Probiotic stool secretory immunoglobulin A modulation in children with gastroenteritis: a randomized clinical trial

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ABSTRACT

Background: We previously conducted the Probiotic Regimen for Outpatient Gastroenteritis Utility of Treatment (PROGUT) study, which identified no improvements in children with acute gastroenteritis (AGE) administered a probiotic. However, the aforementioned study did not evaluate immunomodulatory benefits.

Objectives: The object of this study was to determine if stool secretory immunoglobulin A (sIgA) concentrations in children with AGE increase more among participants administered a Lactobacillus rhamnosus/helveticus probiotic compared with those administered placebo.

Methods: This a priori planned multicenter, randomized, double-blinded, placebo-controlled ancillary study enrolled children presenting for emergency care who received a 5-d probiotic or placebo course. Participants submitted stool specimens on days 0, 5, and 28. The primary endpoint was the change in stool sIgA concentrations on day 5 compared with baseline.

Results: A total of 133 (n = 66 probiotic, 67 placebo) of 886 PROGUT participants (15.0%) provided all 3 specimens. Median stool sIgA concentrations did not differ between the probiotic and placebo groups at any of the study time points: day 0 median (IQR): 1999 (768, 4071) compared with 2198 (702, 5278) (P = 0.27, Cohen’s d = 0.17); day 5: 2505 (1111, 5310) compared with 362 (1122, 4256); (P = 0.77, Cohen’s d = 0.075) and day 5 to day 28 (−1035 (−3130, 499) compared with −1260 (−4437, 843); P = 0.70, Cohen’s d = 0.067), respectively.

Conclusions: We found no effect of an L. rhamnosus/helveticus probiotic, relative to placebo, on stool IgA concentrations. This trial was registered at clinicaltrials.gov as NCT01853124.

Keywords: probiotic, immunoglobulin A, pediatric, gastroenteritis, clinical trial

Introduction

The secretion of immunoglobulin A (IgA) antibody is one of the primary defense mechanisms protecting against enteric infections in both human and animal models (1, 2). IgA provides
protective effects by binding to microbial antigens, toxins, and food proteins, and it inhibits adherence and penetration of the intestinal epithelium (3–5). Animal studies report a substantial increase in intestinal commensal anaerobic bacteria in the absence of normal intestinal secretory IgA (sIgA) concentrations, with intestinal microbiota returning to baseline composition following normalization of sIgA production (6).

The use of probiotics is increasing rapidly despite questionable evidence of efficacy (7), particularly as it relates to pediatric acute gastroenteritis (AGE) (8). However, the interest in probiotics is founded on how they may contribute to immune homeostasis by altering the microbial balance, interacting with the host immune system (9), regulating inflammatory cytokines (10), and increasing sIgA production (11). In mouse models, probiotics protect against enteric infections by inducing intestinal IgA secretion (12, 13), which has been correlated with survival in mice (14). In healthy humans, probiotics increase stool sIgA concentrations across the age spectrum (15–18). However, in children with AGE, conflicting results have been reported (19–21), and no trials have correlated stool sIgA concentrations with clinical outcomes. The possibility that probiotics enhance the protective immune response in the host intestinal tract is a crucial topic requiring clarification (21).

In 2017, we completed the Probiotic Regimen for Outpatient Gastroenteritis Utility of Treatment (PROGUT) clinical trial (22), which randomized children with AGE to receive either a *Lactobacillus rhamnosus/helveticus* probiotic mixture or identical-appearing placebo twice daily for 5 d. Although this trial identified no significant benefits associated with administration of the *L. rhamnosus/helveticus* product (23), we sought to explore the effect of its administration on stool sIgA concentrations by determining if supplementation promotes sIgA secretion as reflected by higher sIgA concentrations in stool specimens relative to those administered a placebo. We also sought to determine if higher day 5 sIgA concentrations would promote pathogen clearance and hence result in less severe disease.

**Methods**

**Study design**

This was an *a priori* planned ancillary study designed within the investigator-initiated, multicenter, PROGUT trial (22). Children with acute diarrhea received a 5-d course of a combination *L. rhamnosus/helveticus* probiotic or placebo. Research ethics boards at each of the 6 participating Canadian, tertiary care, university-affiliated sites approved the trial. Caregivers provided written informed consent for their children to participate.

**Eligibility criteria**

Children aged 3–48 mo were eligible to participate in the PROGUT study if they presented for emergency department (ED) care, experienced ≥3 episodes of watery stools in a 24-h period, had diarrhea or vomiting for <72 h, and were diagnosed as having AGE. Children were excluded if they or a person living in their household had an indwelling vascular-access catheter or if they were immunocompromised, receiving immunosuppressive therapy, or had structural heart disease. Additional exclusion criteria were bilious vomiting, hematochezia, pancreatic dysfunction or insufficiency, a chronic gastrointestinal disorder (e.g., celiac disease, milk allergy), an allergy to soy, probiotic use during the preceding 14 d, and an inability to complete follow-up. Children who had undergone gastrointestinal or oral surgery within the preceding 7 d or previously participated in the trial were also excluded. For this ancillary study, participants submitted stool specimens for sIgA analysis on the day of enrollment (day 0), day 5 (i.e., last day of probiotic/placebo administration), and day 28 following enrollment.

**Randomization and masking**

Random-number–generating software, accessed through a Web-based randomization system (https://www.randomize.net), which used random block sizes of 4 and 6 and a 1:1 trial group assignment ratio stratified according to site, was used to sequentially assign children to the intervention or placebo. Knowledge of the assignment sequence was restricted to the research pharmacy at the coordinating center and Randomize.net until the databases were locked. Participants and their caregivers, trial and clinical staff, and specimen and data analysts were unaware of trial group assignments.

**Illness severity**

Gastroenteritis severity following randomization was quantified using the Modified Vesikari Scale (MVS) score, which ranges from 0 to 20, with higher scores indicating more severe disease (Supplemental Table 1) (24, 25). Scores of 0–8, 9–10, and ≥11 denote mild, moderate, and severe gastroenteritis episodes, respectively. Each participant’s score was assigned based on clinical data collected during the follow-up period. Baseline symptoms that preceded the visit to the ED were not included in the outcome measure.

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Supplemental Tables 1–12 and Supplemental Methods are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/

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Abbreviations used: AGE, acute gastroenteritis; ED, emergency department; IgA, immunoglobulin A; MVS, Modified Vesikari Scale; sIgA, secretory immunoglobulin A.

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Primary and secondary outcomes

The following primary and secondary outcomes were neither altered during the course of this study nor conducted as post hoc analyses. The primary endpoint was the change in stool sIgA concentrations on day 5 compared with baseline (i.e., day 0) concentrations. Secondary endpoints included 1) comparison of the change in stool sIgA concentrations on day 28 compared with day 5; 2) correlation of day 0 stool sIgA concentrations with the baseline MVS score and of days 5 and 28 stool sIgA concentrations with the postrandomization, follow-up MVS scores; and 3) correlation of days 0, 5, and 28 stool sIgA concentrations with identified pathogens in both group (i.e., virus and bacteria) and virus-specific agents (i.e., adenovirus, norovirus, and rotavirus).

Procedures

Rectal swabs, stool specimens, or both were obtained during the enrollment visit (26). If stool was not provided prior to ED discharge, the first stool sample produced following enrollment was collected at home. Additional samples were collected from all children at home on days 5 and 28 postrandomization. Stools collected at home were stored at room temperature for up to 12 h and then retrieved by a study-funded courier and transported to the laboratory on ice packs. Specimens were stored at −80°C until they were thawed for sIgA analyses. Processing was performed as previously described (27).

The investigational preparation employed was a lyophilized powder containing 4.0 × 10⁹ CFUs of 2 bacterial strains—L. rhamnosus R0011 and L. helveticus R0052—in a 95:5 ratio. These strains have been studied (28–33) and demonstrated benefits to consist of live microorganisms that, when administered in adequate amounts, confer a health benefit on the host. The authors of that study reported a between-group difference in stool sIgA concentrations (i.e., prebiotic compared with placebo) of ~300 μg/g of stool (27). Therefore, we sought to detect a minimal difference in stool sIgA concentrations in the probiotic group relative to the placebo group of 300 μg/g with an SD of 500 μg/g. Sample size calculations, employing a power of 80% and a 2-sided type I error of 0.05, yielded a minimal sample size of 50 subjects who provided all 3 stool specimens in each group.

Enteropathogen identification

See Supplemental Methods for a detailed description.

Secretory IgA quantification

Quantification was performed at the Hospital for Sick Children, Toronto, Ontario, Canada. Prior to dilution, stool samples were weighed with extraction buffer and dilution factors calculated for each sample. A lower dilution factor was used for samples with input weights below the manufacturer’s suggested limit to ensure data points would register in the linear range on a standard curve. sIgA stool concentrations were determined using the sIgA ELISA kit (36) (Eagle Biosciences). A 4-parameter logistic standard curve was generated, with samples run in duplicate. Samples were retested if the CV was >10% or absorbance reads at 450 nm against 620 nm were outside the dynamic range of standards provided. The lower limit of detection was 3.1 ng/mL.

Statistical analysis

All analyses were specified a priori (22). Per-protocol analysis was implemented for all patients who submitted specimens on days 0, 5, and 28. For the primary outcome, the change in stool sIgA concentrations (day 0 to day 5) was compared between groups using the Mann–Whitney U test. Effect size estimates for each comparison were calculated as described by Fritz et al. (37) and denoted as Cohen’s d. Within treatment group day 0 and day 5 comparisons were assessed by Wilcoxon’s signed-rank sum test. Factors such as age, sex, frequency of vomiting and diarrhea in the 24-h period before enrollment, recent antibiotic use, breastfeeding status, and the presence of enteropathogens (i.e., virus, bacteria, and codetection) may affect baseline sIgA concentrations. Therefore, secondary analysis of the primary outcome employed generalized linear regression mixed modeling using γ distribution and log link with random effect intercept for subjects adjusted for these a priori identified covariates. We also included the variable “day of stool collection,” measured on a continuous scale, to evaluate the effect of time on sIgA concentrations across the 28-d study period, independent of probiotic treatment, as well as through the inclusion of an interaction term between day of stool collection and treatment assignment. Interaction between treatment assignment and time of stool collection (i.e., day 0 or day 5) was tested in the model.

The secondary outcome of change in stool sIgA concentrations between day 5 and day 28 was analyzed as described for the primary outcome. The association between day 0 stool sIgA concentrations and baseline MVS score was analyzed using a generalized linear model with a log-link function and γ distribution adjusted for age, sex, treatment assignment, recent antibiotic use, breastfeeding status, and enteropathogen status. The same analytic approach was employed to correlate the day 5
and day 28 stool sIgA concentrations with the postrandomization, follow-up MVS score.

Between treatment group sIgA differences were compared at each time point based on enteropathogens identified at group (i.e., virus and bacteria) and individual virus (i.e., norovirus, rotavirus, and adenovirus) concentrations using the Mann–Whitney U test. The independent effects of enteropathogen status on stool sIgA concentrations at baseline, day 5, and day 28 were assessed, respectively, using a generalized linear model with a log-link function and γ distribution to fit skewed stool sIgA data, including the covariates described for the primary outcome. To further evaluate the effect of individual enteropathogens and treatment status on sIgA concentrations, changes from day 0 to day 5 and from day 5 to day 28 were evaluated as described for the primary outcome for participants who were virus positive, bacteria positive, virus–bacteria codetected, adenovirus positive, norovirus positive, and rotavirus positive, respectively. Regression analyses were performed when the minimum number of participants was ≥15.

Analyses were conducted using R version 3.5.6 (R Foundation for Statistical Computing) using packages lme4 and lmne for mixed-model γ regression with log link. Additional descriptive statistics and graphing were performed using SPSS version 24.0.0.1 (IBM) and GraphPad Prism (version 8.0.1). We did not use multiple imputations in the calculations because only 1 participant had incomplete clinical data (38). We calculated 2-tailed P values and set the significance level α at 0.05. To account for the testing of numerous secondary outcomes (n = 27), a Bonferroni correction was applied, and consequently the P value of significance for secondary outcomes was set at 0.002 (39).

Results

Participants

Between November 5, 2013, and April 7, 2017, 886 children were enrolled, of whom 172, 182, and 107 submitted day 0, day 5, and day 28 stool specimens, respectively (Figure 1). A total
of 133 children ($n = 66$ probiotic, $n = 67$ placebo) provided all 3 stool specimens. Study groups did not show a meaningful difference in any baseline characteristics (Table 1). Respective median stool sIgA concentrations on days 0, 5, and 28 did not differ between groups at any of the time points (Table 2).

### Primary outcome

There were no significant changes in stool sIgA concentrations between day 0 and day 5 within *L. rhamnosus*/helveticus- or placebo-treated groups (Figure 2). In addition, stool sIgA concentrations did not differ between the *L. rhamnosus*/helveticus and the placebo groups (Table 2). In the regression model, we found no effects of treatment allocation ($\beta: -0.24 \text{ (} -0.72, 0.24\text{)}$; $P = 0.32$) or interaction between treatment and specimen collection day ($\beta: 0.004 \text{ (} -0.02, 0.02\text{)}$; $P = 0.65$). The only covariate statistically associated with a decrease in stool sIgA concentrations between day 5 and day 28 was a longer time interval between stool collection days ($\beta: -0.03 \text{ (} -0.04, -0.02\text{)}$; $P < 0.001$), indicative of the expected decrease in sIgA concentration following recovery from AGE (Supplemental Table 3).

No relation between baseline stool sIgA concentrations and baseline MVS score (i.e., symptom severity prior to and up to the time of stool specimen submission) was found. With mild disease (MVS score <9) as the reference group, moderate and severe disease were not associated with stool sIgA concentrations: $\beta: 0.51 \text{ (} -0.18, 1.20\text{)}$ ($P = 0.15$) and $\beta: 0.48 \text{ (} -0.14, 1.10\text{)}$ ($P = 0.13$), respectively (Supplemental Table 4).

In the adjusted model evaluating the association between day 5 stool sIgA values and the postrandomization MVS score, we found no relation between moderate $\beta: -0.10 \text{ (} -0.76, 0.56\text{)}$; $P = 0.77$] or severe $\beta: -0.34 \text{ (} -0.99, 0.30\text{)}$; $P = 0.30$] disease

### Secondary outcomes

Although there were significant differences between day 5 and day 28 median stool sIgA concentrations within both the *L. rhamnosus*/helveticus and the placebo treatment groups ($P < 0.001$; Figure 2), no significant differences between treatment groups were found (Table 2).
and day 5 stool sIgA concentrations (Supplemental Table 5). The results were similar with respect to day 28 stool sIgA values, with the exception of a positive but not statistically significant association between day 28 stool sIgA values and the detection of viral pathogens in the stool—that is, higher day 28 values for this group of children \( [\beta = 0.43 (0.05, 0.81); P = 0.03] \) (Supplemental Table 6).

Overall, 67.7% (90/133) of the participants were pathogen positive, with viral pathogens detected most frequently (47.4%; 63/133) (Table 1). There were no differences in baseline, day 5, or day 28 stool sIgA concentrations between treatment groups regardless of pathogens identified (Table 3). Similarly, no differences were noted in the change in stool sIgA between treatment groups, when analyzed by pathogen status, between day 0 and day 5, day 0 and day 28, or day 5 and day 28.

Within the groups of identified individual viral agents, differences between probiotic and placebo groups in stool sIgA concentrations (absolute values or changes over time) were statistically significant after correction for multiple secondary outcomes (Figure 3, Supplemental Table 7). Adjusted models evaluating pathogen-level changes in stool sIgA over time also found no significant treatment group effects (Supplemental Tables 8–11).

Discussion

In this study, we quantified the effect of \( L. \) rhamnosus/helveticus administration on stool sIgA concentrations in preschool-aged children with AGE. Neither the \( L. \) rhamnosus/helveticus group nor the placebo treatment group had

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TABLE 2  Stool sIgA concentrations, change in stool sIgA concentrations, and clinical disease severity scores across study time points

|                | \( L. \) rhamnosus/helveticus (\( n = 66 \)) | Placebo (\( n = 67 \)) | Difference in median | 95% CI of the difference | \( P \) value \(^2\) | Effect size \(^3\) | Test statistic \(^4\) |
|----------------|---------------------------------------------|------------------------|----------------------|--------------------------|----------------|----------------|----------------|
| Day 0, stool sIgA | 1999 (768, 4071)                            | 2198 (702, 5278)       | 199                  | −337, 1101               | 0.27           | 0.17           | 1998           |
| Day 5, stool sIgA | 2505 (1111, 5310)                           | 3207 (982, 7080)       | 701                  | −434, 1551               | 0.19           | 0.16           | 2005           |
| Day 28, stool sIgA| 1377 (697, 2248)                            | 1779 (660, 3977)       | 402                  | −200, 781.2              | 0.27           | 0.19           | 1967           |
| Change day 0 to day 5, sIgA | −500 (−1135, 2362) | 362 (−1122, 4256) | −139                | −989, 1459               | 0.77           | 0.08           | 2115           |
| Change day 5 to day 28, sIgA | −1035 (−3130, 499) | −1260 (−4437, 845) | −225                | −1313, 892.3             | 0.70           | 0.07           | 2125           |
| Change day 0 to day 28, sIgA | −461 (−2001, 723) | −204 (−3798, 1548) | 257                 | −909, 1205               | 0.76           | 0.05           | 2144           |
| Modified Vesikari Scale score, illness onset to day 0 | 11 (9, 13) | 11 (10, 14) | 0.39 | −0.5, 1.3 | 0.39 | 0.13 | 0.853 |
| Modified Vesikari Scale Score, illness day 0 to end of illness | 5 (2, 8) | 5 (3, 8) | 0.47 | −0.8, 1.7 | 0.45 | 0.13 | 0.743 |

\(^1\)Values are medians (IQRs) unless stated otherwise. sIgA concentrations in micrograms per gram. sIgA, secretory immunoglobulin A.

\(^2\)\( P \) values reflect results of Mann–Whitney \( U \) test except for analyses comparing Modified Vesikari Scale scores, which employed the Welch’s \( t \) test.

\(^3\)Effect size reflects the Cohen’s \( d \) effect size estimates, where \( d = 0.2 \), \( d = 0.5 \), and \( d = 0.8 \) refer to small, medium, and large effect sizes, respectively.

\(^4\)Test statistic reflects the Mann–Whitney \( U \) test statistic except for analysis comparing Modified Vesikari Scale scores, which reflects the Welch’s \( t \) statistic.

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FIGURE 2  StoolsIgA concentrations at baseline and 5 and 28 d postrandomization treatment for both the probiotic (A) (\( n = 66 \)) and placebo (B) (\( n = 67 \)) treated groups. An outlier analysis was performed, and outliers were removed; however, because this did not alter the findings, the outliers were retained. The median difference for the day 5 to day 28 change in the probiotic group was 1035 \( \mu \)g/g (95% CI of the difference: 85, 1951). The median difference for the day 0 to day 28 change in the probiotic group was −461 \( \mu \)g/g (95% CI of the difference: −1281, 98). The median difference for the day 5 to day 28 change in the placebo group was 1260 \( \mu \)g/g (95% CI of the difference: 60, 2077). Significance denoted as \( ^* P = 0.01 \), \( ^{**} P = 0.0005 \), and \( ^{***} P = 0.0006 \). sIgA, secretory immunoglobulin A.
detectable increases in stool sIgA concentrations over the 5 d following the ED visit. After controlling for repeated measures and trial site, there was no effect of treatment on stool sIgA concentrations. Although we found significant reductions in stool sIgA concentrations between day 5 and day 28, this was not related to treatment allocation. In addition, we found no relation between disease severity and stool sIgA concentrations.

Only 1 prior study has evaluated stool sIgA in children with AGE; it reported a positive effect on fecal sIgA from the administration of $4 \times 10^8$ CFU/d of *Lactobacillus casei* for 7 d (21). Although it is unclear why our results differed, strain and disease specificity are important characteristics of probiotics (42), and thus strain differences may account for the differential findings. Study rigor may play a role because although the previous study mentioned placebo, the contents were not described, and blinding was not reported. Moreover, the between-group differences, which are reported as $P < 0.05$, appear to be minimal, do not adjust for baseline values, and there

| Pathogen positive (n = 90) | L. _rhamnosus/helveticus_ | Placebo | Difference in median | 95% CI of the difference | P value$^2$ | Effect size$^3$ | Test statistic$^4$ |
|----------------------------|---------------------------|---------|---------------------|--------------------------|------------|--------------|------------------|
| Day 0, stool sIgA          | 2306 (1612, 4931)         | 3394 (1331, 5910) | 1088                  | −884, 2701               | 0.41       | 0.26         | 195              |
| Day 5, stool sIgA          | 1268 (355, 3633)          | 2076 (982, 5750)  | 808                   | −596, 2061               | 0.28       | 0.34         | 185              |
| Day 28, stool sIgA         | 1188 (482, 1875)          | 785 (521, 3977)   | −402                  | −702, 642.7              | 0.97       | 0.02         | 228              |
| Change day 0 to day 5, sIgA| −257 (−1672, 765)         | 27 (−2563, 602)   | 284                   | −1571, 1785              | 0.70       | 0.12         | 214              |
| Change day 5 to day 28, sIgA| −16 (−2529, 742)          | −954 (−3639, 674) | −938                  | −2031, 1662              | 0.67       | 0.13         | 212              |

$^1$Values are medians (IQRs) unless stated otherwise. sIgA concentrations in micrograms per gram. sIgA, secretory immunoglobulin A.

$^2$P value reflects results of the Mann–Whitney U test. Statistical significance set at $P < 0.002$ after correction for multiplicity (n = 27).

$^3$Effect size reflects the Cohen’s d effect size estimates, where $d = 0.2$, $d = 0.5$, and $d = 0.8$ refer to small, medium, and large effect sizes, respectively.

$^4$Test statistic reflects the Mann–Whitney U test statistic.
FIGURE 3 Stool sIgA concentrations at baseline and 5 and 28 d postrandomization treatment for both norovirus- (A) and rotavirus- (B) infected children. Norovirus-infected children: n = 40; probiotic = 17, placebo = 23. Rotavirus-infected children: n = 17; probiotic = 11, placebo = 6. No significant differences were found after correction for multiple comparison. sIgA, secretory immunoglobulin A.

Our study has several limitations. It is possible that stool specimen collection dates did not correspond to the peak timing of L. rhamnosus/helveticus-induced sIgA production. However, in mouse models, pathogen-specific IgA responses are seen as early as 3 d following viral inoculation (50). Despite conducting this study at 6 sites, we obtained repeat stool specimens from only a relatively modest proportion of the PROGUT study cohort. This occurred due to the outpatient nature of the study and the self-limited disease process that made stool retrieval a challenge. Although those who provided all stool specimens were less likely to be infected by rotavirus, they did not differ in any other baseline characteristics from those who did not provide all specimens (Supplemental Table 12). We also did not collect serum nor attempt to correlate fecal sIgA concentrations with IgA concentrations in serum. However, note that individuals with low concentrations of serum IgA likely have sufficient secretory IgA at their mucosal surfaces to remain asymptomatic (51). Also note that other mechanisms of probiotic action were not evaluated, including the impact on the luminal and mucosal microbiomes (52) or via altering fecal metabolites (53). We had intended to conduct bacteria-specific analyses as well; however, given the small numbers of individual pathogens, we are unable to report these findings.

In conclusion, among children with AGE presenting to an ED, L. rhamnosus/helveticus treatment did not increase stool sIgA concentrations relative to placebo. The lack of effect identified in this evaluation of the intestine’s immune system is consistent with the lack of clinical efficacy reported in the recent PROGUT clinical trial.

The authors’ responsibilities were as follows—SBF and PMS: conceived the study and co-wrote the study protocol; JX, SW-U, LC, X-LP, BL, SS, YF, SG, KJF, NP, KH, and DS: designed the study; RH, KJ-H, and PMS: provided major input into the concept and analysis of the study; SBF, JX, SW-U, LC, X-LP, BL, SS, YF, SG, KJF, NP, KH, and DS: conducted the manuscript and revised the manuscript for intellectual content; RH and KJ-H: provided major input into drafting and revision of the manuscript; and all authors: read and approved the final manuscript. SBF reports receiving research support from Lallemand Health Solutions and serves as a consultant for Takeda Pharmaceutical Company, Eligo Bioscience, and RedHill Biopharma. PMS receives research support from Lallemand Health Solutions and honoraria from Abbott Nutrition, Mead Johnson Nutritional, and Nestle Nutrition related to the use of probiotics in humans. All other authors report no conflicts of interest.
Data Availability

The full study protocol, statistical analysis plan, informed consent forms, deidentified individual participant data, and a data dictionary defining each field in the set are available upon request from the corresponding author following the provision of ethics approval, with a signed data access agreement.

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