

# Immune Responses in Rhesus Rotavirus-Challenged Balb/c Mice Treated with Bifidobacteria and Prebiotic Supplements

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## ABSTRACT

*Bifidobacterium* species (*B. bifidum* and *B. infantis*), with or without prebiotic compounds (arabino-galactan, short-chain fructo-oligosaccharide, iso-malto-dextrins), were orally fed to Balb/c pups ( $n = 192$ ) to evaluate their potential synergistic effects on modulating the course of rhesus rotavirus (RRV) infection, as well as their ability to mediate the associated mucosal and humoral immune responses. Rotavirus-specific IgA and IgG in serum, rotavirus antigen, and specific IgA in feces were measured by ELISA. Mucosal total IgA and IgG levels were determined in Peyer's patches by flow cytometry. Significantly delayed onset ( $p = 0.001$ ) and early resolution ( $p < 0.001$ ) of diarrhea were observed in bifidobacteria-treated, RRV-infected mice compared with RRV-infected control mice. Supplementation with prebiotic compounds did not shorten the clinical diarrhea course more than that observed with bifidobacteria treatment alone. Rotavirus-specific IgA in feces was 16-fold elevated on d 5 postinfection in bifidobacteria-treated, RRV-infected mice compared with the RRV-infected alone group. In

addition, the level of rotavirus-specific IgA in serum was four-fold higher in bifidobacteria-treated, RRV-infected litters *versus* mice challenged with RRV alone on 28 and 42 d postinfection. No enhancement of the immune response was found in RRV-infected mice that were treated with both bifidobacteria and prebiotic compounds over those treated with bifidobacteria only. The findings suggest that bifidobacteria may act as an adjuvant by modulating early mucosal and strong humoral rotavirus-specific immune responses, and mitigate severity of rotavirus-induced diarrhea. (*Pediatr Res* 51: 750–755, 2002)

## Abbreviations

**RRV**, rhesus rotavirus  
**PP**, Peyer's patches  
**PI**, postinfection  
**PD**, postdelivery  
**CFU**, colony-forming units  
**PFU**, plaque-forming units

*Bifidobacterium* species and other lactic acid-producing Gram-positive anaerobes dominate the human colonic microflora, and collectively belong to a group of bacteria defined as probiotics (1). These bacteria are considered to be beneficial to human health through their effects in the gastrointestinal tract (1–3). Health effects attributed to probiotics include immune regulation, cholesterol lowering, prevention of cancer recurrence, and amelioration of diarrhea (4–6). Because of their potential benefits to human health, various fermented dairy products (capsules, powders, enriched yogurts and milks) containing bifidobacteria and lactobacilli are commercially available.

Prebiotics are nondigestible food ingredients that can beneficially affect the host by selectively stimulating the growth and/or activity of certain bacteria in the colon that have positive effects on gut physiology (7). Many oligosaccharides are classified as prebiotics. Oligosaccharides are thought to be assembled by glycosyltransferases, which synthesize cell surface glycoconjugates that are often used as receptors by pathogens (8). Some milk oligosaccharides have been documented to protect the nursing infant by acting as receptor homologs, inhibiting the binding of pathogens to their host receptors, *e.g.* enteropathogenic *Escherichia coli* and *Campylobacter jejuni* (9, 10). In addition, several studies have demonstrated that consumption of certain oligosaccharides can increase the endogenous bifidobacteria level in the intestine (11, 12).

Rotavirus still represents a major etiologic agent of viral gastroenteritis in human infants worldwide because of the absence of an effective vaccine [the Rotashield tetravalent

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rhesus-human reassortant rotavirus vaccine was withdrawn from the market because of its potential association with the occurrence of intussusception (13)]. Intensive scientific investigations have shown that certain lactic acid bacteria, including bifidobacteria, can reduce the severity of rotavirus infections in humans (14, 15) and animals (16, 17). However, the combined use of probiotics and prebiotics in the treatment of diarrhea caused by rotavirus has not been studied. Potential additive or even synergistic effects may be produced that positively alter the course of rotavirus disease.

The present study was conducted in Balb/c mice that were inoculated with RRV and treated with bifidobacteria alone or in combination with selected oligosaccharides to investigate whether a synbiotic (mixture of pro- and prebiotic) could effectively modulate the immune response to rotavirus and reduce the severity of rotaviral disease.

## MATERIALS AND METHODS

**Study design.** Midterm pregnant Balb/c dams, certified rotavirus free, at 13–16 d gestation were obtained commercially (Harlan Bioproducts for Science, Indianapolis, IN, U.S.A.). For the series of experiments performed, 42 Balb/c dams with a range in litter size from three to eight pups (total  $n = 192$ ) were used. Pregnant dams and their litters were randomly assigned to one of six experimental groups: 1) saline control; 2) RRV-infected only; 3) bifidobacteria-treated + RRV-infected; 4) bifidobacteria + compound A-treated + RRV-infected; 5) bifidobacteria + compound B-treated + RRV-infected; 6) bifidobacteria + compound C-treated + RRV-infected. Compound A is arabino-galactan (Q Fiber, Quest International, Hoffman Estates, IL, U.S.A.); compound B is a short-chain fructo-oligosaccharide (Rafitose, Orafiti Active Food Ingredients, Tienen, Belgium); and compound C is iso-malto-dextrins (provided by Dr. John Robyt, Iowa State University, Des Moines, IA, U.S.A.). All the animal experiments were conducted in accordance with the Department of Health and Human Services Guidelines for the humane care and use of laboratory animals and were approved by the State University of New York at Buffalo Institutional Animal Care and Use Committee.

**Feeding and RRV inoculation.** As summarized in Table 1, bifidobacteria- (*B. bifidum* ATCC 15696 + *B. infantis* ATCC 15697, American Type Culture Collection, Rockville, MD, U.S.A.) treated pups (groups 3–6) were orally administered a bifidobacteria suspension ( $0.75 \times 10^8$  CFU/mL of *B. bifidum* and  $0.75 \times 10^8$  CFU/mL of *B. infantis*), with or without 125 mg/mL of compound A, B, or C as appropriate per treatment group. Mice in saline control and RRV-infected only groups (groups 1 and 2) were fed saline according to the same schedule as the other groups. Pups in the RRV-infected groups (groups 2–6) were orally inoculated with RRV ( $2 \times 10^7$  PFU/mL) on d 5 PD. RRV was provided by Dr. Taka Hoshino, Department of Infectious Disease, National Institutes of Health, Bethesda, MD, U.S.A.

**Clinical observation and fecal specimen collection.** Animals were observed daily for changes in clinical signs and for changes in color and consistency of stool. The diagnostic

**Table 1.** Treatment and infection schedule by group

Experimental Group	Time PD			
	Inoculation*	Feeding†		
		5 D PD	1–21 D PD	4–5 Wk PD
1. Saline control		S	S	S
2. RRV-infected only	RRV	S	S	S
3. Bifidobacteria + RRV	RRV	B	B	B
4. Bifidobacteria + compound A + RRV	RRV	B + CA	B + CA	B + CA
5. Bifidobacteria + compound B + RRV	RRV	B + CB	B + CB	B + CB
6. Bifidobacteria + compound C + RRV	RRV	B + CC	B + CC	B + CC

\* Inoculum 10  $\mu$ L,  $2 \times 10^7$  PFU/mL of RRV.

† Pups were fed with 10  $\mu$ L of appropriate substance on d 1–21 PD; 20  $\mu$ L once weekly, 4–5 wk PD; 40  $\mu$ L once weekly, 6–7 wk PD.

S, saline; B, bifidobacteria; CA, compound A; CB, compound B; CC, compound C.

criterion for diarrhea was watery or soft stool with mucus. Fecal specimens were collected from 1 through 7 d PI and then once weekly from all litters. Stool samples collected from pups in a given litter at the same time were pooled and stored at  $-70^\circ\text{C}$  for future use. Bacteriology culture was performed on the samples collected at 1, 3, 5, 7, 14, 28, and 42 d PI.

**Animal dissection and sample processing.** Pups were anesthetized with Metofane (Mallinckrodt Veterinary Inc., Mundelein, IL, U.S.A.). Cardiac blood was collected and centrifuged and the serum was stored at  $-70^\circ\text{C}$ . PP were removed from the intestine and placed into a medium containing RPMI 1640, 1% fetal bovine serum, and 0.09%  $\text{NaN}_3$  before analysis by flow cytometry for percentage of IgA- and IgG-positive B cells, as well as the percentage of total B cells. The ileocecal region of the intestine was collected from each pup, placed in sterile saline (0.1 mL per pup), and then minced using a sterile scalpel before bacteriology culture. The intestinal fecal material from the colon was similarly collected and processed. Intestinal fecal and ileocecal samples were cultured and then stored at  $-70^\circ\text{C}$ . All samples collected from a given litter were pooled to obtain sufficient material for assay.

**Isolation of bacteria.** Fecal and intestinal specimens were cultured aerobically on Trypticase soy agar with 5% sheep blood, phenylethyl alcohol agar with 5% sheep blood, and MacConkey II agar (all from BD Diagnostic Systems, Sparks, MD, U.S.A.) at  $36^\circ\text{C}$ . Specimens were also cultured in a  $36^\circ\text{C}$  anaerobic chamber on CDC anaerobe 5% sheep blood agar (BD Diagnostic Systems), CDC anaerobe 5% sheep blood agar with phenylethyl alcohol (BD Diagnostic Systems) and *Bifidobacterium* selective agar (Anaerobe Labs, San Jose, CA, U.S.A.). All cultures were performed using a 0.001-mL calibrated loop (BD Diagnostic Systems, Sparks, MD, U.S.A.) and quantified as CFU per milliliter. Aerobic isolates were identified by conventional bacteriologic techniques. Anaerobic isolates confirmed by Gram's stain to be Gram-positive bacilli were further identified using a RapID ANA II System (Innovative Diagnostic Systems, Inc., Norcross, GA, U.S.A.).

**Flow cytometry.** PP were gently teased apart in media with fine forceps. Samples were diluted in PBS wash buffer (PBS +

1% fetal bovine serum + 0.09% NaN<sub>3</sub>) to give  $2 \times 10^6$  cells/mL. After briefly vortexing and allowing samples to sediment for 5 min at room temperature, 0.05 mL of the upper layer was placed in a new tube for surface receptor labeling. Lymphocyte surface receptor antibodies (Caltag Laboratories, Burlingame, CA, U.S.A.), including CD19, IgA, IgG, and isotype controls, were conjugated with either FITC or phycoerythrin, and paired for appropriate two-color analysis. Surface labeling was performed by adding surface antibodies to each corresponding sample. After 20 min incubation at room temperature, samples were washed with PBS wash buffer, and the cell pellet was gently resuspended in 1 mL of PBS for flow cytometric analysis in an Epics XL flow cytometer (Beckman Coulter, Inc., Company, Fullerton, CA, U.S.A.).

**Serum ELISA.** For determination of mouse rotavirus-specific IgG and IgA, 96-well plates were coated overnight at 4°C with K224 (rabbit anti-human rotavirus, provided by Dr. Lennart Svensson, Swedish Institute for Infectious Disease Control, Stockholm, Sweden) diluted 1:1000 in coating buffer (1.59 g Na<sub>2</sub>CO<sub>3</sub>, 2.93 g NaHCO<sub>3</sub>, 1000 mL distilled H<sub>2</sub>O, pH 9.6). Plates were then back coated with 0.5% bovine albumin fraction V (BAFV, Invitrogen, Carlsbad, CA, U.S.A.) in PBS for 30 min at 37°C. After the plates were washed five times in washing buffer (0.9% NaCl + 0.05% Tween 20), bovine rotavirus (WC3,  $5 \times 10^6$  PFU/mL; provided by Dr. H. Fred Clark, Children's Hospital of Philadelphia, Philadelphia, PA, U.S.A.) diluted 1:200 (0.5% BAFV, 0.05% Tween 20 in PBS) was added, and incubated for 60 min at 37°C. After washing the plates, serum samples were added in serial two-fold dilutions (starting at 1:50 for IgG detection and at 1:8 for IgA). Positive and negative controls were present on every plate and all plates were incubated for 90 min at 37°C. After washing the plates, HRP-goat anti-mouse IgA ( $\alpha$ -chain specific, Southern Biotechnology Associates, Birmingham, AL, U.S.A.), diluted 1:1000, or HRP-goat anti-mouse IgG (H+L) (Bio-Rad, Hercules, CA, U.S.A.), diluted 1:3000 was added and incubated for 60 min at 37°C. Plates were then washed again. Tetramethylbenzidine (TMB) (One-Step Substrate System; DAKO, Carpinteria, CA, U.S.A.) was added at 100  $\mu$ L/well; plates were incubated for 5–10 min at room temperature for IgG, or for 30 min at 37°C for IgA. The reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> (50  $\mu$ L/well) and OD was determined at 450 nm on a Titertek Multiskan Plus MKII ELISA reader (Labsystem, Helsinki, Finland).

**Stool ELISA.** For detection of rotavirus-specific IgA in feces, the procedure entailed the same guidelines as for IgA in serum, with the exception that stool samples in serial two-fold dilutions starting at 1:4 were assayed.

**Detection of rotavirus antigen.** Group A rotavirus antigen was detected by a previously described ELISA (18). Guinea pig anti-human rotavirus serum was provided by Dr. Svensson. Goat anti-rabbit IgG (H+L)-HRP was purchased from Bio-Rad.

**Data analysis.** Descriptive statistics were compiled for all variables, and were stratified by treatment or days PI, as appropriate. The  $\chi^2$  test was used for comparing proportion of individual mice with diarrhea, by treatment, at 1, 3, and 5 d PI. SPSS (SPSS, Inc., Chicago, IL, U.S.A.) was used for analysis.

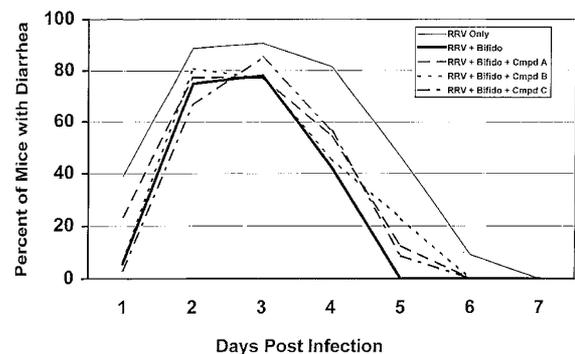
Figures were composed using Microsoft Graph (Microsoft Corp., Redmond, WA, U.S.A.) and SlideWrite Plus (Advanced Graphics Software, Carlsbad, CA, U.S.A.) software programs.

## RESULTS

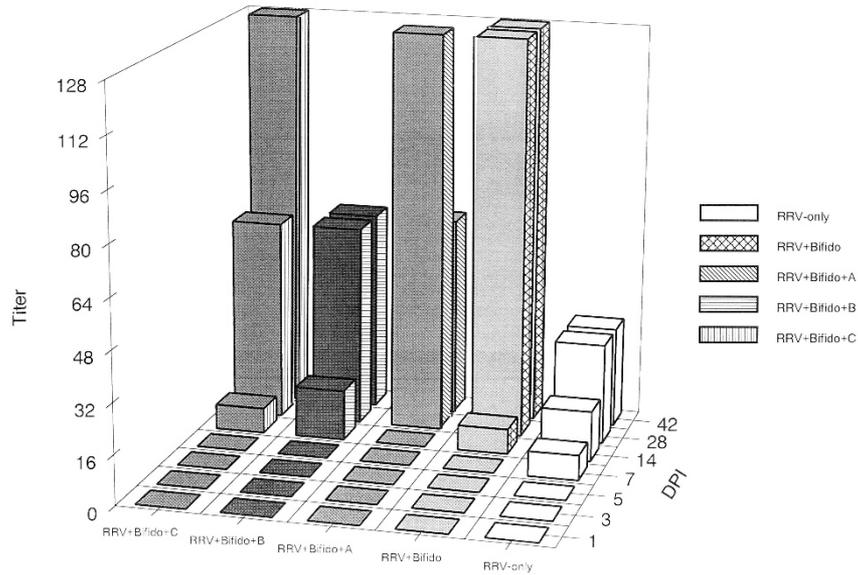
**Clinical outcome.** In total, 42 litters comprised the six experimental groups in the study, with an average of five pups per litter. Figure 1 shows the occurrence of diarrhea in mice challenged with RRV. None of the saline control mice experienced diarrhea during the course of the experiments and are not shown. In the group infected with RRV alone, 39% of pups had diarrhea at 1 d PI, 91% by d 3 PI, and 46% at d 5 PI. In contrast, only 6% of pups in the bifidobacteria-treated + RRV-infected litters had diarrhea at 1 d PI ( $p = 0.001$ ), 78% by d 3 PI ( $p = \text{NS}$ ), and none at d 5 PI ( $p < 0.001$ ). Diarrhea symptoms were also diminished in the experimental litters treated with both bifidobacteria and prebiotic compounds (compounds A, B, and C), but results were not substantially different from that observed in pups treated with bifidobacteria only.

**Serum rotavirus-specific IgA and IgG.** The ELISA results for serum rotavirus-specific IgA are summarized in Figure 2. No rotavirus-specific IgA was detected in the saline control group through the study period (not shown), or in bifidobacteria (with or without prebiotic) treatment groups from 1 to 7 d PI. However, in the bifidobacteria-treated + RRV-infected group, specific IgA titers rose dramatically after 28 d PI, with titers four-fold higher than the RRV-only group at 28 and 42 d PI. The RRV-only group maintained relatively low titers of serum rotavirus-specific IgA throughout the study period. Variable specific IgA responses were noted in the bifidobacteria with prebiotic compound treatment groups between 14 and 42 d PI.

Rotavirus-specific IgG responses (not shown) were fairly consistent in all RRV-infected groups (with or without treatment), with low IgG titers from 1 to 7 d PI, increasing to a titer of 800 by 14 d PI, and peaking at 6400 on d 42 PI.



**Figure 1.** The occurrence of diarrhea in RRV-infected-only Balb/c pups ( $n = 41$ ); bifidobacteria-treated + RRV-infected pups ( $n = 35$ ); bifidobacteria + compound A-treated + RRV-infected pups ( $n = 34$ ); bifidobacteria + compound B-treated + RRV-infected pups ( $n = 29$ ); bifidobacteria + compound C-treated + RRV-infected pups ( $n = 31$ ). Level of significance in bifidobacteria-treated + RRV-infected group vs RRV-infected only group:  $p = 0.001$  on d 1 PI,  $p = \text{NS}$  on d 3 PI,  $p < 0.001$  on d 5 PI. Sample sizes represent the number of mice per group at 1 D PI. Due to the sacrifice schedule, sample sizes decreased at later time points.



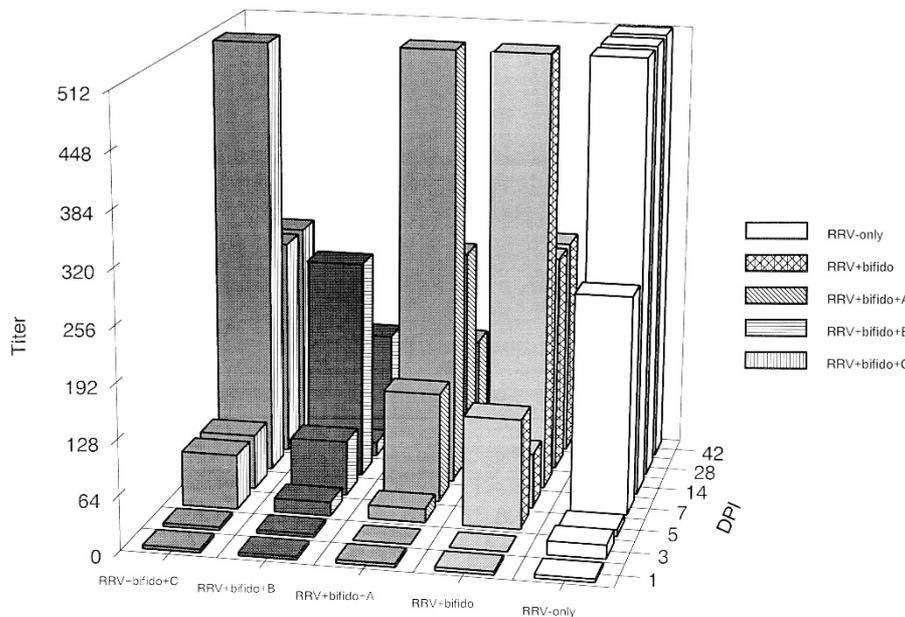
**Figure 2.** Rotavirus-specific IgA levels in serum in RRV-infected litters ( $n = 7$  for each group). A four-fold increase in specific-IgA response was observed in bifidobacteria-treated + RRV-infected group vs RRV-infected-only group at 28 and 42 d PI.

**Intestinal feces rotavirus-specific IgA.** As shown in Figure 3, a markedly elevated rotavirus-specific IgA response was observed in the bifidobacteria-treated + RRV-infected group as early as 5 d PI (titer = 128), which was noticeably higher than that in RRV-infected only mice (titer = 8). However, by 7 d PI, IgA titers rose in the RRV-infected alone group and remained high, showing the most consistently elevated pattern through the end of the follow-up period. The rotavirus-specific IgA levels in the bifidobacteria-treated groups, with or without prebiotic compounds, appeared to peak at 14 d PI and then decrease.

**RRV antigen in stool.** OD was compared across groups for each batch run of the ELISA. The RRV antigen could be

detected by 1 d PI in all infected groups and continued to increase gradually through d 7 PI. However, no consistent differences among the groups were noted. The exact amount of RRV antigen was not determined because the precise viral protein concentration of the positive control was not known.

**Bacteriology results.** Up to 100 colonies were counted per culture plate with counts over 100 being expressed as  $>1 \times 10^5$  CFU/mL, accounting for the dilution (1:1000) factor. *B. bifidum* and *B. infantis* were not routinely detected in samples from most of the experimental groups and only occasionally detected in samples from the bifidobacteria-treated + RRV-infected and bifidobacteria + compound B-treated + RRV-infected groups. There were no major differences in counts of



**Figure 3.** Rotavirus-specific IgA responses in stool in RRV-infected litters ( $n = 7$  for each group). A 16-fold increase in specific-IgA response was detected at the d 5 PI time point in bifidobacteria-treated + RRV-infected group vs RRV-infected-only group.

*Lactobacillus* spp. among the six groups, with all samples having relatively high counts ( $>1 \times 10^5$  CFU/mL) in most of the experiments. Similar culture results for *Enterobacteriaceae* were also noted among all treatment groups, but counts appeared slightly higher (on average) in the RRV-infected alone group compared with the other five groups. No statistical testing of the culture data were feasible because counts  $>1 \times 10^5$  CFU/mL were not further differentiated and only one pooled specimen per litter per time point was available.

**Flow cytometry results.** Because immune markers measured via flow cytometry were not rotavirus-specific and sample sizes were limited, only descriptive results are summarized. The RRV-infected controls did not appear to differ in nonspecific humoral immune responses from treated animals except that IgG-positive B cells dropped sharply at 14 d PI compared with all other groups. The bifidobacteria-treated + RRV-infected mice showed a steep decline in the percentage of total B cells and IgA- and IgG-positive B cells at 28 d PI, but had the highest levels of these markers of any group at 42 d PI. Bifidobacteria + prebiotic compound-treated + RRV-infected groups showed responses similar to the RRV-infected group, except that mice fed with compound B had relatively lower IgA- and IgG-positive B-cell percentages at 28 d PI.

## DISCUSSION

The experimental findings summarized in this report indicate that clinical diarrhea was significantly delayed in bifidobacteria-treated + RRV-infected mice compared with mice challenged with RRV only. In addition, complete convalescence was earlier (5 d PI) for the bifidobacteria-treated + RRV-infected mice compared with the RRV-only group (7 d PI) and the groups supplemented with prebiotic compounds (6 d PI). This finding supports the idea that bifidobacteria therapy is effective in altering the clinical course of rotavirus infectious gastroenteritis, and is consistent with previous animal and human studies (14–17).

The RRV antigen-shedding pattern in stool did not directly parallel the course of clinical diarrhea. Clinically, the diarrhea began to diminish after 4 d PI, with all mice having recovered by 7 d PI. In comparison, the viral shedding continued to increase gradually until 7 d PI, the last time point tested in all infected groups. This virus shedding pattern was different from that observed in a previous study with murine rotavirus (MRV) (17), in which the viral shedding in stool declined on d 7 PI in both bifidobacteria and nonbifidobacteria-treated, MRV-infected mice. One possible reason for this difference could be the fact that RRV has liver tropism (19). The RRV present in the stool during the late course of diarrhea may have been shed into the intestine via the liver (unpublished findings).

The observation of a shortened diarrhea phase has important implications in the treatment of rotavirus diarrhea in animal and human hosts. Although the exact mechanism is unclear, research data has shown that probiotics, including bifidobacteria, have certain properties that may relate to the observed effects in diarrhea disease. Firstly, bifidobacteria can adhere to the epithelial cells and mucosa, and, thus, may exclude or reduce rotavirus attachment and reproduction in mature intes-

tinal epithelial enterocytes. Secondly, some of the by-products of bifidobacteria, including hydrogen peroxide, may inactivate virus (6). In addition to the properties mentioned above, another study reported that lactobacilli can produce antipathogen adhesion substances, crude mixtures termed *biosurfactants*, whose inhibitory effect can extend to a wide range of virulent pathogens (20).

Although bifidobacteria were not routinely isolated in samples from treated mice in these experiments, we cannot summarize conclude that the organisms were not present in the gut, inasmuch as our previous research has shown that *B. bifidum* can successfully colonize the mouse intestine (17). In that study, 80% of minced small intestine samples from *B. bifidum*-treated mice were culture positive for *B. bifidum*, although the ingested strain was not isolated from the intestines of mice challenged with MRV alone or from saline control animals. In addition, when examined under electron microscopy, adherence of bifidobacteria to the small intestinal epithelium was observed in the treated pups. Depending on the sample source, the culture method may not offer optimal sensitivity, and alternative molecular detection methods are being evaluated in a separate study in our laboratories.

It has been shown that probiotics can act as adjuvants in immune responses that involve B and T lymphocytes, blood leukocytes, accessory cells, and mucosal epithelial cells (21). Studies have also shown that increased rotavirus-specific humoral immune responses can occur in children after ingestion of a probiotic formula (22). In the present study, rotavirus-specific IgA levels in serum were four-fold higher in the bifidobacteria-treated + RRV-infected mice on d 28 and 42 PI compared with the RRV-infected only mice. This finding is consistent with studies done in other laboratories with bifidobacteria and lactobacilli (23–25). Rotavirus-specific IgA plays a key role in the protection against rotavirus infection. Rotavirus-specific IgA in human serum can neutralize both unbound and cell-bound rotavirus (26, 27). In experimental animals, the presence of rotavirus-specific IgA correlates with reduced viral shedding when mice are rechallenged with rotavirus (28, 29).

In the present study, mice in the RRV-infected only group had a stronger and more protracted specific IgA response in stool compared with mice in the bifidobacteria-treated (with or without prebiotic compounds) groups. This could be the result of the fact that more severe and prolonged rotavirus infection occurred in the gut of mice that lacked the protection of bifidobacteria. However, at 5 d PI, the bifidobacteria-treated + RRV-infected group had a 16-fold higher specific IgA level in feces versus the RRV-infected-only mice. An earlier elevated specific IgA response in gut mucosa is more important in the protection against acute rotavirus infection than a delayed stronger and more protracted specific IgA response. Rotavirus-specific IgA in the gut may protect mucosal surfaces by inhibition of virus attachment, virus entry, and/or intracellular viral replication (30). It also can act directly against the rotavirus neutralization antigen, VP4, as well as neutralize the virus after transcytosis through epithelial cells and protect mice against diarrhea (31).

We also measured the percentages of total B cells and IgA- and IgG-positive B cells in PP using flow cytometry. No clear difference was observed between RRV-infected alone and bifidobacteria treatment groups. This finding was not totally unexpected inasmuch as nonspecific antibodies were measured. Further studies in which rotavirus-specific antibodies are measured should be undertaken.

In summary, the findings presented in this report suggest that bifidobacteria can shorten the duration of RRV-induced diarrhea in Balb/c mice, whereas addition of the selected prebiotics did not improve the results over bifidobacteria treatment alone. In addition, the rotavirus-specific immune response was not enhanced when mice were treated with these prebiotic compounds compared with the treatment with bifidobacteria alone. Consistent with other investigations (22–25), bifidobacteria may have acted as an adjuvant in the rotavirus-specific immune response by modulating a stronger serum-specific IgA response and triggering an early strong specific IgA response in the gut. The early specific mucosal response, in addition to the antipathogenic properties of bifidobacteria mentioned above, may have contributed to shortening the course of diarrhea caused by rotavirus.

More studies seeking to understand the mechanisms behind the differences noted among the experimental groups regarding the specific IgA response in stool *versus* serum are needed, particularly the clarification of their roles in rotavirus reinfection.

The prebiotic compounds chosen for this study did not show any synergistic effect with bifidobacteria, either in shortening diarrhea or enhancing the immune response. However, the compound doses, which were estimated from human trials, may not have been optimal for the murine model. Currently, more than 100 neutral and acid-containing oligosaccharides have been isolated and identified, providing many other possible candidates for studies in animal models (32). Adjusting the dosage and frequency of administration, as well as testing other combinations of prebiotics, are areas that warrant further investigation.

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