Eur J Nutr
DOI 10.1007/s00394-016-1213-1

ORIGINAL CONTRIBUTION

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

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Received: 23 December 2015 / Accepted: 31 March 2016

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*

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

Electronic supplementary material The online version of this article (doi:10.1007/s00394-016-1213-1) contains supplementary material, which is available to authorized users.

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Published online: 25 April 2016

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), Scheffé'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.

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,

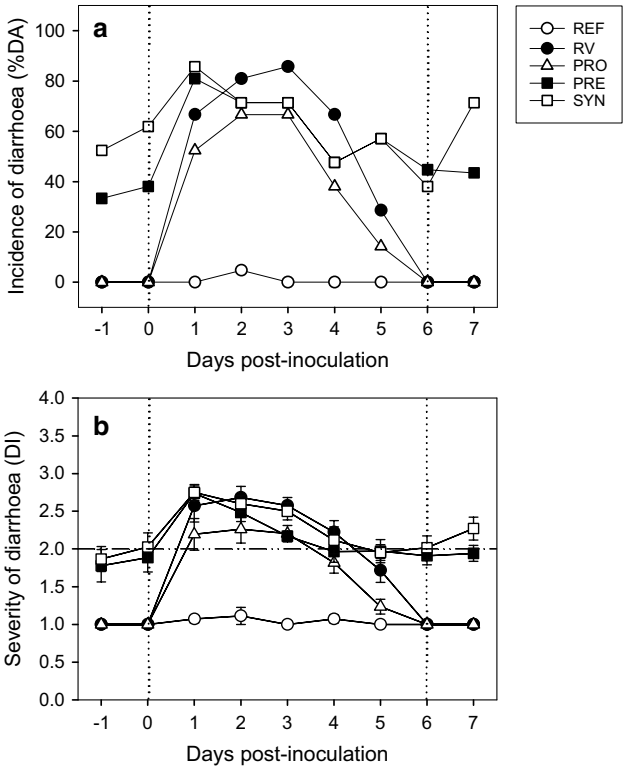

Fig. 1 Clinical indexes. **a** Incidence of diarrhoea: results are expressed as % of diarrhoeic animals. **b** Severity of diarrhoea: faecal samples are scored from 1 to 4 based on colour, texture and amount of stool. Scores of diarrhoea index (DI) ≥2 indicates diarrhoeic faeces. Results are expressed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

%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

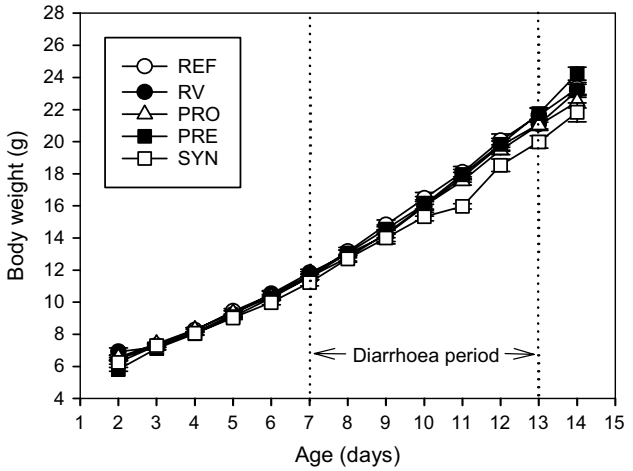

Fig. 2 Body weight (g) during the study, before and after virus inoculation (day 7 of life). Results are expressed as mean ± SEM ($n = 21$ animals/group)

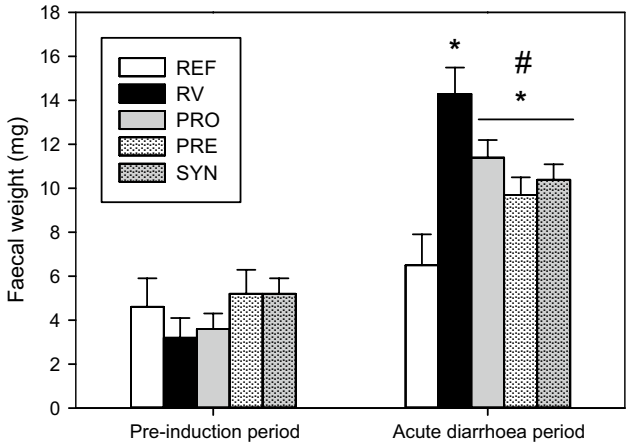

Fig. 3 Faecal weight (mg) during preinduction period (0 DPI) and acute diarrhoea period (pooled data from 1–4 DPI samples). Results are expressed as mean ± SEM ($n = 7$–24 samples/group/period). Statistical significance: * $p < 0.05$ versus REF, # $p < 0.05$ versus RV

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).

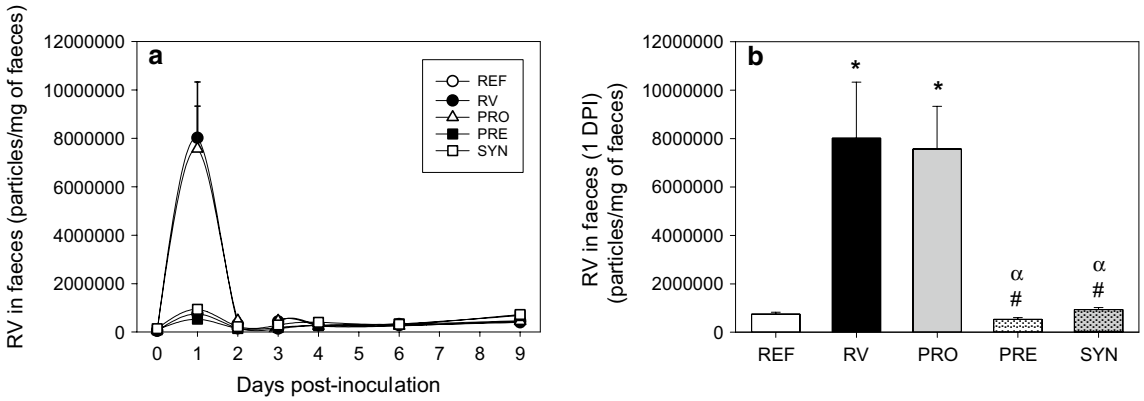

Fig. 4 Viral shedding. RV particles present per faecal sample obtained **a** during the study in each group and **b** on 1 DPI in each group. Results are expressed as mean ± SEM (n = 7–24 samples/group/period). Statistical significance: * $p < 0.05$ versus REF, $^{\#}$ $p < 0.05$ versus RV, $^{\alpha}$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

Acknowledgments This study was supported by a Nutricia Research grant in collaboration with the Fundació Bosch i Gimpera (FBG306349). MRA was the recipient of a fellowship from the Fundació Pedro i Pons. The authors thank Dr. Adele Costabile for her help and advice with the HPLC.

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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