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Host Response to Probiotics Determined by Nutritional Status of Rotavirus-infected Neonatal Mice

Geoffrey A. Preidis^{*}, Delphine M. Saulnier[‡], Sarah E. Blutt[§], Toni-Ann Mistretta[‡], Kevin P. Riehle^{||}, Angela M. Major[†], Susan F. Venable[‡], James P. Barrish[†], Milton J. Finegold[‡], Joseph F. Petrosino^{§,¶}, Richard L. Guerrant[#], Margaret E. Conner[§], and James Versalovic[†]

^{*}Interdepartmental Program in Translational Biology and Molecular Medicine, Baylor College of Medicine, Houston, TX

[‡]the Department of Pathology, Texas Children's Hospital, Houston, TX

[‡]the Department of Pathology & Immunology, Houston, TX

[§]the Department of Molecular Virology and Microbiology, Houston, TX

^{||}the Bioinformatics Research Laboratory, Baylor College of Medicine, Houston, TX

[¶]the Human Genome Sequencing Center, Houston, TX

[#]the Center for Global Health, Division of Infectious Diseases and International Health, University of Virginia School of Medicine, Charlottesville, VA

Abstract

Objectives—Beneficial microbes and probiotics are promising agents for the prevention and treatment of enteric and diarrheal diseases in children; however, little is known about their in vivo mechanisms of action. We used a neonatal mouse model of rotavirus diarrhea to gain insight into how probiotics ameliorate acute gastroenteritis.

Methods—Rotavirus-infected mice were treated with 1 of 2 strains of human-derived *Lactobacillus reuteri*. We assessed intestinal microbiome composition with 16S metagenomic sequencing, enterocyte migration and proliferation with 5-bromo-2'-deoxyuridine, and antibody and cytokine concentrations with multiplex analyses of intestinal explant cultures.

Results—Probiotics reduced diarrhea duration, improved intestinal histopathology, and enhanced intestinal microbiome richness and phylogenetic diversity. The magnitude of reduction of diarrhea by probiotics was strain specific and influenced by nutritional status. *L reuteri* DSM 17938 reduced diarrhea duration by 0, 1, and 2 days in underweight, normal weight, and

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Address correspondence and reprint requests to James Versalovic, MD, PhD, Texas Children's Hospital, Feigin Center, Suite 830, 1102 Bates Ave, Houston, TX 77030, jamesv@bcm.edu.

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overweight pups, respectively. The magnitude of reduction of diarrhea duration correlated with increased enterocyte proliferation and migration. Strain ATCC PTA 6475 reduced diarrhea duration by 1 day in all of the mice without increasing enterocyte proliferation. Both probiotic strains decreased concentrations of proinflammatory cytokines, including macrophage inflammatory protein-1 α and interleukin-1 β , in all of the animals, and increased rotavirus-specific antibodies in all but the underweight animals. Body weight also influenced the host response to rotavirus, in terms of diarrhea duration, enterocyte turnover, and antibody production.

Conclusions—These data suggest that probiotic enhancement of enterocyte proliferation, villus repopulation, and virus-specific antibodies may contribute to diarrhea resolution, and that nutritional status influences the host response to both beneficial microbes and pathogens.

Keywords

epithelial cells; gastroenteritis; immunity; metagenome; mucosal; probiotics

Enteric and diarrheal diseases are responsible for occupying 50% of hospital beds in developing countries (1), for at least 15% of childhood deaths worldwide (2), and for predisposing children to long-term developmental deficits in immune function, fitness, and cognition (3). Present global health strategies to address enteric and diarrheal diseases include provision of potable water and sanitation, antimicrobial therapy, and enteric vaccines; however, these interventions are expensive and fraught with technical challenges. For example, in regions of the world where mortality is highest, new rotavirus vaccines are demonstrating < 50% efficacy (4–6). New low-cost solutions are needed to reduce the global burden of enteric and diarrheal diseases.

Among the more promising new therapeutic strategies are probiotics, live microorganisms that confer a health benefit on the host by altering the intestinal microbial balance (7,8). Multiple randomized, placebo-controlled prospective trials with probiotics report modest benefits in patients with acute gastroenteritis (9–12); however, remarkable probiotic species and strain diversity, along with an insufficient understanding of mechanisms underlying probiosis, prevents selection of optimal therapeutic microbes for infectious diarrhea (13). Thus, it is unclear whether probiotics are ready for integration into routine clinical practice (14,15). Nonetheless, due to the low cost, ease of distribution and administration, and favorable safety profiles, probiotics-based therapies have the potential to play a critical role in comprehensive global health strategies (13).

Among the most-studied probiotics for acute gastroenteritis is *Lactobacillus reuteri*, a commensal bacterium found in the lower gastrointestinal tracts of humans and mice (16). For children hospitalized with acute rotaviral gastroenteritis, *L reuteri* alone or coadministered with *Lactobacillus rhamnosus* reduces the duration of illness by 1 day (17,18). It is unclear how *L reuteri* mediates recovery from diarrhea, or whether this effect can be enhanced. Others have shown that immunomodulation could contribute to the resolution of acute rotaviral gastroenteritis by *L reuteri* in piglets (19–21). Furthermore, we recently found that 2 genotypically and phenotypically distinct strains of *L reuteri*, DSM 17938 and ATCC PTA 6475, known to be safe and effective in treating infantile colic (22,23), increase the rate of intestinal epithelial cell turnover and enhance diversity of the

intestinal microbiome in uninfected mouse pups (24). Therefore, we sought to determine whether any of these mechanisms contribute to the resolution of disease by *L reuteri* strains 17938 and 6475 in a mouse model of acute rotaviral gastroenteritis. We also explored whether these mechanisms of probiosis are relevant to the undernourished host, which suffers a disproportional burden of global enteric and diarrheal diseases of childhood.

Methods

Probiotic Strains and Preparation

Human-derived *L reuteri* strains DSM 17938 and ATCC PTA 6475 (Biogaia AB, Stockholm, Sweden) were grown daily in anaerobic conditions to stationary phase in deMan, Rogosa, Sharpe medium (Difco Laboratories, Detroit, MI), washed 3 times with sterile phosphate-buffered saline (PBS) to remove media, and diluted to a concentration of 2×10^9 cfu/mL in PBS.

Mouse Nutritional States and Rotavirus Infection

Four-day-old CD-1 mice (Charles River Laboratories, Kingston, NY) were pooled and randomly assigned, 10 pups per dam, to the following groups. Malnutrition (“underweight” pups) was induced by separating 5 pups per litter for defined periods (25), increasing to 12 hours/day at 7 days of life and each day thereafter. “Overweight” mice were the 5 littermates of underweight mice that remained with dams to feed all times. “Normal weight” pups were maintained in litters of 10 without separation. Mice received gastric gavages (50 μ L) of probiotics or vehicle daily from days 5 to 14 of life. Rotavirus strain EC_{WT} (1×10^3 ID₅₀) or vehicle was given by gavage on day 8, preceding probiotics by 8 hours on that day. Diarrhea, defined as gold-colored stools that were at least twice the normal volume and >50% liquid, was assessed by an observer blinded to treatment group. All of the protocols were approved by the Baylor College of Medicine institutional animal care and use committee.

16S rRNA Sequence-based Survey of the Distal Gut Microbiome

Distal microbial community profiling was performed as previously described (24). Briefly, genomic DNA was isolated from stool pooled from twenty 11-day-old pups per group, yielding an average of 12.4 mg of stool and 58.9 μ g DNA per group. The V1-V3 and V3-V5 regions of the 16S rRNA gene were amplified by high-fidelity polymerase chain reaction and sequenced in the Genome Sequencer FLX platform (Roche/454 Life Sciences, Branford, CT) at the Human Genome Sequencing Center, Houston, TX. A mean of 20,915 reads per sample (average read length 498 nucleotides) were taxonomically binned by RDP Classifier (Ribosomal Database Project, East Lansing, MI) (26). Species richness, defined as the total number of operational taxonomic units (OTUs), detected in a given sample, Pielou index of community evenness, or the relative abundance of each OTU in the community, and Simpson phylogenetic diversity index, which takes into account both species richness and community evenness, were calculated using the Vegan package of R Statistical Programming (<http://www.r-project.org>).

Histology and BrdU Immunohistochemistry

For histology, 3 mm of distal ileum was fixed in Trump solution, sectioned at 0.5 μ m, and stained with toluidine blue/basic fuchsin. For in vivo labeling studies, 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich, St Louis, MO) was injected intraperitoneally (30mg/kg body weight in 50- μ L PBS) (27) during infection on day 8. Intestines harvested between 4 hours and 4 days postinjection were fixed in 10% formalin, sectioned at 3 μ m, labeled with rat anti-BrdU (1:200; Accurate Chemical, Westbury, NY), and counter-stained with hematoxylin. In 10 well-oriented crypt-villus units, locations of the most superficial and least-progressed BrdU-labeled enterocytes were recorded as the percent of epithelial cell positions from the crypt-villus boundary (0%) to the villus tip (100%). Proliferation was defined as the percent of crypt cells that had incorporated BrdU within 4 hours of BrdU injection. Crypt and villus height were recorded using NIS Elements (Nikon Instruments, Melville, NY). Imaging was performed with an Eclipse 90i microscope (Nikon Instruments).

Ex Vivo Organ Fragment Cultures and Multiplex Assays

Terminal 2 cm of ileum were suspended in individual wells of a 96-well plate with 150 μ L of culture medium consisting of Ex-Cell Hybri-Max Medium (SAFC Biosciences, Lenexa, KS) supplemented with 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% fetal bovine serum, 50 μ g/mL gentamicin, and 1% antibiotic/antimycotic (GIBCO, Carlsbad, CA) (28). Cultures were incubated at 37°C in 95% humidity and 5% CO₂. After 72 hours, media were removed and stored at -20°C until analysis. Samples were evaluated by enzyme-linked immunosorbent assay for total rotavirus-specific antibody (25), using plates coated with reactive guinea pig serum and rotavirus SA11-C13 with goat anti-mouse Ig (Sigma-Aldrich). Infected mouse serum of known titer was used as a positive control. Samples with OD₄₅₀ 0.100 were considered positive. For multiplex analysis, a subset of samples was screened with Mouse Immunoglobulin Isotyping kits and Mouse Cytokine/Chemokine-Premixed 22-plex kits (Millipore, Billerica, MA) in a Luminex 100 system (Luminex Corporation, Austin, TX). Analytes at concentrations exceeding the minimum detectable dose were evaluated in all of the samples. Raw data were obtained with MasterPlex CT version 1.2.0.7 and analyzed with MasterPlex QT version 4.1.5.52 (Hitachi MiraiBio, San Francisco, CA).

Statistical Analysis

Diarrhea duration was recorded as whole-integer days; thus, data were reported as median + interquartile range (IQR), and statistical comparisons were made between the 3 treatment groups with Kruskal-Wallis tests. Sample size was estimated to be 35 mice per group, using previously published methods (29) with a minimum effect size of 1 day. Sample sizes for all other experiments were derived from 1-way analysis of variance calculations based upon the null hypothesis that the means of all of the 3 treatment groups are equal, significance levels are 0.05, and with effect sizes based on data from individual pilot experiments. Antibody and cytokine concentrations were reported as mean + standard deviation (SD) and compared by 1-way analysis of variance, with Tukey multiple comparison tests to detect between-group differences. Pearson chi square test followed by post-hoc pairwise chi square tests with α adjusted via Bonferroni correction determined differences between percentages of

mice with antibodies. For multiplex analyses, α was adjusted using Bonferroni correction for multiple comparisons; only P values $< \alpha$ were considered significant. All of the analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA).

Results

Probiotics Attenuated Rotavirus Diarrhea, Histopathology, and Changes in the Intestinal Microbiome

We first sought to determine whether *L reuteri* modulates disease in rotavirus-infected outbred CD-1 neonatal mice. Daily treatment with either of 2 strains of *L reuteri* reduced the median duration of diarrhea by 1 day compared with vehicle-treated mice ($P < 0.01$, Fig. 1A). In the ileum, where rotavirus pathology is most pronounced (30), infected mice demonstrated swollen villus tips and large supranuclear enterocyte vacuoles; however, treatment with *L reuteri* led to less swollen villi with fewer and smaller vacuoles at 4 days postinfection (Fig. 1B).

Probiotics are thought to function by improving the host's intestinal microbial balance (8). Thus, we examined distal intestinal microbial community composition before and 24 hours after gavage using 16S metagenomic sequencing (Fig. 1C–G). Compared with stools from age-matched uninfected pups, rotavirus-infected stools demonstrated decreased species richness and phylogenetic diversity; however, just 24 hours after a single gavage, probiotics increased species richness, community evenness, and phylogenetic diversity by as much as 63%, 40%, and 163%, respectively, compared with vehicle. These differences were observed despite *L reuteri* representing $<1\%$ of OTUs in samples obtained from treated mice. Thus, *L reuteri* resolved diarrhea, ameliorated histopathology, and replenished intestinal microbiome diversity after rotavirus infection.

Nutritional Status–influenced Probiotic Effects on Diarrhea and the Intestinal Epithelium

Rapid turnover of the intestinal epithelium, a host defense against invasive pathogens (27,31,32), was identified as a potential probiotic effect of *L reuteri* (24). We hypothesized that probiotics would fail to resolve diarrhea in undernourished animals, which demonstrate impaired intestinal epithelial cell turnover (33,34). Compared with vehicle-treated mice, *L reuteri* 17938 reduced diarrhea duration in overweight mice by 2 days ($P < 0.001$) and in normal-weight mice by 1 day ($P < 0.001$), but failed to reduce disease duration in underweight mice. In contrast, strain 6475 reduced diarrhea duration by 1 day, regardless of nutritional status ($P < 0.05$; Fig. 2A). Daily treatment with probiotics did not affect body weight (data not shown).

To determine whether the magnitude of diarrhea reduction by *L reuteri* 17938 reflects different rates of intestinal epithelial cell turnover, we performed in vivo labeling studies with BrdU. Strain 17938 increased enterocyte proliferation from 43% to 51% in overweight mice ($P < 0.05$) and from 35% to 42% in normal weight mice ($P < 0.05$; Fig. 2B), mirroring increases in crypt height and enterocyte migration (Fig. 2C, Supplemental Fig. 1 [<http://links.lww.com/MPG/A95>]). Overweight mice receiving strain 17938 had the fewest BrdU-labeled enterocytes remaining in villus tips 4 days after infection. Strain 6475 increased

migration only in normal-weight mice, and did so without enhancing proliferation. Both probiotic strains failed to increase proliferation or migration in underweight mice (Supplemental Figs. 2–4 [<http://links.lww.com/MPG/A96>, <http://links.lww.com/MPG/A97>, <http://links.lww.com/MPG/A98>). Thus, effects of *L reuteri* on the rotavirus-infected intestinal epithelium are determined by both probiotic strain and nutritional status of the host.

Probiotics Enhanced Intestinal Rotavirus-specific Antibodies and Suppressed Cytokines

In humans and animals, the primary correlate of recovery from rotavirus is virus-specific antibody (35). To determine whether *L reuteri* augments this host response, we measured antibody titers in ileum fragment cultures. Both *L reuteri* strains increased mucosal rotavirus-specific antibodies in overweight and normal weight mice 6 days postinfection, the first day that virus-specific antibodies were detectable (Fig. 3A). This effect was absent in underweight pups. Others have shown that in rotavirus-infected gnotobiotic piglets, *L reuteri* increases total small intestinal IgM and IgG (20). To determine whether *L reuteri* enhances total antibody production in rotavirus-infected mice, we analyzed intestinal explant cultures with a multiplex liquid bead array platform. Treatment with strain 6475 increased immunoglobulin (Ig)-G1 5-fold in overweight mice ($P < 0.05$); however, probiotics did not significantly elevate IgA, which makes up the majority of mucosal rotavirus-specific antibody (36), IgM, or IgG2 (Supplemental Fig. 5, <http://links.lww.com/MPG/A99>). Thus, probiotic ingestion increased rotavirus-specific antibody production in all but underweight mice.

Proinflammatory cytokines are thought to play a role in rotavirus pathogenesis (37). To determine whether probiotics modulated mucosal cytokine concentrations, we used multiplex analysis to quantify 11 detectable proteins. Treatment with either strain of *L reuteri* suppressed multiple proinflammatory cytokines, regardless of nutritional state. Most strongly reduced were macrophage inflammatory protein-1 α (Fig. 3B) and interleukin (IL)-1 β (Fig. 3C). Probiotic suppression of IL-7, IL-10, IL-12, and interferon- γ were also observed (Supplemental Fig. 6, <http://links.lww.com/MPG/A100>), but mucosal quantities of G-CSF, IL-1 α , IL-4, IL-9, and RANTES were not affected by probiotics (Supplemental Fig. 7, <http://links.lww.com/MPG/A101>). Thus, *L reuteri* conferred an anti-inflammatory effect on the mucosa that involved multiple cytokines and was independent of host nutritional status.

Nutritional Status–influenced Host Response to Rotavirus

These studies were initiated to gain insights into mechanisms of probiosis and to determine whether probiotic effects in acute gastroenteritis are influenced by the nutritional status of the host; however, our data also reveal that manipulation of body weight (Fig. 4A) yielded different host responses to rotavirus. Underweight mice had 1 less day of diarrhea than normal-weight or overweight pups (Fig. 4B), but were unable to produce a detectable rotavirus-specific antibody response (Fig. 4C). In overweight mice, enterocytes migrated to villus tips more rapidly (Fig. 4D and Supplemental Fig. 8, <http://links.lww.com/MPG/A103>), and crypts contained increased mitotic activity (Fig. 4E), compared with normal

weight and underweight mice. Thus, host nutrition influences physiologic responses to both beneficial microbes and enteric pathogens.

Discussion

Mechanisms underlying probiosis in diarrheal diseases are poorly understood, but could include hastening repopulation of enterocytes in the intestinal epithelium, altering the structure of intestinal microbial communities, and modulating mucosal immunity (24,38). To determine whether these mechanisms contribute to attenuation of diarrhea, we administered 1 of 2 strains of *L reuteri* to rotavirus-infected neonatal mice. Reduction of diarrhea duration by strain DSM 17938 mirrored probiotic enhancement of crypt proliferation and enterocyte migration in mice and depended on body weight. On the other hand, strain ATCC PTA 6475 reduced diarrhea duration by 1 day in all of the 3 nutritional states without affecting enterocyte proliferation. Both probiotic strains increased mucosal virus-specific antibody in overweight and normal weight mice, conferred an anti-inflammatory effect on the small bowel, and enhanced the phylogenetic diversity of the intestinal microbiome. Intriguingly, nutritional status influenced the response to probiotics and rotavirus. These findings highlight that probiotic strain and host nutritional status should be carefully considered when selecting therapeutic microbes for clinical use.

With *L reuteri* 17938, the magnitude of disease reduction mirrored enhancement of enterocyte proliferation. Underweight mice did not respond to this strain in terms of cell proliferation, crypt height, enterocyte migration, or diarrhea duration. Thus, increased epithelial cell turnover could contribute to resolution of diarrhea. Epithelial cell turnover is a defense mechanism against invasive viruses (27), bacteria (31), and parasites (32), serving to expel pathogens and infected cells from the epithelium. In uninfected mice, *L reuteri* 17938 increases both enterocyte migration and proliferation (24), whereas rotavirus increases enterocyte migration without affecting proliferation (27). Thus, probiotics and rotavirus both increase intestinal epithelial cell turnover, but likely through different mechanisms. Future studies should identify signaling pathways responsible for the epithelial cell responses to both beneficial microbes and pathogens, and should explore other potential probiotic mechanisms of resolving diarrheal disease, such as accelerating the maturation of enterocytes along the crypt-villus axis (39).

Two strains of *L reuteri* differentially modulated diarrhea duration, crypt proliferation, and enterocyte migration, illustrating that genotypic and phenotypic heterogeneity of probiotic species complicates the selection of therapeutic microbes. For example, different lactobacilli induce different gene regulatory networks and signaling pathways in the small bowel mucosa (40). In our studies, strain 6475 (but not strain 17938) reduced median diarrhea duration by 50% in underweight pups, which lacked mucosal virus-specific antibody and the ability to more rapidly replace villus enterocytes. The enhanced ability of strain 6475 to affect the richness of the gut microbiome raises the possibility that effects on the microbiome may be important markers for disease recovery in undernourished children. An important avenue of future research will be discovery of novel probiotic properties that function in states of nutrient deprivation.

Both probiotic strains strongly suppressed multiple proinflammatory cytokines while elevating mucosal titers of rotavirus-specific antibodies. These immunomodulatory data extend findings from rotavirus-infected gnotobiotic pigs, in which treatment with *L reuteri* and *Lactobacillus acidophilus* recruits monocytoïd cells to the mucosal compartment (19), increases nonspecific antibody titers (20), and enhances rotavirus-mediated Toll-like receptor responses (21). Furthermore, we found that probiotic treatment strongly suppresses mucosal concentrations of antibodies in uninfected neonatal CD1 mice (unpublished data). Thus, probiotic antibody enhancement could require simultaneous activation of multiple signaling pathways, such as Toll-like receptor activation by rotavirus (41) along with stimulation of NF- κ B by *L reuteri* (42). Beneficial bacteria may stimulate enterocytes, dendritic cells, or macrophages to produce B-cell stimulatory factors such as APRIL or transforming growth factor- β 1 (43,44), or may increase the affinity or quantities of the polymeric Ig receptor, which transports IgA from the lamina propria into the intestinal lumen (45,46). Whether the modest early increase in rotavirus-specific antibody contributes to amelioration of rotaviral disease by *L reuteri* is unknown; however, antibodies are not essential for probiotic disease attenuation, given that strain 6475 ameliorated diarrhea in underweight mice without enhancing antibody production. Likewise, cytokine suppression does not appear to play an essential role, given that *L reuteri* 17938 reduced cytokine levels in underweight mice without attenuating diarrhea.

Rotavirus decreased microbial community richness and phylogenetic diversity in the neonatal mouse intestine, and probiotics restored diversity with a single gavage during the peak of disease. Perturbations of the human intestinal microbiome are speculated to play a role in rotavirus pathogenesis (47), although it is not known whether microbial population changes contribute to or result from the disease process; however, increased intestinal microbiome diversity appears to be protective against other gastrointestinal diseases (48,49). Probiotics may facilitate expansion of other groups of organisms by providing substrates selectively used by commensal bacteria (50), or they may alter the neonatal intestinal niche to promote colonization (or recolonization) by microbes from the environment. Stabilization of the microbiome by *L reuteri* was observed just 24 hours after treatment, highlighting the vast potential for probiotics to quickly remodel intestinal microbial communities. Interestingly, the magnitude of increased microbial diversity was much greater than recently reported in healthy, uninfected CD-1 mouse pups (24). It is possible that the effects on host physiology may not be specific to *L reuteri*, but could be due to an indirect effect of altered quantities or functions of other microbes in the intestine. Future studies could determine whether microbiome changes are equally profound in underweight mice, for which probiotic ingestion had only minimal effects on host physiology.

Nutritional status alone affected diarrhea duration, with underweight mice demonstrating 1 less day of diarrhea than their larger siblings. These data conflict with studies reporting increased severity and duration of rotavirus diarrhea in neonatal mice (51–54). Contrasting results could be attributed to the differences in mouse and virus strain, infectious dose, or method of inducing malnutrition. Rotavirus-specific antibody was not detected in underweight pups; further study of this malnutrition model may lend insight into why so many undernourished children in developing countries fail to produce sufficient antibody

responses to live oral vaccines (4–6). Importantly, however, just as various strains of *L reuteri* and other commensal-derived probiotics evolved over time to perform specific functions in specific vertebrate hosts (55), these observations should be considered valid only in mice. Additional studies will be needed to determine whether human-derived *L reuteri* has similar physiologic effects in humans. Nonetheless, underweight mice could serve as a basic model to separate the effects of simple energy deficiency from the complex syndrome of environmental enteropathy (56).

Together, our findings reveal that probiotics can alter host physiology, microbiome composition, and disease course, and that the effectiveness of probiotics for enteric and diarrheal diseases is dependent on both probiotic strain and host nutritional status. This knowledge will guide future explorations of host-microbial interactions and seek to uncover new mechanisms of pathogenesis and probiosis for enteric and diarrheal diseases, even in undernourished hosts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. United Nations Development Programme (UNDP). Human Development Report. New York: UNDP; 2006.
2. Black RE, Cousens S, Johnson HL, et al. Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet*. 2010; 375:1969–87. [PubMed: 20466419]
3. Guerrant RL, Oria RB, Moore SR, et al. Malnutrition as an enteric infectious disease with long-term effects on child development. *Nutr Rev*. 2008; 66:487–505. [PubMed: 18752473]
4. Madhi SA, Cunliffe NA, Steele D, et al. Effect of human rotavirus vaccine on severe diarrhea in African infants. *N Engl J Med*. 2010; 362:289–98. [PubMed: 20107214]
5. Zaman K, Anh DD, Victor JC, et al. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in Asia: a randomised, doubleblind, placebo-controlled trial. *Lancet*. 2010; 376:615–23. [PubMed: 20692031]
6. Armah GE, Sow SO, Breiman RF, et al. Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: a randomised, double-blind, placebo-controlled trial. *Lancet*. 2010; 376:606–14. [PubMed: 20692030]
7. Food and Agricultural Organization of the United Nations (FAO)/World Health Organization (WHO). Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. Basel, Switzerland: FAO/WHO; 2001. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.
8. Fuller R. Probiotics in man and animals. *J Appl Bacteriol*. 1989; 66:365–78. [PubMed: 2666378]
9. Szajewska H, Mrukowicz JZ. Probiotics in the treatment and prevention of acute infectious diarrhea in infants and children: a systematic review of published randomized, doubleblind, placebo-controlled trials. *J Pediatr Gastroenterol Nutr*. 2001; 33(suppl 2):S17–25. [PubMed: 11698781]

10. Allen SJ, Okoko B, Martinez E, et al. Probiotics for treating infectious diarrhoea. *Cochrane Database Syst Rev*. 2004;CD003048. [PubMed: 15106189]
11. Huang JS, Bousvaros A, Lee JW, et al. Efficacy of probiotic use in acute diarrhea in children: a meta-analysis. *Dig Dis Sci*. 2002; 47:2625–34. [PubMed: 12452406]
12. Sazawal S, Hiremath G, Dhingra U, et al. Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. *Lancet Infect Dis*. 2006; 6:374–82. [PubMed: 16728323]
13. Preidis GA, Hill C, Guerrant RL, et al. Probiotics, enteric and diarrheal diseases, and global health. *Gastroenterology*. 2011; 140:8–14. [PubMed: 21075108]
14. Tarnow-Mordi WO, Wilkinson D, Trivedi A, et al. Probiotics reduce all-cause mortality and necrotizing enterocolitis: it is time to change practice. *Pediatrics*. 2010; 125:1068–70. [PubMed: 20403934]
15. Soll RF. Probiotics: are we ready for routine use? *Pediatrics*. 2010; 125:1071–2. [PubMed: 20421256]
16. Reuter G. The *Lactobacillus* and *Bifidobacterium* microflora of the human intestine: composition and succession. *Curr Issues Intest Microbiol*. 2001; 2:43–53. [PubMed: 11721280]
17. Shornikova AV, Casas IA, Mykkanen H, et al. Bacteriotherapy with *Lactobacillus reuteri* in rotavirus gastroenteritis. *Pediatr Infect Dis J*. 1997; 16:1103–7. [PubMed: 9427453]
18. Rosenfeldt V, Michaelsen KF, Jakobsen M, et al. Effect of probiotic *Lactobacillus* strains in young children hospitalized with acute diarrhea. *Pediatr Infect Dis J*. 2002; 21:411–6. [PubMed: 12150178]
19. Zhang W, Wen K, Azevedo MS, et al. Lactic acid bacterial colonization and human rotavirus infection influence distribution and frequencies of monocytes/macrophages and dendritic cells in neonatal gnotobiotic pigs. *Vet Immunol Immunopathol*. 2008; 121:222–31. [PubMed: 18006076]
20. Zhang W, Azevedo MS, Gonzalez AM, et al. Influence of probiotic Lactobacilli colonization on neonatal B cell responses in a gnotobiotic pig model of human rotavirus infection and disease. *Vet Immunol Immunopathol*. 2008; 122:175–81. [PubMed: 18023882]
21. Wen K, Azevedo MS, Gonzalez A, et al. Toll-like receptor and innate cytokine responses induced by lactobacilli colonization and human rotavirus infection in gnotobiotic pigs. *Vet Immunol Immunopathol*. 2009; 127:304–15. [PubMed: 19054578]
22. Savino F, Pelle E, Palumeri E, et al. *Lactobacillus reuteri* (American Type Culture Collection Strain 55730) versus simethicone in the treatment of infantile colic: a prospective randomized study. *Pediatrics*. 2007; 119:e124–30. [PubMed: 17200238]
23. Savino F, Cordisco L, Tarasco V, et al. *Lactobacillus reuteri* DSM 17938 in infantile colic: a randomized, double-blind, placebo-controlled trial. *Pediatrics*. 2010; 126:e526–33. [PubMed: 20713478]
24. Preidis GA, Saulnier DM, Blutt SE, et al. Probiotics stimulate enterocyte migration and microbial diversity in the neonatal mouse intestine. *FASEB J*. 2012; 26:1960–9. [PubMed: 22267340]
25. Coutinho BP, Oria RB, Vieira CM, et al. *Cryptosporidium* infection causes undernutrition and, conversely, weanling undernutrition intensifies infection. *J Parasitol*. 2008; 94:1225–32. [PubMed: 18576767]
26. Cole JR, Wang Q, Cardenas E, et al. The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res*. 2009; 37:D141–5. [PubMed: 19004872]
27. Boshuizen JA, Reimerink JH, Korteland-van Male AM, et al. Changes in small intestinal homeostasis, morphology, and gene expression during rotavirus infection of infant mice. *J Virol*. 2003; 77:13005–16. [PubMed: 14645557]
28. Khoury CA, Brown KA, Kim JE, et al. Rotavirus-specific intestinal immune response in mice assessed by enzyme-linked immunospot assay and intestinal fragment culture. *Clin Diagn Lab Immunol*. 1994; 1:722–8. [PubMed: 8556527]
29. Fan C, Zhang D, Zhang CH. On sample size of the Kruskal-Wallis test with application to a mouse peritoneal cavity study. *Biometrics*. 2011; 67:213–24. [PubMed: 20345499]
30. Conner, ME.; Ramig, RF. Enteric viruses. In: Nathanson, N., editor. *Viral Pathogenesis*. Philadelphia: Lippincott-Raven; 1997. p. 713-43.

31. Mulvey MA, Schilling JD, Martinez JJ, et al. Bad bugs and beleaguered bladders: interplay between uropathogenic *Escherichia coli* and innate host defenses. *Proc Natl Acad Sci U S A*. 2000; 97:8829–35. [PubMed: 10922042]
32. Cliffe LJ, Humphreys NE, Lane TE, et al. Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science*. 2005; 308:1463–5. [PubMed: 15933199]
33. Clarke RM. The effect of growth and of fasting on the number of villi and crypts in the small intestine of the albino rat. *J Anat*. 1972; 112:27–33. [PubMed: 5086211]
34. Aldewachi HS, Wright NA, Appleton DR, et al. The effect of starvation and refeeding on cell population kinetics in the rat small bowel mucosa. *J Anat*. 1975; 119:105–21. [PubMed: 1133081]
35. Franco MA, Angel J, Greenberg HB. Immunity and correlates of protection for rotavirus vaccines. *Vaccine*. 2006; 24:2718–31. [PubMed: 16446014]
36. Burns JW, Krishnaney AA, Vo PT, et al. Analyses of homologous rotavirus infection in the mouse model. *Virology*. 1995; 207:143–53. [PubMed: 7871723]
37. Casola A, Estes MK, Crawford SE, et al. Rotavirus infection of cultured intestinal epithelial cells induces secretion of CXC and CC chemokines. *Gastroenterology*. 1998; 114:947–55. [PubMed: 9558283]
38. Preidis GA, Versalovic J. Targeting the human microbiome with antibiotics, probiotics, and prebiotics: gastroenterology enters the metagenomics era. *Gastroenterology*. 2009; 136:2015–31. [PubMed: 19462507]
39. Mariadason JM, Nicholas C, L'Italien KE, et al. Gene expression profiling of intestinal epithelial cell maturation along the crypt-villus axis. *Gastroenterology*. 2005; 128:1081–8. [PubMed: 15825089]
40. van Baarlen P, Troost FJ, van Hemert S, et al. Differential NF-kappaB pathways induction by *Lactobacillus plantarum* in the duodenum of healthy humans correlating with immune tolerance. *Proc Natl Acad Sci U S A*. 2009; 106:2371–6. [PubMed: 19190178]
41. Blutt SE, Crawford SE, Warfield KL, et al. The VP7 outer capsid protein of rotavirus induces polyclonal B-cell activation. *J Virol*. 2004; 78:6974–81. [PubMed: 15194774]
42. Iyer C, Kusters A, Sethi G, et al. Probiotic *Lactobacillus reuteri* promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF-kappaB and MAPK signalling. *Cell Microbiol*. 2008; 10:1442–52. [PubMed: 18331465]
43. He B, Xu W, Santini PA, et al. Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. *Immunity*. 2007; 26:812–26. [PubMed: 17570691]
44. Massacand JC, Kaiser P, Ernst B, et al. Intestinal bacteria condition dendritic cells to promote IgA production. *PLoS One*. 2008; 3:e2588. [PubMed: 18596964]
45. Norderhaug IN, Johansen FE, Schjervén H, et al. Regulation of the formation and external transport of secretory immunoglobulins. *Crit Rev Immunol*. 1999; 19:481–508. [PubMed: 10647747]
46. Hooper LV, Gordon JI. Commensal host-bacterial relationships in the gut. *Science*. 2001; 292:1115–8. [PubMed: 11352068]
47. Zhang M, Zhang C, Du H, et al. Pattern extraction of structural responses of gut microbiota to rotavirus infection via multivariate statistical analysis of clone library data. *FEMS Microbiol Ecol*. 2009; 70:21–9. [PubMed: 19453743]
48. Chang JY, Antonopoulos DA, Kalra A, et al. Decreased diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea. *J Infect Dis*. 2008; 197:435–8. [PubMed: 18199029]
49. Willing BP, Dicksved J, Halfvarson J, et al. A pyrosequencing study in twins shows that gastrointestinal microbial profiles vary with inflammatory bowel disease phenotypes. *Gastroenterology*. 2010; 139:1844.e1–54.e1. [PubMed: 20816835]
50. Sonnenburg JL, Chen CT, Gordon JI. Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. *PLoS Biol*. 2006; 4:e413. [PubMed: 17132046]
51. Noble RL, Sidwell RW, Mahoney AW, et al. Influence of malnutrition and alterations in dietary protein on murine rotaviral disease. *Proc Soc Exp Biol Med*. 1983; 173:417–26. [PubMed: 6867015]

52. Ofor E, Riepenhoff-Talty M, Ogra PL. Effect of malnutrition on rotavirus infection in suckling mice: kinetics of early infection. *Proc Soc Exp Biol Med*. 1985; 178:85–90. [PubMed: 2981423]
53. Riepenhoff-Talty M, Ofor E, Klossner K, et al. Effect of age and malnutrition on rotavirus infection in mice. *Pediatr Res*. 1985; 19:1250–3. [PubMed: 3001627]
54. Riepenhoff-Talty M, Uhnou I, Chegas P, et al. Effect of nutritional deprivation on mucosal viral infections. *Immunol Invest*. 1989; 18:127–39. [PubMed: 2543624]
55. Walter J, Britton RA, Roos S. Microbes and Health Sackler Colloquium: host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm. *Proc Natl Acad Sci U S A*. 2010; 108(suppl 1):4645–52. [PubMed: 20615995]
56. Salazar-Lindo E, Allen S, Brewster DR, et al. Intestinal infections and environmental enteropathy: working group report of the second World Congress of Pediatric Gastroenterology, Hepatology, and Nutrition. *J Pediatr Gastroenterol Nutr*. 2004; 39(suppl 2):S662–9. [PubMed: 15184767]

Figure 1.

Daily gavages with human-derived *L reuteri* ameliorated rotaviral diarrhea, histopathology, and microbial community perturbations in the distal intestine of neonatal mice. A, Duration of diarrhea in rotavirus (RV)-infected mice given daily gavages of *L reuteri* or phosphatebuffered saline (PBS) (n = 35 mice per group; bars show median+ interquartile range). *** $P < 0.001$ and ** $P < 0.01$ compared with PBS-treated mice. B, Representative photomicrographs of villi from the distal ileum of 12-day-old mice (4 days after RV or mock infection), stained with toluidine blue/basic fuchsin at 200× original magnification.

Similar results were observed with both *L reuteri* strains; DSM 17938 is shown. C, Operational taxonomic unit (OTU) assignments revealed by pyrosequencing stool from 80 mice, pools of 20 mice per group of 11-day-old RV-infected or uninfected pups, before and 24 hours after mice received 1 of 2 strains of *L reuteri* or PBS. D, Percentage of OTUs that aligned with *L reuteri*. E, Alpha diversity or species richness, defined as the total number of unique OTUs per pooled sample. F, Pielou index of species evenness. G, Simpson reciprocal index of phylogenetic diversity (β diversity). Data represent the average of 2 sequencing reactions per sample of the microbial 16S rRNA V1 – V3 subunit; similar results were obtained through analysis of the 16S V3–V5 subunits.

Figure 2.

Resolution of diarrhea, increased crypt cell proliferation, and enhanced enterocyte migration by either strain depended on nutritional status. A, Duration of diarrhea in underweight, normal weight, or overweight rotavirus (RV)-infected mice receiving daily gavages of *L reuteri* (strains 17938 or 6475) or phosphate-buffered saline (PBS) ($n = 35$ mice per group; median + interquartile range). B, Crypt cell proliferation in ileum harvested 4 hours after RV infection and BrdU injection ($n = 15$ mice per group; mean + SD). *** $P < 0.001$ and * $P < 0.05$ compared to PBS-treated mice; ## $P < 0.01$, and # $P < 0.05$ between strains. C, Photomicrographs of representative villi in the distal ileum of mice 4 days after BrdU injection following gavage with PBS, *L reuteri* strain 17938, or *L reuteri* strain 6475. Labeled cells were rarely seen at the villus tips in overweight mice treated with *L reuteri* 17938 (arrow). Anti-BrdU immunohistochemistry, 200 $\times$ original magnification; see also Supplemental Figs. 1–4.

Figure 3.

L reuteri modulated mucosal concentrations of antibodies and cytokines from ex vivo ileum cultures of rotavirus (RV)-infected mice. A, Total RV-specific antibody; B, macrophage inflammatory protein (MIP)-1 α ; C, interleukin (IL)-1 β . Percentages indicate mice that had values above the lower limit of detection (n = 13–15 mice per group; mean + standard deviation). *** P < 0.001, ** P < 0.01, and * P < 0.05 compared to phosphate-buffered saline-treated mice; see also Supplemental Figs. 5–7.

Figure 4.

Host response to rotavirus (RV) was dependent on nutritional state. A, Mean body weights of underweight mice were significantly lower than other groups starting on the third day of the experiment, and mean body weights of overweight mice were significantly different from normal weight mice starting on the fifth day of the experiment ($n = 20$ mice per group; mean $\pm$ standard deviation [SD]). B, Significant differences were observed between nutritional states with respect to diarrhea duration ($n = 35$ mice per group; median + interquartile range); C, total RV-specific antibody production ($n = 15$ mice per group; mean); D, enterocyte migration ($n = 7$ mice per group; mean $\pm$ SD); E, crypt-cell proliferation ($n = 15$ mice per group; mean $\pm$ SD). Only RV-infected, phosphate-buffered saline-treated mice were considered in these analyses. For B–E, *** $P < 0.001$ and * $P < 0.05$ compared with underweight mice; ### $P < 0.001$, ## $P < 0.01$, and # $P < 0.05$ between normal-weight and overweight mice.