



Research Signpost
Trivandrum
Kerala, India

Recent Advances in Pharmaceutical Sciences VII, 2017: 19-34 ISBN: 978-81-308-0573-3
Editors: Diego Muñoz-Torrero, Montserrat Riu and Carles Feliu

2. Immunomodulatory role of probiotics in early life

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Abstract. The immune response in early life, as well as the anti-infective capacity of the organism, can be enhanced by some probiotic bacteria, especially those of importance in this neonatal period. The potential effect of these particular strains associated with early life, either isolated from breast milk or from baby faeces, on the immune system should be evaluated by *in vitro* and *in vivo* models of health or infection status before their introduction to babies, for example, in infant formulas.

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Introduction

In the last few years, interest in the mutualism between hosts and their microbiota has increased considerably. The intestinal microbiota affects the human physiology by enhancing the epithelial barrier and immune functions, among others, both directly and indirectly. These beneficial effects are especially relevant in early life, when the immune system is still immature [1]. For this reason, it is important to develop strategies to modulate the intestinal environment and microbiota composition and functionality, which in turn may modulate the mucosal immune system, and therefore the systemic immunity.

Among the dietary strategies used to enhance the anti-infective response of neonates, the use of probiotics is the most studied. It is known that probiotics are exogenous micro-organisms that interact with various cellular components within the intestinal environment and have a positive impact on the host's health as defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) in 2013 [2], based on the initial one suggested by experts in the World Health Organization (WHO) in 2001 and in the Food and Agriculture Organization (FAO) [3]. This concept is supported by several other organizations such as the Codex, the Institute of Food Technologists (IFT), the World Gastroenterology Organization (WGO) and the European Food Safety Authority (EFSA).

The probiotics themselves or their metabolites are responsible for the effects on the immune system. Probiotics can be recognized by the immune cells through pattern-recognition receptors specific to microbial components, such as peptidoglycan or lipoteichoic acid [4]. This direct recognition triggers inflammatory or anti-inflammatory responses, depending on the specific strain [5]. Moreover, probiotics might induce intestinal epithelial cells to secrete an array of cytokines, therefore influencing immune function indirectly [6].

Mechanisms of immunomodulation include the induction of mucus production, short chain fatty acid (SCFA) synthesis, macrophage activation, stimulation of cytokine and secretory IgA production, and elevated production of peripheral immunoglobulins, among others (Fig. 1). During infancy, probiotic interventions could be helpful for the maturation of the immune system and, therefore, in strengthening the defence mechanisms against infections, or even preventing the development of immune-mediated diseases, such as asthma [4].

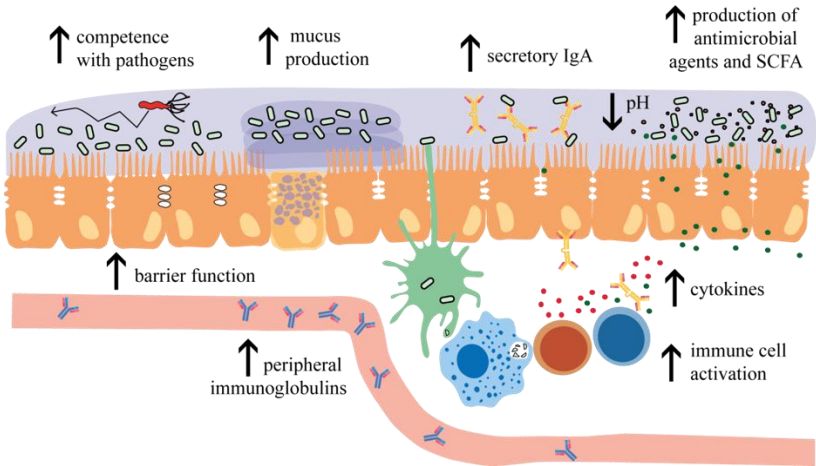


Figure 1. Main mechanisms of probiotics to potentiate the anti-infective capacity and modulate the immune system of the organism.

Not all bacteria induce the same effects in an organism and these effects could be different depending on age. In this case, when the target is the infant, it would be of interest to assess those types of bacteria obtained from a source related to early life, such as probiotics from breast milk or baby faces.

Rotavirus (RV) is the leading cause of severe diarrhoea among infants and young children and, although more standardized studies are needed, nowadays there is enough evidence to show that probiotics can help to fight against RV and other infectious and intestinal conditions.

Despite all the efforts made to evaluate the influence of these probiotic bacteria on infants' immune response, it is difficult to reach a conclusion due to the variability of the physiological or disease status studied, the numerous varieties of the probiotic strains, as well as the limitations in the number of participants. These are the reasons why most currently available data describing 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 supports the current book chapter is that the immune response as well as the anti-infective capacity of the organism in early life can be enhanced by some probiotic bacteria derived from a neonatal source. Therefore, considering this

hypothesis, the main objective of this work is to show, with three particular representative studies, the beneficial effect of probiotic bacteria of importance in early life on the immune development and prevention against RV infections. The potential effect of these particular strains associated with early life, either isolated from breast milk or from baby faeces, on the immune system should be evaluated by *in vitro* and *in vivo* models before their introduction to babies, for example, in infant formulas.

1. *In vitro* immunomodulatory actions of breast milk probiotics

Breast milk has been traditionally considered to be sterile; however, current scientific studies have shown that it contains cultivable strains of at least 19 species of bacteria belonging to at least ten different genera (Table 1). Most of the bacteria isolated belong to the genera *Staphylococcus*, *Streptococcus*, *Lactobacillus* and *Bifidobacterium*, and some of them have already been used in human nutrition for their probiotic activity [7]. Therefore, breast milk constitutes a continuous source of commensal and potentially probiotic bacteria, since an infant that consumes approximately 800 mL of milk /day would ingest between 10^5 and 10^7 bacteria daily [8]. These findings would suggest that breastfeeding helps to shape the immune system's development early in life in order to achieve a competent function of the gut and a balanced immune homeostasis.

Despite all the advances made in probiotic research there is still a lack of a systematic analysis of the immunomodulatory potential of these bacterial strains in human cells and relatively little information is available regarding their mechanisms of action. For this reason, in the study by Pérez Cano *et al.* [9], the effects of two lactobacillus strains isolated from human milk on the modulation of the activation and cytokine profile of peripheral blood mononuclear cell (PBMC) subsets *in vitro* were evaluated. Briefly, *Lactobacillus salivarius* CECT5713 and *Lactobacillus fermentum* CECT5716 at 10^6 bacteria/mL were co-cultured with PBMC (10^6 /mL) from eight healthy donors for 24 h. The activation status (CD69 expression) of natural killer (NK) cells (CD56⁺), total T cells (CD3⁺), cytotoxic T cells (CD8⁺) and helper T cells (CD4⁺) was determined by flow cytometry. Regulatory T cells (Treg) were also quantified by intracellular Foxp3 evaluation [9].

Table 1. Bacteria isolated from human breast milk. Adapted from Fernández *et al.* [8].

Genera	Species	References
<i>Bifidobacterium</i>	<i>adolescentis, bifidum, breve, longum</i>	[10–12]
<i>Enterococcus</i>	<i>faecium, faecalis, durans, hirae, mundtii</i>	[13–16]
<i>Kocuria</i>	<i>rhizophila</i>	
<i>Lactobacillus</i>	<i>acidophilus, fermentum, plantarum, gasseri, crispatus, rhamnosus, salivarius, reuteri, casei, gastricus, vaginalis, animalis, brevis, helveticus,</i>	[13–20]
<i>Lactococcus</i>	<i>lactis</i>	[14, 15]
<i>Leuconostoc</i>	<i>mesenteroides</i>	[14, 15]
<i>Pediococcus</i>	<i>pentosaceus</i>	[11, 12]
<i>Rothia</i>	<i>mucilaginoso</i>	[14, 15]
<i>Staphylococcus</i>	<i>epidermidis, aureus, capitis, hominis</i>	[14, 15, 17]
<i>Streptococcus</i>	<i>mitis, salivarius, oris, parasanguis, lactarius, australis, gallolyticus, vestibularis</i>	[11, 12, 14–17]

To our knowledge this is the first time that the effects of these breast milk probiotics on specific lymphocyte subsets, including Treg cells, were reported. The results obtained in such a study demonstrated that *L. fermentum* CECT5716 and *L. salivarius* CECT5713 – derived from breast milk – were potent activators of NK cells by highly increasing their proportion through the expression of the activation marker CD69. Moreover, both strains were moderate activators of either CD4⁺ or CD8⁺ T cells – even though the increase of CD69 expression was not as evident as the one above. Finally, there was no impact of the breast milk probiotic bacteria on NK-T cell activation status. Thus, both strains have an influence on both innate and acquired immunity (Fig. 2).

Both milk strains *L. fermentum* CECT5716 and *L. salivarius* CECT5713, significantly induced a twofold rise in the Treg proportion with respect to resting cells ($p < 0.05$), although the percentage of Treg did not exceed 1% of the CD4⁺ T-cell population.

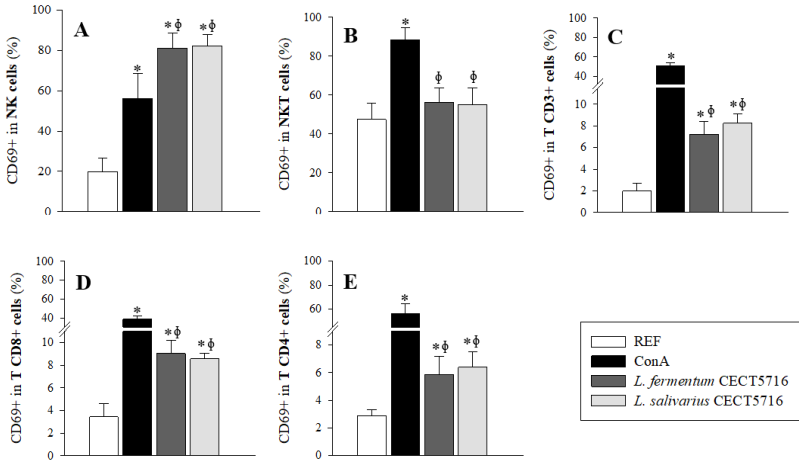


Figure 2. Effect of *L. fermentum* CECT5716 and *L. salivarius* CECT5713 on the expression marker CD69+ of specific lymphocyte subsets from Pérez-Cano *et al.* [9]. Activated **A.** NK cells, **B.** NKT cells, **C.** CD3+ T cells, **D.** CD8+ T cells and **E.** CD4+ T cells. Concanavalin A (ConA) was used as positive control. Data are expressed as mean \pm SEM values of 3–8 healthy donors. Differences between control, ConA and bacterial species were tested by one-way ANOVA. Significance: * $P < 0.05$ vs. control; $\phi P < 0.05$ vs. ConA.

On the other hand, in order to evaluate the induction ability of a wide range of pro- and anti-inflammatory cytokines and chemokines a semi-quantitative method to simultaneously profile the relative levels of 32 selected cytokines and chemokines was used. The Proteome Profiler TM Array with human cytokine array panel A (R&D Systems Europe Ltd., Abingdon, UK) used in the study included C5a, CD40L, G-CSF, GM-CSF, GXCL1,8 and 10–12, CCL1–CCL5, sICAM-1, IFN γ , IL-1a, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32a, MIF, Serpin E-1 and TNF α . Furthermore quantification of IFN γ , IL-1b, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF α , TNF β , MIP-1a and MIP-1b was performed using the human Th1/Th2 plex kit from Bender Medsystems GmbH (Vienna, Austria) and GM-CSF and TGF- β 1/- β 2 by ELISA [21].

The results showed that human PBMC, either in resting conditions, stimulated with LPS or co-cultured with live probiotic bacteria for 24 h displayed different patterns of cytokine secretion (Fig. 3). Unstimulated cells

did not evidence the expression of most of the molecules studied; however, LPS-stimulated cells secreted most of the cytokines and chemokines included in the panel, specifically CCL2, CCL5, MIP-1 α , MIP-1 β , TNF α , Il-1 β , IL-6, IL-18, GRO α and sICAM-. *L. fermentum* CECT5716 and *L. salivarius* CECT5713 promoted the secretion of CCL2, CCL5, GRO α and sICAM-1; the amounts obtained were similar to those induced by LPS (Fig. 3).

In addition, the probiotic bacteria were better inducers of TNF α , MIP-1 β , Il-1 β and IL-18 than LPS and also activated IL-1 α and C5a production in the PBMC, which were not induced by LPS. Overall, two strain-specific effects were found: on the one hand, the *L. fermentum* CECT5716 seem to induce IFN γ , and on the other, *L. salivarius* CECT5713 seem to induce GM-CSF, both in a strong way [21].

Further quantification of most of the cytokines and chemokines assayed above were later confirmed by the human Th1/Th2 plex kit from Bender Medsystems GmbH (Vienna, Austria) and by ELISA [21] (Fig. 4).

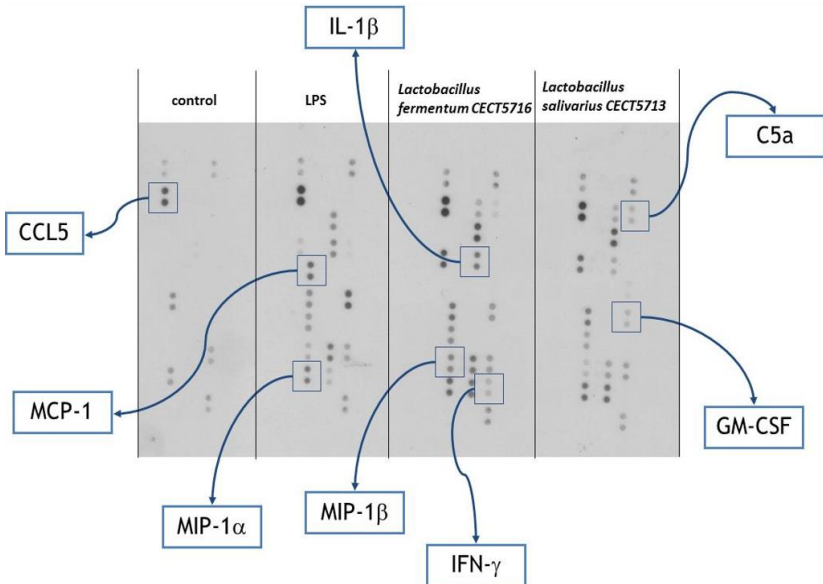


Figure 3. Semi-quantitative determination of relative levels of 32 selected cytokines and chemokines in the presence of *L. fermentum* CECT5716 and *L. salivarius* CECT5713. Results derived from Pérez-Cano *et al.* [9].

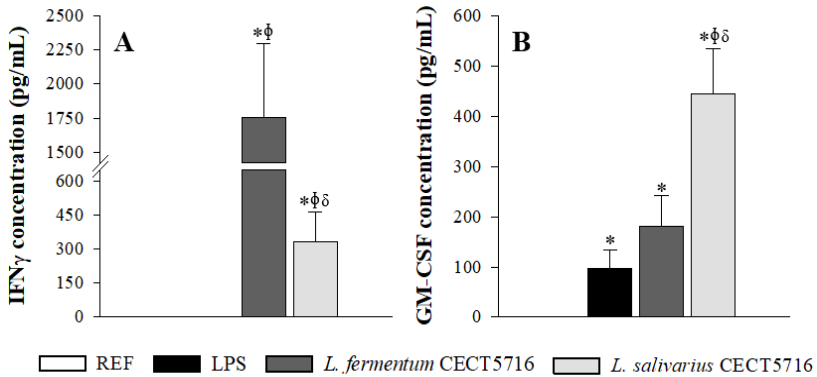


Figure 4. IFN- γ and GM-CSF concentration in PBMC co-cultured with *L. fermentum* CECT5716 and *L. salivarius* CECT5713 media from Pérez-Cano *et al.* [9]. LPS was used as positive control. Data are expressed as mean \pm SEM values of 3–8 healthy donors. Differences between control, LPS and bacterial species were tested by one-way ANOVA. Significance: * P <0.05 vs. control; ϕ P <0.05 vs. LPS; δ vs. *L. fermentum* CECT5716.

In conclusion, this study demonstrates that *L. salivarius* CECT5713 and *L. fermentum* CECT5716 enhanced the activation of NK and T-cell subsets and the expansion of Treg cells, suggesting their ability to strengthen both innate and adaptive immune responses. Moreover, both strains are able to induce a broad array of cytokines in a strain-specific manner. It should be stated that *L. fermentum* and *L. salivarius* from non-breast milk sources also induce the production of a broad array of cytokines [21], and their immunomodulatory importance in early life should also be further studied.

2. *In vivo* effect of probiotics in health: Immune development

The next step after investigating the immunomodulatory potential of early life probiotics *in vitro* consisted of investigating the *in vivo* effect of the supplementation with these types of bacteria on the maturation of the intestinal and systemic immune system during the first stages of development. Very few studies have addressed this issue; in one example, Rigo-Adrover *et al.*, [22] investigated the impact of *Bifidobacterium breve* M-16V supplementation on some aspects of the immune system

development using a neonatal rat as a model. The neonatal rat has been considered as a suitable model for immunonutrition studies, because it allows the characterization of immune changes during suckling in several lymphoid compartments [1].

In the case of *B. breve* M-16V, although not a breast milk-derived probiotic bacteria, it is naturally present in infants' microbiota and has already shown immunomodulatory properties [23–27]. In Rigo Adrover *et al.*'s study, neonatal Lewis rats were supplemented with the probiotic strain or with vehicle during a 13-day period and on day 18 of life, splenocytes, mesenteric lymph node (MLN) cells and intraepithelial lymphocytes (IEL) were isolated as in previous studies adapted to neonatal rats [28, 29]. They were later purified, counted, and stained using immunofluorescence techniques. Main cell subsets were evaluated as well as intestinal aspects such as faecal consistency and immunoglobulin-A (IgA) levels.

Briefly, the study evidenced that *B. breve* M-16V administration during the rat suckling period influences the intestinal and systemic lymphocyte composition, modulates the percentage of cells expressing molecules involved in the interaction with intestinal bacteria such as TLR4, and also potentiates the intestinal IgA production. Regarding the changes in lymphocyte composition, very few changes were observed. Although this nutritional intervention did not seem to potentiate the systemic immune maturation, it increased the proportion of CD8+ NK cells in MLN and reduced that of CD4+ IEL and CD8 $\alpha\beta$ + TCR $\gamma\delta$ + IEL [22].

TLR4 presence in splenocytes was not affected by the nutritional intervention with the probiotic bacteria. On the contrary, it was increased in the MLN cells but not in IEL (Fig. 5A and B). However, the CD4+ T cell subset in the IEL increased the TLR4+ proportion due to the *B. breve* M-16V supplementation, suggesting that this increased bacteria–host interaction may have a role in the preparation of the intestinal immune system for a stronger response against infections. These results are in agreement with other studies conducted in adult animals [30–34]. The $\alpha E\beta 7$ integrin on the lymphocyte surface allows IEL retention in the intestine [35, 36]. For this reason, it was determined in the three compartments and although no changes were found in SPL, the percentage of MLN cells and IEL expressing $\alpha E\beta 7$ integrin was higher in animals fed with the probiotic ($p < 0.05$) (Fig. 5C and D). This result was evidenced in CD4+CD8, CD4–CD8+ and CD4–CD8 cells in both compartments.

Finally, the administration of the *B. breve* M-16V strain for 13 days during the suckling period enhanced the intestinal IgA production (Fig. 6), which is a typical feature of immuno-enhancing probiotic bacteria [22].

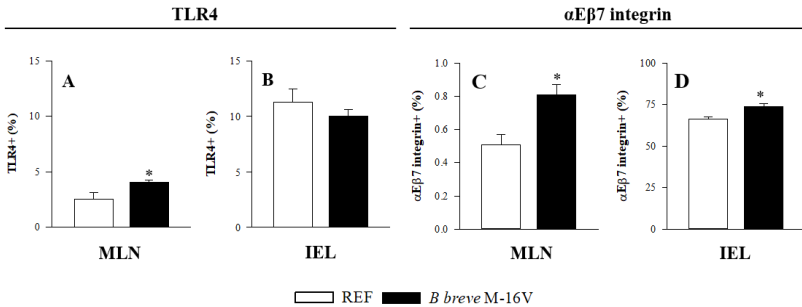


Figure 5. Surface TLR4 and $\alpha E\beta 7$ integrin expression in MLN and IEL lymphocyte in reference and *B. breve* M-16V supplemented rats from Rigo-Adrover *et al.* [22]. Data are expressed as mean \pm SEM (n = 8 animals/group). Significance: *P<0.05 vs. ref. [22].

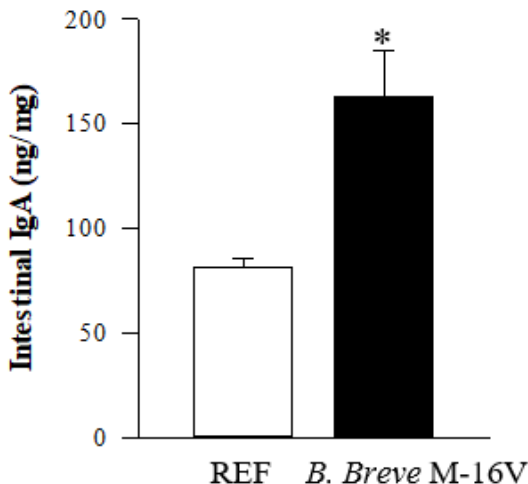


Figure 6. IgA concentration in intestinal washes of 19-day-old rats. Results are expressed as ng of IgA/mg of tissue (mean \pm SEM, n=8 animals/group) from Rigo-Adrover *et al.* [22]. Statistical differences: *p<0.05 vs. ref. [22].

3. *In vivo* effect of probiotics under infection: Rotavirus gastroenteritis

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 RV and other infectious and intestinal pathologies. In this context, due to its immunomodulatory potential, *B. breve* M-16V strain was also tested as a protective agent in such infective processes [22].

Briefly, the neonatal rats received the intervention with the *B. breve* M 16V from the 3rd to the 21st day of life (almost the entire suckling period) by oral gavage. On day 7, RV was orally administered as in previous studies [37]. Clinical variables were evaluated by means of scoring stools from 1 to 4 (diarrhoea index [DI]) based on colour, texture and amount. These scores allow the obtained results to be expressed as incidence and severity, as well as the maximum value of the above variables as indicators.

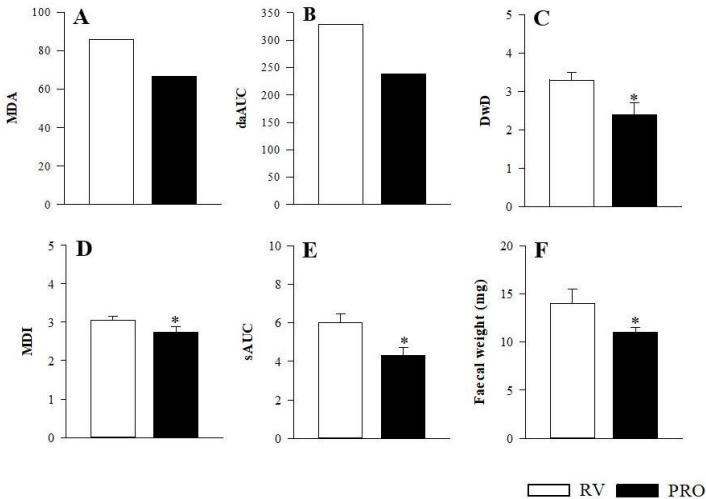


Figure 7. Effect of the supplementation with *Bifidobacterium breve* M-16V in RV-induced diarrhoea animals from Rigo-Adrover *et al.* [22]. Diarrhoea production was studied by different parameters: **A.** proportion of animals with diarrhoea (MDA); **B.** diarrhoeic animals (DA); **C.** duration of the process, **D.** maximum diarrhoea index (MDI); **E.** severity; and **F.** weight of the faecal specimens. Data are expressed as mean \pm SEM (n = 8 animals/group). Significance: *P<0.05 vs. RV.

RV inoculated to 7-day-old animals induced diarrhoea in most of the animals for about 3–4 days (Fig. 7). The supplementation with the probiotic was able to significantly reduce the maximum proportion of animals with diarrhoea (MDA, Fig. 7A) but also the overall course of the diarrhoeic animals (DA, Fig. 7B). The *B. breve* M-16V also reduced the duration of the process (Fig. 7C) as well as its severity, as is observed in the lower values of the maximum diarrhoea index (MDI, Fig. 7D) and the overall severity throughout the process (Fig. 7E). The intervention also reduced the weight of the faecal specimens, which were increased due to the RV infection (Fig. 7F). The study also shows how the probiotic modulates the humoral immune response against the virus as well as the pattern of faecal short-chain fatty acids (SCFA) and the results derived after its use in a synbiotic combination [22].

4. Probiotics in infant formulas

There are several international organizations that are responsible for making recommendations and standards that must be accomplished when preparing formula types 1 and 2, such as the American Academy Committee on Pediatrics Nutrition (AAPCON) and the Committee on Nutrition of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN). A summary of the guidance adapted in Spain can be found in Table 2. It summarizes the main components, including, for example, the proportion of oligosaccharides.

Infant formulas type 1 (0–6 months, 650 kcal/day) and infant formulas type 2 (6–12 months, 850 kcal/day) are quite different in composition with respect to infant formulas type 3 (12–36 months), which do not follow any specific guidance for its formulation.

The probiotics can be optionally added to these formulations in order to better mimic breast milk composition; however, no compilation of data showing a list of probiotics present in these types of products is available. Due to this fact, a pilot evaluation was performed with a total of 40 samples from Spanish stores (10 samples for each type of infant formula 1, 2 and 3 sold in pharmacies and 10 in supermarkets). The study was performed in September–December 2015 (Table 3). Overall, independently of the source providing the formula (pharmacy or supermarket) it can be observed that only a low proportion of them include probiotics (25%, 10/40); a proportion that increases if the synbiotic formulation is considered (30%, 12/40). It must be highlighted that depending on the origin of the product (pharmacy or supermarket) we can observe a high difference: 33.3 % (10/30) of

Table 2. Infant's formula composition. From BOE number. 64. Real Decreto 165/2014 which modifies BOE number. 131. Real Decreto 867/2008, 23 May [38, 39].

Nutrients	Infant formula type 1 (per 100 kcal)	Infant formula type 2 (per 100 kcal)
Energy (Kcal)	60–70/100 mL	60–70/100 mL
Carbohydrates (g)	9–14	9–14
Lactose (g)	>4.5	>4.5
Proteins (g)	1.8–3	1.8–3.5
Whey protein/casein	60/40	20/80
Fat (g)	4.4–6.0	4.0–6.0
Linoleic acid (mg)	300–1200	300–1200
Sodium (mg)	20–60	20–60
Potassium (mg)	60–160	60–160
Calcium (mg)	50–140	50–140
Phosphorus (mg)	25–90	25–90
Iron (mg)	0.3–1.3	0.6–2
Oligosaccharides (g)	<0.8	<0.8
Probiotic bacteria	not mentioned	not mentioned

formulas provided in pharmacies have probiotics (40%, 12/30 if synbiotics are included) whereas none of those found in the supermarkets (0%, 0/10) have probiotics in their composition. Regarding the influence of the type of formula, types 1, 2 and 3 contain probiotics in similar proportions, which comprises between 30 and 40%.

In all cases, the proportion of formulas with probiotics are lower than those with oligosaccharides (prebiotics), which are always higher than 40%, with the exception of the type 3 formulas sold in pharmacies which have only a 20% presence of prebiotics. This pilot study just highlights the low incorporation of probiotics into these types of products.

Table 3. Study of presence of probiotics and prebiotics in infant's formula on the Spanish market (2016). Total samples analysed: 40.

Content	Pharmacy				Supermarket
	Total N=30 (%)	Type 1 N=10 (%)	Type 2 N=10 (%)	Type 3 N=10 (%)	Total N=10 (%)
None	N=6 (20%)	N=2 (20%)	N=0 (0%)	N=4 (40%)	N=6 (60%)
Oligosaccharides	N=12 (40%)	N=5 (50%)	N=5 (50%)	N=2 (20%)	N=4 (40%)
Probiotics	N=10 (33.3%)	N=3 (30%)	N=4 (40%)	N=3 (30%)	N=0 (0%)
Synbiotics	N=2 (6.7%)	N=0 (0%)	N=1 (10%)	N=1 (10%)	N=0 (0%)

5. Conclusions

Overall, early life probiotics have not only demonstrated their immunomodulatory potential *in vitro* and their beneficial effects on immune development but also in the context of infection, as is the case of the rotavirus-induced gastroenteritis in the neonatal rat model. Further studies are needed in order to provide a better understanding of their mechanisms of action and whether they can be considered for inclusion in infant formulas or supplements, to be used as strategies for promoting the maturation of the neonatal immune system or even for protecting against human rotavirus-induced diarrhoea in children. Regardless of their presence in breast milk and the positive effects of this type of probiotic bacteria, they are poorly included in infant formulas.

Acknowledgements

The authors are grateful to Karen Knipping from Nutricia Research and Monica Olivares from Biosearch S.L. for providing the probiotics used in these studies. We also thank Prof. Parveen Yaqoob from the University of Reading where the *in vitro* studies were performed, as well as the Animal Experimental Unit in the Faculty of Pharmacy and Food Science where the animal work was developed. We also thank Prof. Laura Baldomà from the University of Barcelona for her critical revision of the manuscript.

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