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Acció moduladora de prebiòtics i probiòtics sobre la infecció per rotavirus en un model de rates lactants

Maria del Mar Rigo Adrover

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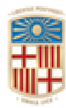

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FACULTAT DE FARMÀCIA I CIÈNCIES DE L'ALIMENTACIÓ
Departament de Bioquímica i Fisiologia

ACCIÓ MODULADORA DE PREBIÒTICS I PROBIÒTICS SOBRE LA INFECCIÓ PER ROTAVIRUS EN UN MODEL DE RATES LACTANTS

MARIA DEL MAR RIGO ADROVER
Barcelona, 2017

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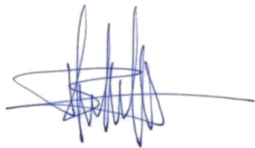
ACCIÓ MODULADORA DE PREBIÒTICS I PROBIÒTICS SOBRE LA INFECCIÓ PER ROTAVIRUS EN UN MODEL DE RATES LACTANTS

Memòria presentada per **Maria del Mar Rigo Adrover** per optar al títol de doctor per la Universitat de Barcelona



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INFORMA

Que la memòria titulada ***Acció moduladora de prebiòtics i probiòtics sobre la infecció per rotavirus en un model de rates lactants*** presentada per **MARIA DEL MAR RIGO ADROVER** per optar al Títol de Doctor amb Menció Internacional per la Universitat de Barcelona, ha estat realitzada sota la meva direcció al Departament de Bioquímica i Fisiologia, i considerant-la conclosa, autoritzo la seva presentació per ser jutjada pel tribunal corresponent.

I perquè així consti, signo el present a

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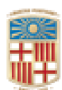

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Abreviatures

AGCC	Àcids grassos de cadena curta
ALA	*Lapin rotavirus strain*
APC	Cèl·lules presentadores d'antígens, *antigen-presenting cells*
B-GOS	Bimuno® GOS
BAP-2	*Lapin rotavirus strain*
BAP_{wt}	*Wild-type lapin rotavirus strain*
BRV	*Bovine rotavirus strain*
C11	*Rabbit rotavirus strain*
CLA	Antigen cutani associat a limfòcits
DP	Grau de polimerització
DTH	Hipersensibilitat retardada, *delayed type hypersensitivity*
EC_{wt}	*Murine wild-type rotavirus strain*
EDIM	*Epizootic diarrhoea of infant mice*
EFSA	*European Food Safety Authority*
EHP	*Murine rotavirus strain*
EHP_{wt}	*Murine wild-type rotavirus strain*
ELISA	*Enzyme-linked Immunosorbent Assay*
EMA	*European Medicines Agency*
ER	Equivalents de trans retinol
ESPGHAN	*European Society for Paediatric Gastroenterology Hepatology and Nutrition*
ET	Equivalent de D-α-tocoferol
EW_{wt}	*Murine wild-type rotavirus strain*
FAO	*Food and Agriculture Organization*
FDA	*Food and Drug Administration*
FM	Postbiòtic derivat de llet fermentada amb *Bifidobacterium breve* i *Streptococcus thermophilus*
FOS	Fructooligosacàrids
GALT	Teixit limfoide associat a l'intestí, *gut-associated lymphoid tissue*
GFA	Barreja prebiòtica scGOS/lcFOS/pAOS

GLM	Ganglis limfàtics mesentèrics
GOS	Galactooligosacàrids
GRAS	*Generally Regarded As Safe*
HAL1166	*Human rotavirus strain*
HBC	Calostre boví hiperimmune, *hyperimmune bovine colostrum*
HEV	Vènules endotelials altes, *high endothelial venule*
HMO	Oligosacàrids de la llet materna, *human milk oligosaccharides*
HRV	Rotavirus humà, *human rotavirus strain*
IEL	Limfòcit intraepitelial, *intraepithelial lymphocyte*
IFNγ	Interferó gamma, *interferon gamma*
Ig	Immunoglobulines
IL	Interleucina
ISAPP	*International Scientific Association for Probiotics and Prebiotics*
LGG	*Lactobacillus rhamnosus* GG
LP	Làmina pròpia
LPS	Lipopolisacàrid bacterià
NCDV	*Bovine rotavirus strain*
NEC	Enterocolitis necrotitzant
NK	*Natural Killer*
OMS	Organització Mundial de la Salut
OSU	*Porcine rotavirus strain*
pAOS	Oligosacàrids acídics derivats de pectina, *pectin-derived acidic oligosaccharides*
PCL	Àcids grassos poliinsaturats de cadena llarga
PP	Plaques de Peyer
PRR	Receptors de reconeixement de patrons, *pattern recognition receptors*
PRV	*Porcine rotavirus strain*
QPS	*Qualified Pressumption of Safety*
R2	*Lapin rotavirus strain*
RF	*Bovine rotavirus strain*
RRV	*Simian rhesus rotavirus strain*
RV	Rotavirus

S-Ig	Immunoglobulines secretores
S-IgA	Immunoglobulina A secretora
SA11	*Simian Agent 11 rotavirus strain*
scGOS/lcFOS	Combinació de galactooligosacàrids de cadena curta i fructooligosacàrids de cadena llarga
Tc	T citotòxics
Th	T col·laboradors o *helper*
TLR	Receptors tipus *Toll*, *Toll-like receptors*
TNFα	Factor de necrosis tumoral alfa, *tumor necrosis factor alpha*
Treg	T reguladors
UFC	Unitats formadores de colònies
W161	*Human rotavirus strain*
Wa	*Human rotavirus strain*
WC3	*Bovine rotavirus strain*
XOS	Xilooligosacàrids
YO	*Human rotavirus strain*

RESUM/*ABSTRACT*

Rotavirus (RV) is the leading cause of severe diarrhoea among infants and young children and, although more standardized studies are needed, there is evidence that probiotics can help to fight against RV and other infectious and intestinal pathologies. It would be of interest to find specific products obtained from a source related to that of young and immature stages, such as probiotics from breast milk or baby faeces, or oligosaccharides reflecting the structure of those contained in the breast milk.

On this basis, the aim of the present thesis was to evaluate the protective effect of probiotics, prebiotics and other related products designed to be suitable for infants against RV infections using the suckling rat as experimental model.

To achieve this objective, an updated model of simple RV infection with some new biomarkers was used. Moreover, a new RV double-infection model in rat, based on early SA11 and post-early-weaning EDIM inoculations, was developed. Modulatory actions during the first infection seem to be relevant to the later immune response – when encountering a second RV infection – as has been demonstrated by means of an RV-hyperimmune bovine colostrum (HBC) administration.

The thesis tested the **probiotic** effect of the supplementation with ***Bifidobacterium breve*** **M-16V** during the rat suckling period. Results showed that this probiotic improved the development of mucosal immunity in early-life rats, by modulating Toll-like receptor expression, enhancing the homing process of naïve T lymphocytes to the mesenteric lymph nodes, and raising the retention of activated lymphocytes in the intraepithelial compartment, as well as enhancing the intestinal IgA synthesis. Moreover, in the single and double RV infection models this probiotic attenuated RV infection and reinfection by ameliorating diarrhoea during first infection but allowing the host to elaborate its own immune response, which seems to help control the second infection.

The thesis also tested the **prebiotic** effect of **scGOS/lcFOS 9:1** (Immunofortis®)**,** throughout the rat suckling period. The prebiotic mixture ameliorated RV infection in the single-infection model and

modulated reinfection in the double RV infection model, showing a high immunomodulatory action, although it had a direct effect on stool consistency. Its effect on the diarrhoea associated with RV infection was comparable to that of the prebiotic **Bimuno® GOS**, allowing protection against further RV infection in this preclinical model. In addition, a **synbiotic** combining the **scGOS/lcFOS** prebiotic with the probiotic ***Bifidobacterium breve*** **M-16V**, was highly effective in modulating RV-induced diarrhoea as well as modulating immune response and RV reinfection in the double-infection preclinical model. Conversely, the combination of Immunofortis® with **pectin-derived acidic oligosaccharides (pAOS)** did not potentiate its preventive effect on the rat gastroenteritis RV model.

On the other hand, the **postbiotic** supplementation with a *Bifidobacterium breve* and *Streptococcus thermophilus*-fermented formula, and its combination with **scGOS/lcFOS** throughout the rat suckling period were able to prevent almost all features derived from the RV-induced diarrhoea and furthermore they also modulated the anti-RV immune response.

Overall, all tested products showed beneficial effects on the RV-induced gastroenteritis in the neonatal rat model, modulating clinical biomarkers and immune system responses early in life, with the probiotic and the postbiotic being the most effective. Further studies are needed in order to better understand their mechanism of action and for them to be considered for inclusion in infant formulas or supplements as strategies for protecting against human RV-induced diarrhoea in children.

OBJECTIUS/*OBJECTIVES*

In the last few years, interest in the mutualism between hosts and their microbiota has increased considerably. In this sense, the intestinal microbiota affects the human physiology by influencing the epithelial barrier and immune function, among others, both directly and indirectly. These beneficial effects are especially relevant in early life, when the immune system is still in development. They are the basis for studying strategies to modulate the intestinal environment and microbiota composition and functionality, and then indirectly to act on the mucosal immune system, which in turn may modulate systemic immunity.

Among the strategies to influence the anti-infective response of neonates, the use of probiotics is the most studied. It is known that probiotics are exogenous microorganisms that interact with various cellular components within the intestinal environment and have a positive impact on the host's health. Moreover, in recent years, prebiotics have attracted increasing interest due to their action in promoting the selective growth of beneficial bacteria. The combination of both – so-called synbiotics – may have synergistic effects. Postbiotics – products of probiotic metabolism – are no longer considered as the metabolic waste of probiotic activity, but instead are now being studied as an alternative to the use of the whole bacteria. It would be of interest to assess those types of products obtained from a source related to early life, such as probiotics from breast milk or baby faeces, or oligosaccharides reflecting the structure of those present in breast milk.

Rotavirus is the leading cause of severe diarrhoea among infants and young children and, although more standardized studies are needed, there is evidence that probiotics can help to fight against rotavirus and other infectious and intestinal pathologies. However, the effects of prebiotics, and to an even lesser degree those of postbiotics, have not been properly addressed in the context of viral infection.

Although there have been many efforts made to evaluate the influence of these microbial modulatory products on infants' immune response, problems exist with the variability of the physiological or disease status studied, regarding the probiotic strains or prebiotic diversity, as well as there being limitations in the number of participants in most of the studies. This is the reason why most currently available data that describe the effects of these compounds on immune response are derived from preclinical and *in vitro* studies.

On the basis of this background, the **HYPOTHESIS** that supported the current thesis was that **the immune response, as well as the anti-infective capacity of the organism, in early life can be enhanced by some microbial modulatory products (i.e. probiotics, prebiotics, postbiotics or their combinations).**

Therefore, considering this hypothesis, the **MAIN OBJECTIVE** of this thesis was:

To establish the beneficial effect of microbial modulatory products of importance in early life on the immunodevelopment and protection against rotavirus infections using the suckling rat as experimental model.

To achieve this main objective, the following specific objectives were proposed:

OBJECTIVE 1. To establish the **immunomodulatory impact of a probiotic strain** isolated from baby faeces in a model of immune development in suckling rats.

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

- Preclinical immunomodulation by the probiotic *Bifidobacterium breve* M-16V in early life. *PlosOne,* 2016; 11(11): e0166082

OBJECTIVE 2. To assess the protective effect of the daily administration throughout the suckling period of different **microbial modulatory products** – one probiotic, several prebiotics, a synbiotic combination and one postbiotic suitable for early life – on the course of a unique rotavirus infection in suckling rats.

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

- A combination of scGOS/lcFOS with *Bifidobacterium breve* M-16V protects suckling rats from rotavirus gastroenteritis. *Eur J Nutr*, 2016; In press.

- A fermented milk concentrate and a combination of scGOS/lcFOS/pAOS protect suckling rats from rotavirus gastroenteritis. *Br J Nutr*, 2017; In press.

- Protection of rotavirus gastroenteritis by a combination of scGOS/lcFOS with a fermented milk concentrate in suckling rats. *Manuscript in preparation (results).*

OBJECTIVE 3. To investigate **the impact of microbial modulatory products** – one probiotic, different prebiotics and a synbiotic combination – **designed for early life action in a rat suckling model of rotavirus infection and reinfection**, as usually occurs in humans. For that reason, a new animal model of reinfection in which to evaluate the nutritional intervention was set up.

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

- Towards a human-like rotavirus gastroenteritis model: double infection in suckling rats. *Submitted to Nutrients (first revision).*

- Nutritional interventions with pro- and prebiotics in a double rotavirus infection model. *Manuscript in preparation.*

INTRODUCCIÓ

1. Desenvolupament de la microbiota intestinal i del sistema immunitari

La microbiota intestinal i el sistema immunitari es desenvolupen de manera paral·lela. El contacte amb microorganismes comensals estimula i modula el sistema immunitari de l'hoste, i alhora, l'adquisició de tolerància per part del sistema immunitari és essencial per a l'establiment de la microbiota (1). La tolerància es coneix com l'estat d'immunosupressió, a nivell local i sistèmic, enfront a antígens innocus (2).

1.1. Microbiota

La microbiota autòctona és el conjunt de microorganismes que colonitza de manera estable la superfície epidèrmica i de les mucoses i que estableix una relació simbiòtica amb l'hoste (3).

1.1.1. Composició

La microbiota inclou bacteris, arqueus, fongs i virus (**Taula 1**), i les diferències en la seva composició als diferents llocs del cos depenen de les condicions de pH, nivell d'oxigen, disponibilitat de nutrients, humitat o temperatura (4). La major part de la microbiota es troba al tracte gastrointestinal, i sobretot, a l'intestí gros (10^{11}-10^{12} unitats formadores de colònies [UFC]/mL).

En quant a la variació interindividual en la composició de la microbiota intestinal, s'han identificat tres enterotips diferents (**Figura 1**). L'enterotip resulta de la classificació dels microorganismes vius presents a l'ecosistema intestinal humà depenent de l'abundància relativa de determinats gèneres (5). Així doncs, l'enterotip 1 es caracteritza per una abundància dels bacteris que pertanyen als gèneres *Bacteroides* i *Parabacteroides*; l'enterotip 2 es caracteritza per una major abundància dels gèneres *Prevotella* i *Desulfovibrio*; i finalment, a l'enterotip 3, que és el més freqüent, hi predominen els *Ruminococcus*, *Staphylococcus*, *Gordonibacter* i *Akkermansia* (6).

La microbiota té un paper important en la salut dels individus, i canvis o alteracions en la seva composició (disbiosi) s'han associat a malalties com l'obesitat, les al·lèrgies, el síndrome d'intestí irritable, la colitis

ulcerativa, la malaltia de Crohn, la malaltia celíaca, la diabetis o el síndrome metabòlic (4,7,8)

Taula 1. Diversitat taxonòmica dels microorganismes que conformen la microbiota autòctona en adults. Modificat de Suárez, 2015 (3)

Domini	**Regne**	**Fila**	**Classe**	**Exemple**
Archaea	*Archaea*	A.II. *Euryarcheota*	*Methanobacteria*	Metanògens intestinals
Bacteria	*Bacteria*	B. XII. *Proteobacteria*	*Gammaproteobacteria*	*Escherichia* (intestí gros)
			Epsilonproteobacteria	*Helicobacter* (estómac)
		B. XIII. *Firmicutes*	*Clostridia*	*Lachnospira*, *Faecalibacterium*, *Roseburia* (intestí gros)
			Bacilli	*Lactobacillus* (vagina, intestí prim), *Staphylococcus* (pell), *Streptococcus* (boca)
		B. XIV. *Actinobacteria*	*Actinobacteria*	*Bifidobacterium* (intestí gros), *Propionibacterium* (pell, intestí gros), *Corynebacterium* (pell), *Gardnerella* (vagina)
		B. XX. *Bacteroidetes*	*Bacteroidetes*	*Bacteroides*, *Prevotella* (intestí gros)
Eukaryota	*Protista*	*Protozoa*	*Rhizopoda*	Amebes comensals (boca, intestí)
			Mastigophora	*Giardia* (duodè)
	Fungi	*Ascomycota*	*Saccharomycetes*	*Candida* (vagina, boca, intestí gros)
		Basidiomycota	*Exobasidiomycetes*	*Malassezia* (pell)
	Animalia	*Arthropoda*	*Arachnida*	*Demodex* (àcars de la pell)

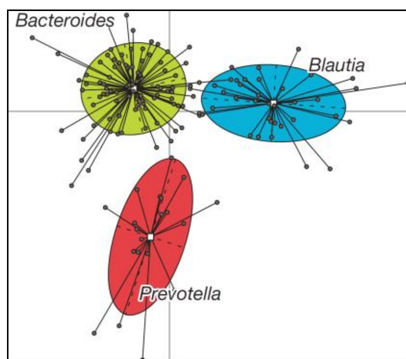


Figura 1. Representació dels enterotips segons les diferències filogenètiques. Modificat d'Arumugam et al., 2011 (6)

1.1.2. Funcions de la microbiota intestinal

Mentre l'intestí proporciona un hàbitat ric en aliments i amb condicions favorables per a la microbiota intestinal, aquesta desenvolupa diverses funcions que són beneficioses per a l'hoste:

- La microbiota és essencial per a la digestió, la motilitat intestinal, les transformacions metabòliques i l'adquisició de nutrients i energia. Aporta enzims capaços de metabolitzar nutrients no digeribles, sintetitza una gran varietat de vitamines i aminoàcids essencials i afavoreix l'absorció de minerals (3,9,10).

- Els components de la microbiota tenen un paper protector enfront a patògens, ja que competeixen amb ells pels llocs d'adhesió i els nutrients, i també secreten substàncies antimicrobianes (3,10).

- La interacció de la microbiota amb el sistema immunitari intestinal promou el desenvolupament d'aquest durant la infància i la seva modulació (1). També s'ha relacionat amb el desenvolupament del cervell i amb el comportament (8,11).

1.1.3. Colonització en les diferents etapes de la vida

La colonització de l'intestí per part de la microbiota comença *in utero*, abans del naixement. Tot i que fins fa poc es creia que aquest medi era estèril, s'han trobat microorganismes presents a la placenta, el líquid amniòtic, les membranes fetals, el cordó umbilical i el meconi de nounats sans i nascuts a terme (4,7,12–14). La microbiota prenatal es caracteritza per una escassa abundància però gran diversitat, el que suggereix que no procedeix principalment de la microbiota vaginal (de baixa diversitat, predominada per *Lactobacillus* spp.), sinó de la microbiota de la cavitat oral materna. I en aquest sentit, la dieta de la mare durant el període de gestació sembla que també pot influenciar l'establiment de la microbiota (12).

Al naixement, tot i la baixa diversitat de la microbiota intestinal, existeix una elevada variabilitat interindividual (**Figura 2**). El pH gàstric relativament elevat i la secreció atenuada de bilis en els nounats afavoreixen l'assentament inicial. El tipus de part, l'edat gestacional en el moment del naixement, l'ús d'antibiòtics o la dieta de la mare durant

la lactància són factors que poden afectar la composició de la microbiota intestinal del nounat (7,8,12–14).

A través de la llet materna, a més d'immunoglobulines (Ig) i oligosacàrids prebiòtics entre d'altres, alguns microorganismes del tracte gastrointestinal de la mare poden arribar al de l'infant per mitjà de la ruta entero-mamària (12). La microbiota de la llet materna, que presenta una elevada variabilitat interindividual, està composta per bacteris dels gèneres *Staphylococcus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Weisella*, *Enterococcus*, *Propionibacterium*, *Lactobacillus*, *Bifidobacterium*, etc. (15).

Durant la primera part de la infància, la microbiota està predominantment formada per microorganismes capaços de digerir oligosacàrids (lactobacils i bifidobacteris), mentre que amb la introducció d'aliments sòlids aquesta canvia i augmenten les espècies capaces de digerir polisacàrids i de sintetitzar vitamines (Firmicutes i Bacteroidetes). Al llarg del primer any de vida hi ha una fluctuació i maduració de la microbiota, que depèn de l'alimentació (llet materna o fórmula) i de l'ambient (ús d'antibiòtics, contacte amb animals de l'entorn, situació geogràfica, etc.).

Fins fa poc, es considerava que l'assoliment de la microbiota madura tenia lloc entre el primer i el quart any de vida, però tot i que ja està més establerta, encara és dinàmica i no del tot madura (11,12). Fins i tot durant l'adolescència s'observen canvis en la microbiota, probablement degut als canvis hormonals. Per tant, no és fins a l'edat adulta que la microbiota és relativament estable. Encara que pot variar a causa d'infeccions, ús d'antibiòtics o canvis en la dieta, té tendència a restablir-se.

La variabilitat interindividual de la microbiota en adults és menor que en els més joves, tot i que factors com la situació geogràfica, el context social, el sexe o factors genètics hi poden influir (12). La microbiota en adults sans està dominada per *Bacteroidetes*, *Firmicutes* i, en menor grau, Actinobacteris (16). Aquesta composició torna a variar en edats avançades.

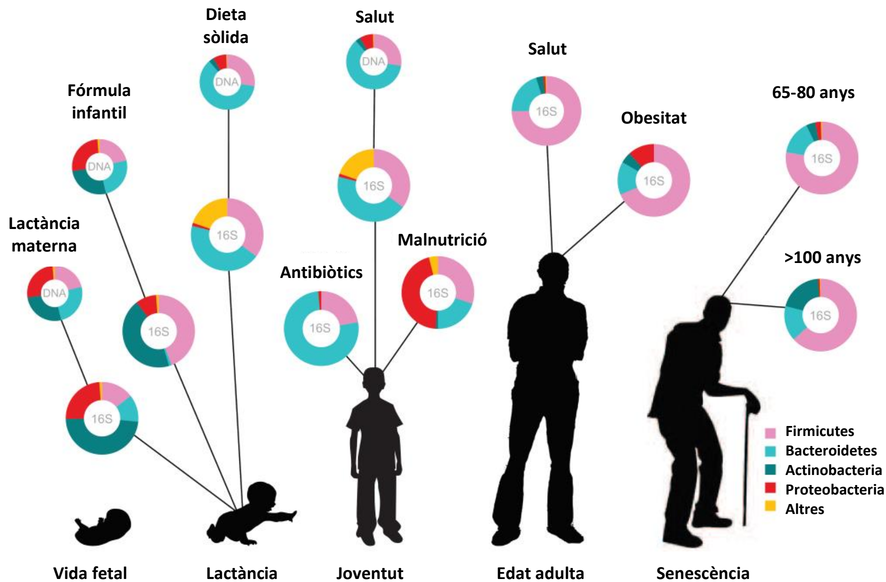


Figura 2. Representació esquemàtica de la variació de la composició microbiana al llarg de la vida -determinada per tècniques moleculars-. Adaptat d'Ottman et al., 2012 (17)

1.2. El sistema immunitari

El sistema immunitari dels infants es troba inhibit durant l'embaràs per tal d'evitar reaccions immunològiques entre la mare i el fetus. En les primeres etapes de vida, la resposta immunitària enfront a patògens és molt variable, però no es pot considerar que es tracti d'un sistema immunitari deficient, ja que en alguns casos s'ha observat que pot donar una resposta igual que en adults (18,19).

La major susceptibilitat a infeccions durant els primers mesos de vida pot ser deguda, per una banda, a la incompleta funcionalitat d'alguns elements de la resposta innata i adquirida al naixement, i per altra banda, a la falta de memòria immunològica al no haver-hi una exposició prèvia a aquests patògens (19). A més, en els nounats la resposta inflamatòria té tendència a estar suprimida (18,19).

1.2.1. El sistema immunitari intestinal

El teixit limfoide associat a l'intestí (GALT, *gut-associated lymphoid tissue*) constitueix la major part del sistema immunitari associat a les mucoses (**Figura 3**). La formació d'estructures limfoides secundàries,

com les plaques de Peyer (PP) o els ganglis limfàtics mesentèrics (GLM), té lloc abans del naixement, però la seva mida i el desenvolupament de centres germinals depèn de la colonització postnatal de l'intestí per microorganismes (20).

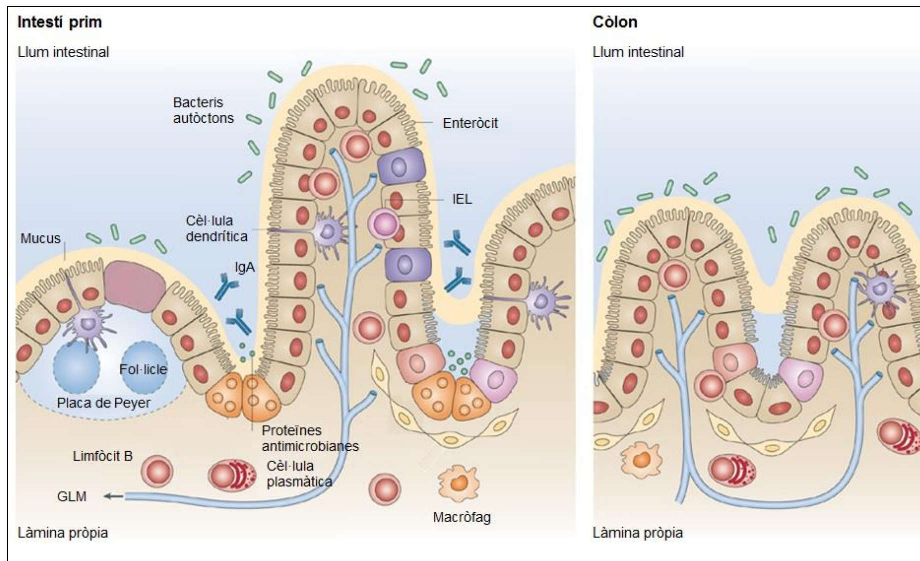


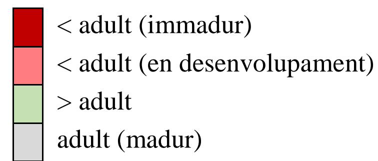
Figura 3. Representació esquemàtica del teixit limfoide associat a l'intestí (GALT). **GLM:** gangli limfàtic mesentèric; **IEL:** limfòcit intraepitelial; **IgA:** immunoglobulina A. Modificat d'Abreu, 2010 (21)

Al moment del naixement, la mucosa de l'intestí prim ja presenta una arquitectura cripta-vellositat madura, amb contínua proliferació de les cèl·lules mare i migració i diferenciació de les cèl·lules epitelials. Així i tot, l'epiteli intestinal és més permeable i la composició de pèptids antimicrobians al lumen intestinal i la composició i la glicosilació del mucus són diferents en nounats respecte als adults (**Figura 4**) (19,20).

La composició de la microbiota, en combinació amb factors genètics i epigenètics té influència sobre la barrera epitelial de les mucoses i la maduració perinatal del sistema immunitari (20). Per una banda, els processos epigenètics tenen una gran importància en el funcionament del sistema immunitari neonatal. Aquests processos comencen *in utero* i regulen l'ontogènia del sistema immunitari (18,22). Per altra banda, els microorganismes intestinals i els antígens alimentaris modulen el desenvolupament del sistema immunitari innat i adquirit a través de l'estimulació de receptors de reconeixement de patrons (PRR, *pattern recognition receptors*) (20). Els receptors tipus *Toll* (TLR, *Toll-like*

receptors) són un tipus de PRR que reconeixen patrons moleculars associats a microorganismes (19).

El tracte gastrointestinal és el lloc amb major interacció entre microorganismes i el sistema immunitari (19). La presència de la microbiota en primeres etapes de vida té un paper crític per a que el desenvolupament morfològic i funcional del sistema immunitari sigui complet (23,24). La microbiota regula l'homeòstasi dels neutròfils, la granulocitosi, augmenta la resistència a la sèpsia i intervé en el desenvolupament d'estructures limfoides secundàries com les PP (22).



	Nounat		Infant		Jove		Adult	
Permeabilitat intestinal	< adult (en desenvolupament)	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Mucines	< adult (en desenvolupament)	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Pèptids antimicrobians	adult (madur)	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Expressió TLR	< adult (en desenvolupament)	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Granulòcits	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Cèl·lules dendrítiques	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Monòcits	> adult	> adult	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Cèl·lules NK	> adult	> adult	> adult	> adult	> adult	adult (madur)	adult (madur)	adult (madur)
Cèl·lules Tc	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Resposta Th1	< adult (immadur)	< adult (en desenvolupament)	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Cèl·lules B	> adult	> adult	> adult	adult (madur)	adult (madur)	adult (madur)	adult (madur)	adult (madur)
Anticossos	< adult (immadur)	< adult (immadur)	< adult (en desenvolupament)	< adult (en desenvolupament)	< adult (en desenvolupament)	< adult (en desenvolupament)	adult (madur)	adult (madur)

Figura 4. Desenvolupament del sistema immunitari. Es representa el grau de maduresa dels principals components del sistema immunitari en diferents etapes de la vida. Basat en dades de Martin et al., 2010; Renz et al., 2011; Pérez-Cano et al., 2005a i 2005b (19,20,25,26)

1.2.2. Resposta immunitària innata

La interacció entre els bacteris intestinals i l'hoste es coneix cam a "*cross-talk*". Com a conseqüència del *cross-talk* entre les cèl·lules epitelials i la microbiota, s'expressen menys els TLR i s'expressa més l'inhibidor del factor nuclear-κB durant el període postnatal (**Figura 4**). D'aquesta manera s'afavoreix la tolerància de la immunitat innata. La sensibilitat de les cèl·lules epitelials dels nounats a l'estimulació de

TLR es troba també disminuïda, però altres vies de senyalització de la immunitat innata són plenament funcionals (20).

L'activació controlada del sistema immunitari innat contribueix a l'absorció de nutrients, l'angiogènesi, la diferenciació de les cèl·lules epitelials i la fortificació de la barrera epitelial (20).

Hi ha menys nombre de granulòcits, amb una producció de superòxid i una expressió de L-selectina reduïdes, i per tant, una quimiotaxi i transmigració deficients (19,22).

La quimiotaxi i la capacitat de fagocitosi i d'endocitosi per part dels monòcits en infants són menors que en adults (**Figura 4**). La quantitat de monòcits és elevada durant les dues primeres setmanes de vida i disminueix a partir de la tercera fins a assolir els nivells de l'adult (22).

Les cèl·lules *Natural Killer* (NK) es troben en major nombre durant la infantesa, gradualment disminueixen per arribar al nivell dels adults al voltant dels 5 anys d'edat i presenten una menor capacitat citotòxica i de desgranulació (19,22,25,26).

1.2.3. Resposta immunitària adquirida

La tolerància de les mucoses està regulada per un conjunt de senyals de les cèl·lules de la immunitat innata que modulen la resposta immunitària adquirida, és a dir, depèn de la correcta interacció entre cèl·lules presentadores d'antígens (APC, *antigen-presenting cells*), els limfòcits T i de la integritat de la barrera epitelial (20).

La producció de citocines i la capacitat de presentar antígens i d'estimulació per part d'APC en infants són menors que en adults (**Figura 4**). La seva expressió d'algunes molècules de superfície, com molècules d'histocompatibilitat o d'adhesió, és menor (22).

Les cèl·lules T dels nounats presenten majoritàriament un fenotip verge però són capaces de respondre a antígens ambientals. Són més susceptibles a apoptosi i la seva producció de citocines és menor que en adults (22).

Els limfòcits T reguladors (Treg) són abundants en els GLM del fetus (19), i la seva migració cap a la mucosa intestinal és especialment activa durant la infància (20). La migració de limfòcits Treg cap a la

mucosa intestinal i la secreció de la citocina anti-inflamatòria interleucina (IL)-10 s'han associat a la colonització bacteriana (20).

Pel que fa a les cèl·lules T citotòxiques (Tc), en els nounats presenten menys activitat i proliferen menys (19).

D'altra banda, els limfòcits T col·laboradors o *helper* (Th) en els nounats tenen una predisposició contra la producció de citocines inflamatòries o de tipus Th1 (**Figura 4**), i per tant, una tendència a les respostes de tipus Th2 (18,19,22).

Es creu que aquesta inclinació a la resposta de tipus Th2 és un mecanisme protector, en primer lloc, perquè les citocines proinflamatòries com factor de necrosi tumoral alfa (TNFα, *tumor necrosis factor alpha*) o IL-1β poden afavorir l'avortament. De fet, la placenta produeix progesterona i prostaglandines que afavoreixen la diferenciació a Th2 en el fetus. En segon lloc, una resposta reguladora permet el desenvolupament de la microbiota sense inflamació (22). D'aquesta manera, la hipometilació de les regions reguladores del locus Th2 de les cèl·lules T CD4+ incrementa les citocines de tipus Th2 com la IL-13 o IL-10, que estimula les cèl·lules Treg, i disminueix la producció de citocines de tipus Th1 com TNFα, IL-6, IL-12, IL-15 o interferó gamma (IFNγ, *interferon gamma*), que repercuteix amb una menor activació de cèl·lules NK i cèl·lules T.

D'altra banda, les cèl·lules B es troben en nombres elevats durant les primeres sis setmanes de vida, van disminuint gradualment a partir dels dos anys, i tenen majoritàriament un fenotip verge. La seva maduració a cèl·lules plasmàtiques és erràtica, i els nivells d'Ig circulants són inferiors (**Figura 4**). La resposta humoral independent de cèl·lules T no és madura fins als 3-5 anys de vida (19,22).

La immunitat humoral contribueix a l'establiment d'una barrera epitelial adequada. La IgA secretora (S-IgA) inhibeix activacions immunitàries inapropiades per part de microorganismes i antígens del lumen intestinal, recobrint-los i restringint la colonització i la penetració d'aquests (20). La interacció amb la microbiota millora la barrera intestinal constituïda per S-IgA i promou la tolerància oral amb una predominança de l'activitat cel·lular Th2 en el període perinatal i un perfil de citocines més equilibrat més endavant (19).

Les cèl·lules plasmàtiques productores d'IgA no són detectables a la làmina pròpia (LP) abans dels deu dies d'edat, i només es troben proporcions ínfimes d'Ig secretores (S-Ig) de tipus S-IgA i S-IgM de síntesi endògena al lumen després del naixement (20,25).

El resultat dels mecanismes que afavoreixen una resposta reguladora en els nounats és una limitada capacitat d'inflamació per combatre els patògens, que pot ser compensada en part per la immunitat passiva amb la transferència d'IgG de la mare durant la gestació, o els bacteris de la microbiota de la glàndula mamària, metabòlits i factors immunitaris del calostre i la llet materna (27). La IgA restringeix l'activació immunitària i prevé l'adhesió de microbis, els oligosacàrids es poden unir als patògens i afavoreixen el creixement de bacteris beneficiosos (bifidobacteris).

2. Rotavirus i diarrea

2.1. Incidència de la diarrea per rotavirus

La diarrea és la quarta causa de mortalitat infantil al món i el rotavirus (RV) és el principal agent etiològic en lactants i nens menors de cinc anys (28,29). Pràcticament tots els nens del món hauran estat infectats per RV, almenys un cop, durant els primers tres anys de vida (30). Aquesta malaltia suposa una càrrega per al sistema de salut i la societat que, tot i que no es coneix amb total exactitud, es preveu molt elevada (31).

El RV és un patogen humà de la família *Reoviridae* ("Reo": "*Respiratory-Enteric-Orfan*") que s'observa principalment en forma de gastroenteritis en lactants i infants menors de cinc anys, tot i que també pot afectar a nens d'edat avançada i adults (32).

El RV és un virus de transmissió principalment fecal-oral, sobretot prevalent en mesos hivernals. La seva denominació la va rebre el 1973 quan Ruth Bishop, Geoffrey Davidson, Ian Holmes i Brian Ruck van identificar unes noves partícules patògenes que es trobaven al citoplasma de cèl·lules epitelials de les vellositats intestinals d'infants. Eren pacients ingressats al Royal Children's Hospital de Melbourne per una pronunciada diarrea i deshidratació. Aquestes partícules virals coincidien amb les que es trobaven a les seves femtes (33). El seu estudi primerenc de la morfologia va permetre donar-li el nom de "rotavirus", ja que "*rota*" significa en llatí "roda", i aquesta és la seva forma.

La seva distribució és universal. De fet, cada any pot causar al voltant de 215.000 morts (31) sobretot en països en vies de desenvolupament -sobretot a la major part d'Àfrica, a Índia, Pakistan o Afganistan- (**Figura 5**).

En el cas d'Europa, la incidència de la diarrea per RV és elevada. Entre un 6 i un 11% dels ingressos hospitalaris de nens menors de cinc anys han estat deguts a una gastroenteritis i d'aquests entre un quart i la meitat són atribuïbles a una infecció per RV.

A Europa, les infeccions nosocomials per RV entre els menors de cinc anys són d'una proporció de 2,5 a un 11,8%. A Espanya s'ha estimat que la incidència anual és de: 86 casos cada 1000 nens menors de 3

anys requereixen atenció domiciliaria; 15 casos per 1000 menors de 5 anys que han de ser atesos a centres d'atenció primària; de 15 a 17 casos per 1000 nens menors de quatre anys han de ser atesos a urgències pediàtriques; i d'1 a 3 casos per 1000 menors de cinc anys requereixen ingrés hospitalari (31).

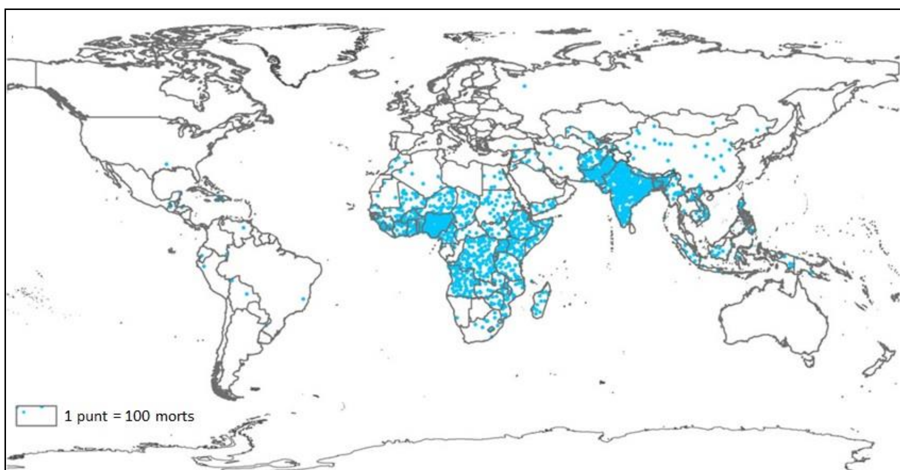


Figura 5. Nombre de morts causades per RV en infants menors de 5 anys el 2013. Adaptat de Tate et al., 2016 (28)

La particularitat que més crida l'atenció de la infecció per RV en països temperats, com el nostre, és la seva estacionalitat. Hi ha un augment de casos a l'hivern i a la primavera, ja que es creu que les característiques climàtiques d'aquests mesos afavoreixen tant la transmissió com l'exacerbació de les manifestacions.

2.2. Estructura del rotavirus

Dins de la família *Reoviridae*, el RV del grup A és el gènere més comú i el principal agent etiològic de la diarrea severa en lactants i nens menors de cinc anys a nivell mundial. El RV es caracteritza per ser un virus d'estructura icosaèdrica, sense embolcall, d'aproximadament 75 nm de diàmetre, i amb un genoma segmentat de RNA de doble cadena que codifica per a sis proteïnes estructurals (VP1-VP4, VP6, i VP7) i per a 6 de no estructurals (NSP1-NSP6) (34,35).

La càpside (**Figura 6**) està formada per 3 capes proteiques concèntriques que contenen el genoma al seu interior. La coberta més externa està formada per les proteïnes estructurals VP4 i VP7, que són

les que permeten infectar les cèl·lules. La coberta intermèdia està formada per la proteïna VP6 i la més interna majoritàriament per la proteïna VP2, i en menor quantitat per les proteïnes VP1 i VP3 (31,35).

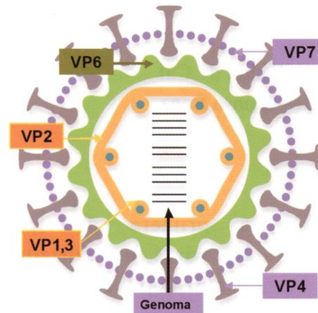


Figura 6. Representació esquemàtica de la partícula de RV amb les proteïnes estructurals. Román Riechmann, 2006 (31)

2.3. Infecció i patogènia de la infecció per rotavirus

Tot i la llarga bibliografia sobre el RV i la seva morfologia, els coneixements sobre el mecanisme exacte pel qual el virus penetra a les cèl·lules és poc conegut (36). Sembla però, que la replicació viral causa canvis fisiològics i morfològics a nivell de les cèl·lules epitelials intestinals, i que la dosi infectiva a la que pot provocar-los és generalment de 10 UFC/mL. El procés fisiopatològic induït és el següent (37):

- **Transmissió:** El RV és altament contagiós. La seva transmissió és principalment fecal-oral, ja que tot i incloure's a la família *Reoviridae*, la detecció del virus en secrecions respiratòries altes no és, de moment, remarcable. La transmissió es pot donar a través del contacte persona-persona, per la ingesta d'aliments i aigua contaminada amb matèria fecal o fòmits, ja que es tracta d'un virus molt resistent al medi ambient i que pot persistir-hi uns mesos si la zona on s'ha establert no es troba desinfectada. Així, el virus penetra a l'organisme per via digestiva -normalment quan els infants es posen les mans a la boca- fins arribar a les cèl·lules epitelials de les vellositats de l'intestí proximal.

- **Penetració cel·lular:** El RV penetra al citoplasma quan s'uneix a integrines de les cèl·lules hoste a través de les proteïnes VP4 i l'àcid siàlic.

- **Canvis intracel·lulars:** Al citoplasma, augmenta la concentració de calci intracel·lular i es debilita el citoesquelet i les unions cel·lulars, de manera que augmenta la permeabilitat.

- **Canvis morfològics de l'intestí:** El virus comença a allisar les cèl·lules apicals de les vellositats intestinals fins arribar a les porcions distals de l'intestí prim (**Figura 7**).

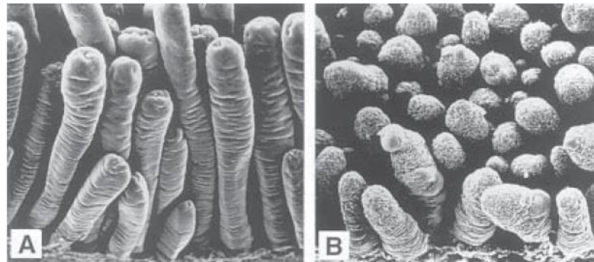


Figura 7. Vellositat de l'intestí prim de vedell. (A) Control d'animals sans. (B) Animals inoculats experimentalment amb RV boví: microvellositats atrofiades i les seves parts apicals desordenades i més gruixudes. Román Riechmann, 2006 (31)

- **Afectació de l'activitat enzimàtica:** Es produeix una pèrdua de la capacitat d'absorció d'aigua, sodi i glucosa. L'activitat de la lactasa i d'altres disacaridases disminueix i es duu a terme la síntesi d'una enterotoxina (NSP4) que provoca una alteració dels canals de clor per un mecanisme calci-depenent.

- **Diarrea:** Augmenta el flux d'aigua i electròlits cap a la llum del tub digestiu, originant els vòmits i les diarrees secretores pròpies de les infeccions per RV.

- **Eliminació viral:** L'eliminació del virus per femta és aproximadament de 10^{10}-10^{11} partícules virals/g. Aquesta pot iniciar-se 3 dies abans de l'aparició de les manifestacions i perllongar-se fins a 10 dies després o 30, segons si es tracta d'una persona sana o immunocompromesa, respectivament.

Davant aquest mecanisme fisiopatològic provocat pel virus, el sistema immunitari de l'hoste sol induir la producció d'anticossos específics contra les proteïnes VP4 i VP7 i l'activació de cèl·lules T específiques.

2.4. Característiques clíniques del procés

El període d'incubació de la infecció per RV és curt, generalment inferior a 48 h i les manifestacions gastrointestinals se solen resoldre entre el tercer i el setè dia de la malaltia.

Tot i presentar signes diarreics propis i semblants a moltes altres patologies intestinals, la clínica que presenta el nen o lactant infectat per RV és variable segons si es tracta d'una primera infecció o d'una reinfecció. Els signes generals principals són la presència de vòmits, la deshidratació greu, un desequilibri electrolític i una pèrdua de pes que sol desencadenar en un ingrés hospitalari. Aquests signes solen anar acompanyats de variacions de l'aspecte físic de l'infant que sol presentar sequedat de la boca o la llengua, una disminució de la turgència cutània, l'absència total o parcial de llàgrimes i l'enfonsament de la fontanel·la i l'abdomen, igual que dels ulls i les galtes (**Figura 8**).

Les complicacions de les infeccions per RV, sobretot en nens o lactants immuno-compromesos per una immunodeficiència congènita o adquirida, poden afectar a diferents òrgans, entre els que cal destacar el ronyó i el fetge.

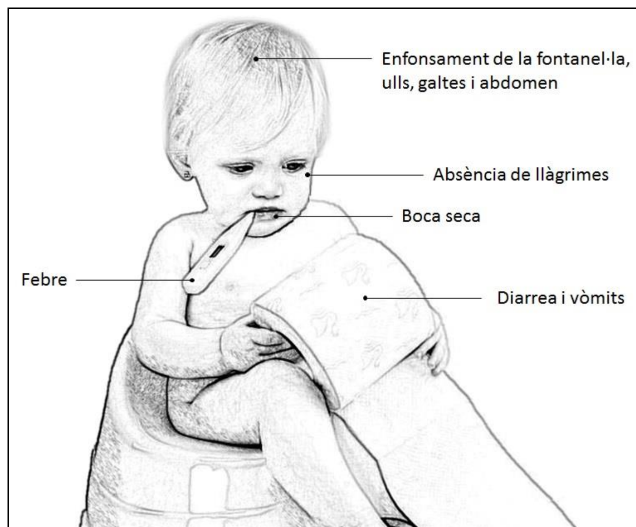


Figura 8. Imatge representativa de les manifestacions clíniques davant una infecció per RV

2.5. Resposta immunitària i protecció enfront del rotavirus

La resposta immunitària que l'organisme desenvolupa enfront d'una infecció per RV és humoral i cel·lular, tant local com sistèmica. Freqüentment un mateix individu pateix varies infeccions per RV al llarg de la vida, encara que la intensitat de les manifestacions disminueix amb l'edat. A més, les infeccions més greus acostumen a ser les infeccions primàries, el que suggereix que després d'una infecció es desenvolupa una protecció immunitària enfront del virus, que en canvi no arriba a ser del tot completa fins transcorregudes varies reinfeccions (31).

La infecció per RV indueix tant una resposta immunitària innata com adaptativa, incloent la producció de citocines i d'anticossos específics contra el virus a través de les cèl·lules immunitàries (38,39). El pas inicial de la resposta immunitària consisteix en la presentació de l'antigen viral de manera local als limfòcits T i B per part dels macròfags i de les cèl·lules dendrítiques, les quals juguen un paper crucial en l'activació de la resposta cel·lular, representant un vincle entre la resposta innata i la resposta adaptativa (40). Les cèl·lules dendrítiques intestinals es troben en diferents compartiments del GALT com les PP, els GLM i la LP, la qual és considerada la zona efectora de la mucosa intestinal. Les cèl·lules NK constitueixen la primera línia de defensa enfront del virus. Aquests, espontàniament, destrueixen les cèl·lules infectades i s'ha demostrat que tenen un paper protector en les infeccions virals. Els limfòcits T citotòxics també presenten una gran capacitat de defensa enfront de la infecció viral i actuen principalment a partir de tres mecanismes: la lisi de les cèl·lules infectades mitjançant les perforines i els gramzims, l'apoptosi cel·lular, i la producció de citocines antivirals (38).

Les citocines són proteïnes de mida petita que secreten principalment els limfòcits T i els macròfags, així com també les cèl·lules epitelials intestinals (41). Aquestes es produeixen durant l'activació cel·lular tant en la resposta immunitària innata com en l'específica. Per una banda, s'ha descrit que les citocines poden causar disfunció de les cèl·lules epitelials degut a la seva activitat citotòxica, així com alteracions en la permeabilitat de les unions estretes o canvis en les secrecions d'ions (**Figura 9**). D'altra banda, també s'ha descrit que algunes citocines com l'IFN-γ i el TNF-α són capaces d'eliminar les partícules virals,

així com de prevenir l'expressió gènica i la replicació del virus sense destruir les cèl·lules infectades, a partir de l'activació d'altres vies immunitàries. Aquest mecanisme podria ser un punt clau en l'eliminació del virus i la protecció de l'hoste en la fase aguda de la malaltia quan encara no s'han activat altres respostes immunitàries com la producció d'anticossos (38,41).

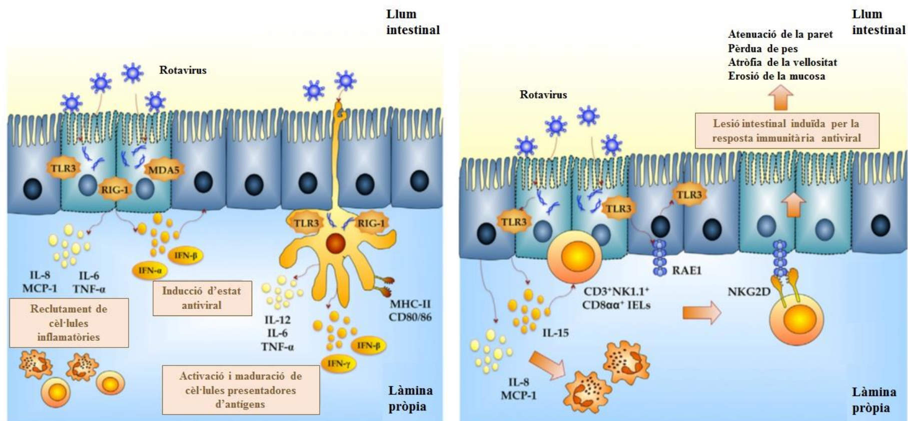


Figura 9. Resposta immunitària innata contra el rotavirus i dany inflamatori induït pel rotavirus via receptor tipus *Toll* 3 (TLR3) a la mucosa intestinal. Modificat de Villena et al., 2016 (41)

En referència a les cèl·lules B i la producció d'Ig, s'ha comprovat en experiments amb ratolins deficients en cèl·lules B que aquestes són necessàries per tal d'aconseguir una protecció de llarga durada enfront de les reinfeccions per RV (42).

La infecció produeix una resposta d'immunoglobulines IgG, IgA, IgM i S-IgA en la mucosa intestinal (LP i PP). Per una banda, la presència de cèl·lules productores d'anticossos en la LP assegura l'eliminació de virus tant en la mucosa com en la llum intestinal. Les cèl·lules B específiques per RV expressen la integrina α4β7, necessària per a la recirculació, migració i assentament en la mucosa intestinal. D'altra banda, la presència de cèl·lules secretores d'anticossos anti-RV en sang perifèrica preveu la presència d'aquestes cèl·lules en la LP, produint localment IgA polimèrica. Aquests anticossos són capaços de neutralitzar partícules víriques i d'inhibir el procés de replicació. Els anticossos sèrics que més predominen durant la fase aguda de la infecció, actuant com a marcadors d'una primera infecció, són els de classe IgM, que són substituïts posteriorment per IgG i en menor concentració per IgA. La resposta d'IgG sèrica és de major durada que

la d'anticossos IgA en saliva o femtes. Les IgA sèriques poden ser indicadors de la presència d'IgA intestinal (43).

Es considera que els anticossos locals de classe IgA constitueixen el mecanisme de protecció més eficaç enfront de la infecció per RV. Diversos autors han demostrat que la IgA intestinal és essencial tant en l'eliminació viral d'una primera infecció per RV com en la protecció immunitària davant de possibles reinfeccions (44). En experiments realitzats amb porcs gnotobiòtics, és a dir, colonitzats per una microbiota coneguda, als que se'ls van inocular per via oral RV humans, es va observar que els anticossos IgA sèrics són els marcadors més fiables de protecció enfront de la infecció (45). Recentment, un estudi realitzat amb ratolins deficients en IgA intestinal ha demostrat que aquesta Ig és crucial en la durada de la primera infecció i és absolutament essencial en l'assoliment d'una completa protecció intestinal enfront de reinfeccions per RV (44). No obstant, altres estudis realitzats amb cohorts d'infants escolaritzats han demostrat que, encara que es pot establir una bona correlació entre els nivells d'IgA en femtes i el nivell de protecció, un nombre significatiu de nens amb títols elevats d'IgA fecal també s'infecten. És possible que en aquests casos els anticossos detectats en femtes procedeixin de la llet materna i tinguin menor eficàcia protectora, tot i que a més d'anticossos IgA, la llet materna conté glucoconjugats amb important activitat antiviral (31).

D'altra banda, els anticossos IgG i IgM específics per RV també sembla que tenen certa capacitat de protecció, com s'ha demostrat en altres estudis amb ratolins deficients en IgA (46). No obstant, hi ha certa controvèrsia sobre el paper d'aquests anticossos sèrics en la infecció per RV, ja que encara no és del tot clar si aquests estan directament relacionats amb la protecció o bé només són un reflex d'una infecció recent. A més, mitjançant estudis experimentals d'immunització passiva en animals s'ha comprovat que els anticossos sèrics neutralitzants per sí sols són insuficients per induir protecció i que es requereix d'anticossos neutralitzants en la mucosa intestinal, especialment de la classe IgA, representant aquesta última el marcador més clar de protecció immunitària enfront de la infecció per RV. Tot i així, encara que es disposa de múltiples estudis sobre les infeccions per RV en la literatura científica, el coneixement actual sobre la resposta immunitària i els mecanismes de protecció enfront de la infecció per RV és encara incomplet, pel que es precisa de més investigació al respecte.

2.6. Vacunes anti-rotavirus

Des de les primeres vacunes dissenyades contra RV durant la dècada dels anys 80 ja es va determinar que es volia desenvolupar una vacuna d'administració oral, degut a la importància de la immunitat local intestinal en la protecció i així aconseguir unes vacunacions més eficients. Aquesta via permet generar una resposta més semblant a la de la infecció habitual.

Les primeres vacunes obtingudes van tenir uns resultats de seguretat bons, però amb una eficàcia protectora escassa i variable. Al principi, les soques utilitzades per aquestes vacunes eren d'origen animal, però actualment ja s'han aconseguit vacunes de soques atenuades de RV humans (34).

2.6.1. Rotashield®

La vacuna Rotashield® (Wyeth) va ser autoritzada l'any 1998 per la *Food and Drug Administration* (FDA). Es va obtenir per recombinació genètica de soques animals i humanes. Es van fer quatre assaigs clínics en els quals no es va observar cap efecte advers. Tot i que hi va haver alguns casos en els que es va produir una invaginació intestinal, aquesta complicació no va ser significativament diferent entre els grups d'estudi.

L'eficàcia demostrada en aquests assaigs era del 80-82% per a la prevenció de patir malaltia greu i d'un 48-68% davant de tots els episodis de diarrea per RV. Aquesta vacuna es va incorporar al calendari de vacunació d'Estats Units l'any 1999. Es recomanava administrar la primera dosi a les 6 setmanes d'edat i se n'havien de fer dues més amb un interval de 3 setmanes.

Un cop aquesta vacuna va superar els assaigs clínics i va arribar a la població general es van detectar 15 casos d'invaginació intestinal que feien pensar que estaven relacionats amb l'administració de la vacuna. El 80% dels casos havien rebut la vacuna la setmana anterior. A l'octubre d'aquell mateix any, l'empresa comercialitzadora va interrompre la producció voluntàriament i poc després el govern va suspendre la comercialització d'aquesta vacuna. Posteriorment, es va observar que durant el període d'administració de la vacuna hi va haver una disminució d'ingressos hospitalaris per gastroenteritis causada per RV.

Des d'aquest episodi, les vacunes contra RV han estat sotmeses a un control més rigorós i continuat dels seus possibles efectes adversos. L'objectiu és mantenir l'eficàcia de la primera però amb una major seguretat. Així, les dues vacunes que s'han desenvolupat posteriorment, RotaRix® (GlaxoSmithKline) i RotaTeq® (Merck), han passat controls de seguretat més estrictes. Ambdues vacunes estan formades per RV vius-atenuats. En general, aquestes dues vacunes tenen uns resultats semblants. La seva implementació ha estat inferior a l'esperada en països en vies de desenvolupament, degut al cost, al requisit d'emmagatzematge amb refrigeració o a la menor eficàcia mostrada en comparació amb els països desenvolupats (41,47).

Ambdues redueixen entre un 29% i un 35% la mortalitat associada a gastroenteritis aguda, també es redueixen entre un 25% i un 64% les visites a emergències i la hospitalització i també hi ha una reducció del nombre d'infeccions diagnosticades pel laboratori per RV entre un 50% i un 86% (31).

2.6.2. Rotarix®

La vacuna RotaRix® és una vacuna monovalent humana atenuada. El fonament d'aquesta vacuna és imitar la infecció natural, la qual protegeix de les reinfeccions i minora la gravetat de les infeccions posteriors, independentment del serotip, i no s'associa amb la invaginació intestinal. A més a més, en ser una vacuna atenuada i oral desenvolupa una resposta immunitària on és més important, al tracte gastrointestinal. La protecció d'aquesta vacuna és millor donat que està més relacionada amb soques humanes de RV (31).

La seguretat d'aquesta vacuna es va estudiar amb 63.225 infants sans. En aquest estudi es van observar sis casos d'invaginació intestinal després de l'administració de la vacuna en 31.673 nens amb una monitorització de 31 dies després de cada dosi, davant de 31.552 que van rebre el placebo i en els que es van observar set casos d'invaginació intestinal. L'eficàcia d'aquesta vacuna per alleugerir la gravetat d'aquesta malaltia lligada amb l'hospitalització és del 85%.

El primer país on es va autoritzar aquesta vacuna va ser Mèxic, al juliol del 2004. L'*European Medicines Agency* (EMA), l'agència descentralitzada de la Unió Europea localitzada a Londres i que és responsable de l'avaluació dels medicaments que s'han de comercialitzar a nivell europeu, va autoritzar la seva comercialització

l'any 2006. No va ser fins al 2009 que es va comercialitzar a l'Estat espanyol. Es recomana l'administració de dues dosis (48).

2.6.3. RotaTeq®

És una vacuna recombinant bovina humana. Conté cinc soques recombinants d'un RV boví. La vacuna ofereix protecció davant de diversos serotips. La seguretat de Rotateq® es va avaluar en 68.038 nens. Al grup en el que se li administrava la vacuna es van observar sis casos d'invaginació intestinal de 28.038 amb un període d'observació de 42 dies després de cada dosi. Al grup que rebia el placebo es van observar cinc casos. En aquest cas, el seguiment posterior a cada dosi de la vacuna es va realitzar durant un any. L'eficàcia d'aquesta vacuna per disminuir la gravetat de la gastroenteritis per RV és del 98%, a més a més, redueix les visites a urgències i les hospitalitzacions associades a la malaltia amb els genotips més normals en humans en un 95% (31). Aquesta vacuna va obtenir l'aprovació de comercialització per l'EMA l'any 2006, any en que també es va iniciar la comercialització a Espanya. Es recomana administrar tres dosis (49).

2.7. Models per a l'estudi de gastroenteritis per rotavirus

El desenvolupament de models experimentals de diverses espècies animals com la vaca, el porc, el gos, el conill, el ratolí i la rata ha estat de gran utilitat per definir els paràmetres de la infecció per RV, la fisiopatologia, el curs de la malaltia, la resposta immunitària i també per poder avaluar l'eficàcia de les vacunes anti-RV i l'efecte modulador d'alguns compostos alimentaris. Els primers estudis amb models experimentals van utilitzar sobretot animals grans, com el porc, en condicions gnotobiòtiques. El porc és un animal monogàstric que presenta una fisiologia intestinal semblant a la dels humans i una gran susceptibilitat per contraure infeccions per diverses soques de RV, pel que se'l posiciona com un dels models d'infecció per RV més adients (50). No obstant, aquest model animal presenta certes limitacions degut a l'elevat cost de manteniment, als problemes logístics d'estabulació i a la gran dificultat en la manipulació experimental (51). Per contra, els animals petits com el ratolí i la rata encara que genèticament són més diferents als humans, se'ls troba àmpliament com a models experimentals gràcies a la seva fàcil manipulació, a la relació cost-

efectivitat i a la capacitat d'incloure múltiples animals en un mateix estudi (51).

Els models experimentals de gastroenteritis per RV en animals nounats o adults més representatius que s'han posat a punt fins al moment han estat els següents (**Taula 2**):

Taula 2. Models animals de gastroenteritis per RV. Llistat d'alguns models de diferents espècies, indicant l'edat, virus inoculats i principals determinacions: incidència (I) i gravetat (G) de la diarrea, detecció de virus en sèrum, teixits o femtes, i avaluació de la resposta immunitària (detecció d'immunoglobulines (Ig) específiques o altres proves funcionals)

Edat	Virus	Avaluació clínica i immunològica						Autor
		Diarrea	Detecció de virus			Resposta immunitària		
			Sèrum	Teixits	Femtes	Ig	Funció	
Ratolí								
d3	SA11	I/G	-	-	X	-	-	Cao, 2014 (52)
>4 setmanes	EC_{wt}	-	X	X	X	X	-	Lopatin, 2013 (53)
d4	RRV	I/G	-	X	-	-	-	Tokuhara, 2013 (54)
d4	Wa	I/G		X	X	X		Zhang, 2013 (55)
-	EC_{wt}	-	X	-	X	X	-	Blutt, 2012 (44)
d7	RRV, EDIM	I/G	-	-	X	X	X	Knipping, 2011 (56)
d4-5	YO	I	-	X	X	X	-	Buragohain, 2010 (57)
d5	EC_{wt}, RRV	-	X	X	X	-	-	Fenaux, 2006 (58)
d7	RRV, EDIM, Wa	I/G	-	-	X	X	X	VanCott, 2006 (59)
d7	EDIM	I/G	-	X	X	-	X	Boshuizen, 2003 (32)
8-16 setmanes	EC_{wt}, RRV	-	-	-	X	X	X	VanCott, 2000 (60)
d7-8	SA11, RF, EHP_{wt}	I	-	-	X	X	X	Fromantin, 1998 (61)
d4	EW_{wt}, EC_{wt}	-	-	-	X	X	X	Franco, 1995 (62)
d5-7	RRV, NCDV, EHP	I	-	-	X	X	-	Feng, 1994 (63)
d5	EDIM	I	-	X	-	-	-	Heyman, 1987 (64)
d1, 7 i 21	EDIM	I	-	X	-	X	X	Sheridan, 1983 (43)
Rata								
d10	Wa	I/G	-	-	X	-	-	Xu, 2016 (65)
d8 o 11	SA11	I/G	-	-	X	X	X	Pérez-Cano, 2007 (66)
d5	RRV, HAL1166	I/G	X	X	-	-	-	Crawford, 2006 (67)

Edat	Virus	Avaluació clínica i immunològica						Autor
		Diarrea	Detecció de virus			Resposta immunitària		
			Sèrum	Teixits	Femtes	Ig	Funció	
Rata								
d5 i >21d	Wa, SA11, HAL1166, WI61, RRV, ALA, WC3, OSU, EC$_{wt}$	I/G	-	X	X	-	-	Ciarlet, 2002 (50)
d2, 5 o 8	SA11	I	-	-	X	-	-	Guérin-Danan, 1998 (68)
d8	Grup B	I	-	-	X	-	-	Salim, 1995 (69)
Porc								
d15	PRV	I/G	-	X	-	X	-	Mao, 2016 (70)
d5	Wa	-	-	-	-	-	X	Wen, 2012 (71)
d3-5	HRV	I/G	-	-	X	-	X	González, 2010 (72)
d3-5	Wa	-	-	-	-	X	X	Yuan, 2002 (73)
Conill								
1 setmana-64 mesos	ALA, SA11, BAP$_{wt}$, BAP-2	I	-	-	X	X	-	Ciarlet, 1998 (74)
d39-55 o 109-142	ALA	I/G	-	-	X	X	-	Conner, 1991 (75)
d32-112	ALA, C11, R2, SA11	I	X	-	X	X	-	Conner, 1988 (76)
3, 8, 12 setmanes	ALA	I/G	-	-	X	X	-	Thouless, 1988 (77)
Vedell								
d2	BRV	I/G	-	-	X	X	X	Parreño, 2010 (78)

ALA: *lapin rotavirus strain;* ***BAP-2:*** *lapin rotavirus strain;* ***BAP$_{wt}$:*** *wild-type lapin rotavirus strain;* ***BRV:*** *bovine rotavirus strain;* ***C11:*** *rabbit rotavirus strain;* ***EC$_{wt}$:*** *murine wild-type rotavirus strain;* ***EDIM:*** *epizootic diarrhoea of infant mouse;* ***EHP:*** *murine rotavirus strain;* ***EHP$_{wt}$:*** *murine wild-type rotavirus strain;* ***EW$_{wt}$:*** *murine wild-type rotavirus strain;* ***HAL1166:*** *human rotavirus strain;* ***HRV:*** *human rotavirus strain;* ***NCDV:*** *bovine rotavirus strain;* ***OSU:*** *porcine rotavirus strain;* ***PRV:*** *porcine rotavirus strain;* ***R2:*** *lapin rotavirus strain;* ***RF:*** *bovine rotavirus strain;* ***RRV:*** *simian rhesus rotavirus strain;* ***SA11:*** *simian agent 11 rotavirus strain;* ***W161:*** *human rotavirus strain;* ***Wa:*** *human rotavirus strain;* ***WC3:*** *bovine rotavirus strain;* ***YO:*** *human rotavirus strain.*

En la posta a punt de models experimentals de gastroenteritis per RV, les principals variables que s’estudien són les manifestacions clíniques (incidència i gravetat de la diarrea), la presència de virus en teixits, l’eliminació viral en femtes i la resposta immunitària. La majoria de

models es basen en una única infecció a edat precoç, sent minoritaris els models de múltiple infecció, i fins i tot inexistents en algunes espècies animals com en el cas de la rata.

Pel que fa a les manifestacions clíniques, la majoria d'autors avaluen la clínica de la patologia a partir de la incidència de diarrea en el període post-inoculació. Alguns autors també avaluen la gravetat de la diarrea a partir d'un criteri de puntuació de les femtes que acostuma a ser de l'1 al 4, anomenat Índex de diarrea. Aquest criteri té en compte el color, la textura i el volum de les mostres fecals obtingudes, considerant-se una femta diarreica amb una puntuació de 2 o superior (56,66).

Referent a la presència de virus en teixits i a la detecció de virus en femtes, aquestes variables indiquen de forma indirecta el grau d'infectivitat del virus a l'organisme i la seva eliminació, respectivament. Aquestes majoritàriament es mesuren mitjançant tècniques d'*enzyme-linked immunosorbent assay* (ELISA), i en menor freqüència a partir de *polymerase chain reaction*, *Focus Fluorescent Assay* o fins i tot per microscòpia confocal.

Pel que fa a la resposta immunitària, en la majoria dels models (**Taula 2**) s'avalua mitjançant la detecció d'anticossos sèrics i/o fecals específics per RV a partir de tècniques d'ELISA. No obstant, alguns autors també estudien la proliferació cel·lular, la producció de citocines i, en menor freqüència, la hipersensibilitat retardada (DTH, *delayed type hypersensitivity*).

3. Probiòtics i prebiòtics

3.1. Conceptes

Els **probiòtics** van ser definits com "microorganismes vius que, quan s'administren en quantitats adequades, confereixen un benefici a la salut de l'hoste" per l'*International Scientific Association for Probiotics and Prebiotics* (ISAPP) al 2013 (79), revisant la definició que havia redactat la comissió d'experts internacionals de la *Food and Agriculture Organization* (FAO) i l'Organització Mundial de la Salut (OMS) al 2001 (80). Aquesta definició ha estat àmpliament acceptada i adoptada per diverses organitzacions, com l'ISAPP, el Codex, l'*Institute of Food Technologists*, la *World Gastroenterology Organization* o l'EFSA (*European Food Safety Authority*). Aquesta definició cobreix aspectes com l'especificitat de la soca i, per tant, que les propietats demostrades per una certa soca no poden ser extrapolats a una altra, ni tan sols de la mateixa espècie. A més, és necessari demostrar científicament la seva eficàcia aportant un benefici per la salut de l'hoste. D'altra banda, no és un concepte restringit a aliments, sinó que també es poden trobar probiòtics en preparacions farmacèutiques d'administració oral o per altres vies. Cal destacar però, que el concepte probiòtic només és aplicable a bacteris vius, no a les cèl·lules mortes o els seus components (81).

Els **prebiòtics** són "ingredients que produeixen una estimulació selectiva del creixement i/o activitat(s) d'un o d'un limitat nombre de gèneres/espècies de microorganismes de la microbiota intestinal conferint beneficis per a la salut de l'hoste" (82). També s'han definit com "ingredients alimentaris que al ser fermentats selectivament produeixen canvis específics en la composició i/o activitat de la microbiota gastrointestinal conferint beneficis a la salut de l'individu" (83) o com "substàncies de la dieta (fonamentalment polisacàrids no amilacis i oligosacàrids no digeribles per enzims humans) que nodreixen a grups seleccionats de microorganismes que habiten a l'intestí afavorint el creixement de bacteris beneficiosos sobre els nocius" (16).

Els **simbiòtics** són productes que combinen al menys un probiòtic i un prebiòtic. Aquestes combinacions poden tenir un efecte sinèrgic i conferir beneficis majors que els de cada un dels components per separat.

El concepte de **postbiòtics** és més recent, i es refereix als productes de fermentació o metabòlits produïts per bacteris, però sense que el producte final contingui bacteris vius, que poden modular la microbiota o el sistema immunitari i conferir beneficis a la salut (19,84). Les llets fermentades en són un exemple, i poden contenir components molt variats, com oligosacàrids o àcids grassos de cadena curta (AGCC). Els AGCC han mostrat molts efectes beneficiosos, com l'estimulació de la barrera intestinal (85). D'altra banda, aquestes preparacions també poden contenir components dels bacteris fermentatius, com peptidoglicans o exopolisacàrids de les parets cel·lulars o el material genètic bacterià. S'ha suggerit que aquests components poden conferir certs efectes beneficiosos, sobretot pel que fa a la immunoestimulació (86–89).

3.2. Probiòtics

Les espècies dels gèneres *Lactobacillus* i *Bifidobacterium* són els probiòtics utilitzats més freqüentment, tot i que el llevat *Saccharomyces cerevisiae* i algunes soques dels gèneres *Streptococcus*, *Enterococcus*, *Pediococcus*, *Propionibacterium*, *Escherichia* i *Bacillus* també s'utilitzen.

3.2.1. Identificació

La identificació a nivell d'espècie i soca és essencial per a la comercialització d'un bacteri. La identificació de la soca possibilita la traçabilitat de proves de laboratori, assajos clínics, estudis epidemiològics i del procés de producció i comercialització. Aquesta identificació és necessària donat que els efectes són soca-específics (90).

Per a la identificació de les espècies són necessàries proves moleculars, basades en la detecció d'empremtes genètiques (*fingerprinting*) o en la seqüenciació de diversos gens com el 16S rRNA o gens essencials pel metabolisme cel·lular (*housekeeping*) com el *pheS*, *rpoA*, *atpD*, *tuf*, *groEL* o *recA*. Les proves fenotípiques (fermentació de carbohidrats, activitats enzimàtiques, etc.) no tenen suficient capacitat de resolució i actualment no són vàlides (91).

Per a la diferenciació de soques, les tècniques d'elecció són les de genotipat, com l'electroforesi en gel de camp pulsat. De cara al futur, la seqüència del genoma complet és la tècnica que major informació pot

aportar per a la identificació d'una espècie/soca (incloent la presència de plàsmids i altres elements extracromosòmics, que poden estar associats a propietats que diferencien una soca d'altres de la seva mateixa espècie).

3.2.2. Seguretat i funcionalitat

Hi ha espècies que, amb una història d'ús segur, han sigut reconegudes com a organismes *Generally Regarded As Safe* (GRAS) i *Qualified Pressumption of Safety* (QPS) per la FDA i l'EFSA, respectivament. Els lactobacils i les bifidobacteris en són un exemple, ja que són microorganismes que colonitzen les nostres mucoses i són innocus sota gairebé qualsevol circumstància (90,91).

L'avaluació de la seguretat dels probiòtics ha de tenir en compte el microorganisme en qüestió, la forma d'administració, el nivell d'exposició, l'estat de salut de l'hoste i les funcions fisiològiques que poden desenvolupar en aquest (92). Els casos en que s'han produït efectes adversos relacionats amb el consum de probiòtics són escassos, incloent assajos clínics amb hostes immunocompromesos (93–95).

Els efectes adversos que s'han de descartar per poder comercialitzar un probiòtic són: la patogenicitat, la producció de metabòlits no desitjables, la possibilitat de transmissió de gens que confereixin resistència a antibiòtics, l'excessiva immunoestimulació o immunosupressió en individus sensibilitzats i els efectes negatius associats als excipients.

Per a que les soques probiòtiques que s'administren per via oral puguin exercir els seus efectes beneficiosos, s'ha de mantenir una concentració adequada de bacteris viables i han de resistir les condicions de l'aparell digestiu (efecte microbicida de la saliva, l'acidesa gàstrica, la bilis, la secreció pancreàtica, etc.) (80).

Els assajos *in vivo* amb models animals permeten l'estudi de mostres, teixits i òrgans que ajuden a identificar els mecanismes d'acció i marcadors relacionats amb els efectes beneficiosos o efectes adversos d'un probiòtic. Degut a les diferències anatòmiques i fisiològiques amb la nostra espècie, els assajos clínics controlats de fase II o III amb persones també són necessaris per determinar finalment el grau d'eficàcia del probiòtic, i per establir la dosificació adequada.

3.2.3. Mecanismes d'acció

Els probiòtics exerceixen efectes beneficiosos sense necessitat de modificar la microbiota de l'hoste (96). Els mecanismes pels quals s'obté l'efecte protector (**Figura 10**) poden ser:

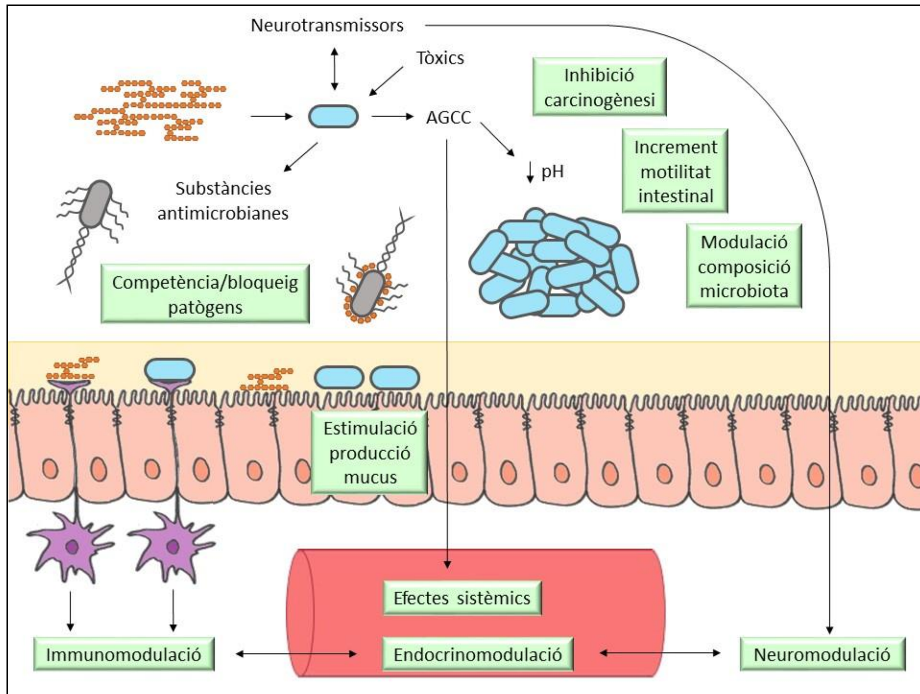


Figura 10. Mecanisme d'acció dels probiòtics i prebiòtics. **AGCC:** àcids grassos de cadena curta

- Generació de compostos antimicrobians, AGCC, bacteriocines, aigua oxigenada o compostos bioactius (16,81,91). La producció d'àcid (làctic, acètic, propiònic o butíric) resultant del metabolisme fermentatiu de sucres és una propietat comuna de la majoria de probiòtics, ja que solen ser organismes anaerobis.

- Neutralització o destoxicació de carcinògens i de contaminants abiòtics. Aquesta propietat s'ha observat en estudis *in vitro* amb varies soques de *Lactobacillus* (97).

- Adhesió a la superfície de les mucoses, coagregació amb certs patògens o exclusió competitiva de patògens. La capacitat d'adherència a les superfícies mucoses o a les cèl·lules epitelials s'ha descrit per a diversos probiòtics en estudis *in vitro* i *in vivo*.

Un exemple de coagregació (**Figura 11**) és la que es va observar per part de la soca probiòtica *Lactobacillus ruminus* amb el patògen *Candida albicans* (98).

- Manteniment o millora de la funció de barrera intestinal mitjançant la modificació de l'expressió dels gens que codifiquen per proteïnes de les zones d'oclusió (ocludina, claudina, etc.), la modificació de la composició de monosacàrids de les mucines, l'augment del gruix de la capa de mucus, la inhibició dels processos d'apoptosi i/o la promoció de la diferenciació cel·lular i d'activitats citoprotectores, incloent la reducció de l'estrès oxidatiu. Els efectes de diversos probiòtics s'han avaluat en aquest sentit en estudis *in vitro* i *in vivo* (84).

- Efectes immunomoduladors. D'igual manera que la microbiota autòctona, els probiòtics poden interactuar amb el sistema immunitari i exercir un efecte sobre diverses poblacions de cèl·lules del sistema immunitari, la producció d'anticossos, citocines, quimiocines o factors de creixement (99). Algunes soques probiòtiques han demostrat aquesta propietat en estudis preclínics i clínics (100,101). Hi ha evidències de que els probiòtics poden millorar la resposta immunitària a les vacunes i reduir el risc d'infecció (90).

- Efectes moduladors dels sistemes endocrí i nerviós a través del sistema nerviós entèric. Aquest sistema és una xarxa nerviosa complexa que es troba al tracte gastrointestinal amb l'objectiu de regular les funcions fisiològiques i de comunicar el cervell, el tracte gastrointestinal, el sistema endocrí i el sistema immunitari. Alguns probiòtics podrien modular aquest eix intestí-cervell mitjançant la interacció amb receptors nerviosos o la biosíntesi i metabolisme de neurotransmissors com la serotonina, la melatonina, l'àcid γ-aminobutíric, catecolamines, la histamina o l'acetilcolina (102,103).

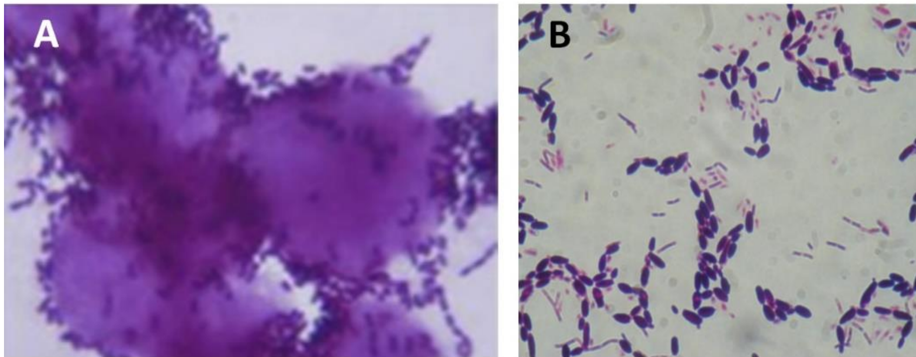


Figura 11. Capacitat dels probiòtics d'adherència a l'epiteli i de coagregació amb patògens. (A) Adherència de *Lactobacillus plantarum* P6 i *L. paraplantarum* P25 a la línia cel·lular COLO 205. (B) Coagregació de *Lactobacillus ruminus* amb *Candida albicans*. Modificat de Gil et al., 2010 i de Pringsulaka et al., 2015 (98,104)

3.2.4. Usos

Els probiòtics s'utilitzen actualment en la prevenció i tractament de diverses afeccions, amb el recolzament d'evidència científica de com a mínim grau 1b (assajos clínics aleatoritzats):

- El control de la diarrea. És l'ús més habitual dels probiòtics (com el *Lactobacillus rhamnosus* GG [LGG], el *Lactobacillus casei* DN-114 001 o el *S. cerevisiae boulardii*, entre altres), tant en la prevenció com en el tractament de diarrea aguda, de la diarrea associada a antibiòtics o de la diarrea nosocomial (principalment per *Clostridium difficile*) en nens i adults (16,105).

- Teràpia coadjuvant de l'eradicació d'*Helicobacter pylori*. Es poden utilitzar probiòtics com el *L. casei* DN-114001 en pacients pediàtrics i l'LGG, el *Lactobacillus acidophilus*, *Lactobacillus reuteri* ATCC 55730 o el *S. cerevisiae boulardii* en adults, per disminuir els efectes secundaris del tractament antibiòtic i per millorar les taxes d'eradicació (105).

- La intolerància a la lactosa. *Streptococcus thermophilus* i *Lactobacillus delbrueckii bulgaricus* milloren la digestió de la lactosa i redueixen les manifestacions de la intolerància a aquest sucre (16).

- La pouchitis i colitis ulcerosa. Alguns probiòtics han demostrat la seva utilitat en la prevenció de pouchitis (VSL#3) i en mantenir la remissió d'aquesta i de la colitis ulcerosa (VSL#3 i *Escherichia coli Nissle*) (16,105).

- L'enterocolitis necrotitzant (NEC). El tractament amb combinacions de probiòtics ha mostrat en assajos clínics una reducció del risc de NEC i del risc de mort en nounats prematurs (*Bifidobacterium bifidum*, *Bifidobacterium infantis* i *L. acidophilus*, entre altres) (16,105).

- La síndrome de l'intestí irritable i el còlic del lactant. S'ha demostrat una reducció de la distensió abdominal i la flatulència, i una millora del dolor i molèsties generals amb l'ús d'alguns probiòtics (com el *L. plantarum*, la combinació VSL#3 o *L. reuteri*, entre altres) (16,105).

Alguns usos prometedors dels probiòtics són:

- La vaginosi i vulvovaginitis, la prevenció d'infeccions urinàries, la correcció de trastorns associats a la menopausa o la mastitis. Alguns probiòtics han demostrat efectes beneficiosos com a coadjuvants del tractament antibiòtic o antifúngic en casos de vaginosi o vulvovaginitis. A més, soques de *Lactobacillus salivarius*, *Lactobacillus fermentum* o *L. reuteri*, entre altres, constitueixen el tractament de primera línia de les mastitis subagudes i subclíniques. Així i tot, calen més assajos clínics aleatoritzats ben dissenyats, amb metodologies estandarditzades i amb major nombre de pacients per a confirmar aquests efectes dels probiòtics, així com la millor via d'administració, la dosi i les pautes de tractament (106).

- Les al·lèrgies. Hi ha evidències de que certes soques probiòtiques poden ser eficaces en el tractament de pacients amb èczema atòpic, però cal més investigació en aquest camp (16). Sembla que un factor important en aquest cas és el consum de probiòtics per part de la mare durant el període perinatal, o bé per part del nounat durant la lactància (90).

- La malaltia de Crohn. Els assajos clínics amb probiòtics han mostrat resultats dispars en quant a la malaltia de Crohn, però

alguns mostren resultats prometedors per a la inducció i manteniment de la remissió (16,105).

- El càncer de còlon. Algun estudi suggereix que l'ús de probiòtics pot disminuir l'expressió de biomarcadors del càncer colorectal, però calen més evidències en aquest sentit (16).

- Hepatopatia grassa no alcohòlica. El tractament d'aquesta patologia amb probiòtics no s'ha confirmat amb assajos clínics aleatoritzats suficients (16).

- Prevenció d'infeccions sistèmiques. Hi ha evidències de la disminució del risc de certes infeccions sistèmiques amb l'ús de probiòtics, però no són suficients per a la seva recomanació de manera pautada (16).

També hi ha diversos estudis amb probiòtics que han mostrat resultats encoratjadors en altres malalties, com les metabòliques (diabetis, obesitat) o les neurològiques (autisme) (107,108).

3.3. Prebiòtics

Els prebiòtics coneguts són carbohidrats no digeribles (oligo- i polisacàrids), que es poden trobar de forma natural en aliments com la llet, la mel, hortalisses i verdures (porro, carxofa, espàrecs, all, ceba, xicoira, etc.), fruites, cereals (blat, civada), llegums i fruites seques, o bé es poden obtenir per mètodes químics i enzimàtics (16).

Els carbohidrats no digeribles arriben al còlon i són utilitzats com a substrat pels microorganismes comensals, originant energia, substrats metabòlics i micronutrients essencials per l'hoste, però per ser considerats prebiòtics, a més han d'estimular el creixement selectiu de determinades espècies beneficioses de la microbiota intestinal, principalment bifidobacteris i lactobacils (90).

3.3.1. Caracterització

L'estructura dels carbohidrats prebiòtics (composició en monosacàrids, tipus d'enllaç glicosídic i pes molecular) té una gran influencia en les propietats que aquests puguin presentar. Per aquest motiu és important que estiguin ben caracteritzats. S'ha de conèixer l'estructura i també la

procedència (origen i font d'obtenció), la puresa i la composició química del prebiòtic (109).

Per a que un aliment es pugui considerar prebiòtic ha de complir les següents característiques:

- No ser hidrolitzat en el tracte gastrointestinal superior, i per tant, ser resistent a l'acidesa gàstrica, a la hidròlisi per enzims digestius i no absorbir-se a l'intestí prim.

- Ser fermentat per bacteris beneficiosos de la microbiota intestinal.

- Ser capaç d'induir efectes fisiològics beneficiosos per a la salut.

Per a la caracterització funcional d'un prebiòtic són imprescindibles assajos de digestibilitat i fermentació *in vitro* i *in vivo* en models animals per comprovar la seva resistència a la digestió i absorció, almenys parcial, al tracte gastrointestinal superior i la seva capacitat moduladora de la composició de la microbiota intestinal i/o la producció de compostos beneficiosos per la salut.

A més, l'eficàcia dels prebiòtics s'ha de demostrar en estudis d'avaluació clínica de fase II en humans, definint prèviament els resultats esperats, la mida de la mostra necessària per demostrar els resultats amb significació estadística, els paràmetres fisiològics a mesurar, i amb un disseny experimental de doble-cec, aleatoritzat i controlat amb placebo (90,109).

Establir una dosi adequada d'aquests carbohidrats és un factor important per evitar efectes adversos. Una ingesta excessiva podria provocar molèsties intestinals, diarrea i flatulències. Per exemple, s'ha demostrat que una ingesta de galactooligosacàrids (GOS) per sobre de 20 g/dia pot provocar diarrea. Tot i que depèn de cada tipus d'oligosacàrid i de la microbiota de cada individu, la major part d'aquests carbohidrats són capaços d'incrementar la població de bifidobacteris amb un consum de 15 g/dia (109).

3.3.2. Tipus

Existeix evidència científica de les propietats prebiòtiques en humans de:

- La inulina, formada per oligosacàrids i polisacàrids de fructosa amb grau de polimerització (DP) entre 2 i 65 unitats.

- Els fructooligosacàrids (FOS), oligosacàrids de fructosa amb un DP de 2 a 10 que s'obtenen de la hidròlisi de la inulina present en productes vegetals o mitjançant transfructosilació enzimàtica a partir de sacarosa, utilitzant fructosiltransferases.

- Els galactooligosacàrids (GOS), formats per 2-10 molècules de galactosa unides a una glucosa terminal, que es diferencien entre sí per la longitud de la cadena i el tipus d'enllaç i que s'obtenen industrialment a partir de la lactosa del permeat de sèrum de formatgeria, mitjançant transglicosilació catalitzada per β-galactosidases (lactases). També es troben de forma natural a la llet humana i animal.

- La lactulosa, un disacàrid sintètic de galactosa i fructosa.

- Els oligosacàrids de la llet materna (HMO, *human milk oligosaccharides*), amb una estructura bàsica que inclou un nucli de lactosa (disacàrid compost per galactosa i glucosa) en l'extrem reductor, allargat per unitats de N-acetil-lactosamina fucosilades i/o sialilades. Això es tradueix en més de 200 estructures d'HMO, que difereixen en la mida, la càrrega i la seqüència. Els HMO es troben en concentracions entre 12-14 g/L en llet humana (**Figura 12**) i entre 22-24 g/L en el calostre. Són considerats els primers prebiòtics en l'alimentació humana i responsables de l'elevat nombre de bifidobacteris presents en els intestins dels lactants.

Altres carbohidrats considerats prebiòtics però dels que no existeix suficient evidència científica són els xilooligosacàrids (XOS), la lactosacarosa, els isomaltooligosacàrids, oligosacàrids de soja i glucooligosacàrids.

Altres carbohidrats no digeribles com els pectooligosacàrids, la polidextrosa, exopolisacàrids bacterians i polisacàrids de macroalgues estan en fases inicials d'estudi (109).

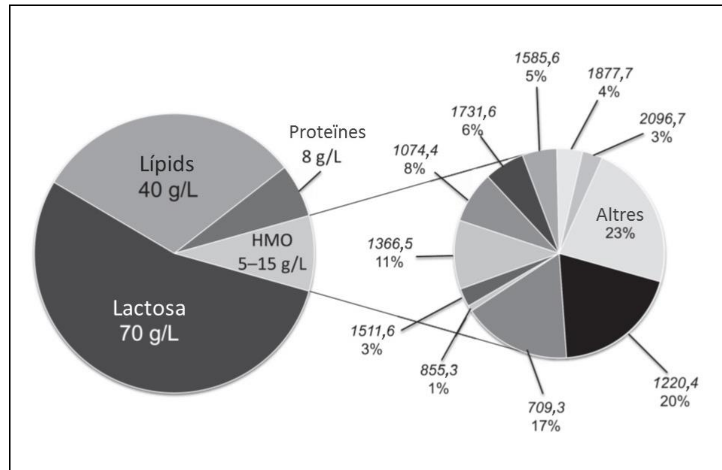


Figura 12. Composició de la llet materna humana. A l'esquerra es mostra la composició dels macronutrients i a la dreta, la composició dels oligosacàrids de llet humana (HMO) més abundants, amb les masses de les estructures d'HMO individuals i la seva abundància relativa. Adaptat de Zivkovic et al., 2011 (110)

3.3.3. Efectes beneficiosos i mecanismes d'acció

Alguns estudis suggereixen que els prebiòtics poden exercir efectes fisiològics beneficiosos per la salut i benestar de l'hoste (**Figura 10**):

- Promoció de la proliferació de microorganismes beneficiosos i modulació de la composició microbiana. Els prebiòtics podrien contribuir a reduir el risc de malalties relacionades amb la disbiosi (malalties intestinals i sistèmiques). Els bacteris fermentatius que estimulen (bifidobacteris i lactobacils) generen AGCC que disminueixen el pH controlant el desenvolupament de certes comunitats de la microbiota amb possibles efectes perjudicials (com algunes espècies dels gèneres *Bacteroides*, *Fusobacterium* i *Clostridium*) (111). El consum de prebiòtics pot tenir un efecte protector contra infeccions intestinals i podria reduir el risc de malalties que cursen amb inflamació. Els FOS han mostrat efectes beneficiosos en models animals d'inflamació intestinal i en estudis en humans amb malaltia inflamatòria intestinal o dermatitis atòpica, tot i que els resultats dels estudis referents al síndrome d'intestí irritable són inconsistents (112). En casos de cirrosi s'han utilitzat XOS i lactulosa per controlar els nivells d'amoni i fenols lliures en sang (113,114). La lactulosa s'ha utilitzat amb èxit pel tractament d'encefalopatia hepàtica que afecta al sistema

nerviós central per l'elevació del contingut d'amoni en sang (16). Els prebiòtics reestableixen la microbiota beneficiosa i frenen el creixement de bacteris productors d'amoni. També hi ha estudis que suggereixen que els prebiòtics podrien reduir el risc de patir càncer de còlon, tot i que calen més evidències en aquest sentit.

- Propietats antiadherents. Els prebiòtics bloquegen els llocs on s'adhereixen els microorganismes patògens o les seves toxines a les cèl·lules epitelials actuant com anàlegs dels receptors de l'epiteli intestinal (19,115). Aquesta propietat contribueix al possible efecte protector contra infeccions intestinals, diarrees del viatger o produïdes per antibiòtics per part dels prebiòtics, que s'ha observat en estudis preclínics *in vivo* i en estudis clínics, amb majors efectes entre la població infantil, però també amb efectes beneficiosos en adults en determinades situacions (112,116).

- Millora del trànsit digestiu. Aquests compostos provoquen un increment del volum fecal i estimulen la motilitat gràcies als AGCC. Alguns prebiòtics s'utilitzen com a laxants en el tractament de la constipació (16,111).

- Afavoriment de l'absorció de calci i altres minerals (magnesi, zinc i ferro), reducció de la pressió arterial, els nivells de glucosa, colesterol, triglicèrids i fosfolípids en sang, així com la síntesi de triglicèrids i àcids grassos en el fetge, prevenint el risc de diabetis, obesitat i aterosclerosi (111,117–119). Els canvis sobre el metabolisme lipídic podrien ser conseqüència de la producció d'AGCC, que poden induir canvis metabòlics en el fetge.

- Efectes immunomoduladors. Alguns prebiòtics han mostrat un efecte potenciador del sistema immunitari (producció d'IgA, modulació de citocines, etc.) (16,119) i la seva capacitat de modificar alguns aspectes de la resposta immunitària tant *in vitro*, com en animals d'experimentació o en estudis clínics amb nens i amb adults, com per exemple, els FOS (112) o els HMO (19). Aquests efectes també estan relacionats amb els beneficis observats en la incidència d'al·lèrgies, infeccions o en la inflamació, en estudis clínics i preclínics.

4. Fórmules infantils

L'OMS, conjuntament amb el *United Nations International Children's Emergency Fund*, recomanen la lactància materna de manera exclusiva durant els primers sis mesos de vida per a uns òptims creixement, desenvolupament i salut. A partir d'aquesta edat, la lactància natural s'hauria de mantenir fins als dos anys d'edat o més tard, però introduint aliments complementaris adequats (120). Així i tot, en les situacions en que les mares no volen o no poden alletar el seu nadó (per malaltia, ús de fàrmacs o drogues per part de la mare, absència d'aquesta o carència de bancs de llet), els nadons s'hauran d'alimentar amb preparats substitutius.

Les fórmules infantils es defineixen com aquell aliment per lactants que serveixen per substituir parcial o totalment la llet humana i que, per tant, han de satisfer les necessitats nutritives del nadó. Habitualment, aquestes fórmules estan desenvolupades a partir de llet de vaca, la qual ha sigut modificada per mimetitzar al màxim la composició en nutrients de la llet humana.

Tot i això, els preparats no poden reproduir les variacions en la composició de la llet materna al llarg de la lactància (calostre, llet de transició i llet madura), ni el tipus i qualitat d'algun dels seus components (121).

Per altra banda, la suplementació de fórmules infantils amb determinats compostos bioactius és un mecanisme interessant per la prevenció i/o tractament d'algunes patologies.

4.1. Regulació de les fórmules infantils

La composició i l'ús de les fórmules per a lactants es van començar a regular durant els anys 70. A nivell internacional, hi ha organismes com la FAO, l'OMS, l'*American Academy of Pediatrics Committee on Nutrition* o l'*European Society for Paediatric Gastroenterology Hepatology and Nutrition* (ESPGHAN), que donen recomanacions de caràcter orientatiu.

D'altra banda, també existeixen normes que són d'obligat compliment. A Estats Units, la FDA és l'agència encarregada de regular la preparació i l'ús dels substituts de llet materna. A la Unió Europea,

actualment s’ha de complir la Directiva 2013/46/CE que modifica els requisits sobre proteïnes dels preparats per a lactants i preparats de continuació de la directiva 2006/141/CE, de 22 de desembre de 2006, relativa als preparats per a lactants i preparats de continuació.

Aquestes directives s’han incorporat a la normativa espanyola per mitjà del Reial Decret 165/2014, de 14 de març, que modifica el Reial Decret 867/2008, de 23 de maig, pel qual es va aprovar la reglamentació tecnicosanitària específica dels preparats per a lactants i dels preparats de continuació.

4.2. Tipus de fórmules per a lactants

Existeixen tres tipus de fórmules infantils per a lactants nascuts a terme sense necessitats especials:

- Els **preparats per lactants o fórmules d’inici**, són productes alimentaris destinats a l’alimentació dels lactants durant els primers mesos de vida, capaços de satisfer per sí mateixos les necessitats nutritives d’aquests lactants fins la introducció d’una alimentació complementària apropiada.

- Els **preparats o fórmules de continuació**, són productes alimentaris destinats a l’alimentació dels lactants quan s’introdueixi una alimentació complementària apropiada, que constitueixin el principal element líquid d’una dieta progressivament diversificada d’aquests lactants.

- Les **fórmules de creixement**, són productes alimentaris destinats a l’alimentació de nens entre 12 i 36 mesos d’edat i que representen un punt d’inflexió entre les fórmules de continuació i la llet de vaca. A diferència de les altres dues, no hi ha cap directiva específica que reguli la seva composició i etiquetatge.

Les fórmules infantils normalment s’obtenen a partir del sèrum desmineralitzat de llet de vaca o cabra, ajustant la relació entre caseïna i proteïnes del sèrum, i afegint lactosa, vitamines, minerals, una barreja adequada d’oli i altres suplements (122). Els aliments elaborats totalment a partir de les proteïnes de llet de vaca o cabra es denominen “llet per a lactants” i “llet de continuació”.

No obstant això, existeixen altres fórmules especials dissenyades per aquells lactants que pateixen alguna patologia o alteració metabòlica, com les fórmules a base d'hidrolitzats de proteïnes o a base d'aïllats de proteïnes de soja.

4.3. Composició de les fórmules infantils

Per a una correcta formulació de les llets de substitució per a lactants s'ha de tenir en compte que la llet humana i la llet de vaca es diferencien tant en composició com en la proporció dels seus components.

La llet de vaca gairebé triplica el contingut proteic de la llet humana i les proporcions de caseïna, proteïnes del sèrum i nitrogen no proteic són de 78:17:5 i de 35:45:25 en la llet de vaca i la llet humana, respectivament (123). A més, la caseïna humana està formada per les formes β i κ, mentre que a la llet de vaca predomina la forma α, el qual la fa menys digerible pels lactants. Pel que fa a les proteïnes del sèrum, a la llet humana predominen l'α-lactoalbúmina, la lactoferrina i la S-IgA, mentre que a la llet de vaca s'hi troben majoritàriament β-lactoglobulina i IgG. Finalment, la llet de vaca presenta un perfil d'aminoàcids diferent al de la llet humana, i un baix contingut en nitrogen no proteic (nucleòtids, àcids nucleics, aminoàcids lliures, àcid ureic, urea i poliamines) (124).

El contingut en carbohidrats és menor a la llet de vaca que a la llet humana. A la llet de vaca hi ha pràcticament absència d'oligosacàrids, i menys de la meitat de lactosa respecte a la llet humana.

En relació a la fracció lipídica, a la llet de vaca predominen els àcids grassos saturats sobre els insaturats, contràriament al que passa a la llet humana. En aquesta última hi ha una major concentració d'àcids grassos poliinsaturats de cadena llarga (123,124).

El contingut de minerals i electròlits és molt superior a la llet de vaca respecte la llet materna. Això suposa un inconvenient pel lactant, degut a la seva immaduresa renal i a que pot provocar obstrucció intestinal (123).

Per tal de corregir algunes d'aquestes diferències i aconseguir preparats substitutius el més semblant possible a la llet materna, el Reial Decret 165/2014, de 14 de març, que modifica el Reial Decret 857/2008, de 23

de maig, imposa uns requisits en la composició d'aquests, tal com es mostra a la **Taula 3**.

Taula 3. Composició de les fórmules infantils. Extret del Reial Decret 165/2014, de 14 de març, i del Reial Decret 857/2008, de 23 de maig, relatius a la reglamentació tecnicosanitària específica dels preparats per a lactants i dels preparats de continuació

Compost	Cas	Fórmula d'inici	Fórmula de continuació
Energia (Kcal/100 mL)		60-70	60-70
	A	1,8-3	1,8-3,5
Proteïnes (g/100 Kcal)	B	1,8-3	1,8-3,5
	C	2,25-3	2,25-3,5
Cistina (per 100 Kcal)		>38	>38
Histidina (per 100 Kcal)		>40	>40
Isoleucina (per 100 Kcal)		>90	>90
Leucina (per 100 Kcal)		>166	>166
Lisina (per 100 Kcal)		>113	>113
Metionina (per 100 Kcal)		>23	>23
Fenilalanina (per 100 Kcal)		>83	>83
Treonina (per 100 Kcal)		>77	>77
Triptòfan (per 100 Kcal)		>32	>32
Tirosina (per 100 Kcal)		>76	>76
Valina (per 100 Kcal)		>88	>88
L-carnitina (mg/100 Kcal)	B/C	>1,2	
Taurina (mg/100 Kcal)		<12	<12
Colina (mg/100 Kcal)		7-50	
Greixos (g/100 Kcal)		4,4-6	4-6
Àcid làuric i mirístic (% del contingut total en greix)		<20	<20
Àcids grassos trans (% del contingut total en greix)		<3	<3
Àcid erúcic (% del contingut total en greix)		<1	<1
Àcid linoleic (mg/100 Kcal)		300-1200	300-1200
Àcid α-linolènic (mg/100 Kcal)		>50	>50
Proporció àcid linoleic i α-linolènic		5-15	5-15
PCL n-3 (% del contingut total en greix)		<1	<1
PCL n-6 (% del contingut total en greix)		<2	<2
Àcid araquidònic 20:4 n-6 (% del contingut total en greix)		<1	<1
Fosfolípids (g/L)		<2	<2
Inositol (mg/100 Kcal)		4-40	
Hidrats de carboni (g/100 Kcal)		9-14	9-14
Lactosa (g/100 Kcal)	*	>4,5	>4,5
Sacarosa (% del contingut total d'hidrats de carboni)	B	<20	
Sacarosa, fructosa, mel (% del contingut total d'hidrats de carboni)			<20
Glucosa (g/100 Kcal)	B	<2	<2
GOS/FOS 9:1 (g/100 mL)		<0,8	<0,8
Midó pretostat o gelatinitzat (g/100 mL)		<2	
Sodi (mg/100 Kcal)		20-60	20-60
Potassi (mg/100 Kcal)		60-160	60-160

Compost	Cas	Fórmula d'inici	Fórmula de continuació
Clor (mg/100 Kcal)		50-160	50-160
Calci (mg/100 Kcal)		50-140	50-140
Fòsfor (mg/100 Kcal)	A/B	25-90	25-90
	C	30-100	30-100
Magnesi (mg/100 Kcal)		5-15	5-15
Ferro (mg/100 Kcal)	A/B	0,3-1,3	0,6-2
	C	0,45-2	0,9-2,5
Zinc (mg/100 Kcal)		0,5-1,5	0,5-1,5
Coure (µg/100 Kcal)		35-100	35-100
Iode (µg/100 Kcal)		10-50	10-50
Seleni (µg/100 Kcal)		1-9	1-9
Manganès (µg/100 Kcal)		1-100	1-100
Fluor (µg/100 Kcal)		<100	<100
Relació calci: fòsfor		1-2	1-2
Vitamina A (µg-ER/100 Kcal)		60-180	60-180
Vitemina D en forma de colecalciferol (µg/100 Kcal)		1-2,5	1-3
Tiamina (µg/100 Kcal)		60-300	60-300
Riboflavina (µg/100 Kcal)		80-400	80-400
Niacina preformada (µg/100 Kcal)		300-1500	300-1500
Àcid pantotènic (µg/100 Kcal)		400-2000	400-2000
Vitamina B_6 (µg/100 Kcal)		35-175	35-175
Biotina (µg/100 Kcal)		1,5-7,5	1,5-7,5
Àcid fòlic (µg/100 Kcal)		10-50	10-50
Vitamina B_{12} (µg/100 Kcal)		0,1-0,5	0,1-0,5
Vitamina C (µg/100 Kcal)		10-30	10-30
Vitamina K (µg/100 Kcal)		4-25	4-25
Vitamina E (mg α-ET/ 100 Kcal)		0,5-5	0,5-5
Nucleòtids (mg/100 Kcal)		<5	<5
Citidina 5'-monofosfat (mg/100 Kcal)		<2,5	<2,5
Uridina 5'-monofosfat (mg/100 Kcal)		<1,75	<1,75
Adenosina 5'-monofosfat (mg/100 Kcal)		<1,5	<1,5
Guanosina 5'-monofosfat (mg/100 Kcal)		<0,5	<0,5
Inosina 5'-monofosfat (mg/100 Kcal)		<1	<1

A: Preparats elaborats a partir de proteïnes de llet de vaca o cabra; **B:** Preparats elaborats a partir d'hidrolitzats de proteïnes; **C:** Preparats elaborats a partir d'aïllats de proteïnes de soja únicament o d'una barreja amb proteïnes de llet de vaca o cabra; *excepte en els preparats per lactants en els que els aïllats de proteïnes de soja suposin més del 50% del total del contingut en proteïnes; **PCL:** àcids grassos poliinsaturats de cadena llarga; **GOS:** galactooligosacàrids; **FOS:** Fructooligosacàrids; **ER:** equivalents de trans retinol; **ET:** equivalent de D-α-tocoferol.

Aquesta norma també indica que només es poden afegir aminoàcids als preparats per a lactants i preparats de continuació per a millorar el valor nutritiu de les proteïnes, i únicament, en la proporció necessària per a aquest fi.

Respecte al contingut en greix, està format per àcids grassos saturats, monoinsaturats i poliinsaturats. A la **Taula 3** s'aprecia una disminució d'aquest a les fórmules de continuació, ja que el nadó incorpora altres aliments a la seva dieta. Per altra banda, es prohibeix la utilització d'oli de sèsam i de cotó als preparats per a lactants i preparats de continuació.

Com a fonts d'hidrats de carboni, en els preparats per a lactants només poden utilitzar-se la lactosa, la maltosa, la sacarosa, la glucosa, la malto-dextrina, el xarop de glucosa o xarop de glucosa deshidratat o el midó pretostat o gelatinitzat originàriament sense gluten. La utilització d'ingredients que continguin gluten està prohibida pels preparats de continuació i, a més, la mel utilitzada s'ha de tractar per destruir les espores de *Clostridium botulinum*. A la **Taula 3** es pot observar que els hidrats de carboni representen la principal font d'energia, essent la lactosa el component majoritari, tal com succeeix a la llet humana. En algunes fórmules aquest sucre és substituït per polímers de glucosa, midó o maltodextrines, l'absorció dels quals no depèn de la lactasa.

En quant a l'adició de taurina, prebiòtics o nucleòtids, és de caràcter opcional. Per a la suplementació amb prebiòtics, es poden utilitzar altres combinacions i nivells màxims de FOS i GOS als recomanats en preparats per a lactants i preparats de continuació, si s'ha comprovat científicament la seva adequació.

4.4. Suplementació de fórmules infantils amb prebiòtics i probiòtics

La inclusió de probiòtics i prebiòtics a fórmules infantils pot estar associada a beneficis clínics com la reducció del risc d'infeccions, el control de les manifestacions d'atòpia, una disminució del risc secundari a l'ús d'antibiòtics i una menor freqüència de còlics o irritabilitat (125).

Alguns estudis han mostrat una disminució en el nombre d'episodis de gastroenteritis aguda i en la seva duració, un increment de la freqüència de deposicions i femtes més toves, una reducció en la incidència d'èczema atòpic i una millora dels símptomes del còlic del lactant en nens alimentats amb una fórmula suplementada amb probiòtics (com LGG*, L. fermentum, L. reuteri, Bifidobacterium lactis, S. thermophilus* o *S. boulardii*) comparant amb nens alimentats amb una fórmula no

suplementada (125,126). A més, per poder ser inclosos a fórmules infantils, s'ha d'haver provat la innocuïtat dels probiòtics. És a dir, a través del reconeixement QPS de la EFSA, estudis de patogenicitat, de la producció de metabòlits, de resistència a antibiòtics, estudis de seguretat preclínics i assajos clínics controlats.

Degut a l'estructura complexa dels HMO, els prebiòtics que es troben a les llets de fórmula són majoritàriament GOS i FOS. S'ha demostrat que la ingesta de preparacions infantils amb GOS/FOS en concentracions entre 0,2-1,5 g/100 mL produeix un augment significatiu de bifidobacteris i lactobacils en femtes respecte a les de nens alimentats amb fórmules sense aquest suplement, aconseguint una microbiota més similar als nadons que reben llet materna. També s'ha associat a un increment de la freqüència de deposicions i femtes més toves. Els diversos estudis realitzats no han mostrat conseqüències nocives al seu consum i, per tant, la ESPGHAN i altres autors han qualificat de segurs els prebiòtics avaluats fins al moment (125–128).

Tot i això, no existeix evidència suficient per a recomanar la seva utilització sistemàtica, i és necessària més investigació en aquest camp per poder establir les proporcions més eficaces dels diferents prebiòtics, les soques probiòtiques més adients, les dosis adequades, la durada i l'edat d'ingesta, els beneficis i seguretat d'ús sobretot en nens prematurs o en circumstàncies especials (125–127).

En l'actualitat es poden trobar fórmules infantils, de venda en farmàcies o grans superfícies, que contenen prebiòtics, probiòtics o la combinació d'ambdós. En un treball de recerca on es van consultar 30 fórmules infantils de diferents marques comercials de venda en farmàcia, es va observar que el 80% incloïen prebiòtics, probiòtics o simbiòtics en la seva composició. El 40% de les marques estudiades contenia exclusivament prebiòtics, majoritàriament de tipus GOS; el 33% contenia probiòtics, essent *L. fermentum*, *B. lactis* i *L. reuteri* les espècies més utilitzades; i només en el 7% de les fórmules es podien trobar simbiòtics, formats per FOS i *Lactobacillus* sp, *B. infantis* i *Bifidobacterium rhamnosus* (129).

Quan es van estudiar aquestes fórmules separadament segons si eren d'inici, de continuació o de creixement, es va poder observar que la majoria de les fórmules d'inici contenien prebiòtics, algunes probiòtics, i d'altres no contenien cap suplement d'aquest tipus (**Figura 13**). No es va trobar cap fórmula d'inici que contingués simbiòtics. Pel que fa a les

llets de continuació, totes les estudiades contenien prebiòtics, probiòtics o simbiòtics, en aquest ordre de predomini (**Figura 13**). En canvi, la majoria de fórmules de creixement no contenien cap d'aquests suplements, i entre les que sí que en contenien, en major proporció contenien probiòtics, seguides de les que contenien prebiòtics, i finalment simbiòtics (129).

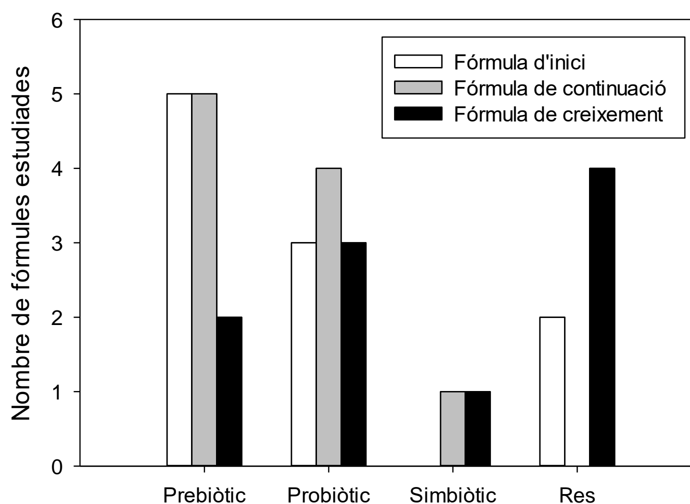


Figura 13. Distribució de 30 mostres de fórmules infantils de venda en farmàcies consultades, segons si contenien prebiòtics, probiòtics, ambdós o cap d'aquests components. Modificat de Sabaté Jofre, 2016 (129)

5. Proteïnes del sèrum de la llet i calostre boví hiperimmune

Com ja s'ha esmentat, la fracció proteínica de la llet està formada per les caseïnes i per les proteïnes del sèrum (com la β-lactoglobulina, l'α-lactoalbúmina, Ig o la lactoferrina). Aquestes proteïnes representen una font nutritiva i d'energia, però també tenen efectes immunomoduladors i protectors enfront a patògens.

Pel que fa al calostre, és una secreció única produïda només durant els primers dies postpart, amb una composició molt específica (major concentració de proteïnes i Ig) i amb la funció de promoure un ràpid creixement i desenvolupament del nadó, protegir-lo contra possibles infeccions i modular el seu sistema immunitari (122).

Quan s'obté el calostre de vaques que han sigut prèviament immunitzades amb un agent patogen concret (calostre boví hiperimmune, HBC), el contingut d'aquest és molt ric en Ig i factors protectors específics contra el patogen.

El capítol de llibre que es presenta a continuació recull les evidències científiques, publicades fins aleshores, en relació als beneficis d'una intervenció nutricional amb proteïnes del sèrum de la llet enfront a la infecció per RV en estudis preclínics.

Aquest tipus de producte ha estat utilitzat en la present tesi com a control positiu de protecció enfront a la infecció per RV.

30. Whey protein is beneficial for rotavirus-induced diarrhoea in preclinical studies

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Abstract

Group A rotavirus (RV) is the leading cause of diarrhoeal disease among young children worldwide. The symptoms range from asymptomatic shedding to severe dehydration and even death. After RV, infection immunity is not complete and reinfections usually occur. Although two oral vaccines are available, the diarrhoea process can be modulated by dietary interventions with bioactive components. Breast milk contains whey proteins (WP) whose capacity to inhibit RV infection has been demonstrated both *in vitro* and *in vivo*. In this context, several animal models, mainly using mice and rats, have been used to explore RV infection and its pathology, clinical and immune response, and to test vaccine efficacy or nutritional modulation of the process. These models are mainly based on homologous or heterologous RV inoculation in suckling rodents, which develop mild-severe diarrhoea, evaluated by clinical and immune variables. On the one hand, hyperimmune colostrums reduce the disease incidence, severity, duration and viral shedding, and modify the immunity response in calves and germfree pigs. In the neonatal mouse model, they are able to prevent diarrhoea and modulate the viral shedding. On the other hand, whey protein concentrates and in particular WP are also able to modulate the diarrhoea process (i.e. incidence and severity) and affect the viral clearance and some aspects of immune response in the neonatal mouse and rat models. In conclusion, as it is well documented that WP are good nutritive supplements due to their capabilities to modulate immunity and given data of rotavirus protection in preclinical studies, the importance of these components in infant formulas should be considered in order to guarantee the same protective function provided by breast milk in breastfed babies.

Keywords: rat, animal models, breast milk

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Summary points

- Rotavirus (RV) belongs to the family *Reoviridae* which are icosahedral and non-enveloped viruses with a double-stranded RNA genome and covered by several protein-derived layers and which are the cause of severe, dehydrating diarrhoeal disease among children in the first five years of life worldwide.
- Several animal models, mainly using mice and rats, have been used to explore rotavirus infection and its pathology, clinical and immune response, and to test vaccine efficacy or nutritional modulation of the process. These models are mainly based on homologous or heterologous RV inoculation in suckling rodents, which develop mild-severe diarrhoea evaluated by clinical and immune variables.
- Milk contains two primary sources of protein: caseins and whey. The whey protein (WP) of bovine whey (β-lactoglobulin, α-lactalbumin, immunoglobulins, glycomacropeptide, bovine serum albumin, lactoferrin and lactoperoxidase) are a good nutritive supplement due to their ability to modulate immunity, which may help in a number of different immune diseases.
- Hyperimmune colostrums, WP concentrates and in particular WP, such as lacthaderin and lactoferrin, have demonstrated *in vitro* their anti-RV activity through interfering in the different steps of virus replication and infection.
- Preclinical studies performed in both the neonatal mouse and rat models have demonstrated the efficacy of both hyperimmune colostrum and WP concentrates in controlling the incidence and severity of diarrhoea.
- The importance of the presence of these components in infant formulas in order to guarantee the same protective function provided by breast milk in breastfed babies should be considered.

Abbreviations

Caco-2	Human epithelial colorectal adenocarcinoma cells
CM3Q3	Protein fraction from macromolecular whey protein
DI	Diarrhea index
Ig	Immunoglobulin
LF	Lactoferrin
MMWP	Macromolecular whey protein
MUC1	Mucin 1
NSP	Non-structural proteins
RV	Rotavirus
SIF	Standard infant formula
WP	Whey protein
WPC	Whey protein concentrates

30.1 Gastroenteritis by rotavirus

Rotavirus infection is the most common cause of severe, dehydrating diarrhoeal disease among children worldwide. Almost every child in the world, in both developed and developing countries will be infected with rotavirus in the first five years of life. Rotaviruses are estimated to be responsible for approximately 527,000 deaths each year, with more than 85% of these deaths occurring in low-income countries in Africa and Asia, and over two million are hospitalized each year with pronounced dehydration.

30.1.1 Rotavirus structure

Rotaviruses belong to the family *Reoviridae* which are 75 mm in diameter, icosahedral and non-enveloped viruses with a genome formed with segments of double-stranded (ds) RNA (670-3,300 base pairs), and covered by several protein-derived layers. Although rotaviruses comprised groups A to G, depending on cross-reactivity and genome similarity among them, viruses from group A are the main human pathogens. Regarding viral genome, each one of the eleven dsRNA segments of rotaviruses encodes for at least one protein. Therefore, they have six structural proteins and six NSPs for which their functional role in viral replication is well characterized (NSP1-NSP6) (Greenberg and Estes, 2009; Hu *et al.*, 2012). The high stability of the triple-layer capsids allows the fecal-oral transmission and for them to reach the small intestine where they show their pathogenic properties (Greenberg and Hestes, 2009).

30.1.2 Pathophysiology of rotavirus infection

RV predominantly infects children and there is a spectrum of disease, ranging from asymptomatic shedding to severe dehydration, seizures and even death. The incubation period for RV disease is approximately 2 days. Typical symptoms of the disease are non-specific and include vomiting,

profuse watery bloodless diarrhoea up to several times per day, and fever and abdominal cramps and pain occur frequently. Symptoms persist for 3 to 8 days and children can spread rotavirus 2 days before and, usually, up to 8 days after they become unwell with diarrhoea. After a RV infection immunity is incomplete, but repeated infections tend to be less severe than the first one. The infection is diagnosed by testing for RV in faecal specimens; detection of strains can be performed by special ELISA or PCR, but is not commonly necessary. The prevention of dehydration and gastroenteritis management has been reviewed by Churgay and Aftab (2012).

The pathogenesis of RV-induced diarrhoea is complex, and it is clear that RV diarrhoea is multifactorial, resulting from the direct effects of virus infection and the indirect effects of infection and the host response. First, RV infects the mature absorptive enterocytes of the villus tip in the proximal two-thirds of the ileum, but some extra-intestinal manifestations may occur (Ramig, 2004). RV alters the function of the small epithelium resulting in diarrhoea, but diarrhoea can occur with no visible tissue damage and, conversely, the histological lesions can be asymptomatic. RV impairs activities of intestinal disaccharidases and Na^+-solute symports coupled with water transport. Maldigestion of carbohydrates and their accumulation in the intestinal lumen and a concomitant inhibition of water reabsorption can lead to a malabsorption component of diarrhoea (Lorrot and Vasseur, 2007).

30.2 Animal models of rotavirus-derived gastroenteritis

Several animal models have been used to explore RV infection and its pathology, clinical and immune response, and to test vaccine efficacy or nutritional modulation of the process. Animal models for the study of the pathogenesis of rotaviruses include on the one hand, long-gestation animals (large animals), such as cows, pigs and sheep, and on the other hand short- gestation animal models, such as rabbits, mice and rats. The early work performed on large animals presented high costs, difficulties in the restricted animal facilities and the necessity of longer periods of study (Pérez-Cano *et al.*, 2012). The rabbit and mouse models of disease (neonatal animals) and infection (adult animals) were then developed but with some difficulties, such as low virus replication or the infectivity restricted to homologous (isolated from the same species) strains. It was then that the neonatal rat model for RV infection was more developed and used for interventional studies (Ciarlet *et al.*, 2002; Pérez-Cano *et al.*, 2007).

30.2.1 Experimental design of the animal models

Focusing on rodents, in the last three decades there have been several studies using these short-gestation-period animals in combination with several types of virus (homologous and heterologous) as models to obtain useful knowledge about RV pathogenesis. Some aspects have to be taken into account when using an animal model of RV diarrhoea, such as the species/strain of the animal, the type and dose of virus used, the age of the animal when it is virus-inoculated, the clinical outcomes based on stool appearance, viremia and viral shedding, and finally markers of immune response or protection.

The first aspects to be taken into account are the species or even the strains of the animals. The mouse model has been widely used to define the pathogenesis of RV against homologous rotaviruses, but heterologous infection only induced limited or absent virus replication (Burns *et al.*, 1995; Ciarlet *et al.*, 2002). Regarding rat, the Lewis strain has the advantage of increased susceptibility to infection by heterologous (non-rat) rotaviruses, including human virus. This is not the case for other rat strains such as Wistar rats which are reported to lack some specific RV receptors or which highly avoid virus replication and therefore no disease is induced (Pérez-Cano *et al.*, 2007).

In rats, similarly to what happens in mice and rabbits, infection can be developed at any age but disease (i.e. diarrhoea) is age-restricted. In this regard, disease is induced after inoculation of different RV A strains in 5-day-old rats but not in older animals such as 21 or 270-day-old-rats (Ciarlet *et al.*, 2002). Moreover, there is clear evidence that diarrhoea is age-dependent when inoculation is performed with only 4 days of delay with a particular type-A RV: incidence at 4th-day post-inoculation is 20 vs. 100%, maximum severity is ~3.5 vs. ~2.0 (in a 1-4 severity scale, see below), or overall diarrhoea process is ~5 vs. ~2 days in 7 and 11-day-old RV-inoculated Lewis rats, respectively (Pérez-Cano *et al.*, 2007).

30.2.2 Clinical evaluation of rotavirus-gastroenteritis in animal models

Clinical outcomes are key data in the assessment of the disease and are mainly based on the presence of diarrhoea. Usually individual faecal samples are obtained by gently pressing the abdomen and either the stool is considered as normal or diarrhoeic, or it is scored in order to obtain more precise information about the severity of the process (Ciarlet *et al.*, 2002; Pérez-Cano *et al.*, 2007) (Figure 30.1b and 30.1c).

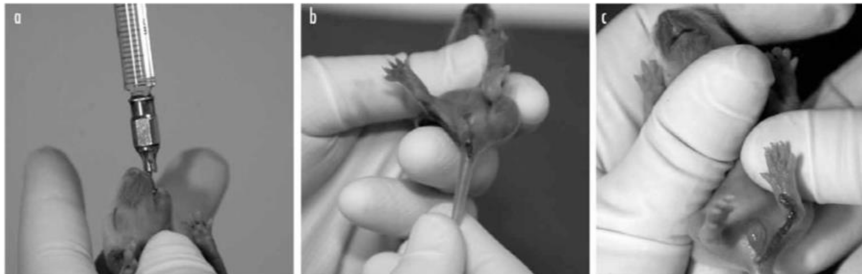

Figure 30.1. Images showing neonatal animal handling in the study of the diarrhoea by rotavirus. (a) Representative image of oral gavaging of the rotavirus (p.o. infection process) in 7-day-old newborns with ophthalmic 23-gauge gavage tubes and a short-volume syringe; (b) example of non-invasive sampling (faeces) from a neonatal non-infected rat, diarrhoea index (DI)=1, no diarrhoea; (c) example of faecal specimen collection during infection, DI=3, clear diarrhoea.

The analysis of the presence or absence of diarrhoea allows the expression of the results as incidence, which can be expressed as the % of animals with diarrhoea with respect to total animals included in the study or with respect to faecal specimens collected each day, since faecal samples are not always collected every day. Overall, all heterologous viruses mentioned in the above studies in rats were able to induce diarrhoea with a very high incidence (up to 100% in most of the instances) (Figure 30.2a).

Regarding severity of illness, the score, sometimes named as DI comprises a scale of 1-3 or 1-4 and is based on colour, texture or degree of soiling and the amount of the collected faecal material (Figure 30.1b and 30.1c) (Ciarlet *et al.*, 2002; Knipping *et al.*, 2011; Pérez-Cano *et al.*, 2007). For example, in a 1-4 diarrhoea score, a DI≥2 is considered as diarrhoea, whereas DI≤2 is considered normal.

The detection of virus in faecal samples is used as an indirect methodology to evaluate viral elimination or clearance (Ciarlet *et al.*, 2002; Guerin-Danan *et al.*, 1998). Regarding the kinetic of viral shedding, the peaks of higher diarrhoea incidence and severity correlate with peak days of virus antigen shedding as can be seen in Figure 30.2b and 30.2c.

30.2.3 Immunity evaluation of rotavirus infection in animal models

Although it is known that humoral and cell immune responses are involved in the protection against RV infection, the precise mechanisms involved are still unclear. However, it is known that, as immune response first takes place at mucosal sites, IgA titres in serum and mucosal samples correlate highly with protection from disease and reinfection (Greenberg and Estes, 2009). In this case, immune response is well characterized in the mice model whereas in rat less data is available. In SA11-inoculated neonatal rats some immune systemic and mucosal markers have been described, such as induction of specific sera antibody titres, specific lymphoproliferation, increased intestinal interferon γ secretion and changes in the intraepithelial lymphocytes phenotype (Pérez-Cano *et al.*, 2007).

Using these animal models, how dietary intervention in early life using several compounds can modulate the course of the disease can be studied. Because RV infection occurs during early age it is logical to focus on a dietary intervention with components that can interact during this period, which mainly include breast milk (Pérez-Cano *et al.*, 2012). It is known that human milk not only supplies nutrients but also a large number of bioactive components that can exert multiple actions on the gastrointestinal tract and beyond. In fact, among the various benefits of breast milk, the reduction in the risk of some infections such as viral and bacterial gastroenteritis should be highlighted (Guo and Hendricks, 2008). The bioactive agents of breast milk, which are of interest in the context of diarrhoea induced by RV, can include carbohydrates, fat- and water-soluble vitamins, lipid components and nucleotides; however, we will focus our attention on the bioactive proteins and peptides present in the aqueous phase of milk, the whey, also known as whey proteins.

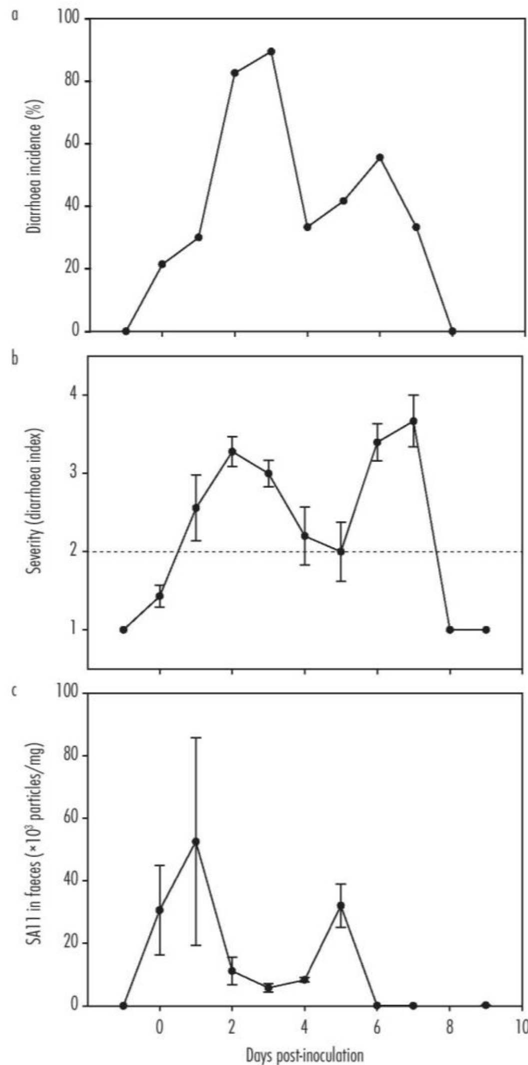

Figure 30.2. Clinical evidence of diarrhoea process in animal models. Representative results after inoculation of ~10^8 infecting SA11 particles in Lewis neonatal rats by means of (a) diarrhoeal incidence, (b) severity of diarrhoea and (c) viral antigen shedding curve.

##should not be in caption but in main text## As performed in Pérez-Cano *et al.* (2007, 2008), virus was inoculated in 7-day-old Lewis rats and after that daily faecal collection was performed to obtain clinical data based on the appearance of the faecal material. It was scored from 1 (normal) to 4 (severe diarrhoea), with values >2 considered as diarrhoeic faeces. The % of animals with diarrhoea with respect to those animals from which a sample was obtained is considered the diarrhoea incidence. The profile of faecal SA11 particles determined by ELISA indicates the clearance of the virus.

30.3 Milk whey proteins as bioactive components

Milk constituents have become recognized as functional components due to their direct and measurable effects on health outcomes. Milk contains two primary sources of protein, the caseins and whey proteins. After processing, the caseins are the proteins responsible for making curds, whereas whey remains in an aqueous state. The protein components of bovine whey include, from the highest to the lowest proportion (% of whey protein), β-lactoglobulin (50-55%), α-lactalbumin (20-25%), immunoglobulins (10-15%), glycomacropeptide (10-15%), bovine serum albumin (5-10%), LF (1-2%), and lactoperoxidase (0.50%). In addition to proteins, whey also contains fat, lactose and minerals (Marshall, 2004).

WP contain all the essential amino acids and have the highest protein quality rating among other proteins (Marshall, 2004; Yalçin, 2006). In addition, WP have a high percentage of branched-chain amino acids – leucine, isoleucine, and valine – which are efficient substrates for synthesizing new proteins and important factors in tissue growth and repair (Abrahao, 2012; Marshall, 2004). WP are also considered to be 'fast proteins' since they reach the jejunum almost immediately and their hydrolysis within the intestine is slower than other proteins. This allows a greater absorption over the length of the small intestine (Beaulieu *et al.*, 2006).

Advances in processing technology, including ultrafiltration, microfiltration, reverse osmosis, and ion-exchange have resulted in the development of several different finished WP products, which differ in the content of protein, lipid, carbohydrate and minerals. Among them, WPC are commonly available as 80% protein and by microfiltration they can achieve 95% protein concentration (Marshall, 2004). WP and their products are used as common ingredients in various products, including, among others, infant formulas, specialized enteral and clinical protein supplements, and sports nutrition products (Yalçin, 2006).

Whey is being recognized as a functional food with a number of health and disease benefits (Abrahao, 2012; Beaulieu *et al.*, 2006; Madureira *et al.*, 2010; Marshall, 2004; Yalçin, 2006). The biological components of whey, including lactoferrin, β-lactoglobulin, α-lactalbumin and immunoglobulins, demonstrate a range of immune-enhancing properties (Beaulieu *et al.*, 2006; Marshall, 2004). In addition, whey has the ability to act as an antioxidant, antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial, and chelating agent (Marshall, 2004). In some cases the benefits of whey proteins and peptides have been demonstrated by clinical trials (Marshall, 2004; Yalçin, 2006).

Whey contains biologically active molecules capable of enhancing intestinal health. There are four beneficial areas of intestinal health modification with whey components: growth promotion of *Bifidobacteria*; antimicrobial properties; improvement of intestinal barrier function; and influences on immunity (Yalçin, 2006). Growth promotion of *Bifidobacterium* species by different whey fractions – containing glycomacropeptide or lactoferrin – has been reported. Whey contains several unique components with wide antimicrobial properties. Immunoglobulins are the best known of the WP that provide antimicrobial action in the intestinal tract. IgG has been shown

to bind the toxin produced by *Clostridium difficile,* thereby reducing the deleterious effects of infection. Lactoferrin has bacteriostatic and bactericidal activity against both Gram-negative and Gram-positive bacteria (Arnold *et al.*, 1977). As well as having a wide antimicrobial spectrum against bacteria and fungi, lactoferrin also has antiviral activity (Valentí and Antonini, 2005). In addition, some peptides generated through proteolytic digestion from α-lactalbumin and β-lactoglobulin, as well as lactoferrin, have high bactericidal activity (Madureira *et al.*, 2010). Recently, it has been reported that whey proteins improve intestinal barrier function, in part, by regulating the expression and functions of claudins (Kotler, 2013).

The majority of the studies concerning the immunomodulatory potential of WP used whey directly and not one individual WP; the effects are therefore associated to WP in general and not to a specific protein (Beaulieu *et al.*, 2006). The immunoenhancing effects of WP on the formation of specific antibodies as well as on the proliferation of non-stimulated splenocytes is well documented. However, some studies indicate that whey can be an immunosuppressive agent in some circumstances. Thus, whey proteins might be a good nutritive supplement due to their capabilities to modulate immunity, which may help in a number of different immune diseases (Beaulieu *et al.*, 2006).

30.4 Whey proteins can modulate rotavirus infection *in vitro*

The ability of milk components to inhibit RV *in vitro* replication first appeared in 1992 (Yolken *et al.*, 1992). These authors isolated a macromolecular component of human milk able to inhibit RV replication *in vitro*; this component was associated with an acidic fraction free of immunoglobulins, and in particular to human milk mucin. This glycoprotein is also responsible for the inhibition of the infectivity of RV produced by milk fat globule membrane glycoproteins (Fuller *et al.*, 2011), although lipid compounds could also be relevant (Fuller *et al.*, 2013). In addition, WP fractions or isolated compounds obtained from human or bovine milk can be responsible for the anti-RV effects.

Considering WP, Kanamaru *et al.* (1999) showed that a high molecular weight fraction from bovine WP inhibited RV replication *in vitro*. Lactadherin seemed to be the major inhibitor of RV infection (Yolken *et al.*, 1992). This inhibitory effect was later demonstrated by human lactadherin in Caco-2 cells infected with the Wa strain of Human RV (Kvistgaard *et al.*, 2004), although, in the same study, bovine lactadherin did not inhibit infectivity of neuraminic acid-sensitive or acid-insensitive strains of RV. This led to the proposal that other components of bovine milk could be responsible for its anti-RV activity. It has been reported that bovine β-lactoglobulin was able to inhibit the replication of RV (Superti *et al.*, 1997), and MUC1 and MMWP fractions from bovine milk showed RV inhibitory effects (Kvistgaard *et al.*, 2004). Bojsen *et al.* (2007) assessed the anti-RV ability of bovine MMWP using four different RV strains (human, bovine, porcine and simian) and two different intestinally derived human cell lines (Caco-2 and human fetal small intestinal cells). MMWP demonstrated an equal capacity to reduce the number of infections produced by all four tested RV strains, regardless of the type of cells used in the assay (Bojsen *et al.*, 2007). The

most RV-inhibitory capacity of MMWP remained after heat (10 min at 85 °C) and protease (1% pepsin for 1 h) treatments. Bovine IgG was shown to be the main constituent of active MMWP fraction; whereas LF did not have any effect on RV infectivity *in vitro* and MUC1 demonstrated an intermediate effect (Bojsen *et al.*, 2007). As a result, it has been proposed that cow's milk can offer passive protection against RV if it contains a high level of specific anti-RV antibodies (Ebina *et al.*, 1992; Song *et al.*, 1999). Nevertheless, the protective efficacy of bovine colostrum from healthy lactating cows in comparison with colostrum whey from cows hyperimmunized with human RV, containing high titres of neutralizing antibodies against HRV, has been evaluated (Inagaki *et al.*, 2010a). These results show that, although antibody-containing colostrum was more potent, the other colostrum also exhibited activity against human RV. This activity was associated with a heat-stable high-M_r glycoprotein fraction from cow's milk whey (Inagaki *et al.*, 2010b).

Studies concerning the RV-inhibitory role of LF are more controversial. Some authors (Bojsen *et al.*, 2007; Kvistgaard *et al.*, 2004) failed to show anti-RV activity for bovine lactoferrin, but Superti *et al.* (1997, 2001) show that bovine LF was able to inhibit infection by the simian neuraminidase-sensitive RV strain SA11 in human colon carcinoma cells. Specifically, these authors demonstrated the role of metal binding, sialic acid and tryptic fragments of LF in the anti-RV activity and concluded that different moieties of the molecule can interfere with different steps of virus replication and then inhibit RV infection (Superti *et al.*, 2001).

30.5 Preclinical evidences of whey protein attenuation of gastroenteritis by rotavirus

Besides the *in vitro* evidences of breast milk components as anti-rotaviral agents some studies performed *in vivo* in the diarrhoea animal models have highlighted the importance of this type of dietary intervention in prevention of disease. The studies can be organized into three groups: (1) those evaluating the effect of bovine hyperimmune colostrums; (2) those studying whey protein concentrates and whey proteins; (3) and those evaluating other components present in breast milk (i.e. probiotics, vitamins, osteopontin, etc.).

30.5.1 Preclinical studies of hyperimmune colostrums on rotavirus gastroenteritis

On the one hand, bovine milk is rich in antibodies, but bovine early milk (i.e. colostrum) has higher concentration of them. On the other hand, cow's milk can be enriched with high-specific and neutralizing anti-RV antibodies by hyperimmunization of pregnant cows with certain RV strains (Inagaki *et al.*, 2010a). Therefore, supplementation with hyper-immunized colostrums during early life may modulate the RV infection in the offspring, similarly to what happens *in vitro*. Some studies have evaluated the efficacy of these types of compounds in different animal models and humans.

Some data is available regarding hyperimmune colostrum protection in calves experimentally challenged with RV (Fernández *et al.*, 1998; Saif *et al.*, 1983; Tsunemitsu *et al.*, 1989), but recently Parreño and collaborators (Parreño *et al.*, 2010) have demonstrated how milk supplemented with immune colostrum can protect against RV diarrhoea and modulate the systemic and mucosal antibody response. In terms of clinical outcomes, a lower disease incidence and severity proportion, duration and viral shedding were induced by the administration of the product at 0.4 or 0.8% proportion in milk in a dose-dependent manner. Moreover, colostrum supplementation induced a delay in the humoral response and modified the profile of the active response, being higher in IgA than in IgM. Similar results regarding disease protection and Ig modulatory effect have been described using the germfree pig model (Hodgins *et al.*, 1999; Nguyen *et al.*, 2006; Parreño *et al.*, 1999).

The neonatal mouse model has also been used to evaluate the effect of RV hyperimmune colostrum (Inagaki *et al.*, 2010a; Knipping *et al.*, 2011). The first article showed how whey from the colostrum of cows hyperimmunized with human RV (rota whey) at a single dose of 0.25 mg/day before neonatal mice inoculation was able to prevent diarrhoea in all animals (Inagaki *et al.*, 2010a). The second one, evidenced the effect of a hyperimmunized colostrum (Gastrogard-R®) administered during the 6 following days after virus inoculation at a dose of 1 mg/day (Knipping *et al.*, 2011). Moreover, it not only demonstrated its efficacy controlling the diarrhoea process but also viral shedding modulation. In fact, this last colostrum has been used as a prophylactic treatment for diarrhoea induced by RV in a clinical trial in children aged <2 years (Davidson *et al.*, 1989).

30.5.2 Preclinical studies of whey proteins and whey protein concentrates on rotavirus gastroenteritis

Besides Ig and specific anti-RV Ig, and as described in the previous section, whey is rich in many other bioactive proteins able to modulate the diarrhoea induced by RV. In this regard, there are some reports showing the beneficial effects of WP or WPC on RV infection in both the neonatal mouse and the neonatal rat models.

Firstly, WPC is able to modulate the course of the diarrhoea process. Wolber *et al.* had already shown in 2005 some relevant data regarding the ability of a whey protein concentrate (IMUCARE™; 3 g/kg of body weight) to control diarrhoea induction by EDIM virus in the neonatal mouse model. Although this supplement over 8 days was not able to reduce the proportion of symptomatic mice, the severity during the post-infection period was significantly reduced. In the same way, Pérez-Cano and collaborators (Pérez-Cano *et al.*, 2008) obtained more clear results by using the neonatal rat model infected with SA11 RV (Figure 30.3). In this study, a WPC administrated daily from day 3 of life at a dose of 0.3 g/kg of body weight was compared to a SIF (3 g/kg) and with another group which, besides WPC, also incorporated 0.1 g/kg of LF. Incidence (Figure 30.3a) and severity (Figure 30.3b) were evaluated throughout the study (from inoculation day and until 10th-day post-inoculation) and both indicators were highly reduced by both WPC and WPC+LF products, the effect induced by the diet enriched with LF being higher. Moreover, other indicators

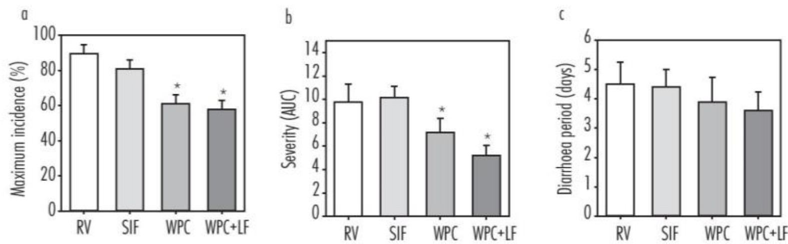

Figure 30.3. Clinical effects of whey protein concentrates (WPC) on rotavirus (RV) diarrhoea. Using the neonatal rat model infected with SA11 rotavirus, Pérez-Cano *et al.* (2008) studied, among others, the effect on (a) incidence (maximum incidence, %), (b) severity (AUC) and (c) duration (diarrhoea period, days) after daily administration of WPC (0.3 g/kg), standard infant formula (SIF; 3 g/kg) and another group which besides WPC also incorporated 0.1 g/kg of lactoferrin (LF). Results are shown as mean±SEM of 10-20 individual values. Statistical differences are shown as **P*<0.05.

or disease were also modified such as the faecal weight or the duration of diarrhoea, which was shortened by almost one day (Figure 30.3c). The high reduction effect of WPC on diarrhoea incidence was also later described by Inagaki and collaborators (Inagaki *et al.*, 2010a). In this case, by using a neonatal mouse model of diarrhoea induced by a human RV they demonstrated the protective efficacy of skimmed and concentrated bovine milk from healthy lactating cows at a single dose of 2.5 mg/day. The incidence reduction throughout the experimental period was up to <10% of the whole number of animals virus-inoculated (1/11).

Secondly, regarding viral clearance of the virus, the viral shedding was affected in mice by WPC, mainly at the end of the studied period (Wolber *et al.*, 2005), and in rats by WPC+LF the day after virus inoculation (Figure 30.4a) (Pérez-Cano *et al.*, 2008). Moreover, some fractions of the WPC or particular WP have also been tested as blocking agents of the RV. Particularly, bovine lactadherin, mucins such as MUC1, MMWP or a particular fraction with high antiviral activity (CM3Q3 fraction) were tested *in vivo* in mice infected with the EMcN murine RV (Bojsen *et al.*, 2007). CM3Q3 fraction was the only product able to modify the viral shedding when preincubated with the virus prior to inoculation.

And finally, the immune response has also been described as being modulated by WP in these preclinical studies. In the neonatal mouse models, the specific antibody levels (i.e. total Ig, IgG or IgA) have been measured in different compartments (sera or gut/faecal fluids) but no significant differences have been found due to WPC diet in the EDIM-infected mice model (Wolber *et al.*, 2005) or the WP fractions preincubated with the HRV in the mice model (Bojsen *et al.*, 2007). However, down modulation of the humoral immune response, by means of quantification of total anti-RV Ig in serum, was observed in SA11 RV-inoculated rats by WPC and WPC+LF treatment (Figure 30.4b) (Pérez-Cano *et al.*, 2008). This attenuating effect was also described before for the hyperimmunized colostrum. These findings indicate a lower systemic viral entrance by both the immune priming effect of some WP and by specific blockage at the intestinal lumen of the virus.

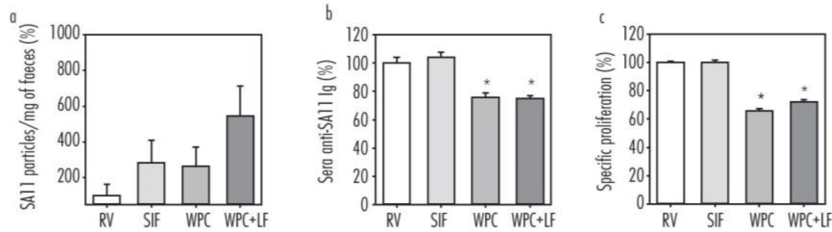

Figure 30.4. Viral elimination and immune response effects of whey protein concentrates (WPC). Using the neonatal rat model infected with SA11 rotavirus (RV), Pérez-Cano *et al.* (2008) studied, among others, the effect on (a) viral clearance (SA11 particles/mg of faeces), (b) sera anti-SA11 antibodies and (c) specific proliferation (%) after daily administration of WPC (0.3 g/kg), standard infant formula (SIF; 3 g/kg) and another group which besides WPC also incorporated 0.1 g/kg of lactoferrin (LF). Data are shown as % with respect the non-supplemented animals (RV group). Results are shown as mean±SEM of 10-20 individual values. Statistical differences are shown as *P<0.05.

However, the effect of WPC on primary and secondary antibody responses has been further studied in an adult mice model of RV infection, but not disease. In this case a boost of the intestinal tract antibody response due to WPC dietary intervention is demonstrated (Low *et al.*, 2003).

Besides specific immune antibody response against RV, WPC has also been described to modulate mucosal immune response by means of modulation of interferon γ secretion *ex vivo* and in biological fluids, lymphocyte phenotype of intraepithelial lymphocytes and proliferative capacity of spleen cells under specific stimulatory conditions (Figure 30.4c) (Pérez-Cano *et al.*, 2008).

Overall, WP have demonstrated their action against RV infection, but other compounds present in breast milk have also been studied in these animal models of disease. To date, the extracellular matrix protein called osteopontein and vitamin A, both found in breast milk, seem to be involved in immune response modulation against RV infection (Maeno *et al.*, 2009; Vlasova *et al.*, 2013). As breast milk contains both prebiotic compounds and probiotic bacteria, the positive effect found using these types of dietary intervention studies may also clarify the protective role of breast milk against infection (Guerin-Danan *et al.*, 2001; Qiao *et al.*, 2002; Ventola *et al.*, 2012).

In summary, preclinical studies show that the administration of isolated components from WP, WP-derived products or WPC are able to modify the course of the RV-induced gastroenteritis. The importance of the presence of these components in infant formulas in order to guarantee the same protective function provided by breast milk in breastfed babies should be considered.

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RESULTATS

Capítol 1

"*Preclinical immunomodulation by the probiotic* Bifidobacterium breve *M-16V in early life*"

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Els resultats del present article han estat presentats a les següents comunicacions:

- *The probiotic strain* B. breve *M-16V promotes immunological maturation in early life*. IXè congrés de la Societat Catalana d'Immunologia (SCI) (Barcelona, Espanya), novembre 2015. Premi al millor pòster del congrés 2015.

RESUM

Objectiu: Estudiar l'efecte de la suplementació amb el probiòtic *Bifidobacterium breve* M-16V durant la lactància sobre la maduració del sistema immunitari intestinal i sistèmic.

Material i mètodes: Rates Lewis lactants van rebre el suplement des del dia 6 fins al dia 18 de vida. Es va controlar diàriament el pes dels animals i es van obtenir mostres de femtes. El dia 19 es van sacrificar les rates i es van obtenir mostres de rentat intestinal, limfòcits aïllats de ganglis limfàtics mesentèrics (GLM), de la melsa i limfòcits intraepitelials (IEL).

Resultats: La suplementació amb el probiòtic no va afectar la corba de creixement ni la maduració del sistema immunitari a nivell sistèmic. No obstant, va incrementar la proporció de cèl·lules portadores de TLR4 als GLM i IEL. També va augmentar la proporció de cèl·lules portadores de la integrina αEβ7 i el marcador CD62L en GLM, i de la integrina αEβ7 en IEL, el que suggereix una millora del procés de reclutament de limfòcits T verges cap als GLM, i la retenció de limfòcits activats al compartiment intraepitelial. El probiòtic també va afavorir la síntesi d'IgA intestinal.

Conclusió: La suplementació amb el probiòtic *Bifidobacterium breve* M-16V durant la lactància millora el desenvolupament de la immunitat de les mucoses en les primeres etapes de vida.

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RESEARCH ARTICLE

Preclinical Immunomodulation by the Probiotic *Bifidobacterium breve* M-16V in Early Life

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Abstract

This study aimed to investigate the effect of supplementation with the probiotic *Bifidobacterium breve* M-16V on the maturation of the intestinal and circulating immune system during suckling. In order to achieve this purpose, neonatal Lewis rats were supplemented with the probiotic strain from the 6th to the 18th day of life. The animals were weighed during the study, and faecal samples were obtained and evaluated daily. On day 19, rats were euthanized and intestinal wash samples, mesenteric lymph node (MLN) cells, splenocytes and intraepithelial lymphocytes (IEL) were obtained. The probiotic supplementation in early life did not modify the growth curve and did not enhance the systemic immune maturation. However, it increased the proportion of cells bearing TLR4 in the MLN and IEL, and enhanced the percentage of the integrin αEβ7+ and CD62L+ cells in the MLN and that of the integrin αEβ7+ cells in the IEL, suggesting an enhancement of the homing process of naïve T lymphocytes to the MLN, and the retention of activated lymphocytes in the intraepithelial compartment. Interestingly, *B. breve* M-16V enhanced the intestinal IgA synthesis. In conclusion, supplementation with the probiotic strain *B. breve* M-16V during suckling improves the development of mucosal immunity in early life.

Introduction

The gut-associated lymphoid tissue (GALT) is a very extensive and complex part of the immune system located in the small intestine and colon. It is organized in inductive sites, which include isolated lymphoid follicles and Peyer's Patches (PP), where antigens are sampled from the lumen by antigen-presenting cells and the synthesis of IgA is stimulated, and the mesenteric lymph nodes (MLN), where the antigens are presented to naïve lymphocytes which will become activated cells. After moving to the blood, activated lymphocytes migrate again to the intestine where they are diffusely distributed in the *lamina propria* lymphocytes (LPL) and the intraepithelial lymphocytes (IEL) [1].

The gut is colonized by intestinal microbiota, composed of hundreds of bacterial species, which play an important role in the host health. The main functions of the microbiota are protective, metabolic and trophic activities [2,3]. By producing bacteriocins and also by competing for nutrients and attachment sites on the intestinal surface, the microbiota protects the host from pathogen microbes. Furthermore, intestinal bacteria are able to ferment non-digestible carbohydrates and to produce short-chain fatty acids (SCFA), and they are also involved in vitamin synthesis and ion absorption. SCFA (such as acetate, propionate and butyrate) are a source of energy, and also regulate the glucose and lipid metabolism [2,4]. SCFA also have a trophic effect, promoting the proliferation and differentiation of intestinal epithelial cells. Furthermore, butyrate seems to inhibit neoplastic cell proliferation. Finally, bacteria in the lumen interact with the immune system through the pattern recognition receptors, such as the toll-like receptors (TLR), which recognize pathogen-associated molecular patterns [5,6].

The host–microbe interaction is of particular importance in early life. The challenge with antigens is necessary for the maturation of intestine and GALT. The intestinal immune system will acquire the ability to induce tolerance against innocuous dietary, commensal bacterial and self-antigens, and to fight pathogen antigens, in order to maintain intestinal homeostasis and avoid immune-mediated disorders [3,7–9]. Thus, the interaction of microbiota with the intestinal epithelial and immune barriers is involved in the development of oral tolerance and allows microbiota to modulate both innate and adaptive immunity, locally and systemically.

Probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts [10]. Different probiotic strains, mainly from *Lactobacillus* and *Bifidobacterium* genera [11], have demonstrated beneficial effects in some disorders, such as diarrhoea, allergy, inflammatory bowel disease (IBD), lactose malabsorption and necrotizing enterocolitis (NEC) in preterm neonates [12]. These results evidence that probiotics, are able to modulate the immune system. Some of the described effects include the modulation of cytokine production by epithelial cells, the increase of mucin secretion, the enhancement of phagocytosis and natural killer (NK) cell activity, the activation of T and NKT cells, the stimulation of IgA production and the reduction of T-cell proliferation [3,5–7,9,13–16]. It has to be taken into account that each effect is strain-dependent, thus a demonstrated action by particular bacteria cannot be attributed to another strain. In this sense, some probiotic strains have shown pro-inflammatory properties (increasingTh1/Th2 ratio), while others have shown anti-inflammatory modulation (decreasingTh1/Th2 ratio). Those stimulating the Th1 cytokine pattern would be useful in preparing the host to fight against infections, and also to ameliorate allergy. But they could be harmful in autoimmune diseases [7], where those promoting anti-inflammatory cytokine patterns would be a better option. There are also probiotic strains stimulating both Th1 and Th2 responses, thus each case needs to be studied individually [7]. The mechanisms of action for this immunomodulation are still not completely known [5,6,13]. Moreover, host microenvironment and enteric commensals, pathogens and vaccines also contributes to diversity of response to identical probiotic strains [17,18].

As explained above, early infancy and childhood are a critical period for immune system programming, and thus they are a window of time of particular interest for the use of probiotics [19,20]. Some authors point out the importance of the establishment of a proper gut microbiota, which depends on several factors, such as the mother's microbiota, the mother's diet, the mode of delivery or the use of antibiotics [9,21]. The administration of probiotics to the child during lactation or after weaning, or even to the mother during pregnancy and lactation, has been proposed as a strategy to prevent dysbiosis and its harmful consequences [22,23]. An example of long-term effects is a reduction in the prevalence of attention-deficit hyperactivity disorder and Asperger's syndrome in 13-year-old boys who were treated with *Lactobacillus rhamnosus* GG (LGG) for the first 6 months of life [24]. Moreover, some effects of the

probiotics seem to be more potent in the neonatal period, and even lost later in life, i.e. the modulation of the IgE response [8,9,13]. Hashemi et al. [25] reviewed the recent animal studies of probiotic supplementation in early life and highlighted some effects, such as the improvement of the microbiota composition, the enhancement of the intestinal maturation and immune response, and the reduction in the prevalence of infections.

Bifidobacterium breve M-16V is naturally present in infants' microbiota and has shown immunomodulatory properties. This probiotic seems to ameliorate allergy symptoms, often in combination with prebiotics [26–29], and rotavirus diarrhoea [30]. The aim of the present study was to evaluate the impact of *B. breve* M-16V supplementation on some aspects of the immune system development using a neonatal rat as a model. The neonatal rat model is suitable for immunonutrition studies, with substantial scientific evidence and an interesting cost-effective ratio [31]. This model allows the characterization of lymphocyte changes during suckling in several lymphoid compartments [32–35].

Materials and Methods

Animals

Four pregnant Lewis rats (G14), obtained from Janvier Labs (Le-Genest-Saint-Isle, France), were housed in individual cages, monitored daily and allowed to deliver naturally. The day of birth was registered as day 1 of life. Litters were unified to 8 pups per lactating dam, with free access to the nipples and rat diet. Dams were fed a commercial diet corresponding to the American Institute of Nutrition 93G formulation and given water *ad libitum*. The animals were housed in controlled conditions of temperature and humidity, in a 12 h:12 h light:dark cycle. Pups were individually identified. They were weighed and monitored daily in order to obtain data regarding the influence of the nutritional intervention on growth and faecal features. This was done after the separation of the pups from their mother, during the handling and before oral administration.

The studies were approved and performed in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethics Committee for Animal Experimentation of the University of Barcelona and the Catalonian Government (CEEA-UB Ref.493/12, DAAM: 6905).

Experimental design and dietary supplementation

The first 21 days of a rat's life corresponds to the lactating 9-month period of humans. The probiotic supplementation began after 1 week of lactation (after the first 1/3 of the period, when strictly only suckling exists) until the end of the study.

The design chosen was that in which the cells from the immune system compartments are still in development (age comprised during the suckling period of the animals) [31–37], but without the interference of breast milk compounds (forcing transition to solid diet on day 17 of life). Moreover, in order to avoid the transitional trapping lymphocytes effects of weaning on immune system [38], last day of experiment was performed on day 19, two days after early weaning.

Suckling rats were distributed into two groups: the reference (REF) and the probiotic (PRO) groups. Each group was composed of 8 pups from two different litters (just half of the pups from each litter were used for this study). The PRO group received *B. breve* M-16V (Morinaga Milk Industry Co. Ltd, Tokyo, Japan) suspension at a dose of $4.5x10^8$ UFC/100 g of body weight/day. The REF group was administered with a matched volume of mineral water. Suckling rats were orally administered, as previously described [36], from day 6 to day 18 of age,

using low-capacity syringes (Hamilton Bonaduz, Bonaduz Switzerland) adapted to forced alimentation tubes of 25 or 23 calibre and 27 mm of length (ASICO, Westmont, IL, USA).

The animals were weighed and faecal samples were collected daily during the study. At the end of the study intestinal wash samples and spleen and MLN cells and IEL were isolated.

Faecal specimen collection and evaluation

Faecal sampling was performed once daily by gently pressing and massaging the abdomen. Faecal samples were scored from 1 to 4 (faecal appearance score [FAS]) based on colour, texture and amount as described: normal faeces (1); soft yellow-green faeces (2); totally loose yellow-green faeces (3); high amount of watery faeces (4). Scores ≥ 2 indicate diarrhoeic faeces [37]. Faecal weight for each specimen obtained was also measured.

Spleen and intestinal samples collection

After ketamine/xylazine injection and exsanguination, the abdomen was opened and MLN, the spleen and the small intestine were collected. The intestine was flushed *in situ* with cold saline solution to remove the content. Thereafter the duodenum was removed. The proximal ¾ portions were used for IEL isolation. The distal ¼ portion of the intestine was used for gut wash for IgA quantification. It was cut in 5 mm pieces, weighed, and incubated with phosphate buffer solution for 10 min in a 37°C shaker. After centrifugation, supernatants were stored at -20°C until analysis [37].

Isolation and purification of spleen and MLN cells and IEL

Spleen and MLN cells were isolated as previously described [39]. IEL suspensions from the proximal ¾ portion of the intestine were obtained following procedures adapted to neonatal rats and established previously in our laboratory [32]. They were later purified and cell number viability was determined using an automated cell counter after staining dead cells with trypan blue (Countess^{TM}, Invitrogen, Madrid, Spain).

Immunofluorescence staining and flow cytometry analysis

Spleen and MLN cells and IEL ($3x10^5$ cells) were stained using immunofluorescence techniques. The mouse anti-rat monoclonal antibody conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein or allophycocyanin used here included anti-CD4 (OX-35), anti-CD8α (OX-8), anti-TCRαβ (R73), anti-TCRγδ (V65) and anti-NKR-P1A (10/78), all from BD Pharmingen (San Diego, CA, USA); anti-CD45RA (OX-33) from Caltag (Burlingame, CA, USA); anti-CD8β (3·41) from Serotec (Kidlington, Oxford, UK); anti-TLR4 (76B357.1) from Novus Biologicals (Littleton, CO, USA); anti-αE integrin (OX-62) and anti-CD62L (OX-85) from BioLegend (San Diego, CA, USA).

For cell subset differentiation four different antibody panels were used: Panel 1: CD45RA/TLR4/CD8α/CD4; Panel 2: αE integrin/CD62L/CD8α/CD4; Panel 3: TCRαβ/NK/CD8α/CD4 and Panel 4: CD8α/CD8β/TCRγδ. Fist aim was to dissect main subpopulations. B cells (CR45RA+ cells, which is a B cell marker in rats) were identified with the first panel. With the Panel 3 it could be differentiated NK cells (TCRαβ-NK+) from NKT cells (TCRαβ+NK+), and also TCRαβ+NK- cells, which in combination with the TCRγδ+ cells (obtained from the Panel 4) constituted the total of T cells. Moreover, the CD8+TCRαβ+NK- cells (Panel 3) plus the TCRγδ+ cells (Panel 4) could be considered as Tc cells. From Panel 3 we obtained the proportion of Th cells (CD4+CD8-TCRαβ+NK- cells). Panel 1 was designed for investigating the frequency of TLR4+ cells in each of the subsets defined in this panel (CD45RA+, CD4+ or CD8

+ cells). The αE integrin/CD62L pattern was studied by Panel 2 in each of the subsets defined by the combination of the markers used (CD4 and CD8). Gating strategy used in the study can be observed in the S1 Fig.

Results are expressed as proportion of positive cells for a certain marker in each particular subset determined by the combination of other markers with respect to the total lymphocytes gated (i.e. proportion of TLR4+ cells in CD4+CD8- cells).

Staining was developed following procedures described in previous studies [35], and results were analysed using a Gallios™ flow cytometer (Beckman Coulter Inc., Madrid, Spain) in the cytometry service of the Scientific and Technological Centres of the University of Barcelona (CCiT-UB). The obtained data were assessed with FlowJo software (Tree Star Inc., Ashland, OR, USA).

ELISA for intestinal IgA quantification

Gut wash IgA concentration was determined using a sandwich ELISA technique with the Rat IgA ELISA Quantitation Set (E110-102) from Bethyl Laboratories (Montgomery, TX, USA). Ninety-six well plates (Nunc MaxiSorp, Wiesbaden, Germany) were coated with adequate dilution of the capture antibody. After incubating standard or gut wash samples, the peroxidase conjugated detection antibody was added. Subsequently, substrate solution (3,3′,5,5′-Tetramethylbenzidine plus hydrogen peroxide in dimethyl sulfoxide and 0.05 M phosphate-citrate buffer, pH 5; Sigma-Aldrich, Madrid, Spain) was added and absorbance was measured at 450 nm, after stopping the enzymatic reaction with 2 M H_2SO_4, on a microtitre plate photometer (Labsystems, Helsinki, Finland). Data were interpolated by means of Multiskan Ascent v.2.6 software (Thermo Fisher Scientific S.L.U, Barcelona, Spain). Dilutions of rat IgA (Bethyl Laboratoires) ranging from 500 to 15.625 ng/mL were used as a standard in each plate. Data are expressed as ng of IgA per mg of intestinal tissue used for the gut wash.

Statistical analysis

The PASW Statistics 18 software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Conventional one-way ANOVA test was performed considering the experimental group as the independent variable. When supplementation had a significant effect on the dependent variable, Scheffé's test was applied. Significant differences were accepted when $p<0.05$. All the results are expressed as mean ± SEM.

Results

Body weight

The growth curves of the REF and PRO groups were very similar and without statistical differences throughout the period, as can be seen in Fig 1. Before the PRO intervention (days 3–6), both groups showed identical weight. From day 6 to day 17 there was a slightly higher weight in the PRO group, but still similar ($p>0.05$). On days 18 and 19, the early weaning effect can be observed in both groups, since there is a slight weight loss during the first days after separation from their dam.

Faecal consistency

The PRO group showed faecal scores slightly higher than 1 (normal faeces) from the second day of supplementation (day 8) until day 14, indicating that the PRO induced soft faeces (Fig 2). However, the faecal weight was 7.60 ± 2.90 mg at the beginning of the supplementation and 10.44 ± 1.83 mg at the end for both groups, without significant differences between groups.

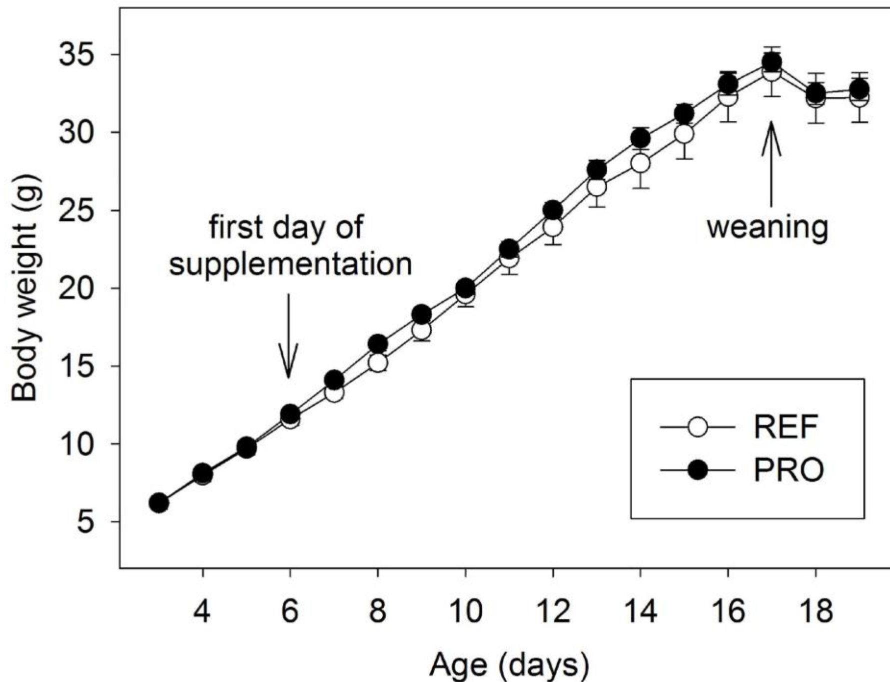

Fig 1. Rats body weight pattern during the study in the reference (REF) and probiotic (PRO) groups. Results are expressed as mean ± SEM (n = 8 animals/group). The first day of nutritional intervention and the weaning day are shown by arrows.

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Phenotype of spleen and MLN cells and IEL

On the last day of the study (day 19) spleen and MLN cells, as well as IEL, were isolated in order to evaluate the influence of the PRO on the lymphocytes. A sufficient number of viable cells from all tissues were obtained (with a viable cell number range of $1.7–4.6x10^7$ and a viability of 73–98% for spleen cells; $1.7x10^5–1.9x10^6$ and 72–91% for MLN cells; $4.5x10^5–1.1x10^6$ and 76–87% for IEL). No differences were observed between the REF and PRO groups.

Main lymphocyte subset proportions. The phenotype of lymphocyte subsets present in systemic (spleen) and intestinal compartments (MLN and IEL) was studied. The percentage of the main subsets present in each compartment is summarized in Table 1.

Among the spleen cells, the PRO nutritional intervention did not affect B cell proportion but was able to significantly reduce the proportion of both T helper (Th) and cytotoxic T (Tc) cells ($p<0.05$). However, the administration of the PRO did not influence any of the TCRγδ + subtypes, nor the NK or NKT cell proportion. In addition, when the percentage of cells

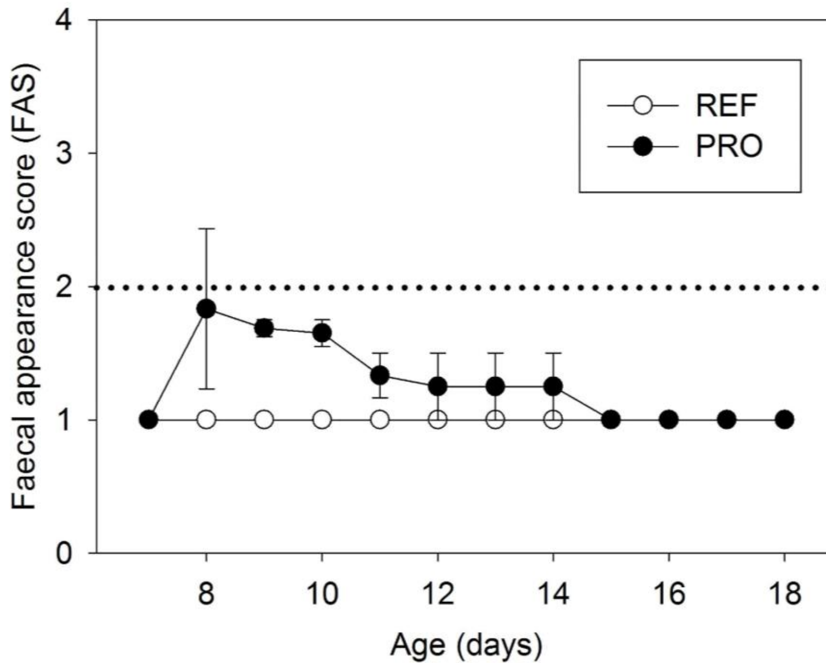

Fig 2. Faecal appearance score (FAS) in the reference (REF) and probiotic (PRO) groups. Faecal samples were scored from 1 to 4 based on colour, texture and amount of stool. Scores of FAS ≥ 2 indicate diarrhoeic faeces. Results are expressed as mean ± SEM (n = 8 animals/group).

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bearing CD8 co-receptor structure was studied, although a decrease of CD8αβ+ cell proportion was observed due to PRO supplementation it did not modify the CD8αα+/CD8αβ+ ratio.

Regarding the MLN, the main subsets proportion (B, Th, Tc, NKT and NK) were not affected by PRO diet, although an increase in the CD8+ NK population was observed ($p<0.05$). Most of the lymphocytes in the MLN with the CD8 co-receptor were CD8αβ+, with no effect caused by the PRO supplementation.

On the other hand, when the IEL composition was studied, it was observed that the Th cell proportion was reduced significantly by the nutritional intervention ($p<0.05$). Although the Tc cell proportion was not modified, the CD8αβ+TCRγδ+ subset percentage was significantly reduced ($p<0.05$). Finally, there was a similar proportion of both CD8αα and CD8αβ IEL in both groups without any effect from the PRO supplementation. This CD8αα/CD8αβ ratio was the highest among the three compartments studied.

Table 1. Main lymphocyte subsets in the spleen, mesenteric lymph nodes (MLN) and intraepithelial lymphocytes (IEL) of the reference (REF) and probiotic (PRO) groups.

				Spleen		MLN		IEL	
				REF	PRO	REF	PRO	REF	PRO
B cells (CD45RA+)				33.00 ± 1.82	33.60 ± 1.41	12.15 ± 1.57	13.99 ± 0.72	-	-
T cells (TCRαβ+NK- and TCRγδ+)				25.85 ± 0.57	22.32 ± 0.62*	74.78 ± 1.56	70.37 ± 1.69	25.85 ± 0.57	25.85 ± 0.57
Th cells (CD4 +CD8-TCRαβ+)				15.95 ± 0.40	13.55 ± 0.60*	55.43 ± 1.32	52.00 ± 1.45	2.55 ± 0.20	1.58 ± 0.11*
	CD4+CD8-			26.11 ± 0.54	23.09 ± 0.49*	59.91 ± 1.58	56.96 ± 1.37	3.35 ± 0.16	2.49 ± 0.14*
	CD4+CD8- TCRαβ-			10.16 ± 0.53	9.54 ± 0.52	4.48 ± 0.40	4.96 ± 0.40	0.80 ± 0.29	0.91 ± 0.17
Tc cells (CD8 +TCRαβ+NK- and TCRγδ+)				9.90 ± 0.20	8.77 ± 0.23*	19.35 ± 0.52	18.37 ± 0.62	20.28 ± 0.21	19.33 ± 0.44
	TCRαβ+ (CD8 + TCRαβ+NK-)			8.17 ± 0.24	7.22 ± 0.16*	17.10 ± 0.55	16.21 ± 0.54	8.17 ± 0.60	7.98 ± 0.24
	TCRγδ+			1.73 ± 0.06	1.55 ± 0.09	2.26 ± 0.03	2.16 ± 0.12	12.11 ± 0.77	11.35 ± 0.34
		CD8-TCRγδ +		0.35 ± 0.03	0.29 ± 0.01	0.38 ± 0.02	0.34 ± 0.02	0.66 ± 0.09	0.57 ± 0.06
		CD8 +TCRγδ+		1.38 ± 0.04	1.26 ± 0.09	1.88 ± 0.03	1.82 ± 0.11	11.45 ± 0.73	10.78 ± 0.35
			CD8αα +TCRγδ+	0.16 ± 0.02	0.17 ± 0.01	0.21 ± 0.02	0.19 ± 0.04	8.04 ± 0.80	7.89 ± 0.27
			CD8αβ +TCRγδ+	1.21 ± 0.04	1.10 ± 0.08	1.67 ± 0.03	1.62 ± 0.09	3.41 ± 0.16	2.89 ± 0.14*
NKT cells (TCRαβ +NK+)				2.91 ± 0.06	2.61 ± 0.23	1.81 ± 0.08	1.87 ± 0.16	21.00 ± 0.57	21.06 ± 0.65
NK cells (TCRαβ-NK +)				7.18 ± 0.19	6.02 ± 0.73	1.18 ± 0.17	1.49 ± 0.15	36.18 ± 0.99	36.89 ± 0.85
	CD8+/TCRαβ- NK+			3.55 ± 0.13	3.23 ± 0.38	0.19 ± 0.02	0.27 ± 0.02*	22.02 ± 0.85	21.29 ± 0.36
CD8αα+ cells				2.49 ± 0.12	2.31 ± 0.24	1.09 ± 0.06	1.27 ± 0.18	21.95 ± 1.49	21.81 ± 0.63
CD8αβ+ cells				10.70 ± 0.11	9.58 ± 0.43*	18.90 ± 0.47	17.49 ± 0.56	21.50 ± 0.61	21.27 ± 0.81
Ratio CD8αα/CD8αβ				0.23 ± 0.01	0.24 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	1.02 ± 0.04	1.03 ± 0.03

Results are expressed as percentage of the total lymphocytes (mean ± SEM, n = 8).

* p<0.05 *vs*. REF.

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TLR4 cell surface expression. The ability of developing lymphocytes to interact with bacteria was investigated by studying the presence of the TLR4 in the main cell subsets in spleen, MLN and IEL (Fig 3). The proportion of spleen B cells expressing TLR4 (~20%) was higher than that in T cells (~10%). No differences in TLR4+ cells were observed between the REF and the PRO groups in the spleen, when explored either in whole lymphocytes or in each cell subset (Tc, Th or B).

In the MLN, the proportion of B cells presenting TLR4 (~15%) was also higher than that in T cells (~5%). In this compartment, the proportion of cells bearing TLR4 was higher in the lymphocytes of the PRO group compared to the REF group. The percentage of TLR4+ cells in the PRO group was higher in Tc, Th and B lymphocytes than that in the REF group, although the difference did only achieve statistical significance for the Th cells ($p<0.05$).

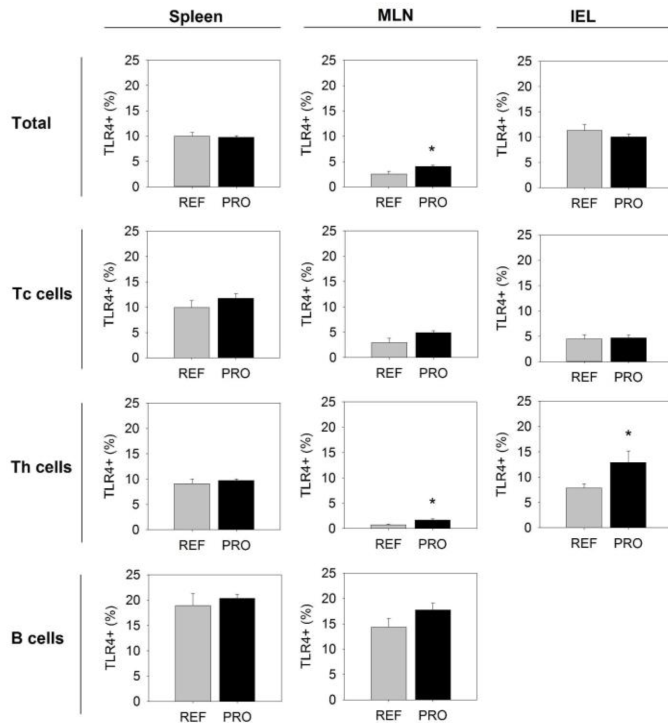

Fig 3. Proportion of TLR4+ cells in the main lymphocyte subsets from the three compartments studied (spleen, mesenteric lymph nodes and intraepithelial lymphocytes) of the reference (REF) and probiotic (PRO) groups. Results are expressed as percentage of positive cells in the indicated subset (mean ± SEM, n = 8 animals/group). Statistical differences: *p<0.05 *vs*. REF.

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Gut IEL had similar proportions of cells expressing TLR4 in both the REF and PRO groups, with the exception of the percentage of TLR4+ Th cells, which was higher in the PRO group (p<0.05).

Cell surface expression of αE integrin and CD62L molecules. The lymphocytes' commitment to the mucosal compartment was studied by means of the proportion of cells expressing two adhesion molecules of importance in the intestinal homing (Fig 4). As expected, the αE integrin/CD62L expression pattern was different among splenocytes, MLN cells and IEL, with CD62L being highly expressed in organized tissues (spleen and MLN) with respect to IEL, and an inverse behaviour found for αE integrin (Fig 4). Focusing on the spleen, no changes were found in the proportion of cells bearing αE integrin and/or CD62L in the REF and PRO groups. The αE

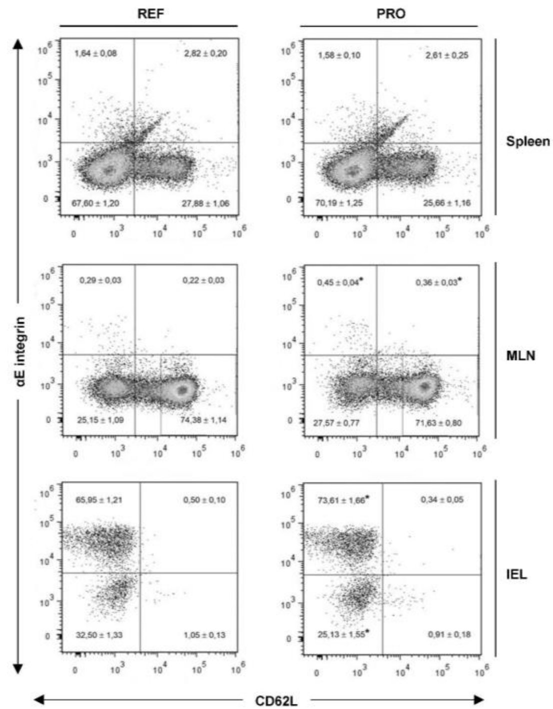

Fig 4. αE integrin/CD62L molecular pattern in the total lymphocytes of the spleen, mesenteric lymph nodes (MLN) and intraepithelial lymphocytes (IEL). Representative histograms for the reference (REF) and probiotic (PRO) groups are shown. In each quadrant the mean ± SEM (n = 8 animals/group) is included. Statistical differences: *p<0.05 *vs*. REF.

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integrin/CD62L pattern was also studied in particular subsets based on the CD4/CD8 cell distribution. In this sense, the proportion of αE+CD62L-, αE+CD62L+, αE-CD62L+ and the αE-CD62L- cells was studied in the CD4+CD8-, CD4+CD8+, CD4-CD8+ and the CD4-CD8- subsets and no statistical differences were found (Table 2).

In the MLN, cells expressing CD62L were ~ 3-fold more abundant than in the spleen. Integrin αE+ cell proportion was very low in comparison with that in the spleen, and the percentage of cells expressing αE integrin, either with or without CD62L co-expression, was higher in the PRO group (0.81 ± 0.06) than in the REF group (0.51 ± 0.06) ($p<0.05$). When the expression of these markers was analysed in particular subsets (CD4+, CD8+ and CD4- CD8-), it was observed that the increase in such a pattern due to the PRO intervention was found in all of them (Table 2).

Table 2. Expression of the integrin αE and the selectin CD62L in CD4+, CD8+ and CD4- CD8- patterns in spleen, mesenteric lymph node (MLN) cells and intraepithelial lymphocytes (IEL) of the reference (REF) and probiotic (PRO) groups.

		αE+CD62L-		αE+CD62L+		αE-CD62L+		αE-CD62L-	
		REF	PRO	REF	PRO	REF	PRO	REF	PRO
Spleen	**CD4+CD8-**	1.83 ± 0.09	2.01 ± 0.16	0.90 ± 0.12	0.71 ± 0.08	42.23 ± 0.91	41.54 ± 2.34	55.08 ± 0.73	55.73 ± 2.27
	CD4+CD8+	11.56 ± 1.08	12.01 ± 0.52	44.63 ± 1.47	45.67 ± 1.65	16.40 ± 1.38	14.06 ± 1.15	27.40 ± 1.81	28.26 ± 1.27
	CD4-CD8+	1.23 ± 0.12	1.41 ± 0.19	2.42 ± 0.48	2.28 ± 0.21	57.58 ± 2.17	52.66 ± 1.62	38.78 ± 1.89	43.66 ± 1.58
	CD4-CD8-	0.87 ± 0.07	0.78 ± 0.03	0.38 ± 0.06	0.27 ± 0.02	17.83 ± 1.89	17.30 ± 0.97	80.95 ± 2.01	81.61 ± 1.00
MLN	**CD4+CD8-**	0.18 ± 0.05	0.24 ± 0.03	0.20 ± 0.05	0.27 ± 0.03	78.73 ± 1.34	77.85 ± 0.60	20.88 ± 1.28	21.65 ± 0.56
	CD4+CD8+	0.24 ± 0.09	1.35 ± 0.35*	1.07 ± 0.23	1.90 ± 0.41	84.63 ± 2.06	81.42 ± 1.18	14.08 ± 2.05	15.35 ± 1.04
	CD4-CD8+	0.47 ± 0.06	0.74 ± 0.11	0.40 ± 0.09	0.51 ± 0.04	77.85 ± 1.37	78.52 ± 0.75	21.30 ± 1.33	20.23 ± 0.63
	CD4-CD8-	0.60 ± 0.04	0.86 ± 0.05*	0.29 ± 0.04	0.47 ± 0.06*	59.65 ± 1.79	52.58 ± 2.16*	39.43 ± 1.71	46.08 ± 2.19*
IEL	**CD4+CD8-**	2.21 ± 1.06	6.31 ± 0.73*	0.46 ± 0.30	0.30 ± 0.20	9.00 ± 2.86	8.09 ± 1.91	88.35 ± 2.14	85.30 ± 2.02
	CD4+CD8+	29.09 ± 10.05	33.21 ± 9.21	9.46 ± 5.90	13.81 ± 4.62	11.90 ± 4.31	8.96 ± 2.53	49.55 ± 8.49	44.01 ± 5.03
	CD4-CD8+	93.58 ± 0.57	95.24 ± 0.54	0.52 ± 0.09	0.37 ± 0.04	1.36 ± 0.25	1.00 ± 0.21	4.54 ± 0.53	3.39 ± 0.51
	CD4-CD8-	50.20 ± 2.67	60.17 ± 1.80*	0.37 ± 0.07	0.27 ± 0.06	0.38 ± 0.05	0.36 ± 0.04	49.03 ± 2.73	39.19 ± 1.76*

* p<0.05 *vs*. REF.

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CD62L was almost completely absent on the surface of the IEL but more than 65% expressed αE integrin. In the PRO group, the integrin αE+ cell proportion (73.95 ± 1.66) was 8% higher than that in the REF group (66.45 ± 1.24) ($p<0.05$). Again, the increase of integrin αE+ IEL in the PRO group was found in all CD4+, CD8+ and CD4- CD8- subsets ($p<0.05$) and therefore it could not be ascribed to one of these cell types (Table 2).

Intestinal IgA

Intestinal IgA was quantified in gut washes from 19-day-old rats. The administration of the PRO only during 2 weeks of the suckling period (starting after the first week of life) was enough to show an enhancing effect on IgA production (Fig 5). In fact, the PRO dietary intervention increased the intestinal IgA concentration 2-fold ($p<0.05$).

Discussion

The current study shows that supplementation with the probiotic strain *B. breve* M-16V during the rat suckling period influences the lymphocyte composition of both intestinal and systemic immune compartments, modifies the proportion of cells expressing molecules involved in the interaction with intestinal bacteria, and also enhances the intestinal antibody synthesis.

In our study, the PRO administration induced no harmful effects because it did not affect the growth curve. Interestingly, a change in the faecal consistency caused by the PRO was found. This effect has already been described for some molecules used as probiotic substrate [40–42], and it is actually beneficial to make faeces more similar to those of breast-fed infants and to reduce constipation.

During suckling, rats undergo phenotypical changes in lymphoid tissues that are a reflex of the immune system's maturation in this period. Thus, immature rat spleen mainly contains B cells, T, NK and NKT cell proportions that are lower than those in adult rats [32]. As the PRO supplementation did not produce an increase but rather a decrease in CD4+ and CD8+ cells, it seems that, apparently, this nutritional intervention does not enhance the systemic immune maturation. However, when considering the MLN, a secondary lymphoid organ located at intestinal level and inducer of immune response in this compartment, the PRO

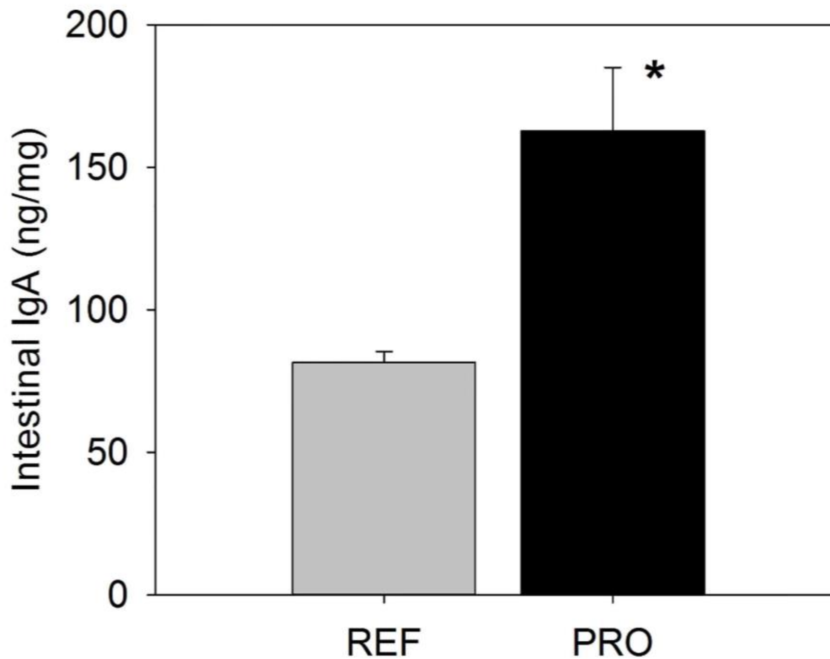

Fig 5. IgA concentration in intestinal washes of 19-day-old rats from the reference (REF) and probiotic (PRO) groups. Results are expressed as ng of IgA/mg of tissue (mean ± SEM, n = 8 animals/group). Statistical differences: *$p<0.05$ *vs.* REF.

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supplementation was able to enhance the proportion of CD8+ NK cells. These cells, although were found in a low proportion in MLN, are key in the innate immunity [35] and therefore the current results show that the PRO administration increased this defensive barrier.

With regard to the IEL, these cells are those most in contact with the intestinal lumen and therefore interventional diets can have major impact on them. This compartment does not contain B cells, and during suckling there is a progressive increase in the proportion of CD8 + IEL co-expressing TCRαβ, whereas the percentage of CD8+ cells expressing the NK receptor decreases [32]. In addition, there is an increase in the proportion of CD4+ cells and TCRγδ + cells during the second half of the suckling period [32]. The PRO administration during suckling did not produce any significant change in the proportion of CD8+ TCRαβ+ IEL or CD8+ NK cells, but lowered the percentage of CD4+ and the proportion of the particular CD8αβ+ TCRγδ+ IEL, which are the least abundant in TCRγδ+ cells in the intraepithelial compartment. These results agree with Gebert et al. [43] who found a decrease in the CD4+ jejunum population of neonatal pigs after *Lactobacillus brevis* supplementation. Some authors also

describe a reduction of CD4+ T cells in the mucosa obtained from patients with Crohn's disease when co-cultured with *Lactobacillus casei* and *Lactobacillus bulgaricus*, but no changes were observed in non-inflamed mucosa [44,45].

In all the three compartments studied, the presence of TLR4 in the main cell subsets was assessed. The TLR4 receptor recognizes the bacterial lipopolysaccharide. It participates in the innate immune response against pathogens, but also in the proliferation, differentiation and development of lymphocytes [46]. TLR4 is essential for the maintenance of the intestinal homeostasis and barrier function [5,8]. The proportion of splenocytes bearing TLR4 was not changed by the nutritional intervention but it was increased due to the PRO supplementation in the CD4+ subset in the MLN and IEL. Therefore, it can be suggested that the PRO can prepare the intestinal immune system for a better response against infections increasing bacteria–host interaction These results agree with those reported after the administration of *Lactobacillus reuteri*, *L. casei* and *L. rhamnosus* in healthy mice [14,47] and by LGG in healthy rats [48]. However, our findings did not match when TLR4 expression was studied in some pathologies. In this context, TLR4 expression was decreased by *L. reuteri* in the intestine of rats with NEC [49], by *L. rhamnosus* and *Lactobacillus acidophilus* in mice with alcoholic liver disease [50], and also by LGG and other *Lactobacillus* and *Bifidobacterium* in the colon of rats with colitis [48,51].

The circulation is important in leading naïve lymphocytes to find their specific antigen, as well as for permitting effector or memory lymphocytes to reach infected or inflamed tissue. Intestinal homing depends on the expression of specific surface molecules [52]. The homing of naïve T lymphocytes in the PP or MLN through the high endothelial venules (HEV) requires the interaction between the CD62L selectin on the lymphocyte surface and the peripheral node addressin on the HEV [53]. Moreover, α4β7 integrin is also important to bind to epithelial cells from the small intestine, and to allow the retention of the IEL [54,55]. Interestingly, the CD62L and αEβ7 integrin pattern in the MLN cells and the IEL of suckling rats changed after the administration of *B. breve* M-16V, which induced a higher percentage of integrin αEβ7+ cells both in the MLN and the intraepithelial compartment. These results seem to indicate an enhancement of the homing process of naïve T lymphocytes to the MLN, and the retention of activated lymphocytes in the IEL compartment [56]. Other studies have evidenced the capacity of probiotics to modulate homing markers. *Lactobacillus plantarum* and its excreted peptide STp have shown stimulation of skin-homing markers *in vitro*, such as cutaneous lymphocyte-associated antigen (CLA), and a decrease in MadCAM-1 and α4β7 integrin expression in colonic mucosa in a mouse model of colitis [57–59]. These effects can be useful to attenuate the intestinal inflammation in IBD. On the other hand, *L. casei* Shirota failed in modulating the homing markers on the stimulated T cells by the dendritic cells of patients with ulcerative colitis, but it did increase the CLA skin-homing marker and β7 gut-homing marker on stimulated T cells by human dendritic cells from healthy controls [60].

On the other hand, and importantly, the PRO administration for 13 days during the suckling period was able to enhance the intestinal IgA synthesis. As expected, the levels of IgA in the intestinal wash samples of the REF animals were very low [20,33], but *B. breve* M-16V doubled the intestinal IgA concentration. Since intestinal IgA is a very reliable marker of the mucosal defence against pathogens and has been clearly described as a useful tool in immunonutrition studies [5], this result indicates the immunomodulatory potential of this probiotic strain. Other studies have reported stimulation of IgA production due to probiotics, such as LGG [61], *Lactobacillus gasseri* [9], *L. casei*, *L. reuteri*, *Bacillus cereus* var. Toyoi, *Bifidobacterium bifidum* and *Lactobacillus kefiri* in mice [23,25,62] or a formula containing *Bifidobacterium lactis* Bb-12 in healthy infants [63].

Finally, it must be considered that in the design of the current study, pups were supplemented from day 6 until the end of the study, and were weaned at day 17, a few days after rats begin to chew solid food. The effects found here demonstrate that supplementation with the specific strain *B. breve* M-16V in this short period is enough to show effect, which is in agreement with a study with mice in which just 2 days of supplementation with *Lactobacillus delbrueckii* subsp. *bulgaricus* or *L. acidophilus*, and 7 days with *L.casei* were necessary to find an increase of IgA in the LP of the small intestine [5]. However, we cannot discount the possibility that a new study design with an earlier and longer administration period would show more significant results.

In summary, the results shown here allow the conclusion to be drawn that the probiotic intervention during suckling seems to have a low, if any, immune-enhancing effect on the systemic compartment (spleen), but *B. breve* M16-V administration enhances the ability to respond against pathogens in both the intestinal inductor (MLN) and effector (IEL) sites and it promotes the intestinal recruitment and retention of the cells in the epithelial compartment. In addition, this probiotic strengthens the humoral intestinal immune response. Thus, supplementation with the probiotic strain *B. breve* M-16V during suckling may be helpful in the development of mucosal immunity in early life.

Supporting Information

S1 Fig. Example of the gating strategy using a mesenteric lymph node lymphocytes' sample. **A)** In Panel 1, B cells (CD45RA+) were identified. The frequency of TLR4+ cells in the B cell subset was investigated, as well as in CD8+CD4- and CD8-CD4+ subsets. TLR4+ cells were also quantified in the total gated lymphocytes (not shown). **B)** In Panel 2, the αE integrin/CD62L pattern was studied in each of the subsets defined by the combination of the markers CD4 and CD8. **C)** In Panel 3, it could be differentiated NK (NK+TCRαβ-) cells from NKT cells (NK+TCRαβ+), and also NK-TCRαβ+ cells, which in combination with TCRγδ+ cells (obtained from the Panel 4) constituted the total of T cells. Moreover, the CD8-CD4+ cells in the NK-TCRαβ+ subset could be considered as Th cells, and the CD8+CD4- cells in this subset plus the TCRγδ+ cells (Panel 4) could be considered as Tc cells. The proportion of CD8+ cells in the NK+TCRαβ- subset (NK cells) was also studied. **D)** In Panel 4, the proportion of CD8αα and CD8αβ cells were studied in the CD8+ cells subset and also in the TCRγδ+ cells subset.
(DOCX)

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Visualization: MRA AF MC FPC.

Writing – original draft: MRA FPC.

Writing – review & editing: AF MC FPC.

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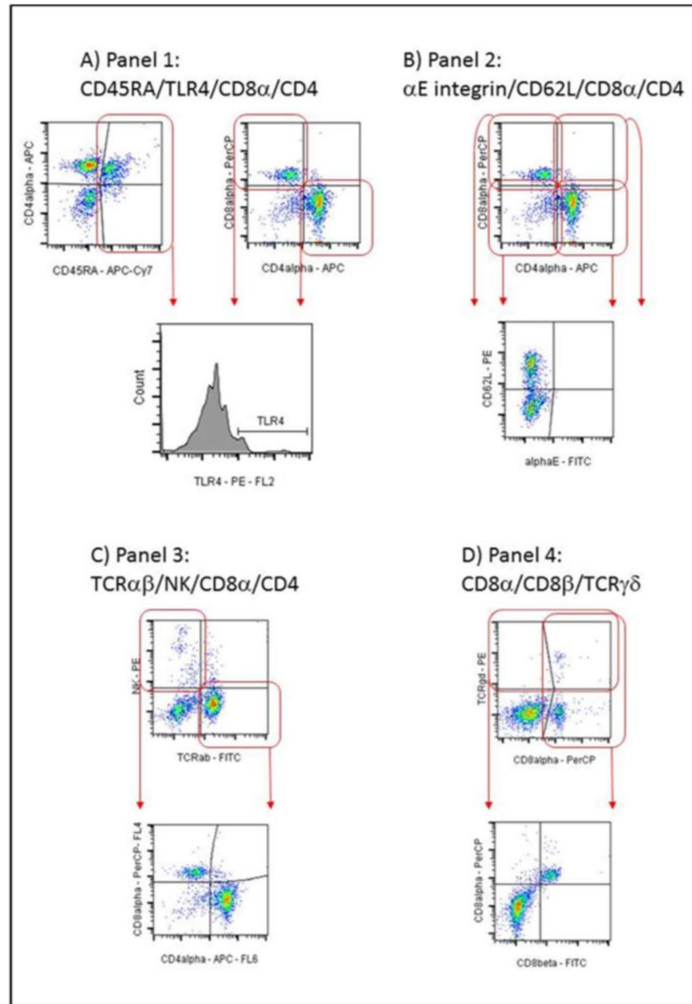

S1 Fig. Example of the gating strategy using a mesenteric lymph node lymphocytes' sample. A) In Panel 1, B cells (CD45RA+) were identified. The frequency of TLR4+ cells in the B cell subset was investigated, as well as in CD8+CD4- and CD8-CD4+ subsets. TLR4+ cells were also quantified in the total gated lymphocytes (not shown). **B)** In Panel 2, the αE integrin/CD62L pattern was studied in each of the subsets defined by the combination of the markers CD4 and CD8. **C)** In Panel 3, it could be differentiated NK (NK+TCRαβ-) cells from NKT cells (NK+TCRαβ+), and also NK-TCRαβ+ cells, which in combination with TCRγδ+ cells (obtained from the Panel 4) constituted the total of T cells. Moreover, the CD8-CD4+ cells in the NK-TCRαβ+ subset could be considered as Th cells, and the CD8+CD4- cells in this subset plus the TCRγδ+ cells (Panel 4) could be considered as Tc cells. The proportion of CD8+ cells in the NK+TCRαβ- subset (NK cells) was also studied. **D)** In Panel 4, the proportion of CD8αα and CD8αβ cells were studied in the CD8+ cells subset and also in the TCRγδ+ cells subset.

Capítol 2

"*A combination of scGOS/lcFOS with* Bifidobacterium breve *M-16V protects suckling rats from rotavirus gastroenteritis*"

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Els resultats del present article han estat presentats a les següents comunicacions:

- *Experimental rotavirus diarrhea is reduced by administration of* Bifidobacterium breve *M-16V during suckling*. 7th International Immunonutrition Workshop: Eating for preventing (Carovigno, Itàlia), maig 2014.
- *Prebiotic supplementation during suckling modulates the course of rotavirus gastroenteritis in rats*. VI Workshop Probióticos, Prebióticos y Salud. Evidencia Científica (Oviedo, Espanya), febrer 2015. Comunicació oral. Publicat a Nutrición Hospitalaria (2015) 31 (1):54.
- *A synbiotic (*B. breve *M-16V and scGOS/lcFOS) supplementation in early life protects from a rotavirus infection*. VII Workshop Probióticos, Prebióticos y Salud. Evidencia Científica (Sevilla, Espanya), gener 2016. Comunicació oral.
- *In vitro anti-rotavirus blocking activity of scGOS/lcFOS*. VII Workshop Probióticos, Prebióticos y Salud. Evidencia Científica (Sevilla, Espanya), gener del 2016. Comunicació oral.
- *Evaluation of "false epithelial pathogen receptor" activity of prebiotics by an* in vitro *assay.* 10th European Mucosal

Immunology Group Meeting (Copenhaguen, Dinamarca), octubre 2016.

RESUM

Objectiu: Avaluar l'efecte protector d'una barreja prebiòtica de galactooligosacàrids de cadena curta i fructooligosacàrids de cadena llarga (9:1) i del probiòtic *Bifidobacterium breve* M-16V, per separat o en combinació (simbiòtic) en un model d'infecció per rotavirus en rata lactant.

Material i mètodes: Els animals van rebre oralment els suplements des del dia 3 fins el dia 21 de vida. El dia 7 van ser infectats amb el rotavirus SA11. Al llarg de l'estudi es van avaluar tant variables clíniques com la resposta immunitària.

Resultats: La intervenció amb el probiòtic va reduir la incidència, la gravetat i la durada del procés diarreic. Les intervencions amb el prebiòtic i el simbiòtic també van millorar les manifestacions clíniques, però una alteració en la consistència de les femtes induïda pel prebiòtic va dificultar l'observació d'aquest efecte. En els grups suplementats amb el prebiòtic i el simbiòtic es va observar una disminució de l'eliminació viral, fet que no va succeir en el grup suplementat amb el probiòtic. Totes tres intervencions van modular la resposta d'anticossos específics en sèrum i rentat intestinal a dies 14 i 21 de vida.

Conclusió: La suplementació diària amb la barreja prebiòtica de galactooligosacàrids de cadena curta i fructooligosacàrids de cadena llarga (9:1), el probiòtic *Bifidobacterium breve* M-16V o la combinació d'aquests, és efectiva en la modulació de la diarrea per rotavirus en aquest model preclínic.

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ORIGINAL CONTRIBUTION

A combination of scGOS/lcFOS with *Bifidobacterium breve* M-16V protects suckling rats from rotavirus gastroenteritis

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Abstract
Purpose Rotavirus (RV) is the leading cause of severe diarrhoea among infants and young children, and although more standardized studies are needed, there is evidence that probiotics can help to fight against RV and other infectious and intestinal pathologies. On the other hand, the effects of prebiotics have not been properly addressed in the context of an RV infection. The aim of this study was to demonstrate a protective role for a specific scGOS/lcFOS 9:1 prebiotic mixture (PRE) separately, the probiotic *Bifidobacterium breve* M-16V (PRO) separately and the combination of the prebiotic mixture and the probiotic (synbiotic, SYN) in a suckling rat RV infection model.
Methods The animals received the intervention from the 3rd to the 21st day of life by oral gavage. On day 7, RV was orally administered. Clinical parameters and immune response were evaluated.
Results The intervention with the PRO reduced the incidence, severity and duration of the diarrhoea ($p < 0.05$). The PRE and SYN products improved clinical parameters as well, but a change in stool consistency induced by the PRE intervention hindered the observation of this effect. Both the PRE and the SYN, but not the PRO, significantly reduced viral shedding. All interventions modulated the specific antibody response in serum and intestinal washes at day 14 and 21 of life.
Conclusions A daily supplement of a scGOS/lcFOS 9:1 prebiotic mixture, *Bifidobacterium breve* M-16V or a combination of both is highly effective in modulating RV-induced diarrhoea in this preclinical model.

Keywords Prebiotic · Probiotic · Synbiotic · Rotavirus · FOS · GOS · *Bifidobacterium breve*

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Introduction

Rotavirus (RV) is the most common pathogen causing severe dehydrating diarrhoeal disease in children younger than 5 years worldwide [1]. RV is a non-enveloped, icosahedral and double-stranded RNA member of the Reoviridae family, which infects mature enterocytes of the small intestine, and has a higher prevalence in the winter season [2]. Virtually every child, in both developed and developing countries, will be infected with RV in the first 3 years of life [3]. RV is estimated to be responsible for millions of hospitalizations and over 450,000 deaths annually (most of them in low-income countries in Africa and Asia) [4, 5]. Current treatment consists basically of oral rehydration [6].

In order to prevent this infection, two live attenuated oral vaccines, RotaTeq (Merck and Co, PA, USA) and Rotarix (GSK Biologicals, Rixensart, Belgium), have been licensed since 2006. RV vaccines have shown safety and efficacy in developed countries, but they are not globally implemented due to cost, refrigerated storage requirements and

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the lower protection offered in developing countries [3, 6]. RV disease also seems to be modulated by nutritional interventions, such as bioactive components of breast milk (i.e. nucleotides or whey proteins), probiotics or prebiotics [7]. Probiotics, live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host, have been widely studied in this regard. *Lactobacillus* and *Bifidobacterium* genera are often present in infant formulas, and they may prevent and modulate RV gastroenteritis. Some in vitro studies have evaluated different probiotic strains and their effects against RV infection in epithelial cell lines, such as some species of *Lactobacillus* [8–14], *Bifidobacterium* [11, 13, 15] and others [9–11, 16]. The mechanism of action described in these in vitro studies is different for each strain: whereas some decrease the secretion of mucin and IL-6 levels induced by RV and increase the TLR2 expression [12], others modify the virus adhesion capacity [9, 10], decrease the virus replication [13], inhibit RV-induced chloride secretion and oxidative stress [16] or modulate the host immune cell response [15]. Overall, probiotics have demonstrated their preventive action [9, 10, 12] and also their effectiveness as an adjuvant treatment [12].

In vivo studies are mostly performed using neonatal gnotobiotic pigs [17–27] or other species such as mice [28–31] or rats [32, 33]. Different types of probiotics have shown modulatory action on clinical symptoms [28, 32] but also immunomodulatory activity [17–20, 22, 25, 27], with *Lactobacillus rhamnosus GG* (LGG) being the most widely studied and effective probiotic in RV infection studies [21, 23, 24, 26, 29, 31, 33].

Some clinical trials with probiotics (mainly randomized, double blind and controlled) have been conducted in children diagnosed with RV infection between 1 month and 5–6 years of age. LGG [34–38], *Lactobacillus acidophilus* [34, 35], *Lactobacillus paracasei* [39], *Lactobacillus sporogenes* [40], *Bifidobacterium lactis* [38, 41], *Bifidobacterium longum* [34, 35], *Streptococcus thermophilus* [38] and *Saccharomyces boulardii* [34, 35, 41–43] are the main probiotics that have been studied. Some of these interventions in RV infection have demonstrated their ability to shorten the duration of diarrhoea [35, 36, 41, 44] and to reduce the incidence of repeated episodes, and also their role in modulating the immune response and viral shedding [37, 38]. Finally, probiotics have demonstrated similar activity to other therapeutic alternatives such as nitazoxanide [34] or zinc [42], suggesting them as interesting adjuvants for treatment.

With regard to prebiotics, fewer studies of their protective role against RV have been carried out. Prebiotics are indigestible food ingredients that reach the colon and promote the growth or activity of certain beneficial species in the intestinal microbiota, thereby generating a health benefit [45, 46]. One study indicates that sialic acid-containing human milk oligosaccharides (HMO) inhibited RV infectivity in vitro (MA-104 cells) [47]. Both neutral and sialic acid with acidic HMO reduced RV replication as measured by the detection of RV non-structural protein 4 in acutely infected piglets [47]. HMO and also a mixture of short-chain galactooligosaccharides (scGOS) and long-chain fructooligosaccharides (lcFOS) are able to decrease the duration of RV-induced diarrhoea in piglets and also to modulate the immune response [48]. Only two clinical studies have tested the efficacy of prebiotics against acute gastroenteritis, including one induced by RV. Aliva (a polyphenol-based prebiotic) [49] and CUPDAY milk (*B. lactis* + Raftilose P95/Acacia gum) [50] offer some benefits to children (and adults as well, in the first study) with diarrhoea, consisting of a decrease in stomach pain and discomfort, gas and bloating or a reduction of the days with four or more stools, respectively. The prebiotics most commonly found in infant formulas are FOS and GOS, which mimic the size, linkage and prebiotic function of HMO present in human milk. FOS and GOS promote beneficial changes in stool consistency and bacterial composition in infants [51]. They increase short-chain fatty acids (SCFA) and lactate in caecum samples [52, 53]. Specific mixtures of scGOS/lcFOS 9:1 reduce the incidence of infections [54, 55], asthma and eczema [56] in infants, induce a beneficial Ig profile in infants at high risk of allergy [57], increase faecal sIgA secretion in infants [58, 59]. Calcium absorption is also improved with FOS and GOS supplementation in rats and infants [60–62], and several effects at the central nervous system level have been observed in rats and humans as well [63, 64].

Moreover, scGOS/lcFOS 9:1 mixture stimulates delayed-type hypersensitivity (DTH) and improves T helper (Th)1-dependent vaccination in mice [65, 66].

In this approach, prebiotics were selected since the effects of prebiotics in general are largely unknown in RV infection. And *Bifidobacterium breve* M-16V was selected due to its natural presence in infants microbiota and its immunomodulatory action (often in combination with prebiotics) observed mainly in allergy studies [67–71]. Moreover, the combination with the scGOS/lcFOS 9:1 prebiotic mixture seems rationale because it mimics the HMO composition of breast milk, and it has shown a synergistic effect, for example, in the allergy model [69, 72].

Taking into account the above comments, the present study aimed to test the effectiveness of a prebiotic mixture, scGOS/lcFOS (9:1), with or without the probiotic *B. breve* M-16V, and the probiotic *B. breve* M-16V alone, in an RV-infected neonatal rat model.

Materials and methods

Animals

G14 pregnant Lewis rats from Harlan (Barcelona, Spain) were housed in individual cages, monitored daily and allowed to deliver at term. The day of birth was registered as day 1 of life. Litters were unified to 7 pups per lactating dam. Pups had free access to the nipples and rat diet. The animals were housed under controlled temperature and humidity conditions, in a 12:12-h light/dark cycle. They were located in a special safe isolated room at the Animal Service of the Faculty of Pharmacy, University of Barcelona, designed and authorized for working under biosecurity level 2 conditions. Dams were fed a commercial diet corresponding to the American Institute of Nutrition (AIN) 93G formulation and given water ad libitum. Pups were individually identified by labelling with a permanent marker after 2 days of environmental adaptation. The animals were weighed and monitored daily in order to obtain data regarding the influence of the virus inoculation, clinical development and nutritional intervention on body weight and growth. This was done after the separation of the pups from their mother, during the handling and before oral administration.

Experimental design and dietary supplementation

Suckling rats were distributed in five different experimental groups: rotavirus (RV), reference (REF), probiotic (PRO), prebiotic (PRE) and synbiotic (SYN). Each group was composed of 3 l with 7 pups each ($n = 21$/group). Animals were orally administered, as previously described [73], with the different products (3 groups: PRO, PRE and SYN) or vehicle (2 groups: RV and REF) beginning on day 3 of life until the end of suckling (day 21), using low-capacity syringes (Hamilton Bonaduz, Bonaduz, Switzerland) adapted to forced alimentation tubes of 25 or 23 calibre and 27 mm of length (ASICO, Westmont, IL, USA). The PRO group received *Bifidobacterium breve* M-16V suspension at a dose of 4.5×10^8 UFC/100 g of body weight/day. The PRE treatment consisted of a combination of scGOS and lcFOS in a 9:1 ratio and was administered in a dose of 0.8 g of prebiotic/100 g of body weight/day in basis of the usual proportion added to an infant formula and taking into account the equivalent amount of food ingested, as in previous studies [73]. The SYN group received both PRO and PRE products at the same concentrations as when administered alone. A group of rats receiving bottled mineral water as vehicle was the inoculated control group (RV group), whilst another group receiving water acted as the non-inoculated control group (REF group).

Animals were inoculated at day 7 of life with an RV strain, with the exception of those from the REF group. Clinical evaluation was performed daily from the day before inoculation until the end of the study. A subgroup of 9 animals in each group was euthanized on day 14 of life, and the rest ($n = 12$), on day 21. Faecal samples were collected daily during the study, and blood and intestinal wash samples on the day of killing. A parallel cohort with non-infected animals receiving the products ($n = 5$/each) was also included (non-infection study, NIS).

Virus inoculation

The RV strain used (simian SA-11) was purchased from the "Enteric Virus Group" of the University of Barcelona (Dr. A. Bosch). Viruses were propagated in foetal African green monkey kidney cells (MA-104) and tittered as $TCID_{50}$/mL (TCID, tissue culture infectious dose) [74, 75]. The production was carried out in compliance with the current principles of GLP (Royal Decree 1369/2000 of July 19th). SA-11 was intragastrically inoculated (2×10^8 $TCID_{50}$ RV/rat in 100 μL of PBS) at day 7 of life, as previously described [75], to suckling rats from the RV, PRO, PRE and SYN groups. The RV was inoculated 1 h after separation from their dams to avoid interferences between RV and milk components. The REF group, which is untreated and uninfected, and therefore constituted the negative control, received the same volume of PBS (100 μL) in the same conditions.

Clinical indexes and faecal specimen collection

SA-11 infection was evaluated on days 1–14 post-inoculation (DPI) by the growth rate and clinical indexes derived from faecal samples. In all groups, faecal sampling was performed once a day by gently pressing and massaging the abdomen. Specimens were immediately scored, weighed and frozen at −20 °C for further analysis. The severity of diarrhoea was expressed by the faecal weight and by scoring stools from 1 to 4 (diarrhoea index [DI]) based on colour, texture and amount as described: normal (1), loose yellow-green (2), totally loose yellow-green (3), high amount of watery (4) faeces. Diarrhoea scores ≥ 2 indicate diarrhoeic faeces, whereas scores of DI <2 indicate absence of diarrhoea [75].

The area under the curve of severity (sAUC) along 0–6 DPI was calculated as a global value of severity. The maximum diarrhoea index (MDI) was defined as the highest score during the diarrhoea period. Incidence of diarrhoea was expressed by the percentage of diarrhoeic animals (%DA, consisting of the percentage of diarrhoeic samples taking into consideration the number of animals

in each group) and by the percentage of diarrhoeic faeces (%DF, consisting of the percentage of diarrhoeic samples taking into consideration the number of total samples collected every day in each group). The AUCs of %DA and %DF (daAUC and dfAUC) along 0-6 DPI were calculated as global values of incidence. AUCs for severity, %DA and %DF were also calculated taking into account the basal values due to intrinsic aspects of each treatment (normalized AUC, AUCn). The maximum percentage of diarrhoeic animals (MDA) and diarrhoeic faeces (MDF) were defined as the highest values during the diarrhoea period. The days when MDI, MDA and MDF were achieved were also used as indicators, called MDId, MDAd and MDFd, respectively. The diarrhoea period (DP) was calculated for each animal as the interval between the first (day of diarrhoea beginning, DDB) and last day (day of diarrhoea ending, DDE) of diarrhoea. The actual days with diarrhoea within the diarrhoea period were also counted (days with diarrhoea, DwD).

Blood and intestinal sample collection

The rats from each group were euthanized, having previously been anaesthetized intramuscular with ketamine/xylazine, at days 14 and 21. Blood was collected by cardiac puncture, and sera stored at −20 °C until analysis. Intestinal tissue was cut into 5-mm pieces and incubated with a phosphate-buffered solution (PBS) for 10 min at 37 °C in a shaker to obtain the gut wash (GW). After centrifugation, supernatants were stored at −80 °C until analysis.

ELISA for specific anti-RV IgA, IgG and IgM antibody quantification in serum and intestinal wash

Ninety-six-well plates (Nunc Maxisorp, Wiesbaden, Germany) were coated with UV-inactivated SA-11 at 10^5 particles/mL. After blocking with PBS-1 % bovine serum albumin (BSA, 1 h, room temperature [RT]), appropriate diluted sera (1/5 for IgA quantification, 1/40 for IgG and 1/20 for IgM) and intestinal wash samples (1/5) were added (3 h, RT). After washing, mouse biotinylated anti-rat IgA (A93-2), IgG1/2a (R19-15) or IgM (G53-238) monoclonal antibodies (Mab) from BD Biosciences (Heidelberg, Germany) were added in 1/300, 1/300 and 1/500 dilutions, respectively. Subsequently, peroxidase-conjugated extravidin (Sigma-Aldrich, Madrid, Spain) was added, followed by substrate solution (*o*-phenylenediamine plus hydrogen peroxide in 0.2 M phosphate, 0.1 M citrate buffer, pH 5; Sigma-Aldrich). Absorbance was measured at 492 nm after stopping the enzymatic reaction with 3 M H_2SO_4 on a microtitre plate photometer (Labsystems, Helsinki, Finland). Data were interpolated by means of Multiskan Ascent v.2.6 software (Thermo Fisher Scientific SLU, Barcelona, Spain). Pooled sera from dams of inoculated litters were used as a standard in each plate. Dilutions of dam sera ranged from 1/2.5 to 1/320. Quadratic polynomial adjustment was used, and dam sera received a value of 1000 arbitrary units (AU)/mL.

Viral shedding

Faecal samples from selected days of interest were diluted in PBS (up to 20 mg/mL) and homogenized using a Polytron (Kinematica, Luzern, Switzerland). Homogenates were centrifuged (200×*g*, 5 min, 4 °C), and supernatants were frozen at −20 °C until use. SA-11 particles in faecal samples were quantified by ELISA using 96-well plates (Nunc Maxisorp), coated with anti-p42 MAb (Meridian Life Science, Memphis, USA) at 5 µg/mL. After blocking the remaining binding, 100 µL of appropriate diluted samples (1/120 dilution in 1 DPI and 1/8 dilution for the rest of the days) in PBS-Tween-1 % BSA was added (3 h, RT). Polyclonal sheep anti-RV peroxidase-conjugated antibody (MyBioSource, San Diego, USA) was added (2 h, RT). Captured SA-11 particles were quantified by adding substrate solution and absorbance measuring as before. Titrated dilutions of SA-11 virus particles, ranging from 10^5 to 10^3 /mL, were used as standard in each plate.

Short-chain fatty acids

SCFAs quantification in faecal samples from 17-day-old rats was performed by HPLC [76, 77]. Faecal samples were diluted to 1:10 (w/v), centrifuged and filtered with Millex® 0.22-µm and 13-mm-diameter sterile filters (Merck Millipore, Darmstadt, Germany). A volume of 200 µL of supernatant was added to 50 µL of 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). Twenty µL of each sample was injected into a 1050 series HPLC System (HP, Crawley, West Sussex, UK), equipped with a Rezex ROA—Organic Acid H+ 8 % column (Phenomenex, Macclesfield, UK) and a SecurityGuard precartridge (Phenomenex, Macclesfield, UK), kept at 85 °C in a 7981 model oven (Jones Chromatography, Lakewood, USA), and a UV detector. The eluent, 2.6 mM sulphuric acid, was supplied at a flow rate of 0.5 mL/min. Peaks were integrated using Agilent ChemStation software (Agilent Technologies, Oxford, UK). SCFAs were identified and quantified using a calibration cocktail which includes acetic, propionic, butyric, lactic and formic acids, in concentrations ranging from 100 to 12.5 mM.

In vitro blocking assay

Dilutions of SA-11 in PBS-Tween 1 % at a concentration of 5×10^4 particles/mL were prepared. Different dilutions of PRO or PRE products of the in vivo-administered concentration were added to the virus (1/2, 1/3, 1/6 or 1/60). The combinations were incubated for 30 min. Free non-coated viral particles were quantified by ELISA, as described above. The standard was an SA-11 dilution at a concentration of 5×10^4 particles/mL.

Statistical analysis

The PASW Statistics 18 software package (SPSS Inc, Chicago, IL, USA) was used for the statistical analysis. Conventional one-way ANOVA was performed considering the experimental group as the independent variable. When virus inoculation/treatment had a significant effect on the dependent variable (body weight or body weight increase), Scheff 's test was applied. The Mann–Whitney *U* test was used for nonparametric analyses (severity, MDI, etc.). Finally, the *Chi*-square test was used to compare frequencies (diarrhoea incidence). Differences were considered significant at *P* values of <0.05. All the results are expressed as mean ± SEM of *n* animals.

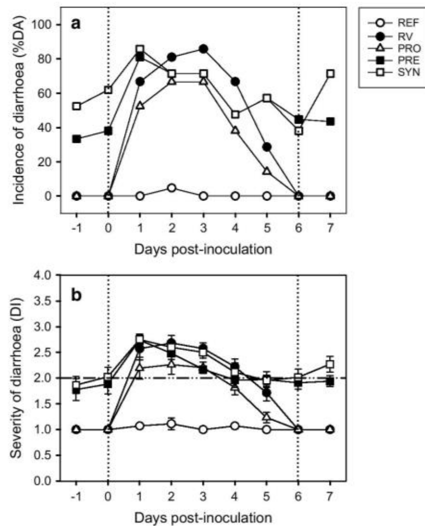

Fig. 1 Clinical indexes. **a** Incidence of diarrhoea: results are expressed as % of diarrhoeic animals. **b** Severity of diarrhoea: faecal samples are scored from 1 to 4 based on colour, texture and amount of stool. Scores of diarrhoea index (DI) ≥2 indicates diarrhoeic faeces. Results are expressed as mean ± SEM ($n = 21$ animals/group). Statistical differences mentioned in the text

Results

Effect of prebiotic supplementation on stool consistency

As shown in Fig. 1, the PRE and SYN diets induced changes in the faecal consistency, thereby increasing the number of faeces considered as diarrhoeic (DI ≥ 2), before the inoculation day and when infection was solved (7 DPI). To better observe these effects, already described for certain prebiotics, a non-infected study (NIS) including suckling rats receiving PRO ($n = 5$), PRE ($n = 5$) and SYN ($n = 5$) diets was performed (see Supplementary Table 1). According to our diarrhoea scores, the animals of the PRE and SYN groups in the NIS had a mean score >1 throughout the study and even >2 for 3 days along the intervention. This direct effect of PRE and SYN on stool consistency was regarded in the main study. No effect of the dietary intervention on body weight was found.

Incidence of diarrhoea

The incidence of RV-induced diarrhoea was evaluated by two approaches. Considering the %DA during the whole period (Fig. 1a), 95–100 % of animals of the inoculated groups developed diarrhoea, whereas only 5 % did so in the REF group (with no RV inoculation). In the RV group, %DA was almost 70 % on 1 DPI; it increased up to 85 % on 3 DPI and decreased to 67 % on 4 DPI. Later, on 5 DPI, only 6/21 animals (29 %) still had some diarrhoea in the RV group and, on day 6 DPI (day 13 of life), none of the animals in the RV group had diarrhoea (Fig. 1a). When the diarrhoea incidence was studied in supplemented animals, some of the groups showed a modulatory effect. In this sense, all supplemented groups had lower %DA than the RV group over the 2–4 DPI period. However, it was only significant for the PRO group on 4 DPI ($p < 0.05$ vs. RV) (Fig. 1a). For the PRE and SYN groups the %DA was higher than that of the RV group in the last days studied (from 5 DPI), as well as before virus inoculation.

If we focus on the maximum percentage of diarrhoeic animals (MDA) induced by RV and the day of MDA (MDAd) (Table 1), all groups had the MDA 1–3 days after the induction. When the AUC of %DA was calculated (daAUC, Table 1), it could be seen that the PRO group presented a lower value than the RV group. In contrast, daAUC for the PRE and SYN groups was higher than that in the RV group. However, when the daAUC was normalized by calculating the AUC of the increment of incidence during

Table 1 Clinical variables determining the diarrhoea process (from day 0 to 6 DPI)

	RV	PRO	PRE	SYN
Incidence				
MDA	85.71	66.67	80.95	85.71
MDAd	3 DPI	2 DPI	1 DPI	1 DPI
daAUC	328.57	238.10	370.00	383.33
daAUCn	328.57	238.10	170.00	80.95
MDF	90.00	70.00	93.75	100.00
MDFd	3 DPI	2 DPI	2 DPI	1 DPI
dfAUC	362.06	262.86	448.33	486.39
dfAUCn	362.06	262.86	215.00	106.11
Duration				
DDB	1.4 ± 0.1	1.8 ± 0.2	1.4 ± 0.2	$1.1 \pm 0.1^{\alpha}$
DDE	4.0 ± 0.2	3.4 ± 0.2	$4.8 \pm 0.3^{\# \alpha}$	$5.0 \pm 0.2^{\# \alpha}$
DP	3.6 ± 0.2	$2.5 \pm 0.3^{\#}$	$4.4 \pm 0.3^{\# \alpha}$	$4.9 \pm 0.2^{\# \alpha}$
DwD	3.3 ± 0.2	$2.4 \pm 0.3^{\#}$	$3.7 \pm 0.3^{\alpha}$	$3.6 \pm 0.2^{\alpha}$
Severity				
MDI	3.05 ± 0.11	$2.74 \pm 0.14^{\#}$	2.87 ± 0.07	3.07 ± 0.08
MDId	2.0 ± 0.3	2.1 ± 0.2	2.0 ± 0.3	2.3 ± 0.4
sAUC	5.99 ± 0.47	$4.32 \pm 0.39^{\#}$	$5.95 \pm 0.29^{\alpha}$	$6.21 \pm 0.36^{\alpha}$
sAUCn	5.99 ± 0.47	$4.32 \pm 0.39^{\#}$	$2.59 \pm 0.23^{\#,\alpha}$	$2.56 \pm 0.25^{\#,\alpha}$

Results are expressed as mean ± SEM ($n = 21$ animals/group)

With regard to incidence: *MDA* maximum percentage of diarrhoeic animals, *MDAd* day with maximum percentage of diarrhoeic animals, *daAUC* area under the curve of diarrhoeic animals, *daAUCn* normalized area under the curve of diarrhoeic animals, *MDF* maximum percentage of diarrhoeic faeces, *MDFd* day with maximum percentage of diarrhoeic faeces, *dfAUC* area under the curve of diarrhoeic faeces, *dfAUCn* normalized area under the curve of diarrhoeic faeces. With regard to duration: *DDB* day of diarrhoea beginning (DPI), *DDE* day of diarrhoea ending (DPI), *DP* diarrhoea period, *DwD* days with diarrhoea. With regard to severity: *MDI* maximum diarrhoea index, *MDId* day of maximum diarrhoea index (DPI), *sAUC* area under the curve of severity, *sAUCn* normalized area under the curve of severity

$^{\#}$ $p < 0.05$ versus RV; $^{\alpha}$ $p < 0.05$ versus PRO

RV infection from the baseline of each group (without counting the non-pathogenic "diarrhoea" induced by the prebiotics in the PRE and SYN groups), it was even lower for the PRE and SYN groups than for the PRO group.

The results corresponding to the incidence of diarrhoeic faeces (%DF) (Table 1), i.e. MDF, MDFd, dfAUC and dfAUCn, followed the same pattern as the %DA.

Duration of diarrhoea

With regard to the duration of the diarrhoea process, in the RV group diarrhoea started at 1.4 ± 0.1 DPI (beginning day of diarrhoea, DDB) and ended at 4.0 ± 0.2 DPI (end day of diarrhoea, DDE). The diarrhoea period (DP) and the days with diarrhoea (DwD) were 3.6 and 3.3, respectively (Table 1). All the nutritional interventions modified this assessment of the process. In the PRO group, the DDE was lower than in the other groups, and the DP and DwD were reduced up to 1 day less (Table 1). In contrast, the PRE and SYN groups significantly increased the length of the diarrhoea period up to 1 day more (Table 1). It should be emphasized that the PRE and SYN groups still had scores >1 until the end of the study.

Severity of diarrhoea

The day after the inoculation (1 DPI) all induced animals had a mean severity (diarrhoea index, DI) of between 2 and 3, without statistical differences among groups. As can be seen in Fig. 1b, the severity curve in the RV group increased from 1 DPI and was maintained at similar values until 4 DPI. At 5 DPI, the mean score was under 2, and therefore, it is not likely that the animals had diarrhoea. Afterwards, no animals from this group had signs of diarrhoea and had a DI = 1. The PRO group already showed a lower severity score than the RV group on day 1 DPI, and this was maintained until the end of the diarrhoea period, although these differences were only significant at 3 and 5 DPI ($p < 0.05$). As in the previous indicators of the pathology (%DA, %DF or the diarrhoea process variables), the effectiveness of the PRE and SYN diets in controlling the RV infection could not be seen through DI data because

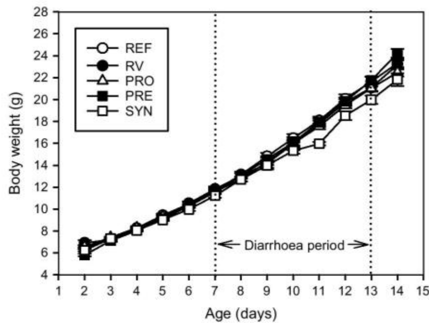

Fig. 2 Body weight (g) during the study, before and after virus inoculation (day 7 of life). Results are expressed as mean ± SEM ($n = 21$ animals/group)

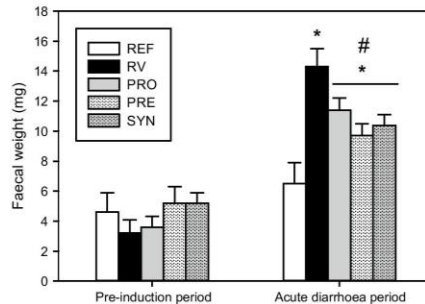

Fig. 3 Faecal weight (mg) during preinduction period (0 DPI) and acute diarrhoea period (pooled data from 1–4 DPI samples). Results are expressed as mean ± SEM ($n = 7$–24 samples/group/period). Statistical significance: * $p < 0.05$ versus REF, # $p < 0.05$ versus RV

the products induced features that occulted their putative action. The RV and PRO groups had no diarrhoea on 6 DPI; however, groups receiving PRE and SYN included some animals with diarrhoea until 9 DPI and some up to the last day studied (data not shown).

The mean MDI for all infected groups was around 3, and in all cases, it was obtained on 2 DPI (Table 1). The PRO group had a lower MDI than the RV group ($p < 0.05$), showing again the ameliorating effect of this compound in the diarrhoea process. The AUC of the severity pattern calculated during the period with diarrhoea (Table 1) showed AUC values of about 6 in inoculated animals, whereas REF animals did not develop diarrhoea and had AUC values around 0 (data not shown). Interestingly, a significant reduction in sAUC (around 30 %) was observed for the PRO group with respect to the RV group, demonstrating an overall reduction in the severity of the disease ($p < 0.05$). The PRE and SYN groups just showed a significant reduction in sAUC when it was normalized (from their baseline DI present before and after the infective process).

Body weight

The first body weight was recorded on day 2 of life and was about 6–7 g, with no significant differences among groups. At the end of the studied clinical period (day 14 of life) all the animals reached a body weight of between 20 and 24 g (Fig. 2). When weight increase (d2–d14) was calculated, all animals had a weight gain of about 205–243 %. No weight loss associated with the viral infection was observed. The growth of the animals was not influenced by the diet, except for the SYN group, in which a lower body weight ($p < 0.05$ vs. REF group on days 10–14) was found.

Faecal weight

The weight of the faecal samples was recorded throughout the study, and data were pooled by distributing them in preinduction period, during the acute diarrhoea period (1–4 DPI) and post-diarrhoea period. Before RV inoculation (preinduction period, 0 DPI), there were no significant differences among groups (Fig. 3). However, in the days just after RV infection, animals from the RV group had a higher faecal weight (~15 mg) than those from the REF group (~6 mg) ($p < 0.05$). The weights of faecal samples from the PRO, PRE and SYN groups were also higher than those from the REF group ($p < 0.05$), but all nutritional interventions were able to decrease the faecal weight with respect to the RV group ($p < 0.05$) (Fig. 3). After this period, there were no differences among groups (data not shown).

Viral shedding

The results obtained for viral shedding during the diarrhoea period (0–9 DPI) in the experimental groups are shown in Fig. 4a. The REF group (non-infected group) had a low background (presence of particles/virus detected in the ELISA as positive for RV). In all RV-inoculated animals, the maximum clearance of the RV was observed on the first day after inoculation (1 DPI). Taking this day into account (Fig. 4b), the PRO group had a similar level to that in the RV group, whereas the PRE and SYN groups had a lower value of RV shedding than that in the RV group ($p < 0.05$) and was even similar to that in the REF group. In fact, the pattern of RV shedding was comparable to that in the REF group throughout the studied period (Fig. 4a).

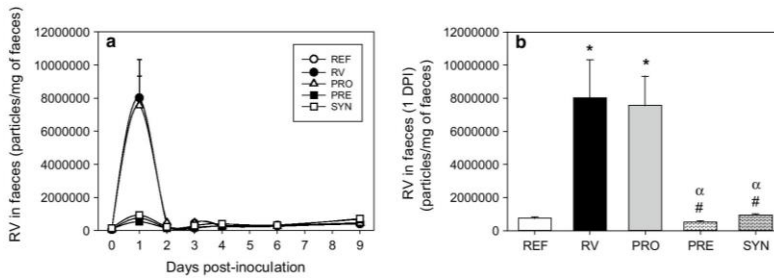

Fig. 4 Viral shedding. RV particles present per faecal sample obtained **a** during the study in each group and **b** on 1 DPI in each group. Results are expressed as mean ± SEM (n = 7–24 samples/group/period). Statistical significance: * $p < 0.05$ versus REF, # $p < 0.05$ versus RV, $^{\alpha}$ $p < 0.05$ versus PRO

Table 2 Specific anti-RV antibodies in serum (IgA, IgG and IgM) and intestinal wash (IgA and IgM) from 14- and 21-day-old rats

	RV	PRO	PRE	SYN
Serum				
d14				
A	313.8 ± 15.8	277.8 ± 27.4	303.6 ± 22.7	272.3 ± 22.5
G	183.6 ± 24.8	116.0 ± 36.1	1288.2 ± 126.4#	161.9 ± 40.6
M	297.4 ± 74.2	529.9 ± 98.5	694.9 ± 112.2#	650.8 ± 158.7#
d21				
A	334.5 ± 23.8	166.0 ± 34.5#	439.0 ± 18.7#	317.9 ± 16.5
G	1615.4 ± 417.0	1168.3 ± 71.5	4144.0 ± 289.5#	235.5 ± 73.4#
M	457.5 ± 143.6	297.4 ± 21.2	351.6 ± 28.9	479.3 ± 141.8
Intestinal wash				
d14				
A	75.7 ± 11.3	111.7 ± 13.9	115.2 ± 5.8#	148.9 ± 35.5
M	96.2 ± 6.2	106.9 ± 10.8	96.0 ± 14.7	107.7 ± 17.6
d21				
A	181.2 ± 24.5	149.0 ± 15.8	25.6 ± 1.9#	158.1 ± 34.7
M	138.2 ± 10.5	131.0 ± 11.1	88.1 ± 8.1#	104.4 ± 16.7

Results are expressed as mean ± SEM (n = 9–12 animals/group) in AU/mL

# $p < 0.05$ versus RV

Anti-RV antibody levels

Specific anti-RV antibodies were quantified in serum (IgA, IgG and IgM) and in intestinal washes (IgA and IgM) from 14- and 21-day-old rats (Table 2). In the RV group, specific IgA, IgG and IgM isotypes were already present in serum at day 14 of life. Only IgG titres increased significantly at day 21 with respect to those from day 14 ($p < 0.05$). In this group, the specific IgA and IgM in intestinal washes were observed at day 14, and both had increased 1 week later. In the PRO group, serum-specific IgA was lower than that of the RV group, whereas it had slightly increased in the intestinal wash at day 14.

In the PRE group, a more pronounced modulation of the systemic and intestinal antibody response was found: serum-anti-RV IgG and IgM levels were significantly higher than those in the RV group at day 14, and anti-RV IgA and IgG titres were higher than those in the RV group at day 21. Specific IgA concentration in intestinal wash from PRE animals was also higher than that in the RV group at day 14 ($p < 0.05$), but, in contrast, intestinal IgA and IgM at day 21 were decreased. In the SYN group, a rise in sera IgM levels at day 14 was observed, but sera-specific IgG concentration at day 21 was decreased.

SCFAs production

The main SCFAs (acetic, propionic and butyric), but also lactic and formic acids, were quantified in the faecal

Table 3 Acetic, propionic, butyric, lactic and formic acid levels, and the sum of all SCFAs (Total), in faecal samples of 17-day-old rats

	REF	RV	PRO	PRE	SYN
Total	2.73 ± 0.83	2.06 ± 0.62	7.96 ± 0.66*#	5.05 ± 2.02	0.85 ± 0.30$^{\alpha}$
Acetic acid	0.54 ± 0.35	0.13 ± 0.06	0.40 ± 0.12	0.07 ± 0.07	0.09 ± 0.09
Propionic acid	0.53 ± 0.07	1.39 ± 0.50	2.96 ± 0.49*	1.44 ± 0.62	0.07 ± 0.04$^{\alpha}$
Butyric acid	0.08 ± 0.05	0.13 ± 0.06	0.25 ± 0.04	0.09 ± 0.00	0.08 ± 0.03
Lactic acid	0.04 ± 0.04	0.03 ± 0.03	0.39 ± 0.12	0.20 ± 0.11	0.00 ± 0.00
Formic acid	1.54 ± 0.53	0.39 ± 0.14	3.96 ± 0.59#	3.25 ± 1.29	0.62 ± 0.24$^{\alpha}$

Results are expressed as mean ± SEM ($n = 5$–10 samples/group)

* $p < 0.05$ versus REF; # $p < 0.05$ versus RV; $^{\alpha}$ $p < 0.05$ versus PRO

Table 4 Percentage of inhibition of RV particle detection after incubation with PRO and PRE at different dilutions of the intervention concentration used in the in vivo study

	Inhibition (%)
PRO	
1/2	13.48 ± 0.87*
1/3	11.75 ± 4.14*
1/6	12.90 ± 3.20*
1/60	11.55 ± 2.43*
PRE	
1/2	39.41 ± 3.79*
1/3	28.44 ± 2.35*
1/6	19.98 ± 2.62*
1/60	10.87 ± 2.86*

Results are expressed as mean ± SEM of duplicates from three independent experiments

* $p < 0.05$ versus % of inhibition without product addition

samples of 17-day-old rats (Table 3). Globally, total and specific SCFAs in the REF group were not statistically modified due to RV infection; however, there was a tendency towards increased SCFAs levels in the PRO and PRE groups (some of them significant only in the PRO group, as in the case of the total SCFAs, propionic and formic acids). The SYN group did not show any increase.

In vitro blocking assay

Due to the in vivo results from the viral shedding, an in vitro approach was used to test the binding capacity to RV particles of the prebiotic and the probiotic (Table 4). We analysed SA-11 after incubation of several dilutions of the PRE and PRO products. SA-11 detection was inhibited with approximately 10 % by PRO preincubation, independently of dilution. However, the previous incubation with the PRE showed a significant dose-dependent blocking effect on virus detection up to 40 % at the highest concentration used, which was half the concentration used in the in vivo study.

Discussion

The main causative agent of acute gastroenteritis in children is rotavirus, and although vaccination and rehydration as preventive and therapeutic interventions are used, the introduction of prebiotics and probiotics is of interest, as a way to develop new and effective strategies for prevention, treatment or both.

Controlled interventional studies in humans, especially in infants, present certain difficulties, and for this reason, an animal model is necessary. In this regard, the neonatal rat is a suitable model, with substantial scientific evidence and cost-effective ratio [78]. In the present study, the RV SA-11 caused diarrhoea in nearly 100 % of the infected animals, and in contrast to a mouse model, a moderate severity of the disease was achieved, similar to our previous studies [75] and to what is normally seen in humans. Moreover, RV-inoculated animals (without treatment) became infected by the virus and synthesized specific antibodies which were found at the systemic and at the intestinal level. Interestingly in this study, the combination of the probiotic and the prebiotic seems to strengthen the antiviral action since the scGOS/lcFOS 9:1 prebiotic mixture enhanced the viral elimination and the host immune response against the virus, and its addition to *B. breve* M16-V ameliorated some diarrhoea indicators such as daAUCn, dfAUCn and sAUCn values, which are lower in the SYN group than in the other groups.

With regard to clinical symptoms, all interventions seem to have a protective role although it is not evident in all the variables analysed. The probiotic was the one with the clearest effect. *B. breve* M-16V reduced the incidence, duration and severity of the experimental diarrhoea. Although this is the first time *B. breve* M-16V has been tested against RV, others have investigated the safety and beneficial effects of this probiotic in premature infants, with results in line with those presented here. To date, an increase of faecal *B. breve* counts and a reduction of the incidence of necrotizing enterocolitis and other infections after its supplementation have been reported [79, 80]. It would be interesting to evaluate its intrinsic anti-RV

diarrhoeic activity in comparison with the other probiotics that have shown effectiveness in these types of preclinical interventions, such as LGG, among others [21, 23, 24, 26, 29, 31, 33]. It would be also interesting to deep into the mechanisms involved in the diarrhoea protection by this probiotic such as the improvement of microbiota composition, immunesupport actions or even an effect on epithelial barrier function.

With regard to the effect of the prebiotic mixture at the dose used here, a masking effect was observed in this study, which is one of the main limitations of this intervention. The prebiotic, and therefore also the synbiotic intervention, induced a softened stool consistency, independently of the presence of the virus. This was observed in the days before inoculation in the main study and in the NIS. It seems that these interventions were able to decrease the incidence and severity of diarrhoea, or at least, the effect on the faecal consistency was not additive with that induced by the RV infection. For this reason, when the severity score was recalculated or normalized on the basis of the basal faecal punctuation in the absence of the virus, a reduction in terms of incidence and severity could be observed. These results are in line with other studies showing that a specific mixture of scGOS/lcFOS prevented infections in infants [54, 55, 60] and caused positive changes in stool consistency, bringing it closer to breast fed infants [51, 60].

Although the faecal score is widely used for this type of studies and conducted in a blinded manner here, it remains a rather subjective evaluation. In this regard, an increase in the faecal weight could be a more objective indicator of the incorporation of water in the total faecal content [75, 81]. This was measured in the present study when the REF and RV faecal weights were compared before and after inoculation. It is noteworthy that all the products tested here, PRE and PRO, alone or in combination, avoided the increase of faecal weight due to RV diarrhoea in the acute phase of the disease.

The RV arrives at the intestine where it binds to the epithelial cells, and starts its infective and replication process. Thus, the viral shedding reflects the viral particles produced due to their replication in the intestine. In previous studies using this model [75], the day with maximum viral shedding in the RV group was just 1 day after the virus inoculation (1 DPI). A similar viral shedding was observed in the group supplemented with the probiotic when compared to the RV group. We can hypothesize that the amelioration in the clinics observed by the PRO diet may not be due to a higher clearance of the virus. Other possible mechanisms could be an improvement of the epithelial barrier or an enhancement of the developing immunity of the suckling rats. In contrast, the peak of viral elimination was substantially reduced (up to 90 %) in the groups supplemented with PRE and SYN, suggesting that the prebiotic is responsible for the effect. This high reduction in the viral shedding may suggest that the mechanism of action of the probiotic and the prebiotic is different. It is significant that the effect of the prebiotic present in the SYN group was still evident regardless of the addition of the probiotic, with the effect being similar to that found in the PRE group. From these results it can be concluded that some part of the GOS/FOS molecules mixture may be able to directly interact with the RV, an action already described for some human milk oligosaccharides [82], and therefore this binding would hinder their detection by ELISA. This reaction may be responsible for the lower adhesion of the virus to the host and consequently lead to a lower infection incidence and severity, as was observed in this study. After obtaining these surprising data, we conducted in vitro blocking assays and the obtained results confirmed that the detection of the virus was lower when the test was performed in the presence of the prebiotic mixture. This would mean that a direct interaction of the GOS/FOS with the virus occurs, which leads to the virus not being detected in vitro. It remains to be elucidated which type, what specificity and stability of this interaction between the RV occur with this particular mixture.

In terms of immune response, although the underlying mechanisms are not totally elucidated, there are several proposed mechanisms of antibody-mediated immunity against the RV that involve both the systemic and the mucosal response by means of monomeric IgG and more importantly dimeric IgA, respectively [3]. In fact, differences in the reactivity of different RV strains in different animal models or humans do not allow clear conclusions to be drawn regarding human protection. To date, rodent models of RV diarrhoea have been centred on early suckling because their natural acquired immune response is able to block the virus after weaning [75, 83]. Despite this limitation, the positive correlation between IgA and protection against the virus seems to be clear. In this context, the model used herein seems to develop protection against the virus after infection as can be seen by the presence of specific antibodies [75], and particularly anti-RV IgA, IgG and IgM in sera, and IgA and IgM in the intestine. However, this effect should be confirmed in the future by using a double infection model in rat, as has been previously developed in mice [84].

With regard to the effect of the supplementation with *B. breve* M-16V on the anti-RV humoral immune response, it barely modified the immune response observed in the RV group. These results contradict with the immunomodulatory effects of this strain at antibody level in food allergy studies in mice [72, 85] and with those of other probiotics such as LGG or *L. acidophilus* NCFM, which increased IgG or IgM in pigs and infants after this type of infection [25, 26, 37]. In contrast, the PRE supplementation in

early life increased local and systemic humoral response against the virus. At day 14 of life, just 1 week after infection, the higher titres of IgM and IgG in serum, and IgA in intestinal wash, compared with the RV group, suggest a modulatory role of this intervention in the maturation of the immune system. In particular, the increase in IgA at the intestinal level indicates higher binding to the RV, which results in higher virus exclusion from the mucosa and therefore infection prevention. In fact, an increase of faecal IgA secretion has also been described after dietary supplementation with a specific mixture of scGOS/lcFOS in infants [54, 55].

At a later time point after infection (day 21), an increase of IgG and IgA in serum was observed. As high values of these isotypes in sera are good indicators of protection [86], it can be suggested that the specific scGOS/lcFOS mixture had some immunomodulatory effects, which led to enhancing the immune response against the virus, not only to allow its elimination but also to maintain protection later in life. It should be emphasized that although the PRE mixture seems to be able to partially bind the virus, blocking its adherence to the intestine and therefore decreasing infection, it was also able not only to maintain, but also to enhance the immune response and protection against the RV. The SYN showed some of these same modulatory effects.

Finally, the SCFAs concentration found in faeces was very low in all groups, a fact that may be due to their high absorption in the colon [87] and might therefore be a limitation in this study in which only faecal samples were analysed. Nevertheless, although a tendency to increase some of the SCFAs can be seen after the PRE supplementation, as seen in other studies with GOS/FOS [52, 53], the highest changes were found after the PRO intervention. It can be suggested that the increase in total SCFA, but especially in propionic and formic acids due to the administration of this strain, may be involved in its protective action against the RV.

In conclusion, all tested products showed beneficial effects on RV-induced gastroenteritis in the neonatal rat model, modulating clinical parameters and immune system response early in life. Further studies are needed in order to better understand their mechanism of action or even to determine the timing and dosage of administration of these compounds to be used as strategies to protect against human RV-induced diarrhoea in children.

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Compliance with ethical standards

Conflict of interest The authors declare that they have a financial relationship with the organization that sponsored the research. K. van Limpt, K. Knipping, J. Garssen and J. Knol are employees of Nutricia Research B.V. The other authors declare that they have no conflict of interest.

Ethical standards The studies were conducted in accordance with the institutional guidelines for the care and use of laboratory animals established by the Ethics Committee for Animal Experimentation of the University of Barcelona and the Catalonian Government (CEEA-UB Ref.165/11, DAAM: 5871).

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Supplementary table 1. Body weight and severity of diarrhoea from the Non-Infected Study (NIS). Faecal samples are scored from 1 to 4 based on colour, texture and amount of stool. Scores of diarrhoea index (DI) ≥ 2 indicate diarrhoeic faeces. Results are expressed as mean ± SEM (n = 5 animals/group).

Age	PRO		PRE		SYN	
	Body weight (g)	DI	Body weight (g)	DI	Body weight (g)	DI
3	7.40 ± 0.18	n.d	6.91 ± 0.19	n.d	7.62 ± 0.41	n.d
4	8.66 ± 0.18	n.d	7.86 ± 0.27	n.d	8.57 ± 0.57	n.d
5	9.70 ± 0.16	n.d	9.11 ± 0.26	n.d	9.78 ± 0.63	n.d
6	10.78 ± 0.18	1.00 ± 0.00	10.00 ± 0.30	1.85 ± 0.15*	11.00 ± 0.66	1.83 ± 0.17*
7	12.17 ± 0.09	1.08 ± 0.08	11.22 ± 0.36	1.67 ± 0.17	12.18 ± 0.68	1.88 ± 0.13*
8	13.41 ± 0.16	1.00 ± 0.00	12.76 ± 0.32	1.70 ± 0.20*	13.91 ± 0.82	1.92 ± 0.30
9	15.09 ± 0.20	1.00 ± 0.00	14.25 ± 0.38	2.08 ± 0.36	15.89 ± 0.93	1.75 ± 0.14*
10	16.65 ± 0.20	1.17 ± 0.17	15.67 ± 0.40	2.13 ± 0.13	17.79 ± 0.98	1.81 ± 0.19*
11	18.25 ± 0.22	1.13 ± 0.07	17.40 ± 0.34	1.50 ± 0.14*	19.63 ± 0.93	2.00 ± 0.00*
12	20.09 ± 0.26	1.00 ± 0.00	19.23 ± 0.29	1.67 ± 0.33	21.41 ± 0.84	1.58 ± 0.30
13	21.87 ± 0.25	1.00 ± 0.00	20.57 ± 0.34	2.65 ± 0.38*	23.17 ± 0.77	2.00 ± 0.27*
14	23.81 ± 0.29	1.00 ± 0.00	22.70 ± 0.25	1.63 ± 0.24	24.77 ± 0.58	2.30 ± 0.25*

*p<0.05 *vs.* REF

Capítol 3

A fermented milk concentrate and a combination of scGOS/lcFOS/pAOS protect suckling rats from rotavirus gastroenteritis”

Maria del Mar Rigo-Adrover, Teresa Pérez-Berezo, Sara Ramos-Romero, Kees van Limpt, Karen Knipping, Johan Garssen, Jan Knol, Àngels Franch, Margarida Castell, Francisco J. Pérez-Cano

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Els resultats del present article han estat presentats a les següents comunicacions:

- B. breve *and* S. thermophilus *fermented milk concentrate protects against rotavirus gastroenteritis in a suckling rat model*. V Workshop Probióticos, Prebióticos y Salud. Evidencia Científica (València, Espanya), gener 2014.

- *Prebiotic supplementation during suckling modulates the course of rotavirus gastroenteritis in rats*. VI Workshop Probióticos, Prebióticos y Salud. Evidencia Científica (Oviedo, Espanya), febrer 2015. Comunicació oral. Publicat a Nutrición Hospitalaria (2015) 31 (1):54.

RESUM

Objectiu: Investigar l'efecte protector contra infeccions gastrointestinals de dos productes: 1) una fórmula fermentada per *Bifidobacterium breve* i *Streptococcus thermophilus* i 2) una combinació de galactooligosacàrids de cadena curta/ fructooligosacàrids de cadena llarga (scGOS/lcFOS) amb oligosacàrids acídics derivats de pectina (pAOS).

Material i mètodes: S'ha utilitzat un model d'infecció per rotavirus en rates lactants per avaluar l'efecte dels suplements sobre el procés infecciós i la resposta immunitària.

Resultats: Els dos productes estudiats han causat una millora de les manifestacions clíniques, tot i que un canvi en la consistència de les femtes induït pels propis productes va dificultar l'observació d'aquests efectes. Ambdós productes també van mostrar una certa modulació de la resposta immunitària, el qual va permetre una resolució més ràpida del procés infecciós. Els resultats d'eliminació viral i de l'assaig de bloqueig *in vitro* van suggerir que els productes són capaços d'unir-se a les partícules virals, resultant en una infecció més moderada.

Conclusió: Ambdós suplements avaluats en aquest estudi van mostrar propietats protectores contra la infecció per rotavirus i, per tant, seria interessant valorar la serva inclusió en preparats infantils per a aquests efectes.

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A fermented milk concentrate and a combination of scGOS/lcFOS/pAOS protect suckling rats from rotavirus gastroenteritis

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A FERMENTED MILK CONCENTRATE AND A COMBINATION OF scGOS/lcFOS/pAOS PROTECT SUCKLING RATS FROM ROTAVIRUS GASTROENTERITIS

Shortened version:

ROTAVIRUS PROTECTION BY PREBIOTICS

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Abstract

Human milk contains bioactive compounds which confer a protective role against gastrointestinal infections. In order to find supplements for an infant formula able to mimic these benefits of breastfeeding, two different concepts have been tested. The products consisted of: 1) a *Bifidobacterium breve* and *Streptococcus thermophilus* fermented formula and 2) a combination of short chain galactooligosaccharides/ long chain fructooligosaccharides (scGOS/lcFOS) with pectin-derived acidic oligosaccharides (pAOS). A rotavirus infection suckling rat model has been used to evaluate improvements in the infectious process and in the immune response of supplemented animals. Both nutritional concepts caused an amelioration of the clinical symptoms, even though this was sometimes hidden by a softer stool consistency in the supplemented groups. Both products also showed certain modulation of the immune response, which seemed to be enhanced earlier and was accompanied by a faster resolution of the process. The viral shedding and the *in vitro* blocking assay suggest that these products are able to bind the viral particles, which can result in a milder infection. In conclusion, both concepts evaluated in this study showed interesting protective properties against rotavirus infection, which deserve to be further investigated.

Keywords

prebiotic, FOS, GOS, pAOS, fermented formula, milk, rotavirus

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Introduction

The World Health Organization (WHO) refers to breastfeeding as the best way of providing food and promoting the healthy development of infants[1]. In fact, human milk is not only a source of energy and nutrients, but also contains bioactive compounds. These compounds, such as oligosaccharides, nucleotides, growth factors, hormones, immunoglobulins, cytokines, lysozyme and lactoferrin, have shown immunomodulatory and bifidogenic effects[2–4]. One of the main benefits of breastfeeding is the protection that it confers against gastrointestinal infections in both developed and developing countries[2]. Despite this, breastfeeding is not always possible, thus the formulation of new infant formulas, which better mimic the composition of human milk and provide its anti-infectious components, is of great importance. In this sense, dietary oligosaccharide structures, which have a prebiotic effect similar to that of human milk oligosaccharides, are usually added to infant formulas. Interventional studies with a specific mixture of short chain galactooligosaccharides (scGOS) and long chain fructooligosaccharides (lcFOS; 9:1 ratio) have been shown to induce a microbiota composition and a stool consistency closer to the breast-fed infant pattern[5,6]. In addition, scGOS and lcFOS supplements produce an increase of short chain fatty acids (SCFAs) in caecum samples[7,8], an increase in intestinal IgA secretion[9,10], an improvement in calcium absorption[11,12], a lower incidence of infections during the first years of life[5,13,14] and also a decreased incidence of food allergy, asthma and eczema[15] in both clinical and preclinical studies. All these effects suggest the importance of scGOS and lcFOS in early life. Recently, prebiotics other than FOS and GOS have been tested in animals and in humans, such as pectin-derived acidic oligosaccharides (pAOS). The pAOS, whose safety has already been assessed[16], are tested in combination with scGOS/lcFOS in order to mimic the presence of neutral and acidic oligosaccharides in human milk. This type of mixture has shown a bifidogenic effect[17]. Moreover, it has demonstrated having influence on the immune system by enhancing the Th1 response, which is weak in early life, in an infection model and in a vaccination mouse model[18,19], as well as in a food allergy model[20]. In infants, this mixture also reduces the occurrence of atopic dermatitis[21]. Some studies point out that this combined formula shows more pronounced immunomodulatory effects than scGOS/lcFOS alone[19]. Therefore, differential mechanisms of action have been suggested[20].

Besides prebiotic- or probiotic-containing formulas, fermented milk (FM)–based infant formulas (FMBIFs) offer an additional means for modulation of gut immunity and/or gut microbiota. These formulas are produced by the fermentation of cow's milk with specific lactic acid bacteria strains, followed by heat treatment. These formulas contain specific products resulting from the

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fermentation process. Additional to the possible modulation of gut microbiota composition, *in vitro* and *in vivo* studies suggest that specific fermentation products can actively participate in the establishment of immune balance and oral tolerance[22]. FMBIFs have also shown a bifidogenic effect associated with an increase in intestinal IgA secretion and a decrease in faecal pH[23,24]. FMBIFs have also demonstrated their ability to reduce the severity of rotavirus (RV) gastrointestinal infection[25,26].

The present study aimed to elucidate the immunomodulatory and the protective effects of a mixture of scGOS/lcFOS/pAOS and of a heat-treated (probiotic-)fermented milk components against RV infection in early life. A neonatal rat model of RV gastroenteritis was applied[4,27,28] because RV is the most common pathogen causing diarrhoea among infants and young children[29].

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Experimental methods

Animals

Twelve G14 pregnant Lewis rats were obtained from Janvier (Le-Genest-Saint-Isle, France) and allowed to deliver at term. The day of birth was registered as day 1 of life. Litters were unified to 7 pups/dam and had free access to the nipples. Dams had access to water and the American Institute of Nutrition (AIN)-93G diet[30] *ad libitum*. Each dam and its litter were housed individually under controlled temperature and humidity conditions, in a 12 h:12 h light:dark cycle. Furthermore, they were located in a safe isolated room, specially designed and authorized for working under biosecurity level 2 conditions at the Animal Service of the Faculty of Pharmacy, University of Barcelona. Pups were individually identified by labelling with a permanent marker after 2 days of environmental adaptation. The animals were weighed and monitored daily in order to obtain data regarding the influence of virus inoculation, clinical development and nutritional intervention on body weight and growth.

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Ethics Committee for Animal Experimentation of the University of Barcelona and the Catalonian Government (CEEA-UB ref. 165/11, DAAM: 5871).

Experimental design

Suckling rats were distributed in four different experimental groups: reference (REF group), rotavirus (RV group), the group receiving heat-treated (probiotic-)fermented milk components (FM group) and the one receiving scGOS/lcFOS/pAOS mixture (GFA group). Each group was

composed of 3 litters with 7 pups each (n=21/group). Diets or vehicle were administered from day 3 of life until the last day of the experiment. The RV inoculation was carried out at day 7 of age in all the experimental groups with the exception of the REF group. Clinical evaluation was performed daily from the day before inoculation until the end of the study. Faecal samples were collected daily throughout the study, and blood and intestinal wash samples on sacrifice day. Animals were euthanized at two different time points: a group of the animals were euthanized on day 14 (n=9), while the others continued receiving the diet until day 21 (n=12). A parallel cohort with non-infected animals receiving the products was also included (non-infection study, NIS).

Dietary supplementation

Animals were administered daily by oral gavage from day 3 to day 21 of age with the different supplements (2 groups: FM and GFA) or vehicle (2 groups: RV and REF), as previously described[31], using low-capacity syringes (Hamilton Bonaduz, Bonaduz, Switzerland) adapted to forced alimentation tubes of 25 or 23 calibre and 27 mm in length (ASICO, Westmont, IL, USA).

The FM diet was obtained by fermentation with *Bifidobacterium breve* C50 ($4.2x10^9$ bacteria per 100 g of powder formula) and *Streptococcus thermophilus* 065 ($3.84x10^7$ bacteria per 100 g of powder formula) during the manufacturing process and then heated to kill ferments (patented technology, Blédina, France). It was administered in a dose of 3 g/100 g of body weight/day. The GFA group received a combination of scGOS, lcFOS and pAOS (76.5:8.5:15) in a dose of 0·8 g/100 g of body weight/day.

Virus inoculation

The RV strain used (simian SA-11) was obtained from the 'Enteric Virus Group' of the University of Barcelona. Viruses were propagated in foetal African green monkey kidney cells (MA-104) and titred as $TCID_{50}$/mL (TCID, tissue culture infectious dose)[4,27]. Production was carried out in compliance with the current principles of GLP (Royal Decree 1369/2000, of July 19th). SA-11 was inoculated ($2x10^8$ $TCID_{50}$/rat in 100 μL of PBS) at day 7 of life, as previously described[27], to suckling rats from the RV, FM and GFA groups. The RV was inoculated after 1 h of separation from their dams to avoid interference between RV and milk components. The REF group received the same volume of phosphate-buffered solution (PBS) in the same conditions.

Clinical indexes and faecal specimen collection

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SA-11 infection was evaluated on days 8–21 by the growth rate and clinical indexes derived from faecal samples as previously described[27]. Faecal sampling was performed once a day by gently pressing and massaging the abdomen. Specimens were immediately scored, in a blinded manner, weighed and frozen at -20 ºC for further analysis.

The severity of diarrhoea was expressed by the faecal weight and by scoring stools from 1 to 4 (diarrhoea index) based on colour, texture and amount as follows: normal (diarrhoea index=1), loose yellow-green (diarrhoea index=2), totally loose yellow-green (diarrhoea index=3), high amount of watery (diarrhoea index=4) faeces. Diarrhoea scores ≥ 2 indicate diarrhoeic faeces whereas scores of diarrhoea index < 2 indicate absence of diarrhoea.

The area under the curve (AUC) of severity during days 7–13 was calculated as a global value of severity. The maximum diarrhoea index was defined as the highest score during the diarrhoea period. Incidence of diarrhoea was expressed by the % of diarrhoeic animals, taking into account the number of animals in each group, and by the % of diarrhoeic faeces, taking into consideration the number of total samples collected every day in each group. The AUCs of the % of diarrhoeic animals and the % of diarrhoeic faeces curves during days 7–13 were calculated as a global value of incidence.

The AUCs for severity, % of diarrhoeic animals and % of diarrhoeic faeces were also calculated taking into account the basal values due to intrinsic aspects of each treatment (normalized AUC). The maximum % of diarrhoeic animals and diarrhoeic faeces were defined as the highest values during the diarrhoea period. The diarrhoea period was calculated for each animal as the interval between the first (beginning day of diarrhoea) and last day (ending day of diarrhoea) of diarrhoea. The actual days with diarrhoea within the diarrhoea period were also counted.

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Faecal pH determination

Frozen faecal samples (5-60 mg) from days 8-12 were diluted in distilled water (up to 200 mg/mL) and softly agitated. Their pH was measured by a pH electrode for surfaces 5207 (Crison Instruments, Barcelona, Spain) and pH-Meter micropH 2001 (Crison), previously calibrated.

Intestinal and blood sample collection

After previous anaesthesia with intramuscular ketamine/xylazine injection, rats from each group were euthanized at days 14 or 21. Blood was collected by cardiac puncture and sera stored at -20 ºC

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until analysis. Small intestine was weighed, cut into 5 mm pieces and incubated with PBS for 10 min at 37 °C in a shaker to obtain the gut wash. After centrifugation, supernatants were stored at -20 ºC until analysis.

ELISA for specific total, IgG, IgA and IgM anti-RV antibody quantification in sera and intestinal washes

Ninety-six well plates (Nunc MaxiSorp, Wiesbaden, Germany) were coated with UV-inactivated SA-11 particles at 10^5/mL. After blocking with PBS-1% bovine serum albumin (BSA, 1 h, room temperature [RT]), appropriate diluted sera or intestinal wash samples were added (3 h, RT). After washing, rabbit anti-rat Ig conjugated to peroxidase from Dako (Barcelona, Spain) or mouse biotinylated anti-rat IgA (A93-2), IgG1/2a (R19-15) or IgM (G53-238) monoclonal antibodies (MAb) from BD Biosciences (Heidelberg, Germany), followed by peroxidase-conjugated extravidin (Sigma-Aldrich, Madrid, Spain), were added. Subsequently, substrate was added, as previously described[27]. Pooled sera from dams of inoculated litters were used as a standard in each plate. Quadratic polynomial adjustment was used. Dam sera received the value of 5000 arbitrary units (AU)/mL for total anti-RV antibodies, and 1000 AU/mL for anti-RV IgG, IgA and IgM antibodies.

ELISA for determination of SA-11 viral load in faeces

Faecal samples from days 7–16 were diluted in PBS (20 mg/mL) and homogenized using a Polytron (Kinematica, Luzern, Switzerland). Homogenates were centrifuged (200 *g*, 5 min, 4 ºC), and supernatants were frozen at -20 °C until use. SA-11 particles in faecal samples were quantified by ELISA, as previously described[27]. Titrated dilutions of inactivated SA-11 virus particles, ranging from 10^5 to 10^3/mL, were used as standard in each plate.

In vitro *blocking assay*

A concentration of 5×10^4 particles/mL of SA-11 in PBS-Tween 1% was prepared. Different dilutions of FM or GFA products of the *in vivo*-administered concentration were added to the virus (from 1/2 to 1/60 dilutions). The combinations were incubated for 30 min. Free non-coated viral particles were quantified by ELISA, as described above (SA-11 viral load).

Statistical analysis

The PASW Statistics 22 software package (SPSS Inc, Chicago, IL, USA) was used for the statistical analysis. Komolgorov-Smirnov test was applied to assess normal distribution, followed by Levene's test in order to determine variance equality. Conventional one-way ANOVA was

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performed considering the experimental group as the independent variable. When virus inoculation/treatment had a significant effect on the dependent variable (body weight, body weight increase, etc.), Scheffé's *post hoc* test was applied. Kruskal-Wallis and Mann–Whitney U tests were used when non-normal distribution or different variance were found (maximum diarrhoea score, severity AUC, etc.). Finally, the chi-square test was used to compare frequencies (diarrhoea incidence). Differences were considered significant at P values of $< 0{\cdot}05$. Results are expressed as mean and SEM or as median and IQR of number of animals.

Results

Effect of the dietary supplementation and virus inoculation on body weight

Body weight was recorded between days 2 and 14 of life. Weight started at about 6–7 g and ended at 22–24 g, with no significant differences among groups. When weight increase (d2–d14) was calculated, all animals had an increase of about 195–255% without statistical differences. The diet had no notable influence on the growth of the animals and no weight loss was associated with the infection process (**Fig. 1**). Only the GFA group had a lower body weight slope than the other groups from day 6 to day 13 ($p<0{\cdot}05$), although at the end of the study no differences were found either in body weight or in weight increase.

Effect of the dietary supplementation on stool consistency

As can be seen in **Figure 2**, before the inoculation day (day 7) and when infection was resolved (day 14), the diets induced changes in faecal consistency (mainly the GFA diet), increasing the number of faeces considered as diarrhoeic (DI≥2). To better observe these effects, already described for certain prebiotics, a non-infected study (NIS) including suckling rats that received FM and GFA diets was carried out. According to our diarrhoea scores, most of the animals in the NIS had scores higher than 1 during the study and in some cases had a diarrhoea index=2 score during the days of supplementation (14 days) (data not shown). Moreover, some animals of the NIS, mainly those from the GFA diet, still had diarrhoea one week later (day 21). Therefore, the direct effect of the diets on stool consistency has to be taken into account.

Incidence of diarrhoea

The incidence of diarrhoea was evaluated by two approaches: % of diarrhoeic animals and % of diarrhoeic faeces. Considering the % of diarrhoeic animals in the whole period (**Fig. 2A**), 95–100% of animals of the inoculated groups developed diarrhoea at some time, whereas <5% did so in the REF group (with no RV inoculation). In the RV group, % of diarrhoeic animals was almost 70% on

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day 8; it increased up to 85% on day 10, decreased later, and after day 12 none of the animals in this group had diarrhoea (**Fig. 2A**). The animals from the FM group showed certain improvement. In this sense, this group had lower % of diarrhoeic animals than the RV group over the day 9–11 period, but it was only significant on day 11 ($p<0·05$ vs. RV) (**Fig. 2A**). The % of diarrhoeic animals for the GFA group was higher than that of the RV group in the last days studied (from day 12), as well as before virus inoculation. If the % of diarrhoea is normalized (subtracting the basal values due to intrinsic aspects of each treatment), a clearer improvement by both supplements is observed (**Fig. 2B**).

All groups had the maximum % of diarrhoeic animals at 1–3 days after the RV induction (**Table 1**). When the diarrhoeic animals AUC was calculated (**Table 1**), it can be observed that the FM group presented a lower value than the RV group. On the contrary, the diarrhoeic animals AUC for the GFA group was higher than that in the RV group. However, when the diarrhoeic animals AUC was normalized by calculating the AUC of the increment of incidence during RV infection from the baseline of each group, it was lower for both supplemented groups than that of the RV group.

The results corresponding to the incidence of diarrhoeic faeces (% of diarrhoeic faeces) (**Table 1**), i.e., maximum % of diarrhoeic faeces, diarrhoeic faeces AUC and normalized diarrhoeic faeces AUC, followed the same pattern as that of % of diarrhoeic animals.

Duration of diarrhoea

Focusing on the duration of the diarrhoea process, in the RV group diarrhoea started 1–2 days after inoculation and ended around the 11th day of life. Both, the diarrhoea period and the days with diarrhoea were between 3 and 4 days (**Table 1**). In the FM group, the ending day of diarrhoea was earlier than in the other groups, and also the diarrhoea period and days with diarrhoea were reduced by up to one day less (**Table 1**). In contrast, the GFA group significantly increased the length of the diarrhoea period when no normalization was applied (**Table 1**). It should be noted that the GFA group still had scores >1 up to the end of the study.

Severity of diarrhoea

In **Figure 2C** the severity curves can be observed. In the RV group the diarrhoea index was about 2·5 on day 8 and maintained similar values until day 11, but later, the mean score was under 2, which means that most animals had no diarrhoea. The FM group showed a lower severity score than the RV group as early as at day 8, and this was maintained until day 12, although these differences

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were only significant at day 10 and 11 ($p<0{\cdot}05$). As in the previous indicators of the pathology (% of diarrhoeic animals, % of diarrhoeic faeces or the diarrhoea process variables), the effectiveness of the GFA diet in controlling the RV infection was hidden because the products induced features that concealed their putative action, but when normalized, a remarkable reduction of diarrhoea severity is shown (**Fig. 2D**).

The mean maximum diarrhoea index for all infected groups was around 3 and was obtained at day 9 in all cases (**Table 1**). The FM group had a lower maximum diarrhoea index than the RV group ($p<0{\cdot}05$). The AUC of the severity pattern calculated during the period with diarrhoea (**Table 1**) was about 6 in the RV group, whereas REF animals did not develop diarrhoea and had AUC values around 0 (data not shown). Interestingly, a significant reduction in severity AUC (34%) was observed for the FM group compared to the RV group, demonstrating an overall reduction in the severity of the process ($p<0{\cdot}05$). The GFA group showed a significant reduction in severity AUC when it was normalized (from its baseline diarrhoea index present before and after the infective process, $p<0{\cdot}05$).

Faecal weight

The weight of the faecal samples was recorded throughout the study. Mean faecal weight in each period was calculated in those samples obtained before the inoculation day (day 7), those during the acute diarrhoea period (days 7–11), and those samples belonging to the post-diarrhoea period (after day 12). Before RV inoculation, there were no differences among the groups (**Fig. 3A**); however, in the days just after RV infection, the faecal weight of animals from the RV group increased (~15 mg) with respect to those from the REF group (~6 mg, $p<0{\cdot}05$). The weights of faecal samples from the FM and GFA groups were also higher than those from the REF group ($p<0{\cdot}05$), but the GFA diet was able to decrease the faecal weight compared to the RV group ($p<0{\cdot}05$) (**Fig. 3A**). After this period, there were no differences among the groups (data not shown).

Faecal pH

Faecal samples from days 8-12 of each group were pooled and pH measured (**Fig. 3B**). The RV group showed a lower faecal pH during the acute diarrhoea period with respect to the REF group ($p<0{\cdot}05$). The FM diet avoided this acidification of faeces ($p<0{\cdot}05$ *vs.* RV). In contrast, the GFA group had an intermediate pH value between those in the REF and RV groups.

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Humoral immune response

Specific anti-RV antibodies were quantified in serum (total, IgG, IgM and IgA) (**Table 2**) and in intestinal washes (IgA and IgM) (**Table 3**) at day 14 and 21 of life. In the RV group, there were already significant levels of specific anti-RV IgA, IgG and IgM in serum and anti-RV IgA and IgM at day 14 of life. The values of each isotype in the RV group on day 14 are considered as 100 AU, and the results for the RV group on day 21 and for the other groups have been proportionally adjusted for each isotype. In the serum samples of the RV group, only anti-RV IgG titres increased significantly from day 14 to day 21 ($p<0·05$). In intestinal washes, the specific anti-RV IgA and IgM levels increased 1 week later.

Both dietary supplementations modulated the humoral immune response against the RV. In the FM group, serum anti-RV antibodies were higher than those of the RV group at day 14, which seems to be due to higher levels of specific IgM, whereas anti-RV IgA concentration was lower than that in the RV group (**Table 2**). In the FM group, specific IgG increased at day 21. The intestinal anti-RV IgA levels were similar to those in the RV group both at day 14 and 21 (**Table 3**). Specific total antibodies in serum from the GFA group were lower than those in the RV group at day 14 ($p<0·05$), which seems to be due to decreased levels of all anti-RV isotypes, in particular IgG, which was also reduced on day 21 ($p<0.05$). In addition, in the GFA group, intestinal anti-RV IgA concentration was increased at day 14 and intestinal anti-RV IgM levels at day 21 were reduced ($p<0·05$).

Viral shedding

The results obtained for viral shedding after inoculation (days 7-16) can be seen in **Figure 4**. The REF group (non-infected group) had a low background (in the ELISA technique). In all RV-inoculated animals, the maximum clearance of the RV was produced on the first day after inoculation (day 8). On this day, both supplemented groups had a lower value of RV shedding than that of the RV group, although it was only significant for the GFA group ($p<0·05$), and was even similar to the background levels found in the REF group.

In vitro *blocking assay*

After observing the results from the viral shedding, an *in vitro* approach was used to test the possible binding ability of the supplements to RV particles (**Fig. 5**). The number of SA-11 particles detected after incubation with several dilutions of the FM and GFA products was analysed. SA-11 detection was inhibited by both products, being the inhibitory activity for both products around 30% at the highest concentration assayed.

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Discussion

Exclusive breastfeeding during the first 6 months of life has a protective, bifidogenic and immunomodulatory effect able to prevent or decrease the incidence and severity of RV diarrhoea, among other infections[2]. In order to mimic these benefits, new supplements to be added to infant formula when infants cannot be breastfed, have been recently investigated. In the present study, both products based on microbial/prebiotic concepts (FM and GFA), have been evaluated on a model of RV-induced diarrhoea in suckling rats.

The FM administration was able to reduce clinical diarrhoea in terms of incidence, duration and severity. A reduction in diarrhoea severity had already been reported in a germ-free suckling rat RV model supplemented with milk fermented by a *Lactobacillus casei* strain[25], and also in infants supplemented with a cell-free fermented infant formula[26]. This last study demonstrated that the presence of live microorganisms is not essential to obtain probiotic-like effects. The FM also avoided the RV-induced acidification of faeces. But this result, similar to that found in the REF group, contrasts with the reduction of faecal pH in healthy mice and humans receiving a cell-free fermented formula[23,24]. This could be due to the effect of breastfeeding in our study, which may already acidify the intestinal content. In RV-induced groups, viral shedding was mainly produced on day 8, agreeing with previous studies using this model[27]. However, in the FM group, viral shedding was slightly lower than that in the RV group, which suggests that the FM has a certain capacity to bind the RV particles. In fact, interactions between enterocyte surface glycan and VP7/VP4 RV proteins are crucial for infection[32], and glycoproteins have been characterized as active compounds of a fermented milk [23]. In consequence, it seems reasonable to think that some FM components could bind the virus. That is why we performed the blocking assay, which showed an inhibition of around 30% of virus detection in the presence of this FM.

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The GFA group showed a softer consistency of faeces throughout the study, which were even sometimes considered as diarrhoeic. This effect has already been described for some prebiotics, and it is actually useful to make formula-fed infants' faeces more similar to those from breast-fed infants and to reduce constipation[5,6,33]. However, the difficulty of differentiating the softer stool consistency induced by the probiotic from the RV-induced diarrhoea is a limitation of this suckling rat model. In spite of that, this diet showed clinical improvements similar to those of FM administration and in line with other studies, where a specific mixture of scGOS/lcFOS supplementation prevented infections in infants[5,13,14,34]. Although the faeces were scored in a

blinded manner, faecal weight seemed to be a more objective measure of the water content in the faeces. In this context, a reduction of faecal weight in the acute diarrhoea period in the group supplemented with GFA also evidenced the improvement induced by this diet. In addition, the faecal pH in the GFA group had an intermediate value between those of the REF and RV groups. This fact does not discard that this product may avoid the RV-induced electrolytic disorder, but a reduction of pH could be because of the pAOS own acidity and/or caused by scGOS/lcFOS directly. The increase of bacterial fermentation can lead to an increase of SCFAs production, and thus to a pH reduction. In fact, lower pH has been reported as a beneficial effect associated with prebiotic oligosaccharides[7,33]. In the group supplemented with GFA, the viral shedding was greatly reduced; in fact, it was comparable to that in the REF group. The blocking assay indicated a certain inhibition of the detection of the virus in the presence of GFA, similarly to FM, which may indicate a lower infection of the epithelial cells in the intestine and a lower replication. In this sense, acidic oligosaccharides are known to be able to interact with the epithelial surface and to prevent the pathogen adhesion[3].

Overall, both concepts tested showed protective effects against RV-induced diarrhoea in suckling rats. A possible mechanism responsible for the reduction of clinical signs produced by both concepts could be the direct interaction with viral particles and their blocking, avoiding the entry into enterocytes and, consequently, their replication. However, we cannot discard the possibility that the concepts could also interact with the intestinal microbiota, or directly with the cell surface, stimulating changes in the mucus and reinforcing the epithelial barrier.

Moving to the immune response against RV, this infection induces local and systemic, humoral and cellular immune responses. B cells presenting the $\alpha 4\beta 7$ homing integrin, and T CD4+ cells are crucial for the specific antibodies production, and T CD8+ cells constitute the cell-mediated immune response, which by means of inducing cytotoxicity shortens the infection and promotes viral clearance. Like most humoral immune responses, specific anti-RV antibodies are firstly IgM, and then IgG and IgA isotypes, being the levels of anti-RV IgA, either intestinal or systemic, better correlated with protection[35–37]. Nevertheless, the acquirement of protection against RV is not fully understood[35–37]. In the present study, the RV infection caused an increase of specific antibodies in serum and intestinal wash samples. The FM diet did cause some changes in anti-RV antibodies concentration. Some studies have suggested that the increase of intestinal IgA could be the mechanism of action of a fermented milk[26], but our results do not agree with that suggestion because intestinal IgA levels in the FM group were similar to those found in the RV group. On the

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other hand, the GFA diet's results suggest an enhancement in the early response as shown in intestinal IgA, which agrees with other studies showing that GFA activated the immune response[17–21] and which allows us to hypothesize that these antibodies may solve the infection rapidly, with fewer antibodies needed later on. This early increase in intestinal anti-RV IgA synthesis seems to have been enough to fight against the virus.

In conclusion, both microbial/prebiotic-related concepts tested here showed some beneficial effects on RV-induced diarrhoea in a neonatal rat model and can be considered for inclusion in infant formulas or supplements. Further studies are needed to understand their mechanism of action. A more in-depth investigation into the direct interaction of the products with the virus would be of interest, as well as their effect on the intestinal microbiota.

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Financial Support

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Conflict of Interest

The authors declare that they have a financial relationship with the organization that sponsored the research. K. van Limpt, K. Knipping, J. Garssen and J. Knol are employees of Nutricia Research B.V. The other authors declare that they have no conflict of interest.

Authorship

The authors' contributions were as follows: FJPC, AF, MC, KL, KK, JG and JK designed the study; MRA, TPB and SRR conducted the research and acquired data; FJPC and MRA reviewed data, interpreted results and drafted the manuscript; all the authors contributed to the critical review and revision of the manuscript.

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Tables

Table 1. Clinical variables determining diarrhoea process from day 7 to 13 of life. Results are expressed as mean and SEM or as median and IQR (n = 21 animals/group).

		RV		FM		GFA	
		Mean	SEM	Mean	SEM	Mean	SEM
Incidence	Maximum % of diarrhoeic animals	85·71		70·00		95·00	
	Diarrhoeic animals AUC	328·57		208·85		440·63	
	Normalized diarrhoeic animals AUC	328·57		178·97		232·26	
	Maximum % of diarrhoeic faeces	90·00		87·50		100·00	
	Diarrhoeic faeces AUC	362·06		274·68		511·67	
	Normalized diarrhoeic faeces AUC	362·06		183·33		290·61	
		Median	IQR	Median	IQR	Median	IQR
Duration	Beginning day	8·0	1·0	8·0#	0·0	8·0#	0·0
	Ending day	11·0	2·0	10·5	2·0	12·0#α	1·0
	Diarrhoea period	4·0	1·0	2·0#	2·0	5·0#α	1·0
	Days with diarrhoea	4·0	2·0	2·0#	1·0	5·0#α	1·0
Severity	Maximum diarrhoea index	3·00	0·25	2·74#	0·56	3·00	0·50
	Severity AUC	5·75	3·50	4·31#	1·75	6·88α	2·69
	Normalized severity AUC	5·75	3·50	3·60#	1·52	3·16#	1·98

AUC, area under the curve. Statistical significance: #p<0·05 *vs.* RV; αp<0·05 *vs.* FM

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Table 2. Specific anti-RV antibodies in serum and intestinal wash from 14- and 21-day-old rats. The values of each isotype in the RV group on day 14 are considered as 100 AU, and the results for the RV group on day 21 and for the other groups have been proportionally adjusted for each isotype. Results are expressed as mean and SEM (n = 9-12 animals/group) in AU/mL.

	Antibodies	Age (d)	RV		FM		GFA	
			Mean	SEM	Mean	SEM	Mean	SEM
Serum	Anti-RV Ig	14	100·00	17·75	154·69#	21·26	66·93#	18·48
		21	236·67	17·34	244·89	13·06	203·44	4·50
	Anti-RV IgG	14	100·00	13·51	88·16	47·74	38·77#	9·92
		21	879·79	227·12	1620·86#	117·94	86·20#	16·72
	Anti-RV IgM	14	100·00	24·96	162·35	41·96	65·85	10·52
		21	153·84	48·28	95·53	24·71	82·66	10·37
	Anti-RV IgA	14	100·00	5·03	74·62#	7·71	83·10	8·87
		21	106·61	7·60	127·43	9·77	118·41	9·63
Intestinal wash	Anti-RV IgA	14	100·00	14·94	91·07	18·61	182·54#	47·41
		21	239·22	32·33	195·90	22·30	166·92	41·06
	Anti-RV IgM	14	100·00	6·46	90·31	13·76	85·29	18·69
		21	143·64	10·89	132·56	19·81	101·33#	5·29

Statistical significance: #$p<0·05$ *vs.* RV at same time point

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Figure legends

Fig. 1 Body weight (g) during the study, before and after virus inoculation on day 7. Results are expressed as mean values ± SEM. Statistical significance is explained in the text (n=21 animals/group).

Fig. 2 Diarrhoea incidence. (A) Percentage of diarrhoeic animals. (B) Normalized percentage of diarrhoeic animals (subtracting the basal values due to intrinsic aspects of each treatment). (C) Severity of diarrhoea in a scale from 0 to 4. Scores of diarrhoea index ≥2 indicate diarrhoeic faeces. (D) Normalized severity of diarrhoea (subtracting the basal values due to intrinsic aspects of each treatment). Arrows in each graph indicate the RV inoculation day (day 7 of life). Results are expressed as mean ± SEM. Statistical significance is explained in the text (n=21 animals/group).

Fig. 3 Mean faecal weight (mg) from samples collected during the pre-inoculation period (before day 7) and the acute diarrhoea period (days 8-11) (A). Faecal pH from day 8-12 pooled samples (diarrhoea period) (B). Results are expressed as mean values of obtained samples from the 21 animals/group within the detailed period (n= 11-62 samples/group for faecal weight and n= 4-6 samples/group for pH) with their SEM. Statistical significance: *p>0·05 *vs.* REF, #p>0·05 *vs.* RV.

Fig. 4 Viral shedding curves of faecal samples. Results are expressed as mean values of obtained samples from the 21 animals/group within the detailed period (n= 11-62) with their SEM. Statistical significance: *p>0·05 *vs.* REF, #p>0·05 *vs.* RV.

Fig. 5 Percentage of inhibition of RV particle detection after incubation with FM and GFA at different dilutions of the product concentration used in the *in vivo* study. Results are expressed as mean with their SEM of duplicates from three independent experiments.

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Figures

Figure 1

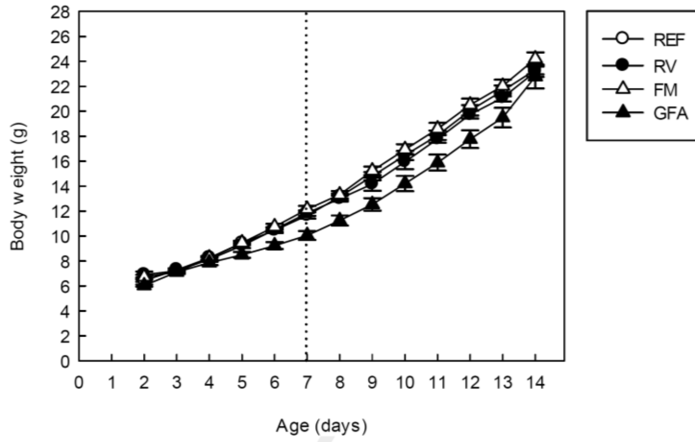

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Figure 2

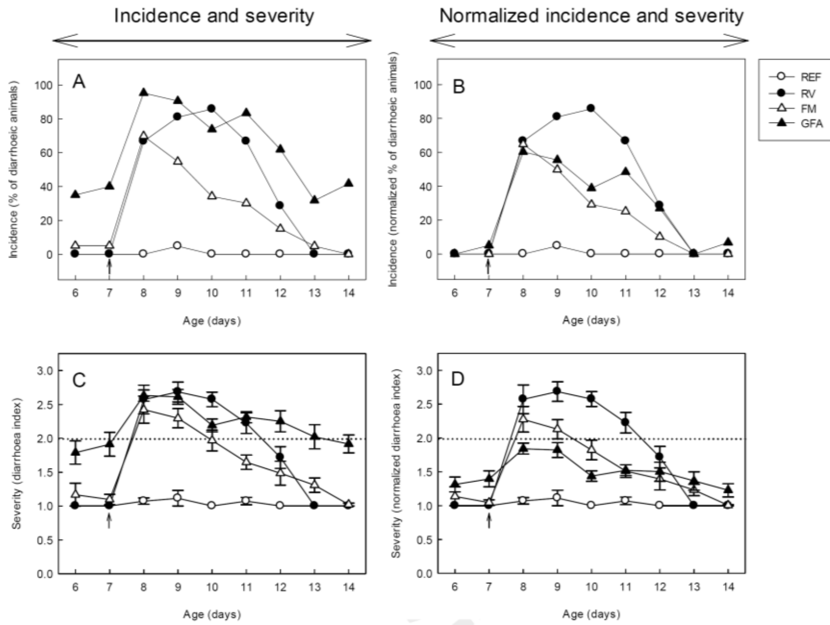

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Figure 3

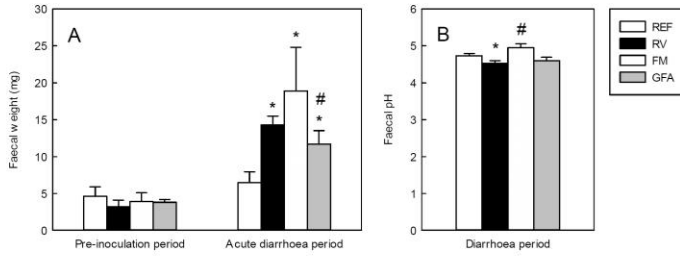

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Figure 4

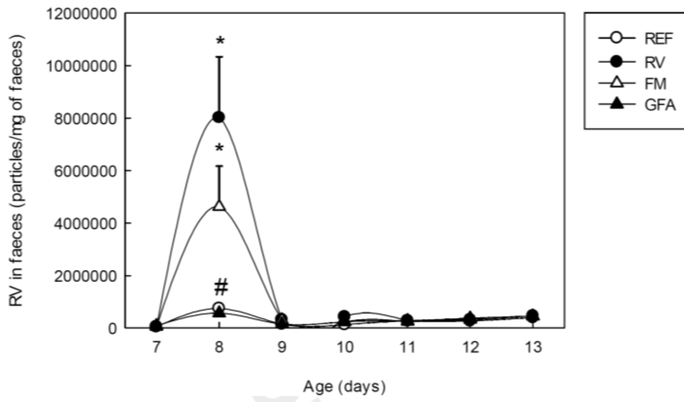

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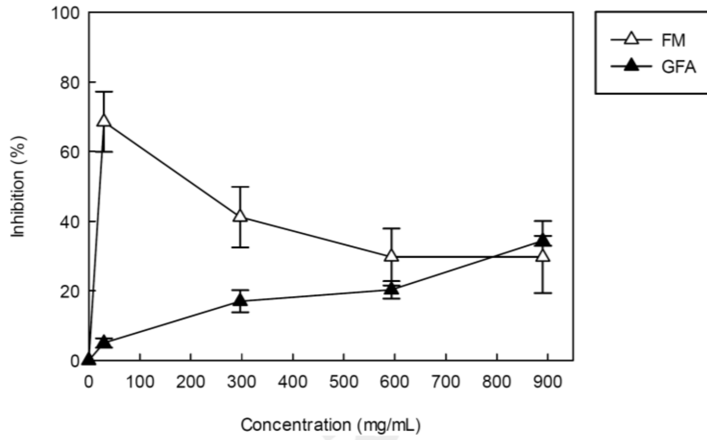
Figure 5



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Capítol 4

"Protection of rotavirus gastroenteritis by a combination of scGOS/lcFOS with a fermented milk concentrate in suckling rats"

Rigo-Adrover M, van Limpt K, Knipping K, Garssen J, Knol J, Franch À, Castell M, Pérez-Cano FJ

Manuscrit en preparació (resultats)

RESUM

Objectiu: Avaluar l'efecte protector de la combinació de galactooligosacàrids de cadena curta i fructooligosacàrids de cadena llarga (9:1) amb un postbiòtic a base d'una fórmula fermentada per *Bifidobacterium breve* i *Streptococcus thermophilus* en un model d'infecció per rotavirus en rata lactant.

Material i mètodes: Els animals van rebre oralment el suplement des del dia 3 fins el dia 21 de vida. El dia 7 van ser infectats amb el rotavirus SA11. Al llarg de l'estudi es van avaluar tant variables clíniques com la resposta immunitària.

Resultats: La intervenció va millorar les manifestacions clíniques, però una alteració en la consistència de les femtes induïda pel prebiòtic va dificultar l'observació d'aquest efecte. També es va observar una disminució de l'eliminació viral i una modulació de la resposta d'anticossos específics en sèrum i rentat intestinal a dies 14 i 21 de vida. Els resultats de l'assaig de bloqueig *in vitro* van suggerir que la combinació d'ambdós productes presenta una elevada capacitat per unir-se a les partícules virals, conduint a una infecció més moderada.

Conclusió: La suplementació diària amb la combinació del postbiòtic i dels galactooligosacàrids de cadena curta i fructooligosacàrids de cadena llarga (9:1) en aquest estudi ha mostrat efectes protectors contra la diarrea per rotavirus en aquest model preclínic.

Protection of rotavirus gastroenteritis by a combination of scGOS/lcFOS with a fermented milk concentrate in suckling rats

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Material and methods

Animals were administered daily by oral gavage from day 3 to day 21 of age with a microbial modulatory product (MMP group, n = 21), which consisted in a combination of short chain galactooligosaccharides/long chain fructooligosaccharides (scGOS/lcFOS, 9:1 ratio) with a fermented milk concentrate obtained by fermentation with *Bifidobacterium breve* C50 ($4.2x10^9$ bacteria per 100 g of powder formula) and *Streptococcus thermophilus* 065 ($3.84x10^7$ bacteria per 100 g of powder formula) during the manufacturing process and then heated to kill ferments (patented technology, Blédina, France) in a dose of 2.24 g of mixture/100 g of body weight/day consisting in a daily administration of 16.85 µL/g. The animals from the reference (REF) group and rotavirus-inoculated (RV) group (n = 21/group) are the same as those of previous papers (Rigo-Adrover et al., 2016, 2017). The experimental proceedings are the same as those explained in the previous study (Rigo-Adrover et al., 2017) (**Fig. 1**).

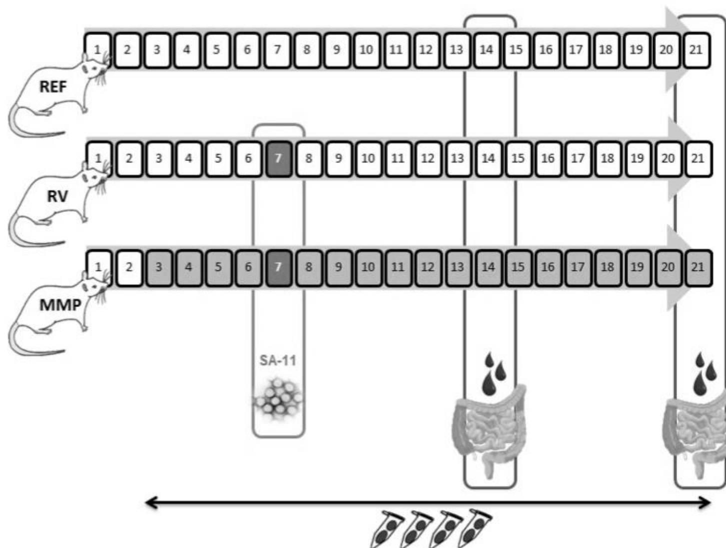

Fig. 1 Representation of the experimental design. Animals from the RV and SP groups were inoculated with SA-11 at day 7 of life. The SP group received a supplementation from day 3 to day 21. Faecal samples were collected during the study period and blood and intestinal samples were obtained on the sacrifice days (day 14 and 21).

Results

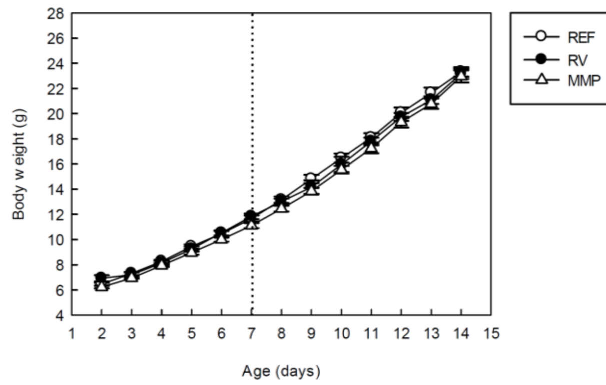

Fig. 2 Body weight (g) during the study, before and after virus inoculation on day 7. Results are expressed as mean values ± SEM (n=21 animals/group).

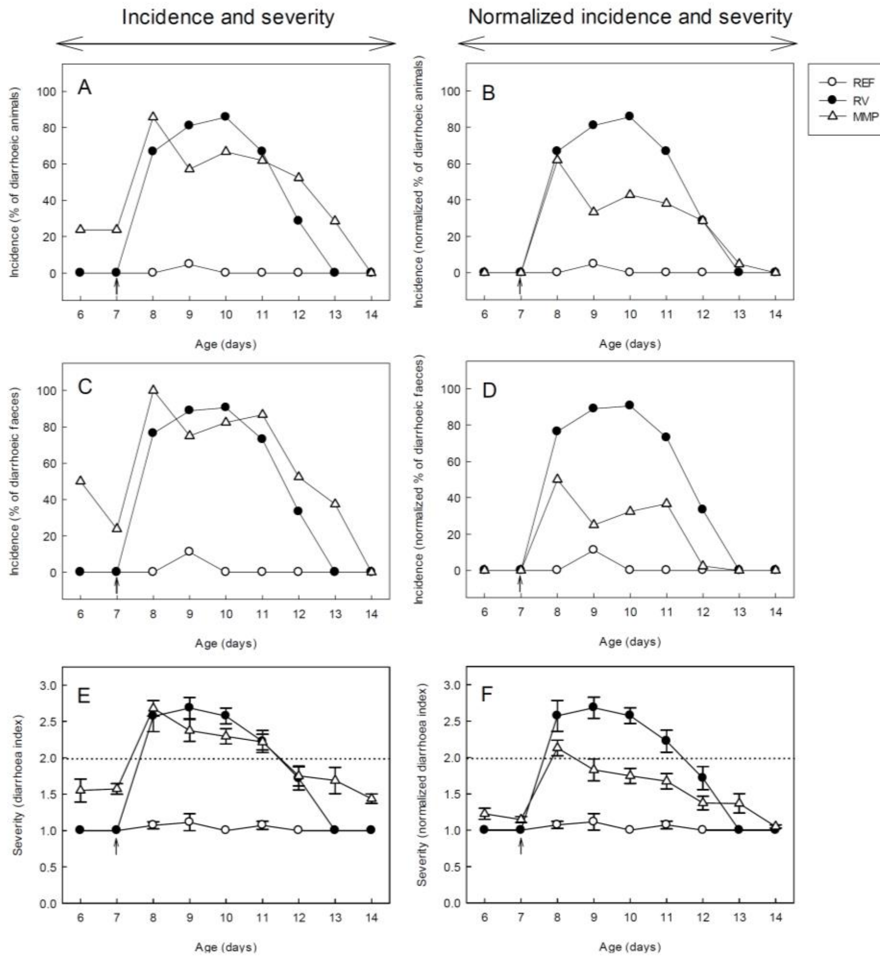

Fig. 3 Diarrhoea incidence and severity. (A) Percentage of diarrhoeic animals. (B) Normalized percentage of diarrhoeic animals (subtracting the basal values due to intrinsic aspects of each treatment). (C) Percentage of diarrhoeic faeces. (D) Normalized percentage of diarrhoeic faeces (subtracting the basal values due to intrinsic aspects of each treatment). (E) Severity of diarrhoea in a scale from 0 to 4. Scores of diarrhoea index ≥2 indicate diarrhoeic faeces. (F) Normalized severity of diarrhoea (subtracting the basal values due to intrinsic aspects of each treatment). Arrows in each graph indicate the RV inoculation day (day 7 of life). Results are expressed as mean ± SEM (n=21 animals/group).

Table 1. Clinical variables determining diarrhoea process from day 7 to 13 of life. Results are expressed as mean and SEM (n = 21 animals/group).

		RV		MMP	
Incidence	**Maximum % of diarrhoeic animals**	85.71		90.00	
	Diarrhoeic animals AUC	328.57		350.00	
	Normalized diarrhoeic animals AUC	328.57		207.14	
	Maximum % of diarrhoeic faeces	90.00		100.00	
	Diarrhoeic faeces AUC	362.06		432.98	
	Normalized diarrhoeic faeces AUC	362.06		107.86	
		Mean	**SEM**	**Mean**	**SEM**
Duration	**Beginning day**	8.4	0.1	8.1	0.1
	Ending day	11.0	0.2	11.8#	0.3
	Diarrhoea period	3.6	0.2	4.5#	0.4
	Days with diarrhoea	3.3	0.2	3.5#	0.3
Severity	**Maximum diarrhoea index**	3.05	0.11	2.78	0.12
	Severity AUC	9.00	0.25	5.70	0.46
	Normalized severity AUC	5.99	0.47	3.27#	0.34

AUC, area under the curve. Statistical significance: #$p<0.05$ *vs.* RV

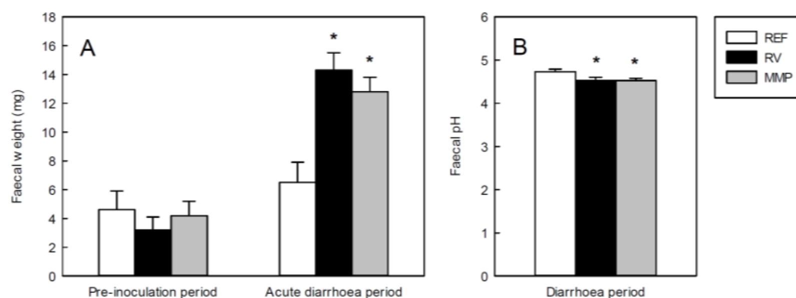

Fig. 4 Mean faecal weight (mg) from samples collected during the pre-inoculation period (before day 7) and the acute diarrhoea period (days 8-11) (A). Faecal pH from day 8-12 pooled samples (diarrhoea period) (B). Results are expressed as mean values of obtained samples from the 21 animals/group within the detailed period (n= 66-77 samples/group for faecal weight and n= 16-23 samples/group for pH) with their SEM. Statistical significance: *p>0.05 *vs.* REF.

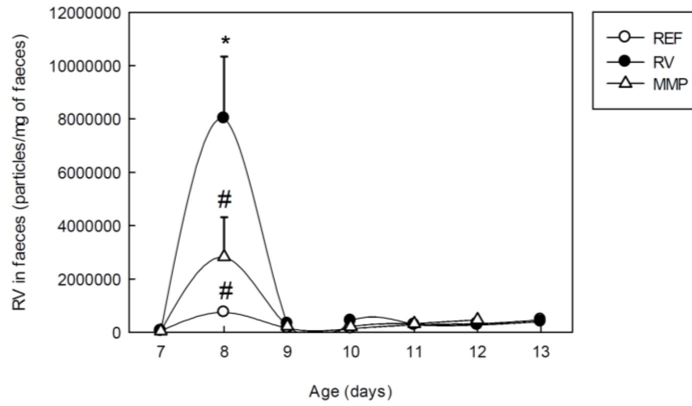

Fig. 5 Viral shedding curves of faecal samples. Results are expressed as mean values of obtained samples from the 21 animals/group within the detailed period (n= 27-63) with their SEM. Statistical significance: *p>0·05 *vs.* REF, #p>0·05 *vs.* RV.

Table 2. Specific anti-RV antibodies in serum and intestinal wash from 14- and 21-day-old rats. The values of each isotype in the RV group on day 14 are considered as 100 AU, and the results for the RV group on day 21 and for the other group have been proportionally adjusted for each isotype. Results are expressed as mean and SEM (n = 9-12 animals/group) in AU/mL.

	Antibodies	**Age**	**RV**		**MMP**	
		(d)	**Mean**	**SEM**	**Mean**	**SEM**
Serum	Anti-RV Ig	14	100.00	17.75	126.04	11.88
		21	236.67	17.34	212.55	6.48
	Anti-RV IgG	14	100.00	13.51	235.26#	33.24
		21	879.79	227.12	64.44#	12.79
	Anti-RV IgM	14	100.00	24.96	163.00	34.15
		21	153.84	48.28	190.09	29.62
	Anti-RV IgA	14	100.00	5.03	70.04#	11.59
		21	106.61	7.60	87.65	7.65
Intestinal wash	Anti-RV IgA	14	100.00	14.94	178.17	23.50
		21	239.22	32.33	119.61	20.53
	Anti-RV IgM	14	100.00	6.46	97.13	10.04
		21	143.64	10.89	105.55#	6.70

Statistical significance: #$p<0.05$ *vs.* RV at same time point

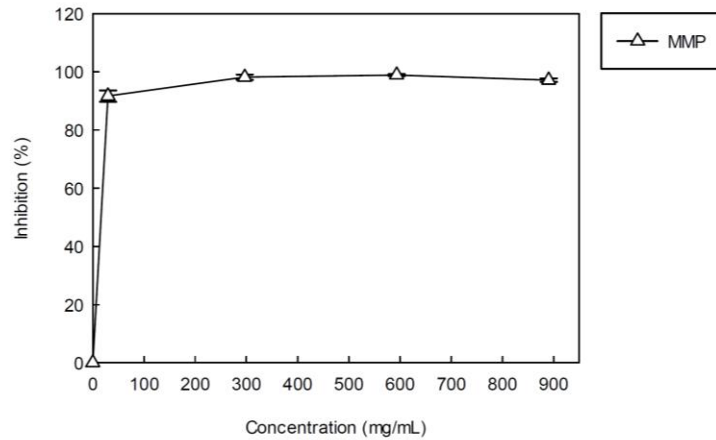

Fig. 6 Percentage of inhibition of RV particle detection after incubation with MMP at different dilutions of the product concentration used in the *in vivo* study. Results are expressed as mean ± SEM of duplicates from three independent experiments.

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Rigo-Adrover, M. del M., Pérez-Berezo, T., Ramos-Romero, S., van Limpt, K., Knipping, K., Garssen, J., Knol, J., Franch, À., Castell, M., Pérez-Cano, F.J., 2017. A fermented milk concentrate and a combination of scGOS/lcFOS/pAOS protect suckling rats from rotavirus gastroenteritis. Br. J. Nutr. In press.

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Capítol 5

"Towards a human-like rotavirus gastroenteritis model: double infection in suckling rats"

Maria del Mar Rigo-Adrover, Karen Knipping, Johan Garssen, Sandra Saldaña-Ruíz, Àngels Franch, Margarida Castell, Francisco José Pérez-Cano

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Els resultats del present article han estat presentats a les següents comunicacions:

- *Study of immune anti-rotavirus response in a double infection rat model*. VIIè congrés de la Societat Catalana d'Immunologia (SCI) (Barcelona, Espanya), novembre 2013. Premi al millor pòster del congrés 2013.

- *Development of a rotavirus double infection model in suckling rats*. 10th European Mucosal Immunology Group Meeting (Copenhaguen, Dinamarca), octubre 2016.

RESUM

Objectiu: Desenvolupar un model de doble infecció per rotavirus en rata, que mimetitzi la realitat clínica i que sigui útil per avaluar la possible modulació per part de compostos nutricionals sobre la gastroenteritis i la resposta immunitària induïda per rotavirus.

Material i mètodes: Es van portar a terme tres dissenys experimentals amb l'objectiu d'aconseguir un comportament diferencial en animals doble infectats respecte als animals infectats només amb un rotavirus. Es van fer servir dos rotavirus del grup A amb elevada reactivitat creuada (SA11 i EDIM). Es va fer un seguiment de la progressió d'ambdues infeccions mitjançant l'avaluació de variables clíniques, el pes corporal, el pH fecal, la temperatura corporal i les respostes d'anticossos anti-RV i DTH.

Resultats: Es va observar que l'edat d'inoculació i el deslletament prematur eren punts clau pel desenvolupament del model de doble infecció. Només es va observar diarrea durant la primera infecció, acompanyada d'una reducció del pH fecal i de febre. Després de la segona infecció també es va trobar un increment de la temperatura corporal, però no cap altra manifestació clínica. La resposta immunitària contra la segona infecció està modulada per la primera infecció, en termes d'anticossos específics i resposta DTH.

Conclusió: S'ha aconseguit desenvolupar un model de doble infecció per rotavirus en rata, adequat per a dur a terme estudis intervencionals. L'acció moduladora de la primera infecció sembla ser rellevant en la resposta immunitària de futures infeccions.

1 *Article*

2 Towards a human-like rotavirus gastroenteritis 3 model: Double infection in suckling rats

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15 Received: date; Accepted: date; Published: date

16 **Abstract:** Rotaviruses (RV) are the most common cause of acute diarrhoea among children under the
17 age of five worldwide. This study aimed to set up a double-infection model in rat which mimics the
18 clinical reality and would be useful to test a possible modulation by nutritional compounds on the
19 RV-associated disease and immune response. Three experimental designs were conducted in order
20 to achieve a differential behaviour in the double-infected animals compared to the single-infected
21 ones. Two RV of the group A – SA11 and EDIM – with high cross-reactivity were inoculated. Clinical
22 variables, body weight, faecal pH and body temperature changes, anti-RV antibodies and DTH
23 response were determined to assess the infection progression during both the first and the second
24 infection. Early inoculation and early weaning were critical for the successful development of the
25 double-infection model. Diarrhoea was only observed after the first infection, with a reduction of
26 faecal pH and fever. After the second infection an increase in body temperature was also found, but
27 no other clinical symptoms. The immune response against the second infection is modulated after
28 the first infection in terms of specific antibodies and DTH. A rotavirus double-infection rat model
29 has been developed and it is suitable to be used for future studies, such as nutritional interventions.
30 Dietary modulatory actions of first infection would be relevant for the immune response thereafter.

31

32 **Keywords:** rotavirus; diarrhoea; rat; model; double-infection
33

34 1. Introduction

35 Diarrhoea is the fourth cause of mortality among children under the age of five worldwide [1],
36 and rotavirus (RV) is the aetiological agent responsible for 37 % of these deaths [2]. Almost every
37 child in the world will be infected with RV in the first 3 years of life [3], however, it can also infect
38 adults [4], and the presence of RV particles in extra-intestinal tissues has also been reported [5–8]. RV
39 belongs to the family *Reoviridae*, which are non-enveloped, icosahedral, double-stranded RNA
40 viruses covered by triple-layer capsids. Their viral genome encodes for six structural proteins (VPs)

and six non-structural proteins (NSPs) [6,9–11]. Group A RV are the main human pathogens and their transmission is faecal-oral, with a higher prevalence in winter. They infect the mature absorptive enterocytes of the small intestine, even though the exact entry mechanism is still unknown [12]. The main symptoms are fever, vomiting, abdominal cramps and diarrhoea in children, lasting for 3 to 8 days [6,13], and the virus can be spread from 2 days before and up to 8 days after the onset of diarrhoea. Oral rehydration is the most usual treatment [14,15].

Immunity after an RV infection is incomplete and several re-infections usually occur, which tend to be less severe than the first [13]. Innate and adaptive immune responses are induced by RV infections, including cytokine and specific antibody (Ab) production [16,17]. The initial step of the immune response is the antigen presentation to T and B lymphocytes by macrophages and dendritic cells (DC). DC seem to be crucial for cellular response activation and represent a link between innate and adaptive immunity [18]. Natural killer (NK) lymphocytes constitute the first line of defence against the virus and destroy infected cells. T cells also lyse infected cells and produce cytokines. Finally, B cells produce Ab, locally and systemically, and are required for long-term protection [19]. Protection against RV seems to be positively correlated with IgA [20,21], although IgG and IgM also confer certain protection [22].

The introduction into routine of two oral vaccines against RV (RotaTeq, Merck & Co and Rotarix, GSK Biologicals) has shown a reduction of the health burden of severe childhood diarrhoea [2,9], but their implementation is still not as large as had been expected, due to cost and refrigerated storage requirements, among others [3,15]. Thus, it is necessary to develop alternative approaches to control RV disease.

Interventional studies in humans, and particularly in infants, present certain difficulties. For this reason, several animal models have been used to better understand RV pathology and infection, and also to study the vaccines' efficacy or the modulation of the disease course by a dietary intervention in early life with bioactive compounds (such as whey proteins, prebiotics and probiotics) [23–26]. Most of the studies have focused on the evaluation of clinical markers, such as the incidence and severity of diarrhoea, while others have also studied the presence of the virus in serum, tissues and/or faeces, and evaluated the immune function by titres of Ab, among others [25]. In the RV models context, large animals (such as cow, pig and sheep) have been used as experimental subjects, although these studies involve high costs and require of long periods of study. For example, the severe diarrhoea obtained usually in the RV suckling mouse models makes the observation of the benefits of certain nutritional interventions difficult. However, the neonatal rat model, with a more moderate severity of disease [27,28], has demonstrated to be suitable for immunonutrition studies and has substantial scientific evidence and a cost-effective ratio [24].

Most RV animal models include just one RV infection (i.e. single-infection models), but due to the frequency of re-infection in human infants, a double-infection model would provide added information. At present, rabbit, mouse and calf RV double-infection models already exist [29–33], but not a rat model. In the double-infection models, it would also be interesting to evaluate in depth the differential response of both infections together compared to the single ones, and even how the protection against the first infection, i.e. by a nutritional intervention, will affect the second infection, mainly in terms of immune response. Therefore, the aim of the present study was to set up and characterize a neonatal rat double RV infection model suitable for further interventional studies.

2. Materials and Methods

2.1. Animals

G14 pregnant Lewis rats were obtained from Harlan (Barcelona, Spain) and Janvier Labs (La Plaine Saint Denis Cedex, France). They were housed in individual cages, monitored daily and

91 allowed to deliver at term. The day of birth was registered as day 1 of life. Pups had free access to the
92 nipples and rat diet. The animals were housed under controlled temperature and humidity
93 conditions, in a 12:12 h light/dark cycle. They were located in a special safe isolated room at the
94 Animal Service of the Faculty of Pharmacy, UB, designed and authorized for working under
95 biosecurity level 2 conditions. Dams were fed with a commercial diet corresponding to the American
96 Institute of Nutrition 93M formulation and given water *ad libitum*. The RV were intragastrically
97 inoculated as previously described [28], 1 h after separation from their dams to avoid interference
98 between RV and milk components in the stomach.
99
100 The studies were approved and conducted in accordance with the institutional guidelines for
101 the care and use of laboratory animals as established by the Ethical Committee for Animal
102 Experimentation of the UB and the Catalonia Government (CEEA-UB Ref.493/12, DAAM: 6905 for
103 studies in rats and CEEA-UB Ref.494/12, DAAM: 6875 for the studies in mice for virus *in vivo*
104 production).

105 *2.2. Viruses*

106 Two different type-A viruses have been used for the experiments: the simian agent 11 (SA11)
107 and the epizootic-diarrhoea infant-mouse virus (EDIM). The virus selected for the first infection in
108 the model was the SA11, an RV strain produced by the 'Enteric Virus Group' of the UB as in
109 previous studies [23,26,28]. The virus selected for the second infection in the model was the EDIM
110 strain, which is not able to grow in cell culture. For that, in order to obtain enough quantity of EDIM,
111 an *in vivo* obtainment design was developed using an initial inoculum, which was a kind gift from
112 Karen Knipping, Nutricia Research, The Netherlands. Neonatal BALB/c mice from 6 litters (n=31)
113 (Janvier) were inoculated at the age of 3 days with 5 μL of EDIM 0.9×10^8 viral particles/mL. Stool
114 samples were collected from day 4 to day 15, pooled and homogenized using the Polytron®
115 (Kinematica, Luzern, Switzerland). EDIM was extracted with Genetron®
116 (1,1,2-trichloro-1,2,2-trifluoroethane, Sigma-Aldrich, Madrid, Spain) [33]. The quantification of
117 EDIM particles was performed by ELISA (1.3×10^8 viral particles/mL) as described in previous
118 studies [33]. Its infectivity was later confirmed in mice: 5 μL of the new EDIM stock were inoculated
119 to 3-day-old BALB/c mice from 3 litters (n=15), causing diarrhoea in all the animals (100 % of
120 incidence), between day 4 and 14 of life. Moreover, at the age of 21 days, splenocytes were isolated
121 from some animals to test their specific proliferative response, which was significantly positive
122 against RV particles (data not shown).

123 *2.3. Experimental designs*

124 Several experimental designs (ED) were conducted to select the optimal conditions for the
125 double RV infection (DRI) model. SA11 was selected as a first infective virus because previous
126 studies allowed us to obtain a rat model of mild diarrhoea in early life. EDIM was used as second
127 infective virus, and as no previous literature about this RV strain in rats was found, a preliminary
128 study was designed to confirm the infectivity of EDIM in early-life rats (**Table S1 and Figure S1**).
129 Moreover, the cross-reactivity against both types of viruses was also confirmed by means of ELISA
130 and ELISPOT for anti-RV Ab levels and secreting cells (SC) quantification in infected rats,
131 respectively (**Figure S2**).
132
133 We established three critical points to organize these designs. Key criteria to consider; the first
134 infection was always performed in the first week of life (between day 6 and day 7), as our previous
135 studies demonstrated to us that later in life no clinical symptoms are obtained [28]; the second
136 infection was induced early in the third week of life (between days 16–18) in order to try to induce
137 infection when the intestinal immune system is still in maturation [34]. Finally, conscious of the
138 importance of the bioactive factors present in breast milk in protecting the babies from infection, the
139 weaning day was either physiologically performed on day 21 or, in order to induce a lower

140 defensive situation in the babies, carried out the same or the previous day of the second infection.
141 The ED's nomenclature indicates the pattern of weaning with respect to the day of the second
142 infection in the DRI groups of each design: ED normal weaning (NoW), ED same day weaning
143 (SDW) and ED day before weaning (DBW).
144
145 In all of them, the DRI groups of rats were inoculated with SA11 (~$1.8x10^8$ $TCID_{50}$ RV/rat in 100
146 μL of phosphate-buffered solution [PBS]) at day 6–7 of life. The second inoculation was induced
147 with 100 μL of EDIM (~$1.3x10^8$ RV/mL) at days 16–18 of life in still suckling rats or early weaned
148 (same or previous day) rats in order to obtain differential second-infection patterns.
149
150 Each one of the ED was composed of four different experimental groups: a non-inoculated
151 reference (REF) group; a single infected with SA11 (SA11) group; a single infected with EDIM
152 (EDIM) group; and a double RV infection (DRI) group infected with both SA11 first and later with
153 EDIM (n = 2 dams/group with 6-9 pups each dam).
154
155 In addition to these ED, an extra group was added to be used as a control group of protection
156 during first infection. This group was constituted by DRI animals, which received 'anti-RV
157 hyperimmune bovine colostrum' (HBC, kindly provided by Dr Viviana Parreño, Institute of
158 Virology, CICV and A-INTA, Castelar, Argentina) in a concentration of 50 mg/day previous to and
159 during first infection (from day 5 to day 13). This HBC was titred to be effective in blocking the virus
160 *in vitro* in concentrations higher than 10 μg/mL.
161
162 Clinical evaluation was performed daily from the day before the first inoculation until the end
163 of the study. Faecal samples were collected daily during the study and blood samples on day 28,
164 when animals were euthanized (by cardiac puncture and exsanguination after ketamine/xylazine
165 injection [Imalgene 100 mg/mL, Merial Laboratorios, Barcelona, Spain / Rompun® 20 mg/mL, Bayer
166 Hispania, Sant Joan Despí, Spain]). Faecal pH and body temperature were measured after the
167 infections. The delayed-type hypersensitivity (DTH) response and specific anti-RV Ab in sera were
168 determined at the end of the study.

169 *2.4. Clinical indexes and faecal specimen collection*

170 SA11 and EDIM infections were evaluated at 1 to 10 days post-inoculation (DPI) by the growth
171 rate and clinical indexes that require daily faecal sampling as previously described [28]. Faecal
172 sampling was performed once a day by gently pressing and massaging the abdomen. Specimens
173 were immediately scored for severity from 1 to 4 (diarrhoea index [DI]), weighed and frozen at -20
174 ºC for further analysis. The DI is based on colour, texture and amount as described: normal (1); loose
175 yellow-green (2); totally loose yellow-green (3); high amount of watery (4) faeces. Diarrhoea scores ≥
176 2 indicate diarrhoeic faeces, whereas scores of DI < 2 indicate absence of diarrhoea. Incidence of
177 diarrhoea was expressed by the percentage of diarrhoeic animals (%DA, consisting of the percentage
178 of animals with diarrhoeic faeces taking into consideration the number of animals in each group)
179 and by percentage of diarrhoeic faeces (%DF, consisting of the percentage of diarrhoeic samples
180 taking into consideration the number of total samples collected every day in each group).

181 *2.5. Faecal pH and body temperature determination*

182 pH from faecal diluted samples (up to 200 mg/mL in distilled water) from the peri-inoculation
183 period was measured by a pH electrode for surfaces 5207 and pH-meter micropH 2001 (Crison
184 Instruments, Barcelona, Spain). Body temperature was measured with the TEMP JKT thermometer
185 (Oakton, Vernon Hills, IL, USA) and the RET-3-ISO rectal probe for neonatal rats (Physitemp,
186 Clifton, NJ, USA). This measure was taken during the peri-inoculation period of the virus. Results
187 were expressed as the relative increase of temperature compared to the temperature the day before
188 the inoculation as basal.

189 *2.6. ELISA for specific anti-RV total antibody quantification in serum*

190 Ninety-six-well plates were coated with UV-inactivated SA11 particles at 10^5/mL, and after
191 blocking and incubating the sample in appropriate dilution, a rabbit peroxidase-conjugated anti-rat
192 Ig (Dako, Barcelona, Spain) was used for detection, as in previous studies [28]. Pooled sera from
193 dams of inoculated litters were used as standard in each plate.

194 *2.7. Delayed-type hypersensitivity*

195 One day before sacrifice, the thickness of both the right and left ears of every animal were
196 measured to constitute the basal values, using a pocket thickness gauge 7309 (Mituyoto, Hampshire,
197 UK). For virus priming, animals were anaesthetized with isofluorane (Abbott Laboratories,
198 Berkshire, UK) and a volume of 20 µL of UV-inactivated virus (~0.5×10^6 RV particles/mL) was
199 injected into the right ear (RE) and the same volume of PBS was injected into the left ear (LE). After
200 24 h and prior to sacrifice, an evaluation of the ear thickness was performed again. Results are
201 expressed as the increase of thickness (in mm) of the RE subtracting the increase of the thickness of
202 the LE (in mm).

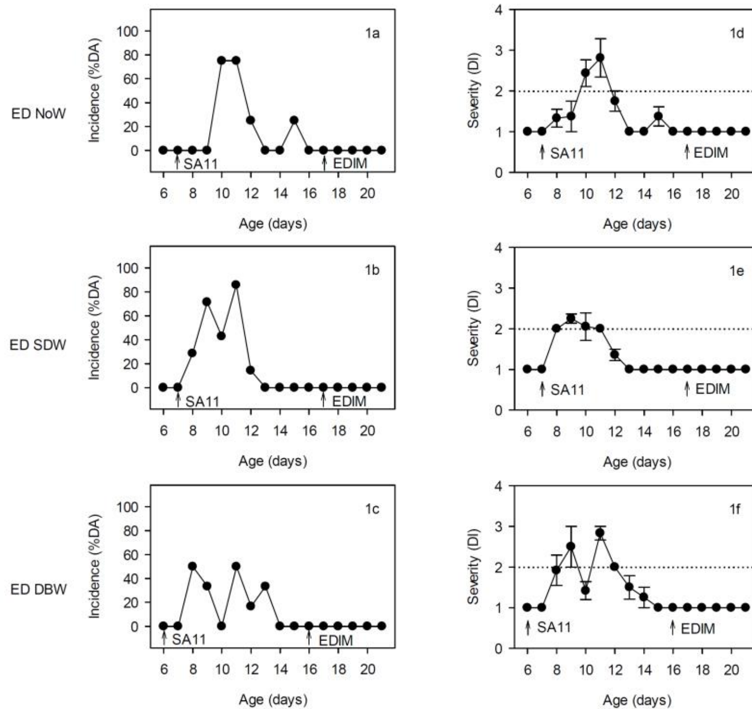
203 *2.8. Statistical analysis*

204 The PASW Statistics 22 software package (SPSS Inc, Chicago, IL, USA) was used for the
205 statistical analysis. Komolgorov-Smirnov test was applied to assess normal distribution, followed by
206 Levene's test in order to determine variance equality. Conventional one-way ANOVA test was
207 performed considering the experimental group as the independent variable. The results from
208 different groups were sometimes pooled for a unique variable (i.e. SA11-infected rats from different
209 groups vs. non-infected rats). When virus inoculation had a significant effect on the dependent
210 variable, Scheffé's *post hoc* test was applied. Kruskal-Wallis and Mann-Whitney U tests were used
211 when non-normal distribution or different variance were found. Finally, chi-square test was used to
212 compare frequencies. Differences were considered significant at P values of <0.05. All the results are
213 expressed as mean ± SEM of n animals.

214 **3. Results**

215 *3.1. Clinical variables and body weight during first RV infection*

216 First infection with SA11 during the first week of life induced diarrhoea in all the inoculated
217 groups in the three ED conducted here, as can be observed from the incidence (%DA and %DF) and
218 severity (DI) data. In **Figure 1**, only data from the DRI group of each ED is represented, but results
219 from the single-infected group (SA11 group) of each ED followed a similar pattern than that in the
220 DRI group. The %DA in the DRI group appeared from 1–3 DPI in all ED, reaching up to 50–100 %
221 between 1 and 4 DPI, and none of the animals in these ED still had diarrhoea after 8 DPI. All ED
222 showed a biphasic disease with two peaks of infection (**Figure 1a-c**). Overall, 70–100 % of the
223 SA11-infected animals developed diarrhoea at some point. The results corresponding to the %DF
224 followed a similar pattern as the %DA, achieving in all ED a %DF of 100 % at some point (data not
225 shown).



226

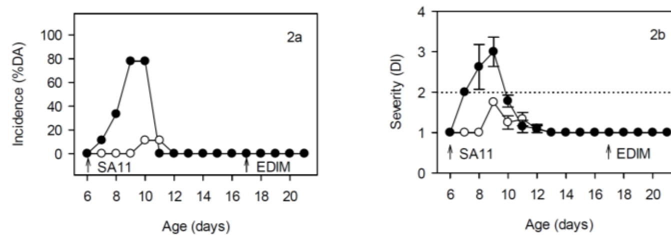
227 **Figure 1.** Evaluation of the incidence (**1a–1c**) and severity of diarrhoea (**1d–1f**) of the DRI groups of
228 the different experimental designs (ED). Incidence is expressed as % of diarrhoeic animals (%DA)
229 and severity as mean ± SEM of the diarrhoea index (DI) (n = 6–12 animals/group). Arrows in each
230 graph indicate the RV inoculation day, first with SA11 and second with EDIM.

231 With regard to the severity, the SA11-inoculated groups in all ED developed mild diarrhoea
232 (**Figure 1d-f**). The maximum diarrhoea index ranged from 2.25–3 in all cases and it was achieved
233 around 2–4 DPI.
234
235 In all ED the diarrhoea was also objectivized by a faecal weight increase during the 4 days after
236 infection when compared to REF animals without differences among ED. The average weight of the
237 faecal output during this period was of 12.05 ± 1.71 g (mean ± SEM) in the SA11-infected groups
238 from all ED, whereas in the REF group it was 4.05 ± 0.94 ($p<0.05$). Finally, the viral infection and the
239 consequent diarrhoea did not significantly affect the growth curve of the animals in any of the three
240 ED, the overall body weight increase between d4 and d14 being about 190.44 ± 7.67 % in the REF
241 groups and 177.82 ± 3.35 % in the SA11-infected groups.

242 *3.2. HBC protection during first infection*

243 The intervention with the HBC from day 5 to day 13 significantly reduced the incidence and
244 severity of diarrhoea. For instance, a maximum of 11 %DA and 17 %DF on days 4–5 DPI in the HBC
245 group was achieved, whereas in SA11-infected animals, the values were of about 80 % and 100 %,

246 respectively (**Figure 2a**). Moreover, the severity of the few animals that developed diarrhoea was
247 also controlled, with a maximum diarrhoea index of 1.75 ± 0.00 on 3 DPI (**Figure 2b**), a value lower
248 than that of the SA11-infected rats (3.00 ± 0.36, $p<0.05$). The duration of the disease was also greatly
249 shortened (the diarrhoea period of the SA11 group was 1.67 ± 0.30 days and that of the HBC group
250 was 0.22 ± 0.15 days). Faecal weight during the diarrhoea period (d7–10) was also significantly
251 reduced in comparison with the SA11-infected animals, achieving values of 4.64 ± 0.99 g ($p<0.05$ vs.
252 SA11). Body weight was not affected due to the HBC intervention.



253

254 **Figure 2.** Evaluation of the incidence (**2a**) and severity of diarrhoea (**2b**) of DRI animals (black circles)
255 and DRI animals which have received an 'anti-RV hyperimmune bovine colostrum' (HBC)
256 supplement (white circles). Incidence is expressed as % of diarrhoeic animals (%DA) and severity as
257 mean ± SEM of the diarrhoea index (DI) (n = 9 animals/group). Arrows in each graph indicate the RV
258 inoculation day, first with SA11 and second with EDIM.

259 *3.3. Clinical variables and body weight after second RV infection*

260 Second infection with EDIM during the third week of life failed to induce diarrhoea in all the
261 inoculated groups – both the single (EDIM group, data not shown) and the double-infected (DRI
262 group) groups – in the three ED conducted here, as can be observed from the incidence and severity
263 data (**Figure 1**).
264

265 Moreover, there were no differences in faecal weight among groups after the second infection
266 (data not shown). The absence of diarrhoea after the EDIM inoculation, either in the single or the
267 double context, is parallel with the absence of changes in the body weight gain during this period,
268 being similar to those animals that did not receive any virus in this period (the body weight increase
269 between d6 and d27 was 367.58 ± 6.54 % in the REF groups and 353.66 ± 9.18 % in the SA11 groups).
270 Conversely, the early weaning in the ED SDW and ED DBW avoided the physiological increase of
271 body weight during the 2–3 days after separation from their dams. As expected, due to the
272 age-dependent insusceptibility to RV, no clinical data evidenced the second infection with EDIM
273 and therefore new variables were included in the study.

274 *3.4. Faecal pH after RV infections*

275 The pH of faecal samples, as a possible marker of gastrointestinal alteration, from 0–5 DPI was
276 measured after the onset of both RV infections. Samples from SA11-infected animals (pooled results
277 of SA11 and DRI groups of each ED) showed a significantly lower faecal pH in two of the three ED
278 during the acute diarrhoea period compared to the REF group (**Table 1**). The faecal pH of the HBC
279 supplemented animals was also similar to that in the RV group (data not shown). The faecal pH in
280 the peri-infection period of the second infection with EDIM was also measured and, as expected due
281 to the lack of changes in the faecal score, no differences were found between the infected groups and
282 the REF group (data not shown).

283 **Table 1.** Faecal pH in the peri-inoculation period (first infection). Results are expressed as mean ±
284 SEM (n = 4–8 samples/group).

	1st infection	
	REF	**SA11**
ED NoW	4.83 ± 0.05	4.56 ± 0.03*
ED SDW	5.07 ± 0.16	4.48 ± 0.07*
ED DBW	4.36 ± 0.05	4.76 ± 0.21

285 *p<0.05 vs. REF group.

286 *3.5. Body temperature after RV infections*

287 Rats' body temperature in both SA11 and EDIM infections was monitored from 0–5 DPI as a
288 possible marker of disease. The relative increase of temperature for the maximum value obtained
289 after infection with respect to the 0 DPI value was calculated for each individual only in weaned
290 animals' designs (SDW and DBW), because temperature from suckling pups -in touch with their
291 mother (NoW)- cannot be obtained (**Table 2**). After the first infection with SA11, an increase of rectal
292 temperature was observed among the infected animals when compared to the REF group, only
293 being significant in the case of ED SDW (p<0.05). Even though diarrhoea was not observed in the
294 second infection, an increase of body temperature was found in both the EDIM and the DRI groups
295 (p<0.05 vs. REF group in ED SDW and DBW), suggesting the presence of infection. On the other
296 hand, the HBC supplementation did not have any effect on the temperature, either after the first
297 infection or later in the second infection (data not shown).

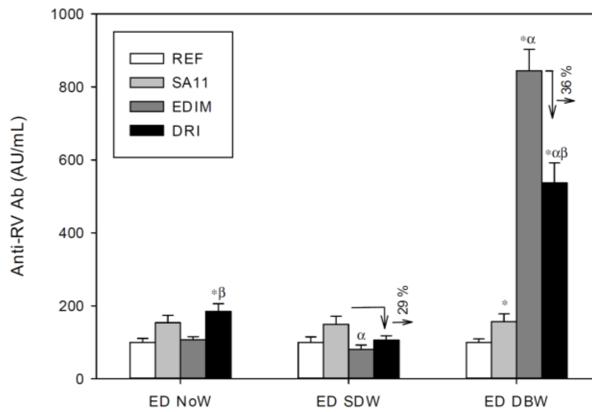
298 **Table 2.** Rectal temperature of the animals in the peri-inoculation period (first and second infection).
299 Results are expressed as mean ± SEM (n = 6–12 animals/group) of the relative increase of temperature
300 with respect to the temperature the day before virus inoculation (basal value).

	1st infection (2 DPI)		**2nd infection (1 DPI)**		
	REF	**SA11**	**REF**	**EDIM**	**DRI**
ED SDW	0.47 ± 0.32	4.35 ± 1.22*	0.12 ± 0.12	4.40 ± 0.77*	3.60 ± 0.42*
ED DBW	0.40 ± 0.32	0.83 ± 0.46	3.24 ± 0.81	5.53 ± 0.38*	7.50 ± 0.72*

301 *p<0.05 vs. REF group.

302 *3.6. Anti-RV antibodies generated after SA11 and EDIM infections*

303 In all three ED the total serum specific anti-RV Ab were studied in 28-day-old rats when their
304 immune response was more mature (**Figure 3**). The anti-RV Ab concentrations in SA11-infected
305 groups were significantly increased up two fold from those in the REF groups in all ED. The
306 different conditions tested for the EDIM inoculation (i.e. keeping breastfeeding or early weaning the
307 same day of infection) failed in inducing significant Ab response against the virus, with exception of
308 the ED DBW (early weaning the day before of infection) in which the anti-RV Ab concentrations
309 increased more than eight times with respect to the REF group (p<0.05).



310

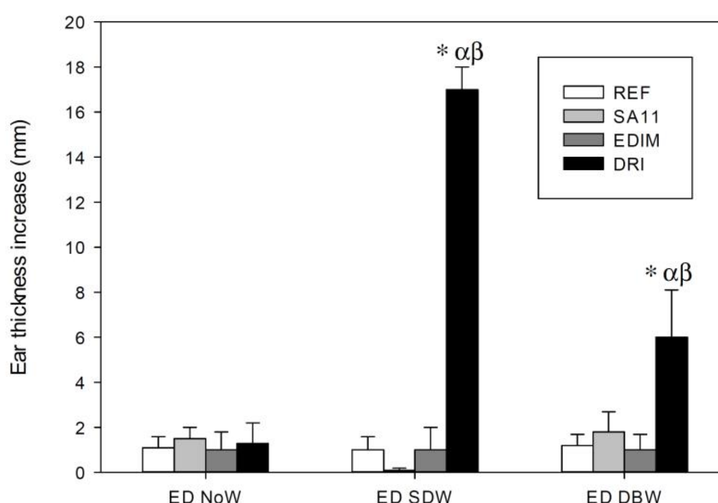
311 **Figure 3.** Specific anti-RV total antibodies (Ab) in serum from 28-day-old rats from the different
312 experimental designs (ED). Results are expressed as mean ± SEM (n = 6–12 animals/group) in
313 AU/mL. Statistical differences: *p<0.05 vs. REF group; $^{\alpha}$p<0.05 vs. SA11 group; $^{\beta}$p<0.05 vs. EDIM
314 group.

315 When the immune response after the combination of both viruses, in the three different designs,
316 was studied, two different patterns were found for the DRI with respect to a unique infection (either
317 SA11 alone or EDIM alone): no effect (ED NoW) or down-modulatory effect (ED SDW and ED
318 DBW). Apart from the lack of modulation in ED NoW (Ab response in DRI group were similar to
319 that in SA11 group), the other two conditions generated an Ab response in animals from the DRI
320 group, infected with both SA11 and EDIM, that was significantly lower than that when infection was
321 only performed with SA11 (ED SDW) or EDIM (ED DBW), suggesting a down-modulatory effect of
322 the second infective agent: a decrease of 29–36 % of Ab levels was produced in these cases.
323

324 Regarding HBC intervention during first infection, the Ab synthesis due to the SA11 infection
325 was significantly down-modulated (up to three times).

326 *3.7. Delayed type hypersensitivity*

327 The DTH response at 24 h was studied in all three ED, showing a clear up-modulatory effect of
328 double infection in the early weaned animals (**Figure 4**). The groups infected with SA11 and EDIM
329 alone did not show an increase in this response when compared to the REF group, but the DTH
330 response increased in the DRI group in the ED involving early weaning, either the same day of
331 infection (ED SDW) or the day before (ED DBW) (p<0.05).



332
333 **Figure 4.** Delayed-type hypersensitivity (DTH) response in some of the experimental designs (ED) in
334 28-day-old animals. Results are expressed as mean ± SEM of the 24 h-increase of thickness of the
335 RV-injected ear subtracting the increase of thickness of the PBS-injected ear (n = 6–12 animals/group).
336 Statistical differences: *$p<0.05$ vs. REF group; $^{\alpha}p<0.05$ vs. SA11 group; $^{\beta}p<0.05$ vs. EDIM group.

337 **4. Discussion**

338 Group A RV are the most common cause of acute diarrhoea among children under the age of
339 five worldwide [35,36]. Due to its impact on the health system and society, it is of interest to explore
340 the RV infection and its pathology, along with the clinical and immune response, and to test its
341 possible modulation by nutritional compounds. Several RV infection animal models are already
342 available, but most of them are single-infection models, which do not reflect the multiple
343 re-infections that humans have during our early life. That's why the purpose of this study was to
344 develop a double-infection model in rat, which is more similar to the clinical reality than a
345 single-infection model.
346
347 First, rats from the Lewis strain were selected, having a high susceptibility to infection by RV,
348 while the Wistar strain has mechanisms to avoid virus replication [28]. Age is also an important
349 factor, because rodents can be infected at any age, but they do not always develop disease. In
350 particular, rats only present clinical symptoms when they are infected before 21–27 days of life, and
351 diarrhoea is age-dependent [27,28,36].
352
353 Two RV inoculations were performed in the different experimental designs tested in the present
354 study. The first one, with SA11, was at the end of the first week of life, in line with the previous
355 studies [26,28]. A 5-day mild diarrhoea was achieved, without body weight loss, but with faecal
356 weight increase in the acute diarrhoea period, as in previous studies [26,28]. In other animal models

357 with a more severe diarrhoeal disease, such as in mice, body weight loss has been described [37]. For
358 the second infection, mimicking re-infections that often occur in infants, the RV EDIM was chosen
359 and inoculated at day 17 of life, as previously established in mice [33], confirming its infectious
360 capacity on rats. In this case, diarrhoea was not observed due to the older age of the animals, as in
361 the other models of RV double-infection in other species [29–33]. To enhance the RV infection, the
362 inoculation was performed on day ~17 of life in the different ED, but with early weaning introduced
363 on the same day, or even one day before, which implies a physiological stress for the animal. This
364 change in the weaning day still did not provoke the apparition of clinical features and therefore,
365 other markers needed to be assessed.
366
367 The faecal pH seems to decrease with an RV infection, but only when diarrhoea is obtained
368 (first infection). This may be because of the body electrolyte imbalances caused by the diarrhoea [13].
369 However, Li et al. [38] observed an increase in pH in the colonic content of RV-infected piglets. In
370 our case, the pH measure was not useful in assessing the second infection. We confirmed a
371 temperature rise after both the first infection and the second infection, even though no clinical
372 symptoms were observed in this last situation. However, no different pattern was observed between
373 the single and the double infection. Few studies have evaluated the body temperature. For example,
374 Parreño et al. [32] measured temperature after RV infection (they measured it only after the first
375 infection, not after the second one) in a calf model and observed fever. Overall, pH and temperature
376 measures seem to be useful as disease indicators in this type of models, although the results are
377 more evident in the first infection. A current limitation of this study that have to be considered in the
378 future was to not have evaluated the viral shedding, a tool used in previous studies [23,26] and by
379 others, which could have allowed us to observe the impact of first infection on the prevention of the
380 second one.
381
382 Finally, changes in immune response were assessed in order to obtain differential patterns in
383 the double infection in terms of specific Ab and DTH response. These variables were determined at
384 the end of the study, when the immune system of the animals was more developed.
385
386 The DTH response after second infection was found to be modulated by a first infection.
387 Whereas single infection did not promote a DTH response, a clear effect in the early weaned DRI
388 animals was found. These results are contrary to what has been observed in other studies, where the
389 DTH response was detected after an infection at day 17 in mice, but was supressed after a
390 re-infection in mice who had previously received a primary infection [33,39]. Our results may
391 suggest that animals that have already been in contact with the virus (first infection), are able to
392 respond to the DTH stimuli after the second infection in a stronger way. This result is justified by the
393 fact that it is known that RV specific CD8+ T cells developed after a first infection mediate the
394 resolution of the second infection [19,40].
395
396 In the SA11 groups of all the ED, increased levels of Ab compared to the REF groups were
397 already found, similarly to previous studies [28,29,31]. With regard to the second infection, the
398 results from the ED NoW (physiological weaning) suggested that the second infection was not
399 strong enough to modify the Ab levels (those from the DRI group were similar to those from the
400 SA11 group), but when an early weaning was performed, the second infection modulated this
401 variable (ED SDW) and more significantly if the animals had been weaned one day before the EDIM
402 inoculation (ED DBW). Thus, a down-modulatory action on humoral response on the second
403 infection after the first one was found. It has also been shown by Sheridan et al. in a model of
404 re-infection in adult mice [39]. In contrast with these results, Knipping et al. did not find a
405 differential Ab pattern between single and double infections [33]. Overall, the low titres of anti-RV
406 Ab in the DRI animals may be linked to the higher cell response achieved after first infection which
407 could be protecting against the virus without needing to highly activate the humoral immune
408 response. However, it has to be highlighted that Ab in the breast milk are variable and can confer

409 protection to the pups. We found a high level of RV-specific Ab in dams' serum and breast milk and
410 we even obtained different levels according to the animal supplier, presumably due to the previous
411 contact of the dams with RV (data not shown). For this reason, we concluded that, although the
412 importance of evaluating anti-RV Ab titres in pups' sera it is also important to determine those of the
413 dams before starting an interventional study with this type of model.
414
415 Moreover, the HBC is an effective protection in the control of diarrhoea in this and other RV
416 models. In other studies, the administration of Gastrogard-R® (colostrum containing RV-specific
417 Ab) also protected suckling mice against the first RV infection, but the faecal-viral load after the
418 second infection was as high as if it was the first one, but allowing the development of B and T cell
419 responses [33]. Parreño et al. described full protection by HBC after a first and second virus exposure
420 in calves [32]. A combination of HBC and the probiotic strain *Lactobacillus rhamnosus* GG also
421 protected mice successfully from RV diarrhoea [41]. Other authors have also found diarrhoea
422 protection or a reduction of rotaviral disease in mice with the administration of bovine colostrum
423 from normal cows [42,43] or whey protein concentrates [23,25,44], sometimes with diminished Ab
424 titres [23] and sometimes with no differences among groups [44]. In the case of multiple infections,
425 HBC intervention may help in understanding the influence of modulatory interventions during
426 these processes. In our case the protection from the first infection by HBC leads to the induction of a
427 lower immune response after the second infection (in terms of Ab response), suggesting that HBC, in
428 the first RV infection, is able to effectively block the virus, promote its elimination and therefore
429 diminish its infectivity and disease but also the priming of immunity in this sense. Future studies
430 should be directed to define an intermediate dose or to find protective agents that may control
431 disease but also allow the development of own immunity against the pathogen.
432
433 In summary, the early weaning and an early age for both infections are important features for
434 the model to achieve a valid design candidate. The model includes clinical signs during first
435 infection, but not in the second, and modulation of immune response due to the first RV contact. The
436 diarrhoea index and the faecal pH are suitable tools for assessing the first infection, body
437 temperature is an appropriate clinical variable for both infections, and specific Ab in serum at day 28
438 and the DTH response are useful variables for evaluating the second RV infection. We can conclude
439 that the RV double-infection rat model is suitable for studying the influence of interventions
440 performed to modulate first infections (e.g. by vaccination or nutritional supplementation) on the
441 onset of a future re-infection, as happens in humans.

442 **Supplementary Materials:** The following are available online at www.mdpi.com/link, **Table S1:** Evaluation of
443 the infectious capacity of EDIM on rats. One litter of 3-day-old rats (EDIM d3 group, n=8) was inoculated with
444 1.3×10^5 viral particles/animal and another litter of 6-day-old rats (EDIM d6 group, n=8) was inoculated with
445 2×10^6 viral particles/animal of the EDIM aliquot generated *in vivo* in mice. A differential dose was used at
446 different ages due to the age-dependent resistance to infection of suckling rats. Evaluation of the incidence and
447 severity of diarrhoea when EDIM was inoculated at days 3 or day 6 of life. In both cases, there was a low but
448 significant incidence of diarrhoea during the following days after the virus inoculation, which in any case was
449 higher than 50 % of diarrhoeic animals (%DA). The mean severity of the inoculated animals did not achieve
450 values ≥ 2, overall meaning faeces with not normal appearance (loose, watery, trend to yellow-green) but with
451 no high diarrhoeic features. This pattern was extended from the day after virus infection (1 DPI) to at least 4–5
452 days later, with the effect being more pronounced the earlier the infection was introduced (d3 vs. d6), even
453 though the virus dose was higher in the older animals. This mild diarrhoea did not induce any body weight loss
454 (data not shown); **Figure S1:** Evaluation of the infectious capacity of EDIM on rats. One litter of 3-day-old rats
455 (EDIM d3 group, n=8) was inoculated with 1.3×10^5 viral particles/animal and another litter of 6-day-old rats
456 (EDIM d6 group, n=8) was inoculated with 2×10^6 viral particles/animal of the EDIM aliquot generated *in vivo* in
457 mice. A differential dose was used at different ages due to the age-dependent resistance to infection of suckling
458 rats. Proliferative response against RV on 21-day-old rats that were infected with EDIM at day 3 of life.
459 Regarding the immune response at the end of the study, there was a higher proliferative response in
460 splenocytes from EDIM d3 rats when stimulated with the RV than in those from the REF group, indicating that
461 EDIM was able to infect and prime the neonatal rat immune system. However, no increase was found in EDIM

462 d3 and EDIM d6 serum anti-RV antibodies with respect to REF animals (data not shown). NS, non-stimulated
463 cells; EDIM, cells stimulated with 10^4 particles EDIM/well. Proliferating cells were identified by means of BrdU
464 Cell Proliferation Assay Kit (Merck Millipore, Darmstadt, Germany), following manufacturer's instructions.
465 Results are expressed as mean ± S.E.M. Statistical difference: *$p<0.05$ vs. NS; **Figure S2:** SA11 and EDIM
466 cross-reactivity. In order to ensure that a first infection with SA11 would be able to modulate a subsequent
467 infection with EDIM, the cross-reactivity among both viruses was studied. (**S2a**) Proliferative response against
468 different RV stimulus in rats infected with SA11. The specific proliferative capacity under diverse RV stimuli
469 (SA11 or EDIM) was studied in SA11-infected rat splenocytes. Cells were able to proliferate in both conditions,
470 achieving a significant three-fold increase of proliferation when compared with non-stimulating conditions
471 ($p<0.05$). NS, non-stimulated cells; EDIM, stimulated with 10^4 EDIM particles/well; SA11, stimulated with 10^4
472 SA11 particles/well. Results are expressed as mean ± SEM. Statistical differences: *$p<0.05$ vs. NS. (**S2b**)
473 Quantification of antibody titres from SA11-infected rats in SA11 or EDIM-coated ELISA wells at different
474 concentrations. To test the specificity of serum anti-RV Ab developed by SA11-infected animals, their
475 cross-reactivity was analysed by ELISA. There was an increasing positive signal depending on the amount of
476 virus coated to the plate but it did not depend on the type of virus (SA11 or EDIM). The increasing signal of the
477 Ab present in the serum was dependent on the amount of virus coated to the well and therefore is
478 dose-dependent and, indeed, has a similar pattern between both types of virus. Results are expressed as mean ±
479 SEM. (**S2c**) Representative images of detection by ELISPOT of spontaneous anti-RV IgM-SC either from the
480 spleen or the MLN of SA11-infected rats. Specific anti-RV Ab-SC from SA11-infected animal spleen or MLN
481 were detected by the ELISPOT technique. Anti-RV IgM-SC were clearly detected – with more signal in the
482 spleen than in the MLN cells – against both SA11 and to EDIM, showing the capacity of SA11-sensitized
483 animals to also recognize EDIM. 200,000 cells plated in each well, SA11 or EDIM at the top indicate the coating
484 antigen (10^5 particles/mL) used in each nitrocellulose well. Conditions used as in previous publications of the
485 group.

486 **Table S1.** Evaluation of the infectious capacity of EDIM on rats. One litter of 3-day-old rats (EDIM d3
487 group, n=8) was inoculated with 1.3×10^5 viral particles/animal and another litter of 6-day-old rats
488 (EDIM d6 group, n=8) was inoculated with 2×10^6 viral particles/animal of the EDIM aliquot
489 generated *in vivo* in mice. A differential dose was used at different ages due to the age-dependent
490 resistance to infection of suckling rats. Evaluation of the incidence and severity of diarrhoea when
491 EDIM was inoculated at days 3 or day 6 of life. In both cases, there was a low but significant
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494 achieve values ≥ 2, overall meaning faeces with not normal appearance (loose, watery, trend to
495 yellow-green) but with no high diarrhoeic features. This pattern was extended from the day after
496 virus infection (1 DPI) to at least 4–5 days later, with the effect being more pronounced the earlier the
497 infection was introduced (d3 vs. d6), even though the virus dose was higher in the older animals.
498 This mild diarrhoea did not induce any body weight loss (data not shown).

	EDIM d3		EDIM d6	
	DI	**%DA**	**DI**	**%DA**
d3	1.00 ± 0.00	0.00	1.00 ± 0.00	0.00
d4	1.50 ± 0.29	22.22	1.00 ± 0.00	0.00
d5	1.50 ± 0.14	11.11	1.00 ± 0.00	0.00
d6	1.25 ± 0.25	0.00	1.50 ± 0.22	33.33
d7	1.08 ± 0.08	0.00	1.38 ± 0.24	16.67
d8	1.21 ± 0.10	0.00	1.71 ± 0.16	50.00
d9	1.11 ± 0.04	0.00	1.35 ± 0.22	16.67
d10	1.75 ± 0.09	33.33	1.00 ± 0.00	0.00
d11	1.38 ± 0.13	0.00	1.00 ± 0.00	0.00

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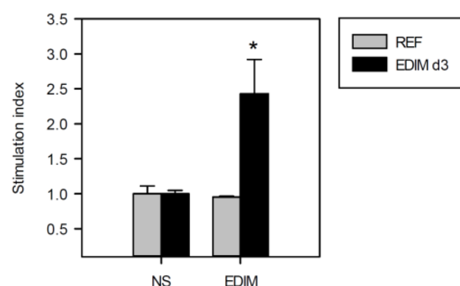

500

501 **Figure S1.** Evaluation of the infectious capacity of EDIM on rats. One litter of 3-day-old rats (EDIM
502 d3 group, n=8) was inoculated with $1.3x10^5$ viral particles/animal and another litter of 6-day-old rats
503 (EDIM d6 group, n=8) was inoculated with $2x10^6$ viral particles/animal of the EDIM aliquot
504 generated *in vivo* in mice. A differential dose was used at different ages due to the age-dependent
505 resistance to infection of suckling rats. Proliferative response against RV on 21-day-old rats that were
506 infected with EDIM at day 3 of life. Regarding the immune response at the end of the study, there
507 was a higher proliferative response in splenocytes from EDIM d3 rats when stimulated with the RV
508 than in those from the REF group, indicating that EDIM was able to infect and prime the neonatal rat
509 immune system. However, no increase was found in EDIM d3 and EDIM d6 serum anti-RV
510 antibodies with respect to REF animals (data not shown). NS, non-stimulated cells; EDIM, cells
511 stimulated with 10^4 particles EDIM/well. Proliferating cells were identified by means of BrdU Cell
512 Proliferation Assay Kit (Merck Millipore, Darmstadt, Germany), following manufacturer's
513 instructions. Results are expressed as mean ± S.E.M. Statistical difference: *$p<0.05$ vs. NS.

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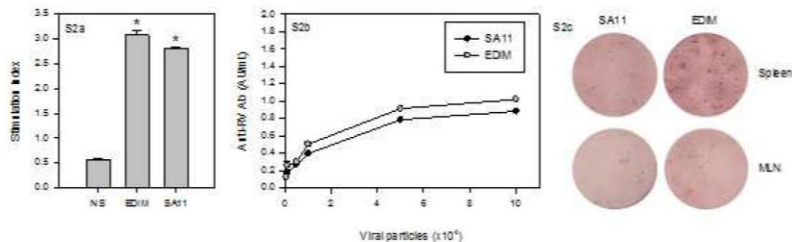

515

516 **Figure S2.** SA11 and EDIM cross-reactivity. In order to ensure that a first infection with SA11 would
517 be able to modulate a subsequent infection with EDIM, the cross-reactivity among both viruses was
518 studied. (**S2a**) Proliferative response against different RV stimulus in rats infected with SA11. The
519 specific proliferative capacity under diverse RV stimuli (SA11 or EDIM) was studied in
520 SA11-infected rat splenocytes. Cells were able to proliferate in both conditions, achieving a
521 significant three-fold increase of proliferation when compared with non-stimulating conditions
522 ($p<0.05$). NS, non-stimulated cells; EDIM, stimulated with 10^4 EDIM particles/well; SA11, stimulated
523 with 10^4 SA11 particles/well. Results are expressed as mean ± SEM. Statistical differences: *$p<0.05$ vs.
524 NS. (**S2b**) Quantification of antibody titres from SA11-infected rats in SA11 or EDIM-coated ELISA
525 wells at different concentrations. To test the specificity of serum anti-RV Ab developed by
526 SA11-infected animals, their cross-reactivity was analysed by ELISA. There was an increasing
527 positive signal depending on the amount of virus coated to the plate but it did not depend on the
528 type of virus (SA11 or EDIM). The increasing signal of the Ab present in the serum was dependent on
529 the amount of virus coated to the well and therefore is dose-dependent and, indeed, has a similar
530 pattern between both types of virus. Results are expressed as mean ± SEM. (**S2c**) Representative
531 images of detection by ELISPOT of spontaneous anti-RV IgM-SC either from the spleen or the MLN
532 of SA11-infected rats. Specific anti-RV Ab-SC from SA11-infected animal spleen or MLN were
533 detected by the ELISPOT technique. Anti-RV IgM-SC were clearly detected – with more signal in the
534 spleen than in the MLN cells – against both SA11 and to EDIM, showing the capacity of
535 SA11-sensitized animals to also recognize EDIM. 200,000 cells plated in each well, SA11 or EDIM at
536 the top indicate the coating antigen (10^5 particles/mL) used in each nitrocellulose well. Conditions
537 used as in previous publications of the group.

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542 performed the experiments; F.J.P.C. and M.R.A. analysed the data, interpreted results and drafted the paper; all
543 the authors contributed to the critical review and revision of the manuscript.

544 **Conflicts of Interest:** The authors declare that they have a financial relationship with the organization that
545 sponsored the research. K Knipping and J Garssen are employees of Nutricia Research BV. The other authors
546 declare that they have no conflict of interest.

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Capítol 6

“*Nutritional interventions with pro- and prebiotics in a double rotavirus infection model*”

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Manuscrit en preparació

Els resultats del present article han estat presentats a les següents comunicacions:

- *Modulation of the suckling rat rotavirus infection by a preventive hyperimmune bovine colostrum administration*. VIIIè congrés de la Societat Catalana d’Immunologia (SCI) (Barcelona, Espanya), novembre 2014.

- *Probiotic modulation of immune response in a pre-clinical model of a double rotavirus infection*. IXè congrés de la Societat Catalana d’Immunologia (SCI) (Barcelona, Espanya), novembre 2015.

- B. breve *M16-V modulates rotavirus-associated disease in a double infection model in suckling rats*. 10th European Mucosal Immunology Group Meeting (Copenhaguen, Dinamarca), octubre 2016.

RESUM

Objectiu: Estudiar el possible paper protector de dos prebiòtics (scGOS/lcFOS i GOS), el probiòtic *Bifidobacterium breve* M-16V i la combinació del primer prebiòtic amb aquest probiòtic, com un simbiòtic, en un model de doble infecció per rotavirus (RV) en rata lactant.

Material i mètodes: Els animals van rebre els suplements nutricionals des del dia 3 fins al dia 14 de vida. Es va fer servir com a control positiu de protecció un calostre boví hiperimmune contra RV (HBC). La primera infecció per rotavirus es va induir amb la soca SA11 i la segona amb EDIM. Les variables clíniques i immunològiques es van avaluar després d'ambdues infeccions.

Resultats: Les intervencions nutricionals van millorar les manifestacions clíniques després de la primera infecció. El simbiòtic va mostrar un efecte modulador de la febre durant la primera infecció. Els prebiòtics i el simbiòtic van reduir significativament l'excreció del virus després de la primera infecció, però totes les intervencions van mostrar una excreció viral més elevada que la del grup RV després de la segona infecció. Totes les intervencions van modular la producció d'Ig específiques i citocines *ex vivo*, els nivells de citocines en rentat intestinal i l'expressió gènica en intestí prim.

Conclusió: Un suplement diari dels productes avaluats en aquest estudi és efectiu en la modulació de la diarrea induïda per RV i les seves reinfeccions en aquest model preclínic, suggerint que aquests productes són potencials agents moduladors de la infecció per rotavirus en infants.

1 *Article*

2 Nutritional interventions with pro- and prebiotics in 3 a double rotavirus infection model

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21 **Abstract:** Rotavirus (RV) causes morbidity and mortality among infants worldwide and there is
22 evidence that probiotics and prebiotics can have a positive influence against infective processes
23 such as that due to RV. The aim of this study was to evidence a preventive role of two prebiotics
24 (scGOS/lcFOS and GOS), the probiotic *Bifidobacterium breve* M-16V and the combination of the first
25 prebiotic and the probiotic, as a synbiotic, in a suckling rat double RV infection model. An
26 hyperimmune bovine colostrum was used as protection control. Fist infection was induced with
27 the RV SA11 and the second one with EDIM. Clinical variables and immune response were
28 evaluated after both infections. The dietary interventions ameliorated clinical symptoms after the
29 first infection. The prebiotics and the synbiotic significantly reduced viral shedding after the first
30 infection but all the interventions showed higher viral load than the RV group after the second one.
31 All interventions modulated *ex vivo* Ig and CK production, gut wash CK levels and small intestine
32 gene expression. In conclusion, a daily supplement of the products tested here is highly effective in
33 modulating RV-induced diarrhea and its reinfection in this preclinical model, suggesting these
34 components as potential agents to modulate RV infection in infants.

35

36 **Keywords:** Prebiotic; Probiotic; Synbiotic; Rotavirus; FOS; GOS; *Bifidobacterium breve*

37 **PACS:** J0101
38

39

40 **1. Introduction**

41 Rotavirus (RV) is the most common etiological agent of severe dehydrating diarrhea among
42 children under the age of 5 worldwide [1]. The main symptoms are fever, vomiting and diarrhea in
43 children, for 3-8 days [2,3]. Virtually, every child in the world will be infected with RV in the first 3
44 years of life [4], and several reinfections usually occur, although they tend to be less severe than the
45 first one [3]. RV is a non-enveloped, icosahedral, double-stranded RNA and covered by triple-layer
46 capsids member of the *Reoviridae* family. Its viral genome encodes for six structural (VPs) and six
47 non-structural proteins (NPs) [2,5–7]. RV are classified in groups (A-G), subgroups (I and II) and
48 serotypes/genotypes (based on the antigenic differences among the external capsid). RV from the
49 group A are the main human pathogens and their transmission is fecal-oral, with higher prevalence
50 in the winter season. It infects mature enterocytes of the small intestine, but the entry mechanism is
51 not still well known [8].
52
53 Once the RV infects, the innate and adaptive immune responses are induced and will lead to
54 cytokine and specific antibodies production [9,10]. Natural killer (NK) lymphocytes and dendritic
55 cells (DC) are crucial for response activation of both innate and adaptive immunity, respectively
56 [11]. Besides T cell action on infected cells and cytokine production, B cell antibodies production, at
57 local and systemic level, is required for long term protection, specially IgA [12–15]. Even though,
58 immunity after an RV infection is not fully complete and re-infections usually occur, although less
59 severe than the first [3].
60
61 Several interventional approaches can be addressed, since oral rehydration, which is the
62 commonest treatment [16,17], to immune modulator interventions such as vaccination or dietary
63 supplementation with active agents. In this sense live attenuated oral vaccines, Rotateq (Merck &
64 Co, PA, USA) and Rotarix (GSK Biologicals, Rixensart, Belgium), are available since 2006. RV
65 vaccines have shown safety and efficacy [5,18–20], but their implementation is not already global
66 [4,17]. Regarding nutritional interventions, bioactive components from breast milk -which are in
67 high proportion in colostrum-, probiotics or prebiotics are the leading studied products.
68
69 To date, whey protein concentrates and bovine colostrum (with or without RV-specific
70 antibodies) have shown effective protection against RV disease [21–27], and thus, are candidates for
71 protection control in dietary interventional studies. Moreover, hyperimmune bovine colostrums
72 (HBC) have been also tested in different RV infection models with very satisfactory results [24,25,28].
73
74 Probiotics are live microorganisms that, when administered in adequate amounts, confer a
75 health benefit on the host [29]. Several species of the *Lactobacillus* and the *Bifidobacterium* genera have
76 been studied and have demonstrated some effects against RV infection by modulating several
77 mechanisms: chloride secretion and oxidative stress [30], virus replication and adhesion capacity
78 [31,32] and host immune cell response [33]. Due to these actions, probiotics lead to clinical
79 amelioration in animal models [34,35], as well as some clinical and immunological benefits in
80 RV-infected babies in clinical trials [36–39]. On the other hand, prebiotics are indigestible food
81 ingredients that reach the colon and promote the growth or activity of certain beneficial species in
82 the intestinal microbiota, thereby generating a health benefit [40,41]. Besides their beneficial role in
83 modulating short chain fatty acids (SCFAs) or even intestinal IgA secretion, just a few interventional
84 studies have been conducted in order to investigate the role of prebiotics against RV infection. In this
85 sense, prebiotics with importance in early life, such as human milk oligosaccharides (HMO) or
86 specific mixtures of short chain galactooligosaccharides (scGOS) and long chain
87 fructooligosaccharides (lcFOS), widely used in infants formulas, have been the most studied in both
88 preclinical [42,43] and clinical [44,45] studies involving RV infection.
89

90 In previous studies, the effectiveness of a prebiotic mixture, scGOS/lcFOS in a 9:1 proportion
91 (Immunofortis®, Danone Baby Nutrition), with or without the probiotic *B. breve* M-16V, and the
92 probiotic *B. breve* M-16V alone, have been tested in an RV-infected neonatal rat model [43]. However,
93 a limitation of such study was that the frequency of reinfection in humans is very high and the
94 impact of these active compounds on the host immunity against a new RV infective process is not
95 known. In this sense, a double infection model will provide significant information of how the
96 protection against the first infection due to the prebiotics or probiotics interventions will affect the
97 second infection, mainly in terms of immune response. For that reason, a rat double RV infection
98 model has been recently set up in our laboratory in order to study this type of effects due to
99 interventional approaches in early life [28]. Moreover, in order to evaluate the importance of
100 combining both type of prebiotics used, scGOS and lcFOS, we also included a prebiotic just based in
101 GOS, the Bimuno® GOS (Clasado Biosciences Ltd, UK) with 65 % GOS, 10.1 % lactose, 22 % glucose
102 and 1.8 % galactose, which also has demonstrated immunomodulatory activities [46,47].
103
104 Taking into account the above comments, the present study aimed to test the effectiveness of a
105 prebiotic mixture, scGOS/lcFOS (9:1), with or without the probiotic *B. breve* M-16V, the probiotic *B.*
106 *breve* M-16V alone, and another prebiotic, Bimuno® GOS, in a double RV infection neonatal rat
107 model.

108 **2. Materials and Methods**

109 *2.1. Animals*

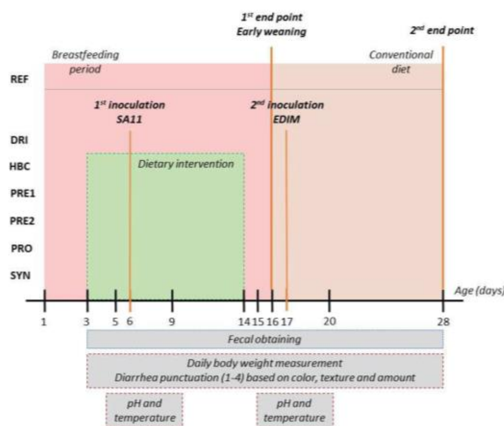
110 Twenty-seven G14 pregnant Lewis rats were obtained from Harlan (Barcelona, Spain). They
111 were housed in individual cages, monitored daily and allowed to deliver at term. The day of birth
112 was registered as day 1 of life. Litters were unified to 7 pups per lactating dam. Pups had free access
113 to the nipples and rat diet. The animals were housed under controlled temperature and humidity
114 conditions, in a 12:12 h light/dark cycle. They were located in a special safe isolated room at the
115 Animal Service of the Faculty of Pharmacy, University of Barcelona, designed and authorized for
116 working under biosecurity level 2 conditions. Dams were fed with a commercial diet corresponding
117 to the American Institute of Nutrition 93G formulation [48] and given water *ad libitum*. The animals
118 were weighted and monitored daily in order to obtain data regarding the influence of the virus
119 inoculation, clinical development and nutritional intervention on growth and fecal features. This
120 was done after the separation of the pups from their mother, during the handling and before oral
121 administration.
122
123 The studies were conducted in accordance with the institutional guidelines for the care and use
124 of laboratory animals as established by the Ethical Committee for Animal Experimentation of the
125 University of Barcelona and the Catalonia Government (CEEA-UB Ref.493/12, DAAM: 6905).

126 *2.2. Viruses*

127 Two different type A viruses have been used for the experiments: the simian agent 11 (SA11)
128 and the epizootic-diarrhea infant-mouse virus (EDIM). The virus selected for the first infection was
129 the SA11, a rotavirus strain produced by the "Enteric Virus Group" of the University of Barcelona, as
130 in previous studies [22,43,49]. The virus selected for the second infection was the EDIM, obtained *in*
131 *vivo* from inoculated neonatal BALB/c mice (Janvier, La Plaine Saint Denis Cedex, France) with an
132 initial inoculum of the virus (kind gift from K.Knipping, Nutricia Research, The Netherlands) [28].
133 Briefly, stool samples were collected twice a day from day 3 to day 13, pooled and homogenized by
134 using the Polytron® (Kinematica, Luzern, Switzerland). EDIM was extracted with Genetron®
135 (1,1,2-trichloro-1,2,2-trifluoroethane, Sigma-Aldrich, Madrid, Spain) and later quantified by ELISA
136 as described in previous studies [25].

137 *2.3. Experimental design and dietary supplementation*

138 Suckling rats were distributed in seven different experimental groups: reference (REF), double
139 rotavirus infection (DRI), "hyperimmune bovine colostrum" (HBC), prebiotic 1 (PRE1), prebiotic 2
140 (PRE2), probiotic (PRO) and synbiotic (SYN) and followed the design showed in the **Figure 1**.



141

142 **Figure 1.** Experimental design. Inoculation, weaning and end point days are indicated for the
143 different groups. The duration of the dietary interventions, the evaluation of fecal samples, body
144 weight, pH and temperature is also represented.

145 Each group was composed of 3 litters with 7 pups each (n = 21/group). Animals were orally
146 administered, as previously described [50], using low-capacity syringes (Hamilton Bonaduz,
147 Bonaduz, Switzerland) adapted to forced alimentation tubes of 25 or 23 caliber and 27 mm in length
148 (ASICO, Westmont, IL, USA), with the different products (HBC, PRE1, PRE2, PRO and SYN groups)
149 or vehicle (DRI and REF groups) from day 3 until day 14 of life. The HBC group received "anti-RV
150 hyperimmune bovine colostrum" in a 50 mg/animal/day; this HBC was tittered to be effective
151 blocking the virus *in vitro* in concentrations higher than 10 μg/mL (gently given up by Dr. Viviana
152 Parreño, Institute of virology, CICV and A-INTA, Castelar, Argentina) and to be effective protecting
153 RV infection in the neonatal rat model at such concentration [28]. The PRE1 supplement consisted of
154 Immunofortis®, a combination of scGOS and lcFOS in a 9:1 ratio, and was administered in a dose of
155 0.8 g/100 g of body weight/day. The PRE2 group received Bimuno® GOS in a dose of 0.8 g/100 g of
156 body weight/day. The PRO group received *Bifidobacterium breve* M-16V (Morinaga Milk Industry Co,
157 Ltd, Tokyo, Japan) suspension at a dose of 4.5×10^8 UFC/100 g of body weight/day. The SYN group
158 received both PRE1 and PRO products at the same concentrations as when administered alone. A
159 group of rats receiving bottled mineral water as vehicle was the inoculated control group (DRI
160 group), whilst another group receiving water acted as the non-inoculated control group (REF
161 group).

162

163 The RV inoculations were carried out in all the experimental groups, with the exception of the
164 REF group, as previously described [49]. It was done 1 h after separation from their dams to avoid
165 interference between RV and milk components. SA11 was selected as a first infective virus because
166 previous studies allowed us to obtain a rat model of mild diarrhea in early life. It was inoculated at
167 day 6 of life in a dose of ~2×10^8 $TCID_{50}$ RV/rat in 100 μL of phosphate-buffered solution (PBS). EDIM
168 was used as a second infective virus, inoculated at day 17 in a dose of ~1.3×10^8 RV/mL in 100 μL,
169 when the intestinal immune system is still in maturation [51]. Furthermore, because of the

170 importance of the bioactive factors present in breast milk in protecting the pups from infection, the
171 weaning day was on day 16, in order to induce a lower defensive situation in the babies, as
172 previously demonstrated in the setup of the model [28]. In addition, there were two more groups:
173 those single infected either with SA11 (SA11) or with EDIM. Moreover, selected animals from the
174 supplemented groups were not submitted to the second infection with EDIM and therefore
175 preliminary data from single infected animals with SA11 and dietary interventions (SA11+HBC,
176 SA11+PRE1, SA11+PRE2, SA11+PRO and SA11+SYN) was also obtained at the end of the study.
177
178 Clinical evaluation was performed daily from the first day of supplementation (d3) until the
179 end of the study (d28). One third of the animals from each group were euthanized on day 16 of life,
180 and the rest, on day 28. Fecal samples were collected daily during the study, and blood, small
181 intestine, intestinal wash samples, small intestinal tissue and isolated spleen and mesenteric lymph
182 node (MLN) cells at the end point. Body temperature and fecal pH were measured during the
183 peri-inoculation period of both infections. The delayed type hypersensitivity (DTH) response was
184 determined at the end of the study.

185 *2.4. Clinical indexes and fecal specimen collection*

186 SA11 and EDIM infections were evaluated from day 2 to day 28 of life by the growth rate and
187 clinical indexes that require daily fecal sampling as previously described [49]. Briefly, fecal sampling
188 was performed once a day by gently pressing and massaging the abdomen. Specimens were
189 immediately scored (1-4), weighed and frozen at -20 ºC for further analysis. The severity of diarrhea
190 was expressed by the fecal weight and by scoring stools from 1 to 4 (diarrhea index [DI]) based on
191 color, texture and amount as follows: normal (DI=1), loose yellow-green (DI=2), totally loose
192 yellow-green (DI=3), high amount of watery (DI=4) feces. Diarrhea scores ≥ 2 indicate diarrheic feces
193 whereas scores of DI < 2 indicate absence of diarrhea. The area under the curve of severity (sAUC)
194 during 0-6 DPI was calculated as a global value of severity. The maximum diarrhea index (MDI) was
195 defined as the highest score during the diarrhea period. Incidence of diarrhea was expressed by the
196 percentage of diarrheic animals (%DA, consisting of the percentage of diarrheic animals taking into
197 account the number of animals in each group) and by the percentage of diarrheic feces (%DF,
198 consisting of the percentage of diarrheic samples taking into consideration the number of total
199 samples collected every day in each group). The AUCs of %DA and %DF (daAUC and dfAUC)
200 during 0-6 DPI were calculated as a global value of incidence. The AUCs for severity, %DA and %DF
201 were also calculated taking into account the basal values due to intrinsic aspects of each treatment
202 (normalized AUC, AUCn). The maximum percentage of diarrheic animals (MDA) and diarrheic
203 feces (MDF) were defined as the highest values during the diarrhea period. The days when MDI,
204 MDA and MDF were achieved were also used as indicators, called MDId, MDAd and MDFd,
205 respectively. The diarrhea period (DP) was calculated for each animal as the interval between the
206 first (day of diarrhea beginning, DDB) and last day (day of diarrhea ending, DDE) of diarrhea. The
207 actual days with diarrhea within the diarrhea period were also counted (days with diarrhea, DwD).

208 *2.5. Fecal pH determination*

209 Fecal samples from the peri-inoculation period of the virus were diluted in distilled water (up
210 to 200 mg/mL) and softly agitated. Their pH was measured by a pH electrode for surfaces 5207
211 (Crison Instruments, Barcelona, Spain) and a pH-Meter micropH 2001 (Crison Instruments),
212 previously calibrated.

213 *2.6. Body temperature determination*

214 Rat's body temperature was measured with the TEMP JKT thermometer (Oakton, Vernon Hills,
215 IL, USA) and the RET-3-ISO rectal probe for neonatal rats (Physitemp, Clifton, NJ, USA) and with
216 the aid of peanut oil (Acofarma, Terrassa, Spain) to lubricate. This measure was taken during the

217 peri-inoculation period of the virus. Results were expressed as the relative increase of temperature
218 with respect to the initial temperature, which was obtained the day before the inoculation.

219 *2.7. Viral shedding*

220 Fecal samples from selected days of interest were diluted in PBS (10 mg/mL) and homogenized
221 using a FastPrep (MP Biomedicals, Santa Ana, California, USA). Homogenates were centrifuged
222 (19000 *g*, 3 min), and supernatants were frozen at -20 °C until use. SA11 and EDIM particles in fecal
223 samples were quantified by ELISA, as previously described [49]. Titrated dilutions of inactivated
224 SA11 virus particles, ranging from $4x10^5$ to $2,5x10^4$/mL, were used as standard in each plate.

225 *2.8. DTH*

226 Two days before sacrifice (day 26), the both right and left ears thickness of every animal were
227 measured to constitute the basal conditions by using the pocket thickness gauge 7309 (Mituyoto,
228 Hampshire, UK). For virus priming, animals were anesthetized with isoflurane (Abbott
229 Laboratories, Berkshire, UK) and the virus was injected in the ear using low volume Hamilton
230 syringes (100 µL) connected to needles (30 G ½ 0.3x13). A volume of 20 µL of UV-inactivated virus
231 (~$0.5x10^6$ RV particles/mL) was injected in the right ear (RE) and same volume of PBS was injected in
232 the left ear (LE). After 24 h and 48 h, prior to sacrifice, an evaluation of the ear thickness was
233 performed again. Results are expressed as the increase of thickness of the RE subtracting the increase
234 of thickness of the LE (to eliminate the intrinsic increase due to the puncture).

235 *2.9. Blood, spleen and intestinal sample collection*

236 After previous anesthesia with intramuscular ketamine/xylazine injection (Imalgene
237 100 mg/mL, Merial Laboratorios, Barcelona, Spain / Rompun® 20 mg/mL, Bayer Hispania, Sant Joan
238 Despí, Spain), rats from each group were euthanized at days 16 or 28. Blood was collected by cardiac
239 puncture and sera stored at -20 ºC until analysis. Small intestine was weighed, cut into 5 mm pieces
240 and incubated with PBS for 10 min at 37 °C in a shaker to obtain the gut wash (GW). After
241 centrifugation, supernatants were stored at -80 ºC until analysis. One centimeter of tissue from the
242 middle of the small intestinal was obtained and kept in RNA later from Ambion (Thermo Fisher
243 Scientific, Barcelona, Spain) at -20 ºC for further determinations. The spleen and MLNs were
244 removed under sterile conditions. Spleen and MLN cells were isolated as previously described [52].
245 Cell numbers and viability were determined using an automated cell counter after staining dead
246 cells with trypan blue (CountessTM, Invitrogen, Madrid, Spain). Isolated mononuclear cells from the
247 spleen and MLNs were cultured during 72 h under SA11/EDIM mix stimulatory conditions (10^5 viral
248 particles/mL). After incubation, the 24-well plate was centrifuged and the supernatants were
249 collected and kept at -80 ºC until cytokine determination.

250 *2.10. ELISA for total IgA and IgM antibody quantification in serum and gut wash*

251 Ninety-six-well plates (Nunc MaxiSorp, Wiesbaden, Germany) were coated with purified
252 mouse anti-rat IgA or IgM (BD Biosciences, Heidelberg, Germany). After blocking with PBS-1%
253 bovine serum albumin (BSA, 1 h, room temperature [RT]), appropriate diluted sera or intestinal
254 wash samples were added (3 h, RT). After washing, biotin anti-rat IgA or IgM from BD Biosciences,
255 followed by peroxidase-conjugated extravidin (Sigma-Aldrich), were added. Subsequently,
256 substrate was added, as previously described [49]. Dilutions of purified rat IgA or IgM (BD
257 Biosciences) ranging from 80 to 1.25 ng/mL were used as a standard in each plate. Quadratic
258 polynomial adjustment was used.

259 *2.11. ELISA for specific anti-RV total and IgM antibodies quantification in serum and gut wash*

260 Ninety-six-well plates (Nunc MaxiSorp) were coated with UV-inactivated SA11 particles at
261 10^5/mL. After blocking with PBS-1% bovine serum albumin (BSA, 1 h, RT), appropriate diluted sera

262 or intestinal wash samples were added (3 h, RT). After washing, rabbit anti-rat Ig conjugated to
263 peroxidase from Dako (Barcelona, Spain) or mouse biotinylated anti-rat IgM (G53-238) monoclonal
264 antibody (MAb) from BD Biosciences, followed by peroxidase-conjugated extravidin
265 (Sigma-Aldrich), were added. Subsequently, substrate was added, as previously described [49].
266 Pooled sera from dams of inoculated litters were used as a standard in each plate. Quadratic
267 polynomial adjustment was used.

268 *2.12. ELISPOT for specific anti-RV antibody production*

269 An ELISPOT technique was used to quantify anti-RV Ig-secreting cells (SC) from spleen and
270 MLN by following previous experience [53]. Ninety-six-well nitrocellulose plate (Merck Millipore)
271 were coated with viral particles of SA11 or EDIM (10^5 particles/mL) in sterile conditions (overnight,
272 4 ºC). The remaining binding sites were blocked with complete media for 1 h at 37 ºC. Freshly
273 isolated cells were plated at serial dilutions (2×10^5, 1×10^5, 0.5×10^5 and 0.25×10^5 cells/well) and
274 incubated for 3 days (37 ºC, 5% CO_2). Cells were then removed by washing 10 times with PBS
275 containing 0.25 % of Tween 20, and once with distilled water. Biotin conjugated anti-rat IgM Ab (BD
276 Pharmingen, San Diego, CA, USA, 2 mg/L in PBS) was added and incubated for 2 h. The plate was
277 washed again, and then incubated with extravidin-peroxidase conjugate (Sigma-Aldrich, 4 mg/L) for
278 1 h. Spots, each one corresponding to one anti-RV Ig-SC, were visualized after adding the substrate
279 solution (3-amino-9-ethyl-carbazole plus H_2O_2 in 0.1 mol/L acetate solution). The reaction was
280 stopped using tap water. Spots were counted automatically by the computer-assisted ELISPOT
281 image analysis (ELISPOT reader system, AID, Strasberg, Germany) and expressed as the number of
282 Ig-SC per 10^6 cells.

283 *2.13. Bead immunoassay*

284 Cytokine concentration in supernatants from stimulated spleen cells and from gut wash
285 samples was measured. An antiviral and Th1 (IFNγ), a Th2 (IL-4), an anti-inflammatory and
286 regulatory (IL-10) and a pro-inflammatory (TNFα) cytokines were evaluated. Molecules
287 determinations were performed by BD™Cytometric Bead Assay Rat Soluble Protein Flex Set (BD
288 Biosciences, Madrid, Spain) as detailed in previous studies [54]. A FacsAria SORP sorter (BD, San
289 José, CA, USA) from the cytometry service of the Scientific and Technological Centers of the
290 University of Barcelona (CCiT-UB) was used. Data analysis was performed using the FlowJo 10.0.7
291 software (Tree Star, Inc., Ashland, OR, USA).

292 *2.14. Real time PCR for small intestinal gene expression*

293 Gene expression of different genes that could be of interest was evaluated. Specifically, we
294 selected target genes expressed in the small intestine at day 28 which could be representative of the
295 possible effect that the dietary intervention could modulate: receptors from the TLR family (i.e. TLR2
296 and TLR4), Th1 and Th2 responses (IFNγ and IL-4, respectively), molecules involved in the Tight
297 Junction (TJ, i.e. Occludin, Claudin-2), innate defenses (mucin) and regulatory and tolerogenic
298 response (IL-10, TGF-β and FoxP3).
299
300 On sacrifice day, the intestinal tissue was obtained and kept in RNA later from Ambion
301 (Thermo Fisher Scientific) at -20 ºC until analysis. Homogenization and RNA isolation were
302 performed as previously described [55]. A Nanodrop spectrophotometer and Nanodrop IVD-1000
303 v.3.1.2 software (Nanodrop Technologies, Wilmington, DE, USA) were used to quantify the amount
304 of RNA obtained. The RNA integrity number (RIN) was evaluated, in the Genomic Service of the
305 SCT-UB (GS-SCT-UB), in order to ensure the quality and integrity of the material. RNA was
306 reverse-transcribed, as previously described [55]. PCR Taqman® primers and probes specific for rat
307 target genes and HPRT as endogenous control were used (Assays on Demand,™ Gene Expression
308 Products, AB). The amount of target mRNA, normalized with an endogenous control (HPRT) and
309 relative to a calibrator (tissue samples from REF group as control group), was given by the $2^{-\Delta\Delta Ct}$

310 method, as previously described [55]. Results are expressed as the mean ± SEM of the percentage of
311 these values for each experimental group compared to its reference group, which represents 100 % of
312 gene expression.

313 *2.15. Short-chain fatty acids*

314 SCFAs quantification in fecal samples from 21 and 28-day-old rats was performed by HPLC, as
315 previously described [43]. SCFAs were identified and quantified using a calibration cocktail which
316 includes acetic, propionic, butyric, lactic and formic acids, in concentrations ranging from 100 to
317 12.5 mM.

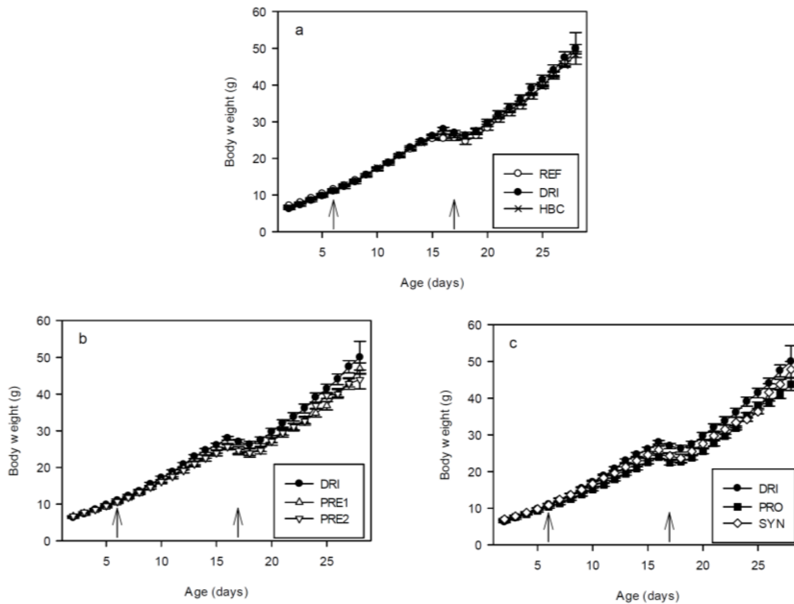
318 *2.16. Statistical analysis*

319 The PASW Statistics 22 software package (SPSS Inc, Chicago, IL, USA) was used for the
320 statistical analysis. Komolgorov-Smirnov test was applied to assess normal distribution, followed by
321 Levene's test in order to determine variance equality. Conventional one-way ANOVA test was
322 performed considering the experimental group as the independent variable. When virus inoculation
323 or dietary interventions had a significant effect on the dependent variable, Scheffé's *post hoc* test was
324 applied. Kruskal-Wallis and Mann-Whitney U tests were used when non-normal distribution or
325 different variance were found. Finally, the chi-square test was used to compare frequencies.
326 Differences were considered significant at P values of <0.05. All the results are expressed as
327 mean ± SEM of a certain number of animals.

328 **3. Results**

329 *3.1. Body weight*

330 Body weight was recorded between days 2 and 28 of life. The first one was about 6-7 g and the
331 last one about 44-49 g. As it can be observed in **Figure 2**, the inoculation with the first virus (SA11)
332 was performed on pups with around 10-11 g of body weight and it had no impact on this variable.
333 With regard to the second inoculation with EDIM, a weight loss was observed on day 17. However,
334 it cannot be ascribed to the infection, but it is due to the early weaning, since the REF group showed
335 the same pattern. The HBC intervention did not induce changes in the body weight course
336 compared to the REF and DRI groups (**Figure 2a**).
337
338 Regarding the effect of dietary supplementation on body weight, **Figure 2b** shows that both
339 prebiotic interventions (PRE1 and PRE2) induced a slight decrease of the weight of the animals
340 before fist inoculation (first week of life) when compared to the REF group ($p<0.05$). The SA11
341 inoculation did not affect the growth of these animals in the next days (when compared to REF or
342 DRI) because the pattern was similar as before. After the day 13, both prebiotic groups started to
343 have also lower weight than the DRI group ($p<0.05$). The EDIM inoculation did not affect this
344 variable either, but the early weaning did cause a loss of body weight as in the REF and DRI groups.
345



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348 **Figure 2.** Body weight (g) during the study, before and after the virus inoculations (indicated by
349 arrows) on day 6 and day 17 (**a**) for REF, DRI and HBC groups; (**b**) for DRI, PRE1 and PRE2 groups;
350 (**c**) for DRI, PRO and SYN groups. Results are expressed as mean as values ± SEM. Statistical
351 significance is explained in the text (n=12-21 animals/group).

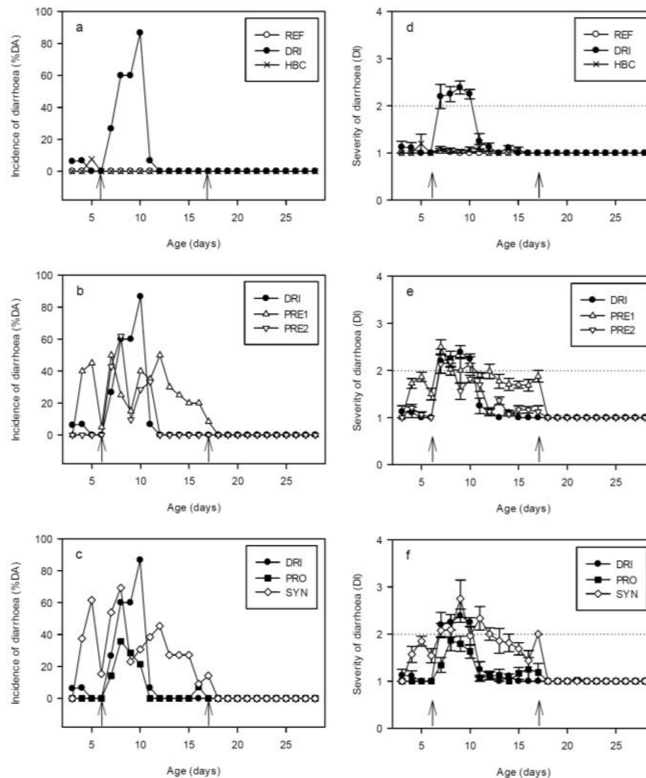
352 The dietary supplementation with the PRO and the SYN (**Figure 2c**) induced a pattern of body
353 weight similar to that found for the prebiotics administration. Along life, the animals from PRO
354 group had lower body weight than the REF and the DRI groups ($p<0.05$), although this difference
355 was not so evident for the animals in the SYN group. Same body weight loss after early weaning
356 (d17) was again observed, but not effect due to SA11 nor EDIM inoculation.
357

358 When the global behavior during the post-infective period was studied after the first RV
359 inoculation, the percentage of body increase during d7-d17 of all groups was between 123-155 %,
360 being all the inoculated animals' values higher than those from the REF group ($p<0.05$). After the
361 second infection with EDIM (d17-d27) no differences among groups were found.

362 *3.2. Incidence of diarrhea*

363 As can be seen in **Figure 3**, diarrhea appeared only after first infection (day 6 with SA11), but
364 not after the second infection (day 17 with EDIM), as was expected. Focusing on the first one, the
365 incidence of SA11-induced diarrhea was evaluated by two approaches which behaved very similar.
366 Considering the %DA, in the DRI group it was about 27 % on 1 DPI (**Figure 3a**); it increased up to 60
367 % on 2 and 3 DPI, achieving the maximum value of 87 % on 4 DPI (day 10 of life). Later, on 5 DPI, it
368 decreased to 7 % and, on day 6 DPI, none of the animals in the DRI group had diarrhea (**Figure 3a**).
369 The inoculation of EDIM on day 17 did not induce any modification in the fecal appearance the

370 following days and until day 28 (**Figure 3a**). When the diarrhea incidence was studied in
371 supplemented animals, all the groups showed a modulatory effect. In this sense, all supplemented
372 groups had lower %DA than the DRI group over the 3-4 DPI period (days 9 and 10 of life). The HBC
373 group was totally protected along all the studied period (**Figure 3a**).
374



375

376 **Figure 3.** Clinical indexes. Incidence of diarrhea (**a**) for REF, DRI and HBC groups; (**b**) for DRI, PRE1
377 and PRE2 groups; (**c**) for DRI, PRO and SYN groups: results are expressed as % of diarrheic animals.
378 Severity of diarrhea (**d**) for REF, DRI and HBC groups; (**e**) for DRI, PRE1 and PRE2 groups; (**f**) for
379 DRI, PRO and SYN groups: fecal samples are scored from 1 to 4 based on color, texture and amount
380 of stool. Scores of diarrhea index (DI) ≥2 indicates diarrheic feces. The inoculation days are indicated
381 by arrows. Results are expressed as mean ± SEM. Statistical differences mentioned in the text
382 (n = 12-21 values/group).

383 As it can be observed in **Figure 3b**, the PRE1 induced a certain %DA even before the SA11
384 inoculation (20-45 %) and throughout the nutritional intervention period. However, the MDA in this
385 group was 50 % (**Table 1**), achieved on day 7 and much lower than the 87 % of the DRI group
386 ($p<0.05$), and the %DA during days 9 and 10 (the highest in the DRI group) was reduced up to 15 %
387 and 40 %, respectively ($p<0.05$).
388

Table 1. Clinical variables determining the diarrhea process (from day 0 to 6 DPI). With regard to incidence: MDA, maximum percentage of diarrheic animals; MDAd, day with maximum percentage of diarrheic animals; daAUC, area under the curve of diarrheic animals; daAUCn, normalized area under the curve of diarrheic animals; MDF, maximum percentage of diarrheic feces; MDFd, day with maximum percentage of diarrheic feces; dfAUC, area under the curve of diarrheic feces; dfAUCn, normalized area under the curve of diarrheic feces. With regard to duration: DDB, day of diarrhea beginning (DPI); DDE, day of diarrhea ending (DPI); DP, diarrhea period; DwD, days with diarrhea. With regard to severity: MDI, maximum diarrhea index; MDId, day of maximum diarrhea index (DPI); sAUC, area under the curve of severity; sAUCn, normalized area under the curve of severity. Results are expressed as mean ± SEM (n = 12-21 animals/group). Statistical differences: * $p<0.05$ *vs.* REF; # $p<0.05$ *vs.* DRI.

		REF	DRI	HBC	PRE1	PRE2	PRO	SYN
Incidence	**MDA**	0.00	86.67	0.00#	50.00#	61.90#	35.71#	69.23#
	MDAd	-	10	-	7	8	8	8
	daAUC	0.00	225.00	0.00	192.50	176.19	100.00	245.80
	daAUCn	0.00	225.00	0.00	92.50	61.90	100.00	89.16
	MDF	0.00	100.00	0.00#	100.00	81.25#	38.46#	81.82#
	MDFd	-	9	-	7	8	8	8
	dfAUC	0.00	368.96	0.00	376.03	280.03	122.90	425.63
	dfAUCn	0.00	368.96	0.00	167.50	96.50	122.90	184.89
Duration	**DDB**	-	8.2 ± 0.3	-	7.7 ± 0.3	7.9 ± 0.3	8.0 ± 0.2	7.4 ± 0.4
	DDE	-	9.9 ± 0.1	-	11.0 ± 0.4#	9.4 ± 0.3	8.8 ± 0.4#	10.2 ± 0.7
	DP	0.0 ± 0.0	2.7 ± 0.3*	0.0 ± 0.0#	3.6 ± 0.5*	2.4 ± 0.3*	1.1 ± 0.3*#	3.5 ± 0.7*
	DwD	0.0 ± 0.0	2.4 ± 0.2*	0.0 ± 0.0#	2.2 ± 0.3*	1.8 ± 0.2*#	1.0 ± 0.3*#	2.9 ± 0.7*
Severity	**MDI**	1.05 ± 0.03	2.60 ± 0.11*	1.15 ± 0.05#	2.58 ± 0.11*	2.37 ± 0.10*	2.14 ± 0.22*	2.53 ± 0.19*
	MDId	-	8.40 ± 0.27	-	8.61 ± 0.42	7.95 ± 0.24	8.33 ± 0.22	8.60 ± 0.54
	sAUC	0.03 ± 0.02	3.46 ± 0.22*	0.25 ± 0.09*#	3.27 ± 0.39*	2.75 ± 0.32*	2.29 ± 0.37*#	4.00 ± 0.92*
	sAUCn	0.03 ± 0.02	3.46 ± 0.22*	0.25 ± 0.09*#	2.28 ± 0.30*#	2.46 ± 0.30*#	2.29 ± 0.37*#	2.69 ± 0.72*

The PRE2 group had lower %DA than the DRI group during all the process. The MDA of this group was about 60 % on day 8 ($p<0.05$ *vs.* DRI group) and the %DA on days 9 and 10 was 10 % and 30 %, respectively ($p<0.05$ *vs.* DRI group).

Figure 3c shows %DA from those groups receiving the PRO and SYN supplement. PRO only induced a clear %DA after SA11 inoculation, being this value always lower than that of the DRI group. It has to be highlighted that the MDA in this group was 36 % (**Table 1**), achieved on day 8 and much lower than the 87 % of the DRI group, and that the %DA during days 7-11 was statistically lower as well ($p<0.05$).

The SYN induced a certain %DA before and after the SA11 inoculation in a similar way as PRE1, but achieving higher values (30-60 %). The MDA in this group was 70 % on day 8 ($p<0.05$ *vs.* DRI group) and the %DA during days 9 and 10 was reduced to 23 % and 30 %, respectively ($p<0.05$ *vs.* DRI group).

Thus, as before and after the SA11 infection, the PRE1 and SYN diets induced changes in the fecal consistency, increasing the number of feces considered as diarrheic. To better dissect this direct effect, already described for certain prebiotics, a normalization of the results was performed in basis of the average punctuation of the products obtained in the period previous to infection (d3-d6) and after infection was solved (d12-d16). This procedure has demonstrated to be similar to that performed using data from a non-infection study [43]. After normalization of the data, the effect of

421 PRE1 and its combination with the probiotic were even more evident (**Table 1** and **Supplementary**
422 **Figure S1**)
423
424 When the AUC of %DA was calculated (daAUC, **Table 1**), it could be seen that all the
425 supplemented groups, with exception of the SYN group, presented a lower value than the DRI
426 group. In contrast, daAUC for the SYN group was higher than that in the DRI group. However,
427 when the daAUC was normalized by calculating the AUC of the increment of incidence during SA11
428 infection from the baseline of each group (without counting the non-pathogenic "diarrhea" induced
429 by the prebiotics in the PRE1, PRE2 and SYN groups), it was significantly lower.
430
431 When the incidence data is calculated by using the second approach, results corresponding to
432 the incidence of diarrheic feces (%DF) (**Table 1**), and its consequent parameters, i.e. MDF, MDFd,
433 dfAUC and dfAUCn, it can be observed the same pattern as the %DA.

434 *3.3. Duration of diarrhea*

435 With regards to the duration of the diarrhea process, in the DRI group diarrhea started at day
436 8.2 ± 0.3 (beginning day of diarrhea, DDB) and ended at day 9.9 ± 0.1 (end day of diarrhea, DDE). The
437 diarrhea period (DP) and the days with diarrhea (DwD) were 2.7 and 2.4, respectively (**Table 1).** The
438 PRE1 and SYN diets did not modify these variables, but the PRE2 was able to reduce the DwD, and
439 the PRO reduced both DP and DwD ($p<0.05$) (**Table 1**). It should be emphasized that the PRE1 and
440 SYN groups still had scores >1 until the end of the study and that these duration variables are also
441 influenced by the direct effect on stool consistency from PRE1, thus the normalized data allow us to
442 better observe the positive effect of such product (data not shown).

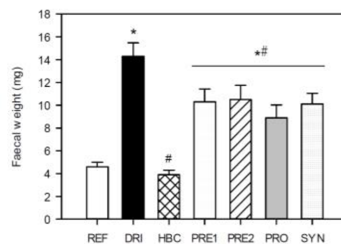
443 *3.4. Severity of diarrhea*

444 Effect on stool consistency was only found after SA11 infection in early life, but not after EDIM
445 infection on day 17, or combination of both (DRI) during the second infection. Regarding first
446 infection, as it can be seen in **Figure 3d**, the severity curve in the DRI group increased from day 7 (1
447 DPI) and was maintained at similar values until day 10 (4 DPI). A day 11, the mean score was under
448 2, and therefore, it is not likely that the animals had diarrhea. Afterwards, no animals from this
449 group had signs of diarrhea and had a DI = 1. This diarrhea was totally prevented by the HBC
450 intervention, as can be observed by the low punctuations obtained along the process which were all
451 around 1 (**Figure 3d)**. In contrast, the PRE1 was not able to prevent this clinical variable of diarrhea
452 and the score values were similar to those from the DRI group (**Figure 3e**). The direct effect of this
453 compound on fecal texture is evidenced due to scores higher than 1 before infection and after the
454 diarrhea period (days 7-12), as previously commented. The PRE2 modulated the severity of the
455 process by decreasing the scores on specific days (9 and 10, $p<0.05$). As also observed for the
456 incidence and duration variables, the PRO intervention was the most effective in reducing the
457 severity of diarrhea ($p<0.05$ *vs.* DRI group on days 7, 9 and 10). However, when the probiotic is
458 administered with the PRE1 (SYN group) this protective action disappeared (**Figure 3f**). The
459 effectiveness of the PRE1 and SYN diets in controlling the RV infection could not be seen through
460 direct scoring data because the products induced features that occulted their putative action. The
461 mean MDI for all infected groups was around 3, with exception of that from the HBC group, which
462 was lower. In all cases, it was obtained around day 8 (**Table 1**). The AUC of the severity pattern
463 calculated during the period with diarrhea (**Table 1**) showed AUC values of about 3 in inoculated
464 animals, whereas REF animals did not develop diarrhea and had AUC values around 0, as well as
465 the HBC group. Interestingly, a significant reduction in sAUC was observed for the PRO group with
466 respect to the DRI group, demonstrating an overall reduction in the severity of the disease ($p<0.05$).
467 The PRE1 and PRE2 groups just showed a significant reduction in sAUC when it was normalized
468 (from their baseline DI present before and after the infective process), and the SYN did not modified
469 this variable.

470

471 *3.5. Fecal weight*

472 The weight of the fecal samples was recorded throughout the study. During the acute diarrhea
473 period (1-4 DPI), animals from the DRI group had a higher fecal weight (~14 mg) than those from the
474 REF group (~5 mg) (p<0.05). The fecal weight from the HBC group in this period was significantly
475 lower than that from the RV animals (p<0.05) and similar to that from the REF group (~4 mg). The
476 weights of fecal samples from the PRO, PRE1, PRE2 and SYN groups were higher than that from the
477 REF group (p<0.05), but all nutritional interventions were able to decrease the fecal weight with
478 respect to the DRI group (p<0.05) (**Figure 4**). Before and after this period, the fecal weight was
479 similar among groups and only the PRE1 and SYN groups had a slightly higher value (data not
480 shown). The infection with EDIM alone on day 17 did not induce any change in fecal weight.
481 Moreover, in all the studied groups, those double infected without being supplemented (DRI) or
482 those after the dietary intervention displayed a fecal weight of 27.4 ± 1.0 mg in the 1-4 days post
483 EDIM inoculation (data not shown).



484

485 **Figure 4.** Fecal weight (mg) during the acute diarrhea period (pooled data from 1-4 DPI samples).
486 Results are expressed as mean ± SEM (n = 32-49 samples/group). Statistical significance: *p<0.05 *vs.*
487 REF, #p<0.05 *vs.* DRI.

488 *3.6. pH changes*

489 The pH of fecal samples from 1-5 DPI in each of the two infections was measured. Regarding
490 SA11 infection, the DRI group showed a higher fecal pH (7.18 ± 0.14) on day 7 (1 DPI) with respect to
491 the REF group (mean 5.54 ± 0.44) (p<0.05). All the nutritional interventions avoided this change and
492 had pH values similar to that in the REF group (of about 5.5-6). However, the infection with EDIM
493 did no caused significant changes in pH and neither the diets (data not shown).

494 *3.7. Temperature changes*

495 Rats' body temperature was measured from 0 to 3 DPI of both SA11 and EDIM infections as a
496 possible new marker of disease. The relative increase of temperature for the maximum value
497 obtained after infection with respect to the 0 DPI value was calculated for each animal and it is
498 shown in **Table 2**. After the first infection with SA11, an increase of rectal temperature was observed
499 among the infected animals when compared to the REF group (p<0.05). Even though diarrhea was
500 not observed in the group of animals infected with the EDIM at d17 (data not shown), an increase of
501 body temperature was found in this group (2.61± 0.97 ºC, p<0.05 *vs.* REF group), suggesting the
502 presence of infection. However, the presence of the first infection in the DRI animals seems to
503 prevent such increase when the second infection is performed. In addition, the double infected
504 supplemented groups did not have any effect on the temperature after the second infection (**Table**
505 **2**).

506

507 **Table 2.** Body temperature of the peri-inoculation period (first and second infection). Results are
508 expressed as mean ± SEM (n = 12-21 animals/group). Statistical differences: *p<0.05 *vs.* REF.

	1st infection	2nd infection
REF	0.90 ± 0.29	0.21 ± 0.14
DRI	2.11 ± 0.41*	0.00 ± 0.00
HBC	4.85 ± 1.23*	1.15 ± 0.65
PRE1	3.18 ± 0.95*	0.27 ± 0.18
PRE2	3.16 ± 0.70*	0.03 ± 0.03
PRO	3.42 ± 0.75*	1.02 ± 0.45
SYN	1.10 ± 0.42	0.40 ± 0.40

509 *3.8. Viral shedding*

510 In all RV-inoculated animals, the maximum viral shedding was observed on the first day after
511 inoculation (1 DPI) for both SA11 and EDIM infections. Taking day 7 (1 DPI for SA11) into account,
512 the HBC group had a similar RV shedding than the DRI group (101.36 % with respect to the DRI
513 group's viral shedding). The PRO group showed a viral clearance of the 135.82 % with respect to that
514 of the DRI group. On the contrary, the PRE1, PRE2 and SYN groups had a lower viral shedding than
515 the DRI group (59.33, 70.10 and 57.70 %, respectively), a difference only significant for the SYN
516 group (p<0.05).
517
518 As for day 18 (1 DPI for EDIM), the DRI group had lower viral shedding than that in animals
519 only infected with EDIM at day 17, without the previous infection with SA11 (171.46 % with respect
520 to the DRI group's viral shedding). However, in this case all the supplemented groups showed
521 higher viral clearance than the DRI group (1272.39 % for the HBC group; 474.25 % for the PRE1
522 group; 138.13 % for the PRE2 group; 302.86 % for the PRO group; and 434.34 % for the SYN group),
523 but without statistical significance due to the high inter-individual variability found.

524 *3.9. DTH*

525 The DTH response was studied on day 28, after 24 and 48 h post-ear priming. At 24 h, the REF
526 group obtained an ear thickness increase of 0.86 ± 0.46. The EDIM group (without the first infection
527 with SA11) had an increase of 1.13 ± 0.52 (p<0.05 vs. REF), however the DRI group had similar values
528 than those obtained from the group REF (0.71 ± 0.57). None of the supplemented groups showed
529 statistical differences (0.86 ± 0.70 for HBC; 1.91 ± 0.77 for PRE1; 2.00 ± 0.65 for PRE2; 0.88 ± 0.64 for
530 PRO; and 2.00 ± 0.77 for SYN group) with respect to the REF or the DRI groups. The DTH response at
531 48 h followed a similar pattern (data not shown).

532 *3.10.* Ex vivo *specific anti-RV antibody production*

533 The ability of sensitized cells to produce specific Ig in the systemic and the mucosal
534 compartments has been studied after dietary treatment in animals only infected with SA11 or double
535 infected with SA11 and EDIM (**Table 3**). It can be observed that none of the single infections (SA11 at
536 day 6 alone or EDIM at day 17 alone) were able to increase this variable of spontaneous ability to
537 have natural defenses against the RV. However, the double infection induced an increase in the
538 number of the Ig-SC in the systemic compartment (p<0.05). It did not happen in the mucosal
539 compartment.
540
541
542
543

544 **Table 3.** *Ex vivo* anti-RV Ig producing spleen and MLN cells from 28-day-old animals from SA11
545 and double (SA11 and EDIM) infected groups with dietary intervention. Results are expressed as
546 Mean Ig-SC/10^6 cells ± S.E.M (n= 3-12 animals/group). Statistical differences: *p<0.05 *vs.* REF; #p<0.05
547 *vs.* SA11 or DRI.

	SA11 infection		SA11 and EDIM infections	
	Spleen	**MLN**	**Spleen**	**MLN**
REF	204.7 ± 40.8	245.1 ± 43.9	204.7 ± 40.8	245.1 ± 43.9
DRI	228.0 ± 21.2	318.3 ± 36.3	302.9 ± 18.5*	296.7 ± 29.3
EDIM	-	-	223.4 ± 34.6	205.5 ± 36.7
HBC	340.0 ± 75.1*#	443.3 ± 43.3*#	242.6 ± 51.1	215.1 ± 42.9
PRE1	435.1 ± 72.1*#	188.3 ± 16.4#	253.1 ± 23.4	246.0 ± 26.9
PRE2	380.8 ± 42.3*#	377.5 ± 22.1*	261.6 ± 19.8	242.8 ± 28.1
PRO	356.7 ± 114.0*#	320.0 ± 45.8	174.6 ± 46.0#	216.5 ± 51.9
SYN	396.7 ± 112.7*#	375.0 ± 62.1*#	250.9 ± 57.3	276.2 ± 54.8

548
549 Focusing on the first infection (**Table 3**), all dietary interventions induced an increase in the
550 anti-RV Ig-SC with respect to the REF and SA11 groups, with exception of the PRE1 on the MLN
551 (p<0.05 *vs.* SA11 group).
552
553 A different pattern was found when animals were subjected to a double infection (**Table 3**). All
554 dietary interventions down-modulated the Ig-SC number and therefore, had an immune response
555 similar to the non-infected group.

556 *3.11.* Ex vivo *cytokine production*

557 The levels of *ex vivo* production of IFNγ by splenocytes after 72 h were under the limit of
558 detection in some groups. This is the case of the REF, DRI and HBC (double infected) groups
559 (**Figure 5a**). However, the simple infected groups, either with SA11 at day 6 or EDIM at day 17, had
560 cells able to respond to the challenge and produced some amount of IFNγ. Concerning the dietary
561 interventions in single infected animals, any of them greatly modified the levels of IFNγ induced by
562 SA11 *in vitro* stimulation, with exception of the SYN group, which had twice that value. After the
563 second infection, although the DRI group had no IFNγ, all the pre- and probiotic interventions
564 induced detectable levels, being the higher effect in the SYN group again (**Figure 5a**).

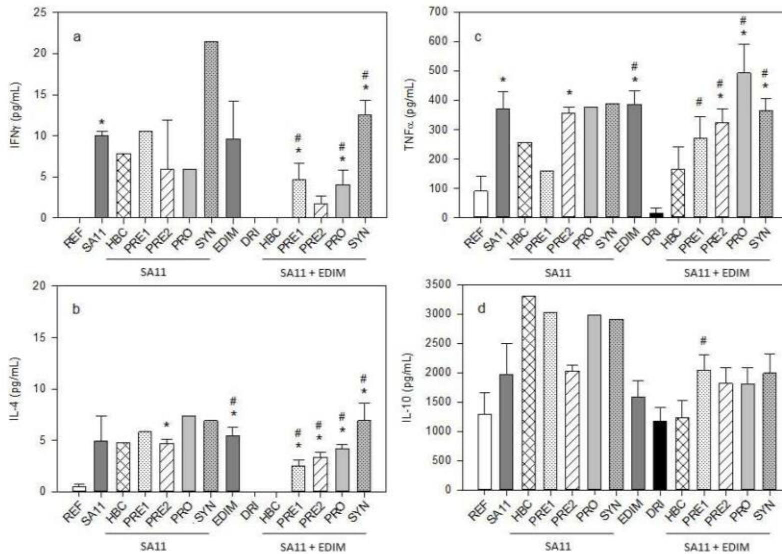


Figure 5. Cytokine *ex vivo* production in single and double infected 28-day-old animals. Results are expressed as mean ± SEM (n = 3-12 animals/group). Statistical significance: *$p<0.05$ *vs.* REF, #$p<0.05$ *vs.* SA11 or DRI (first and second infection, respectively).

IL-4 was also studied (**Figure 5b**). This cytokine was lowly produced in cells from the REF group and not detected after double infection with both RV (DRI group). However, the single infections did induce IL-4, similarly to that described for IFNγ. The pre- and probiotic supplementations did not significantly modify the IL-4 production in SA11 infected animals, although the PRO and SYN groups showed higher levels (**Figure 5b**). After the double infection, the DRI group had no detectable IL-4, as well as the HBC group, but the rest of interventions induced the production of this cytokine, being the SYN group the one with the highest production (**Figure 5b**).

As for TNFα, the levels of this cytokine were low in the REF group and the infection with either SA11 or EDIM alone induced an increase up to four times (**Figure 5c**). However, this increase was not observed in the DRI group. The dietary intervention did not affect the production of TNFα when SA11 infection was performed alone, but they did induce an increase in such levels after a double infection when compared to DRI group levels (**Figure 5c**).

Finally, there was no statistical difference between the IL-10 *ex vivo* production in the REF, single infected groups and the DRI group (**Figure 5d**). It can be also observed a non-significant increase of the IL-10 production due to the supplements in double infected animals, with respect to the DRI group.

3.12. Mucosal IgA and serum IgM global antibody levels

The mucosal immune status was studied in the animals with different type of infections and after receiving the products at the first and second end point. The total IgA was measured in

intestinal wash after the first (day 16) and second infection (d28). On day 16, the SA11 group had 2367.73 ± 689.70 arbitrary units (AU) of total IgA in gut wash and all dietary interventions increased this value, although only the PRE1 and SYN groups were significantly different (7411.41 ± 3714.63 AU for HBC; 14236.02 ± 3855.70 AU for PRE1; 2728.46 ± 588.06 AU for PRE2; 2998.60 ± 1678.05 AU for PRO; and 27800.00 ± 5809.04 AU for SYN group). On day 28, the DRI group displayed values of 923.94 ± 112.22 AU of total IgA in gut wash, which was similar to this antibody's levels in the SA11 group at this age (1003.60 ± 178.12), but higher than that in the EDIM group (451.90 ± 66.85, $p<0.05$). In this case, the supplements did not affect the IgA levels, with exception of the HBC and the PRO groups, which had lower levels (460.14 ± 97.29 AU for HBC; 1193.12 ± 131.11 AU for PRE1; 681.31 ± 120.25 AU for PRE2; 591.59 ± 96.51 AU for PRO; and 631.49 ± 142.09 AU for SYN group).

Additionally, the systemic immune status in such early life was also assessed by evaluation of total IgM measurement in serum after the first (day 16) and second infection (d28). Age influences the production of this Ig, being values on day 28 two-times higher than those at day 16 (data not shown). On the other hand, the infections did not influence such values, and only the SYN diet was able to increase the IgM titters ($p<0.05$) (data not shown).

3.13. Anti-RV Ig in the intestine and serum

Specific anti-RV antibodies were quantified in intestinal wash from 16- and 28-day-old rats as well (**Table 4**).

At mucosal level, the DRI group at day 16 of life had similar titters of anti-RV antibodies than the REF group or even lower. Although no statistical significance was found on d16, the pre- and probiotic supplementations showed a tendency to increase these levels. Regarding second infection, there was an age-dependent increase of this titters, as can be seen on day 28 of life when compared with day 16, but again without significant differences between REF and DRI groups. A group of only infected with EDIM animals had 119.27 ± 50.14 AU of anti-RV antibodies, slightly higher than that of the DRI group (with a previous RV infection). The nutritional interventions did not affect this variable either (**Table 4**).

Table 4. Specific anti-RV antibodies in gut wash (total Ig) and serum (total Ig and IgM) from 16- and 28-day-old rats. Results are expressed as mean ± SEM (n = 6-12 animals/group). Statistical significance: *$p<0.05$ *vs.* REF; ǂ$p<0.05$ *vs.* DRI.

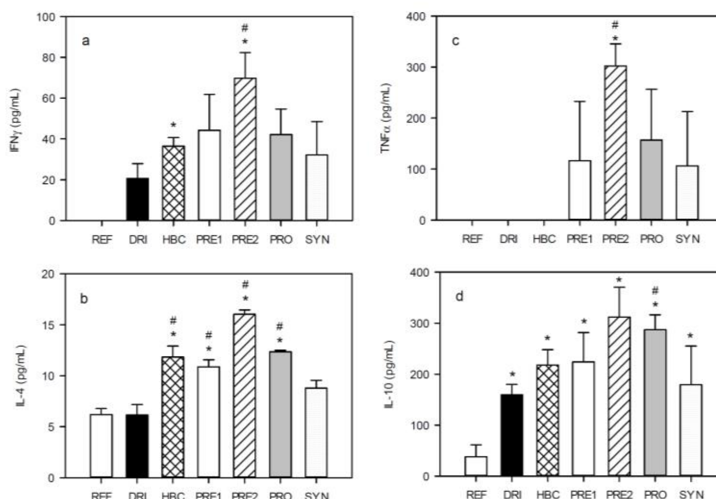
			REF	DRI	HBC	PRE1	PRE2	PRO	SYN
d16	GW	Ig	14.85 ± 3.00	7.14 ± 2.43	6.09 ± 1.82*	13.05 ± 3.92	11.34 ± 3.00	22.97 ± 6.94	23.45 ± 6.75
	Serum	Ig	1624.96 ± 71.22	954.78 ± 155.66*	1268.11 ± 195.28	1234.32 ± 193.24	1144.40 ± 222.92	980.19 ± 68.19*	1537.90 ± 64.49ǂ
		IgM	442.64 ± 41.28	388.02 ± 61.10	540.19 ± 68.59	419.68 ± 47.22	404.05 ± 48.24	500.79 ± 58.51	451.96 ± 99.81
d28	GW	Ig	27.37 ±27.25	54.88 ± 35.31	109.08 ± 56.77	35.24 ± 23.21	52.00 ± 26.85	71.58 ± 50.20	31.99 ± 31.82
	Serum	Ig	1055.90 ± 81.64	1048.51 ± 84.14	1510.03 ± 276.22	883.89 ± 77.37	1033.92 ± 139.09	800.36 ± 64.38*ǂ	1041.69 ± 89.86
		IgM	575.59 ± 28.71	711.26 ± 95.59	1067.42 ± 196.08*	700.77 ± 71.57	746.42 ± 83.82	774.38 ± 89.41	746.52 ± 75.46

Specific anti-RV antibodies were quantified in serum (total and IgM) from 16- and 28-day-old rats (**Table 4**). The REF group had already levels of anti-RV Ig, which were even higher than those after SA11 infection at d6 or EDIM infection at day 17 (682.43 ± 28.86 AU and 1154.41 ± 116.44 AU, respectively). The EDIM infection alone and the DRI behaved quite similar. Interestingly, the titters did not increase with age, and even decreased in some cases. Regarding the dietary interventions,

629 just the PRO and SYN groups modified the anti-RV Ig (p<0.05). The PRO diet down-modulated the
630 titters in both time points, and the SYN diet up-modulated it on day 16 (**Table 4**).
631
632 Specific anti-RV IgM followed a similar pattern than total IgM in serum. Levels increased with
633 age, and REF values were similar to those of infected animals, in this case, with exception of EDIM
634 alone (905.03 ± 87.80, p<0.05 *vs.* REF group). The HBC seemed to potentiate the production of these
635 antibodies (p<0.05 on day 28), but not clearly the rest of dietary interventions.

636 *3.14. Cytokine levels in gut wash*

637 Cytokines in gut wash samples at the end of the study (day 28) were determined. In healthy
638 animals (REF group), IFNγ was not detected. However, all the infected animals (double infected
639 with SA11 and EDIM) had quantifiable amounts of this molecule (**Figure 6a**). All dietary
640 interventions induced also higher levels than the DRI animals, however, only HBC and PRE2
641 achieved statistical significance.



642

643 **Figure 6.** Cytokine levels in gut wash from 28-day-old rats. Results are expressed as mean ± SEM
644 (n = 3 animals/group). Statistical significance: *p<0.05 *vs.* REF, #p<0.05 *vs.* DRI.

645 Regarding the IL-4, it was detected in all samples from REF and infected groups (**Figure 6b**).
646 Although IL-4 levels are similar between the REF and DRI groups, the animals with dietary
647 interventions displayed higher values, being statistically different than those in REF (p<0.05) and
648 DRI groups (p<0.05), with exception of the SYN group. The PRE2 intervention was the most effective
649 in enhancing IL-4 production.
650
651 TNFα is also a cytokine measured in this fluid that was not detected in all samples. To date,
652 none of the samples in the REF, DRI and HBC group had detectable levels. All dietary interventions
653 with pre- or probiotics induced detectable levels of TNFα, but only the PRE2 group induced a
654 statistical increase (**Figure 6c**).
655

With respect to IL-10, it was found in all samples at levels ranging from 40-300 pg/mL. REF group had values of ~40 pg/mL, and these values were increased up to four times in the DRI group and even more due to the HBC treatment. All infected groups displayed significantly higher values than non-infected animals, being only significant vs. group DRI those animals from the group PRO (**Figure 6d**).

3.15. Gene expression changes

Gene expression of several genes involved in immune response and intestinal barrier were studied, as it can be observed in **Table 5**.

Regarding TLRs, neither TLR2 nor TLR4 were modified by DRI or HBC interventions. However, the four dietary interventions modulated this pattern similarly. Although some methodological problems with PRE1, the dietary treatments with PRE2, PRO or SYN significantly increase TLR2 levels with respect to REF or DRI animals. On the contrary, TLR4 gene expression (**Table 5**) was reduced by all four interventions when compared with the REF group, suggesting a down-modulatory action on the activation of the immune response.

Table 5. Colonic gene expression from 28-day-old rats. Results are expressed as mean ± SEM (n = 6 animals/group). Statistical significance: *p<0.05 *vs*. REF, ‡p<0.05 *vs*. DRI.

	TLR2	TLR4	Occludin	Claudin-2	Mucin	IL-10	TGFβ
REF	100.00 ± 17.14	100.00 ± 19.46	100.00 ± 17.14	100.00 ± 17.10	100.00 ± 29.31	100.00 ± 17.65	100.00 ± 15.79
DRI	99.21 ± 55.51	87.09 ± 38.38	119.48 ± 15.28	117.52 ± 23.67	144.33 ± 26.46	103.86 ± 28.57	101.58 ± 5.95
HBC	84.55 ± 34.39	70.58 ± 16.75	80.13 ± 25.88	283.25 ± 182.97	240.58 ± 170.19	145.85 ± 47.41	106.09 ± 16.06
PRE1	-	59.86	36.76 ± 12.00*‡	-	-	135.03 ± 62.54	102.54 ± 30.13
PRE2	205.00 ± 49.74*	46.00 ± 16.88*	58.97 ± 16.30*‡	1951.01 ± 982.50	110.05 ± 56.62	115.94 ± 33.81	63.19 ± 7.99*‡
PRO	252.19 ± 62.42*‡	39.33 ± 7.00*	65.67 ± 12.67*‡	622.36 ± 322.50	100.41 ± 18.57	148.34 ± 74.31	62.85 ± 10.34*‡
SYN	678.41 ± 77.09*‡	39.10 ± 15.88*	50.35 ± 8.87*‡	1115.81 ± 360.78*	103.92 ± 44.01	92.23 ± 6.41	48.14 ± 3.16*‡

The analysis of IL-4 revealed that it was very low expression in this tissue and conditions (in most of the samples was not detected and in some others with a Ct>38). However, with the low number of samples in which it was detected the results showed that the DRI group had a 143.74 ± 63.91 % (with respect to the 100 % in REF group), whereas the HBC and the PRO diets reduced up to 38.33 ± 14.44 and 29.10 ± 7.23 %, respectively. The gene expression of IFNγ in this intestinal tissue was even lower, with only some samples with quantifiable expression in the REF, DRI and HBC groups, but with Ct>39.

Occludin and claudin-2 were also evaluated (**Table 5**). Similarly to the other genes studied, the gene expression of both TJ proteins was not affected in the DRI or the HBC animals. However, the dietary treatments induced a decrease of the occluding gene expression (p<0.05) and an increase of the claudin gene expression, which was only significant in the case of the SYN group. Mucin gene expression (**Table 5**) was not modified by either the infective process or the dietary interventions.

Finally, IL-10, TGF-β and foxp3 were also quantified. The gene foxp3 was not detected in any of the analyzed samples (data not shown). However, the cytokines IL-10 and TGF-β were detectable (**Table 5**). In the case of IL-10, its gene expression was very low and it was unaffected by the infection or the dietary supplementations. On the contrary, the gene expression of TGF-β was not affected in the DRI or HBC groups, as well as in PRE1 group but, PRE2, PRO and SYN induced a significant reduction.

3.16. SCFAs production

695 The main SCFAs (acetic, propionic and butyric), but also lactic and formic acids, were
696 quantified in the fecal samples of 21 and 28-day-old rats. Globally, total and specific SCFAs in the
697 REF group were not statistically modified due to RV infections or dietary interventions, except for
698 an increase of acetic acid in the DRI group at day 28 ($p<0.05$) (**Supplementary Figure S2**).

699 **4. Discussion**

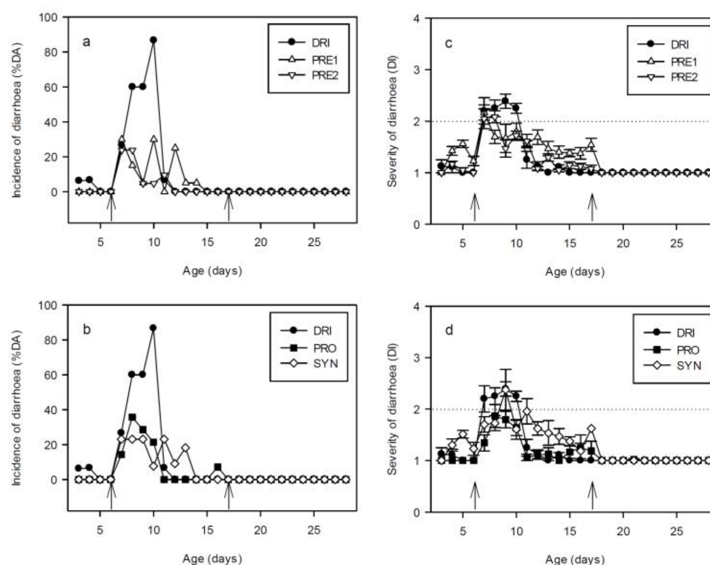
700 Rotavirus is a major cause of acute and severe gastroenteritis in children, and although
701 appropriate rehydration is the election therapeutic intervention, the introduction of other strategies
702 such as prebiotics and probiotics are of interest. In this regard, by using the double RV infection
703 model in neonatal rat, which is more similar to the clinical reality than a simple infection model, this
704 study has evidenced the potential of different microbial modulator products of importance in early
705 life (two type of prebiotics and a probiotic strain) to modulate first infection and the host defenses
706 for a better second infection resolution.
707
708 Regarding the model used, it has the appropriate features expected. To date, the first infection
709 induces mild diarrhea, which was very similar than that in previous studies (with the simple SA11
710 infection model) [22,43,49]. The incidence, duration and severity of diarrhea, and the fecal weight
711 results from the DRI group after SA11 infection in this study, have been consistent with those in the
712 RV group in previous studies [43]. Additionally, changes in pH and temperature has been associated
713 to first viral infection. Moreover, as expected, no diarrhea or changes in pH and temperature has
714 been observed after the second infection, if first is performed, due to the maturity of immune system
715 as described already in the setup of the model [28]. Concerning other variables studied, the
716 maximum viral shedding of SA11 has been on 1DPI after both first and second infections.
717 Furthermore, a presence of specific antibodies at the systemic and at the intestinal level was found at
718 day 14 in the previous study [43] as well as at day 16 in this one, increasing with age in both studies.
719 Overall several changes due to SA11 and EDIM infections
720
721 In order to evaluate the effect of modulating first infection on the outcomes in the second viral
722 infection a positive protection control (HBC) was used. A complete protection from diarrhea was
723 achieved after the first infection with SA11 and it avoided the increase of fecal weight induced by RV
724 in the acute phase of the disease. However, it increased the viral shedding after the second infection
725 with EDIM, but still allowed the development of immune response, among other changes. These
726 results, which are in line with those observed in other studies [24,25,28], allow us to confirm its
727 suitability as a protective control in this design. This intervention allows us to guess the behavior
728 that a protective nutritional intervention during first infection has to induce, providing us a pattern
729 of changes in the variables analyzed to be compared.
730
731 As the second infection did not induce diarrhea we have to focus on first infection results in
732 order to evaluate the prebiotic and probiotic intervention on the control of clinical manifestations.
733 Overall, all diets seem to have a protective role somehow in some of the variables analyzed. On the
734 one hand, the scGOS/lcFOS mixture, at the dose used here, as in previous studies [43], has a masking
735 effect on the fecal consistency, which is one of the main limitations of this compound. This effect,
736 also present in the SYN intervention, induced a softened stool consistency, which is not dependent
737 of the presence of the virus. Even though the scGOS/lcFOS (Immunofortis®) supplementation was
738 able to decrease the incidence and severity of diarrhea, or at least, the impact on the fecal consistency
739 was not added to that derived from the gastroenteritis, as is observed after normalization of the
740 results. On the other hand, the GOS based prebiotic (Bimuno® GOS) had some effect on the fecal
741 consistency, but lower than the previous one, showing a clearer protection from diarrhea. These
742 changes in stool consistency, bringing it closer to breast fed infants [56,57], is then a desirable effect.
743 Anyway, the clinical amelioration by these prebiotics are in agreement with other research showing
744 anti-infective effect of this type of products in infants [56,58,59]. The PRO was the one with the

745 clearest effect. *B. breve* M-16V reduced the incidence, duration and severity of the experimental
746 diarrhea, effects that seem to be hidden by the prebiotic addition when the synbiotic is formulated.
747 These results are in line with those obtained in the previous study [43]. In this line, the increase in the
748 fecal weight seem to be a clear indicator of the incorporation of water in the total fecal content [49,60]
749 and therefore, it has been used as objective marker of diarrhea in this model. All nutritional
750 interventions here avoided the fecal weight increase associated to the acute phase of the diarrhea.
751 The effect due to scGOS/LcFOS and *B. breve* M-16V agree with those obtained in the previous study
752 [43] and seem to be as effective as the new prebiotic tested, the Bimuno® GOS.
753
754 In addition, fecal pH and body temperature were measured in the present study, as new
755 non-invasive clinical markers. The fecal pH did only change during the first infection. It may be
756 because of the electrolyte imbalances caused by the diarrhea [3]. Li et al. also observed an increase of
757 the pH in the colonic content of RV infected piglets when compared to the non-infected animals [42].
758 The dietary interventions avoided the RV-induced increase of fecal pH. On the other hand, the rectal
759 temperature increased after the first RV inoculation with SA11 in the DRI group, but not after the
760 second inoculation with EDIM. However, the EDIM inoculation on day 17 as a primary infection
761 was able to induce fever on that day, so this variable shows a differential pattern between the simple
762 and the double infection model, suggesting that the first infection allows setting up mechanisms
763 involved in second infection control. Few studies have evaluated the body temperature. For
764 example, Parreño et al. measured it after the first infection of the double infection calf model and
765 observed fever [24]. The HBC and the dietary interventions with pre- and probiotics showed a
766 similar behavior than the DRI group, which means that, even the interventions conferred protection
767 during the first RV inoculation, this infection was still able to allow mechanisms to control the
768 second infection.
769
770 The viral shedding has been suggested as a marker of protection where higher presence of the
771 virus in feces would mean higher elimination. However, this statement is not fully applicable in our
772 model and interventions, because we have demonstrated that the scGOS/lcFOS mixture has high
773 capacity to bind the RV and block its infection but at the same time did not allow the viral detection
774 by our ELISA technique [43]. In accordance to this, the peak of viral elimination after the first
775 inoculation with SA11 was substantially reduced in the groups supplemented with scGOS/lcFOS
776 alone or in combination with the *B. breve* M-16V, but levels were only slightly diminished in the
777 Bimuno® GOS group, suggesting an alternative mechanism to control the infection rather than the
778 "false receptor" one displayed by the former. In contrast, a similar viral shedding was observed in
779 the group supplemented with the probiotic when compared to the DRI group after the first
780 inoculation, as well as happened in the previous study [43]. However, after the second inoculation,
781 all dietary supplemented groups, even that with scGOS/lcFOS mixture, shed higher viral load than
782 the DRI group, maybe as a consequence of the protection conferred during the first infection, and in
783 line with the results obtained with HBC [25].
784
785 With regard to humoral immune response, it is described that the antibody-mediated immunity
786 against RV involve both systemic and mucosal responses [4]. This is a limitation of rodent models of
787 RV infection because diarrhea only appears on early suckling due to that their natural acquired
788 immune response against the virus is highly effective already in the weanling period [49,61]. In this
789 context, the presence of specific antibodies in the model used herein did not allow to clearly observe
790 a development of protection against the virus after the first infection (although on day 16 the animals
791 were already weaned, the maternal influence can still be of importance in this day), neither after the
792 second infection. Any of the supplementations showed a clear effect on the antibody titters either.
793 These results contradict those obtained in the previous study [43], in which scGOS/lcFOS alone or in
794 combination with the *B. breve* M-16V supplementation in early life increased local and systemic
795 humoral response against the virus, suggesting a modulatory role of this intervention in the
796 maturation of the immune system. However, some enhancing effects on the humoral immune status

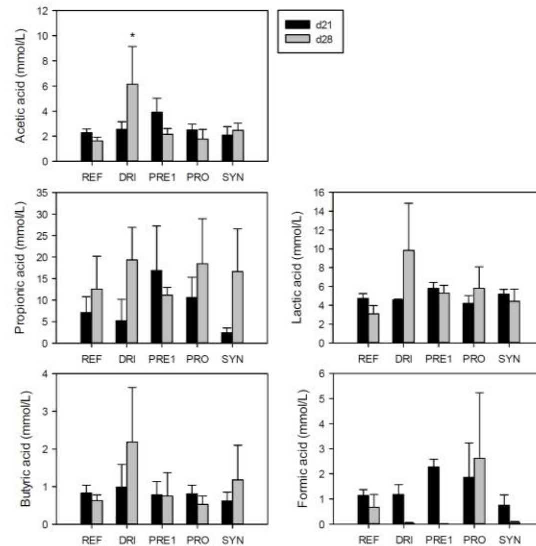
797 associated to certain dietary interventions (i.e. *B. breve* M-16V on systemic IgM) are in line with our
798 results related to the supplementation with *B. breve* M-16V in the previous study [43] and those
799 derived from other probiotics such as LGG or *L. acidophilus* NCFM, which increased IgM in pigs and
800 infants after this type of infection [62–64]. In fact, a previous study was conducted to demonstrate
801 the high immunomodulatory potential of *B. breve* M-16V in early life using the rat as a model of
802 immune development [65].
803
804 In contrast, the *ex vivo* Ig production determination allowed to observe a differential response
805 between the single infections and the double infection model. The effect of the nutritional
806 interventions could be evaluated, and even they enhanced the immune response against the first
807 virus, a down-modulation was observed in the second infection. This result may indicate that the
808 dietary reinforcement during the first infection at cellular level may lead to higher protection in the
809 second one and therefore intense humoral immune response is not required. An increase of IgA-SC
810 or IgG-SC was observed in other studies, when LGG or *L. acidophilus* NCFM were administered in
811 vaccinated and challenged with HRV (human rotavirus) pigs [62,64].
812
813 Regarding cellular response, several immune variables have been evaluated such as the DTH
814 response and the production of several cytokines. Particularly, the DTH response has been described
815 to be different depending on the model used in this context. Whereas other studies showed that
816 response after an infection at day 17 in mice and later suppression after a re-infection in mice who
817 had received a primary infection before [25,66], our previous results in rats showed the opposite
818 result, suggesting that the primary contact with the virus was able to enhance the DTH response
819 against the second [28]. However, in this study this variable behaved as the mice model and did not
820 allow obtaining clear conclusions.
821
822 Regarding cell mediators, RV triggers an immune response on the host, which is responsible for
823 the timely resolution of the disease and the subsequent acquisition of immunity against reinfections.
824 The cytokines produced act as mediators of immune and inflammatory responses, leading to the
825 recruitment and activation of different populations of leukocytes, which ultimately produce
826 cytokines in response to and against the RV. The potential part played by cytokines in the cellular
827 response to RV has highlighted the importance of this aspect of host defense. The determination of
828 intestinal cytokines during the peak of the diarrheic process, when it is already solved or after *in*
829 *vitro* challenge could give an insight of the state of immune activation.
830
831 A differential pattern between the single infections and the double infection model was also
832 found in the *ex vivo* cytokine production, meaning that first infection causes different immune
833 response in the second infection. In this case, the dietary interventions enhanced the production of
834 these cytokines when only one infection occurs but also in the double infected animals. This effect
835 was observed for all Th1 (IFNγ), Th2 (IL-4), pro-inflammatory (TNFα) and anti-inflammatory (IL-10)
836 cytokines. Several probiotics have shown the ability to enhance cytokines production *in vitro* [67].
837 Wen et al. [64,68] found an increase of IFNγ production by T cells and a decrease of IL-10 production
838 by Treg in vaccinated with HRV and administered with LGG pigs. On the other hand, the intestinal
839 wash is a fluid with components from the mucosa layer which reflects the activity of the intestine.
840 Cytokines from intestinal wash were only determined in double infected animals and followed a
841 similar pattern than the cytokines produced *ex vivo*.
842
843 Regarding the gene expression, TLR2 and TLR4 genes were selected to be studied because TLR2
844 recognizes, among other ligands, the cell-wall components such as peptidoglycan, lipoteichoic acid
845 and lipoprotein from Gram-positive bacteria, whereas the second one binds to the bacterial
846 lipopolysaccharide (LPS), which is the major structural component of the outer wall of all
847 Gram-negative bacteria and a potent activator of the immune system. IFNγ and IL-4 were the
848 selected cytokines, involved in the Th1 and Th2 responses, respectively, for this assay. Besides

849 immune molecules, others such those from the TJ, which have been described to be modulated by
850 probiotics *in vitro* and *in vivo* elsewhere [69], were also quantified. Mucin is a high molecular weight
851 and heavily glycosylated protein produced by the intestinal epithelial tissues in order to form a gel
852 that acts in lubrication, cell signaling or in chemical barriers formation [69]. Finally, molecules
853 involved in regulatory and tolerogenic response, such as IL-10, TGF-β and FoxP3, were also
854 measured.
855
856 No differences in levels of these molecules in the double-infected group (DRI) and HBC
857 treatment were observed at day 28. This result may indicate that at this age, more than two weeks
858 after the second viral infection, no effects if any, due to the RV infection persisted. However, the
859 effect of the supplementation with some of the products seem to still be evident. On the one hand,
860 the presence of prebiotics and probiotics (or both) in the gut, may increase the proportion of
861 Gram-positive bacteria (i.e. bifidobacteria and/or lactobacilli) and therefore the TLR in charge of its
862 detection too, the TLR2. On the contrary, Wang et al. [70] did not found a significant increase in
863 TLR2 gene expression in the intestinal mononuclear cells of rotavirus vaccinated and administered
864 with LGG pigs, but they did find an increase in TLR4 gene expression. Other studies have observed
865 the ability of some probiotics to improve the barrier function [69], which is not clear in the present
866 work because whereas occludin is downmodulated, claudin expression is enhanced. The results
867 obtained here also suggest that the dietary interventions maintain or even down-modulate the
868 regulatory and tolerogenic immune response, in disagreement with Wang et al. [70], that also found
869 an increase of IL-10 gene expression.
870
871 Finally, the SCFAs concentration found in feces was very low in all groups, as well as happened
872 in the previous study [43] and in contrast with the increase of the SCFAs seen in other studies with
873 GOS or GOS/FOS [71–73]. This lack of positive outcome in the fecal samples analyzed be due to the
874 prebiotic supplementation can be a reflect of their high absorption in the colon [74] that might
875 therefore affect their content in fecal samples.
876
877 In conclusion, both Immunofortis® and Bimuno® GOS prebiotic products, as well as *B. breve*
878 M-16V supplementation in early life are able to ameliorate RV-induced gastroenteritis whereas
879 allow the host to elaborate own immune responses that would be of importance to control a second
880 infection. The results obtained help in identifying key prebiotics and probiotics with modulatory
881 effects on the maturation of defense mechanisms of the newborn, especially in the prevention and
882 treatment of the RV infections. Further studies are needed in order to get a deeper insight on the
883 effects of these compounds, and to understand if such effects could be transmittable from the rat
884 model to humans. Moreover, the timing and dosage of administration of these microbial modulator
885 compounds is also to be further determined. All these determinations may lead to conclude if they
886 are suitable for strengthening the mechanisms of defense of the newborn and if this scientific
887 knowledge generated by these results at a preclinical level could permit, at mid-time, the
888 incorporation of these types of functional supplements into infant formulas.

889 **Supplementary Materials:** The following is available online at www.mdpi.com/link, Figure S1: Normalized
890 clinical indexes. Normalized incidence of diarrhea (a) for DRI, PRE1 and PRE2 groups; (b) for DRI, PRO and
891 SYN groups: results are expressed as % of diarrheic animals. Normalized severity of diarrhea (c) for DRI, PRE1
892 and PRE2 groups; (d) for DRI, PRO and SYN groups: fecal samples are scored from 1 to 4 based on color, texture
893 and amount of stool. Scores of diarrhea index (DI) ≥2 indicates diarrheic feces. The inoculation days are
894 indicated by arrows. Results are expressed as mean ± SEM. Statistical differences mentioned in the text
895 (n = 15-21 values/group); Figure S2: SCFAs concentrations in feces from the different experimental groups at
896 day 21 and 28. Results are expressed as mean ± SEM (n = 3-7 samples/group). Statistical differences: *$p<0.05$ vs.
897 REF.



898
899 **Figure S1.** Normalized clinical indexes. Normalized incidence of diarrhea (**a**) for DRI, PRE1 and
900 PRE2 groups; (**b**) for DRI, PRO and SYN groups: results are expressed as % of diarrheic animals.
901 Normalized severity of diarrhea (**c**) for DRI, PRE1 and PRE2 groups; (**d**) for DRI, PRO and SYN
902 groups: fecal samples are scored from 1 to 4 based on color, texture and amount of stool. Scores of
903 diarrhea index (DI) ≥2 indicates diarrheic feces. The inoculation days are indicated by arrows.
904 Results are expressed as mean ± SEM. Statistical differences mentioned in the text (n = 15-21
905 values/group).



906

907 **Figure S2.** SCFAs concentrations in feces from the different experimental groups at day 21 and 28.
908 Results are expressed as mean ± SEM (n = 3-7 samples/group). Statistical differences: *p<0.05 vs. REF.

909

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915 SS performed the experiments; FJPC and MRA analyzed the data, interpreted results and drafted the paper; all
916 the authors contributed to the critical review and revision of the manuscript.

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919 B.V. The other authors declare that they have no conflicts of interest.

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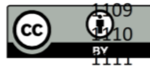

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DISCUSSIÓ

Els principals agents causals de gastroenteritis aguda en nens menors de cinc anys arreu del món són els RV del grup A (130,131). A causa del seu impacte en el sistema de salut i la societat, és interessant explorar la infecció per RV i la seva patologia, juntament amb la resposta clínica i immunitària, així com la seva possible modulació per part de compostos nutricionals.

Dur a terme estudis d'intervenció controlats en humans, especialment en lactants, presenta certes dificultats, i per aquesta raó, són necessaris els models animals. Existeixen diversos models animals d'infecció per RV, però la majoria d'ells són models d'infecció simple, que no reflecteixen les múltiples reinfeccions que els humans pateixen en les primeres etapes de vida.

La rata lactant és un model útil, amb evidència científica substancial i amb un ràtio cost-efectivitat adequat (51). De fet, la rata constitueix un bon model de desenvolupament immunitari, ja que, degut al seu període curt de gestació, les rates lactants presenten un sistema immunitari més immadur que aquelles espècies amb un període de gestació més llarg. Aquest fet, permet avaluar els canvis progressius en els diferents elements del sistema immunitari en primeres etapes de vida. Donat això, ha estat el model experimental d'elecció en aquesta tesi, tant en context de salut (**capítol 1**), com de malaltia (**capítols 2-6**).

Així, en aquesta tesi s'han utilitzat dos models de RV en rata lactant, un d'infecció simple amb el RV *simian agent 11* (SA11) (**capítols 2-4**), i un altre de doble infecció amb els RV SA11 i *epizootic diarrhoea of infant mice* (EDIM) (**capítols 5 i 6**). En ambdós models es van seleccionar les rates de la soca Lewis, que tenen una alta susceptibilitat a la infecció per RV, ja que altres soques, com la Wistar, tenen mecanismes per evitar la replicació del virus (66). L'edat també és un factor important, ja que els rosegadors poden infectar-se a qualsevol edat, però no sempre desenvolupen la malaltia. En particular, les rates només presenten manifestacions clíniques quan s'infecten durant les primeres setmanes de vida, i la diarrea és edat dependent (50,66,131).

En quant al model d'infecció simple, aquest ja s'havia desenvolupat i utilitzat en estudis previs al nostre laboratori (66,132). El RV SA11 sol causar una diarrea d'elevada incidència i de gravetat moderada, sense pèrdua de pes corporal, com ha passat en aquests treballs i en estudis previs (66,132), i semblant al que normalment té lloc en humans, en contrast amb altres models animals amb una diarrea de gravetat més

elevada, com ara en ratolins, on s'ha descrit pèrdua de pes corporal (64). Tot i que la puntuació fecal s'utilitza àmpliament per a aquest tipus d'estudis, i que es va dur a terme de manera cega, segueix sent una avaluació sotmesa a la percepció humana, i per tant amb un component subjectiu. En aquest sentit, un augment del pes fecal, utilitzat en aquests i en estudis previs, sembla ser un indicador més objectiu de la incorporació d'aigua en el contingut fecal total (66,68).

A més, amb l'objectiu de millorar el model establert es va incorporar la mesura de nous marcadors de la malaltia com a marcadors clínics no invasius, com van ser el pH fecal i la temperatura rectal.

En aquest cas, el pH fecal ha estat un marcador diferencial associat a la diarrea per RV, tot i que en els primers estudis (**capítols 3-5**) va disminuir amb la infecció per SA11, mentre que, sorprenentment, en el darrer estudi (**capítol 6**) el pH va incrementar en els animals infectats. De fet, Li et al. (133) van observar un augment del pH en el contingut del còlon en porcs infectats per RV. Aquesta diferència deu estar associada al curs diferencial de la pròpia infecció i als desequilibris electrolítics del cos causats per la diarrea (31), o degut a l'ús d'alíquotes de virus diferents, l'origen dels animals o altres factors del procés experimental. Ara bé, en qualsevol cas, la diarrea altera el pH de les femtes i sembla ser un marcador a avaluar en aquest tipus d'estudis.

D'altra banda, pocs estudis han avaluat la temperatura corporal durant el procés infecciós, tot i que ha resultat ser un valuós marcador (**capítols 5-6**). L'increment de temperatura corporal observat coincideix amb l'obtingut per Parreño et al., els quals van mesurar la temperatura després de la infecció per RV en un model de vedell i van observar febre (78).

Respecte al mecanisme d'infecció, el RV arriba a l'intestí, on s'uneix a les cèl·lules epitelials i inicia el seu procés infecciós i de replicació. L'excreció viral, que reflecteix aquesta replicació, a l'igual que en estudis previs utilitzant aquest model (66), va mostrar un pic màxim tan sols un dia després de la inoculació del virus (**capítols 2, 3, 4 i 6**). Aquest fet implica que la majoria del virus excretat és el propi inoculat. Cal tenir present que degut a que és un model que utilitza un virus heteròleg per la rata i a dosis molt elevades, era d'esperar aquest tipus de patró.

Pel que fa a la resposta immunitària contra el RV, aquesta infecció indueix resposta immunitària humoral i cel·lular, local i sistèmica. Les cèl·lules B que presenten la integrina de reclutament intestinal α4β7 i les cèl·lules T CD4+ són crucials per a la producció d'anticossos específics, i les cèl·lules T CD8+ constitueixen la resposta immunitària mediada per cèl·lules que, per mitjà de la inducció de citotoxicitat, escurça la infecció i promou l'excreció del virus. Com la majoria de les respostes immunitàries humorals, els anticossos específics anti-RV són en primer lloc de l'isotip IgM, i després IgG i IgA, essent els nivells d'IgA anti-RV, ja sigui a nivell intestinal o sistèmic, els que presenten millor correlació amb la protecció (30,31,37,131). No obstant això, el mecanisme d'adquisició de protecció contra el RV no és completament conegut (30,31,37,131). De fet, les diferències en la reactivitat de diferents soques de RV en diferents models animals o en humans no permeten establir conclusions clares pel que fa a la protecció en humans. Per exemple, els models de diarrea per RV en rosegadors s'han centrat en l'etapa primerenca de la lactància ja que la seva resposta immunitària adquirida natural és capaç de bloquejar el virus després del deslletament (50,66). Tot i aquesta limitació, la correlació positiva entre la IgA i la protecció contra el virus sembla ser clara. En aquest context, el model utilitzat aquí sembla desenvolupar protecció contra el virus, com es pot veure per l'increment d'anticossos específics en sèrum i mostres de rentat intestinal (particularment IgA, IgG i IgM anti-RV en sèrum i IgA i IgM anti-RV a l'intestí) després de la infecció per RV (**capítols 2-4**), així com també va succeir en estudis previs (59,66,75).

Per altra banda, es va desenvolupar un nou model de doble infecció (com a fase prèvia en l'**objectiu 3**), més semblant a la realitat clínica humana que el model d'infecció simple. En aquest cas es van dur a terme dues inoculacions de RV en diferents dissenys experimentals realitzats. La primera, amb SA11, al final de la primera setmana de vida, com en el model d'infecció simple (66,132). Per a la segona infecció, imitant les reinfeccions que succeeixen sovint en infants, es va escollir el RV EDIM i es va inocular el dia 17 de vida, de forma similar a un model en ratolins (56), confirmant prèviament la seva capacitat infecciosa en rates. En aquest cas, no es va observar diarrea, a causa de l'edat més tardana dels animals, així com passa en els altres models de doble infecció per RV en altres espècies (56,59,63,75,78). Per tal de potenciar aquesta segona infecció, la inoculació es va realitzar al voltant del dia 17 de vida, però amb un deslletament precoç introduït el mateix dia, o fins i tot un dia abans, fet que implica un estrès fisiològic per a l'animal. Aquest canvi en el dia del deslletament va provocar una

pèrdua de pes durant els dies 17-18 (que no va ser deguda a les infeccions per RV), però no l'aparició de manifestacions clíniques i, per tant, van ser necessaris altres marcadors per a l'avaluació del procés infecciós.

És en aquest context que variables com el pH fecal i la temperatura corporal, aplicats en el model simple, podien ser d'utilitat. La mesura del pH no va ser útil per a l'avaluació de la segona infecció ja que, en no haver-hi diarrea, tampoc va aparèixer el canvi electrolític causant de modificacions en el pH (**capítols 5 i 6**). Ara bé, tot i que una infecció durant la tercera setmana de vida amb EDIM indueix un augment de la temperatura corporal, indicant l'existència d'un procés infecciós, la inoculació del virus EDIM després d'una primera infecció amb SA11 (model de doble infecció) no va causar canvis, de manera que aquesta variable mostra un patró diferencial entre el model d'infecció simple i el model de doble infecció.

Una limitació de l'estudi per al desenvolupament del model de doble infecció (**capítol 5**) va ser la de no avaluar l'excreció viral, una eina que sí que va ser utilitzada en el model simple, en estudis anteriors (132) i per altres, i que també es va mesurar posteriorment en l'estudi per avaluar intervencions nutricionals en aquest model (**capítol 6**). A l'igual que en el cas de la primera infecció, després de la segona inoculació amb EDIM, es va eliminar una elevada quantitat del virus el dia posterior a la inoculació i amb una càrrega viral més baixa que els animals infectats amb només EDIM, mostrant una modulació de la segona infecció per part de la primera.

Pel que fa als canvis en la resposta immunitària, per tal d'obtenir patrons diferencials en la doble infecció es van avaluar marcadors de resposta humoral específica com els anticossos anti-RV i de resposta cel·lular, com la resposta d'hipersensibilitat retardada o DTH. Aquestes variables es van determinar al final de l'estudi, quan el sistema immunològic dels animals ja es trobava més desenvolupat.

Tot i que la resposta DTH no va permetre obtenir conclusions clares (**capítol 6**) en l'estudi de les intervencions nutricionals en el model de doble infecció, durant l'estudi per al desenvolupament del model sí que s'havia observat que la resposta DTH després de la segona infecció estava modulada per la primera infecció (**capítol 5**). Mentre que la infecció simple no va promoure una resposta DTH, els animals doble infectats i deslletats prematurament van mostrar un efecte clar. Aquests

resultats són contraris al que s'ha observat en altres estudis, on es va detectar una resposta DTH després d'una infecció a dia 17 en ratolins, però que va ser suprimida després d'una reinfecció en els ratolins que havien rebut prèviament una infecció primària (43,56). Els nostres resultats poden suggerir que els animals que ja han estat en contacte amb el virus (primera infecció), són capaços de respondre als estímuls de DTH després de la segona infecció d'una manera més forta. Aquests resultats es poden justificar pel fet que se sap que les cèl·lules T CD8+ específiques per RV desenvolupades després d'una primera infecció són responsables de la resolució de la segona infecció (134,135).

En quant als anticossos específics, després de la primera infecció ja es van trobar nivells més alts d'anticossos en comparació amb els grups de referència, de manera similar al model d'infecció simple i a estudis previs (59,66,75), però que en el cas de l'estudi d'intervencions nutricionals en el model de doble infecció no són suficients per permetre observar clarament el desenvolupament de protecció contra el virus després de la primera infecció (**capítol 6**).

No obstant això, cal destacar que els anticossos de la llet materna són variables i poden conferir protecció a les cries (encara que a dia 16 els animals ja eren deslletats, la influència materna encara pot ser important en aquest dia). Es va trobar un elevat nivell d'anticossos específics anti-RV en el sèrum i la llet materna de les rates mares, i fins i tot vam obtenir diferents nivells segons el proveïdor dels animals, presumiblement a causa del contacte previ de les rates mares amb RV (resultats pendents de publicació). Per aquesta raó, es va arribar a la conclusió que, tot i la importància d'avaluar els títols d'anticossos anti-RV en el sèrum de les cries, també és important determinar els de les rates mares abans d'iniciar futurs estudis d'intervenció amb aquest tipus de model.

Pel que fa a la segona infecció, els resultats del disseny experimental amb deslletament fisiològic van suggerir que la segona infecció no era prou forta com per modificar els nivells d'anticossos (els del grup doble infectat van ser similars als del grup amb infecció simple), però quan es va realitzar un deslletament precoç, la segona infecció va modular aquesta variable, i més significativament si els animals havien estat deslletats un dia abans de la inoculació de l'EDIM. El que es va trobar va ser una disminució de la resposta humoral en la segona infecció després d'una primera infecció. Aquest fet també el van observar Sheridan et al. en un model de reinfecció en ratolins adults (43). En

contrast amb aquests resultats, Knipping et al. no va trobar un patró d'anticossos diferencial entre infecció simple i doble infecció (56). Tot i això, en el cas de l'estudi d'intervencions nutricionals en el model de doble infecció (**capítol 6**), aquesta variable no va permetre observar clarament el desenvolupament de protecció contra el virus després de la segona infecció. En general, els baixos títols d'anticossos anti-RV trobats en els animals doble infectats poden estar relacionats amb una major resposta cel·lular desenvolupada després d'una primera infecció i que podria protegir enfront del virus sense necessitat d'activar gaire la resposta immunitària humoral.

En l'últim estudi, d'avaluació d'intervencions nutricionals en el model de doble infecció (**capítol 6**), es van afegir, a més, algunes determinacions noves per a obtenir més informació sobre l'estat d'immunitat, com la producció d'Ig i citocines *ex vivo*, els nivells de citocines en rentat intestinal i l'expressió d'alguns gens rellevants. La determinació de la producció d'Ig *ex vivo* va permetre observar una resposta diferencial entre el model d'infecció simple i el model de doble infecció, augmentant només després de la doble infecció, i no en els grups infectats només amb SA11 o només amb EDIM (infeccions simples).

També es va trobar un patró diferencial entre el model d'infecció simple i el model de doble infecció en referència a la producció de citocines *ex vivo*, el que indicaria que la primera infecció influeix sobre la resposta immunitària davant una segona infecció. Concretament, la producció de citocines *ex vivo* de tipus Th1 (IFNγ), Th2 (IL-4), proinflamatòries (TNFα) i antiinflamatòries (IL-10) incrementen en les infeccions simples, bé per SA11 o bé per EDIM, però no després de la doble infecció. D'altra banda, el rentat intestinal és un fluid amb components de la capa de moc que reflecteix l'activitat de l'intestí. Les citocines presents al rentat intestinal només es van determinar en els animals doble infectats i van seguir un patró similar al de les citocines produïdes *ex vivo*. Per tant, aquestes variables semblen ser també útils en el context del model i demostren la influència d'una primera infecció sobre una futura resposta immunitària a un nou RV.

Pel que fa a l'estudi de l'expressió gènica associada a la infecció per RV, es van seleccionar els gens dels receptors TLR2 i TLR4 per a ser estudiats, ja que el TLR2 reconeix, entre d'altres lligands, components de la paret cel·lular com ara el peptidoglicà, l'àcid lipoteicoic i la lipoproteïna de bacteris Gram-positius, mentre que el segon s'uneix al

lipopolisacàrid bacterià (LPS), que és el component estructural principal de la paret externa de tots els bacteris Gram-negatius i un potent activador del sistema immunitari. L'IFNγ i la IL-4 van ser les citocines seleccionades per a aquest assaig, implicades en les respostes Th1 i Th2, respectivament. A més de molècules immunitàries, també es van quantificar altres com les de les unions estretes, de les quals s'ha descrit que poden ser modulades per probiòtics *in vitro* i *in vivo*. En aquest sentit, es van seleccionar l'ocludina i la claudina, components principals de les unions estretes. La mucina és una proteïna d'alt pes molecular i altament glicosilada produïda pels teixits epitelials intestinals per tal de formar un gel que actua en la lubricació, la senyalització cel·lular o en la formació d'una barrera química. Finalment, les molècules implicades en la resposta reguladora i tolerogènica, com ara la IL-10, el TGF-β i el FoxP3, també es van mesurar. Ara bé, les infeccions no van afectar l'expressió gènica de cap de les molècules estudiades quan s'avaluen als 28 dies d'edat, és a dir, setmanes després de la darrera infecció amb RV. Aquest fet indica que els possibles canvis associats a les infeccions s'han d'avaluar durant el pic de la infecció, com per exemple 2-3 dies posteriors a la inoculació. A partir d'aquests resultats, en estudis portats a terme en l'actualitat en aquests models en el nostre grup, s'està estudiant l'expressió gènica just els dies posteriors a les infeccions.

Finalment, en el curs del desenvolupament dels models, la utilització d'un HBC contra RV ha demostrat ser un protector eficaç per al control de la diarrea (**capítols 5 i 6**). En altres estudis, l'administració de GastroGard-R® (calostre que conté anticossos específics contra RV) també va protegir a ratolins lactants contra una primera infecció per RV, però la càrrega viral en femtes després de la segona infecció era tan alta com en la primera. No obstant això, va permetre el desenvolupament de les respostes de cèl·lules B i T (56). Parreño et al. van descriure una protecció completa per part de l'HBC en vedells després d'una primera i una segona exposició al virus (78). Una combinació d'HBC i de la soca probiòtica LGG també va protegir amb èxit a ratolins de la diarrea per RV (136). Altres autors també han trobat protecció o una reducció de la diarrea per RV en els ratolins administrats amb calostre boví de vaques normals (137,138) o amb concentrats de proteïnes del sèrum de la llet (132,139), de vegades observant una disminució dels títols d'anticossos (132) i de vegades sense diferències entre grups (139). En el cas de les infeccions múltiples, la intervenció amb HBC pot ajudar a entendre la influència d'intervencions moduladores durant aquests processos. En el nostre cas,

la completa protecció enfront a la primera infecció per part de l'HBC, i la seva capacitat per evitar l'augment del pes fecal induït per RV durant la fase aguda de la malaltia, va conduir a la inducció d'una menor resposta humoral i a una major excreció del virus després de la segona infecció, el que suggereix que l'HBC, en la primera infecció per RV, és capaç de bloquejar efectivament el virus, promoure la seva eliminació i, per tant, disminuir la seva infectivitat i la malaltia, però també el desenvolupament de la immunitat en aquest sentit. Així i tot, va permetre el desenvolupament d'una certa resposta immunitària. Aquests resultats estan en línia amb els observats en altres estudis (56,78). D'altra banda, el tractament amb HBC no va afectar l'expressió gènica de cap de les molècules estudiades analitzades setmanes després d'haver finalitzat amb la intervenció.

D'altra banda, encara que s'utilitzen la vacunació i la rehidratació com a intervencions preventives i terapèutiques, és d'interès desenvolupar noves estratègies eficaces per a la prevenció i/o tractament de la infecció per RV. En aquest sentit, l'interès per la relació simbiòtica entre els hostes i la seva microbiota ha augmentat considerablement en els últims anys. La microbiota intestinal afecta la fisiologia humana, influint en la barrera epitelial de moltes maneres, i influint en la funció immunitària, entre d'altres, tant directament com indirectament. Aquests efectes beneficiosos són especialment rellevants en les primeres etapes de la vida, quan el sistema immunològic està encara en desenvolupament. Aquestes són les bases per a l'estudi d'estratègies per modular l'entorn intestinal i composició i funcionalitat de la microbiota, i després indirectament actuar sobre el sistema immunitari de la mucosa, que alhora pot modular la immunitat sistèmica i la resposta enfront de patògens.

Entre aquestes estratègies, l'ús dels probiòtics és de les més estudiades. Se sap que aquests microorganismes exògens poden interactuar amb diversos components cel·lulars dins de l'entorn intestinal i tenir un impacte positiu sobre la salut de l'hoste. D'altra banda, en els últims anys, els prebiòtics han guanyat interès a causa de la seva acció en la promoció del creixement selectiu de bacteris beneficiosos. No obstant això, els efectes dels prebiòtics no s'han abordat adequadament en el context d'una infecció per RV. La combinació de probiòtics i prebiòtics, anomenada simbiòtics, pot tenir efectes sinèrgics. Recentment, els postbiòtics, productes del metabolisme de probiòtics, ja no es consideren com els residus metabòlics de rebuig de l'activitat probiòtica, sinó que ara s'estan investigant com una alternativa a la

utilització de bacteris viables. En el context de les primeres etapes de vida, seria d'interès avaluar els tipus de productes obtinguts a partir de fonts més relacionades amb l'etapa de desenvolupament, com ara els probiòtics de la llet materna o de la femta dels nadons; o oligosacàrids que reflecteixin l'estructura dels que es troben presents a la llet materna.

Tot i els esforços per avaluar la influència d'aquests productes moduladors microbians sobre la resposta immunitària del lactant, existeixen problemes de variabilitat en les condicions fisiològiques o de malaltia estudiades, en les soques probiòtiques o diversitat de prebiòtics, així com limitacions de poder imposades pels nombres reduïts de participants en la majoria dels estudis. Aquesta és la raó per la que la majoria de les dades disponibles en l'actualitat que descriuen els efectes d'aquests compostos sobre la resposta immunitària deriven d’estudis *in vitro* i en models animals.

En base a aquests fets la **hipòtesi** que sustenta la part d’intervenció nutricional d’aquest treball, ha estat que la resposta immunitària, així com la capacitat antiinfecciosa de l’organisme en estat de maduració poden ser potenciades per diversos components moduladors microbians -de rellevància en primeres etapes de vida- de tipus probiòtics, prebiòtics, simbiòtics i postbiòtics.

Concretament, i per tal d’avançar en aquest camp d’estudi i de satisfer la necessitat de compostos moduladors de la infecció per RV, en aquesta tesi es van avaluar diferents productes d’interès en el context neonatal: el probiòtic *B. breve* M-16V; una barreja prebiòtica de scGOS/lcFOS/pAOS (GFA); el prebiòtic Bimuno® GOS (B-GOS); la barreja prebiòtica Immunofortis® (scGOS/lcFOS 9:1); el simbiòtic format per *B. breve* M-16V i Immunofortis®; i un postbiòtic derivat de llet fermentada amb *Bifidobacterium breve* i *Streptococcus thermophilus* (llet fermentada, FM) de forma aïllada o en combinació amb l’Immunofortis®.

Com a **primer objectiu** es va estudiar el paper del probiòtic *B. breve* M-16V, aïllat de femtes d’infant, en el desenvolupament del sistema immunitari neonatal. I així, es van trobar importants efectes immunomoduladors deguts a la soca probiòtica *B. breve* M-16V quan s’administrava en rates sanes durant la lactància, com un efecte sobre la composició dels limfòcits o la modificació de la proporció de cèl·lules que expressen molècules implicades en la interacció amb els bacteris intestinals (**capítol 1**).

Un dels aspectes importants d'estudi van ser els canvis fenotípics associats al procés maduratiu del sistema immunitari. En aquest sentit, durant la lactància, les rates travessen un procés de canvis fenotípics als seus teixits limfoides que són un reflex de la maduració del sistema immunitari en aquest període. D'aquesta manera, la melsa immadura de les rates conté principalment cèl·lules B, T, NK i NKT, en menors proporcions que les de rates adultes (26). Com que l'administració de *B. breve* M-16V no va produir un augment, sinó més aviat una disminució, de cèl·lules CD4+ i CD8+, sembla que aquesta intervenció nutricional no va potenciar la maduració del sistema immunitari sistèmic. No obstant això, quan es consideren els GLM, un òrgan limfoide secundari situat a nivell intestinal i inductor de la resposta immunitària en aquest compartiment, la suplementació amb *B. breve* M-16V va ser capaç d'incrementar la proporció de cèl·lules NK CD8+. Aquestes cèl·lules, encara que es troben en una baixa proporció als GLM, són claus per la immunitat innata (140) i, per tant, aquests resultats mostren que l'administració de *B. breve* M-16V va millorar aquesta barrera defensiva.

Pel que fa als limfòcits intraepitelials (IEL, *intraepithelial lymphocytes*), aquestes cèl·lules són les que estan més en contacte amb el lumen intestinal i, per tant, es poden veure afectades de manera més significativa per les intervencions dietètiques. Aquest compartiment no conté cèl·lules B, i durant la lactància hi ha un augment progressiu de la proporció d'IEL CD8+ que coexpressen TCRαβ, mentre que el percentatge de cèl·lules CD8+ que expressen el receptor NK disminueix (26). A més, hi ha un increment en la proporció de cèl·lules CD4+ i cèl·lules TCRγδ+ durant la segona meitat del període de lactància (26). L'administració de *B. Breve* M-16V durant la lactància no va produir cap canvi significatiu en la proporció d'IEL CD8+ TCRαβ+ o de cèl·lules NK CD8+, però va provocar una disminució del percentatge d'IEL CD4+ i de la proporció dels IEL CD8αβ+ TCRγδ+, que són els menys abundants entre les cèl·lules TCRγδ+ del compartiment intraepitelial. Aquests resultats coincideixen amb els de Gebert et al. (141), que va trobar una disminució de la població CD4+ al jejú de porcs nounats després de l'administració *Lactobacillus brevis*. Alguns autors també han descrit una reducció de les cèl·lules T CD4+ a la mucosa obtinguda de pacients amb malaltia de Crohn cocultivada amb *L. casei* i *L. bulgaricus*, però no es van observar canvis en mucosa no inflamada (142,143).

En tots tres compartiments estudiats, es va avaluar la presència de TLR4 en els principals subgrups de cèl·lules. El receptor TLR4 reconeix, tal i com abans s'ha mencionat, l'LPS, participa en la resposta immunitària innata contra els patògens, però també en la proliferació, la diferenciació i el desenvolupament dels limfòcits (144). El TLR4 és essencial per al manteniment de l'homeòstasi intestinal i de la funció barrera (145,146). La intervenció nutricional no va ser capaç de canviar la proporció d'esplenòcits portadors de TLR4, però sí va causar un increment de les cèl·lules CD4+ portadores de TLR4 en GLM i IEL. Per això, es pot suggerir que la suplementació amb *B. breve* M-16V prepara el sistema immunitari intestinal per a una millor resposta contra infeccions augmentant la interacció bacteri-hoste. Aquests resultats concorden amb els descrits després de l'administració de *L. reuteri*, *L. casei* i *L. rhamnosus* en ratolins sans (147,148) i de LGG en rates sanes (149). No obstant això, els nostres resultats no coincideixen amb altres en que l'expressió de TLR4 es va estudiar en algunes patologies. En aquest sentit, l'expressió de TLR4 va disminuir a l'intestí de rates amb NEC després de l'administració de *L. reuteri* (150), en ratolins amb malaltia hepàtica alcohòlica després de l'administració de *L. rhamnosus* i *L. acidophilus* (151), i també al còlon de rates amb colitis després de l'administració de LGG i d'altres lactobacils i bifidobacteris (149,152).

D'altra banda, la circulació és important per a que els limfòcits verges puguin trobar el seu antigen específic, així com per a que els limfòcits efectors o de memòria puguin arribar als teixits infectats o inflamats. El reclutament intestinal depèn de l'expressió de molècules de superfície específiques (153). El reclutament dels limfòcits T verges a les PP o als GLM a través de les vènules endotelials altes (HEV, *high endothelial venule*) requereix de la interacció entre la selectina CD62L de la superfície de limfòcits i l'adresina de nòduls perifèrics de les HEV (154). D'altra banda, la integrina $\alpha 4\beta 7$ també és important per retenir els IEL a l'intestí prim (155,156). Curiosament, el patró de la selectina CD62L i de la integrina $\alpha E\beta 7$ a les cèl·lules dels GLM i als IEL de les rates lactants va canviar degut a l'administració de *B. breve* M-16V, induint un major percentatge de les cèl·lules portadores de la integrina $\alpha E\beta 7$ tant en els GLM com en el compartiment intraepitelial. Aquests resultats semblen indicar una millora del procés de reclutament dels limfòcits T verges als GLM i de la retenció de limfòcits activats en el compartiment dels IEL (157). Altres estudis han posat de manifest la capacitat dels probiòtics per modular marcadors de reclutament. El *L. plantarum* i el pèptid STp que excreta aquest, han demostrat una

estimulació dels marcadors de reclutament a la pell en estudis *in vitro*, com ara l'antigen cutani associat a limfòcits (CLA), i una disminució de l'expressió de la molècula d'adhesió cel·lular MadCAM-1 i de la integrina α4β7 a la mucosa colònica en un model de colitis en ratolí (158–160). Aquests efectes poden ser útils per atenuar la inflamació intestinal en la malaltia inflamatòria intestinal. Contràriament, el *L. casei* Shirota no va modular els marcadors de reclutament en les cèl·lules T estimulades per les cèl·lules dendrítiques de pacients amb colitis ulcerosa, però sí que va incrementar el marcador de reclutament a la pell CLA i el marcador de reclutament intestinal β7 a les cèl·lules T estimulades per cèl·lules dendrítiques humanes de controls sans (161).

Cal tenir en compte que segons el disseny d'aquest estudi, les cries van rebre el suplement des del dia 6 fins al final de l'estudi, i van ser deslletades el dia 17, uns dies després de que les rates comencin a mastegar aliments sòlids, i amb aquest curt període ja va ser capaç d'incrementar la IgA intestinal. Els resultats obtinguts demostren que la suplementació amb la soca *B. breve* M-16V en aquest curt període és suficient per causar efectes, igual que en un altre estudi, amb ratolins, en el qual només 2 dies de suplementació amb *L. delbrueckii* subsp. *bulgaricus* o *L. acidophilus*, o 7 dies de suplementació amb *L.casei* van ser suficients per trobar un augment d'IgA a la LP de l'intestí prim (145). No obstant això, no podem descartar que amb un disseny diferent de l'estudi que inclogués un període d'administració més temprà o més llarg, es poguessin obtenir resultats més significatius.

Així, globalment, es pot concloure que es millora el desenvolupament de la immunitat de la mucosa en rates durant les primeres etapes de vida, per la modulació de l'expressió de TLR, millorant el procés de reclutament dels limfòcits T verges, i la retenció de limfòcits activats en el compartiment intestinal, així com la millora de la síntesi d'IgA intestinal. Aquests efectes positius de la soca *B. breve* M-16V poden ser d'utilitat en la defensa per part de l'hoste contra patògens com el RV. Per tant, es va procedir a determinar la influència d'aquest probiòtic en un model d'infecció simple amb RV (**objectiu 2**) i en un model de doble infecció (**objectiu 3**), desenvolupament dels quals va donar lloc a les **capítols 2** i **6.**

La suplementació amb *B. breve* M-16V va mostrar un clar efecte de protecció enfront a la primera infecció per SA11 tant en el model d'infecció simple, com en el model de doble infecció. Aquest probiòtic

va reduir la incidència, la durada i la gravetat de la diarrea experimental, i va evitar l'augment de pes fecal a causa de la diarrea per RV en la fase aguda de la malaltia. Aquesta intervenció també va evitar l'augment del pH fecal induït per l'SA11 en el model de doble infecció, i pel que fa a la temperatura corporal, el grup suplementat amb el probiòtic va mostrar un comportament similar al del grup doble infectat (només es va observar febre durant la primera infecció), el que significa que el probiòtic va conferir protecció durant la primera infecció per RV, i fins i tot, aquesta infecció encara va ser capaç de modular la segona. Tot i que aquesta és la primera vegada que *B. breve* M-16V ha estat avaluat contra el RV, altres estudis han investigat la seguretat i els efectes beneficiosos d'aquest probiòtic en nadons prematurs, obtenint resultats en línia amb els obtinguts aquí. Per exemple, s'ha observat un augment dels recomptes fecals de *B. breve* i una reducció de la incidència de NEC i altres infeccions després de l'administració d'aquest probiòtic (162,163). A més, aquest microorganisme està descrit com a GRAS per la FDA (164). En els presents estudis, també s'ha observat que l'administració de *B. breve* M-16V en rates lactants sanes no va induir efectes nocius, ja que no va afectar la corba de creixement. Curiosament, es va trobar un lleuger canvi en la consistència fecal causada per la suplementació amb *B. breve* M-16V. Aquest efecte ja s'havia descrit per alguns probiòtics i prebiòtics (126,165–167), el qual és beneficiós per a que les femtes siguin més similars a les dels lactants alimentats amb llet materna i reduir el restrenyiment. Com a limitacions del disseny, cal a destacar que seria interessant avaluar la seva activitat anti-diarreica per RV en comparació amb altres probiòtics que han mostrat eficàcia en aquest tipus d'intervencions preclíniques, com ara LGG, entre d'altres (55,168–173). També seria interessant aprofundir en els mecanismes implicats en la protecció enfront de la diarrea per part d'aquest probiòtic, com ara la millora de la composició de la microbiota.

En el grup suplementat amb el probiòtic es va observar una excreció viral similar a la del grup RV després de la primera inoculació (tant en el model d'infecció simple com en el model de doble infecció). Es pot postular doncs, que la millora clínica observada en el grup suplementat amb el probiòtic pot no ser deguda a una major eliminació del virus. Altres possibles mecanismes podrien ser una millora de la barrera epitelial o una millora de la immunitat en desenvolupament de les rates lactants, tal i com s'observa al **capítol 1**. No obstant això, després de la segona inoculació, la càrrega viral excretada va ser més elevada que la del grup doble infectat, potser com a conseqüència de la protecció

conferida durant la primera infecció, i en línia amb els resultats obtinguts amb l'HBC (56).

Pel que fa a l'efecte de la suplementació amb *B. breve* M-16V sobre la resposta immunitària humoral anti-RV, aquesta dieta pràcticament no va modificar la resposta observada en el grup RV en cap moment, ni en el model d'infecció simple ni en el model de doble infecció. Aquests resultats contradiuen els efectes immunomoduladors d'aquesta soca a nivell d'anticossos en estudis d'al·lèrgia als aliments en ratolins (174,175) i els d'altres probiòtics com ara LGG o *L. acidophilus* NCFM, que va augmentar les IgG o les IgM en porcs i en infants després d'aquest tipus d'infecció (173,176,177). També estan en controvèrsia amb el que s'ha trobat quan la soca probiòtica s'administra en rates sanes durant 13 dies en el període d'alletament, un efecte de potenciació de la síntesi d'IgA intestinal (**capítol 1**). En aquest estudi, els nivells d'IgA a les mostres de rentat intestinal dels animals de referència van ser molt baixos, com era d'esperar (19,25), però la suplementació amb *B. breve* M-16V va duplicar la concentració d'IgA intestinal. Com que la IgA intestinal és un marcador fiable de la defensa de les mucoses contra els patògens i s'ha descrit clarament com una eina útil en estudis de immunonutrició (145), aquest resultat suggereix un potencial immunomodulador d'aquesta soca probiòtica. Altres estudis també han descrit una estimulació de la producció d'IgA per part de probiòtics, com ara LGG (178), *Lactobacillus gasseri* (101), *L. casei*, *L. reuteri*, *Bacillus cereus* var. *Toyoi*, *Bifidobacterium bifidum* i *Lactobacillus kefiri* en ratolins (179–181) o una fórmula que conté *Bifidobacterium lactis* Bb-12 en infants sans (182).

En quant a la concentració d'AGCC trobada en femtes (**capítols 2 i 6**), va ser molt baixa, igual que en el grup RV, tant en el model d'infecció simple com en el model de doble infecció. No obstant això, es va poder veure una tendència a augmentar alguns dels AGCC després de la intervenció amb *B. breve* M-16V. Es pot suggerir que l'augment del total d'AGCC i, especialment, dels àcids propiònic i fòrmic a causa de l'administració d'aquesta soca, pot estar implicat en la seva acció protectora contra el RV.

D'altra banda, la intervenció amb el probiòtic va potenciar la producció d'Ig *ex vivo* després de la primera infecció (**capítol 6**), però la resposta va ser inferior a la segona infecció, i per tant, similar a la del grup EDIM (és a dir, implicant protecció durant la primera infecció). En altres estudis es va observar un increment cèl·lules secretores d'IgA o

d'IgG en porcs administrats amb LGG o *L. acidophilus* NCFM i vacunats i infectats amb la HRV (rotavirus humà) (173,176).

En referència a la producció de citocines *ex vivo* (en el model de doble infecció, **capítol 6**), es va trobar augmentada en el grup suplementat quan es produeix només una infecció, però també en els animals doble infectats, de manera similar o de vegades encara més que el grup RV. Diversos probiòtics han demostrat la capacitat d'augmentar la producció de citocines *in vitro* (99). Wen et al. (173,183) van trobar un augment de la producció d'IFNγ per part de les cèl·lules T i una disminució de la producció d'IL-10 per part de les cèl·lules Treg en porcs administrats amb LGG i vacunats amb HRV. Les concentracions de citocines en el rentat intestinal també eren més elevades en el grup d'aquesta intervenció després de la doble infecció, respecte al grup RV (**capítol 6**).

Pels resultats obtinguts en la determinació de l'expressió gènica, sembla que la presència del probiòtic a l'intestí, pot incrementar la proporció de bacteris Gram-positius (com els bifidobacteris i els lactobacils) i per tant, el TLR encarregat de la seva detecció també. Per contra, Wang et al. (184) no van trobar un augment significatiu en l'expressió del gen del TLR2 en les cèl·lules mononuclears intestinals de porcs vacunats amb RV i administrats amb LGG, però sí van trobar un augment en l'expressió del gen de TLR4. Altres estudis han observat la capacitat d'alguns probiòtics per millorar la funció de barrera (84), que no està clara en aquest estudi. Els resultats obtinguts aquí també suggereixen que la intervenció amb aquest suplement manté, o fins i tot disminueix, la resposta immunitària reguladora i tolerogènica, en desacord amb Wang et al. (184), que també va trobar un augment de l'expressió gènica de la IL-10.

En qualsevol cas, la suplementació amb *B. breve* M-16V durant primeres etapes de vida ha demostrat ser capaç de controlar la diarrea experimental induïda per SA11, i alhora permetre un correcte desenvolupament de la resposta immunitària, que juntament amb la seva capacitat immunomoduladora, sembla dotar al nounat de capacitat protectora enfront una posterior infecció.

Una vegada establerta la utilitat del probiòtic assajat en la infecció per RV, es va procedir a estudiar el paper de barreges de substàncies prebiòtiques i en combinació amb altres components (**capítols 2-4**). Concretament, l'estudi va voler centrar la seva recerca en una barreja

prebiòtica d'elevada importància en primeres etapes de vida, scGOS/lcFOS 9:1 (Immunofortis®), que pretén mimetitzar els oligosacàrids presents a la llet materna, i de la qual s'han descrit nombroses accions beneficioses pels lactants en assajos *in vitro*, *in vivo* i en humans. Aquesta substància és un dels productes pertanyents a l'empresa que ha subvencionat aquests estudis. Ara bé, pel que fa a la capacitat immunomoduladora de l'Immunofortis®, cal destacar que són necessaris més estudis, com el present, per reforçar els resultats, ja que l'EFSA (185) va considerar al 2010 que encara no hi havia prou evidència que avalés aquesta al·legació.

La barreja prebiòtica Immunofortis® va ser estudiada en tots dos models, d'infecció simple i doble, de forma aïllada o conjuntament amb el probiòtic *B. breve* M-16V (**capítols 2 i 6**). Amb la dosi utilitzada, es va observar un efecte d'emmascarament en la consistència fecal, que suposa una de les principals limitacions d'aquesta intervenció. El prebiòtic, i per tant també la intervenció amb les barreges que el contenien, com en el cas del simbiòtic, va induir una consistència més tova en les femtes, independentment de la presència del virus. Sembla que aquestes intervencions van ser capaces de disminuir la incidència i la gravetat de la diarrea, o almenys, l'efecte sobre la consistència de la femta no va ser additiu amb l'induït per la infecció per RV. Per aquesta raó, quan la puntuació de la gravetat es va tornar a calcular o normalitzar tenint en compte la puntuació fecal basal en absència del virus, es va poder observar una reducció en termes d'incidència i de gravetat. Aquests resultats estan en línia amb altres estudis que van mostrar la capacitat d'una barreja específica de scGOS/lcFOS per prevenir infeccions en infants (165,186,187), i de causar canvis positius en la consistència de les femtes, de manera que s'assemblava més a la dels lactants alimentats amb llet materna (165,166). Cal destacar que aquest prebiòtic, sol o en combinació amb el probiòtic, va evitar l'augment de pes fecal a causa de la diarrea per RV en la fase aguda de la malaltia. A més, en el model de doble infecció, aquesta dieta (i la dieta amb el simbiòtic) també va evitar l'augment del pH fecal induït pel RV. Les intervencions dietètiques amb Immunofortis® amb i sense l'addició del probiòtic, van mostrar un comportament similar al del grup doble infectat pel que fa a la temperatura corporal (només es va observar febre durant la primera infecció), i per tant, el suplement va conferir protecció durant la primera infecció per RV i, així i tot, aquesta infecció encara va ser capaç de modular la segona.

El pic de l'excreció viral en el model simple (**capítol 2**) i en el model de doble infecció (**capítol 6**) per a l'SA11 va ser substancialment inferior en el grup que rebia Immunofortis® i el simbiòtic, en comparació amb el grup RV (fins al 90%), el que suggereix que el prebiòtic és responsable d'aquest efecte. Aquesta alta reducció en l'excreció viral suggereix que el mecanisme d'acció del probiòtic i el del prebiòtic són diferents. És significatiu que l'efecte del prebiòtic present al simbiòtic fos evident, tot i l'addició del probiòtic. A partir d'aquests resultats es pot concloure que una part de la barreja de molècules de GOS/FOS pot ser capaç d'interactuar directament amb el RV, una acció ja s'ha descrit per a alguns oligosacàrids de llet materna (188), i per tant aquesta unió podria dificultar la seva detecció per ELISA. Aquesta reacció pot ser la responsable de la menor adhesió del virus a l'hoste i, en conseqüència, d'una menor incidència i gravetat de la infecció, com es va observar en aquest estudi. Amb objectiu de confirmar aquest possible mecanisme d'acció, es va portar a terme un estudi de bloqueig *in vitro*, posat a punt al nostre laboratori i que ha permès confirmar que la detecció del virus era inferior quan la prova es realitzava en presència de la barreja prebiòtica. Això significaria que es produeix una interacció directa entre els GOS/FOS i el virus, el que fa que el virus no sigui detectat *in vitro*. Queda per dilucidar quin tipus, quina especificitat i quina estabilitat té aquesta interacció entre el RV i aquesta barreja prebiòtica.

D'altra banda, l'administració d'aquest prebiòtic en el model d'infecció simple va augmentar la resposta humoral local i sistèmica contra el virus. A dia 14 de vida, només 1 setmana després de la infecció, els títols més elevats d'IgM i IgG en sèrum i d'IgA en rentat intestinal, en comparació amb el grup RV, suggereixen un paper modulador d'aquesta intervenció en la maduració del sistema immunitari. En particular, l'augment d'IgA a nivell intestinal indica una major unió al RV, que resulta en una major exclusió del virus de la mucosa i, per tant, la prevenció d'infeccions. De fet, un augment de la secreció d'IgA fecal també s'ha descrit després de la suplementació dietètica amb una barreja específica de scGOS/lcFOS en infants (186,187). En un punt més tardà després de la infecció (dia 21), es va observar un augment d'IgG i IgA en sèrum. Ja que valors elevats d'aquests isotips en sèrum són bons indicadors de protecció (131), es pot suggerir que la barreja específica scGOS/lcFOS va presentar alguns efectes immunomoduladors que van permetre potenciar la resposta immunitària contra el virus, no només per permetre la seva eliminació, sinó també per mantenir la protecció en el futur. Cal destacar que la barreja prebiòtica, amés de que sembla ser capaç d'unir-se parcialment

al virus, de bloquejar la seva adhesió a l'intestí i per tant, de disminuir la infecció, també va ser capaç no només de mantenir, sinó també de millorar la resposta immunitària i la protecció contra el RV (**capítol 2**). El simbiòtic va mostrar algun d'aquests mateixos efectes moduladors. En el model de doble infecció, en canvi, aquests suplements no van poder mostrar un efecte clar sobre els títols d'anticossos (**capítol 6**).

Curiosament, la combinació del probiòtic i el prebiòtic sembla reforçar l'acció antiviral de la barreja prebiòtica scGOS/lcFOS 9:1, que millora l'eliminació viral i la resposta immunitària de l'hoste contra el virus, i l'addició de *B. breve* M-16V aporta la millora d'alguns indicadors de diarrea, que són inferiors en el grup suplementat amb el simbiòtic respecte als altres grups.

Pel que fa a la concentració d'AGCC en femtes, malgrat va ser molt baixa en tots els grups, es pot veure una lleugera tendència a augmentar alguns dels AGCC després de l'administració del prebiòtic, com s'ha vist en altres estudis amb GOS/FOS (189,190).

La producció d'Ig *ex vivo* es va veure potenciada per la suplementació amb Immunofortis® i amb el simbiòtic després de la primera infecció, però la resposta va ser inferior a la segona infecció, i per tant, similar a la del grup EDIM. En referència a la producció de citocines *ex vivo* estudiada en el model de doble infecció, aquesta es va trobar augmentada en els grups suplementats amb aquest prebiòtic i el simbiòtic quan es produeix només una infecció, però també en els animals doble infectats, de manera similar o de vegades encara més que la del grup RV. Les concentracions de citocines en el rentat intestinal també eren més elevades en els grups suplementats amb aquest prebiòtic i el simbiòtic després de la doble infecció, respecte al grup RV. Pels resultats obtinguts en la determinació de l'expressió gènica, sembla que aquest prebiòtic, amb o sense el probiòtic, pot incrementar la proporció de bacteris Gram-positius, i per tant, el TLR encarregat de la seva detecció; que la capacitat per millorar la funció de barrera per part d'aquestes intervencions no està clara; i que la intervenció amb aquests suplements no requereixen incrementar la resposta immunitària reguladora i tolerogènica.

Els bons resultats obtinguts amb aquesta barreja prebiòtica i amb la seva combinació amb el probiòtic van portar a assajar en aquest model l'efectivitat d'una nova barreja prebiòtica a base de scGOS/lcFOS.

Concretament, en combinació amb oligosacàrids acídics derivats de la pectina (pAOS) (**objectiu 2**).

Així, el suplement amb la barreja prebiòtica scGOS/lcFOS/pAOS (GFA), avaluat en el model d'infecció simple (**capítol 3**) va provocar una consistència més tova de les femtes durant tot l'estudi, que fins i tot va ser considerada a vegades com diarreica. Aquest efecte, ja descrit anteriorment i probablement degut a la presència dels GOS/FOS, van dificultar el poder diferenciar la consistència més tova de la femta induïda pel prebiòtic de la diarrea induïda per RV. Així i tot, el grup suplementat amb aquesta dieta va mostrar certes millores clíniques, que també es van posar de manifest per una reducció del pes fecal, respecte al grup RV, en el període de diarrea aguda. A més, el pH fecal en el grup suplementat amb GFA tenia un valor intermedi entre els dels grups de referència i RV. Aquest fet no descarta que aquest producte pugui evitar el trastorn electrolític induït per RV, ja que una certa reducció del pH podria ser deguda a la pròpia acidesa dels pAOS i/o causada pels scGOS/lcFOS directament.

En el grup suplementat amb GFA, l'excreció viral es va reduir en gran mesura, de fet, era comparable a la del grup de referència. A partir d'aquests resultats es pot concloure que una part de la barreja de molècules de GOS/FOS/pAOS pot ser capaç d'interactuar directament amb el RV i, per tant, aquesta unió podria dificultar la seva detecció per ELISA. Després d'obtenir aquestes dades, es van realitzar assajos de bloqueig *in vitro*, i els resultats obtinguts van confirmar una certa inhibició de la detecció del virus en presència de GFA, el que pot indicar una menor infecció de les cèl·lules epitelials de l'intestí i una replicació més baixa, tot i que aquest efecte pot ser degut de nou a la presència de GOS/FOS en la barreja. No obstant això, no podem descartar altres possibles mecanismes d'acció d'aquest producte, que també podria interactuar amb la microbiota intestinal, o directament amb la superfície cel·lular, estimulant canvis en el moc i reforçant la barrera epitelial.

D'altra banda, els resultats de la determinació d'IgA intestinal en el grup suplementat amb GFA suggereixen una millora en la resposta immunitària primerenca, d'acord amb altres estudis que van mostrar que la barreja de GFA activa la resposta immunitària (191–195) i, per tant, permet hipotetitzar que aquests anticossos poden resoldre la infecció ràpidament, amb menor necessitat d'anticossos més endavant. Aquest increment de la síntesi d'IgA anti-RV intestinal primerenca

sembla haver estat suficient per la defensa contra el virus. En tot cas, la barreja no ha originat una potenciació molt evident dels seus components i per això no s'ha assajat en el context del model de doble infecció.

D'altra banda, i donat el potencial de la barreja prebiòtica a base de scGOS/lcFOS, es va voler avaluar si altres prebiòtics similars també podien ser efectius en la infecció per RV, i amb aquest objectiu es va emprar el prebiòtic B-GOS, que conté GOS, però no FOS, en la seva composició.

En aquest cas, el B-GOS va ser avaluat directament en el model de doble infecció (**objectiu 3**, **publicació 6**), el qual ja permet observar la capacitat moduladora en la diarrea infecciosa originada en la primera infecció. Aquest suplement va causar un cert efecte sobre la consistència fecal, però tot i això va mostrar una protecció enfront a la diarrea per RV i també va evitar l'augment de pes i del pH fecal degut a la diarrea per RV durant la fase aguda de la malaltia. En quant a la temperatura corporal, el grup suplementat amb B-GOS va mostrar un comportament similar al del grup doble infectat (només es va observar febre durant la primera infecció), i per tant, el suplement va conferir protecció durant la primera infecció per RV i, així i tot, aquesta infecció encara va ser capaç de modular la segona. El pic de l'excreció viral després de la primera inoculació amb SA11 va ser lleugerament inferior en el grup suplementat amb B-GOS.

D'altra banda, aquest prebiòtic no va mostrar un efecte clar sobre els títols d'anticossos en sèrum i rentat intestinal, ni sobre la concentració d'AGCC en femtes. La producció d'Ig *ex vivo* es va veure potenciada per la suplementació amb B-GOS després de la primera infecció, però la resposta va ser inferior a la segona infecció, i per tant, similar a la del grup EDIM. En referència a la producció de citocines *ex vivo*, es va trobar augmentada en el grup suplementat quan es produeix només una infecció, però també en els animals doble infectats, de manera similar o de vegades encara més que la del grup RV. Les concentracions de citocines en el rentat intestinal també eren més elevades en el grup d'aquesta intervenció després de la doble infecció, respecte al grup RV. Pel que fa als resultats obtinguts en la determinació de l'expressió gènica, es va comportar de manera molt similar a la barreja Immunofortis®.

Així, aquest prebiòtic ha mostrat una bona capacitat moduladora enfront a la infecció per RV, fet que reforça el seu caràcter prebiòtic, ja que en altres estudis, ja s'havia observat la seva capacitat de prevenir la diarrea del viatger en adults sans i efectes beneficiosos sobre la composició de la microbiota intestinal *in vitro* i en persones grans, així com la millora de la funció immunològica d'aquestes últimes (196–198).

Finalment, també es va voler centrar l'atenció en altres tipus de components més novedosos, com són els postbiòtics (**objectiu 2**). Concretament, en aquest estudi es va escollir un postbiòtic derivat de llet fermentada amb *B. breve* i *S. thermophilus* (llet fermentada, FM) que ja havia mostrat activitat anti-RV en assajos *in vitro*.

L'efecte del postbiòtic (FM) es va avaluar en el model d'infecció simple (**capítol 3**). L'administració d'FM va ser capaç de reduir la clínica diarreica en termes d'incidència, durada i gravetat. En aquest sentit, ja s'havia descrit la reducció de la gravetat de la diarrea en un model de RV en rata lactant suplementat amb un altre postbiòtic a base de llet fermentada per una soca de *L. casei* (199), i també en infants suplementats amb una fórmula infantil fermentada lliure de cèl·lules bacterianes (200). Aquest últim estudi va demostrar que la presència de microorganismes vius no és essencial per obtenir efectes similars als dels probiòtics. L'FM també va evitar l'acidificació de les femtes induïda per RV. Però aquest resultat, similar a l'observat al grup de referència, contrasta amb la reducció del pH fecal en ratolins sans i en humans que rebien una fórmula fermentada lliure de bacteris (201,202). Això podria ser degut a l'efecte de la lactància materna en el nostre estudi, que ja pot acidificar el contingut intestinal.

L'excreció viral en el grup suplementat amb l'FM va ser lleugerament menor que la del grup RV, el que suggereix que l'FM té una certa capacitat d'unir-se a les partícules de RV. De fet, les interaccions entre els glicans de la superfície dels enteròcits i les proteïnes VP7/VP4 del RV són crucials per a la infecció (188), i les glicoproteïnes han estat caracteritzades com a compostos actius d'una llet fermentada (201). En conseqüència, sembla raonable pensar que alguns components de l'FM podrien unir-se al virus. L'assaig de bloqueig *in vitro* va mostrar una inhibició del voltant del 30% de la detecció de virus en presència d'aquesta FM. Un dels possibles mecanismes responsables de la reducció de les manifestacions clíniques produïda per aquest producte podria ser la interacció directa i el bloqueig de les partícules virals,

evitant la seva entrada als enteròcits i, per tant, la seva replicació. No obstant això, no es poden descartar l'existència d'altres mecanismes.

La dieta FM va causar alguns canvis en la concentració d'anticossos anti-RV. Alguns estudis han suggerit que l'augment de la IgA intestinal podria ser el mecanisme d'acció d'una llet fermentada (200), però els resultats aquí obtinguts no concorden amb aquest suggeriment, ja que els nivells intestinals d'IgA en el grup FM van ser similars als del grup RV.

D'altra banda, es va investigar el possible efecte sinèrgic de la combinació de l'FM amb Immunofortis® (**capítol 4**). El grup suplementat amb aquesta combinació va presentar l'efecte sobre la consistència de les femtes que s'havia trobat en el grup suplementat amb Immunofortis®, i tot i que quan es van normalitzar els resultats d'incidència i gravetat es va poder observar una millora respecte al grup RV, aquests resultats van ser similars als de l'FM i l'Immunofortis® per separat (**capítols 2 i 3**). Mentre que els valors del pes fecal en el període de diarrea aguda i de l'excreció viral en aquest grup són intermedis entre els del grup FM i els del grup suplementat amb Immunofortis®, s'ha trobat alguna diferència a nivell d'anticossos. En aquest sentit, la concentració d'IgG anti-RV en sèrum a dia 14 és més elevada en el grup suplementat amb la combinació de prebiòtic-postbiòtic que en el grup RV, tot i que no tant com al grup suplementat amb l'Immunofortis®. D'altra banda, mentre el prebiòtic i el postbiòtic per separat incrementen els nivells d'IgG anti-RV en sèrum a dia 21, la combinació d'ambdós els disminueixen significativament. Aquest fet podria estar relacionat amb l'elevat potencial de bloqueig del virus per part de la combinació de prebiòtic-postbiòtic que s'ha observat a l'assaig de bloqueig *in vitro*, amb una inhibició de la detecció del virus molt més elevada que amb l'FM o Immunofortis® per separat.

En resum, el model d'infecció simple ha permès avaluar l'efecte de les intervencions nutricionals sobre les manifestacions clíniques i sobre la immunitat generada per una primera infecció. Per altra banda, el model de doble infecció, que inclou manifestacions clíniques durant la primera infecció, però no en la segona, ens ha permès a més, i tot i la falta de reproductibilitat d'algunes determinacions, avaluar la influència dels suplements sobre la resposta immunitària en futures reinfeccions, com passa en els éssers humans, quan s'ha modulat la primera infecció.

En conclusió, tant els productes prebiòtics GFA, Immunofortis® i Bimuno® GOS, com la suplementació amb *B. breve* M-16V o el postbiòtic FM en primeres etapes de vida, són capaços de millorar la gastroenteritis induïda per RV mentre que permet a l'hoste elaborar la resposta immunitària pròpia, que podria ser d'importància per controlar una segona infecció. Els resultats obtinguts ajuden a la identificació de prebiòtics i probiòtics clau amb efectes moduladors sobre la maduració dels mecanismes de defensa del nounat, especialment en la prevenció i tractament de les infeccions per RV. Ara bé, es necessiten estudis addicionals per tal d'obtenir una visió més profunda sobre els efectes d'aquests compostos i per entendre si aquests efectes poden ser extrapolables des del model de rata als éssers humans. D'altra banda, la pauta i la dosi d'administració d'aquests compostos moduladors microbians estan encara per determinar. Totes aquestes determinacions poden conduir a la conclusió de si són adequats per a la potenciació dels mecanismes de defensa del nadó i si el coneixement científic generat per aquests resultats a nivell preclínic podria permetre, a mig termini, la incorporació d'aquest tipus de suplements funcionals en els preparats per lactants.

CONCLUSIONS/*CONCLUSIONS*

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

Regarding the suitability of the rotavirus **(RV) preclinical model,**

- A better characterization of the existing SA11-induced diarrhoea model in suckling rats has been performed by adding non-invasive objective variables such as faecal pH and rectal temperature. Moreover, the anti-RV hyperimmune bovine colostrum administration in this model confers total protection and, therefore, it is a suitable intervention as a control protective agent.

- A new RV double-infection model in rat, based on early SA11 and post-early weaning EDIM inoculations, has been developed. Several immune variables, such as anti-RV humoral and cell immune responses evaluation, have been demonstrated as being suitable for performing interventional studies. In this model, modulatory actions on the first infection seem to be relevant for the later immune response when encountering a second RV infection.

Regarding the **probiotic** supplementation with the ***Bifidobacterium breve*** **M-16V** strain during the rat suckling period,

- It improves the development of mucosal immunity in early life rats, by modulating Toll-like receptor expression, enhancing the homing process of naïve T lymphocytes to the mesenteric lymph nodes, raising the retention of activated lymphocytes in the intraepithelial compartment, as well as intensifying the intestinal IgA synthesis.

- It modulates RV infection (in the single-infection model) and reinfection (in the double RV infection model), by ameliorating diarrhoea during the first infection but allowing the host to elaborate its own immune response, which provides the ability to control the second infection.

Regarding the **prebiotic** supplementation with **scGOS/lcFOS 9:1** prebiotic mixture (Immunofortis®) during the rat suckling period,

- It ameliorates RV diarrhoea, despite its direct effect on stool consistency, and modulates reinfection in the double RV infection model by showing a high immunomodulatory action.

- Its action on the prevention of diarrhoea associated with RV infection is comparable to that of the prebiotic **Bimuno® GOS**, allowing the development of protection against further RV infections in this preclinical model.

- In the **synbiotic** combination with the probiotic ***B. breve*** **M-16V**, it is highly effective in attenuating RV-induced diarrhoea as well as modulating immune response and RV reinfection in the double-infection preclinical model.

- In contrast, the combination of Immunofortis® with **pectin-derived acidic oligosaccharides (pAOS)** does not enhance its preventive effect on the rat gastroenteritis RV model.

Regarding the **postbiotic** supplementation with a *Bifidobacterium breve* and *Streptococcus thermophilus* fermented formula during rat suckling,

- It is able to prevent almost all features derived from the RV-induced diarrhoea; moreover, it also modulates the anti-RV immune response.

- The addition of Immunofortis® to this postbiotic does not show a clear synergistic effect.

Overall, all tested products showed beneficial effects on RV-induced gastroenteritis in the neonatal rat model, modulating clinical biomarkers and immune system response in early life, the probiotic and the postbiotic being the most effective. Further studies are needed in order to provide a better understanding of their mechanism of action and whether they can be considered for inclusion in infant formulas or supplements, to be used as strategies for protecting against human RV-induced diarrhoea in children.

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