the integrin αE (CD103+ cells) was established. In Th cells, there was a significant effect for the interaction between oral sensitization and cocoa ($p = 0.000$ by two-way ANOVA), and the Bonferroni test revealed that there was a higher percentage of CD103+ cells in Th lymphocytes only in oral sensitized animals fed the cocoa diet ($p = 0.013$) (Figure 4c). Considering Tc cells, there was a higher percentage of CD103+ induced by the cocoa diet ($p = 0.028$ by two-way ANOVA).

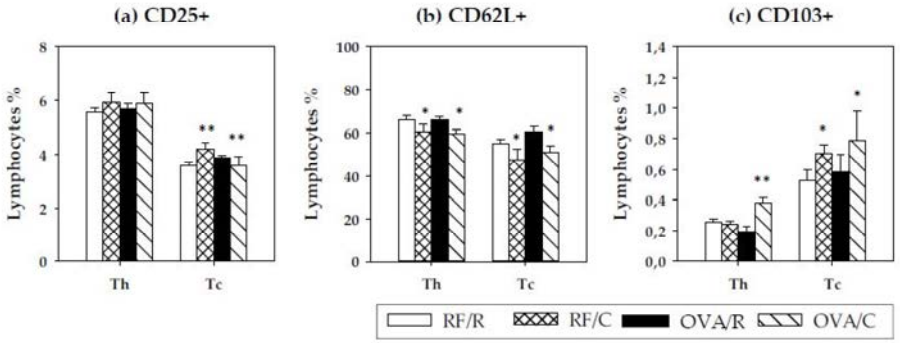

Figure 4. Percentage of cells bearing CD25 (IL2rα), CD62L (L-selectin) and CD103 (integrin αE) in Th and Tc subsets. (**a**) Proportion of CD25+ cells; (**b**) proportion of CD62L+ cells; (**c**) proportion of CD103+ cells. Values are expressed as mean ± standard error ($n = 9$). Statistical differences: * means a statistical significant difference induced by the cocoa diet ($p < 0.05$ by two-way ANOVA); ** means a statistical difference with respect to reference diet ($p < 0.05$ by Bonferroni test). Groups: RF/R = reference group; RF/C = reference group fed cocoa diet; OVA/R = sensitized group; OVA/C = sensitized group fed cocoa diet.

3.5. Gene Expression and Cytokine Production in MLN Cells

The possible influence of the oral sensitization and the cocoa diet on gene expression of some molecules and on cytokine secretion in the MLN lymphocytes was also established.

The relative gene expression of molecules associated with dendritic cells (OX40L, CD11c) and representative of an inflammatory response (IL-1β, IL-17α), the regulation of the immune response (NF-κB), the response to antigens (IL-12), and the regulatory function (IL-33) are shown in Figure 5.

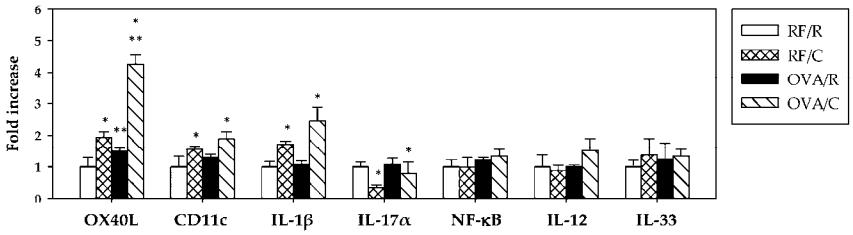

Figure 5. mRNA gene expression in MLN lymphocytes. Values are expressed as mean ± standard error (n = 6–9). Statistical differences: * means a significant difference induced by the cocoa diet ($p < 0.05$ by two-way ANOVA), ** means a significant difference with respect to the reference diet or induced by the oral sensitization process ($p < 0.001$ by Bonferroni test). Groups: RF/R = reference group; RF/C = reference group fed cocoa diet; OVA/R = sensitized group; OVA/C = sensitized group fed cocoa diet.

Oral sensitization increased the gene expression of OX40L (p = 0.000 by two-way ANOVA) and did not modify any of the remaining genes studied. The cocoa diet produced a higher expression of OX40L, CD11c, and IL-1β genes (p = 0.000, p = 0.018, and p = 0.001, respectively, according to two-way ANOVA). There was a significant interaction between oral sensitization and diet for the values of OX40L gene expression (p = 0.001 by two-way ANOVA), and further analysis revealed that the expression of this gene was higher in the OVA/C group compared to the OVA/R group (p = 0.000 by Bonferroni test).

On the other hand, the cocoa diet decreased IL-17α gene expression (p = 0.049 by two-way ANOVA).

To establish the effect of the oral sensitization and the cocoa diet on the cytokine pattern, MLN cells were incubated with OVA (10 μg/mL) for 72 h. From these supernatants IL-4, IL-10, TNF-α, and IFN-γ were quantified (Table 4). Neither oral sensitization nor cocoa intake significantly modified the levels of these cytokines released from MLN cells in the applied conditions, although a tendency to increase IFN-γ and IL-10 was observed in OVA/R animals compared with the RF/R group.

Table 4. Cytokine production from OVA-stimulated MLN lymphocytes and from gut lavage. Values are expressed as mean ± standard error (n = 6–9). Statistical difference: ** means a statistical difference with respect to reference diet ($p < 0.01$ by two-way ANOVA); # $p < 0.05$ compared with RF/R group by Bonferroni test. N.D. means non detectable levels.

Sample	Cytokine	Group [1]			
		RF/R	RF/C	OVA/R	OVA/C
MLN	IFN-γ	1.000 ± 0.110	1.484 ± 0.661	2.158 ± 0.843	1.521 ± 0.323
MLN	IL-4	1.000 ± 0.080	0.807 ± 0.113	0.895 ± 0.083	0.768 ± 0.110
MLN	TNF-α	1.000 ± 0.065	0.860 ± 0.040	1.033 ± 0.087	0.901 ± 0.089
MLN	IL-10	1.000 ± 0.042	1.106 ± 0.133	1.584 ± 0.302	1.241 ± 0.033
gut lavage	IFN-γ	N.D.	N.D.	N.D.	N.D.
gut lavage	IL-4	N.D.	N.D.	N.D.	N.D.
gut lavage	TNF-α	N.D.	N.D.	N.D.	N.D.
gut lavage	IL-10	1.000 ± 0.586	17.512 ± 3.783 #	25.177 ± 3.151 **	17.060 ± 3.739

[1] Groups: RF/R (reference group: vehicle and AIN-93M diet); RF/C (reference cocoa group: vehicle and 10% cocoa diet); OVA/R (sensitized group: OVA plus CT and AIN-93M diet); and OVA/C (sensitized cocoa group: OVA plus CT and 10% cocoa diet).

Cytokines were also analyzed in gut lavage. No detectable levels of IL-4, IFN-γ, and TNF-α were found in any group. However, IL-10 was found in the gut lavage of reference animals and it increased because of the oral sensitization process ($p = 0.000$ by two-way ANOVA). There was a significant interaction between oral sensitization and diet ($p = 0.001$ by two-way ANOVA) and further analysis revealed that values of the RF/C group were significantly higher than those in the RF/R group (0.009 by Bonferroni test).

4. Discussion

The current study demonstrates that a cocoa diet is able to prevent oral immune sensitization in young Lewis rats. This effect is associated with changes in composition as well as the gene expression of some molecules in MLN that could reflect the induction of tolerance to oral antigens, *i.e.*, the ability to suppress immune reaction to food proteins, through cocoa intake.

In the oral sensitization model used, Lewis rats received, by oral route, OVA as allergen and CT as adjuvant to breakdown oral tolerance, as developed previously [20]. For four weeks, rats were fed either a reference diet or a 10% cocoa diet. This amount of cocoa was chosen because previous reports demonstrated the immunomodulatory effect of cocoa at this dose [12,13,28]. The oral sensitization was evidenced by the synthesis of specific antibodies. The antibodies produced in the present study mainly belong to the isotypes related to Th2 responses (IgG1 and IgG2a) [29,30], although a certain amount of anti-OVA IgG2b, related to Th1 responses, was also synthesized. Unlike other animal models that use CT as a mucosal adjuvant with allergens that achieve specific IgE development, such as peanut [31], buckwheat [32], lupin [33], and OVA [34], the model used here does not develop IgE antibodies [20]. The different animal species, the various allergenic molecules, the amount of the adjuvant, or the dosage of immunogen may be responsible for the current lack of specific IgE production. Nevertheless, in the sensitization protocol applied, the cocoa diet was able to attenuate the development of specific IgG1, IgG2b, and IgM, although its effect on anti-OVA IgG2a did not achieve significant differences. These results partially agree with previous studies in food allergy models [13,28] in which cocoa attenuated specific IgG1 and IgG2a antibodies in Brown Norway rats, a rat strain that has shown a different susceptibility to a cocoa diet [35]. In any case, it can be confirmed that a 10% cocoa diet attenuates the production of antibodies and therefore prevents oral sensitization.

Focusing on the intestinal and serum IgA, no differences were seen due to the oral sensitization process, which does not agree with other studies in which CT increases serum and intestinal IgA levels [36]. However, in the current study, a cocoa diet downregulates the production of this immunoglobulin in both compartments and in either reference or sensitization conditions, as in previous studies [11,26,35,37]. Polyphenol content is partially responsible for this effect on intestinal IgA [35]. The attenuation of this immunoglobulin by a cocoa diet seems to be a consequence of a lower homing and activation of IgA+ B cells to the intestinal lamina propria in part due to changes in the gene expression of several molecules [37]. Although intestinal IgA has been associated with oral tolerance [38], our results suggest that it could also be achieved with low levels of this immunoglobulin.

To gain insights into the mechanisms induced by a cocoa diet, we focused on the composition and some functional aspects of MLN cells, due to their important role in oral tolerance [39,40]. The transport of the antigen captured by antigen-presenting cells from lamina propria into MLN is a key point in the induction of oral tolerance [17]. In this compartment, no changes in lymphocyte composition were observed due to the oral sensitization process, which is similar to what was observed in a food allergy model in Brown Norway rats combining an intraperitoneal immunization plus oral administration of OVA [29]. However, the intake of the 10% cocoa diet increased the proportion of B, TCRγδ+, and NK cells, whereas it decreased that of TCRαβ+ lymphocytes, similar to previous studies [11,41].

Interestingly, the cocoa diet induced a higher proportion of TCRγδ+ cells in MLN, which can be attributed to the higher amount of CD8αα+ cells, a typical intestinal phenotype [42], suggesting a possible migration from the intraepithelial compartment to MLN [43]. TCRγδ+ lymphocytes play a

crucial role in the mucosal immune system and several studies suggest their function in the induction of tolerance to oral antigens [43–45]. In particular, it has been described that the intestinal CD8αα+ TCRγδ+ cells favor tolerance [46], and that the blockade of TCRγδ+ cells results in elevated food allergic responses upon oral sensitization using CT as adjuvant [43]. Moreover, a recent study associates a subset of TCRγδ+ cells with an attenuating effect on the synthesis of antibodies by B lymphocytes [47]. These data could explain why, although we observed a relative increase of B cells in MLN due to the cocoa diet, the levels of specific antibodies in the serum of these animals were low. Therefore, it could be suggested that the increase of TCRγδ+ lymphocytes due to cocoa intake could be partially responsible for the prevention of specific antibody synthesis produced by this diet. In addition, NK cells, which also increased in proportion by cocoa diet, could also contribute to the regulation of antibody synthesis [48].

On the other hand, the cocoa diet decreased the proportion of TCRαβ+ lymphocytes and produced an imbalance in the two main subsets, Th and Tc, in favor of Tc cells, which is also in line with previous studies [11,41,49]. In addition, Th and Tc lymphocytes were characterized according to the surface expression of molecules related to lymphocyte homing (CD62L and CD103) and cell activation (CD25). CD62L, also called L-selectine, is involved in lymphocyte rolling on endothelium and the homing to peripheral lymphoid tissues [50]. The oral sensitization did not modify the expression of this molecule in MLN cells, but the cocoa diet decreased the proportion of both Th and Tc cells bearing CD62L. These results could mean that the cocoa diet decreased the arrival of lymphocytes at MLN and, consequently, their activation, thus avoiding lymphocyte activation and then promoting tolerance. In this context, the study of activated CD25+ cells revealed that cocoa intervention only produced significant changes in the proportion of CD25+ Tc lymphocytes, with opposite effects depending on whether the rats were sensitized or not. Although the proportion of CD25+ cells in Tc increased in healthy conditions, the cocoa diet in oral-sensitized animals decreased the proportion of CD25+ cells in Tc lymphocytes, which could also reflect a decrease in the arrival of these cells, as reflected in the proportion of CD62L+ Tc lymphocytes.

Regarding the molecule CD103, a subunit of the α3β7 integrin that can mediate cell adhesion, migration, and signaling [51], recent studies have demonstrated that it is also important in some resident memory CD8+ cells in various tissues, including the gut [52]. Our data show that the 10% cocoa diet in sensitized animals enhanced the proportion of CD103+ cells, both in Th (CD4+) and Tc (CD8+) cells. Both CD4+103+ and CD8+CD103+ cells have been associated with a regulatory role because their proportion increases after treatment with immunosuppressive agents [53]. Therefore, the increase of these cells in the MLN could contribute to the tolerogenic effect induced by a cocoa diet.

In order to shed some light on the role of MLN in the tolerogenic effects of a cocoa diet in rats, we quantified some genes related to the oral sensitization process, including those of molecules associated with dendritic cells (OX40L, CD11c), and representative of an inflammatory response (IL-1β, IL-17α), the regulation of the immune response (NF-κB), the response to antigens (IL-12), and the regulatory function (IL-33). Firstly, it has been taken into account that MLN gene expression did not produce significant results with regard to the sensitization protocol, with the exception of OX40L. We studied OX40L and CD11c related to dendritic cells because it has been described that there was a selective migration and activation of a unique subset of dendritic cells (CD11c+, CD103+, and CD8+) to the MLN in a model using CT as adjuvant, which seems responsible for Th2 polarization in this model [21]. In spite of these data, our results do not agree with such studies because no increase in cells bearing CD103 and no upregulation of CD11c were found in MLN of orally sensitized rats. In addition, it has been described that CT induces an upregulation of OX40L [21,54] and that OX40L–OX40 interactions led to the generation of Th2 responses during antigen presentation [55,56]. However, other data contradict this role [57]. In the current study, OX40L expression was upregulated by the oral sensitization process, which agrees with data relating this molecule to the induction of a Th2 response [54]. However, surprisingly, the expression of OX40L was also enhanced in rats fed cocoa and had an additive effect on rats administered orally with OVA plus CT. These results would suggest

that although OX40L–OX40 interaction is enhanced by oral challenge with a mucosal adjuvant, the role of a cocoa diet could be placed downstream of the Th2 immune responses that would eventually inhibit antibody synthesis. Otherwise, it has been described that the activation of the OX40 pathway can also promote Th1 responses [57], which is in line with the gene expression of IL-1β found to be elevated in cocoa-fed animals. Nevertheless, cocoa has been reported to also possess anti-inflammatory properties [58].

Our results regarding the gene expression of NF-κB do not agree with other studies that report that CT breaks oral tolerance by stimulating the production of NF-κB-dependent proinflammatory cytokines [23]. In agreement with the finding that there is no modification of NF-κB gene expression, we also found that the IL-1β mRNA and the level of TNF-α released by MLN cells from OVA-sensitized animals, typical proinflammatory cytokines, did not change with oral sensitization. The gene expression of IL-12 and IL-33 was also determined because IL-12 is related to the response to antigens and decreases after CT administration [59], and IL-33 is important in the induction of Th2 immune responses [60]. Similar to the above molecules, they were not affected by either oral sensitization or the cocoa diet. Nevertheless, we found that the cocoa diet downregulated the gene expression of IL-17α, which seems to be beneficial to oral tolerance because this cytokine could inhibit the tolerance to antigens [61].

Another aspect studied in the MLN was the release of some cytokines after *in vitro* stimulation. Although we expected to find increased Th2-related cytokines due to the oral sensitization protocol, we found no changes due to either sensitization or the cocoa diet. In this context, Singh *et al.* [62] did not detect changes in the concentrations of IL-4, IL-5, IL-10, and IFN-γ released from OVA-stimulated MLN cells in an oral murine model of food allergy, although a tendency to increase IL-10 and IFN-γ was observed, similar to the results presented here. On the other hand, in gut lavage from the small intestine, we found a rise in IL-10 levels in orally sensitized animals and also after cocoa intake. IL-10 is a multifunctional cytokine that is secreted in Th2 responses [63], in line with our increase in the sensitization protocol, and also plays a role regulating immune response and mucosal tolerance [64], agreeing with the results obtained showing the IL-10 increase in the cocoa-sensitized group.

Finally, although further research is necessary to establish the cocoa component responsible for its effects on the immune system and the prevention of oral sensitization, the possible role of flavonoids must be considered, particularly the flavanols, which are abundant in cocoa. In this context, the preventive effects of several flavonoid compounds and classes in allergy have been described [8] and, more recently, it has been reported that epicatechin and also a cocoa extract rich in epicatechin are able to decrease allergic symptoms, including the attenuation of specific antibodies, in a model of orally sensitized mice with OVA together with CT [62]. Therefore, even though more studies are necessary, the epicatechin present in cocoa appears to be one of the cocoa compounds able to prevent oral sensitization in rats. On the other hand, further studies must also be carried out in allergic humans in order to extrapolate the tolerogenic effect of cocoa on this process.

5. Conclusions

In conclusion, the cocoa diet, due to its flavanol content such as epicatechin or other compounds, is able to induce tolerance in an oral sensitization model in rats. Changes in mesenteric lymph node lymphocytes, particularly a higher proportion of TCRγδ+ (CD8αα+) and CD103+CD8+ cells and a lower proportion of CD62L+CD4+ and CD62L+CD8+ cells, together with the regulation of some immune-related genes, could contribute to this effect. These results show the ability of a cocoa diet to prevent the breakdown of oral tolerance and its potential as a nutraceutic in food allergies.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

APC	allophycocyanin
BSA	albumin from bovine serum
CT	cholera toxin
DMEM	Dulbecco's Modified Eagle Medium
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
IFN	interferon
I	inventoried
Ig	immunoglobulin
IL	interleukin
MLN	mesenteric lymph nodes
NF	nuclear factor
OPD	*o*-phenylenediamine
OVA	ovalbumin
OVA/C	sensitized group fed cocoa diet
OVA/R	sensitized group
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridininchlorophylla protein
RF/C	reference group fed cocoa diet
RF/R	reference group
Tc	T cytotoxic
TCR	T cell receptor
Th	T helper cells
TNF	tumor necrosis factor

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ARTICLE 3

"Effect of a cocoa diet on the small intestine and gut-associated lymphoid tissue composition in an oral sensitization model in rats"

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Resum ARTICLE 3

Objectiu: Després de conèixer els canvis induïts per la sensibilització oral i per la dieta rica en cacau sobre la producció d'anticossos i en els ganglis limfàtics mesentèrics, el següent propòsit va consistir en establir l'efecte de la dieta amb un 10% de cacau en el sistema immunitari intestinal (plaques de Peyer, limfòcits intraepitelials i de làmina pròpia de l'intestí prim) en el mateix model de sensibilització oral en rata.

Material i mètodes: Rates Lewis de tres setmanes d'edat es varen distribuir en quatre grups en funció de la dieta (10% cacau o estàndard) i de si se'ls hi va induir la sensibilització oral o no. Després de 4 setmanes, es van analitzar els limfòcits de plaques de Peyer (PP), intraepitelials (IEL) i de làmina pròpia (LPL) de l'intestí prim (citometria de flux). A més, es va determinar l'expressió gènica de diferents molècules (PCR a temps real) i, mitjançant tècniques histològiques i immunofluorescència es va quantificar la presència de cèl·lules caliciformes, cèl·lules IgA+ i cèl·lules granzim B+ a la làmina pròpia intestinal.

Resultats: A PP, la dieta amb cacau va produir un increment de la proporció de cèl·lules TCR$\gamma\delta$+, NKT, CD4+CD25+, CD4+CD103+ i CD8+CD103+, i una disminució de la proporció de limfòcits Th i de cèl·lules CD4+CD62L+. En els IEL, la dieta va produir un increment en la proporció de limfòcits TCR$\gamma\delta$+ i de les cèl·lules NK, i una disminució de la proporció de limfòcits TLR4+. A nivell de LPL, es va detectar un increment de la proporció de Th en el grup sensibilitzat i alimentat amb cacau. La sensibilització oral i la dieta cacau varen disminuir la proporció de cèl·lules IgA+ a la làmina pròpia. En condicions de referència, la dieta cacau també va induir una disminució de la proporció de granzim B a la làmina pròpia. Pel que fa a l'expressió gènica, la sensibilització va disminuir els nivells de mRNA d'IL-10 i la dieta rica en cacau l'expressió gènica d'IgA, de TGF-β1, de CD11b i de CD11c.

Conclusions: La dieta amb un 10% de cacau en un model de sensibilització oral en rata produeix canvis en la composició de limfòcits de plaques de Peyer, de l'epiteli i de la làmina pròpia intestinals. Entre d'altres canvis, la dieta indueix un increment de la proporció dels limfòcits TCR$\gamma\delta$+ i de les cèl·lules NK a PP i IEL, cèl·lules que podrien estar implicades en la prevenció de la sensibilització oral. Així mateix, la dieta rica en cacau redueix l'expressió gènica de molècules relacionades amb les cèl·lules dendrítiques i amb la formació i nombre de cèl·lules productores d'IgA i granzim B presents a la làmina pròpia. Tots aquests canvis poden contribuir en promoure la tolerància oral i evitar el desenvolupament de la sensibilització en les rates alimentades amb cacau.

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Effect of a cocoa diet on the small intestine and gut-associated lymphoid tissue composition in an oral sensitization model in rats☆

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Abstract

Previous studies have attributed to the cocoa powder the capacity to attenuate the immune response in a rat oral sensitization model. To gain a better understanding of cocoa-induced mechanisms at small intestinal level, 3-week-old female Lewis rats were fed either a standard diet or a diet containing 10% cocoa for 4 weeks with or without concomitant oral sensitization with ovalbumin (OVA). Thereafter, we evaluated the lymphocyte composition of the Peyer's patches (PPL), small intestine epithelium (IEL) and lamina propria (LPL). Likewise, gene expression of several immune molecules was quantified in the small intestine. Moreover, histological samples were used to evaluate the proportion of goblet cells, IgA+ cells and granzyme+ cells as well. In cocoa-fed animals, we identified a five-time reduction in the percentage of IgA+ cells in intestinal tissue together with a decreased proportion of TLR4+ IEL. Analyzing the lymphocyte composition, almost a double proportion of TCRγδ+ cells and an increase of NK cell percentage in PPL and IEL were found. In addition, a rise in CD25+, CD103+ and CD62L− cell proportions was observed in CD4+ PPL from cocoa-fed animals, along with a decrease in gene expression of CD11b, CD11c and IL-10. These results suggest that changes in PPL and IEL composition and in the gene expression induced by the cocoa diet could be involved, among other mechanisms, on its tolerogenic effect.

Keywords: Cholera toxin; Cocoa; Ovalbumin; TCRγδ+ cells; Tolerance

1. Introduction

The intestinal tract is the largest surface of the body protecting the internal toward the external environment. The primary function of the intestine is digestion and absorption of nutrients [1], but it is also recognized as the major immune organ, with the gut-associated lymphoid tissue (GALT) playing a central role in immune system homeostasis [2]. The GALT comprises approximately 70% of immune cells from the entire immune system [2], protecting the enormous intestinal surface (200 m^2 in humans) [3], which is in contact every day with a vast number of potentially harmful antigens [4]. The GALT is structurally and functionally classified into two different compartments: the organized inductive site and the diffuse effector site. The organized compartment is composed of Peyer's patches (PP), mesenteric lymph nodes (MLN) and isolated lymphoid follicles, whereas the diffuse compartment is formed by lamina propria lymphocytes (LPL) and intraepithelial lymphocytes (IEL). PP lymphocytes (PPL) and MLN lymphocytes (MLNL) are considered to be responsible for inducing oral tolerance or initiating immune response to antigens [5,6]. Situated in the lamina propria, LPL also contribute to oral tolerance, respond to antigen uptake and initiate migration of dendritic cells (DC) to the MLN [7]. Located between the epithelial cells, IEL regulate intestinal homeostasis, defend against infection and protect the integrity of the epithelial barrier [8,9].

Food allergies are currently considered a major public health concern due to their increasing prevalence. Food allergies affect approximately 5% of the general population and 8% of children worldwide [10]. According to current understanding, multiple pathways, cells and molecules are involved in the generation of an

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allergic response. Upon oral sensitization, allergens resisting denaturation and digestion in the gastrointestinal tract [11] reach the intestinal epithelium in an intact form and penetrate the first barrier of defense, the mucus layer, produced by the goblet cells and then the intestinal epithelial cell barrier. Afterwards, DC, mainly found in PP, acting as antigen-presenting cells, pick up the allergen and present it to T helper (Th) cells in PP or MLN [12]. Later, Th cells proliferate and differentiate into Th1, Th2, Th17 or regulatory T (Treg) cells according to different cytokine patterns [13]. In most food allergies, an imbalance is evident toward Th2 response [14]. The immune response ends with the activation of effector cells such as B cells, which later turn into IgA-secreting cells. Nevertheless, it still remains a matter of debate which cells are the driving forces for initiating sensitization in the gut.

In a healthy immune response, ingestion of food proteins results in the development of oral tolerance, that is, the suppression of an immune response [7,10]. This immune unresponsiveness affects different immune compartments and is associated with the suppression of antibody production [7]. In contrast, a food allergy develops when there is either a failure in the induction or a breakdown of oral tolerance [15,16]. Due to its increasing frequency, researchers worldwide are focusing on new food allergy preventive measures with increasing awareness of a potential beneficial role of nutraceuticals.

Previous studies have indicated the ability of a cocoa-enriched diet to influence the GALT functionality in rats. Accordingly, we have shown that cocoa consumption modifies the PPL composition in rats [17,18]. The tolerogenic influence of cocoa in a rat oral sensitization model has recently been demonstrated [19]. A 10% cocoa-enriched diet inhibited the synthesis of serum specific anti-ovalbumin (OVA) antibodies and attenuated intestinal IgA. In addition, this nutritional intervention induced changes in the lymphocyte composition and gene expression of MLN [19]. Specifically, in MLN, a cocoa diet increased the proportion of TCRγδ+ cells, playing a crucial role in the tolerance to oral antigens and CD8+ CD103+ cells, associated with regulatory functions. A decrease in CD4+CD62L+ and CD8+CD62L+ cell percentage was additionally observed, indicating a reduced influx of lymphocytes in MLN [19]. Together, these results show the capacity of a cocoa diet to induce oral tolerance and its potential role as a nutraceutical in food allergies.

Despite these previous studies, the influence of cocoa at the intestinal level remains unknown. We hypothesized that cocoa-enriched diet might regulate intestinal lymphoid tissue because it is the first compartment of the immune system in contact, and these changes could thereby contribute to the avoidance of the oral sensitization in rats. On the basis of this hypothesis, the present study aimed to analyze the effects of a cocoa diet on the small intestinal immune system in a rat oral sensitization model. Therefore, intestinal samples from rats orally sensitized with OVA were evaluated for lymphocyte composition in three functional compartments of the small intestinal immune system (PP, IEL and LPL) and also for the effect of a cocoa diet on representative molecules produced by GALT.

2. Materials and methods

2.1. Chemicals, reagents and diets

Albumin from chicken egg white (OVA; grade V), cholera toxin (CT), collagenase, 1,4-dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), Hanks balanced salt solution (HBSS), Roswell Park Memorial Institute (RPMI), Mayer's hematoxylin solution, eosin Y solution, Percoll®, Trizma® base (Tris Base) and Tween 20 were purchased from Sigma-Aldrich (Madrid, Spain). Donkey serum was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). NaN_3, p-formaldehyde, tri-sodium citrate dihydrate and citric acid were provided by Merck (Darmstadt, Germany) and RNAlater® by Ambion (Applied Biosystems, Austin, TX, USA). Xylene and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific (Vienna, Austria) and ethanol absolute from VWR (Vienna, Austria). Fluoromont-G was provided by SouthernBiotech (Birmingham, AL, USA). Ketamine was obtained from Merial Laboratories S.A. (Barcelona, Spain) and xylazine from Bayer A.G. (Leverkusen, Germany). Natural Forastero cocoa was obtained from Idilia Foods SL (formerly Nutrexpa S.L., Barcelona, Spain) and AIN-93 M diet and basal mix from Harlan Teklad (Madison, Wisconsin, USA).

2.2. Animals and experimental food intervention

Female Lewis rats were obtained from Janvier Labs (Saint Berthevin Cedex, France) and maintained under conditions controlled for temperature and humidity in a 12-h light/dark cycle. The present experimental design and procedure were approved by the local Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref.5988).

After a week of acclimatization at the Faculty of Pharmacy animal facilities (UB), 3-week-old rats were housed in cages (three per cage) and given *ad libitum* access to water and solid food during the 28 days of the study. The rats were randomly distributed into four experimental groups: reference group (RF/R), reference group fed cocoa diet (RF/C), OVA-sensitized group (OVA/R) and OVA-sensitized group fed cocoa diet (OVA/C) (n=9 each). The RF/R and the OVA/R groups were fed a standard diet (AIN-93 M), whereas the RF/C and the OVA/C groups were fed an isoenergetic diet containing 10% cocoa (Table 1). The OVA/R and OVA/C groups were orally sensitized as described [19,20], receiving 50 mg of OVA plus 30 μg of CT in 1 mL of distilled water by oral gavage three times per week (days 0, 2, 4, 7, 9, 11, 14, 16, 18 and 21). The RF/R and OVA/R groups received 1 mL of distilled water accordingly.

2.3. Sample collection and processing

On day 28, the animals were euthanized, and the small intestine was carefully collected. After discarding the duodenum, the intestine was rinsed with phosphate buffer saline (PBS) solution in order to remove fecal content. A 0.5 cm portion of the middle of the intestine was immediately conserved in RNAlater®, and the consecutive following segment was placed in cassettes and fixed in 4% paraformaldehyde. The rest of the intestine was opened lengthwise along the mesenteric line; PP were collected and stored in RPMI medium. Finally, the remaining intestine was cut into 2 cm pieces and immersed in HBSS supplemented with 10% heat-inactivated FBS to isolate IEL and LPL.

2.4. Peyer's patches lymphocyte isolation

PP were incubated with 1 mM DTT in RPMI medium under continuous agitation (55 u/min, 5 min, 37 °C). Consecutively, DTT medium was discarded, and PP were washed and passed through a sterile 70 μm mesh with a syringe plunger. The suspension obtained was centrifuged (538 g, 5 min, 4 °C) and resuspended with RPMI-10% FBS medium. Thereafter, cells were counted and viability was determined by staining with trypan blue using a Countess™ Automated Cell Counter (Invitrogen, Thermo Fisher Scientific).

2.5. Intraepithelial and lamina propria lymphocyte isolation

IEL and LPL isolation was carried in accordance with previous studies [21,22]. Briefly, small pieces of intestine, without PP, were incubated with a 5 mM DTT solution

Table 1
Composition of the experimental diets

Components	Standard diet [1]	10% cocoa diet [2]	
		Basal mix	Cocoa powder
Proteins	140.73	118.27	23.05
Lipids	38.71	27.06	11.53
Carbohydrates	721.93	692.41	16.76
Soluble fiber	–	–	8.91
Insoluble fiber	50.00	24.52	26.72
Minerals	35.86	27.83	6.29
Vitamins	10.20	7.92	0.04
Choline bitartrate	2.56	1.98	–
Antioxidant	0.01	0.01	–
Theobromine	–	–	2.50
Phenolic compounds [3]	–	–	4.02
Total	1000.0	1000.0	

All values are expressed as g/kg of diet.
[1] AIN-93 M formula was used as standard diet.
[2] The 10% cocoa diet was prepared from a basal mix in which 100 g cocoa/kg was added.
[3] Reversed-phase high-performance liquid chromatography coupled to a diode array detector revealed that cocoa phenolic compounds were epicatechin (2.34 mg/g), catechin (0.4 mg/g) and procyanidins.

in HBSS-10% FBS under continuous agitation (55 u/min, 20 min, 37 °C). The first supernatants were then collected by decanting the tubes. Afterwards, a solution of 5 mM EDTA in HBSS-10% FBS was added to the remaining intestinal tissue and incubated twice (55 u/min, 15 min, 37 °C). The supernatants were collected together with the first ones and centrifuged (538 g, 5 min, 4 °C). The resulting cell suspensions were subjected to IEL purification.

For LPL collection, the remaining intestinal tissue from the above incubation was washed with RPMI-10% FBS (55 u/min, 20 min, 37 °C). The supernatants were discarded, and intestinal samples were cut into small pieces for 60 min of incubation with 300 U/mL of collagenase in RPMI-10% FBS at 85 u/min and 37 °C. Afterwards, 10 mL of medium was added to each sample to stop the reaction. The supernatants were filtered through a stainless steel mesh. Finally, the suspensions containing LPL were centrifuged (538 g, 5 min, 4 °C).

The resulting suspensions of both IEL and LPL were subjected to a cell purification gradient using 44–67.5% Percoll. Lymphocytes were resuspended in medium, and cell number and viability were determined using a Countess™ Automated Cell Counter.

2.6. Flow cytometry analysis

For flow cytometric analysis, 5×10^5 PPL, IEL and LPL were labeled with mouse anti-rat monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridininchlorophylla protein (PerCP) or allophycocyanin (APC). The antibodies used were anti-CD4, anti-CD8α, anti-CD8β, anti-TCRαβ, anti-TCRγδ, anti-NKR-P1A, anti-CD25 (BD Biosciences, Oxford, UK), anti-CD62L, anti-CD103 (Biolegend, San Diego, CA, USA) and anti-TLR4 (Novus Biologicals, Littleton, CO, USA). The cells were stained as previously described [19]. Briefly, lymphocytes were incubated with saturating amounts of antibodies in PBS-0.2% FBS-0.1% NaN_3 (darkness, 20 min, 4 °C). Consecutively, the cells were washed and fixed with 0.5% p-formaldehyde (darkness, until analysis, 4 °C). A negative control staining was included in each cell sample. Analyses were performed with a Gallios Cytometer (Beckman Coulter, Miami, FL, USA) in the Scientific and Technological Centers of the University of Barcelona (CCiTUB).

2.7. Gene expression in small intestine

As previously described [20], intestinal samples conserved in RNA later® were transferred into a lysing matrix tube (MP Biomedicals, Illkirch, France) for 30 s of homogenization by a FastPrep-24 (MP Biomedicals). RNA was obtained using an RNeasy® mini kit (Qiagen, Madrid, Spain) following the manufacturer's instructions. RNA purity and concentration were determined by a NanoPhotometer (BioNova Scientific, S.L. Fremont, CA, USA). Subsequently, cDNA was obtained in a thermal cycler PTC-100 Programmable Thermal Controller using TaqMan® Reverse Transcription Reagents (Applied Biosystems, AB, Weiterstadt, Germany).

The specific PCR TaqMan® primers (AB) used to perform the PCR quantitative assay (ABI Prism 7900 HT, AB) were IgA (331,943, made to order), TGF-β1 (Rn00572010_m1, Inventoried (I)), CD11c (Rn01511082_m1, I), CD11b (Rn00709342_m1, I), OX40-L (Rn00585582_m1, I), IL-10 (Rn00563409_m1, I), FoxP3 (Rn01525092_m1, I) and Muc2 (Rn01498195_m1, I). The relative gene expression of genes of interest was normalized with the housekeeping genes β-actin (Rn00667869_m1, I) or HPRT1 (Rn01527840_m1, I) using the $2^{-\Delta\Delta Ct}$ method [20]. For FoxP3, IL-10 and OX40-L, the gene expression of HPRT1 was used as housekeeping gene, whereas for the rest of the gens, β-actin expression was used. Results are expressed as percentage of values of each experimental group normalized to the mean value obtained for the reference group, which was set at 100%.

2.8. Hematoxylin eosin and periodic acid Schiff stainings

Fixed intestinal tissues were dehydrated, paraffin-embedded and cut into 4 μm sections using a microtome (Thermo Scientific Microtome® HM355-S). Subsequently, the sections were mounted on glass slides and dried overnight at 37 °C.

For hematoxylin eosin (HE) staining, the samples were deparaffinized and rehydrated. Afterwards, intestinal tissues were stained with hematoxylin for 7 min, washed with running tap water and stained with eosin 5% for 4 min. Slides were again washed twice in distilled water and mounted with coverslips using Fluoromount-G.

For Periodic Acid Schiff (PAS) staining, deparaffinized and rehydrated samples were immersed in a 0.5% periodic acid solution for 5 min and washed in distilled water. Afterwards, sections were submerged in Schiff's reagent for 15 min, washed in running tap water and counterstained with Mayer's hematoxylin solution for 1 min. To finish, slides were rinsed in running tap water and in distilled water and mounted with coverslips.

After histological staining, microscopy analysis was performed using the TissueFaxs technology platform (TissueGnostics, Vienna, Austria). By means of the TissueFAXS 4.6.6245.1019 BF software, acquisition was done with a 200× magnification objective. Afterwards, PAS staining samples were analyzed using HistoQuest v4.04.0131 software (TissueGnostics, Vienna, Austria). Five microvilli of each intestine were selected as regions of interest (ROIs). The number of goblet cells was counted manually. Goblet cells are expressed as PAS-positive cells per mm^2 of area. All analyses were done twice, examining different sections and different microvilli to obtain representative results. The counting of goblet cells was done by two independent investigators.

2.9. Immunofluorescence staining of small intestinal tissue

For immunofluorescence (IF) staining, sections were deparaffinized and rehydrated. Successively, antigen retrieval was done followed by permeabilization with PBS-0.2% Tween and a blocking with serum. Sections were incubated for 1 h with the primary antibody and, consecutively, washed and incubated with the corresponding secondary antibody for 1 h. After washing, the samples were incubated for 10 min with DAPI to stain the nuclei and finally mounted with fluoromount-G.

For IgA IF, 10 mM Tris-1 mM EDTA (pH 9) was used for antigen retrieval and 5% donkey serum for blocking. Polyclonal goat anti-rat IgA α chain (Abcam, Cambridge, UK) was used as primary antibody and Alexa Fluor 568 donkey anti-goat IgG (Life Technologies, Austin, TX, USA) as secondary.

For granzyme B (GzmB) IF staining, 10-mM citrate buffer (pH 6) was used for antigen retrieval and 10% of normal goat serum for blocking. The primary antibody used was polyclonal rabbit anti-rat GzmB (Novus Biologicals), with Alexa Fluor 647 goat anti-rabbit IgG (Life Technologies) being used as secondary antibody. In both stainings, a negative control was done omitting the primary antibody.

After IF stainings, pictures were acquired by TissueFaxs 4.2.6245.1019 FL software using a 200× magnification objective. Stainings were analyzed using TissueQuest 4.0.1.0128 software (TissueGnostics). Five microvilli of each intestine were selected as ROIs to be analyzed together and to obtain the scattergram of positive cells. All analyses were done twice, examining different sections and different microvilli for representative results.

2.10. Statistical analysis

Data are expressed as means ± standard errors. All statistical analyses were performed using the IBM Statistical Package for the Social Sciences (SPSS, Version 22.0, Chicago, IL, USA).

Prior to the analysis, the Levene's and Kolmogorov–Smirnov test were performed in order to assess variance equality and normal distribution, respectively. When all the results indicated equality of variance and normal distribution, a two-way ANOVA test was performed (oral sensitization and diet). When the interaction between oral sensitization and diet was statistically significant, a one-way ANOVA with Bonferroni's *post hoc* test was carried out to detect differences among groups.

The results that had different variance and/or different distribution were analyzed by nonparametric tests; Kruskal–Wallis followed by Mann–Whitney *U* tests were performed. In all cases, significant differences were accepted when $P\leq.05$.

3. Results

At the end of the study, the body weight increase of the animals fed standard diet was about 186%, whereas animals fed cocoa showed an increase of almost 157% [19]. This lower body weight increase, also observed in previous studies [17,18], was not associated with less food intake. Considering the average food consumption throughout the study and the amount of cocoa in the food, the daily amount of cocoa powder ingested was 11.75 g/kg.

3.1. Effect of a cocoa diet on the intestinal structure and the proportion of goblet cells in orally sensitized rats

HE staining (Fig. 1a–d) did not reveal morphological changes due to the nutritional intervention with cocoa or with the administration of OVA plus CT. In addition, no significant variations were detected among groups concerning the proportion of goblet cells (Fig. 1e–i).

3.2. Effect of a cocoa diet on Peyer's patches lymphocyte composition in orally sensitized rats

PP from the RF/R group were characterized by having about 70% CD45RA+ cells, 20% TCRαβ+ cells, and less than 3% TCRγδ+ lymphocytes and NK cells (Fig. 2a). Likewise, in the RF/R group, there was a similar proportion of TCRγδ+ CD8αα+ and TCRγδ+ CD8αβ+ cells (Fig. 2b and c), and among all TCRαβ+ cells, 65% were Th (TCRαβ+CD4+), 30% Tc lymphocytes (TCRαβ+CD8+) and about 5% NKT cells (Fig. 2d and e). Oral sensitization decreased the total proportion of TCRαβ+ cells (Fig. 2a) without modifying their main subsets (Th, Tc and NKT cells) (Fig. 2d and e). On the other hand, a cocoa-enriched diet increased the proportion of TCRγδ+ cells (both CD8αα+ and CD8αβ+) and NK cells (Fig. 2a and c). Although the total proportion of TCRαβ+ cells was

not influenced by a cocoa-enriched diet, there was an increase in the percentage of NKT cells and a decrease in the proportion of Th lymphocytes (Fig. 2d), producing a reduced Th/Tc ratio (Fig. 2e).

The percentage of cells bearing toll-like receptor 4 (TLR4) on PP was also established, but it did not reveal any modifications by oral sensitization or a cocoa diet (Fig. 2f).

Further analysis of CD4+ and CD8+ PPL with the activation marker (CD25+), the marker of intestinal homing (CD103+) and the marker of secondary lymphoid tissue homing (CD62L+) was carried out. Rats fed a cocoa diet showed an increase in the proportion of CD25+ (Fig. 2g) and CD103+ (Fig. 2h) cells at intestinal level. Moreover, a decrease in the proportion of CD4+ CD62L+ lymphocytes (Fig. 2i) was detected, indicating an increase in CD4+CD62L− cell percentage.

3.3. Effect of a cocoa diet on intraepithelial lymphocyte composition in orally sensitized rats

In the RF/R animals, the small intestinal IEL population was composed of about 40% TCRαβ+, 25% NK cells and 15% TCRγδ+ lymphocytes (Fig. 3a). Almost 80% TCRγδ were CD8αα+ (Fig. 3b and c). Within the TCRαβ+ IEL, we found 79% CD8+, 14% CD4+ and 6% NKT cells (Fig. 3e). Oral sensitization with OVA did not induce significant changes in these IEL proportions, in spite of a tendency toward a higher CD4+/CD8+ ratio (Fig. 3f). However, similarly to PPL, an increase in the percentage of TCRγδ+ and NK cells was observed as a result of being fed a cocoa diet. The increase in TCRγδ+ IEL was due to both the CD8αα+ major population and CD8αβ+ minor population (Fig. 3b and c). A decrease in the TCRαβ+ cell proportion occurred in the OVA/C group (Fig. 3a); however, these changes could not be attributed to any of the CD4+, CD8+ and NKT subsets considered (Fig. 3e).

With regard to NK cells, the cocoa diet induced an increase in the MFI of CD90+ (Thy-1) expression of NK cells, whereas no changes were observed in the MFI of CD25 in this cellular type (Fig. 3d).

With respect to TLR4+ IEL, the cocoa diet reduced their proportion by up to a half (Fig. 3g), affecting cells both with high and low TLR4+ expression (data not shown).

Evaluating both the activation and the homing marker expressions in CD4+ and CD8+ IEL, no significant differences were detected in the CD4+ CD25+ and CD8+ CD25+ subsets in OVA-sensitized animals (Fig. 3h). As expected, most of the CD8+ IEL expressed the intestinal CD103 marker, whereas only about 2% were CD62L+. In contrast, in the smaller fraction of CD4+ IEL, less than 15% were CD103+ and about 25% were CD62L+. In CD8+ IEL, oral sensitization reduced the proportion of CD103+ cells and increased that of CD62L+ cells. Similarly, the sensitization procedure induced a higher percentage of CD62L+ cells in CD4+ IEL which was prevented by the cocoa diet (Fig. 3j). An increase in CD4+ CD103+ cells was observed due to the cocoa diet.

3.4. Effect of a cocoa diet on lamina propria lymphocyte composition in orally sensitized rats

Focusing on the LPL composition in the RF/R group, the distribution of the main lymphocyte subsets was about 40% TCRαβ+ cells, 35% CD45RA+ lymphocytes, 15% NK cells and less than 3% TCRγδ+ cells (Fig. 4a). In addition, in the total TCRαβ+ LPL population, about 69% were Th cells, 28% were Tc cells and 3% were NKT cells (Fig. 4c). No significant differences were detected after oral

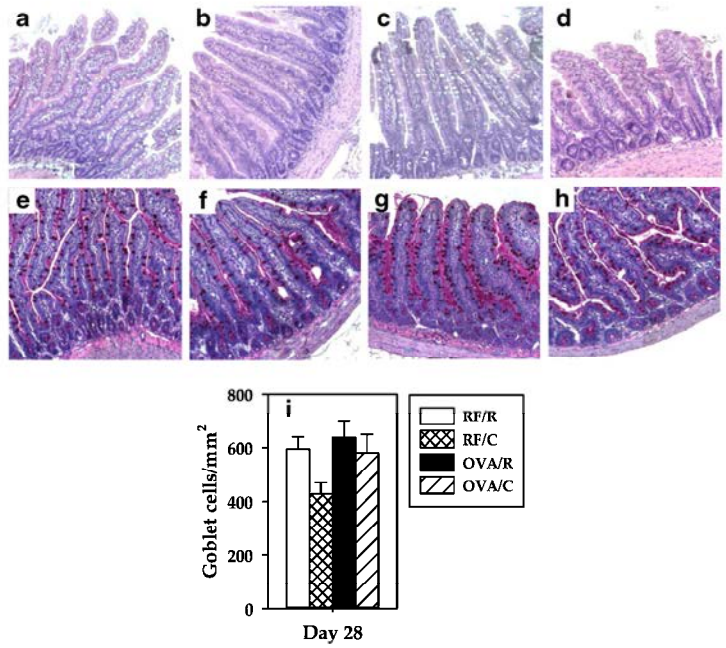

Fig. 1. HE and PAS staining. HE staining of small intestine section from a representative rat belonging to (a) RF/R, (b) RF/C, (c) OVA/R, and (d) OVA/C groups. PAS staining showing goblet cells of small intestine from a representative rat belonging to (e) RF/R, (f) RF/C, (g) OVA/R and (h) OVA/C groups. (i) Goblet cells number/mm^2 (values are expressed as mean ± standard error, $n=6$).

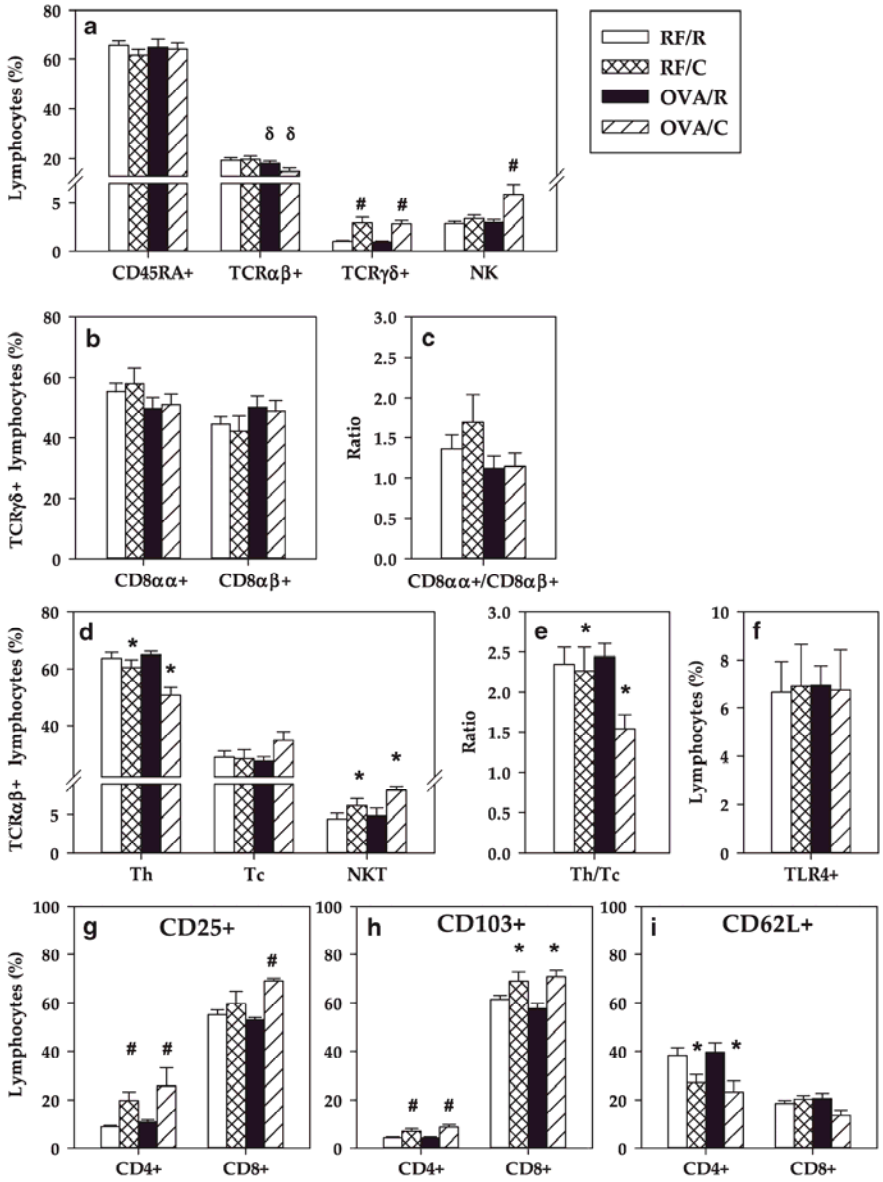

Fig. 2. PP lymphocyte composition. (a) Percentage of the main lymphocyte subsets; (b) percentage of TCRγδ+ PPL subsets; (c) CD8αα+/CD8αβ+ ratio in TCRγδ+ cells; (d) percentage of TCRαβ+ PPL subsets; (e) Th/Tc ratio; (f) percentage of TLR4+ PPL; proportion of (g) CD25+, (h) CD103+ and (i) CD62L+ cells in CD4+ and CD8+ PPL. Values are expressed as mean ± standard error (n=6–9). Statistical differences: δ P<.05 (two-way ANOVA) induced by the oral sensitization * P<.05 (one-way or two-way ANOVA) induced by the diet; # P<.05 (Mann–Whitney U) *versus* the corresponding reference group.

sensitization. However, the OVA/C group had higher proportion of Th LPL (Figs. 4c and d). Moreover, the cocoa diet decreased the percentage of NKT cells in TCRαβ+ LPL.

A cocoa-enriched diet was associated with an increase in the expression of CD90 in NK cells, and no changes were observed in the expression of the CD25 molecule (Fig. 4b).

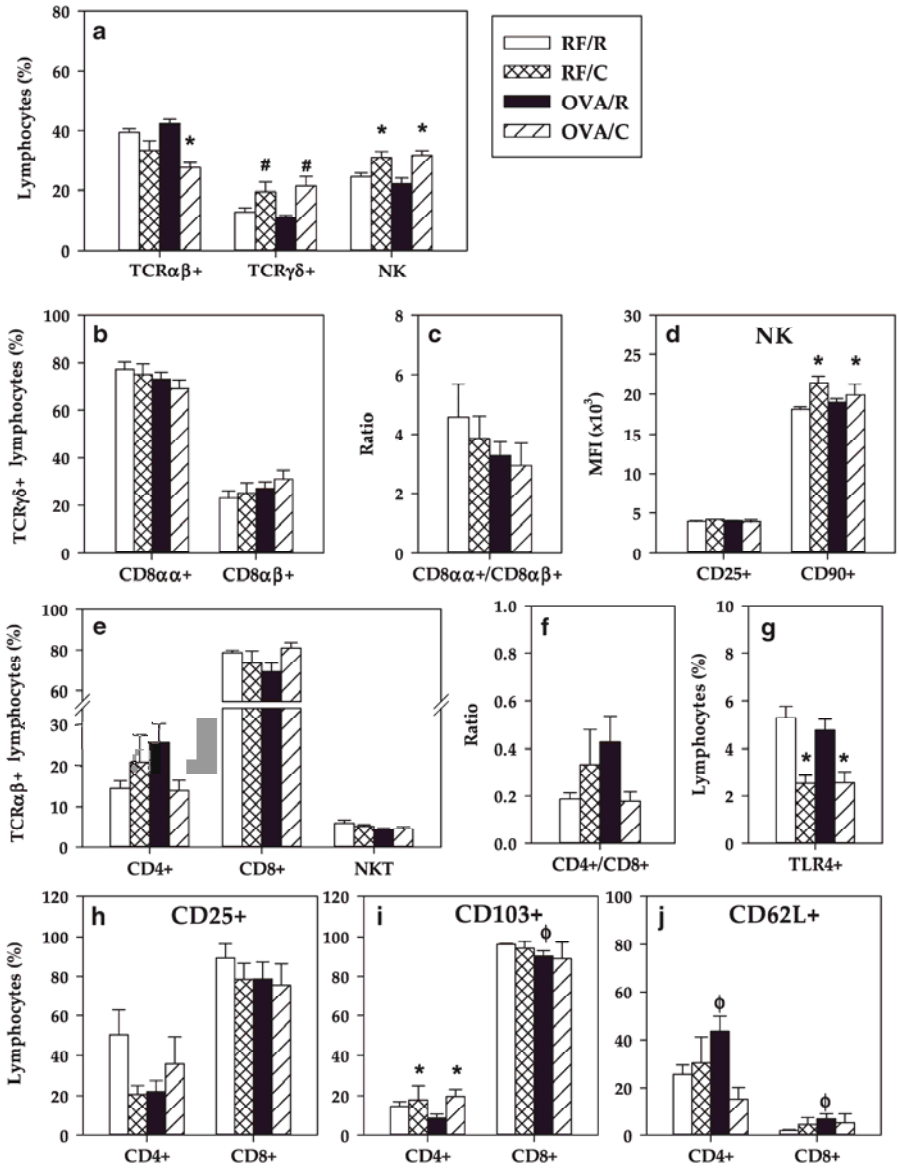

Fig. 3. IEL lymphocyte composition. (a) Percentage of the main lymphocyte subsets; (b) percentage of TCRγδ + IEL subsets; (c) CD8αα+/CD8αβ + ratio in TCRγδ + cells; (d) mean fluorescence intensity (MFI) of CD25+ and CD90+ in NK cells; (e) percentage of TCRαβ + IEL subsets; (f) Th/Tc ratio; (g) percentage of TLR4+ IEL; proportion of (h) CD25+ (i) CD103+ (j) and CD62L+ cells in CD4+ and CD8+ IEL. Values are expressed as mean ± standard error (n=6–9). Statistical differences: * P<.05 (one-way or two-way ANOVA) induced by the diet; # P<.05 (Mann–Whitney U) *versus* the corresponding reference group; ϕ P<.05 (Mann–Whitney U) *versus* RF/R group.

CD25 expression was highly variable on CD4+ and CD8+ cells from LPL (data not shown). No changes were observed due to the cocoa diet either through the sensitization procedure in the expression of TLR4 (Fig. 4e), CD103 (Fig. 4f) and CD62L (Fig. 4g) in LPL.

3.5. Effect of a cocoa diet on small intestine IgA+ cells in orally sensitized animals

RF/R rats showed a percentage of about 2.5% of IgA+ cells located in the lamina propria (Fig. 5). Oral sensitization decreased this

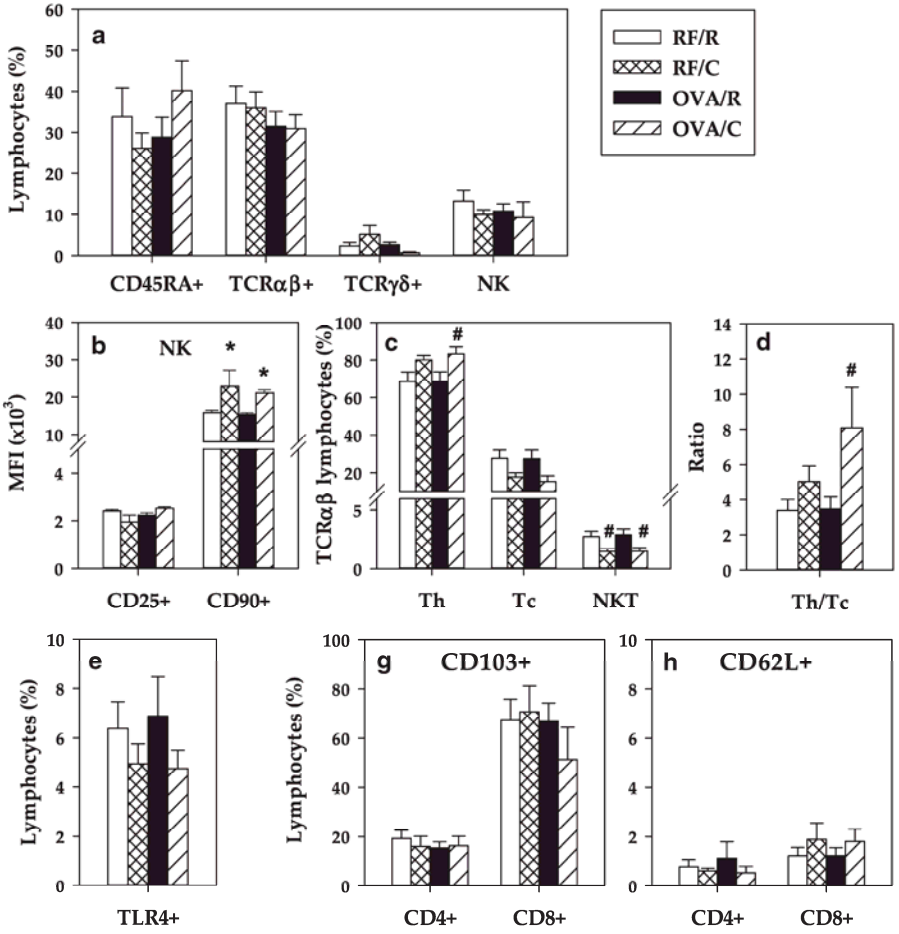

Fig. 4. LPL lymphocyte composition. (a) Percentage of the main lymphocyte subsets; (b) mean fluorescence intensity (MFI) of CD25 and CD90 MFI in NK cells; (c) percentage of TCRαβ + LPL subsets; (d) Th/Tc ratio; (e) percentage of TLR4+ LPL; proportion of (f) CD103+ and (g) CD62L+ cells in CD4+ and CD8+. Values are expressed as mean ± standard error (n=6–9). Statistical differences: # P<.05(Mann–Whitney U) *versus* the corresponding reference group.

proportion up to 15%. A cocoa-enriched diet was associated with a reduction in that percentage to 0.5% IgA+ LP cells both in the RF/C and the OVA/C groups (Fig. 5a–i).

3.6. Effect of a cocoa diet on small intestine granzyme B+ cells in orally sensitized animals

In the RF/R group, the percentage of GzmB+ cells in LP was about 1.3% (Fig. 6). No differences were observed after sensitization; however, the cocoa-enriched diet induced a decrease in the GzmB+ cell percentage to 0.4% in the RF/C group, whereas changes in the OVA/C group did not achieve statistical significance.

3.7. Effect of a cocoa diet on the small intestine gene expression of orally sensitized animals

The gene expression of IgA, TGF-β1, CD11b and CD11c was not modified by oral sensitization but was reduced for IgA, CD11b and CD11c in the RF/C and OVA/C groups. TGF-β1 expression was decreased only in the RF/C group.

On the other hand, oral OVA sensitization did not modify the gene expression of Muc2, FoxP3 and OX40-L but decreased that of IL-10. In addition, cocoa diet intake was associated with reduced levels of IL-10 mRNA (Fig. 7).

4. Discussion

In a previous study, we demonstrated that a cocoa diet prevents antibody synthesis and modifies mesenteric lymph node composition and functionality using the same rat oral sensitization model [19]. These results prompted us to study the influence of a cocoa diet on intestinal tissue composition. The data presented here demonstrate that a cocoa diet modifies the small intestinal compartment mainly by increasing the presence of TCRγδ+ cells and NK cells in PPL and IEL. Moreover, a cocoa diet increased the proportion of CD4+CD25+, CD4+CD103+, CD8+CD103+ and CD4+CD62L− cells in PP. These

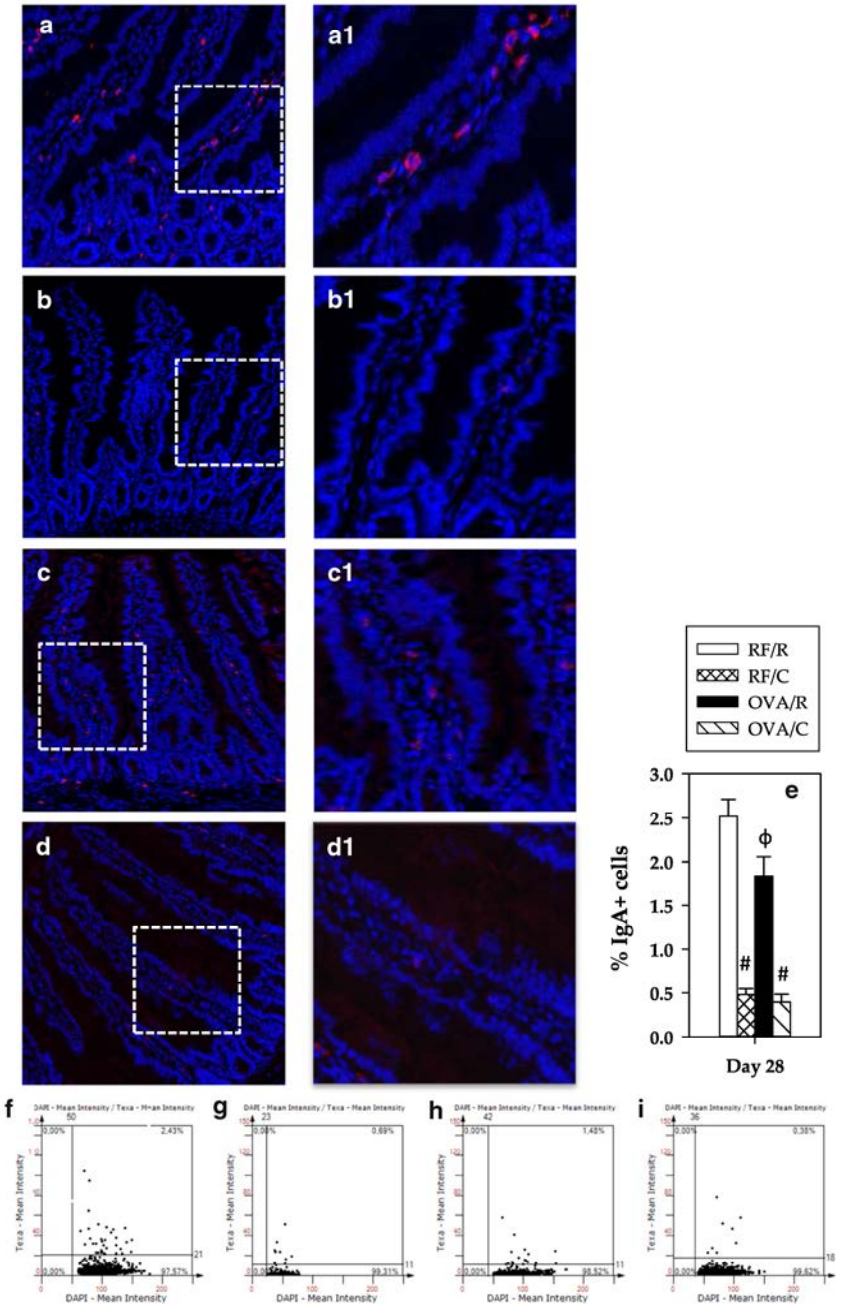

Fig. 5. Percentage of IgA+ cells. Immunofluorescence IgA staining of sections from small intestinal samples from a representative rat belonging to (a) RF/R, (b) RF/C, (c) OVA/R, or (d) OVA/C groups. A magnification of the specified area for each representative sample is shown (a1, b1, c1 and d1). The images show IgA+ cells in red (Texas red). The DAPI blue fluorescence indicates the nuclei. (e) Percentage of IgA+ cells in small intestine of the rats (values are expressed as mean ± standard error, $n=6–9$). Statistical differences: # $P<.05$ (Mann–Whitney *U*) respect to the corresponding reference group; ϕ $P<.05$ (Mann–Whitney *U*) *versus* RF/R group. Scattergrams of the expression of IgA+ from a representative rat of each group of study: (f) RF/R, (g) RF/C, (h) OVA/R and (i) OVA/C.

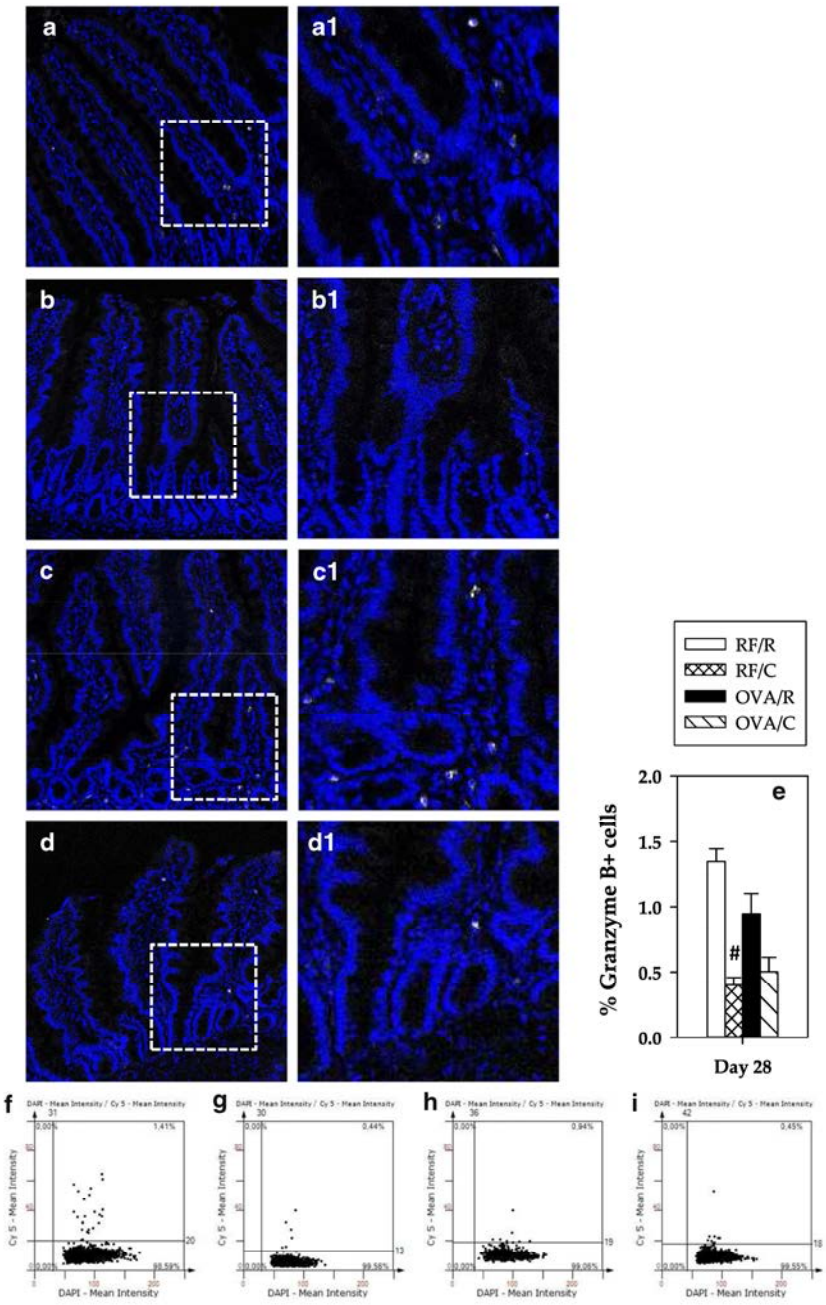

Fig. 6. Percentage of GzmB+ cells. Immunofluorescence GzmB staining of sections from small intestinal samples from a representative rat belonging to (a) RF/R, (b) RF/C, (c) OVA/R, or (d) OVA/C group. A magnification of the specified area for each representative sample is shown (a1, b1, c1 and d1). The images show GzmB+ cells in white (Cy5). The DAPI blue fluorescence indicates the nuclei. (e) Percentage of GzmB+ in small intestine of the rats (values are expressed as mean ± standard error, n=6–9). Statistical difference: # P<.05 (Mann–Whitney U) *versus* RF/R group. Scattergrams of the expression of GzmB+ from a representative rat of each group of study: (f) RF/R, (g) RF/C, (h) OVA/R and (i) OVA/C.

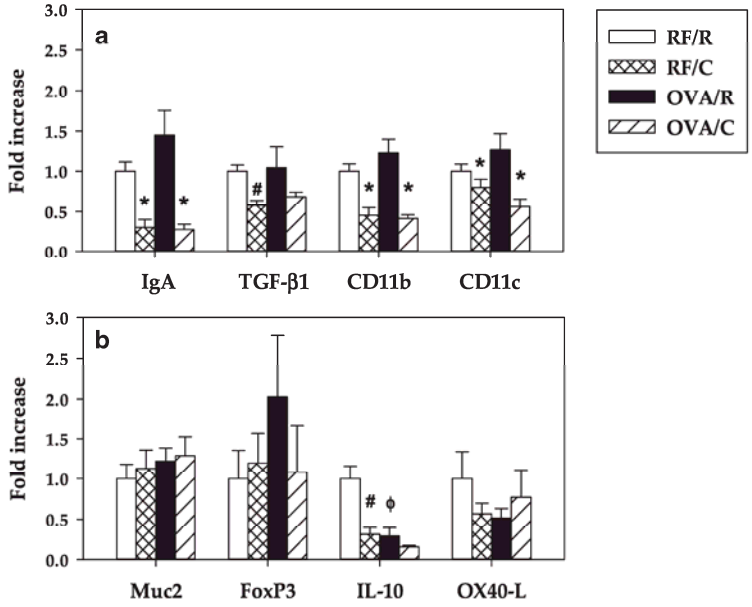

Fig. 7. Gene expression of some molecules in small intestinal samples. The relative mRNA gene expression was calculated assigning the value of 1 to the mean of the rats from the RF/R group. Values are expressed as mean ± standard error (n=6). Statistical differences: * P<.05 (two-way ANOVA) induced by the cocoa diet compared with its reference group, # P<.05 (Mann–Whitney U) *versus* RF/R group, ϕ P<.05 (Mann–Whitney U) *versus* RF/R group.

changes were accompanied by a decrease in the proportion of IgA+ cells and GzmB+ cells in LP and in the gene expression of IgA, TGF-β1, CD11b, CD11c and IL-10 in the small intestine.

In contrast with other studies using CT as intestinal adjuvant that reported an increase in intestinal IgA levels [23,24], the oral sensitization procedure used here decreased the proportion of intestinal IgA+ cells in LP, although no differences were seen at the secreted intestinal IgA levels in these conditions [19]. Furthermore, with this sensitization protocol, no changes were found in the gene expression of IgA and TGF-β1 in line with previous studies [20]. In the current study, a cocoa diet was also associated with decreased numbers of intestinal IgA+ cells. Previously, we demonstrated that a 10% cocoa diet attenuated intestinal IgA levels at different ages and in different rat strains, such as Wistar [25,26], Brown Norway [18] and Lewis [19,27]. With the current results regarding IgA+ cells on LP, we can conclude that the reduction of intestinal IgA was due to a lower number of IgA+ cells at this level. These results are in line with the lower IgA mRNA levels found in this work as well as in a previous study [26]. Multiple cytokines such as TGF-β1 [28] and a vast number of cell interactions are required to activate and differentiate B cells in PP [16] and to promote their migration to the MLN and the eventual gut homing [29]. The reduction in TGF-β1 gene expression at small intestinal level suggests that, among other possibilities, a 10% cocoa diet influences IgA+ B cell differentiation, thereby involving a reduction of IgA+ B cells in LP. These results are in line with changes in the chemokines CCR9, CCL25, CCL28, RARα and RARβ, required for gut homing, as previously demonstrated after cocoa feeding [25]. On the other hand, in the current study, a cocoa-enriched diet was also associated with a decrease in the proportion of TLR4+ IEL, the receptor binding the bacterial endotoxin lipopolysaccharide (LPS). This reduction in TLR4+ IEL was in line with previous data detecting a decrease in TLR4 gene expression in the small intestine due to cocoa diet [25] and could be related to changes in intestinal microbiota as previously reported in cocoa-fed rats [30]. TLR4 signaling also has implication in the production of intestinal IgA as activation of TLR4 pathways is associated with an increase of diverse chemokines (mainly CCL20, CCL28 and CXCL16) related to the recruitment and differentiation of IgA+ B cells to the intestine [31]. Accordingly, the decrease in the TLR4+ IEL in animals fed a 10% cocoa diet could be seen in the context of lower numbers of LP IgA+ cells and the consequently lower intestinal IgA levels.

In the current study, neither 10% cocoa diet nor the administration of OVA plus CT modified the intestinal structure. In order to initiate the immune response, the first barrier that antigens must penetrate is the mucus layer. This barrier in the intestine prevents food allergy, enhancing gut homeostasis and oral tolerance [32]. To determine whether oral sensitization and the cocoa diet were interacting at that point, evaluation of goblet cells and one of their products, Muc2, was considered. Neither the sensitization procedure nor the cocoa diet modified the goblet cell proportion and Muc2 gene expression. These results are in partial agreement with those obtained in a food allergy model in Brown Norway rats fed with two different cocoa-enriched diets with unchanged Muc2 gene expression but lower numbers of goblet cells [33]. Overall, these results lead us to suggest that the tolerogenic effect of a cocoa diet is not due to a modification of that barrier.

Even though no changes were detected at the structural level of small intestine, several modifications were found in the proportions of lymphocyte populations isolated from PP, IEL and LPL, representing essential sites involved in oral tolerance induction [7,34]. A cocoa diet induced an increase in the proportions of TCRγδ+, both CD8αα+ and CD8αβ+, cells in PPL and IEL, essential subsets for the induction of oral tolerance. Our results are in line with those of Akiyama *et al.* [35], who reported that unripe apple polyphenols inhibit oral sensitization associated with a rise in the proportion of TCRγδ+ IEL. In line with

these studies, it was described that a decrease in the number of TCRγδ+ cells induced by anti-TCRγδ antibody treatment facilitated oral sensitization in mice [36].

Furthermore, a cocoa diet increased the NK cell percentage in both PP and IEL compartments. Although NK cells are primarily involved in innate immunity, they also have regulatory functions and can contribute to the inhibition of allergic disease [37,38]. Several subsets of NK cells have been defined in humans and mice [39,40], but less information about rat NK cells is available. In a recent study focused on mice NK cells [41], activation of NK cells involved, among other changes, a lower expression of granzyme B, CD11b and CD11c but a higher expression of CD90 in these cells. It is worth noting that the increase in NK IEL due to a cocoa diet found here was accompanied by a higher CD90 surface expression. In addition, cocoa diet decreased GzmB+ cell percentage in the intestinal LP and down-regulated CD11b and CD11c intestinal gene expression. Although further studies are needed for an in-depth evaluation of the NK subset and function associated with a cocoa diet, the current results suggest that some intestinal NK cells (perhaps related to high CD90 surface expression, low GzmB content and low CD11b and CD11c gene expression) might contribute to the tolerogenic effect of a cocoa diet. In addition, the decrease in CD11b and CD11c gene expression may be related to a lower presence of dendritic cells in the intestinal wall. These cells could migrate from LP to MLN, where we have previously observed higher CD11c gene expression [19] and where they could promote tolerance as suggested [12].

A cocoa diet additionally modified the proportion of CD25+ cells in CD4+ and CD8+ PPL. The CD25 molecule is, among others, a marker of Treg cells, which induce tolerance against dietary antigens [42]. Thus, the increased proportion of CD25+ cells in PP cells after consumption of a cocoa diet might contribute to the tolerogenic effect of cocoa.

Consuming cocoa also changed the proportions of cells expressing CD103 and CD62L molecules in PPL. Cocoa-fed animals showed a higher proportion of CD103+ cells in CD4+ and CD8+ PPL, and a lower proportion of CD62L+ (and consequently a higher CD62L− percentage) in CD4+ PPL. CD103 (also known as αE integrin) is a marker of gut homing cells [8] with a role in controlling the homeostasis of the intestinal immune system and inducing the expansion of Treg cells [43]. Therefore, the increase of CD103+ cells in the CD4+ and CD8+ PPL subsets could also contribute to the tolerogenic effect of the cocoa diet. As a marker of lymph node homing, CD62L is constitutively expressed in naive lymphocytes and is down-regulated after cell activation [44,45]. $CD62L^{low}$ Treg cells found in secondary lymphoid tissues guide T cells to migrate to nonlymphoid tissues in order to maintain immune homeostasis [46]. Our results suggest that a cocoa diet induced more cell activation, and consequently, more effector cells were retained in the intestinal compartment with the potential to enhance the tolerogenic response. Taking all these results into consideration, we suggest that a cocoa diet induces the activation of tolerogenic cells migrating to the intestinal PP compartment, thereby avoiding oral sensitization.

Another modification found here was the down-regulation of IL-10 gene expression in intestinal tissue both in orally sensitized animals and in cocoa-fed rats. IL-10 has been shown to induce Treg cells mostly of the Tr1 type [47] which agrees with lower IL-10 gene expression in sensitized animals, thereby promoting the loss of oral tolerance as reported in a murine model of food allergy [48]. However, cocoa-fed animals also down-regulated IL-10 gene expression. Despite these contradictory data, our results are in line with those reported for flavonoids such as quercitrin, flavones and those found in an apple extract in orally sensitized mice [49–51]. Therefore, it might be concluded that flavonoids modulated immune response without enhancing IL-10 tolerogenic effects.

In the current study, several markers of healthy immune status of GALT were considered. Unexpectedly, in the applied experimental design, not so many changes were seen due to the oral sensitization procedure used, although it was able to induce the production of Th2-antibodies, as previously described [19]. This could be due to the implication of other mechanisms such as specific dendritic cells at intestinal level that would enhance antigen presentation, the up-regulation of some makers in the lymphocytes enhancing either the antigen presentation or the activation of plasma cells, among others. Moreover, the time point in which of these biomarkers were studied could be too late to observe their alterations.

In conclusion, the data presented here showed that consumption of a diet containing 10% of cocoa for 4 weeks either in healthy conditions or in a rat oral sensitization model was associated with similar substantial changes in small intestinal lymphocyte subsets located in Peyer's patches, epithelium and lamina propria. A cocoa-enriched diet induces a rise in the proportion of TCRγδ+ cells and NK PPL and IEL, suggesting a contribution to the prevention of oral sensitization. In line with this, the nutritional intervention with cocoa induces an increase of CD25+, CD103+ and CD62L− cells in PP and reduces CD11b, CD11c and IL-10 gene expression, together with a lower number of IgA+ LP cells. In summary, these changes might contribute to enhancing oral tolerance, thereby underlining the role of cocoa in preventing oral sensitization.

Conflicts of interest

None of the authors have any conflicts of interest to declare.

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ARTICLE 4

"Gut microbiota in a rat oral sensitization model: effect of a cocoa-enriched diet"

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- VIII Workshop de la Sociedad Española de Probióticos y Prebióticos (SEPyP), Santiago de Compostela, febrer de 2017. Camps-Bossacoma M, Pérez-Cano FJ, Franch A, Castell M. "Estudio metagenómico de la microbiota intestinal tras una sensibilización oral y una dieta rica en cacao en rata".

Resum ARTICLE 4

Objectiu: Tant la sensibilització oral com la ingesta de cacau influeixen sobre el sistema immunitari intestinal i, considerant la relació que existeix entre aquest compartiment en la microbiota intestinal, seguidament es va voler establir l'efecte de la sensibilització i de la dieta rica en cacau sobre la microbiota intestinal.

Material i mètodes: Rates femelles Lewis de 3 setmanes d'edat varen ser sensibilitzades com els anteriors estudis (articles 2 i 3). Després de 4 setmanes d'intervenció nutricional, es va portar a terme l'anàlisi metagenòmica de les mostres fecals. A més, es va quantificar la IgA intestinal a partir d'homogenats fecals (tècnica ELISA).

Resultats: La sensibilització oral va induir canvis a la microbiota intestinal de les rates, amb una disminució en l'abundància relativa de l'ordre *Erysipelotrichales*, en concret, d'una espècie del gènere *Allobaculum*, de *Clostridium metallolevans* i d'una espècie de l'ordre de *Bacteroidales*. A més, entre altres, va afavorir l'aparició d'espècies dels gèneres *Bacillus, Christensenella* i *Anaeroplasma*. Per altra banda, les rates sensibilitzades oralment i alimentades amb la dieta rica en cacau, varen presentar diversos canvis quantitatius i qualitatius. El cacau va modificar l'abundància relativa i absoluta dels diferents fílums detectats, en concret, va incrementar la proporció de *Tenericutes* i *Cyanobacteria*, va afavorir l'aparició de *Prevotella copri, Anaerostipes* sp., *Ralstonia* sp. i la desaparició de *Clostridium perfringens* i *Blautia productia*, entre altres. La dieta 10% cacau va atenuar la creixent concentració d'IgA intestinal sent evident a partir del 7è dia d'estudi.

Conclusions: La dieta amb un 10% de cacau en un model de sensibilització oral en rata produeix canvis quantitatius i qualitatius a la microbiota intestinal. A més, inhibeix la síntesi d'anticossos específics i la producció d'IgA intestinal. Per tant, les modificacions sobre la microbiota intestinal podrien contribuir a l'efecte tolerogènic prèviament establert del cacau.

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Research Article

Gut Microbiota in a Rat Oral Sensitization Model: Effect of a Cocoa-Enriched Diet

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Increasing evidence is emerging suggesting a relation between dietary compounds, microbiota, and the susceptibility to allergic diseases, particularly food allergy. Cocoa, a source of antioxidant polyphenols, has shown effects on gut microbiota and the ability to promote tolerance in an oral sensitization model. Taking these facts into consideration, the aim of the present study was to establish the influence of an oral sensitization model, both alone and together with a cocoa-enriched diet, on gut microbiota. Lewis rats were orally sensitized and fed with either a standard or 10% cocoa diet. Faecal microbiota was analysed through metagenomics study. Intestinal IgA concentration was also determined. Oral sensitization produced few changes in intestinal microbiota, but in those rats fed a cocoa diet significant modifications appeared. Decreased bacteria from the Firmicutes and Proteobacteria phyla and a higher percentage of bacteria belonging to the Tenericutes and Cyanobacteria phyla were observed. In conclusion, a cocoa diet is able to modify the microbiota bacterial pattern in orally sensitized animals. As cocoa inhibits the synthesis of specific antibodies and also intestinal IgA, those changes in microbiota pattern, particularly those of the Proteobacteria phylum, might be partially responsible for the tolerogenic effect of cocoa.

1. Introduction

Apart from its nutritional role, food intake influences intestinal tissue and increasing evidence exists regarding the interaction among diet, immune system, and microbiota. Food intake determines the composition of microbiota and the function of gut-associated lymphoid tissue (GALT). These last two factors are also closely related, so a vast number of diets alter bacteria composition, thereby affecting immune homeostasis, and vice versa [1]. Gut microbiota, which in the adult human tract contains more than 100 trillion bacteria and more than 150 times the number of genes compared to the host genome [2, 3], provides several benefits to the intestinal immune system. Therefore, intestinal bacteria are critical for the regulation of the immune system and barrier function [3] and play an important role in the development of both innate and acquired response, promoting the expansion of B and T cells in Peyer's patches and mesenteric lymph nodes [4]. The intestinal immune system characteristically produces antibodies belonging to the immunoglobulin A (IgA) isotype. IgA is the most abundant immunoglobulin in the body and is considered to be the first line of defence in protecting the intestine against ingested pathogens [5].

Among the most prevalent diseases related to a faulty immune system function are allergic diseases. In Western countries, the prevalence of allergic diseases, including food allergy, is increasing and has become a major public health concern [3]. An allergic response generally occurs when antigen-presenting cells present the antigen to T helper (Th) lymphocytes, which once activated, proliferate and turn mainly into Th2 effector cells, secreting their characteristic cytokine pattern [6]. Nowadays, an association between changes in microbiota and high susceptibility to allergy is recognized [7, 8]. Therefore, the hygiene hypothesis suggests

that the later the microbial exposure, the greater the prevalence of allergic diseases [9]. It has been reported that germ-free mice undergo an increase in the development of oral allergic sensitization, which is the first step in allergy disease [10]. Therefore, microbiota is important for the induction of oral tolerance that protects from food allergies [11]. In particular, the administration of a main human bacterium, *Bacteroides fragilis* [12], and a mixture of *Clostridia* strains [13] can increase the development of regulatory T (Treg) cells and, therefore, inhibit the development of oral allergy.

As previously stated, food influences microbiota and the intestinal immune system. Among the bioactive components with this recognized action are flavonoids, a kind of polyphenols, which, besides their antioxidant properties, modulate bacterial growth and composition and which influence toll-like receptor (TLR) activation as well as inflammatory and immune response activation [14]. Oligomeric and polymeric polyphenols can reach the colon [15], and published data in human and in vitro and in vivo animal models indicate their role in changing microbiota composition (reviewed in [16, 17]). In addition, some flavonoids have shown their antiallergic potential (reviewed in [18]), a fact that could be related to their impact on the composition of gut microbiota [19]. One food relatively rich in flavonoids is cocoa, which also contains carbohydrates, proteins, lipids, fibre, minerals, and methylxanthines. A few studies have been published that discuss the effect of cocoa on gut microbiota. Feeding of 10% cocoa diet for 6 weeks in Wistar rats decreased the proportion of *Bacteroides*, the *Staphylococcus* genus, and the *Clostridium histolyticum* subgroup [20]. Another study in which three different amounts of cocoa polyphenols were given to the same rat strain for 4 weeks described the age-dependent inhibition of the growth of *Staphylococcus*, *Streptococcus*, *Clostridium histolyticum*, and *Clostridium perfringens*, which was partially attributed to their polyphenol content [21].

Recently we demonstrated the tolerogenic effect of a 10% cocoa diet on a rat oral sensitization model [22]. These results led us to ascertain whether a cocoa diet may exert its effects, at least partially, by influencing the microbiota composition in this rat oral sensitization model [22, 23]. Taking all these facts into consideration, the aim of the present study was to establish the influence of an oral sensitization model, both alone and together with a cocoa-enriched diet, on gut microbiota.

2. Materials and Methods

2.1. Animals and Diets. Female Lewis rats were obtained from Janvier (Saint-Berthevin Cedex, France) and housed in cages under controlled temperature and humidity in a 12 h light-12 h dark cycle in the Faculty of Pharmacy and Food Sciences' animal facility. The procedures used in the current study were approved by the Ethics Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref. 5988).

Three-week-old rats were randomly distributed into three groups ($n = 6$ each) according to the diet and the oral sensitization procedure: the reference (RF) group (standard diet and no oral sensitization), the ovalbumin (OVA) group (standard diet and oral OVA sensitization), and the OVA/C group (10% cocoa diet and oral OVA sensitization). The diet lasted for four weeks during which the animals had free access to food and water. AIN-93M (from Harlan Teklad, Madison, Wisconsin, USA) formula was used as the standard diet and a 10% cocoa diet was produced through modification of the standard formula, adjusting the amounts of carbohydrates, proteins, lipids, and fibre in accordance with the 10% of cocoa powder (from Idilia Foods SL, formerly Nutrexpa SL, Barcelona, Spain) as described previously [22]. The diets were isoenergetic and had the same proportion of macronutrients and micronutrients. The cocoa diet contained 40.18 mg/g of total polyphenols (expressed as catechin) determined according to Folin-Ciocalteu method.

2.2. Oral Sensitization. Rat oral sensitization was induced as previously described [22]. In brief, rats from sensitized groups received orally 50 mg of OVA (grade V; Sigma-Aldrich, Madrid, Spain) with 30 μg of cholera toxin (CT; Sigma-Aldrich) as adjuvant in 1 mL of distilled water by oral gavage, three times per week for three weeks (on days 0, 2, 4, 7, 9, 11, 14, 16, 18, and 21). However, the RF group received just 1 mL of the vehicle with the same frequency of administration. This procedure is able to induce the synthesis of specific anti-OVA antibodies [22, 23].

2.3. Sample Collection and Processing. Faecal samples were collected before oral sensitization and once per week afterwards (days 0, 7, 14, 21, and 28) and processed in order to obtain faecal homogenates as previously described [24]. Briefly, faecal samples were dried and weighed, and phosphate-buffered saline (PBS, pH 7.2) was added to obtain a final concentration of 20 mg/mL. Immediately, the mix was homogenized with a Polytron® (Kinematica, Lucerne, Switzerland) and centrifuged, and supernatants were frozen at −20°C until total IgA quantification. Moreover, fresh faecal samples from day 23 were, on the one hand, weighed, dried for 5 h at 37°C, and weighed again in order to determine the percentage of humidity as an indicator of faecal consistency and, on the other hand, used for faecal pH determination using a surface electrode (Crison Instruments, SA, Barcelona, Spain).

2.4. Quantification of Intestinal IgA. IgA from faecal homogenates was quantified using a sandwich enzyme-linked immunoabsorbent assay (ELISA) technique with a Rat IgA ELISA Quantification Set (E110-102) from Bethyl Laboratories (Montgomery, TX, USA). Briefly, 96-well plates (Nunc MaxiSorp®, Wiesbaden, Germany) were coated with 2 μg/mL of the capture antibody in carbonate buffer (pH 9.6). After blocking, the standard and the samples were incubated. Finally, an adequate dilution of the peroxidase-conjugated detection antibody was added and, after washing, an *o*-phenylenediaminedihydrochloride-H_2O_2 (OPD-H_2O_2) (Sigma-Aldrich) solution was added. Absorbance was measured in a microplate photometer. Data were interpolated by Multiskan Ascent v2.6 software (Thermo Fisher Scientific SLU, Barcelona, Spain) according to the concentration of the standard.

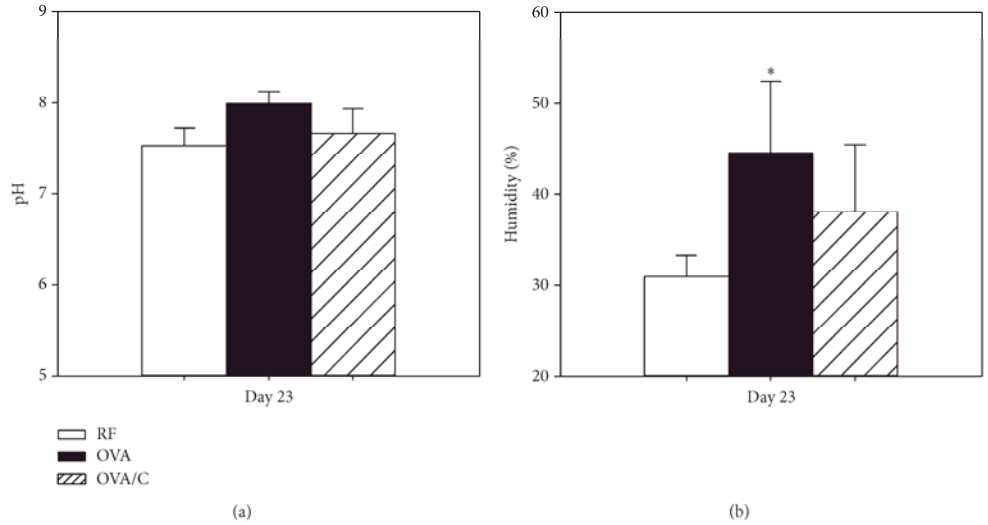

Figure 1: Faecal pH and humidity at day 23 of the study. Values are expressed as mean + standard error ($n = 6$). Percentage of humidity was calculated as follows: ((initial faecal weight − dry faecal weight)/initial faecal weight) × 100. Dry faecal weight was considered after 5 h at 37°C. Statistical differences: $^*p < 0.05$ versus RF group (Mann–Whitney U test).

2.5. Faecal Metagenomic Analysis. DNA from three representative faecal samples from each group from the 28th day of the experimental design was extracted using a FastDNA® SPIN kit (MP Biomedicals, Solon, Ohio, USA) according to the manufacturer's instructions. An Ion 16S™ Metagenomics kit (Life Technologies, Madrid, Spain) was used for the metagenomic study carried out by Bioarray Genetic Diagnosis (Bioarray, Alicante, Spain).

After confirming that all DNA samples had good levels of concentration, purity, and integrity, a massive sequentiation was carried out with the platforms QIIME v1.8.0 and USEARCH v.7.0.1090. In order to assign the taxonomy, the different sequences with 97% similarity were assembled into operational taxonomic units (OTUs) using the data base GreenGenes v13_8 with the UCLUST method.

2.6. Statistical Analysis. Statistical analysis was performed using IBM's Statistical Package for Social Sciences program (SPSS, version 22.0, Chicago, IL, USA). Differences were considered statistically significant when $p < 0.05$.

In order to determine equality of variance and normal distribution, the Levene and Kolmogorov-Smirnov tests, respectively, were carried out. One-way ANOVA and Bonferroni's post hoc test were performed on the results with equality of variance and normal distribution. The nonparametric Mann–Whitney U test was performed on the data that did not have equality of variance and/or normal distribution.

Bivariate Pearson correlation was used to determine whether an association exists between intestinal IgA concentration and either relative abundance, absolute abundance, or the number of detected bacterial species.

3. Results

3.1. Effect of Cocoa on Faecal pH and Humidity in Orally Sensitized Rats. Faecal pH and humidity were determined on day 23 of the study (Figure 1). The RF group had a faecal pH average of 7.52 and no differences were detected due to the oral sensitization or the cocoa diet (Figure 1(a)). In contrast, the orally sensitized group showed a higher faecal humidity (Figure 1(b)), exhibiting more water content than the RF group, whereas no significant differences with respect to the OVA/C group were found.

3.2. Effect of Cocoa on the Intestinal IgA Concentration in Orally Sensitized Rats. Faecal IgA determination revealed that the animals fed the standard diet, whether or not they received the oral sensitization, increased IgA concentration during the study. However, this time-dependent increase was inhibited from day 7 due to the 10% cocoa diet (Figure 2).

3.3. Effect of Cocoa on Gut Metagenome in Orally Sensitized Rats

3.3.1. Quantitative Metagenomic Study. As shown in Figure 3(a), from the total microbiota detected in reference rats, 61% of the bacteria belonged to the Firmicutes phylum, 33% to Bacteroidetes, 6% to Proteobacteria, and less than 1% to the Tenericutes, Actinobacteria, Cyanobacteria, Verrucomicrobia, and TM7 phyla. From these phyla, no significant differences were found in the OVA group with respect to the RF group. However, those sensitized rats fed a cocoa-enriched diet (OVA/C group) showed a higher proportion

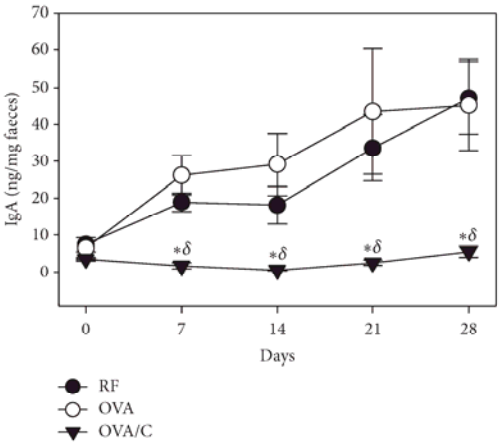

FIGURE 2: Intestinal IgA concentration during the study. Values are expressed as mean ± standard error ($n = 6$). Statistical differences: $^{*}p < 0.05$ versus RF group and $^{\delta}p < 0.05$ versus OVA group (Mann–Whitney U test).

of bacteria belonging to the Tenericutes and Cyanobacteria phyla compared to those from the RF and OVA groups.

The study of absolute bacterial abundance also revealed significant changes in animals from the OVA/C group (Figure 3(b)). The orally sensitized group fed a cocoa diet had a lower amount of total bacteria compared to the RF group, which could be attributed to a reduction in the number of Firmicutes and Proteobacteria. However, a higher proportion of Tenericutes with respect to the RF and OVA groups was observed.

Furthermore, a deeper analysis revealed significant changes in the relative abundance inside each phylum (Table 1). Oral sensitization decreased the proportion of bacteria belonging to the Erysipelotrichales order (Firmicutes phylum) in animals fed with either a standard or cocoa diet. Moreover, the cocoa diet lowered the proportion of bacteria from the RF32 order belonging to the Alphaproteobacteria class (Proteobacteria phylum). However, this diet favoured the presence of Chloroplast class (Cyanobacteria phylum), particularly the Streptophyta order, and increased the percentage of the Mollicutes class, specifically the RF39 order.

Table 2 shows the changes found at family, genus, and species level. OVA sensitization with both standard and cocoa diets decreased the relative abundance of an unknown species of the Bacteroidales order, *Clostridium metallolevans*, and *Allobaculum* sp. Moreover, animals from the OVA/C group had lower percentages of *Ruminococcus flavefaciens*, one species belonging to the Erysipelotrichaceae family, *Holdemania* sp., and one specific species of the RF32 order, compared to the RF and/or OVA groups. On the other hand, the proportion of three species of the Prevotellaceae family, a species of the Streptophyta order, *Lactobacillus reuteri*, *Anaerostipes* sp., a species of the Mogibacteriaceae and Erysipelotrichaceae families, and a species of the Mollicutes class had a higher percentage in the cocoa-fed animals (OVA/C group) with respect to the RF and/or OVA groups.

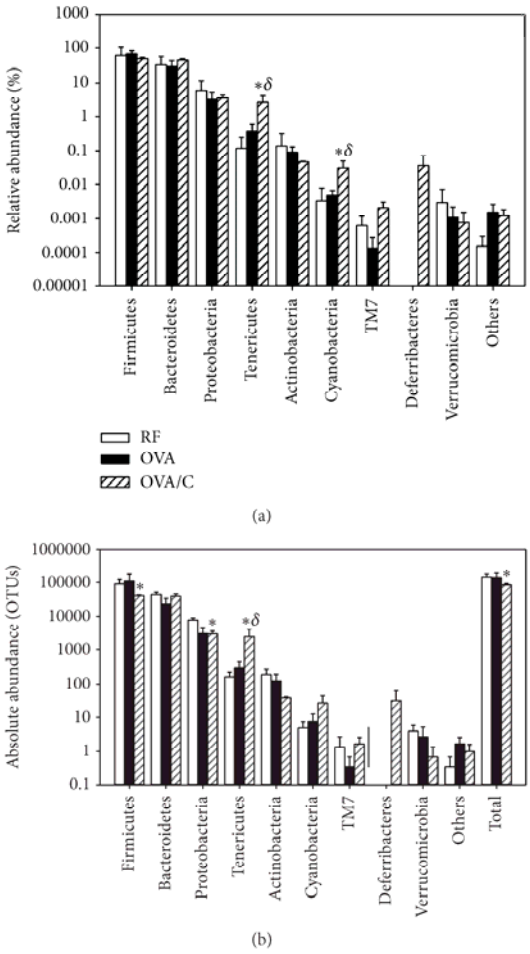

FIGURE 3: Bacteria phyla on faecal gut microbiota. (a) Relative abundance (%) and (b) absolute abundance in the groups of study. Values are expressed as mean ± standard error ($n = 3$). OTUs: operational taxonomic units. Statistical difference: $^{*}p < 0.05$ versus RF group and $^{\delta}p < 0.05$ versus OVA group (Mann–Whitney U test).

3.3.2. Qualitative Metagenomic Study. The metagenomic analysis also provides us with qualitative information about the gut bacterial pattern. The number of species present in at least two of the three rats from each group was counted. A total of 90 species were detected in the RF group, 84 species in the OVA group and 86 species in the OVA/C group. The number of different species classified into the different phyla is shown in Figure 4(a). For all animals, the highest bacteria richness was found in the Firmicutes, Bacteroidetes, and Proteobacteria phyla.

In order to establish the differences among the bacteria species found in each group, a Venn diagram was plotted (Figure 4(b)). Out of all the faecal detected species, 74 were present in the three studied groups. However, some

Table 1: Bacteria order on faecal gut microbiota. Relative abundance (%) of the statistically different significant orders of bacteria. Values are expressed as mean ± standard error ($n = 3$). Statistical difference: $^{*}p < 0.05$ versus RF and $^{\delta}p < 0.05$ versus OVA group (Mann–Whitney U test).

Phylum	Class	Order	RF	OVA	OVA/C
Cyanobacteria	Chloroplast	Streptophyta	0.000 ± 0.000	0.000 ± 0.000	0.017 ± 0.010$^{*\delta}$
Firmicutes	Erysipelotrichi	Erysipelotrichales	4.033 ± 1.368	0.999 ± 0.494*	0.716 ± 0.241*
Proteobacteria	Alphaproteobacteria	RF32	0.905 ± 0.234	0.842 ± 0.555	0.278 ± 0.065*
Tenericutes	Mollicutes	RF39	0.083 ± 0.037	0.270 ± 0.193	2.536 ± 1.662$^{*\delta}$

modifications were detected due to the oral sensitization, the cocoa diet, or both. In reference conditions, eight different species were unique in the RF group, meaning that these species disappeared due to the oral sensitization (OVA and OVA/C groups). Four of these belonged to the Firmicutes phylum, three to the Proteobacteria phylum, and one to the Verrucomicrobia phylum (Table 3). Three species from the Firmicutes phylum were included in the *Staphylococcus* genus (e.g., *S. equorum*), whereas the other one corresponded to *Clostridium metallolevans*. With regard to the Proteobacteria phylum, the three species that disappeared due to the oral sensitization procedure belonged to either the Alphaproteobacteria class (Rhodospirillales order), the Deltaproteobacteria class (*Spirobacillales* order), or the Gammaproteobacteria class (Vibrionales order). In addition, *Akkermansia muciniphila*, from the Verrucomicrobia phylum, was not found in orally sensitized groups.

In the OVA group, four new species were detected with respect to the RF animals. Three of them were only found in sensitized animals fed a standard diet and one was also present after the cocoa diet. From these new species, two belonged to the Firmicutes phylum, one to the Tenericutes phylum, and one to the Actinobacteria phylum. The Firmicutes phylum species included *Bacillus* and *Christensenella* genera, the Tenericutes phylum included the *Anaeroplasma* genus (Table 3), and the Actinobacteria phylum species also found in the OVA/C group was *Bifidobacterium pseudolongum* (Table 4).

With regard to the sensitized group fed a cocoa diet, nine different species were found with respect to the RF and OVA groups (Table 3). Two species belonged to the Bacteroidetes phylum, one of those being *Prevotella copri*. From the Cyanobacteria phylum, one species from the Streptophyta order was present. As regards the Firmicutes phylum, three species from the Clostridiales order were detected, belonging to the Dehalobacteriaceae, Lachnospiraceae and Veillonellaceae families. Moreover, two new species appeared from the Proteobacteria phylum (*Ralstonia* sp. and *Desulfovibrio* sp.), and a new TM7 bacterium was also found in the OVA/C group.

It is worth noting that two bacterial species were not found in the OVA group but were present in both the RF and OVA/C groups, suggesting that the cocoa diet failed to eliminate these species due to the oral sensitization. These bacteria belonged to the Bacteroidetes phylum, *Bacteroides uniformis* and *Prevotella* sp. in particular (Table 4). Moreover, six species present in both the RF and OVA groups disappeared with the cocoa diet: five of those belonged to the Firmicutes phylum, in particular the *Clostridia* (e.g., *Clostridium perfringens, Blautia producta,* and *Epulopiscium* sp.) and Erysipelotrichi (*Coprobacillus* sp.) classes and one to the Proteobacteria phylum, specifically *Desulfovibrio* sp. (Table 4).

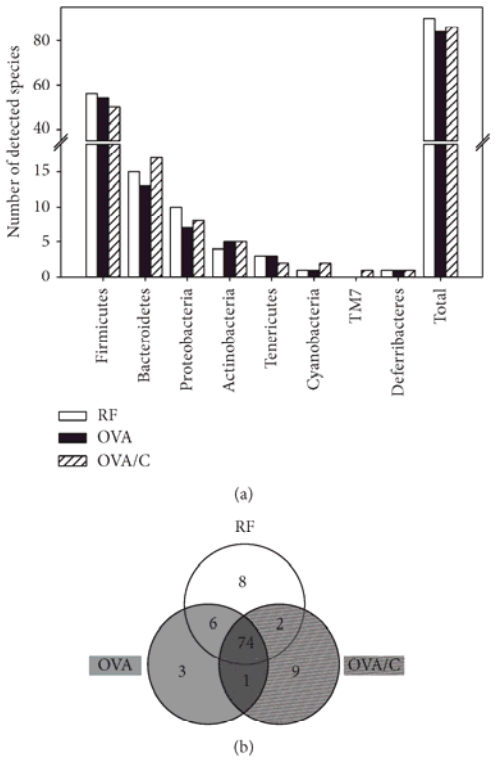

Figure 4: Number of detected species in faeces from each studied group. (a) Data is expressed as the total number of species detected. A species was detected if present in at least two of the three rats of each group. (b) Venn diagram of the different detected species. The diagram shows the absolute number of detected species that belong to each group, the detected species in common between each pair of groups, and, in the centre, the detected species in common among all three groups.

3.4. Intestinal IgA and Microbiota Associations. In order to determine whether microbiota was associated with intestinal IgA, a linear regression analysis was performed between IgA

Table 2: Summary of the significant results of relative abundance of the three groups of study. Arrows indicate significant changes ($p < 0.05$) of the first group with respect to the second one.

Phylum	Class	Order	Family	Genus	Species	OVA versus RF	OVA/C versus RF	OVA/C versus OVA
Bacteroidetes	Bacteroidia	Bacteroidales				↓	↓	=
	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	*uniformis*	=	=	↑
	Bacteroidia	Bacteroidales	Prevotellaceae	Others	Others	=	↑	↑
	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	Others	=	↑	↑
	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*		=	↑	↑
Cyanobacteria	Chloroplast	Streptophyta				=	↑	↑
Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	*reuteri*	=	↑	↑
	Clostridia	Clostridiales	Lachnospiraceae	*Anaerostipes*		=	↑	↑
	Clostridia	Clostridiales	Peptostreptococcaceae	*Clostridium*	*metallolevans*	↓	↓	=
	Clostridia	Clostridiales	Ruminococcaceae	*Ruminococcus*	*flavefaciens*	=	=	↓
	Clostridia	Clostridiales	Mogibacteriaceae			=	=	↑
	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	Others	Others	=	=	↑
	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae			=	↓	=
	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	*Allobaculum*		↓	↓	=
	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	*Holdemania*		=	↓	=
Proteobacteria	Alphaproteobacteria	RF32				=	↓	=
Tenericutes	Mollicutes	RF39				=	↑	↑

Table 3: Bacteria exclusively detected in one of the groups.

Group	Phylum	Class	Order	Family	Genus	Species
RF	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	Others
		Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	
		Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	*equorum*
		Clostridia	Clostridiales	Peptostreptococcaceae	*Clostridium*	*metallolevans*
	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		
		Deltaproteobacteria	Spirobacillales			
		Gammaproteobacteria	Vibrionales	Vibrionaceae	*Vibrio*	Others
	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	*Akkermansia*	*muciniphila*
OVA	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	
		Clostridia	Clostridiales	Christensenellaceae	*Christensenella*	
	Tenericutes	Mollicutes	Anaeroplasmatales	Anaeroplasmataceae	*Anaeroplasma*	
OVA/C	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Other	Others
		Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	*copri*
	Cyanobacteria	Chloroplast	Streptophyta			
	Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae		
		Clostridia	Clostridiales	Lachnospiraceae	*Anaerostipes*	
		Clostridia	Clostridiales	Veillonellaceae		
	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	*Ralstonia*	
		Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*	Others
	TM7	TM7-3	CW040	F16		

Table 4: Bacteria present in two of the groups.

Phylum	Class	Order	Family	Genus	Species	Groups		
						RF	OVA	OVA/C
Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	Others	Yes	Yes	No
	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*perfringens*	Yes	Yes	No
	Clostridia	Clostridiales	Lachnospiraceae	*Blautia*	*producta*	Yes	Yes	No
	Clostridia	Clostridiales	Lachnospiraceae	*Epulopiscium*		Yes	Yes	No
	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	*Coprobacillus*		Yes	Yes	No
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	*Desulfovibrio*		Yes	Yes	No
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	*uniformis*	Yes	No	Yes
	Bacteroidia	Bacteroidales	Prevotellaceae	*Prevotella*	Others	Yes	No	Yes
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*	*pseudolongum*	No	Yes	Yes

values and data from relative and absolute abundance of bacteria and the number of detected species of each phylum. As shown in Table 5, although no significant correlation for total values was found in any of the above variables, a strong positive correlation between intestinal IgA levels and Proteobacteria phylum relative abundance was found. Apart from that, no significant correlations were seen between the relative abundance, absolute abundance, or the number of detected species from each phylum and intestinal IgA concentration.

Table 5: Correlation between intestinal IgA and microbiota. Pearson's correlation between intestinal IgA concentration and data from the absolute and relative abundance of phylum and the number of detected species of each phylum ($n = 9$). Statistical difference: $^{*}P = 0.017$ (Pearson's correlation).

	Relative abundance	Absolute abundance	Number of detected species
Firmicutes	0.318	0.402	0.427
Bacteroidetes	−0.403	−0.400	−0.621
Actinobacteria	−0.375	0.253	−0.111
Proteobacteria	0.843*	0.731	0.351
Cyanobacteria	−0.535	−0.483	−0.640
Tenericutes	−0.475	−0.440	−0.522
TM7	−0.342	−0.570	−0.243
Deferribacteres	−0.402	−0.402	−0.402
Verrucomicrobia	−0.130	0.304	−0.136
Total	0.500	0.332	0.650

4. Discussion

In healthy conditions, cocoa components are able to produce some modifications in both human and rat intestinal microbiota as previously demonstrated by using FISH technique [20, 25]. The current study, by means of a metagenomic approach, was able to go more deeply into establishing the effect of a cocoa diet and also an oral sensitization procedure

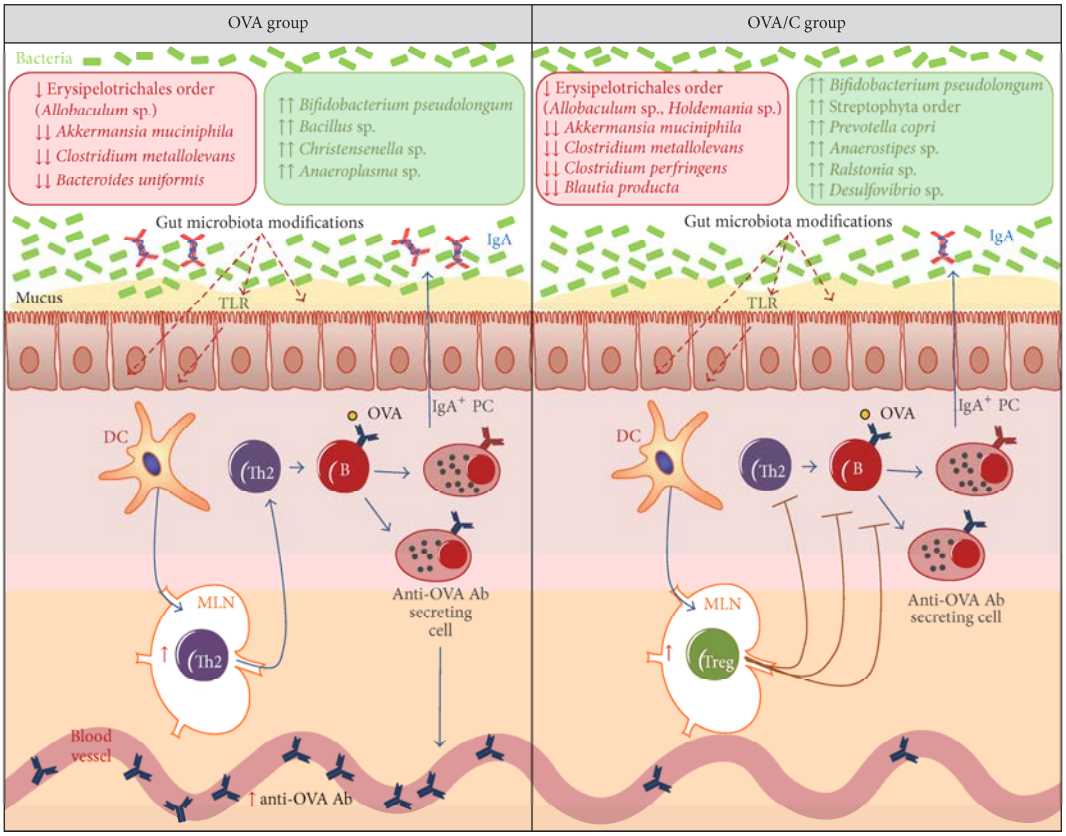

Figure 5: Summary of the changes on gut microbiota on a rat oral sensitization model fed either a standard diet or a 10% cocoa diet and its possible effects on the immune system. Discontinuous red arrows show the possible mechanism induced by the altered gut microbiota on the immune system. ↓ = decrease on the relative or absolute abundance. ↑ = increase on the relative or absolute abundance. ↓↓ = disappearance of the species. ↑↑ = appearance of the species. Ab: antibody; B: B lymphocyte; DC: dendritic cell; MLN: mesenteric lymph node; OVA: ovalbumin; PC: plasma cells; Th: T helper; TLR: toll-like receptor; Treg: T regulatory lymphocyte.

on rat gut microbiota. In this study we describe microbiota changes appearing in orally sensitized animals fed both a standard and cocoa diet (Figure 5), which means that the changes must be due to oral sensitization; moreover, we found microbiota alterations only in the orally sensitized animals fed the standard diet meaning that the cocoa diet prevented such effects induced by OVA and CT; and finally we observed microbiota modifications only in animals fed cocoa, which suggest these were mainly due to cocoa diet.

The oral sensitization did not induce any significant change at the phyla level. These results do not match studies demonstrating alterations in microbiota due to food allergy, such as increases in the abundance of bacteria from the Firmicutes phylum and decreases in those belonging to the Bacteroidetes, Proteobacteria, and Actinobacteria phyla [26, 27]. Although no significant modifications at phyla level were observed in our sensitization model, in the Firmicutes phylum, the OVA plus CT administration in both diets decreased the relative abundance of bacteria belonging to the Erysipelotrichales order, which is in line with data obtained after the oral sensitization of Il4raF709 transgenic mice [28]. A deeper analysis revealed that the oral sensitization reduced the relative abundance of the Erysipelotrichaceae family and the *Allobaculum* genus. It must be noted that the Erysipelotrichi class, and particularly the *Allobaculum* genus, have been associated with a better mucus layer in the colon [29], suggesting that their decrease reflects the alteration of the mucus layer by oral sensitization that could not be prevented by the cocoa diet. On the other hand, some qualitative changes in the microbiota composition appeared due to sensitization: new bacteria colonized the damaged mucosa (four new species) and some others could not resist the new environment (ten species disappeared), which also suggests lower diversity, which is in accordance with what happened in children with eczema [30]. With regard to the bacteria species that were not found in orally

sensitized animals, the absence of *Akkermansia muciniphila*, from the Verrucomicrobia phylum, is of particular interest. This Gram-negative anaerobic bacterium plays a role in host immune response and the restoration of mucus layer thickness and mucus production, secreting important proteins to the mucus [31], and is decreased in many diseases, such as intestinal disorders, inflammatory diseases, obesity, and type 2 diabetes [32]. *A. muciniphila* has recently been proposed as a new functional microbe with probiotic properties [33] and its absence in orally sensitized animals found here affirms its protective role.

On the other hand, the altered intestinal environment induced by the oral sensitization procedure in both standard and cocoa-fed animals led to the new colonization of the *Bifidobacterium pseudolongum*, which belongs to the less predominant *Bifidobacteria* in infants, representing in those around 2% of the *Bifidobacterium* count [34]. It has been described that *B. pseudolongum* increased differentially in rats fed two kinds of prebiotic diets [35]. Therefore, we suggest that the sensitization procedure may affect rat's diet components biodisponibility and lead to a significant difference in the gut environment that selectively enhances this particular bacteria's growth. In addition, our results are in line with the absence of these bacteria in 18-week-old healthy Wistar rats and with their abundance in animals under two other dysbiotic conditions: exercise and obesity [36].

Considering the effect of a cocoa diet on orally sensitized animals, a vast number of modifications were seen with respect to animals fed standard diets both in healthy and in sensitized conditions. The cocoa diet in this sensitization model decreased the total bacterial count similarly to healthy rats fed cocoa containing 2% polyphenols [21]. Specifically, the cocoa diet favoured the reduction of the absolute abundance of the Firmicutes and Proteobacteria phyla, whereas more Tenericutes were observed. Moreover higher relative abundance of Tenericutes and Cyanobacteria spp. was found. With regard to the increase in Cyanobacteria, this was accompanied by the appearance of bacteria belonging to the Streptophyta order in rats fed cocoa, but not in rats fed a standard diet. As far as we know, the role of such bacteria in the intestinal microbiota remains to be elucidated, and further studies must be carried out to establish the relationship between these specific bacteria and the tolerance effects of cocoa. On the other hand, the increase in the Tenericutes phylum, partially due to bacteria belonging to RF39 order (Mollicutes class), together with the appearance of a species belonging to the TM7 phylum, could be an adaptation to the fibre content of the cocoa diet because both phyla have been associated with crude fibre digestibility in pigs [37]. In addition, bacteria from the Tenericutes phylum could provide some beneficial effects in the intestinal integrity because lower counts of these bacteria were found in intestinal inflammation induced by dextran sodium sulphate [38].

Although a cocoa diet did not influence the absolute abundance of the Bacteroidetes phylum, it increased some families from this phylum. Thus, orally sensitized rats fed a coca diet increased the relative abundance of the *Prevotella* genus and *Bacteroides uniformis*. These results could be associated with cocoa's polyphenol content since they are found elevated in humans who consume red wine polyphenols daily [39], and *Prevotella* is more common in people consuming a plant-rich diet [40]. Moreover, *B. uniformis* is able to secrete antimicrobial proteins that antagonize strains of the same species [41], which could explain why the cocoa diet decreased other Bacteroidales bacteria. With regard to the *Prevotella* genus, *P. copri*, which has been associated with improvements of glucose tolerance in mice [40], appeared in orally sensitized rats fed cocoa. This could partially explain the effect on glucose tolerance by a similar cocoa diet on Zucker diabetic fatty rats [42].

The cocoa diet also influenced the bacterial pattern of the Firmicutes phylum. The cocoa diet decreased the absolute counts of these bacteria, which was accompanied not only by decreases but also increases in some particular families of bacteria. In animals fed cocoa there was a higher proportion of *Lactobacillus reuteri*, beneficial bacteria that when administered orally in humans induced the expression of proinflammatory Th1 cytokines but not the anti-inflammatory Th2 ones [43]. This effect, which is in line with the attenuation of Th2 responses by cocoa [44], might contribute to the prevention of sensitization observed here and demonstrated with an oral treatment with live *L. reuteri* in a model of airway allergy [45]. On the other hand, the cocoa diet decreased the counts of *Ruminococcus flavefaciens* and some bacteria of the Erysipelotrichaceae family, although an unknown species from the latter family increased significantly. *R. flavefaciens* are bacteria able to degrade plant cell-wall polysaccharide [46], but they were found to be decreased after a particular condensed tannins diet in bovine rumen, which suggests again that cocoa components can modify the bacterial growth pattern in the gut [47].

On the other hand, as previously described in the same oral sensitization procedure, a cocoa diet is able to induce oral tolerance and inhibit the synthesis of specific anti-OVA antibodies [22]. These effects were accompanied by an increase in TCR$\gamma\delta$ cells and CD103+CD8+ cells in mesenteric lymph nodes from cocoa-fed animals [22], cells associated with a regulatory function. In addition, as gut microbiota enhance Treg development and function [48], changes effected in the gut microbiota by cocoa could also contribute to oral tolerance throughout Treg cells (Figure 5).

Finally, here we found that both groups of rats fed a standard diet produced increasing amounts of intestinal IgA during the study period. On the other hand, the oral sensitization increased faecal water content in line with results obtained by using CT as an oral adjuvant [49]. The cocoa diet partially avoided the increase in faecal humidity and also reduced the time-dependent increase in intestinal IgA. This last effect is in line with previous results obtained in healthy conditions [50] and also confirms those derived from gut lavage and serum in the same rat oral sensitization procedure [22]. It is worth noting the correlation between intestinal IgA and the Proteobacteria phylum, whereby the more relative abundance of Proteobacteria, the higher IgA levels. These results agree with suggestions that bacteria from the Proteobacteria phylum are the main inducers of IgA by B cells [51]. B cells are responsible for the regulation of commensal bacteria producing IgA [52], so the more

relative abundance of Proteobacteria could activate B cells for IgA production, evidencing higher levels of these mucosal antibodies. Previous studies have associated the effect of a 10% cocoa diet on the reduction of IgA with gene expression modifications of several genes involved in the differentiation and maturation of B cells [53, 54]. In this sense, IL-6 gene expression is reduced by the cocoa diet [53], which could reflect a lower IL-6 secretion by dendritic cells, thus partially explaining the possible dendritic cell involvement in that process. Anyway, our results allow us to suggest that oral tolerance can be achieved with low levels of IgA, although this antibody has been associated with this kind of unresponsiveness [55].

5. Conclusions

This study demonstrates that a cocoa diet, by means of its content of antioxidant polyphenols, fibre, or other bioactive compounds, such as theobromine, is able to modify the microbiota bacterial pattern in orally sensitized animals. As cocoa inhibits the synthesis of specific antibodies and also the production of intestinal IgA, those changes in microbiota composition, particularly those of the Proteobacteria phylum, might be partially responsible for this tolerogenic effect of cocoa.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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ARTICLE 5

"Influence of hesperidin on the systemic and intestinal rat immune response"

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Els resultats del present article es presentaran al congrés següent:

- 10th Anniversary International Symposium on Immunonutrition, Madrid, juliol de 2017. Camps-Bossacoma M, Franch A, Pérez-Cano FJ, Castell M. "Immunomodulatory effect of hesperidin in immunized rats".

Resum ARTICLE 5

Objectiu: El cacau, ric en flavonoides, ha demostrat un efecte immunoregulador en rata. Per això, en aquest estudi, es va voler ampliar el coneixement del possible potencial dels flavonoides sobre el sistema immunitari i es va utilitzar un flavonoide purificat, no present en el cacau. L'objectiu d'aquest estudi va consistir en establir l'efecte de l'hesperidina sobre els teixits limfoides i la producció d'anticossos sistèmics i intestinals.

Material i mètodes: Per assolir aquest propòsit, es van realitzar dos dissenys experimentals diferents. En el primer, rates Lewis de 3 setmanes d'edat es varen immunitzar de forma intraperitoneal (i.p.) amb ovoalbúmina (OVA) i l'hesperidina es va donar per via oral (p.o.) tres vegades la setmana durant 4 setmanes a les dosis de 100 mg o 200 mg/kg. En aquest disseny, es va analitzar el fenotip dels ganglis limfàtics mesentèrics (GLM, citometria de flux), es varen estudiar les citocines produïdes per aquests limfòcits i les que hi havia a nivell intestinal (ProcartaPlex® Multiplex Immunoassay). També, es varen determinar els anticossos anti-OVA sèrics, així com la IgA intestinal (tècnica ELISA). En el segon disseny experimental, les rates varen ser sensibilitzades oralment utilitzant el model desenvolupat en el primer article de la tesi, i varen ser alimentades, durant 4 setmanes, amb una dieta que contenia 0,5% d'hesperidina. En aquest model, es va analitzar la composició de limfòcits aïllats de GLM, plaques de Peyer (PP), l'epiteli i la làmina pròpia intestinals (IEL i LPL, respectivament) (citometria de flux). També es varen obtenir mostres de sèrum i fecals on es varen determinar els anticossos específics i la IgA mitjançant tècniques d'ELISA.

Resultats: En el primer disseny experimental, l'hesperidina, administrada oralment, va reduir la proporció de limfòcits B i va incrementar la dels TCR$\alpha\beta$+ als GLM. Aquesta immunització va activar la producció de diverses citocines per part dels limfòcits de GLM, i l'hesperidina, en les dues dosis estudiades, va incrementar la síntesi d'IFN-γ. Aquest flavonoide no va modificar la producció d'anticossos específics ni la d'IgA total.

En el segon disseny experimental, la dieta amb 0.5% d'hesperidina va produir canvis en la composició de IEL i LPL de l'intestí prim. En concret, va produir un increment dels IEL TCR$\gamma\delta$+ i una disminució d'aquests a la làmina pròpia. A més, entre altres, es va observar una disminució en la proporció de LPL CD4+CD103+ i CD8+CD103+. L'administració d'hesperidina de forma continuada va provocar un increment de la IgA intestinal total, però, no va modificar la síntesi d'anticossos sèrics anti-OVA.

Conclusions: L'hesperidina en un model d'immunització i.p. modifica la composició i la funcionalitat dels limfòcits de GLM. Per altra banda, en un model de sensibilització oral, aquest flavonoide canvia la composició d'IEL i LPL i incrementa la concentració d'IgA intestinal. Tot i així, l'efecte immunomodulador de l'hesperidina no modifica la producció d'anticossos sèrics específics.

nutrients

MDPI

Article

Influence of Hesperidin on the Systemic and Intestinal Rat Immune Response

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Abstract: Polyphenols, widely found in edible plants, influence the immune system. Nevertheless, the immunomodulatory properties of hesperidin, the predominant flavanone in oranges, have not been deeply studied. To establish the effect of hesperidin on in vivo immune response, two different conditions of immune system stimulations in Lewis rats were applied. In the first experimental design, rats were intraperitoneally immunized with ovalbumin (OVA) plus *Bordetella pertussis* toxin and alum as the adjuvants, and orally given 100 or 200 mg/kg hesperidin. In the second experimental design, rats were orally sensitized with OVA together with cholera toxin and fed a diet containing 0.5% hesperidin. In the first approach, hesperidin administration changed mesenteric lymph node lymphocyte (MLNL) composition, increasing the TCRαβ+ cell percentage and decreasing that of B lymphocytes. Furthermore, hesperidin enhanced the interferon (IFN)-γ production in stimulated MLNL. In the second approach, hesperidin intake modified the lymphocyte composition in the intestinal epithelium (TCRγδ+ cells) and the lamina propria (TCRγδ+, CD45RA+, natural killer, natural killer T, TCRαβ+CD4+, and TCRαβ+CD8+ cells). Nevertheless, hesperidin did not modify the level of serum anti-OVA antibodies in either study. In conclusion, hesperidin does possess immunoregulatory properties in the intestinal immune response, but this effect is not able to influence the synthesis of specific antibodies.

Keywords: antibody; flavanone; flavonoids; hesperidin; immune system; immunoregulatory; polyphenol

1. Introduction

Polyphenols are secondary metabolites of plants that are widely distributed in fruits (e.g., apple, grape, pear, cherry, berries), vegetables, nuts, flowers, cereals, legumes, chocolate, and beverages (tea, coffee, and wine) [1]. Polyphenols, named thus for the presence of various phenolic groups [2], are mainly classified according to their chemical structure into flavonoids (isoflavones, neoflavonoids, chalcones, flavones, flavonols, flavonones, flavononols, flavanols, proanthocyanidins, and anthocyanidins) or non-flavonoids (phenolic acids or phenolic amides) [3].

In the last 20 years, polyphenols have gained attention mainly due to their antioxidant properties [3,4], and a large number of beneficial effects have been reported such as on degenerative disease, cardiovascular disease, cancer, osteoporosis; and their influence on the immune system has also been shown [2,5,6]. Focusing on polyphenol immunomodulatory properties, a number of in vitro, in vivo, and clinical studies have confirmed the influence of various flavonoids on the innate and acquired immune response by attenuating immune function, thus showing their beneficial role in

immune hypersensitivity [7]. Accordingly, flavonoid administration has been demonstrated to be useful in the prevention of allergic asthma and rhinitis [8].

Hesperidin (5,7,3-trihydroxy-4-methoxyflavanone-7-rhamnoglucoside) is a flavonoid belonging to the flavanone class [9], its aglycone form being hesperetin [10]. Hesperidin is mainly found in the fruits of the genus *Citrus* [1], particularly in the epicarp, mesocarp, endocarp, and juice of citrus fruits [11] and it is the predominant flavanone found in oranges [12,13]. The majority of the flavonoids found in citrus fruits are glycosides and just a little quantity of hesperitin is present [12].

To date, several pharmacological effects of hesperidin have been reported. It prevents hypercholesterolaemia and fatty liver [14], osteoporosis [15], hypertension, and cerebral thrombosis, among others [16]. In terms of its effects on the immune system, the role of hesperidin has been described in reducing Th2 cytokines in mouse models of asthma [9,17] and in stimulated macrophages [18]. Nevertheless, there are no in-depth studies concerning hesperidin's effect on immune tissues, including the intestinal lymphoid tissue, and on specific antibody synthesis. In this line, the study of such effects on animal models is of interest because it allows the arrival of hesperidin or its metabolites to the lymphoid tissues, and its analysis will contribute to a better understanding of a flavanone-enriched diet on human health. For this reason, the aim of the current study was to spotlight the effects of hesperidin on Th2 antibody production and on lymphoid tissues, focusing on the gut-associated lymphoid tissue (GALT), which is the first line of defence encountered by the hesperidin present in food. We have investigated this action under two different conditions triggering Th2 immune responses and using three different hesperidin dosages.

2. Materials and Methods

2.1. Chemicals

Hesperidin was provided by Ferrer HealthTech (Murcia, Spain), with a purity of 95.5% (High Performance Liquid Chromatography) containing 2% isonaringine, 1.5% didimine, and other impurities.

Carboxymethylcellulose (CMC), cholera toxin (CT), fetal bovine serum (FBS), L-glutamine, ovalbumin (OVA, grade V), penicillin-streptomycin, toxin from *Bordetella pertussis* (Bpt), and RPMI 1640 medium were provided by Sigma-Aldrich (Madrid, Spain). Imject™ alum adjuvant was obtained from Thermo Fisher Scientific (Barcelona, Spain). Biotin-conjugated anti-rat immunoglobulin (Ig)A, IgG1, IgG2a, IgG2b, and IgG2c monoclonal antibodies, anti-rat IgE monoclonal antibody, and anti-rat fluorochrome-conjugated monoclonal antibodies (detailed later) were purchased from BD Biosciences (Madrid, Spain), Biolegend (San Diego, CA, USA), or Novus Biologicals (Littleton, CO, USA). Peroxidase conjugated and unconjugated goat anti-rat IgA antibody and IgA standard were provided by Bethyl Laboratories (Montgomery, TX, USA). Peroxidase-conjugated anti-rat Ig was from DakoCytomation (Glostrup, Denmark). 2-β-mercaptoethanol was from Merck (Darmstadt, Germany). Ketamine was provided by Merial Laboratories S.A. (Barcelona, Spain) and xylazine by Bayer A.G. (Leverkusen, Germany).

2.2. Animals and Experimental Designs

Three-week-old Lewis rats (Janvier Labs, Saint Berthevin CEDEX, France) were maintained at the animal facility of the Faculty of Pharmacy and Food Science (University of Barcelona) housed in cages (three rats per cage) and kept under controlled conditions of temperature and humidity in a 12 h light-dark cycle. Animal procedures were approved by the Ethical Committee for Animal Experimentation at the University of Barcelona (CEEA/UB ref. 5988) and conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

The effect of hesperidin on systemic and intestinal immune response was studied in two experimental designs (Figure 1). The first design studied the influence of hesperidin in a systemic immune response that was triggered by an intraperitoneal (i.p.) immunization, as previously

described [19]. Briefly, rats received an i.p. injection with 0.5 mg of OVA plus 50 ng of *Bordetella pertussis* toxin (Bpt) in 0.5 mL of alum emulsion (1:3 alum:OVA+Bpt solution). Hesperidin was given by oral gavage three times per week at doses of 100 or 200 mg/kg of rat body weight (BW). Therefore, the first experimental design included three groups: the reference immunized group (OVAip group), the immunized group given 100 mg/kg hesperidin (H100 group), and the immunized group given 200 mg/kg hesperidin (H200 group). Hesperidin was prepared daily in 0.5% CMC as vehicle. The OVAip group received the vehicle. In the second design, the effect of hesperidin on the intestinal immune response was triggered in orally sensitized rats and was included in the rat food. For this, rats were orally sensitized with OVA and CT, as previously described [20], and animals were fed either a standard diet (AIN-93M, Harlan Teklad, Madison, WI, USA) (reference sensitized group: OVAoral group), or a diet containing 0.5% hesperidin (H0.5 group). In both designs, the animals had free access to water and food throughout the study. The consumption of water and food per cage was periodically registered and referred to as water or food consumed per 100 g of BW of the rats included in the cage.

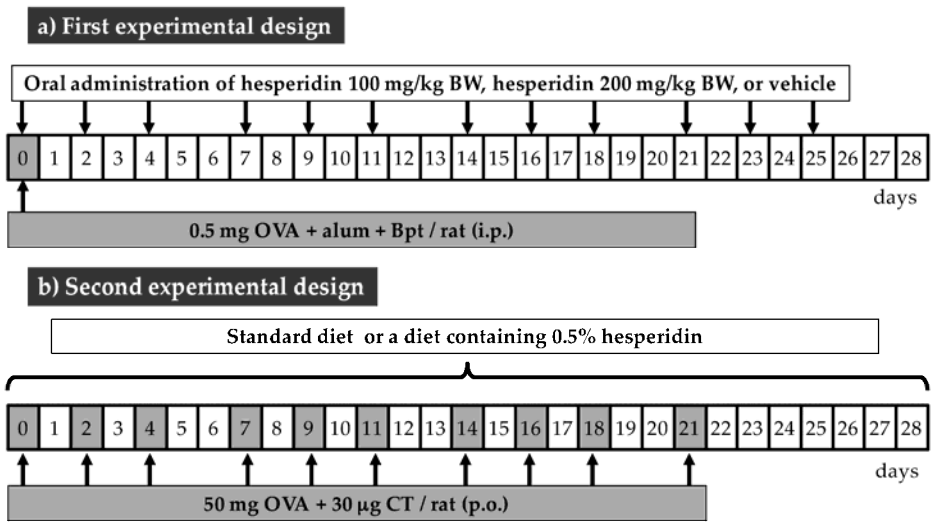

Figure 1. Experimental designs. (**a**) First experimental design: rats were immunized by intraperitoneal (i.p.) route the first day of the study (day 0), and hesperidin was given by oral gavage three times per week (indicated by arrows) for 4 weeks; (**b**) Second experimental design: rats were sensitized by oral route (*per os*, p.o.) three times per week (indicated by arrows), and hesperidin was included in the rat food throughout the 4 weeks. BW, body weight; OVA, ovalbumin; CT, cholera toxin.

2.3. Sample Collection and Processing

At the end of both studies, animals were anaesthetized by subcutaneous route with ketamine-xylazine. Apart from faecal and blood samples, the mesenteric lymph nodes (MLN) and the small intestine were collected. In the second design, the duodenal part of the intestine was discarded and the rest was opened lengthwise in order to separate the Peyer's patches (PP). From the ileum, intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were isolated, as reported previously [21]. From the resting tissue, gut lavage was obtained as previously described [21] and kept at −20 °C for IgA quantification and at −80 °C for cytokine determination.

The lymphocytes from MLN (MLNL) and from PP (PPL) were also isolated, as detailed in prior research [21,22]. Isolated lymphocyte counts and viability were determined by a Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific, Barcelona, Spain) in order to proceed with the staining for the flow cytometric analysis or the culture of MLNL.

Blood samples were centrifuged and serum was kept at −20 °C for antibody determination. From faeces, faecal homogenates supernatants were obtained and kept at −20 °C for intestinal IgA quantification. Briefly, faeces were dried, weighed, diluted with PBS pH 7.2 (20 mg/mL), homogenized with a Polytron (Kinematica, Lucerne, Switzerland), and, finally, the supernatants obtained after centrifugation were kept at −20 °C for intestinal IgA quantification.

2.4. Lymphocyte Phenotypic Analysis

MLNL, PPL, IEL, and LPL were stained with fluorescent-labelled antibodies, as previously described [22]. The following fluorochrome-conjugated antibodies were used: FITC-TCRαβ, FITC-CD8β, FITC-CD25, FITC-TLR4, FITC-CD103, PE-NKR-P1A, PE-TCRγδ, PE-TLR4, PerCP-CD8α, APC-CD4, and APC-Cy8-CD45RA. Data were acquired by Gallios Cytometer (Beckman Coulter, Miami, FL, USA) in the Scientific and Technological Centers of the University of Barcelona (CCiTUB) and the analysis was performed with FlowJo v.10 software (Tree Star, Inc., Ashland, OR, USA). Results are expressed as percentages of positive cells in the lymphocyte population selected according to their forward and side scatter characteristics.

2.5. Specific Anti-OVA Antibodies and Intestinal IgA Quantification

The levels of the specific anti-OVA antibodies (total, IgG1, IgG2a, IgG2b, and IgG2c isotypes) were determined by an indirect Enzyme-Linked ImmunoSorbent Assay (ELISA), as previously described [22]. Specific anti-OVA IgE was measured with a modified ELISA, as formerly reported [19]. In all cases, a pool of sera from immunized rats was used as positive control and all data were calculated in accordance with the arbitrary units (A.U.) assigned to this pool.

Total IgA concentration from serum, gut lavages, or faecal homogenates was determined with a sandwich ELISA using a Rat IgA ELISA Quantification Set (E110-102) from Bethyl Laboratories (Montgomery, TX, USA).

2.6. Cytokine Quantification

MLNL (6×10^6/mL) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 IU/mL penicillin-streptomycin, 2 mM L-glutamine, and 0.05 mM 2-β-mercaptoethanol and stimulated with 200 mg/mL of OVA in vitro. After 72 h, supernatants were collected to assess cytokine production.

The cytokines secreted by MLNL and from gut lavage were evaluated by ProcartaPlex® Multiplex Immunoassay (Affymetrix, eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. The analysed cytokines were interleukin (IL)-10, IL-4, monocyte chemoattractant protein (MCP)-1, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ, their detection limits being 11.08, 1.03, 17.99, 3.91, and 4.64 pg/mL, respectively.

2.7. Statistical Analysis

Statistical analysis of the data was performed with the software package SPSS version 22.0 (IBM Statistical Package for the Social Sciences, Chicago, IL, USA).

To assess the homogeneity of variance and the distribution of the results, Levene's and Shapiro-Wilk tests were performed, respectively. One-way ANOVA followed by Bonferroni's post hoc test were carried out in cases with homogenized and normally distributed variance from the data. Kruskal-Wallis and Mann-Whitney U tests were performed in cases with non-homogenized and/or non-normally distributed variance from the data. Significant differences were considered when $p \leq 0.05$.

3. Results

3.1. Effect of Hesperidin on Food and Water Intake and Body Weight

The administration of 100 or 200 mg/kg hesperidin by oral gavage altered neither food nor water consumption in comparison to the reference group (OVAip group) (Table 1). Likewise, the inclusion of hesperidin in the food did not produce any change among groups in food or water intake (Table 2). Moreover, the administration of hesperidin, both by oral gavage or in the food, did not affect BW increase (data not shown).

Table 1. Food and water intake in the first experimental design. These values were established per day and per cage and referred to 100 g of the total BW in the cage. Data are expressed as the range between the two values obtained from two cages. OVAip, the reference immunized group; H100, the immunized group given 100 mg/kg hesperidin; H200, the immunized group given 200 mg/kg hesperidin.

	Food Intake (g/100 g BW/Day)			Water Intake (mL/100 g BW/Day)		
	OVAip	**H100**	**H200**	**OVAip**	**H100**	**H200**
Day 4	13.06–13.97	12.80–13.07	13.59–13.92	11.73–11.74	11.52–15.78	11.95–12.11
Day 11	13.51–13.57	7.75–8.64	10.20–10.47	11.31–12.66	11.26–12.39	12.15–12.74
Day 18	11.60–12.03	11.94–12.02	11.82–12.24	10.39–11.03	11.07–13.83	11.42–12.11
Day 25	9.32–9.34	9.35–9.35	9.07–9.08	11.01–11.20	12.68–14.55	13.19–14.85
Day 28	9.58–10.16	9.38–10.03	9.73–9.85	11.77–12.87	13.95–15.61	14.05–15.05

Table 2. Food and water intake in the second experimental design. These values were established per day and per cage and referred to 100 g of the total BW in the cage. Data are expressed as the range between the two values obtained from two cages. OVAoral, animals were fed a standard diet; H0.5, a diet containing 0.5% hesperidin.

	Food Intake (g/100 g BW/Day)		Water Intake (mL/100 g BW/Day)	
	OVAoral	**H0.5**	**OVAoral**	**H0.5**
Day 7	10.93–11.37	10.74–10.97	16.84–24.85	14.93–20.67
Day 14	11.65–11.70	11.28–11.52	11.39–14.45	11.05–16.23
Day 21	10.25–10.60	10.76–10.77	9.01–10.57	9.95–14.33
Day 28	8.33–8.55	7.90–8.57	9.57–10.52	8.24–12.44

3.2. Effect of 100–200 mg/kg Hesperidin on Mesenteric Lymph Node Lymphocyte Composition and Functionality

The influence of hesperidin administration on the lymphocyte composition of mesenteric lymph nodes was established (Figure 2). In comparison to the OVAip group, hesperidin, in both tested doses, increased the proportion of TCRαβ+ cells (107% in both doses) in MLNL and, consequently, decreased the proportion of B (CD45RA+) lymphocytes (81% and 77% for H100 and H200 doses, respectively) (Figure 2a), thus increasing the ratio of TCRαβ+/B cells (Figure 2b). The changes were not dose-dependent. No significant differences were seen in the two TCRαβ subsets, Th (TCRαβ+CD4+) and Tc (TCRαβ+CD8+) cells, meaning that both subsets were increased by hesperidin administration (Figure 2c–d). The expression of CD25 (a cell activation marker) was also determined in CD4+, CD8+, and B cells. A decrease in the proportion of CD8+CD25+ cells was observed only in the rats receiving the highest dose of hesperidin with respect to the OVAip group (Figure 2e).

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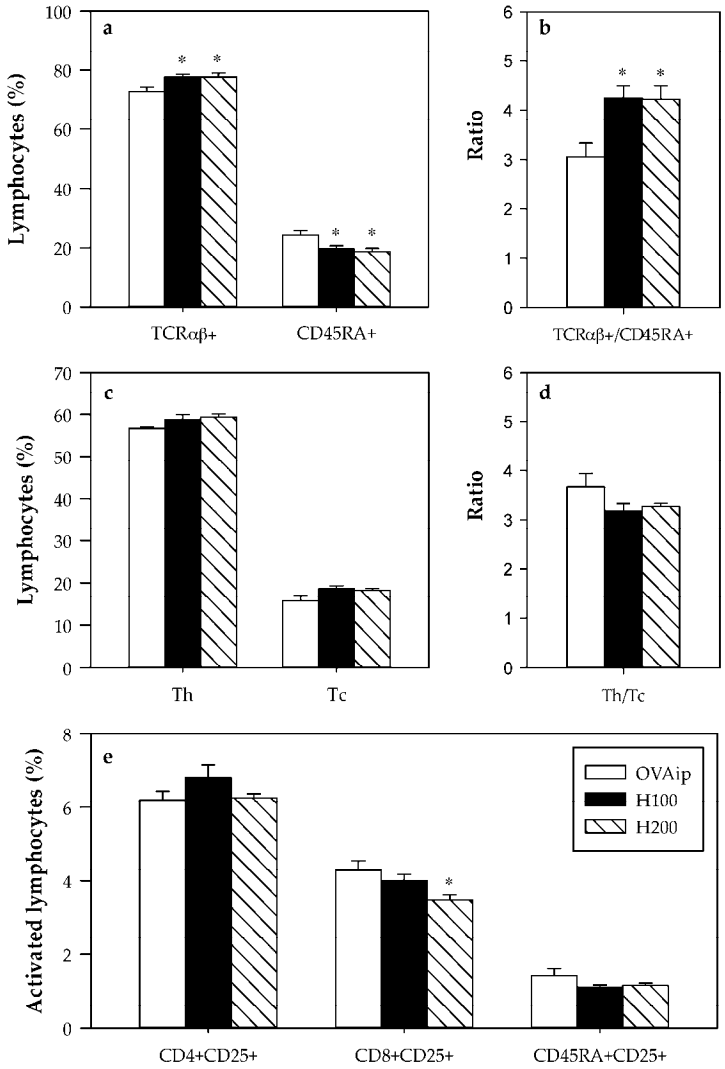

Figure 2. Proportion of mesenteric lymph node lymphocytes (MLNL) according to their phenotype in the first experimental design. (**a**) TCRαβ+ and CD45RA+ lymphocytes; (**b**) TCRαβ+/CD45RA+ ratio; (**c**) Th (TCRαβ+CD4+) and Tc (TCRαβ+CD8+) lymphocytes; (**d**) Th/Tc ratio; (**e**) CD25+ cells in CD4+, CD8+, and CD45RA+ lymphocytes. Data are expressed as mean ± standard error (n = 6). Statistical difference: * $p < 0.05$ (by Mann-Whitney U).

To establish the function of MLNL, the cytokine pattern secreted by these cells after in vitro stimulation with OVA was determined (Figure 3a). Hesperidin administration, in both doses, induced an increase in the release of IFN-γ (145% and 150% with respect to the OVAip group, for H100 and H200 doses, respectively), a Th1-related cytokine. No differences in the secretion of IL-4, IL-10, TNF-α, and MCP-1 were observed.

In addition, cytokines in gut lavage from the first experimental design were also determined, reflecting their spontaneous secretion (Figure 3f–j). In this compartment, hesperidin did not modify the production of the considered cytokines.

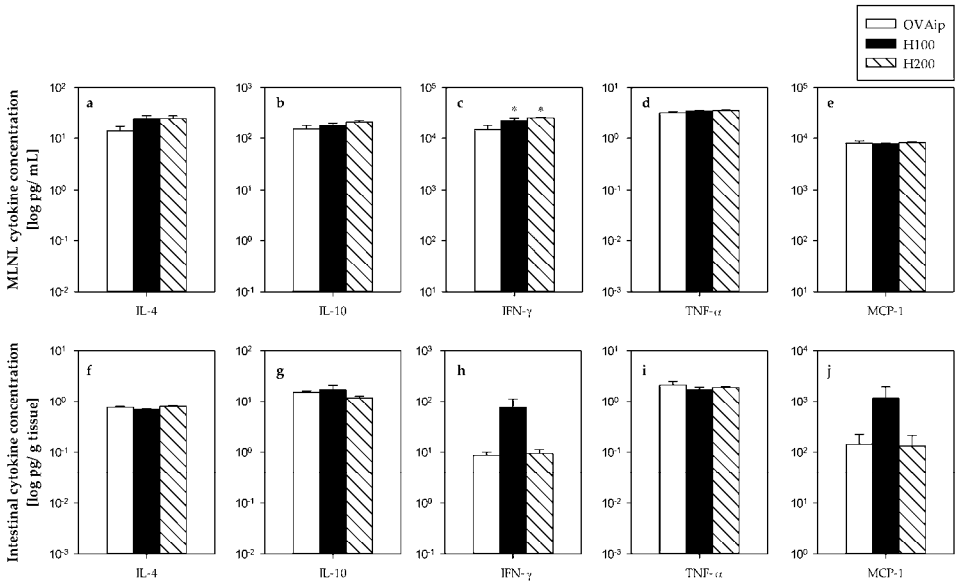

Figure 3. Cytokine concentrations in the second experimental design. Cytokines from stimulated MLNL (**a**–**e**) and gut lavage (**f**–**j**). Data are expressed as mean ± standard error (n = 6). Statistical difference: * $p < 0.05$ (by Mann-Whitney U).

3.3. *Effect of 100–200 mg/kg Hesperidin on Antibody Synthesis and Intestinal IgA*

The untreated i.p. immunized group (OVAip) developed systemic anti-OVA antibodies (Figure 4a) and no changes were seen after hesperidin administration. No serum IgE anti-OVA antibodies were detected in any of the studied groups.

Additionally, intestinal IgA was determined in gut lavage and in faeces (Figure 4b,c) but no modifications were produced as a result of the hesperidin administration.

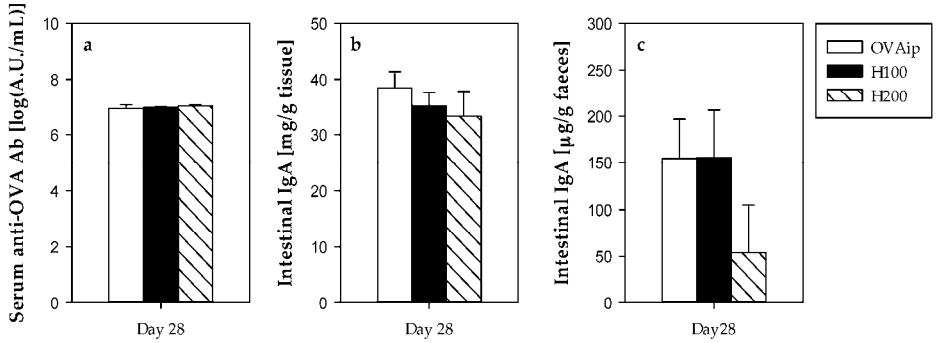

Figure 4. Anti-OVA antibodies (Ab) and total immunoglobulin (Ig)A levels from the first experimental design. (**a**) Serum anti-OVA Ab at the last day of the study; Total IgA from (**b**) gut lavage and (**c**) faecal homogenates from the last day of the study. Data are expressed as mean ± standard error (n = 6).

3.4. *Effect of 0.5% Hesperidin on Intestinal Lymphocyte Composition*

A second experimental design was then carried out focusing on the intestinal immune system, including both inductive (MLN, PP) and effector (IEL, LPL) compartments and using an oral sensitization process that challenged these specific sites. As the hesperidin intake in the first experimental design did not affect the synthesis of anti-OVA antibodies, the second approach applied hesperidin in a more continuous manner (included in the food) and using a higher dose. Therefore, the effect of 0.5% hesperidin intake on lymphocyte composition in a rat oral sensitization model was studied, analysing the phenotype of both inductive (MLNL and PPL) and effector sites (IEL and LPL) of the GALT.

No differences were seen as a result of the intake of the 0.5% hesperidin diet on MLNL or PPL (Table 3). In particular, in the mesenteric lymph nodes, the proportion of B (CD45RA+), T (TCRαβ+ and TCRγδ+), and natural killer (NK) cells, as well as that of TCRαβ+CD4+, TCRαβ+CD8+, TCRαβ+NK, CD4+CD25+, CD4+CD62L+, CD8+CD25+, and CD8+CD62L+ cells, was similar between the OVAoral and the H0.5 groups. Likewise, in the Peyer's patches, the proportion of B, T, and NK cells did not differ between groups, and that of TCRαβ+CD4+, TCRαβ+CD8+, TCRαβ+NK, TLR4+ (including CD45RA+TLR4+, CD4+TLR4+, CD8+TLR4+), CD45RA+CD25+, CD4+CD25+, and CD8+CD25+ cells also remained unchanged. Interestingly, the 0.5% hesperidin diet modified the proportion of the lymphocytes in the effector sites of the GALT (Table 3). In particular, hesperidin intake increased the percentage of TCRγδ+ cells in IEL (140%) in comparison to the reference group (OVAoral group), which was due to an increase in both TCRγδ+CD8αα+ and TCRγδ+CD8αβ+ subsets, although, in this compartment, hesperidin did not significantly modify other important lymphocytes such as TCRαβ+ (and any of their CD4+, CD8+, and natural killer T –NKT- cell subsets) and NK cells. With regard to LPL, the 0.5% hesperidin diet increased the proportion of B (CD45RA+) cells to 180% and decreased that of TCRγδ+ and NK cells (35% and 29%, respectively) with respect to orally sensitized animals (OVAoral group). In addition, although the total TCRαβ+ population was not significantly modified, the percentage of TCRαβ+CD4+ cells increased (132%), whereas that of TCRαβ+CD8+ cells and NKT cells decreased (52% and 42%, respectively) with respect to the OVAoral group. Likewise, the hesperidin-enriched diet intake decreased the percentage of both CD4+ and CD8+ LPL expressing the CD103+ (50% and 60%, respectively, from that found in the OVAoral group).

3.5. *Effect of 0.5% Hesperidin on Antibody Synthesis and Total IgA*

The oral sensitization procedure applied induced the development of serum anti-OVA antibodies. However, as in the first experimental design, this immune response was not modified by hesperidin (Table 4). In order to find out what happened in Th1/Th2-associated antibody isotypes, the concentration of specific IgG1, IgG2a, IgG2b, IgG2c, and IgE antibodies was determined. The oral sensitization caused the synthesis of antibodies belonging to IgG1, IgG2a, and IgG2b isotypes, as previously reported [20,22]. The 0.5% hesperidin-enriched diet did not significantly modify the levels of these anti-OVA antibodies (Table 4). Specifically, IgG2c and IgE were not detectable in any group.

In addition, intestinal and serum IgA was assessed after four weeks of the nutritional intervention. In this approach, hesperidin intake produced an increase in intestinal IgA, whereas no changes were found with respect to serum IgA (Table 4).

Table 3. Proportion of MLNL, Peyer's patches lymphocytes (PPL), intraepithelial lymphocytes (IEL), and lamina propria lymphocytes (LPL) according to their phenotype in the second experimental design. Data are expressed as mean ± standard error ($n = 6$). Statistical difference: * $p < 0.05$ (by Mann-Whitney U).

	MLNL		PPL		IEL		LPL	
Lymphocytes (%)	**OVAoral**	**H0.5**	**OVAoral**	**H0.5**	**OVAoral**	**H0.5**	**OVAoral**	**H0.5**
CD45RA+	12.30 ± 0.38	12.20 ± 0.29	58.90 ± 4.29	59.81 ± 3.81	10.54 ± 2.88	10.89 ± 0.88	25.86 ± 6.25	46.10 ± 6.11 *
TCRαβ+	77.21 ± 0.66	77.15 ± 0.72	16.16 ± 0.61	17.74 ± 1.12	41.59 ± 2.27	37.72± 3.13	29.65 ± 4.12	21.00 ± 2.78
TCRγδ+	1.50 ± 0.04	1.45 ± 0.03	0.67 ± 0.09	0.92 ± 0.10	11.60 ± 1.21	16.22 ± 1.63 *	3.81 ± 0.60	1.32 ± 0.40 *
TCRγδ+CD8αα+					73.12 ± 4.60	80.50 ± 3.39		
TCRγδ+CD8αβ+					26.88 ± 4.60	19.49 ± 3.39		
NK	0.32 ± 0.03	0.26 ± 0.03	3.31 ± 0.45	2.92 ± 0.27	24.33 ± 2.52	22.24 ± 1.82	13.81 ± 2.14	3.97 ± 1.06 *
TCRαβ+CD4+	77.03 ± 0.66	77.22 ± 0.62	65.28 ± 1.38	68.69 ± 1.76	22.10 ± 6.99	13.07 ± 3.60	60.45 ± 6.92	80.08 ± 5.55 *
TCRαβ+CD8+	22.76 ± 0.69	22.58 ± 0.49	24.73 ± 1.17	23.37 ± 1.32	71.88 ± 6.43	81.00 ± 3.45	34.63 ± 5.99	18.09 ± 1.45 *
TCRαβ+NK	0.61 ± 0.02	0.58 ± 0.04	6.95 ± 0.98	5.33 ± 0.59	4.95 ± 0.63	4.47 ± 0.54	3.47 ± 0.76	1.46 ± 0.23 *
TLR4+			37.76 ± 2.33	33.04 ± 2.74	5.43 ± 0.65	7.83 ± 1.12	6.88 ± 1.60	7.39 ± 2.41
CD45RA+TLR4+			41.41 ± 5.34	32.46 ± 4.10				
CD45RA+CD25+			3.96 ± 0.32	3.65 ± 0.61				
CD4+CD25+	5.58 ± 0.15	5.38 ± 0.21	10.79 ± 0.96	9.22 ± 0.86				
CD4+CD62L+	66.99 ± 2.15	64.42 ± 4.29			5.99 ± 3.46	1.75 ± 0.81	1.77 ± 1.09	0.81 ± 0.21
CD4+CD103+					91.09 ± 4.56	96.03 ± 0.98	19.02 ± 2.30	9.62 ± 1.59 *
CD4+TLR4+			13.41 ± 2.12	11.15 ± 1.43				
CD8+CD25+	3.95 ± 0.13	4.21 ± 0.32	53.01 ± 0.98	54.87 ± 3.98				
CD8+CD62L+	63.78 ± 2.99	60.35 ± 5.99			38.92 ± 8.70	19.18 ± 7.76	1.34 ± 0.49	1.77 ± 0.61
CD8+CD103+					11.83 ± 2.74	15.36 ± 2.37	77.70 ± 5.86	46.69 ± 11.97 *
CD8+TLR4+			36.48 ± 5.30	34.03 ± 3.89				

Table 4. Serum and intestinal anti-OVA and total antibodies from the second experimental design after 0.5% hesperidin diet. Data are expressed as mean ± standard error (n = 6). ND: not detectable. Statistical difference: * $p < 0.05$ (by ANOVA).

	Group	
	OVAoral	**H0.5**
Serum total Ig anti-OVA [log(U.A./mL)]	3.45 ± 0.32	3.16 ± 0.34
Serum IgG1 anti-OVA [log(U.A./mL)]	1.52 ± 0.26	1.86 ± 0.29
Serum IgG2a anti-OVA [log(U.A./mL)]	1.40 ± 0.34	1.21 ± 0.50
Serum IgG2b anti-OVA [log(U.A./mL)]	1.48 ± 0.05	1.65 ± 0.23
Serum IgG2c anti-OVA	ND	ND
Serum IgE anti-OVA	ND	ND
Serum total IgA (µg/mL)	5.72 ± 0.15	5.36 ± 0.24
Intestinal total IgA (µg/g faeces)	34.60 ± 6.79	58.07 ± 7.61 *

4. Discussion

The current study shows the effect of hesperidin in two different approaches related to Th2 immune responses to ovalbumin: an i.p. immunization with the allergen Bpt and alum, and an oral sensitization with the allergen plus cholera toxin. It was found that the administration of hesperidin in i.p. immunized rats modified MLNL composition and functionality. Moreover, in orally sensitized rats, this flavanone changed the proportions of IEL and LPL and increased intestinal IgA content. However, hesperidin did not affect anti-OVA antibody production in any of the studied immune system stimulations.

First of all, we wanted to establish the effect of the hesperidin administration in an i.p. immunization model, triggering a systemic immune response. The hesperidin doses in this case were in accordance with the quantity given to rats to protect against gentamicin nephrotoxicity [23]. The i.p. immunization was performed using the adjuvants alum (enhancer of Th2 response [24]) and Bpt (considered a potent agent to elicit IgE response [25]), as was previously reported in Brown Norway rats [19]. However, this i.p. immunization in Lewis rats was not able to induce the production of anti-OVA IgE, contrary to when using the Brown Norway strain, a high IgE responder [26]. In addition, although a specific antibody response was induced, hesperidin did not modify the levels of such antibodies.

In order to establish the influence of hesperidin on the lymphocyte composition and function, MLNL were analysed. We observed that both doses of hesperidin used here were able to increase TCRαβ+ cell percentage and decrease that of B lymphocytes. This effect was opposite to results observed after cocoa flavonoids intake [22]. The imbalance between the proportions of TCRαβ+ cells and B cells could be due to an increase in the number of TCRαβ+ lymphocytes and/or a decrease in the number of B lymphocytes. In agreement with these results, some flavonoids have demonstrated their ability to reduce B cell viability [2,27]. In particular, catechin, a green tea flavanol, induces apoptosis of human malignant B cells [28]. Therefore, the effect of hesperidin on reducing B cell numbers may not be disregarded, although it is not reflected in antibody production. Consequently, further studies are necessary to confirm the potential of hesperidin in expanding TCRαβ+ cells or reducing B cell numbers.

Apart from the TCRαβ+ and B lymphocyte proportions, other TCRαβ+ subsets were determined. No changes in the Th (TCRαβ+CD4+) and Tc (TCRαβ+CD8+) cells were observed as a result of hesperidin administration, but a significant decrease in the proportion of CD8+CD25+ cells was found after the administration of 200 mg/kg hesperidin. The surface CD25 molecule is expressed in activated cells, and an increased number of blood T CD25+ cells in asthmatic patients has been reported [29]. Although we did not use a model of asthma, our results in terms of Tc CD25+ cell proportion would correspond with the anti-asthmatic effects of hesperidin [9].

On the other hand, stimulated MLNL induced the production of cytokines related to Th1 and Th2 responses. The administration of either 100 or 200 mg/kg hesperidin increased the amount of IFN-γ released from stimulated MLNL, although no changes were found in Th2 cytokines. IFN-γ is a product of Th1 cells that exerts inhibitory properties on Th2 differentiation [30], and its downregulation seems crucial for the development of allergic diseases [31]. Therefore, the increase of this type of Th1 cytokine suggests another hesperidin mechanism involved in the attenuation of allergic asthma. In addition, it has been reported that the administration of 5 mg/mL of hesperidin in a mouse model of allergic asthma inhibited the IL-4 production in splenocytes and the IL-5 concentration in the bronchoalveolar fluid [9]. Overall, these data suggest that hesperidin displays an anti-allergic action by increasing Th1 cytokines or decreasing Th2 cytokines.

Despite the results found after inducing a systemic immune response, we aimed to focus on the intestinal immune response, studying the effect of hesperidin on the GALT using a previously established model of oral sensitization [20]. As the applied doses of hesperidin did not influence antibody synthesis, we increased the dosage of flavanone, including it in the diet (0.5% hesperidin). This dosage was chosen because it has been used in previous reports, such as in the inhibition of bone loss in androgen-deficient male mice [15], in ovariectomized rats [32], and in senescent rats [33]. Considering the amount of food intake per rat, the diet with 0.5% hesperidin meant a consumption of about 360 mg/100 g BW of hesperidin per week, which was higher than that provided in the first experimental design (30 and 60 mg/100 g BW per week for 100 and 200 mg/kg hesperidin doses, respectively).

In the second approach, in contrast to the first, the hesperidin-enriched diet did not modify the composition of MLNL. Although in this second design we used a higher amount of flavanone than that used in the first approach (6- or 12-fold times higher), the inclusion of hesperidin in the food (meaning a slow intake) compared with the oral gavage (meaning a fast intake) and/or a different stimulation of MLNL (by i.p. route or by intestinal route) may affect the cellular composition of this lymphoid tissue differently. In addition, in this second approach, hesperidin did not affect the lymphocyte composition of another inductive site of the GALT, the PP. Interestingly, however, the hesperidin intake changed the proportion of cells found in the intraepithelial and the lamina propria compartments, the effector sites of the GALT. In particular, the hesperidin diet increased the proportion of TCRγδ+ lymphocytes in the intestinal epithelium, which is in line with previous results described after the intake of some polyphenol-enriched foods such as unripe apple [34] and cocoa [21]. TCRγδ+ IEL have an important role in maintaining the epithelial homeostasis, and several studies have related this cellular type with mucosa-associated tolerance [21,34–37]. However, it has been recently suggested that TCRγδ+ IEL triggered by CT exhibit antigen-presenting cell activity in recipient mice fed an oral antigen and contribute to breaking tolerance and inducing a Th2 response [38]. In particular, the oral administration of CT in mice caused the migration of TCRγδ+ IEL to LPL, where they produce IL-10 and IL-17 [38]. Our results regarding rats orally sensitized with OVA together with CT showed that hesperidin produced an opposite effect in the proportion of TCRγδ+ cells both in IEL and LPL, suggesting a protective action of hesperidin in the migration of these cells induced by CT. The hesperidin decrease in TCRγδ+ LPL is in line with the lower proportion of CD8+CD103+ cells in this compartment. The integrin CD103 (also known as αE) binds to E-cadherin and mediates T cell adhesion to the intestine [39], and is highly expressed at the mucosal sites [40], thus suggesting that CD8+CD103+ LPL would move to the intraepithelial compartment (although CD8+CD103+ IEL proportion did not significantly increase).

In addition, after the intake of 0.5% hesperidin diet, LPL showed a relative higher proportion of B cells, which could be related to the enhanced action of hesperidin in the antibody intestinal response, thereby increasing intestinal IgA content, which is in line with other dietary polyphenols [41,42]. In addition, a rise of the Th/Tc proportion in the hesperidin-fed group was found, in agreement with the results reported for LPL after a cocoa-enriched diet using the same rat oral sensitization model [21].

Despite all these lymphocyte composition changes, the specific antibody immune response was not modified in the animals fed the hesperidin diet. In addition, when Th1- and Th2-related antibodies

were studied (IgG2b, and IgG1 and IgG2a, respectively) [19], no particular effects were observed, contrary to the impact of the intake of cocoa, a rich source of flavonoids, in the same oral sensitization model [21,22]. A wide range of polyphenols has been demonstrated to play a role in decreasing specific antibody production in allergy models [8] and it has also been reported that hesperidin attenuated the OVA-specific IgE production in asthma models [9,17,43]. Nevertheless, in the current study, no differences were detected in serum anti-OVA antibodies in either the first or the second experimental designs.

5. Conclusions

In summary, the present results show that hesperidin administration in i.p. immunized rats influences MLNL composition (increasing TCRαβ+ lymphocyte proportion and decreasing that of B and CD8+CD25+ cells) and functionality (increasing IFN-γ synthesis). Moreover, a diet containing 0.5% hesperidin in orally sensitized rats increases intestinal IgA content and also modifies IEL and LPL composition, suggesting the prevention of cell changes triggered by oral CT. However, this hesperidin immunomodulation is not associated with the attenuation of specific antibodies induced by both systemic and intestinal sensitization. Finally, it must be taken into account that this study shows the properties of hesperidin alone; further studies must focus on establishing the effect of hesperidin-enriched food and also the dosage of such foods to achieve these immune effects.

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Conflicts of Interest: The authors declare no conflict of interest.

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ARTICLE 6

"Effect of cocoa's theobromine on intestinal microbiota of rats"

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Molecular Nutrition and Food Research

En premsa

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Categoria: Food Science & Tecnology, Q1 (5/125)

Els resultats del present article han estat presentats al congrés següent:

- VIII Workshop de la Sociedad Española de Probióticos y Prebióticos (SEPyP), Santiago de Compostela, febrer de 2017. Martín-Peláez S, Camps-Bossacoma M, Massot-Cladera M, Rigo-Adrover M, Franch A, Pérez-Cano FJ, Castell M. "¿Es la teobromina la responsable de los efectos del cacao sobre la microbiota intestinal en rata?"

Resum ARTICLE 6

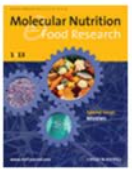
Objectiu: En estudis previs del grup de recerca s'ha observat que la dieta rica en cacau influeix sobre la microbiota intestinal i que aquest efecte no es pot atribuir al seu contingut en fibra. Per això, l'objectiu del present treball va consistir en establir si la teobromina era la responsable dels efectes del cacau sobre la composició de la microbiota intestinal i dels seus productes de fermentació.

Material i mètodes: Rates Lewis de 3 setmanes d'edat varen ser alimentades, durant 15 dies, amb una dieta estàndard, una dieta amb un 10% de cacau o una dieta amb un 0,25% de teobromina (la mateixa quantitat de teobromina que conté la dieta amb un 10% de cacau). Al final de l'estudi es va determinar la microbiota intestinal mitjançant la tècnica de FISH acoblada a citometria de flux (FISH-FCM) i mitjançant una anàlisi metagenòmica. A més, es van quantificar els àcids grassos de cadena curta i el percentatge de bacteris units a IgA en les mostres fecals.

Resultats: Les dietes cacau i teobromina van produir canvis quantitatius i qualitatius en els diferents fílums bacterians intestinals (*Firmicutes, Bacteroidetes, Tenericutes, Actinobacteria i Cyanobacteria*) detectats per FISH-FCM o per l'anàlisi de metagenòmica. Alguns d'aquests canvis es varen detectar només en el grup de teobromina, i d'altres només en el grup de cacau. Les dues intervencions nutricionals varen incrementar la producció d'àcids grassos de cadena curta, principalment per un increment en la producció d'àcid butíric. A més, les dues dietes varen disminuir la proporció de bacteris units a IgA.

Conclusions: La teobromina del cacau és la responsable de la disminució de bacteris units a IgA i de l'increment d'àcids grassos de cadena curta produïts per la ingesta de cacau. Per altra banda, el cacau i la teobromina modifiquen de forma diferent la microbiota intestinal suggerint que altres components del cacau també actuen a aquest nivell, incrementant o disminuint els efectes de la teobromina.

Molecular Nutrition and Food Research



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Effect of cocoa's theobromine on intestinal microbiota of rats

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Keywords:	FISH, metagenomics, methylxanthines, short chain fatty acids, microbiota

TITLE

Effect of cocoa's theobromine on intestinal microbiota of rats

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KEYWORDS

FISH; metagenomics; methylxanthines; microbiota; short-chain fatty acids

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ABBREVIATIONS

CC: cocoa

F/B: Firmicutes/Bacteroidetes ratio

FBS: fetal bovine serum

FCM: flow cytometry

FISH: fluorescence *in situ* hybridization

OTU: operational taxonomic units

PI: propidium iodide

RF: reference

TB: theobromine

ABSTRACT

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

METHODS AND RESULTS: Lewis rats were fed either a standard diet (RF diet), a diet containing 10% cocoa (CC diet) or a diet including 0.25% theobromine (TB diet) for 15 days. Gut microbiota (fluorescence *in situ* hybridization coupled to flow cytometry and metagenomics analysis), SCFA and IgA-coated bacteria were analyzed in fecal samples.

CC and TB diets induced lower counts of *E. coli* whereas TB diet led to lower counts of *Bifidobacterium* spp., *Streptococcus* spp. and *Clostridium histolyticum-C. perfingens* group compared to RF diet. Metagenomics analysis also revealed a different microbiota pattern among the studied groups. The SCFA content was higher after both CC and TB diets, which was mainly due to enhanced butyric acid production. Furthermore, both diets decreased the proportion of IgA-coated bacteria.

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

1. Introduction

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

Cocoa powder contains macronutrients, fiber, minerals, polyphenols (flavonoids, mainly flavanols) and methylxanthines [9]. The most abundant xanthine found in cocoa is theobromine, followed by caffeine. In fact, cocoa is the richest natural source of theobromine [10, 11]. While the effects of

flavonoids present in cocoa have been thoroughly studied, less attention has been paid to the presence of theobromine in cocoa. Even so, a few studies have related its content to a variety of properties attributed to cocoa powder [10, 12]. As theobromine is able to reach the gut [13, 14], we hypothesized that this methylxanthine could contribute to the effects of cocoa intake on gut microbiota. Therefore, the purpose of the present work was to establish the role of cocoa theobromine in the composition of gut microbiota and fermentation products after cocoa consumption in rats.

2. Material and methods

2.1. Animals and diets

Lewis rats (3 week old) obtained from Janvier Labs (Saint-Berthevin Cedex, France) were housed in cages (2-3 animals/cage on days 0-8, and individually on days 8-15) under controlled temperature and humidity in a 12:12 h light:dark cycle. The rats were randomly distributed into three dietary groups (n=7 per group): the reference (RF) group ingested a standard diet AIN-93M (Teklad, Madison, USA), the cocoa (CC) group ingested a standard diet with 10% of natural Forastero cocoa (Idilia Foods S.L., Barcelona, Spain) containing 2.5% theobromine, and the theobromine (TB) group ingested a standard diet including 0.25 % of theobromine (Sigma-Aldrich, Madrid, Spain), i.e. the content of theobromine present in the CC diet. The two experimental diets were elaborated on the basis of the AIN-93M formula by subtracting the amount of carbohydrates, proteins, lipids and insoluble fiber provided by the corresponding supplements. The resulting diets were isoenergetic and contained the same proportion of macronutrients and insoluble fiber as the RF diet (**Table 1**). Animals were provided with feed and water *ad libitum* for 2 weeks. Animal procedures were approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (ref. 5988).

Table 1. Composition of diets used in the study

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

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

2.2. Fecal samples collection and pre-analytical procedures

Fresh feces were collected at days 0, 8 and 15 and processed according to the specific variables to be analyzed. Some fresh fecal samples were used to determine fecal pH, using a surface electrode (Crison Instruments, S.A., Barcelona, Spain). The rest of the fecal samples were directly frozen either at -20 °C until the metagenomics analysis, the bacterial characterization by fluorescence *in situ* hybridization, and the IgA-coated bacteria quantification, or at -80 °C until SCFA analysis. For these determinations, fecal homogenates were later obtained following procedures previously described [5].

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

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

(Serva, Heidelberg, Germany) prior to the hybridization process [16]. All samples were hybridized at the specific probe hybridization temperature, as described [15], and kept in the dark at 4 °C overnight until FCM analysis.

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

2.4. Determination of the proportion of bacteria coated with IgA

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

2.5. Flow cytometry analysis

For FISH and IgA-coated bacteria quantification, FCM analysis was performed using a FacsAria SORP sorter (BD, San José, CA, USA) as previously described [5]. Commercial Flow Check™ 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 are expressed as the $\log_{10}$ of specific probe labeled bacteria counts/g of feces in each sample. Moreover, the *Firmicutes* to *Bacteroidetes* (*F/B*) ratio was calculated taking into account the analyzed bacterial groups belonging to the *Firmicutes* phylum (those hybridized by Chis150, Erec482, Lab158, Staphy and Strept probes) and those belonging to the *Bacteroidetes* phylum (those hybridized by the Bac303 probe). IgA-coated bacteria results are expressed as the percentage of bacteria coated with IgA with respect to the total bacteria.

2.6. Lactic acid and SCFA analysis

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

propionic, butyric and formic acids (12.5-100 mM). Results were expressed as mM (for total SCFA) and relative increases of the total and individual SCFA with respect to those values found in the RF group.

2.7. Metagenomics analysis

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

2.8. Statistical analysis

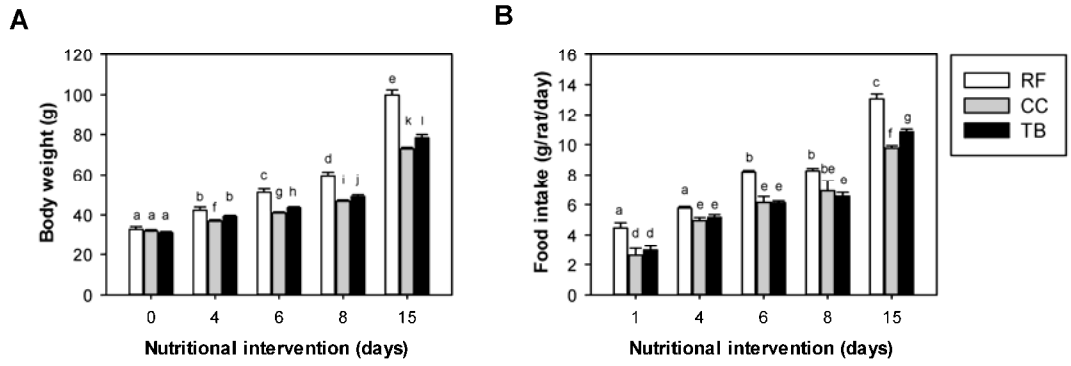
The normality of continuous variables was assessed by normal probability plots and the Shapiro–Wilk test, and the variance equality by Levene's test. Non-normally distributed variables were analyzed by non-parametric tests, specifically Kruskal–Wallis and Mann–Whitney U tests. Normally distributed variables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

3. Results

3.1. Body weight and food intake

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

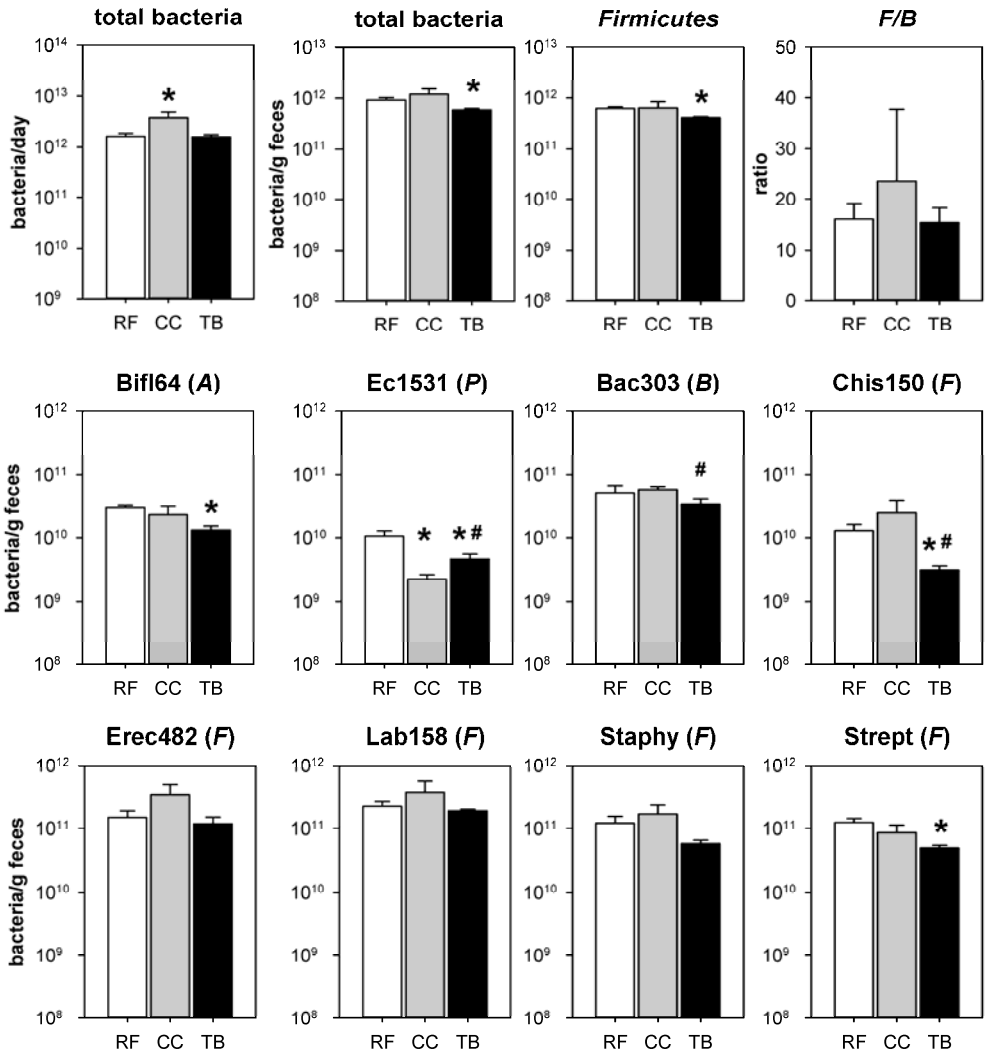
Figure 1. Body weight (**A**) and food intake (**B**) throughout the study. The amount of food intake showed in each day was calculated considering the amount fed in each interval divided into the number of days in each period. Values are expressed as mean ± SEM (n=7). RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. * P<0.05 *vs* RF group; # P<0.05 *vs* CC group.



3.2. Gut bacterial populations by FISH–FCM

After 15 days of dietary intervention, significant differences in the gut microbiota composition were observed (**Figure 2**). Concerning total bacteria counts, the CC diet caused the elimination of higher number of bacteria per day than the RF diet. This increase could be associated with the stool amount per day, which was higher in CC rats (3.07 ± 0.11 g) than that from RF rats (1.78 ± 0.10 g) (P<0.05). Nevertheless, the total bacteria counts relative to fecal weight from CC fed rats were similar to those in the RF group. In the case of TB diet, although it also resulted in a higher stool amount per day (2.73 ± 0.24 g), it did not modify the number of bacteria in feces. Therefore the TB group showed lower counts than the other groups when considering the number of bacteria per fecal weight (P=0.021 and P=0.055 compared to the RF and CC groups, respectively).

Figure 2: Total bacteria counts, total *Firmicutes* counts, *Firmicutes/Bacteroidetes* ratio, and bacteria counts detected with selected probes indicated in the top determined by FISH–FCM from fecal samples. RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. *A: Actinobacteria, B: Bacteroidetes; F: Firmicutes, P: Proteobacteria.* Total bacteria counts are expressed as bacteria/day and bacteria/g feces. Bacterial groups and phylum counts are given as means of $\log_{10}$ bacteria/g feces ± SEM (n=7). * P<0.05 *vs* RF group; # P<0.05 vs CC group.

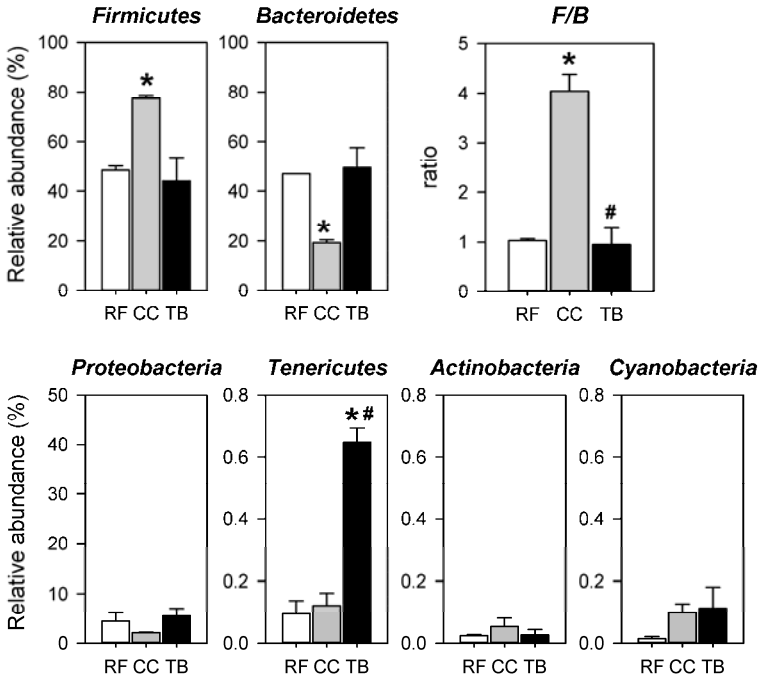


Regarding particular bacterial groups, both the CC and TB groups presented lower counts of *E. coli* than the RF group, with the counts being even lower in the CC group than in the TB one. The TB diet also led to significantly lower counts of *Bifidobacterium* spp., *Streptococcus* spp. and *Clostridium histolyticum-C. perfringens* than the RF group. The decrease in the *Clostridium* group, together with a reduction in the *Bacteroidaceae-Prevotellaceae* group, was also significant compared to the CC group. As a result, the *Firmicutes* counts were lower in feces from the TB group than those from RF rats (P=0.005). Even so, the *F/B* ratio was not significantly modified in the feces of the studied groups.

3.3. Quantitative metagenomics analysis of gut bacterial populations

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

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



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

3.4. Qualitative metagenomics analysis of gut bacterial populations

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

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

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

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

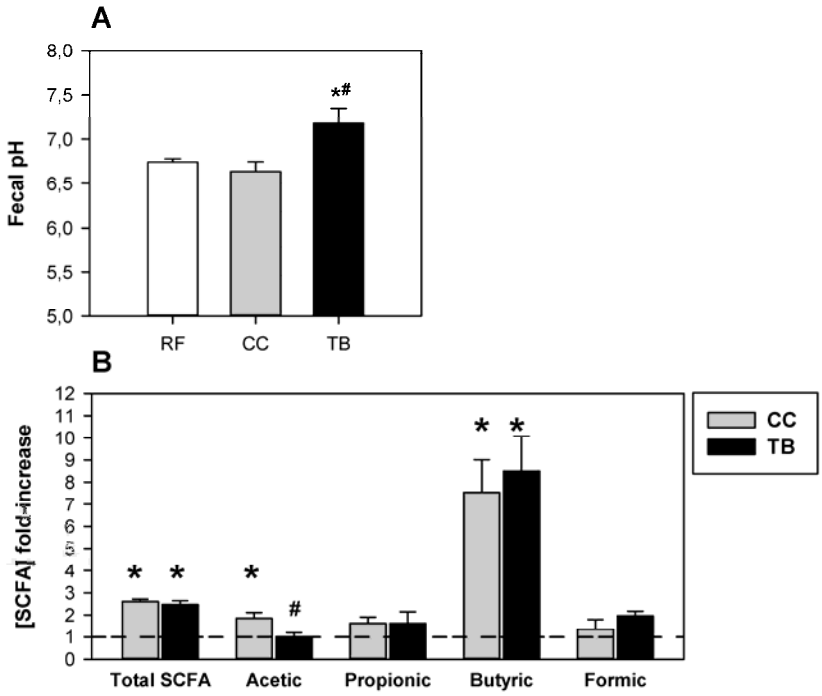
Phylum	Class	Order	Family	Genus	Specie	RF	CC	TB
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*					
Firmicutes	*Erysipelotrichi*	*Erysipelotrichales*	*Erysipelotrichaceae*	*Coprobacillus*				
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*	*Ruminococcus*	*flavefaciens*			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

3.5 Fecal pH, lactic acid and SCFA

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

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

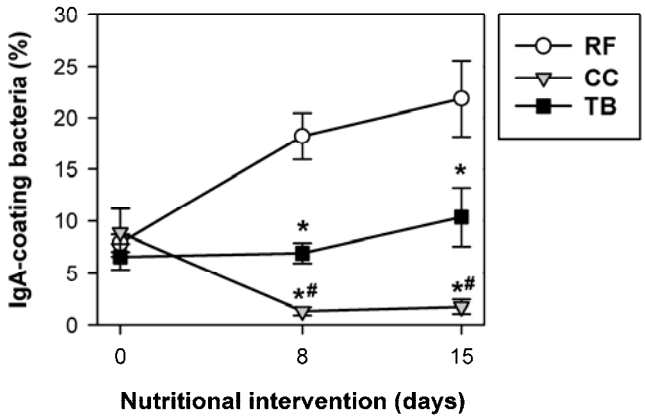
Figure 4. A) Fecal pH. **B**) Fold change of the total and the individual SCFA analyzed compared to the RF diet which was considered as 1. Values are expressed as mean ± SEM (n=7). RF, reference group; CC, group fed diet containing 10% cocoa; TB, group fed diet containing 0.25 % theobromine. * P<0.05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



3.6 Percentage of fecal bacteria coated with IgA

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

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



4. Discussion

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

In vitro, *in vivo* and clinical studies demonstrate that cocoa is able to modulate the growth of gut microbiota [5, 7, 15, 18]. Previous studies in rats show that the intake of cocoa-enriched diets for at least three weeks modifies the intestinal microbiota pattern [5, 7, 15]. In the present study, the ingestion of the cocoa diet for two weeks was not able to significantly modify most of the bacterial groups analyzed by FISH–FCM, probably because of the shorter length of this nutritional

intervention. However, some changes were observed when theobromine was ingested alone, indicating that theobromine by itself is able to directly or indirectly modify gut microbial populations. The metagenomics analysis, even though it was carried out in a small number of samples, allows to have an idea of particular genera and/or species from gut microbiota modified by CC and TB diets and thus, by using both techniques in a complementary manner, we obtained a wider approach of the gut microbiota changes.

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

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

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

One important finding of our study is that theobromine (both in the CC and TB groups) lowered the counts of *E. coli*. This agrees with the reported inhibitory effects of theobromine on Gram-negative bacteria [29], suggesting an inhibitory effect on the growth of potential gut pathogens. This inhibition was enhanced with the CC diet, suggesting the role of polyphenols in this effect [30]. In the same phylum, *Ralstonia* sp. seems to appear due to the CC and TB diets. *Ralstonia* sp. was formerly included in the *Pseudomonas* genera, which includes species able to degrade methylxanthines [31, 32]. Therefore, its presence may reflect the adaptation of gut microbiota to diets rich in methylxanthines.

The impact of theobromine on gut microbiota was also patent in the *Tenericutes* phylum, which increased almost fourfold with theobromine ingested alone. This was associated with a higher number of bacteria belonging to the *RF39* order (*Mollicutes* class). A study reported a similar effect with the ingestion of cocoa for 4 weeks [8]. The absence of effects on *Tenericutes* with the CC diet suggests that other cocoa compounds delayed the theobromine effect on this phylum.

With regard to *Actinobacteria*, a prebiotic effect of cocoa polyphenols in humans [33] and of cocoa fiber in rats [7] by increasing the counts of *Bifidobacterium* group has been reported. As TB diet, but not CC diet, decreased the proportion of *Bifidobacterium* spp., it can be suggested that theobromine is counteracting the prebiotic effects of cocoa fiber. However, the metagenomics results suggested no changes in the relative abundance of *Actinobacteria* species, either in the TB or CC diet, although it seems that CC diet leads the appearance of one species of the *Actinomycetales* order. In line with these results, blueberries increased the relative abundance of *Actinomycetales* order in rats, which

allows us to suggest the role of polyphenols in such an effect [34]. Finally, the appearance of one species of the *Streptophyta* order (*Cyanobacteria* phylum) with the ingestion of cocoa, in agreement with the reported effect of a CC diet for 4 weeks [8], must be related to the cocoa's polyphenol or fiber content. Nevertheless, the role of such bacteria in the intestinal microbiota remains to be elucidated.

Overall, this study reveals the impact of theobromine on gut microbiota. The effects were different depending on whether theobromine was ingested alone or when forming part of cocoa, although few common characteristics were found. Some changes observed exclusively in the TB group would have been due to the action of this methylxanthine, which were counteracted by other cocoa compounds, such as fiber and polyphenols. Other changes in the TB group agree with previous results reported with a longer CC diet, suggesting that these other compounds included in the CC diet could delay the TB effect. The modifications exclusively found in the CC group must be related to the cocoa's fiber or polyphenol content.

The effect of theobromine on gut microbiota has also been reflected by the changes observed in SCFA in both theobromine-containing diets. The enhanced generation of SCFA was mainly due to the butyric acid. Butyrate is considered the main energy source for colonocytes, and is also important for the regulation of gene expression, the intestinal barrier and the immune system, among others [35, 36]. However, whereas butyric acid increased with both diets, the increase in the proportion of acetic acid was only observed after cocoa ingestion. This disagreement could be due to the fermentation of different substrates with both interventions. After cocoa intake, SCFA would come directly from polyphenol and/or fiber fermentation [7], whereas for the TB diet, changes in the generation of SCFA would be indirectly due to the inhibition of some bacterial populations and thus contribute to enhancing the amount of substrate available for other bacteria. The differential patterns in the SCFA generated support the idea that the ingestion of theobromine alone or as part of cocoa has a different impact on gut microbiota. Furthermore, the unexpected higher fecal pH when theobromine was ingested alone deserves further studies focusing on microbial metabolites which could explain the observed fecal pH changes.

The current results evidence that theobromine (both in the TB and CC diets) contributes to the lower proportion of bacteria coated with IgA found after the cocoa diet, in line with previous results [5, 7, 15]. As rats fed the CC diet even showed a lower proportion of IgA-coated bacteria, the combination of cocoa polyphenols with theobromine in the CC diet could have an additive or a synergistic effect

on reducing their proportion. On the other hand, the effect of cocoa fiber must be discarded because it was associated with an increase in the percentage of IgA-coated bacteria [7].

Results regarding body weight suggest that theobromine present in cocoa was the main reason for a slower body weight increase produced by the 10% cocoa diet. In fact, there was a lower food intake per animal already in the first day of diet, which could affect the body weight increase and it can also influence gut microbiota. On the other hand, body growth could be affected by TB influence on metabolism. In this sense, it has been demonstrated that caffeine has a stimulatory effect on thermogenesis [37] and has been associated with bone mass loss [38].

In conclusion, here we demonstrate that cocoa theobromine plays a relevant role in some effects related to cocoa intake, such as lower body weight increase and the proportion of IgA-coated bacteria. In addition, theobromine modifies gut microbiota, although other cocoa compounds –such as cocoa polyphenols or fiber– also act on the intestinal bacteria, attenuating or enhancing the theobromine effects, that overall leads to the global effect of cocoa on microbiota which differs from that of each particular cocoa component.

Author contributions

The authors' contributions were as follows: À.F., F.J.P.-C. and M.C. conceived and designed the study; M.C.-B. and M.M.-C. were responsible for the animal experiments and sampling; S.M.-P. carried out the FISH–FCM and IgA-coated bacteria analyses; S.M.-P, M.C.-B., M.M.–C., F.J.P.-C. and M.C. carried out the metagenomics data analysis; M.R.-A. analyzed the SCFA; S.M –P. and M.C.-B were mainly responsible for the interpretation of the results and the writing of the final manuscript; F.J.P.-C and M.C. contributed to the critical revision of the manuscript. All authors have read and approved the final version of the manuscript for publication.

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SUPPLEMENTARY MATERIAL

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

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

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

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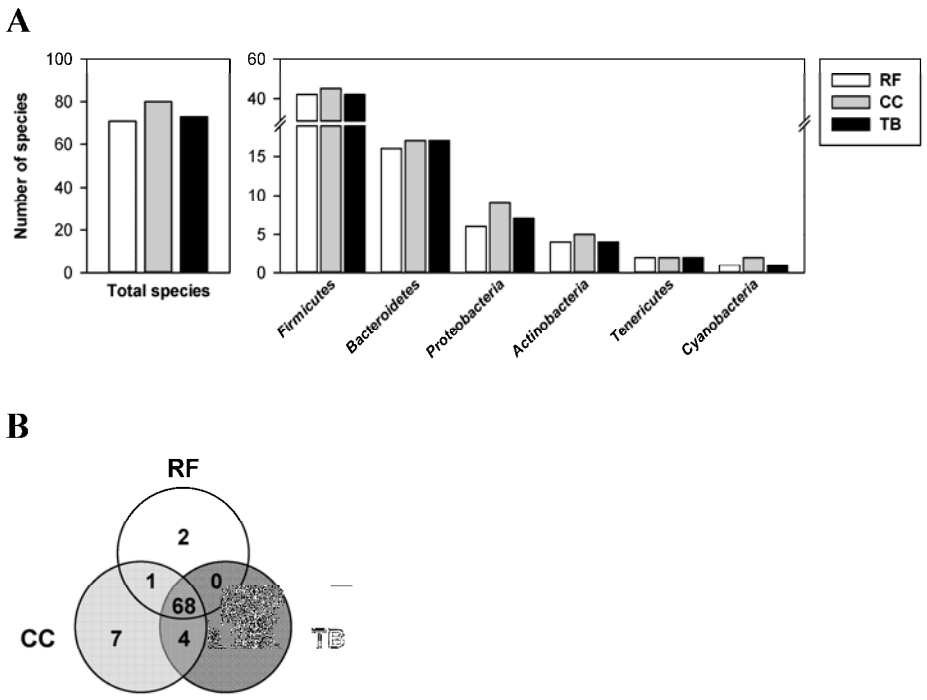
Supplementary Figure 1. Diversity of bacterial species found in feces by metagenomics analysis. **A**) Richness of bacterial species; **B**) Venn diagram of differentially detected species. The diagram shows the absolute number of detected species that belonged to each of the individual nutritional interventions,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



ARTICLE 7

"Theobromine is responsible for the cocoa effects on rat antibody immune response"

Mariona Camps-Bossacoma, Francisco J. Pérez-Cano, Àngels Franch, Margarida Castell

Sotmès a publicació

Els resultats del present article han estat presentats al congrés següent:

- 2nd International Congress of Chocolate and Cocoa in Medicine, Barcelona, setembre de 2015. Camps-Bossacoma M, Bitlloch-Obiols M, Abril-Gil M, Franch A, Pérez-Cano FJ, Castell M. "Short term effect of cocoa theobromine on primary and secondary lymphoid tissues in rats". Communicació oral. Publicat a *Nutrients*. Second International Congress on Chocolate and Cocoa in Medicine Held in Barcelona, Spain, 25-26th September 2015. 2015; 7(12): 9785-9803.

Resum ARTICLE 7

Objectiu: Després d'observar el poder immunoregulador de la dieta rica en cacau, l'objectiu final de la tesi va ser establir si la teobromina era la responsable dels seus efectes sobre la producció d'anticossos sistèmics i intestinals i caracteritzar la seva influència sobre en la composició limfocítica de teixits limfoides primaris i secundaris a curt termini.

Material i mètodes: Rates Lewis de 3 setmanes d'edat varen ser alimentades durant 19 o 8 dies amb una dieta estàndard, una dieta amb un 10% de cacau o una dieta amb 0.25% de teobromina (la mateixa quantitat que conté la dieta de cacau). Es varen obtenir mostres sèriques i fecals al llarg de l'estudi per tal de determinar les concentracions d'immunoglobulines en aquests teixits (tècnica ELISA). A més, al final de l'estudi de 8 dies, es varen aïllar els limfòcits del timus, melsa i ganglis limfàtics mesentèrics per tal d'establir-ne la seva composició (citometria de flux).

Resultats: La dieta amb un 10% de cacau va disminuir la concentració sèrica d'IgG després de 19 dies d'intervenció, degut de la reducció d'IgG2c, IgG2b i IgG1. Aquesta dieta també va reduir la concentració sèrica d'IgM i d'IgA, després de 4 i 8 dies d'intervenció, respectivament. A nivell intestinal, el cacau va disminuir la concentració d'IgA després de 4 dies d'ingesta. La dieta amb teobromina va produir exactament els mateixos efectes sobre les immunoglobulines sèriques i intestinals. A nivell tissular, al timus, les dues dietes van incrementar la proporció de cèl·lules DP i de les SP. Als GLM, les dues dietes van disminuir els Th i incrementar els Tc. A més, la dieta cacau va disminuir la proporció de CD45RA+CD62L+. A melsa, les dues dietes varen disminuir els TCR$\gamma\delta$, les cèl·lules NK i NKT, i incrementar les Th. A més, la dieta amb teobromina va incrementar la proporció de les cèl·lules CD4 i CD8 amb el marcador CD62L.

Conclusions: La dieta 10% cacau degut el seu contingut amb teobromina produeix una disminució dels anticossos sistèmics i intestinals i modifica la composició de limfocíts del timus, ganglis limfàtics mesentèrics i melsa. La majoria dels canvis, ja són evidents sols després d'una setmana d'ingesta de cacau.

Theobromine is responsible for the cocoa effects on rat antibody immune response

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Running title: Cocoa's theobromine effects on antibody response

Abbreviations

APC: allophycocyanin; CC: Cocoa group, fed 10% cocoa diet; ELISA: Enzyme-Linked ImmunoSorbent Assay; FITC: fluorescein isothiocyanate; GALT: Gut-associated lymphoid tissues; Ig: Immunoglobulin; MLN: Mesenteric lymph nodes; PARP-1:Enzyme-Linked ImmunoSorbent Assay; PE: phycoerythrin; perCP: peridinin-chlorophyll-protein; RF: Reference group, fed standard diet; sIgA: Secretory IgA; sIgM: Secretory IgM; TB: Theobromine, fed 0.25% theobromine diet; Treg: T regulatory lymphocytes

Conflicts of interest

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

Funding disclosure

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Abstract

Background: A 10% cocoa-enriched diet influences the immune system functionality including the attenuation of the antibody response. However, neither cocoa polyphenols nor cocoa fiber can totally explain these immunoregulatory properties.

Objective: In the present study, we firstly aimed to establish the role of the cocoa theobromine in systemic and intestinal immunoglobulin production and, secondly, we aimed to ascertain the promptness of cocoa influence on the lymphoid tissues and immune functionality carrying out a study lasting a single week.

Methods: For 19 or 8 days, three-week-old Lewis rats received either a standard diet, a 10% cocoa diet (CC) or a 0.25% theobromine diet (the same amount provided by the CC diet). Throughout the 19 or 8 days, serum IgG, IgG isotypes, IgM, IgA concentrations and intestinal secretory IgA (sIgA) levels were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA). In addition, after 8 days, the thymus, mesenteric lymph nodes (MLN) and spleen lymphocyte populations were analyzed by flow cytometry.

Results: Both cocoa- and theobromine-enriched diets prompted a similar attenuating response in serum IgG, IgG isotypes, IgM and IgA and intestinal sIgA. In addition, the cocoa diet changed the composition of the thymus (e.g. increasing double-negative and CD4+ thymocyte proportions), MLN (e.g. decreasing the percentage of Th lymphocytes) and spleen (e.g. increasing the proportion of Th lymphocytes) as early as after a single week of diet. Most of these changes were identical to those produced by theobromine intake.

Conclusions: The theobromine content in cocoa is responsible for cocoa's regulatory effect on both systemic and intestinal antibody production and also for modifying thymus, MLN and spleen lymphocyte composition. The majority of these changes can be observed after a single week of diet.

Keywords: cocoa; immune system; immunoglobulins; lymphoid tissues; mesenteric lymph node; methylxanthine; spleen; theobromine; thymus

1. Introduction

Cocoa, derived from *Theobroma cacao* fermented seeds, was introduced by the Mayan and Aztec civilizations as dietary and medicinal food and was diffused to Europe in the mid-1500s [1]. At present, cocoa products are consumed worldwide [2], mainly as a snack due to its pleasurable taste. Furthermore, an increasing number of health properties have been attributed to its consumption, such as promoting cardiovascular health, preventing metabolic and endocrine disorders and improving cognition, mood and behavior [2–7].

In previous studies, we extensively reported that the feeding of a cocoa-enriched diet has an immunoregulatory effect on rats. Specifically, a 10% cocoa diet in rats influences systemic and intestinal immune function and lymphoid tissue composition. In the thymus, a cocoa diet increases the proportion of mature single positive (SP) CD4+ cells and decreases the immature double positive (DP) cells [8]. In mesenteric lymph nodes (MLN), cocoa intake increases the proportion of TCR$\gamma\delta$ cells and Tc cells [9], whereas in the spleen, this nutritional intervention induces a higher percentage of B cells together with a reduction in the Th lymphocyte proportion [10]. With regard to immunoglobulins, a cocoa diet to six-week-old female Wistar rats for three weeks decreases the concentration of serum IgG2a and IgM and secretory IgA (sIgA) and secretory IgM (sIgM) [11]. Similar results were observed after two weeks of cocoa diet in eight-week-old male Lewis rats feeding different cocoa flavonoid-enriched diets [12]. In this vein, the immunoregulatory influence of cocoa has been used to prevent the development of both allergy and oral sensitization in rat models, in which it has effectively prevented specific antibody synthesis [13–15].

Cocoa contains carbohydrates, proteins, lipids, fiber, minerals, polyphenols and methylxanthines [16]. Among polyphenols, flavonoids are the most important and include procyanidins, epicatechin and catechin. Over the last decades, a great number of cocoa benefits have been described as a result of the antioxidant and anti-inflammatory properties of these polyphenols [2,17–19]. However, cocoa is also a source of methylxanthines. These compounds are derived from xanthines and are found in several vegetal derivatives [20], such as coffee, tea and cocoa, which contain caffeine, theophylline and theobromine as the most relevant methylxanthines, respectively [21]. Cocoa contains both theobromine and caffeine, the first being the most abundant [22]. Currently, several theobromine health effects have been described. In this context, among other benefits, theobromine acts on oral health, suppresses cough, produces bronchodilation in asthma patients, has a psycho-stimulant action, protects against neurodegenerative diseases (Parkinson's and Alzheimer's), and inhibits acid uric crystallization [21–24]. Today, there is increasing evidence regarding the important role played by methylxanthine in the healthy properties of cocoa [22,23].

In recent years, interest in the identification of those cocoa compounds that play an immunoregulatory role has grown. For that reason, several experimental designs have been carried out in order to

ascertain whether polyphenols or cocoa fiber were responsible for the immunomodulatory properties of cocoa. Nevertheless, in both cases, the results showed that these components only partially explained the impact of cocoa on the immune system and, therefore, other cocoa compounds might contribute to the attenuation of the humoral immune response [12,25,26]. In fact, the effect of theobromine on the immune system is not yet known. In this regard, we can hypothesize that theobromine could have a role in the immunoregulatory action of cocoa. On the basis of these previous studies, the first aim of the current study was to establish the influence of cocoa theobromine in systemic and intestinal immunoglobulin production. In addition, as given that theobromine was responsible for these effects, the second goal of the study was to ascertain the promptness of cocoa influence on both the primary and secondary lymphoid tissues and also on antibody production, carrying out a study lasting a single week.

2. Materials and Methods

2.1. Animals and experimental nutritional intervention

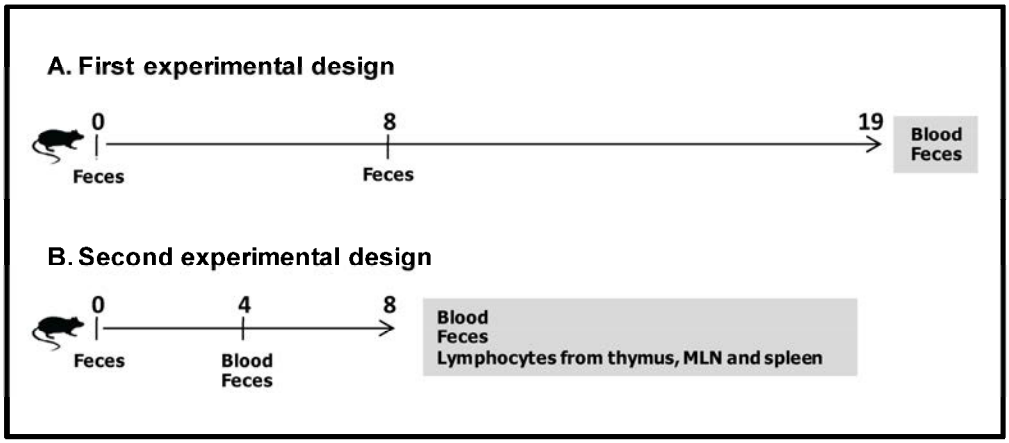
Three-week-old female Lewis rats obtained from Janvier Labs (Saint Berthevin Cedex, France) were housed (2-3 rats/cage) under controlled conditions of temperature and humidity in a 12 h / 12 h light/dark cycle. The rats were randomly assigned into three dietary groups (n = 6-7 each): the reference group (RF), fed with the standard diet AIN-93M; the cocoa group (CC), fed a 10% cocoa diet; and the theobromine group (TB) fed a standard diet with 0.25% theobromine, which was the same amount provided by the 10% cocoa diet (**Table 1**). Animals were given free access to water and chow. In order to achieve the first aim of the study, an experimental design lasting 19 days was carried out, and to achieve the second aim, an experimental design lasting one week was conducted (**Figure 1**). All the experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals, reviewed and approved by the Ethical Committee for Animal Experimentation of the University of Barcelona (CEEA/UB ref. 5988).

TABLE 1. Composition of the experimental diets. RF: reference group, CC: cocoa group, TB: theobromine group (n= 6 rats per group).

Components	Diets[a] [g/kg diet]		
	RF	CC	TB
Proteins	141	141	140
Lipids	38.7	38.5	38.6
Carbohydrates	722	711	720
Insoluble fiber	50.0	50.0	49.9
Soluble fiber	-	8.5	-
Minerals	35.9	34.1	35.8
Vitamins	10.2	8.0	10.2
Choline bitartrate	2.5	2.0	2.5
Antioxidant	0.01	0.01	0.01
Theobromine	-	2.5	2.5
Phenolic compounds	-	4.02	-
Total	1000	1000	1000

[a]RF, standard diet (AIN-93M); CC, 10% cocoa diet; TB, 0.25% theobromine diet.

FIGURE 1. Experimental designs. The first experimental design was carried out to establish the influence of cocoa theobromine in immunoglobulin production. The second experimental design was carried out to ascertain the promptness of cocoa influence on lymphoid tissues and immune function.



2.2. Sample collection and processing

Blood and fecal samples were collected at the time points established in **Figure 1**. Serum was kept at -20°C until immunoglobulin (IgG, IgG isotypes, IgM and IgA) quantification. Fecal samples were collected and treated as in previous studies in order to obtain fecal homogenates (20 mg/mL), which were kept at -20°C until sIgA quantification [27].

At the end of the second experimental design, the animals were euthanized and the thymus, MLN and spleen were carefully removed. Thymus and spleen were immediately weighed. For lymphocyte isolation, tissue samples were passed through a sterile mesh cell strainer (40 µm, ThermoFisher Scientific) and the resultant cell suspensions were used for thymus and MLN samples, whereas spleen samples were submitted to the osmotic lyses of erythrocytes. Cell counting and viability of the isolated cells were determined with a Countess™ Automated Cell Counter (Invitrogen™, Thermo Fisher Scientific) after staining with trypan blue.

2.3. Immunoglobulin determination

Serum IgG, IgG1, IgG2a, IgG2b, IgG2c, IgM, IgA and intestinal sIgA concentrations were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) from Bethyl Laboratories (Montgomery, TX, USA), following the manufacturer's instructions. Absorbance was measured at 492 nm by a photometer (Labsystems Multiskan, Helsinki, Finland) and data were interpolated with Multiskan Ascent v.2.6 software (Thermo Fisher Scientific S.L.U, Barcelona, Spain) according to the concentration of the corresponding standards.

2.4. Assessment of lymphocyte composition by flow cytometry analysis

Lymphocytes from thymus, MLN and spleen were stained with the mouse anti-rat CD4, CD8α, CD8β, TCRαβ, TCRγδ, NKR-P1A (BD Biosciences, Oxford, UK) and CD62L (Biolegend, San Diego, CA, USA) monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll-protein (PerCP) or allophycocyanin (APC), as previously described [14]. Briefly, cells were labeled with saturating amounts of the antibodies in phosphate buffered saline (PBS) containing 2% of fetal bovine serum (FBS) and 0.1% NaN_3 (darkness, 20 min, 4°C). Consecutively, the cells were washed and fixed with 0.5% p-formaldehyde and kept in darkness at 4°C until analysis. A negative control staining was included for each sample. Analyses were performed using a Gallios Cytometer (Beckman Coulter, Miami, FL, USA) in the Scientific and Technological Centers at the University of Barcelona (CCiTUB). All results were assessed by the Flowjo v.10 software (Tree Star Inc., Ashland, OR, USA).

2.5. Statistical analysis

Data are expressed as mean ± standard error. For the statistical analysis, results were evaluated with the software package SPSS 22.0 (IBM Statistical Package for the Social Sciences, version 22.0, Chicago, IL, USA). The Levene test was performed to assess the homogeneity of variance (homoscedasticity) of the results, and the Shapiro-Wilk test their distribution. Homogeneity of variance and normal distributed data were analyzed by the parametric test one-way ANOVA followed by Bonferroni's post hoc test. In contrast, the results having different variance and/or different distribution were evaluated by the Kruskal-Wallis and Mann-Whitney U nonparametric tests. Significant differences were established when $p \leq 0.05$.

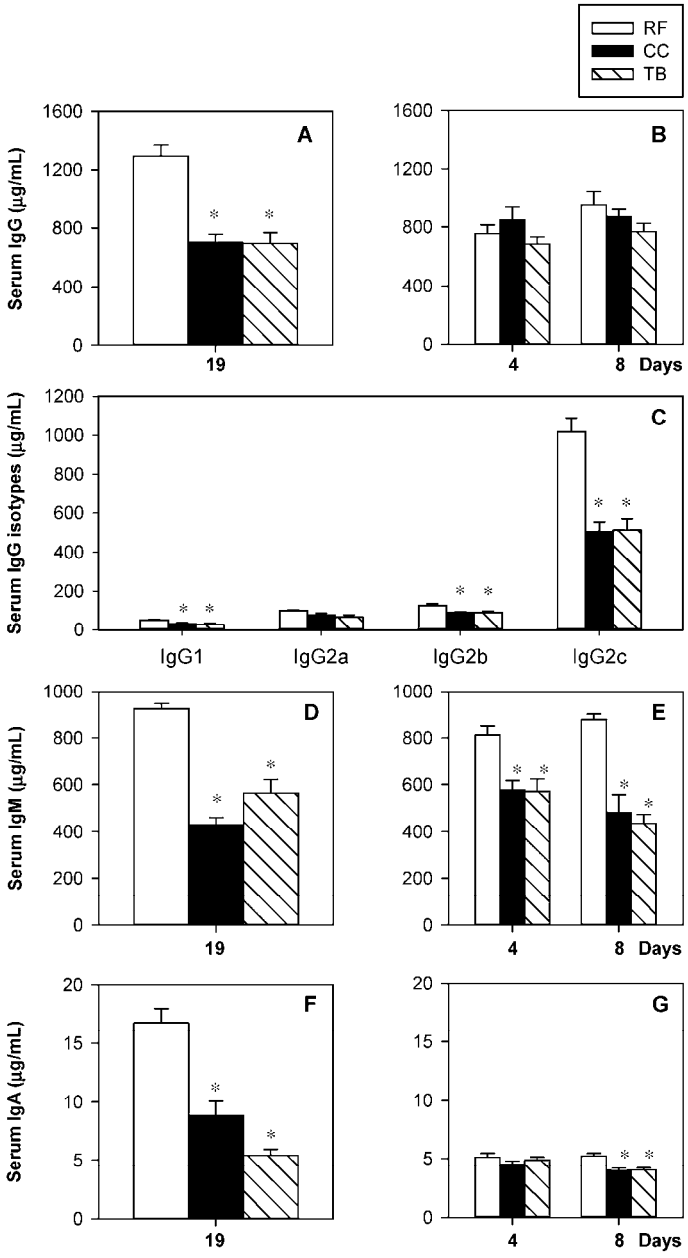
3. Results

3.1. Effect of cocoa theobromine on serum immunoglobulins

IgG, the most abundant serum immunoglobulin, was reduced after 19 days of cocoa or theobromine diets to the same degree (**Figure 2**A). However, no differences were seen earlier (**Figure 2**B). The total IgG reduction on day 19 was mainly due to a decrease in IgG1, IgG2b and IgG2c isotypes (**Figure 2**C).

Total serum IgM and IgA on day 19 were also decreased by the two nutritional interventions (**Figure 2**D-**2**E). The analysis of the second experimental design revealed that IgM and IgA were already reduced at day 4 and 8, respectively (**Figure 2**F-**2**G).

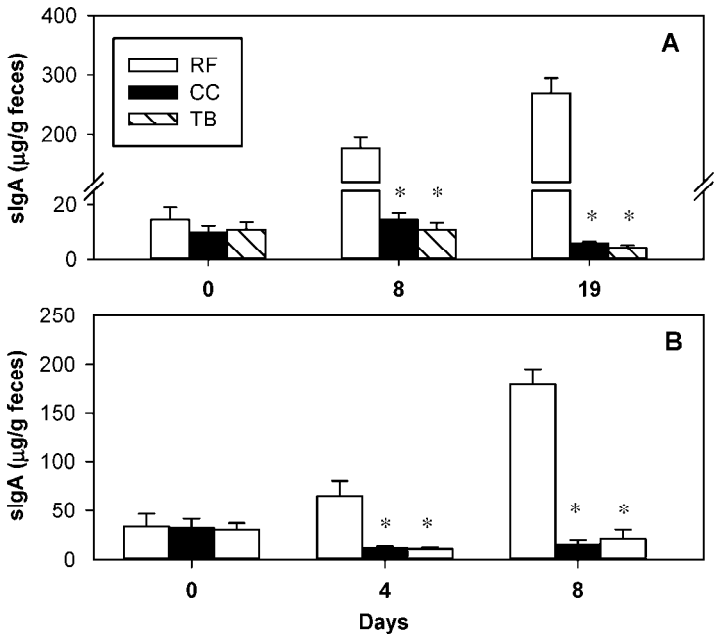
FIGURE 2. Serum IgG, IgM and IgA concentrations. (A-B) IgG in the 19-day experimental design and the 8-day experimental design; (C) IgG isotypes in the 19-day design; (D-E) IgM in the 19-day design and the 8-day design; (F-G) IgA in the 19-day design and the 8-day design. RF: reference group, CC: cocoa group, TB: theobromine group (n= 6-7 rats per group). Values are expressed as mean ± standard error. Statistical difference: * p≤0.05 by Mann-Whitney U test.



3.2. Effect of cocoa theobromine on intestinal sIgA

In order to ascertain the contribution of the theobromine in cocoa in the intestinal compartment, sIgA was also quantified in feces samples throughout both studies (**Figure 3**). Cocoa and theobromine groups showed a similar decrease in intestinal sIgA content in both designs. In the first one, the decrease was detected after 8 days of intake (**Figure 3**A) and in the second, it was evident from day 4 (**Figure 3**B).

FIGURE 3. Intestinal sIgA concentration. sIgA content in feces from (A) the 19-day experimental design and (B) the 8-day experimental design. RF: reference group, CC: cocoa group, TB: theobromine group (n= 6-7 rats per group). Values are expressed as mean ± standard error. Statistical difference: * $p \leq 0.05$ by Mann Whitney U test.



3.3. Effect of cocoa theobromine on thymus, MLN and spleen

As shown in previous studies, cocoa diet was able to modify the lymphocyte composition of lymphoid tissues [8–10]. To ascertain the promptness of cocoa influence on these tissues, thymus, MLN and spleen were collected in the second experimental design. **Table 2** summarizes the relative weight of the thymus and spleen after the second experimental design. Cocoa and theobromine were able to similarly decrease the weight of both lymphoid tissues after a single week of diet intake.

To determine whether theobromine content was also responsible for the changes in lymphocyte composition of primary and secondary lymphoid tissues, the phenotype of lymphocytes from thymus, MLN and spleen was studied after one week of the nutritional intervention.

TABLE 2. Relative weight of thymus and spleen (% with respect to BW) after 8 days of nutritional intervention. RF: reference group, CC: cocoa group, TB: theobromine group (n= 6 rats per group). Values are expressed as mean ± standard error (n = 6). Statistical difference: * $p \leq 0.05$ by one-way ANOVA.

Group	Relative weight (%)	
	Thymus	Spleen
RF	0.39 ± 0.009	0.30 ± 0.009
CC	0.22 ± 0.008*	0.24 ± 0.009*
TB	0.20 ± 0.018*	0.25 ± 0.006*

Lymphocytes from thymus are classified into four subsets according to the expression of CD4 and CD8 molecules (**Figure 4**A-4D). The most immature population are double-negative cells (DN, CD4-CD8-), then these cells turn into DP cells (CD4+CD8+) to finally become SP cells (CD4-CD8+ or CD4+CD8-), corresponding to the most mature thymocytes which eventually migrate to the peripheral lymphoid organs [28,29]. Both cocoa and theobromine diets increased the relative amount of DN cells, whereas they decreased that of DP cells and CD4+CD8- cells (**Figure 4**D). In addition, in the thymocyte maturation, there is a gradual increase in the expression of TCRαβ, TCRαβ^{high} being the most mature cells. The expression of this marker on the four thymocyte subsets was studied (**Table 3**). A decrease in the proportion of DN TCRαβ+ was observed in both nutritional interventions, with the proportion of TCRαβ^{high} being reduced (**Table 3**). Otherwise, the proportion of CD8+CD4- TCRαβ+ cells (both TCRαβ^{high} and TCRαβ^{low} thymocytes) was increased by cocoa and theobromine diets (**Table 3**).

FIGURE 4. Thymus lymphocyte composition after 8 days of nutritional intervention. Representative flow cytometry histograms of CD4/CD8 expression on lymphocytes from (A) reference group; (B) cocoa group; and (C) theobromine group. (D) Percentage of cells expressing or not CD4 and CD8 molecules. Values are expressed as mean ± standard error (n=6). Statistical difference: * $p \leq 0.05$ by ANOVA or Mann-Whitney U test.

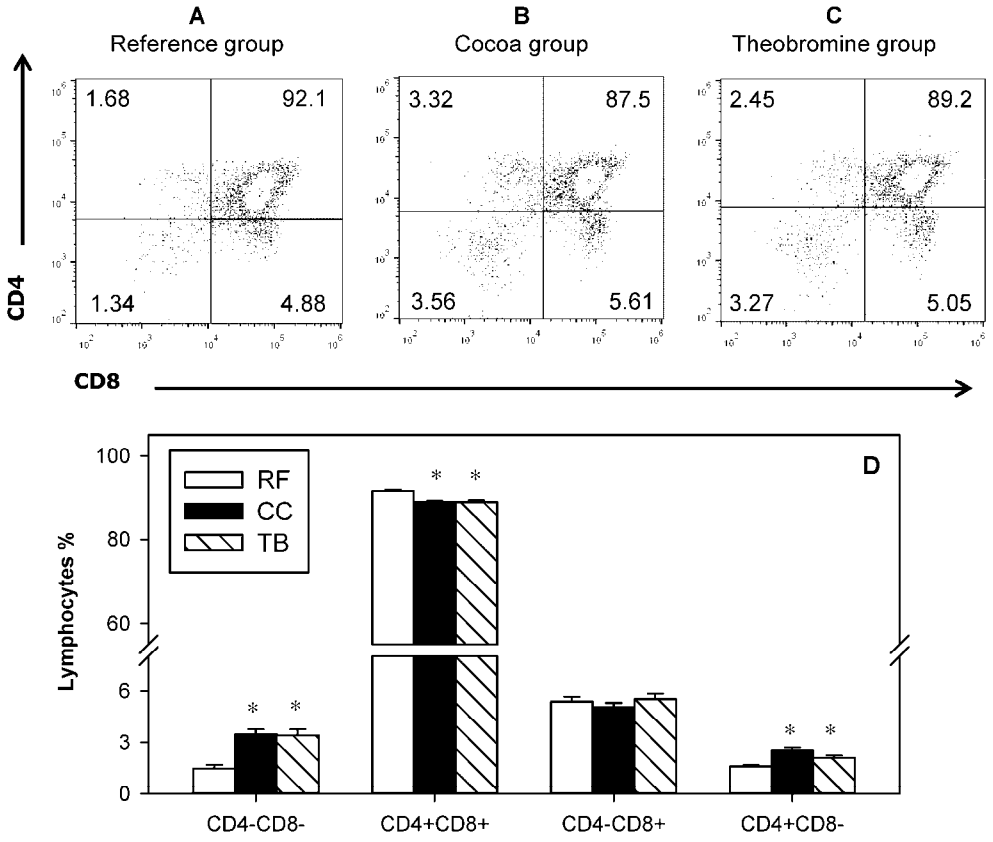


TABLE 3. TCRαβ expression on thymus lymphocytes according to CD4 and CD8 expression. RF: reference group, CC: cocoa group, TB: theobromine group (n= 6-7 rats per group).Values are expressed as mean ± standard error. Statistical difference: * $p \leq 0.05$ by ANOVA.

	TCRαβ+			$TCR\alpha\beta^{low}$			$TCR\alpha\beta^{high}$		
	RF	CC	TB	RF	CC	TB	RF	CC	TB
DN	44.5 ± 3.61	31.8 ± 3.56*	30.5 ± 2.07*	29.8 ± 3.67	25.5 ± 2.52	22.1 ± 1.62	14.7 ± 1.38	6.24 ± 1.09*	8.36 ± 0.95*
DP	85.0 ± 0.68	85.90 ± 0.87	85.0 ± 0.94	81.0 ± 0.7	82.0 ± 0.83	81.0 ± 0.88	3.95 ± 0.22	3.90 ± 0.27	3.98 ± 0.17
CD8 SP	97.0 ± 0.35	98.6 ± 0.34*	98.6 ± 1.04*	24.0 ± 2.48	10.1 ± 1.45*	12.5 ± 1.46*	72.8 ± 2.66	88.5 ± 1.53*	86.2 ± 1.75*
CD4 SP	89.7 ± 0.81	88.8 ± 1.14	89.4 ± 1.39	30.1 ± 1.83	30.5 ± 2.07	26.0 ± 2.59	59.5 ± 1.75	58.3 ± 2.73	63.4 ± 2.69

With regard to the MLN, the proportions of the main lymphocyte subsets, e.g. CD45RA+ (B lymphocytes), TCRαβ+, TCRγδ+ and NK cells, were not modified after 8 days of either the cocoa- or theobromine-enriched diets (**Figures 5**A-**5**B). However, both interventions decreased the proportion of TCRαβ+CD4+ (Th) lymphocytes and increased that of TCRαβ+CD8+ (Tc) cells (**Figure 5**C). Consequently, Th/Tc ratio was similarly reduced after the cocoa or theobromine diets (**Figure 5**D). In the case of TCRγδ+ cells, no significant differences were observed as a result of the diets in either the CD8αα+ or CD8αβ+ subsets (**Figure 5**E-**5**F). With regard to the proportion of those Th, Tc and B cells expressing CD62L (L-selectin), a different pattern was found after the cocoa-enriched diet only, which reduced the proportion of CD45RA+CD62L+ cells and augmented that of CD45RA+CD62L- (**Figure 5**G-**5**I).

The spleen was also affected after 8 days of either the cocoa or theobromine diet. In particular, both nutritional interventions decreased the proportion of TCRγδ+ and NK cells and no significant differences were found in TCRαβ+ and CD45RA+ lymphocytes (**Figures 6**A-**6**B). However, studying in depth the TCRαβ+ subsets, in contrast to what happened in MLN, both diets increased the proportion of Th cells, whereas they decreased those of Tc and NKT cells (**Figure 6**C). As a result, the Th/Tc ratio was significantly higher than the reference group (**Figure 6**D). The percentage of TCRγδ+CD8αα+ and TCRγδ+CD8αβ+ cells in the three groups was similar (**Figure 6**E-**6**F). In the case of the CD62L marker on CD4+, CD8+ and CD45RA+ cells, only theobromine increased CD62L+ in CD4+ and CD8+ lymphocyte proportion (**Figure 6**G-**6**I).

FIGURE 5. Mesenteric lymph node lymphocyte composition after 8 days of nutritional intervention. (A) Percentage of the main lymphocyte subsets; (B) TCRαβ+/CD45RA+ ratio; (C) percentage of TCRαβ+ subsets; (D) Th/Tc ratio; (E) percentage of TCRγδ+ subsets; (F) CD8αα+/CD8αβ+ ratio; proportion of CD62L+ and CD62L- cells in (G) CD4+, (H) CD8+ and (I) CD45RA+ lymphocytes. Values are expressed as mean ± standard error (n=6). Statistical difference: * $p \leq 0.05$ by one way ANOVA or Mann-Whitney U test.

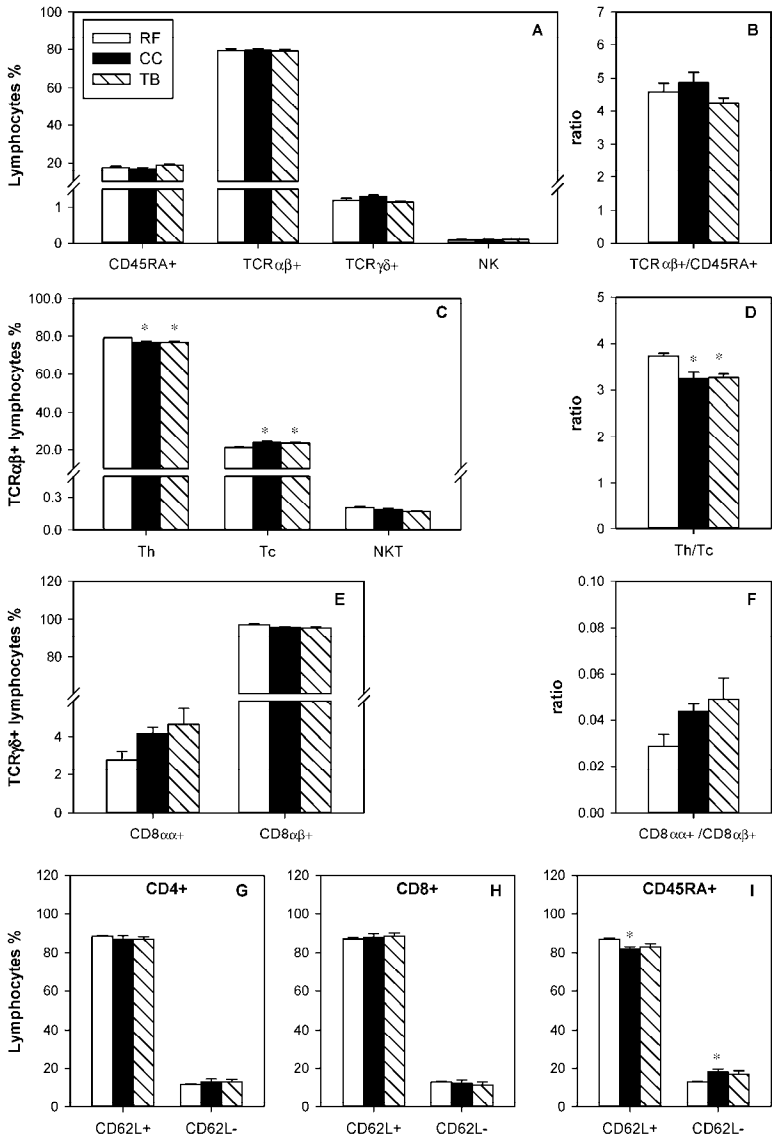
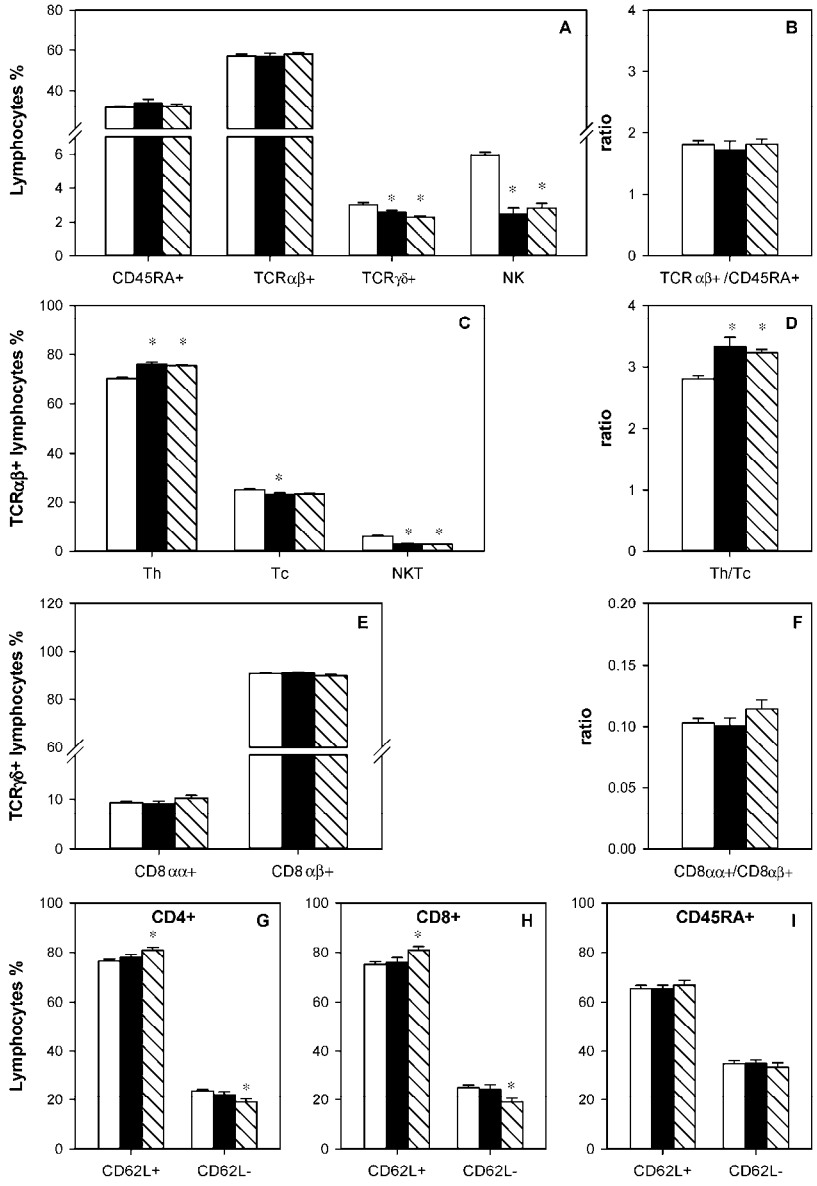


Figure 6. Spleen lymphocyte composition after 8 days of nutritional intervention. (A) Percentage of the main lymphocyte subsets; (B) TCRαβ+/CD45RA ratio; (C) percentage of TCRαβ+ subsets; (D) Th/Tc ratio; (E) percentage of TCRγδ+ subsets; (F) CD8αα/CD8αβ ratio; proportion of CD62L+ and CD62L- cells in (G) CD4+ (H) CD8+ and (I) CD45RA+ lymphocytes. Values are expressed as mean ± standard error (n=6). Statistical differences: * $p \leq 0.05$ by one way ANOVA or Mann-Whitney U test.



4. Discussion

Previous studies carried out in rats demonstrated that a 10% cocoa diet has the potential to regulate the immune function, attenuating the antibody synthesis both in systemic and intestinal compartments [9–11,30,31], and modifying lymphoid tissue composition mainly in the gut-associated lymphoid tissue (GALT) [8–11]. These effects could not be totally attributed to the cocoa polyphenol or fiber content [12,32,33]. The present data show, for the first time, the role of theobromine in cocoa's effects on antibody immune response and on lymphoid tissues. In addition, this study reveals the effects of cocoa on lymphoid tissue after only one week of intervention.

The current results demonstrate that the cocoa diet influenced the production of IgG when the intake lasted for more than 8 days. Specifically, we observed a decrease after 19 days of diet and, at this time, this was due to the reduction of IgG2c>IgG2b>IgG1. The total IgG decrease was in accordance with the previous 10% cocoa reported effects for 3 weeks on 3-week-old Wistar rats [10,32], whereas it did not match with a study carried out in older animals, in particular in 8-week-old Lewis rats [12]. These results suggest the importance of age in cocoa's effects on IgG. Regarding the sensitivity of each IgG isotype, previous studies modified them differently [11,25,31], reflecting that, among others, factors such as age, rat strain and environment could also be important in the influence of cocoa on IgG. Nonetheless, it is worth noting that theobromine on its own produced an identical effect on serum IgG and its isotypes to that of cocoa.

With regard to other serum immunoglobulins, theobromine or 10% cocoa diet also decreased the concentrations of IgM and IgA by similar proportions. These results partially or completely agree with those found after 3 or 7 weeks of 10% cocoa diet in 3-week-old or 6-week-old Wistar rats [10,11,31,32], and after 2 weeks of different cocoa-polyphenol-enriched diets in 8-week-old Lewis rats [12]. In addition, the current data show that IgM was affected earlier than IgA and both immunoglobulins earlier than IgG. In any case, most importantly, we can conclude that theobromine is responsible for cocoa's influence on systemic immunoglobulins.

With respect to the effect on intestinal antibodies, sIgA was also reduced by the cocoa diet as a result of its theobromine content. This down-regulatory effect was already observed after 4 days of the nutritional intervention and corresponded with prior data obtained after one [11,12], two [9,25], three [25,32], six [30] or seven weeks [31] of cocoa feeding using different rat strains. As reported previously, cocoa intake decreases the gene expression of cytokines and chemokines required for gut homing including TGF-β1, CCR9, CCL25, CCL28, RARα and RARβ in small intestinal tissue [31,34], which contributes to the lower proportion of IgA-secreting cells quantified in this compartment [34]. The results obtained here suggest that some or all of these mechanisms would be quickly modified after 4 days from the start of the diet.

On the other hand, this study also focused on the effect of the diets containing methylxanthines on the lymphoid tissues. These are classified into primary and secondary tissues, the primary lymphoid organs (bone marrow and thymus) being responsible for the development of lymphocytes [35], and the secondary ones (lymph nodes, spleen and mucosa-associated lymphoid tissues) involved in the regulation and the beginning of immune responses [36,37]. Previous studies have reported the influence of cocoa diet on MLN after at least 3 or 4 weeks of diet [9,14,38,39], whereas studies on the spleen and the thymus are very limited [8,10]. Indeed, in any of the studied lymphoid organs, there are no results regarding so short a diet period. First of all, it must be taken into consideration that both cocoa and theobromine intake decreased the weight of spleen and thymus after only one week of the experimental diet, suggesting that these compounds could inhibit the proliferation of lymphocytes that will eventually reduce lymphoid tissue organ weight, in a similar way to that described by a corticosteroid treatment [40]. In addition, the study of the thymus composition revealed that cocoa and theobromine intake increased the proportion of DN cells (CD4-CD8-) and CD4+ SP, whereas it decreased that of DP. Furthermore, both nutritional interventions delayed the expression of TCRαβ on DN cells. The relatively higher amount of the less mature cell type (DN cells) is in line with previous data obtained with a longer diet period [8], and leads us to think that theobromine may delay T cell maturation together with the low proliferation ratio suggested by the thymus weight. Nevertheless, this lymphocyte maturation delay after 8 days of diet was not associated with a lower proportion of T cells (TCRαβ+ cells) in either MLN or spleen. However, longer intake studies have demonstrated a decrease in the proportion of TCRαβ+ cells in lymph nodes [14], which could be due to this effect already observed in the thymus after one week of nutritional intervention.

In the current study, the balance of the main populations in the MLN and the spleen, i.e. B lymphocytes and TCRαβ+ cells, was not modified by the diet. These results do not agree with those found in MLN after 4 weeks of cocoa intake, which showed a higher B cell proportion and lower TCRαβ+ cell percentage [14], or with those found in spleen, which reported an increase in the percentage of B cells after feeding cocoa diet for 3 weeks [10]. Therefore, we can suggest that these previously observed changes would need a longer cocoa intake. However, the current results in MLN showed a reduction in the proportion of Th cells and an increase in that of Tc cells after one week of cocoa or theobromine intake, as found in longer studies [9,14]. Nevertheless, in the case of the spleen composition, there was a contrary effect, i.e. an increased proportion of Th cells and a decrease in Tc cell percentage, in line with the results found in thymus. In addition, in the spleen, there was a higher percentage of CD4+CD62L+ and CD8+CD62L+ cells in animals fed cocoa or theobromine. CD62L (L-selectin) is a cell adhesion molecule found in lymphocytes [41] that induces homing to lymphoid tissues and it is implicated in inflammatory leukocyte trafficking [42]. The increase of such splenocytes could indicate the arrival of Th cells to this lymphoid organ, presumably coming from the thymus and the MLN. In particular, Th cell production in the thymus was increased by the cocoa diet, and these cells could then move to thespleen. Moreover, we found a lower Th cell proportion in MLN,

therefore the emigration of these cells to the spleen, induced directly by cocoa intake, could also be suggested.

In the spleen, the cocoa and theobromine diets induced a relative reduction of the TCRγδ+ and NK cells that did not agree with the reported effect on the spleen in a longer study [10]. On the other hand, in the current study, TCRγδ+ cell and NK cell proportions in MLN were not modified by the nutritional interventions. However, other studies reported a higher proportion of TCRγδ+ (and sometimes NK cells) in MLN [9,14], Peyer's patches and intestinal intraepithelial lymphocytes [34], which could indicate that these cells leave the spleen to go to these peripheral lymphoid organs, although our study in MLN was not long enough to demonstrate such an increase.

The comparison of the effects of the cocoa and theobromine diets on the composition of both primary and secondary lymphoid tissues revealed that both diets produced almost the same degree of action in the modified lymphocyte subsets. Overall, these results indicate that theobromine is the mainly responsible for the immunoregulatory effect of cocoa on the lymphoid tissues, as stated before for the attenuation of systemic and intestinal antibody synthesis. Until now, different effects of theobromine have been published, but, as far as we know, no previous results suggesting the role of theobromine as an immunoregulatory agent have been published. In this context, here we show that theobromine has an immunoregulatory potential and could be indicated in situations, such as in organ transplantation to prevent rejection [43], in autoimmune disorders (multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus) [44] and in hypersensitivity reactions (allergic disease and asthma) [45]. With regard to its action mechanism, it has been described that theobromine is a potent inhibitor of the poly(ADP-ribose)polymerase-1 (PARP-1) [46]. PARP-1 is a nuclear enzyme that has an essential role in DNA repair [46] and relevant immunological functions, including the regulation of gene transcription in dendritic cells, macrophages and lymphocytes [47]. In this context, PARP-1 activation has been associated with pathologic conditions such as in inflammatory response in murine asthma models [48,49], and its inhibition prevents airway eosinophilia and suppresses Th2 cytokine production [50,51]. It has also been reported that PARP-1 negatively regulates Treg cell function [52,53] and higher numbers of CD4+CD25+FoxP3+ Treg cells appear in thymus, spleen and lymph nodes of PARP-1 knockout mice [54]. Altogether, these facts lead us to think that the reported inhibition of PARP-1 by theobromine [46] could have an important implication in the immunoregulatory role described here. Nevertheless, further studies are required to establish the exact mechanisms and to ascertain the minimum dose required of this methylxanthine for it to be able to produce the observed effects.

Overall, the current results suggest that cocoa, due to its theobromine content, affects thymocyte proliferation, producing a delay in lymphocyte maturation and favoring the presence of CD4+ cells, effects that are also reflected in the spleen. In addition, cocoa may also act directly on MLN, reducing the proportion of Th cells involved in B cell activation and, consequently on antibody production. It

remains to be ascertained whether the increase in CD4+ thymocyte could be due to an increase in CD4+CD25+ Treg cells, as previously reported [54].

In conclusion, a 10% cocoa-enriched diet is able, due to its theobromine content, to decrease systemic and intestinal immunoglobulin synthesis and modify thymus, mesenteric lymph nodes and spleen lymphocyte composition after a single week of dietary intervention.

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Authors' contributions to the manuscript

F.J.P-C, À.F and M.C. designed research; M.C-B conducted research, analyzed data and wrote the paper. F.J.P-C and M.C. contributed to the critical revision of the manuscript. All authors read and approved the final manuscript.

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Discussió

L'al·lèrgia alimentària constitueix un problema creixent de salut pública degut, principalment, a l'augment de la seva prevalença durant les darreres dècades. Per això, és important disposar de models animals d'aquest tipus d'al·lèrgia que permetin identificar els mecanismes responsables del desenvolupament de la sensibilització i, també, estudiar nous tractaments (immunoteràpies, medicaments o intervencions nutricionals) que regulin o evitin el procés [152]. Aquests últims anys ha augmentat l'ús de nutrients com a moduladors de la salut, ja sigui en forma d'aliment funcional o com a nutricèutic [153]. En aquest àmbit, el grup d'Autoimmunitat i Tolerància ha establert els efectes del cacau sobre el sistema immunitari sistèmic i intestinal [124,154–159], fet que ha permès definir l'objectiu general d'aquesta tesi, que consisteix en aprofundir sobre les propietats immunomoduladores del cacau mitjançant l'establiment dels efectes i dels possibles mecanismes induïts per una dieta rica en cacau en un model de sensibilització oral en rata, i en identificar el/s component/s responsable/s d'aquests efectes.

Per tal d'aconseguir aquest propòsit, el **primer objectiu** de la present tesi va ser desenvolupar un model de sensibilització oral en rata. Fins ara, s'havien descrit diversos models d'al·lèrgia alimentària en rosegadors, majoritàriament en ratolí [94]. Aquests models utilitzen diferents vies de sensibilització, diverses soques, l'administració conjunta d'adjuvants, diferents tipus d'antígens i un ampli ventall de dosis amb una durada variable del període de sensibilització [91,92,94,96]. Tot i així, es disposa de pocs models de sensibilització induïts únicament per via oral, via d'entrada que mimetitza millor el procés de sensibilització en humans. És per això, que, a l'inici de la tesi es van provar diversos procediments d'inducció de la sensibilització en els que es va utilitzar l'ovoalbúmina com al·lergogen alimentari (**Article 1**). Les diferències entre els protocols es basaven en la soca i el gènere de les rates (Brown Norway o Lewis, mascles o femelles), la seva edat a l'inici de la sensibilització (3, 5 o 8 setmanes), la dosi de l'al·lergen, la utilització d'adjuvant (toxina colèrica o sense adjuvant) i la pauta d'administració (d'una sola administració setmanal a una dosi diària). Els resultats obtinguts d'aquesta primera part de la tesi, permeten concloure que el desenvolupament d'un model de sensibilització oral depèn de l'ús d'adjuvants, la posologia de l'al·lergogen i de l'edat i el sexe dels animals (**Article 1**). En concret, es demostra que l'administració d'ovoalbúmina (50 mg) juntament amb l'adjuvant toxina colèrica (30 µg), tres vegades per setmana durant tres setmanes, en rates femelles Lewis de tres setmanes d'edat, indueix el desenvolupament d'una resposta immunitària que s'evidencia per la formació d'anticossos específics. Tal com es mostra en els **Articles 1 i 2,** aquests anticossos pertanyen als isotips IgG1, IgG2a, IgG2b i IgM, però no es desenvolupen anticossos antiovoalbúmina dels isotips IgE ni IgG2c. El model de sensibilització posat a punt a l'**Article 1**, ha estat emprat en els estudis posteriors, i ha permès una caracterització més profunda del model (**Articles 2, 3 i 4)**.

La toxina colèrica és un potent adjuvant [160] que evita el desenvolupament de la tolerància oral per diferents mecanismes més o menys establerts. Per una banda, la toxina colèrica incrementa la permeabilitat intestinal i, per tant, augmenta el pas d'antígens a la submucosa [161] alhora que afavoreix la pèrdua d'aigua, dada que es relaciona amb l'increment d'humitat en femtes detectat en el model (**Article 4**). A més a més, aquest adjuvant produeix un increment de l'expressió de les molècules del complex principal d'histocompatibilitat de classe II en els limfòcits B [162], activa la maduració de cèl·lules

dendrítiques [163] i la mobilització d'aquestes cap als ganglis limfàtics mesentèrics a on augmenta l'expressió de diferents molècules involucrades en la síntesi de citocines Th2 [164]. La toxina colèrica també activa el NF-κB en cèl·lules de ganglis limfàtics mesentèrics i de plaques de Peyer [165] i incrementa l'expressió gènica de molècules proinflamatòries [166]. Totes aquestes accions provoquen una elevada presentació antigènica amb activació de respostes Th2 que promouen la producció d'IgE i IgG1, tot afavorint l'activitat inflamatòria [163,165–168]. Encara que, en el model de sensibilització oral escollit, l'ús d'aquest adjuvant no ha comportat la producció d'IgE específica, els anticossos majoritaris sintetitzats s'associen a les respostes Th2 (IgG1 i IgG2a) en rata [97,169] (**Articles 1 i 2**). Per altra banda, s'ha descrit que la toxina colèrica potencia la formació d'IgA intestinal [170]; tanmateix, en el protocol de sensibilització establert, no es varen detectar variacions en el contingut intestinal d'aquesta immunoglobulina en cap dels experiments realitzats (**Articles 1, 2 i 4**). A més, a l'**article 3**, on s'analitza la presència de cèl·lules productores d'IgA a la paret intestinal, es mostra que la sensibilització oral s'acompanya d'un nombre inferior d'aquestes cèl·lules. De fet, la falta d'una resposta en IgA pot afavorir altres efectes de la toxina colèrica [171], com l'augment de la presentació antigènica i el desenvolupament de la sensibilització tal i com s'ha descrit en ratolins amb manca d'IgA intestinal [172].

Els **Articles 2 i 3**, de forma col·lateral als seus objectius, han permès conèixer la composició limfocítica de diferents teixits limfoides i l'expressió gènica de molècules involucrades en la resposta immunitària en el model de sensibilització posat a punt. Pel que fa al teixit limfoide associat a l'intestí, la sensibilització oral modifica la composició dels limfòcits intraepitelials i dels limfòcits de plaques de Peyer a l'intestí prim (**Article 3**), encara que no produeix cap canvi significatiu en la composició dels limfòcits de la làmina pròpia intestinal (**Article 3**) ni dels ganglis limfàtics mesentèrics (**Article 2**). Concretament, la sensibilització oral redueix el nombre relatiu de limfòcits TCRαβ+ a plaques de Peyer i modifica les proporcions de limfòcits que expressen molècules d'adhesió en el compartiment intraepitelial. En concret, disminueix la proporció de limfòcits CD8+CD103+ i incrementa la proporció de limfòcits CD62L+, tant CD4+ com CD8+ (**Article 3**). El marcador CD103 (integrina αE) promou l'adhesió de limfòcits a les cèl·lules epitelials intestinals [173], mentre que la molècula CD62L (selectina L) activa la migració a teixits limfoides perifèrics [174]. Els canvis induïts per la sensibilització oral en els limfòcits intraepitelials i en les plaques de Peyer indiquen que aquests limfòcits són els més sensibles al protocol de sensibilització usat, tot induint canvis relacionats amb la mobilització dels limfòcits entre diferents compartiments limfoides.

L'estudi de l'expressió gènica de diferents molècules en els teixits limfoides permet apropar-se als mecanismes implicats en la sensibilització oral. A ganglis limfàtics mesentèrics, s'ha observat que la sensibilització comporta un augment de l'expressió gènica d'OX40L (**Article 2**). OX40L s'expressa en cèl·lules dendrítiques i s'ha vist que la interacció OX40L-OX40 durant la presentació antigènica, afavoreix la inducció de respostes Th2 en ratolins quan se'ls hi administra toxina colèrica [164]. Aquest resultat permet suggerir la implicació dels ganglis limfàtics mesentèrics en la inducció de la sensibilització oral, encara que, la composició limfocítica en aquest compartiment no s'ha vist modificada (**Article 2**). Per altra banda, a nivell intestinal, la sensibilització oral

provoca una disminució de l'expressió gènica de la citocina IL-10 (**Article 3**) i d'un dels receptors tipus *toll*, receptors implicats en la interacció entre cèl·lules de l'hoste i microorganismes, concretament del TLR5 (**Article 1**). Cal assenyalar que tant la IL-10 com el TLR5 (receptor de la flagel·lina, present en cèl·lules dendrítiques intestinals) han mostrat un paper protector en un model d'al·lèrgia alimentària en ratolí [175] i, per tant, la seva baixa expressió gènica pot ser un mecanisme desencadenant de la sensibilització oral. Malgrat això, les rates sensibilitzades oralment presenten un augment del contingut intestinal d'IL-10 (**Article 2**) que podria ser degut a l'increment de la permeabilitat induït per la toxina colèrica.

L'anàlisi metagenòmica de la microbiota intestinal revela que la sensibilització oral també origina canvis en la microbiota intestinal (**Article 4**). Així, la sensibilització produeix una disminució de l'abundància relativa de l'ordre *Erysipelotrichales (Firmicutes),* per una reducció del gènere *Allobaculum*, gènere associat amb una adequada barrera mucosa [176]. A més, la sensibilització oral també indueix diferents canvis qualitatius i provoca una menor diversitat bacteriana, tot fent desaparèixer, entre altres, el bacteri *Akkermansia muciniphila,* responsable de la restauració del gruix de la barrera mucosa i de la producció de moc [177]. Tots aquests resultats junt amb altres presents a l'**Article 4** permeten concloure que la sensibilització oral amb toxina colèrica com adjuvant afecta a la microbiota intestinal i, tot i que són necessaris més estudis per tal de determinar el paper exacte que té cada una de les modificacions a l'hoste, les variacions microbianes suggereixen que l'administració d'ovoalbúmina i toxina colèrica afecten a diversos bacteris relacionats amb la producció i qualitat de la barrera intestinal.

Una vegada aconseguit el primer objectiu, el **segon objectiu** de la tesi es va centrar en establir l'efecte i els possibles mecanismes exercits per una dieta amb un 10% de cacau en el model de sensibilització oral desenvolupat.

Primer de tot, cal remarcar l'efecte modulador de la dieta rica en cacau sobre la síntesi d'IgA intestinal (**Articles 2, 3 i 4**). Aquest resultat s'observa de forma independent a la inducció de la sensibilització oral i es correspon amb els resultats d'altres estudis en rates de diferents soques alimentades amb una dieta similar [156,157,159,178]. Cal remarcar que els efectes del cacau en la IgA intestinal en les rates sensibilitzades oralment ja són evidents després d'una setmana d'administració de la dieta (**Article 4**) i després de quatre dies en animals no sensibilitzats (**Article 7**). Aquesta reducció, associada amb una menor expressió gènica d'IgA a l'intestí (**Articles 3**) és deguda a canvis en l'expressió gènica de diferents molècules involucrades en la formació d'IgA: des de l'atenuació de la citocina TGF-β1 (**Article 3**), implicada en l'activació de limfòcits B i la seva diferenciació a cèl·lules secretores d'IgA [179], fins a la reducció de molècules associades amb la migració intestinal de limfòcits, tals com CCR9, CCL25, CCL28, RARα i RARβ [25], que comporten finalment un menor nombre de cèl·lules IgA+ a la paret intestinal (**Article 3**). A més a més, la dieta rica en cacau comporta una menor proporció de limfòcits intraepitelials TLR4+ (**Article 3**), el que coincideix amb una reducció de la seva expressió

gènica, observada en estudis previs [156]. L'activació de TLR4 (receptor del lipopolisacàrid bacterià) s'ha associat amb diverses citocines que participen en el reclutament i diferenciació de les cèl·lules B IgA+ [180]. Així doncs, la dieta amb un 10% de cacau modula la proporció de cèl·lules TLR4+, modifica diverses citocines i marcadors involucrats en el reclutament intestinal i disminueix la proporció de cèl·lules IgA+ a la làmina pròpia intestinal (**Article 3**), el que comporta, finalment, una menor concentració d'aquest anticòs a la llum intestinal (**Articles 2, 3 i 4**) i una reducció en la proporció de bacteris units a IgA en femtes (**Article 6**).

El resultat més important observat després de la ingesta d'una dieta rica en cacau en el model de sensibilització oral, és l'efecte inhibitori sobre la producció d'anticossos específics sistèmics. Concretament, la dieta rica en cacau evita la formació d'anticossos antiovoalbúmina dels isotips IgG1, IgG2b i IgM (**Article 2**). Aquests resultats mostren els efectes tolerogènics del cacau en aquest model de sensibilització oral, de forma similar als observats prèviament en un model d'al·lèrgia alimentària i en una immunització sistèmica [181,182]. Per tal de caracteritzar els mecanismes que podrien estar implicats en l'adquisició de la tolerància, es va estudiar la composició limfocítica dels teixits limfoides intestinals inductors (ganglis limfàtics mesentèrics i Plaques de Peyer) i efectors (limfòcits intraepitelials i limfòcits de làmina pròpia). La dieta rica en cacau incrementa la proporció de cèl·lules NK i de limfòcits TCR$\gamma\delta$+ tant en els teixits inductors, és a dir, plaques de Peyer (**Article 3**) i ganglis limfàtics mesentèrics (**Article 2**), com en el compartiment intraepitelial de l'intestí prim (**Article 3**). Cal esmentar que hi ha estudis que associen l'increment de cèl·lules NK amb un efecte inhibitori sobre malalties al·lèrgiques [88,183], i d'altres, que reconeixen el paper dels limfòcits TCR$\gamma\delta$+ en la inducció de la tolerància oral [184–187]. A més a més, s'ha descrit que l'administració oral de la toxina colèrica a ratolins causa la migració del TCR$\gamma\delta$+ de l'epiteli a la làmina pròpia [167], el que suggereix que la dieta rica en cacau podria evitar aquesta migració i provocar la seva acumulació en el compartiment intraepitelial. Tots aquests resultats suggereixen la implicació de les cèl·lules NK i dels limfòcits TCR$\gamma\delta$+ en el procés de tolerància oral induït per la dieta rica en cacau.

El cacau, a més de promoure la presència de cèl·lules NK i de cèl·lules TCR$\gamma\delta$+, també modifica, de forma similar, la proporció d'altres limfòcits en els mateixos teixits limfoides inductors (plaques de Peyer i ganglis limfàtics mesentèrics). En ambdós teixits, la ingesta de cacau produeix una disminució en el nombre relatiu de limfòcits Th (**Articles 2 i 3**), fet que permet suggerir que aquesta davallada pot contribuir a una menor activació dels limfòcits B i, conseqüentment, a una menor producció d'anticossos tant a nivell intestinal (IgA) com a nivell sistèmic (anticossos específics a l'al·lergen). De forma general, en aquests dos compartiments, també s'observa que la dieta rica en cacau indueix un increment relatiu de cèl·lules CD25+ i CD103+ i una reducció de limfòcits CD62L+ (**Articles 2 i 3**). CD25 és un dels marcador de les cèl·lules T reguladores [26] i, encara que en la tesi no s'ha estudiat l'expressió de Foxp3, la major proporció de cèl·lules CD25+ podria indicar la proliferació de limfòcits reguladors implicats en el procés de tolerància. A més a més, els dos compartiments estudiats mostren l'increment relatiu de cèl·lules CD4+ i CD8+ amb expressió de CD103, cèl·lules que durant aquesta darrera dècada se'ls hi ha atribuït un important paper regulador [188,189]. Per altra banda, tant els resultats de ganglis

limfàtics mesentèrics (**Article 2**) com els de plaques de Peyer (**Article 3**) mostren una disminució en la proporció de limfòcits CD62L+ (molècula que potencia la migració a ganglis) el que podria indicar una menor arribada de limfòcits efectors als ganglis i, d'aquesta manera, s'evitaria l'activació de la resposta immunitària en aquest teixit, amb conseqüent reducció de la producció d'anticossos.

L'estudi de l'expressió gènica de diferents molècules a ganglis limfàtics mesentèrics i a l'intestí prim ha demostrat que la dieta rica en cacau incrementa els nivells de mRNA de CD11c en ganglis limfàtics mesentèrics (**Article 2**), mentre que es produeix una reducció de l'expressió gènica de CD11b i CD11c a nivell intestinal (**Article 3**). CD11b i CD11c són molècules presents a les cèl·lules dendrítiques [190], i els resultats obtinguts en ambdós compartiments suggereixen la mobilització d'aquestes cèl·lules intestinals cap a ganglis limfàtics mesentèrics, compartiment important en la tolerància oral [19]. Tanmateix, la ingesta de cacau redueix l'expressió gènica d'IL-17α en els ganglis, citocina inhibidora de la tolerància oral que actua mitjançant la inhibició dels limfòcits T reguladors [191], tot reforçant la participació d'aquest compartiment en el paper tolerogènic del cacau.

L'**Article 4** demostra els efectes del cacau i la sensibilització oral sobre la microbiota intestinal. Entre els efectes del cacau, cal destacar un increment en l'abundància relativa del gènere *Prevotella*, associat a efectes beneficiosos sobre l'intestí [192], i de l'espècie *Bacteroides uniformis,* similar al que s'ha trobat en humans que consumien polifenols del vi [193]. La intervenció nutricional també incrementa la proporció de *Lactobacillus reuteri,* bacteri àmpliament utilitzat com a probiòtic i que presenta una important activitat moduladora de les citocines intestinals [194,195]. A més a més, la reducció de la IgA intestinal per la dieta rica en cacau es correlaciona directament amb la davallada de *Proteobacteria*, bacteris associats amb la immaduresa i inflamació intestinal que constitueixen una gran part dels bacteris units a IgA en femtes i estimulen la producció d'IgA [196]. Per altra banda, cal remarcar que la dieta rica en cacau en els animals sensibilitzats no és capaç de revertir canvis induïts per la sensibilització oral, com són la disminució de l'ordre *Erypelotrichales,* la desaparició dels bacteris *Akkermansia muciniphila* i *Clostridium metallolevans* i l'aparició de *Bifidobacterium pseudolongum*. Això podria indicar que les modificacions de la microbiota induïdes per la sensibilització oral no intervenen en la formació d'anticossos contra l'al·lergen oral. Ara bé, de forma particular, la dieta rica en cacau en animals sensibilitzats provoca canvis a la microbiota que no s'observen en els animals sensibilitzats. Entre aquests canvis, es detecta una desaparició de *Clostridium perfringens* i de *Blautia producta*, mentre que apareix *Prevotella copri,* associada a dietes riques en vegetals [192], i d'altres espècies dels gèneres *Anaerostipes*, *Ralstonia* i *Desulfovibrio*. Algun d'aquests canvis específics de la dieta de cacau podrien estar implicats en la seva activitat tolerogènica.

Una vegada estudiats els efectes que realitza la dieta amb un 10% de cacau sobre el model de sensibilització desenvolupat, es va dur a terme el **tercer objectiu** d'aquesta tesi, centrat en identificar els components bioactius del cacau responsables del seu efecte

immunomodulador i en determinar l'efecte d'un flavonoid no present en el cacau sobre la resposta immunitària. Estudis previs havien evidenciat que els polifenols del cacau podien ser responsables d'algun dels efectes immunoreguladors d'aquest [178]. Aleshores, es va voler saber si tots els polifenols tenien una influència similar sobre el sistema immunitari. Per tal d'avaluar un polifenol diferent, es va escollir l'hesperidina (**Article 5**). Cal recordar que el cacau conté diversos tipus de flavonoides, essent els flavanols els majoritaris: catequina, epicatequina i procianidines [103]. La dieta de cacau emprada durant els Articles **2, 3 i 4**, amb un 10% de cacau en pols, proporciona una quantitat de polifenols del 0,4%. L'hesperidina és un flavonoide que pertany a la família de les flavanones i es troba principalment en les fruites cítriques tals com la taronja dolça, la llimona i la clementina [197]. Diversos autors han demostrat el paper immunomodulador de l'hesperidina, mitjançant la reducció de la síntesi de citocines Th2 en models d'asma [198,199] i, per això, es va escollir aquest flavonoide i es va analitzar el seu efecte en dos tipus de sensibilització en rata: intraperitoneal i oral (**Article 5**).

En el disseny de sensibilització intraperitoneal es va emprar l'al·lergen ovoalbúmina i dos adjuvants (hidròxid d'alumini i toxina de *Bordetella pertussis*) que estimulen una resposta immunitària Th2 [200,201]. Els resultats obtinguts amb la intervenció nutricional d'hesperidina difereixen bastant dels resultats previs observats amb la dieta rica en cacau en rates Wistar immunitzades per aquesta mateixa via [182]. En primer lloc, cap de les dues dosis d'hesperidina estudiades afecta la síntesi d'anticossos antiovoalbúmina desenvolupats (**Article 5**), al contrari de l'efecte observat pel cacau en estudis previs [182]. A més a més, l'anàlisi de la composició dels limfòcits de ganglis limfàtics mesentèrics mostra que l'hesperidina augmenta la proporció de limfòcits TCR$\alpha\beta$+ i disminueix la dels limfòcits B (**Article 5**), contràriament a l'efecte de la ingesta de flavonoides de cacau (**Article 2**). L'avaluació de la funcionalitat dels limfòcits ganglionars revela que l'hesperidina potencia la producció d'IFN-γ, citocina relacionada en respostes Th1 i inhibidora de respostes Th2 [202], resultats que no s'observen per l'efecte del cacau en l'actual tesi (**Article 2**), però si per la ingesta d'una dieta amb 4% i 10% de cacau en rates immunitzades en un estudi anterior [182].

Per altra banda, dins d'aquest mateix estudi (**Article 5**), s'ha analitzat la influència de l'hesperidina en el model de sensibilització oral posat a punt a l'**Article 1** que permet conèixer la resposta immunitària intestinal i en el teixit limfoide associat a l'intestí. En aquest cas, es va incloure l'hesperidina a la dieta, barrejada en el pinso, per tal d'arribar a una dosificació superior i continuada. En concret, la dieta amb hesperidina va proporcionar una quantitat de flavonoide de 0,5% (relatiu al pes del pinso), quantitat de flavonoide similar a la proporcionada amb la dieta amb un 10% de cacau. Els resultats d'aquest estudi també difereixen dels obtinguts amb la dieta rica en cacau (**Articles 2 i 3**). En el model de sensibilització oral, l'hesperidina incrementa el contingut d'IgA intestinal i no modifica la producció d'anticossos antiovoalbúmina ni la composició de limfòcits dels teixits inductors intestinals (ganglis limfàtics i plaques de Peyer) (**Article 5**). Per altra banda, la ingesta d'hesperidina produeix canvis en la composició dels compartiments intestinals efectors (limfòcits intraepitelials i de làmina pròpia): augmenta la proporció de limfòcits intraepitelials TCR$\gamma\delta$+, tal com prèviament s'havia observat amb la dieta rica amb cacau (**Article 3**) i amb altres polifenols [203] però, per contra, provoca

una disminució de la proporció d'aquest tipus cel·lular a la làmina pròpia. En aquest sentit, els resultats obtinguts suggereixen que l'hesperidina, de forma similar al cacau, pot tenir un efecte contrari a l'acció que presenta la toxina colèrica sobre la migració d'aquest tipus cel·lular [167] i, per tant, protector d'aquesta mobilització cel·lular.

En resum de l'estudi realitzat amb hesperidina, encara que molts polifenols han demostrat tenir un efecte atenuador de la producció d'anticossos específics en models d'al·lèrgia [204,205] (**Revisió 1**), i que s'ha descrit el paper d'aquesta flavanona en la reducció de la síntesi d'IgE específica en models d'asma [198,199], aquestes propietats no s'han pogut reproduir en els nostres models en rata. Així doncs, l'hesperidina influeix sobre el sistema immunitari intestinal de forma diferent a com ho fa la dieta rica en cacau, el que permet suggerir que, els polifenols, en funció de la seva estructura, actuen diferencialment sobre el sistema immunitari i, a més, altres components del cacau influeixen en el seu poder immunoregulador.

El cacau, a més de polifenols, conté metilxantines. Concretament és ric en teobromina [126]. Per això, per completar el tercer objectiu, els estudis següents es van centrar en l'efecte de la teobromina, emprada a la mateixa concentració que es troba en la dieta rica en cacau, en la microbiota intestinal i en el sistema immunitari (**Articles 6 i 7**). Els resultats de l'anàlisi de les poblacions bacterianes intestinals (**Article 6**) permeten concloure que la ingesta de teobromina altera quantitativament i qualitativament la microbiota intestinal, encara que de forma diferent a com ho fa la dieta rica en cacau. Aquesta discrepància podria ser deguda a la interacció d'altres components del cacau, com la fibra o els flavonoides, sobre la microbiota intestinal. En aquest sentit, estudis previs realitzats amb fibra de cacau han demostrat el seu paper prebiòtic [206]. Per tant, els efectes de la dieta rica en cacau sobre la microbiota resulten del balanç dels efectes individuals dels seus components (fibra, flavonoides i teobromina). Tanmateix, altres modificacions observades per la ingesta de cacau també s'han trobat per la ingesta de teobromina, resultats que suggereixen el paper d'aquesta metilxantina en aquestes accions. Així, tant la dieta rica en cacau com la dieta amb teobromina augmenten la concentració fecal d'àcids grassos de cadena curta, principalment d'àcid butíric (**Article 6**). Aquest increment, que també es produeix per la ingesta de fibra de cacau [206], pot ser molt beneficiós ja que l'àcid butíric presenta un paper important en la regulació de la barrera intestinal i del sistema immunitari intestinal [207,208]. A més, l'àcid butíric i l'àcid acètic (augmentat només amb la dieta rica en cacau tal com es mostra a l'**Article** 6 i, sobre tot, quan la dieta és a base de fibra de cacau [206]) poden potenciar el desenvolupament de la tolerància oral tal com s'ha descrit prèviament [71].

Finalment, es va dur a terme l'estudi de la influència de la teobromina del cacau sobre el sistema immunitari, tant pel que fa a la concentració d'immunoglobulines (sistèmiques i intestinals), com a la composició dels teixits limfoides primaris i secundaris (**Article 7**). Amb aquest estudi s'evidencia que la teobromina és la responsable dels efectes del cacau en l'atenuació de la concentració sistèmica d'IgG, d'IgA i d'IgM i del contingut intestinal d'IgA. A més a més, la majoria d'aquests efectes ja són evidents a partir de la primera setmana d'administració, tal com s'havia observat en la IgA intestinal a l'**Article 3**. També es va veure que la dieta rica en cacau i la dieta amb teobromina provoquen efectes

similars sobre les poblacions limfocítiques dels teixits limfoides estudiats després de 8 dies d'intervenció nutricional, el que indica que la teobromina és la responsable dels canvis en les poblacions limfocítiques. Pel que fa al timus (teixit limfoide primari), l'increment observat en la proporció de cèl·lules immadures doble negatives (CD4-CD8-) i la reducció del pes de l'òrgan suggereixen que la ingesta de cacau, degut al seu contingut en teobromina, és capaç d'inhibir la proliferació de timòcits i alentir la seva maduració. Aquest efecte no s'acompanya d'una menor proporció de limfòcits TCR$\alpha\beta$+ (ni T CD4+ ni T CD8+) en els teixits limfoides secundaris estudiats després d'una setmana d'intervenció, però podria repercutir més tard, tal com es detecta després de 4 setmanes d'ingesta de cacau en la població ganglionar TCR$\alpha\beta$+ (**Article 2**). En aquest mateix sentit, altres poblacions limfocítiques ganglionars no s'han modificat després de 8 dies d'intervenció nutricional (**Article 7**), a diferència del que s'ha trobat després de 4 setmanes (**Article 2**), el que suggereix que aquestes poblacions necessiten més temps de dieta per tal de veure's modificades.

L'**Article 7** també ha inclòs l'estudi de la melsa, teixit limfoide secundari no considerat en altres estudis de la tesi i del que només es disposava d'un antecedent [155]. A aquest nivell, s'ha detectat que la dieta rica en cacau i la ingesta de teobromina redueixen el nombre relatiu de limfòcits TCR$\gamma\delta$+ i NK esplènics. Aquests efectes, juntament amb els obtinguts als **Articles 2** i **3** que mostren un increment de la proporció d'aquests dos tipus cel·lulars a ganglis limfàtics mesentèrics, plaques de Peyer i en el compartiment intraepitelial, suggereixen que la dieta rica en cacau, a causa del seu contingut en teobromina, provoca la migració de limfòcits TCR$\gamma\delta$+ i cèl·lules NK des de la melsa cap a aquests òrgans limfoides, on podrien desenvolupar el seu paper tolerogènic, tal com s'ha proposat anteriorment.

En general, els resultats derivats de l'**Article 7** plantegen el paper immunoregulador de la teobromina. Aquest potencial es podria aprofitar en situacions on l'increment d'anticossos té un paper patogènic, com és per evitar el rebuig de trasplantaments [209], els trastorns autoimmunitaris [210] i en reaccions d'hipersensibilitat [211]. Pel que fa al mecanisme d'acció, s'ha descrit que la teobromina presenta una acció inhibidora de la poli-ADP ribosa polimerasa-1 (PARP-1) [212]. La PARP-1 és un enzim nuclear, expressat en la majoria de les cèl·lules, relacionat amb la reparació del DNA a través de la modulació de la transcripció i l'estructura de la cromatina i, també, s'ha associat amb inflamació i carcinogènesi, entre d'altres [213,214]. A més a més, s'ha descrit el paper de PARP-1 en el sistema immunitari, tant a nivell d'immunitat innata (macròfags i granulòcits, entre d'altres) com d'immunitat adaptativa (cèl·lules dendrítiques, limfòcits B i T) [215]. En aquest sentit, s'ha observat un increment de l'activació de PARP-1 a la resposta inflamatòria de l'asma [216,217] i, per això, aquest enzim es pot considerar una diana important en la modulació de respostes Th2, tal com s'ha descrit en diferents estudis [215,218–220]. Per altra banda, la inhibició d'aquest enzim altera la immunocompetència de les cèl·lules dendrítiques, comporta una menor proliferació dels limfòcits T [221] i altera diferents mecanismes de la generació de la resposta Th2 (disminució d'IL-5, inhibició de l'activació del NF-κB, etc.) [217]. A més, ratolins *knockout* per PARP-1 presenten un nombre superior de limfòcits T reguladors (CD4+CD25+Foxp3+) en timus, melsa i ganglis limfàtics [222]. Per tot això, es pot postular que la teobromina present en

la dieta rica en cacau interacciona amb PARP-1 i produeix la seva inactivació. Aquest mecanisme pot disminuir la proliferació i diferenciació de limfòcits T en el timus, el que podria repercutir en la composició limfocítica de la melsa, a on també podria tenir un efecte directe. A més a més, la teobromina del cacau, inhibint aquest enzim, pot afectar el teixit limfoide intestinal on podria provocar l'acumulació de limfòcits TCR$\gamma\delta$+ i cèl·lules NK (**Articles 2 i 3**). En aquest sentit, són necessaris més estudis per tal d'identificar tots els mecanismes implicats, la mínima dosi necessària i desenvolupar estudis clínics amb aquestes intervencions nutricionals. Com que l'homologia de la PARP-1 d'humans amb la seqüència d'aminoàcids de ratolins és del 92% [215], es pot postular que la influència en humans pot ser similar.

En resum, el cacau, degut el seu contingut en teobromina, és capaç de modular la resposta immunitària sistèmica i també influeix sobre el teixit limfoide associat a l'intestí, accions que comporten una atenuació de la resposta immunitària intestinal i contribueixen al desenvolupament de la tolerància oral. Així doncs, el cacau (o la teobromina) podria usar-se com a potent nutricèutic en la prevenció d'una sensibilització oral, i en general, en la de les al·lèrgies alimentàries.

Conclusions

The results obtained from the current thesis led us to conclude that:

- The administration of 50 mg of ovalbumin and 30 μg of cholera toxin, three times per week for three weeks, in female Lewis rats is able to breakdown oral tolerance, producing the synthesis of anti-ovalbumin antibodies (IgG1, IgG2a, IgG2b and IgM). The development of the oral sensitization depends on the use of cholera toxin as adjuvant, the rat strain, the dosage, the age and the sex of the animals. This oral sensitization causes modifications in the intestinal microbiota, particularly in the number of bacteria related to the mucus production and its quality, and produces changes in the lymphocytes present in the Peyer's patches and in the gene expression of regulatory molecules, such as interleukin 10 and toll-like receptor 5.

- A 10% cocoa diet prevents the synthesis of systemic anti-ovalbumin antibodies (IgG1, IgG2b and IgM) and intestinal IgA in the induced rat oral sensitization model.

- A 10% cocoa diet modifies the lymphocyte subsets present in the mesenteric lymph nodes, Peyer's patches and small intestine epithelium and lamina propria. It is noteworthy that the cocoa intake produces a relative expansion of TCR$\gamma\delta$+ lymphocytes and NK cells in three of the previous compartments, together with a decrease in the proportion of Th cells on the inductive sites of the gut-associated lymphoid tissue, suggesting their role in cocoa's tolerogenic effect.

- In the small intestine and mesenteric lymph nodes, a 10% cocoa diet modifies the presence of dendritic cells together with the gene expression of cytokines, surface markers and molecules related to IgA synthesis that could be implicated in the cocoa's tolerogenic effect.

- Hesperidin intake in intraperitoneally or orally stimulated rats influences the gut-associated lymphoid tissue composition and functionality, although it does not modify the specific antibody synthesis.

- A 10% cocoa diet influences quantitatively and qualitatively the microbiota composition in a rat oral sensitization model. These effects seem not to be attributable to cocoa's theobromine content, although this methylxanthine produces a similar action in terms of decreasing IgA-coated bacteria and in increasing the metabolic microbiota products, the short-chain fatty acids.

- The theobromine intake attenuates systemic and intestinal immunoglobulin synthesis and modifies lymphoid tissue composition in a similar way to the cocoa diet. Therefore, the theobromine content in cocoa seems to be the main component responsible for the immunoregulatory properties of cocoa.

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