Effects of Early Prebiotic and Probiotic Supplementation on Development of Gut Microbiota and Fussing and Crying in Preterm Infants: A Randomized, Double-Blind, Placebo-Controlled Trial

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Objective To evaluate the impact of early prebiotic and probiotic intervention on preterm infants' well-being, crying, growth, and microbiological programming.

Study design Ninety-four preterm infants (gestational age 32-36 weeks and birth weight >1500 g) randomized to receive prebiotics (mixture of galacto-oligosaccharide and polydextrose 1:1), probiotics (*Lactobacillus rhamnosus* GG), or placebo during the first 2 months of life were followed up for 1 year. Infants were categorized based on the extent of crying and irritability during the first 2 months of life, and their gut microbiota was investigated by fluorescence in situ hybridization (n = 66) and quantitative polymerase chain reaction (n = 63).

Results A total of 27 of 94 infants (29%) infants were classified as excessive criers, significantly less frequently in the prebiotic and the probiotic groups than in the placebo group (19% vs 19% vs 47%, respectively; $P = .02$). The placebo group had a higher percentage of *Clostridium histolyticum* group bacteria in their stools than did the probiotic group (13.9% vs 8.9%, respectively; $P = .05$). There were no adverse events related to either supplementation.

Conclusions Early prebiotic and probiotic supplementation may alleviate symptoms associated with crying and fussing in preterm infants. This original finding may offer new therapeutic and preventive measures for this common disturbance in early life. *(J Pediatr 2013;163:1272-7)*.

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During early infancy, balanced host–microbe interaction is essential for healthy intestinal and immunological development.[1] Microbiological programming begins in utero and proceeds gradually during birth and infancy.[2] The process is determined by environmental influences such as mode of delivery, perinatal antibiotic exposure, mode of feeding, and amount of skin-to-skin contact.[3] Risk factors associated with perturbation of the compositional development of the gut microbiota tend to accumulate in infants born preterm. As a consequence, their encounter with beneficial bacteria is often delayed. Thus far, deviations in gut microbiota composition in term infants have been associated with various inflammatory and functional gastrointestinal disorders such as colicky crying, cow's milk allergy, infantile diarrhea, and even celiac disease.[4] In such clinical conditions, the use of probiotics (ie, live microorganisms that, when administered in adequate amounts, confer a health benefit on the host) and prebiotics (ie, nondigestible food ingredients that stimulate the growth and/or activity of the indigenous intestinal bacteria) provide promising tools to alleviate specific disorders and hence enhance infant well-being.[5,6]

In preterm infants, one of the most promising targets of probiotic intervention is prevention of necrotizing enterocolitis, a devastating immunoinflammatory intestinal disease often resulting in severe impairment.[2] However, in everyday life, much more frequent sources of frustration to parents of preterm infants are benign functional gastrointestinal disorders related to feeding, crying, and irritability. Data on the effects of prebiotic and probiotic interventions on the prevention or treatment of these common problems are needed.

To improve our understanding of the impact of prebiotics and probiotics on preterm infant well-being and gut microbiota composition, we conducted a

Cy3	Carbocyanine 3
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction

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Supported by the Juho Vainio Foundation, the Päivikki and Sakari Sohlberg Foundation, the Foundation for Pediatric Research, an EVO grant from Turku University Hospital and Satakunta Central Hospital, and Mead Johnson Nutrition (covered the costs of the prebiotic and probiotic products [prepared by Turku University Hospital Pharmacy] and part of the salary for R.L.). The sponsors had no influence on the design or conduct of the study, data management and analysis, writing of the report, or the decision to submit the manuscript for publication. The authors declare no conflicts of interest.

Registered with ClinicalTrials.gov: NCT00167700.

 http://dx.doi.org/10.1016/j.jpeds.2013.05.035

randomized, double-blind, prebiotic and probiotic intervention study in preterm infants. We hypothesized that early modification of the gut microbiota with specific prebiotics and probiotics can enhance infant well-being by reducing the risk of infant crying and fussing. The overall aims were to balance the gut microbiota composition with prebiotic or probiotic supplementation and consequently improve infant well-being and reduce their disease risk. We describe the impacts of prebiotic and probiotic intervention on infant crying, fussing, and irritability and on their gut microbiota development.

Methods

This randomized, double-blind placebo-controlled study involved 94 infants who were recruited between days of life 1 and 3 in the Department of Pediatrics, Turku University Hospital, between June 2008 and May 2011. To be eligible for enrollment, infants had to meet the following criteria: gestational age between 32 + 0 and 36 + 6 weeks, birth weight >1500 g, and absence of any congenital defects in the gastrointestinal system or other defects preventing enteral nutrition. The study was approved by the Ethics Committee of the Hospital District of South-West Finland. Written informed consent was obtained from the infants' parents.

The participants were randomly assigned to 1 of the 3 study groups according to computer-generated block randomization by an independent statistician (J.M.). To avoid disproportionate numbers of patients in each group, randomization was performed in blocks of 6 subjects. Randomization was implemented with SAS software package PROC PLAN (SAS Institute, Cary, North Carolina). To ensure allocation concealment, a member of a group not involved in the conduct or reporting of this study was responsible for the packaging and labeling of the study products. Study personnel, staff in the neonatal ward, and parents were blinded to the randomization. The study nurse allocated the next available product on entry to the trial, and each patient received the study product directly from the department. The code was revealed to the investigator once recruitment, data collection, and analysis were completed. The study data were collected on printed case record forms, and the members of the research group entered the data. All data were kept confidential.

Preterm infants were randomly assigned to receive orally a prebiotic mixture (polydextrose [Danisco Sweeteners, Surrey, United Kingdom] and galacto-oligosaccharides [Friesland Foods Domo, Zwolle, Netherlands] 1:1; 600 mg/d in 1 dose from day 1 to day 30 and 600 mg twice daily from day 31 to day 60), probiotic (*Lactobacillus rhamnosus* GG [ATCC 53103; Mead Johnson & Co, Evansville, Indiana] 10^9 colony-forming units/d in 1 dose from day 1 to day 30 and 10^9 colony-forming units twice daily from day 31 to 60 day), or placebo (microcrystalline cellulose and dextrose anhydrate [Chr. Hansen, Hoersholm, Denmark]). All study products were prepared by the Turku University Hospital Pharmacy. The products were identical in appearance, taste, and smell and were stored in identical closed capsules. The viability of the probiotic was confirmed by regular blinded analysis in the Functional Foods Forum, Turku University. Parents were taught by the study nurse to mix the products immediately before administration to the infant with ~10 mL of breast milk or formula given by spoon or bottle once a day. The parents were instructed to inspect the product for any signs of damage before use and to keep it in a refrigerator when not in use.

After the enrollment day, follow-up consultations were conducted by the same study nurse at the ages of 1, 2, 4, 6, and 12 months. The infants were clinically examined by a physician (R.L. or A.P.) at the age of 12 months and otherwise as needed. During all study visits, parents reported the infant's behavior patterns, including sleeping patterns, fussing, crying, irritability, feeding, vomits, stools (consistency [normal, loose, firm, hard, atypical] and frequency [1/wk, 2-3/wk, daily, 1-2/d, 2-3/d, >3/d], infection and other diseases, and medication use. An infant was classified as an excessive crier if parents and study nurse reported excessive crying and irritability (>3 h/d) causing clinical concern without underlying medical causes during the 1- and 2-month study visits and resolving by the age of 6 months. The parents kept a structured diary on each child's infectious disease symptoms and pharmacological treatment during the follow-up year. Weight, height, and head circumference were measured, and growth charts were drawn at each visit. Adverse events were asked from the parents during all visits. Fecal samples were collected from diapers after defecation at the age of 1 month, immediately frozen to −20°C, and delivered to the study clinic within 24 hours.

Homogenized fecal samples were fixed overnight in 4% paraformaldehyde and stored in phosphate-buffered saline:ethanol (1:1) at −20°C until analyzed (maximum 3 months). Fluorescence in situ hybridization (FISH) with a flow cytometer was performed as previously described.[7,8] Samples were hybridized at specific temperatures in hybridization buffer (containing 26 mM Tris-HCl, 1.2 M NaCl, and 0.1% sodium dodecyl sulfate, pH 7.2) with specific probes at a concentration of 5 ng/μL. After overnight hybridization, samples were washed with buffer without sodium dodecyl sulfate, pelleted, and resuspended in phosphate-buffered saline. The EUB 338 probe was covalently linked at the 5′ end with fluorescein isothiocyanate (FITC) and other probes with carbocyanine 3 (Cy3). The sequences of the probes are presented in **Table I** (available at www.jpeds.com). All oligonucleotides were purchased from Thermo Electron Corporation (Thermo Bioscienses, Ulm, Germany).

Data acquisition was performed with an LSR II flow cytometer equipped with an HTS 96-well-plate reader (Becton Dickinson, San Jose, California). The 15-mV argon ion laser (488 nm) was used to measure forward and side scatter (488 ± 10 nm)—green fluorescent for FITC (530 ± 30 nm) and red fluorescent for Cy3 (575 ± 26 nm); 40 μL of specimens of each sample was collected in duplicate. Data were analyzed with use of BD FACSDiva software (Becton Dickinson). The

total amount of bacteria was determined with use of the EUB 338 FITC probe. Determination of specific bacteria was made by combining each of the group-specific Cy3 probes with the EUB 338 FITC probe and counting double-positive cells.[9]

For the quantitative polymerase chain reaction (PCR) (qPCR) analysis, fecal samples were stored at −80°C until analyzed. Samples were pretreated and DNA was extracted using an automated KingFisher DNA extraction system (Thermo Fisher Scientific Oy, Vantaa, Finland) and InviMag Stool DNA kit (Stratec Molecular, Berlin, Germany), as previously described.[10] The standard DNA for qPCR was prepared as previously described.[10] All DNA samples were stored at −20°C until analyzed. qPCRs were conducted as previously described.[7,11] PCR amplification and detection were performed with an ABI PRISM 7300-PCR sequence detection system (Applied Biosystems, Foster City, California).

Statistical Analyses

Univariate associations between clinical characteristics and the dichotomous outcome variable of excessive crying were studied using logistic regression analysis. All continuous variables such as microbiota variables and stool frequency were compared between the groups using Kruskal-Wallis test. Associations between study group and categorical clinical characteristics were studied using χ^2 test. All analyses were performed on data from the intention-to-treat population. P values <.05 were considered statistically significant.

Results

A total of 94 infants were eligible for inclusion and were equally distributed between the intervention groups (Figure; available at www.jpeds.com). All participants were white. The mean (range) gestational age of the infants was 34.6 (32-36) weeks and the mean (range) gestational weight was 2393 (1550-3965) g. Clinical characteristics were comparable among the study groups (Table II). The 1-year study was completed by 68 of the 94 infants (72%) (Figure). At the age of 1 year, the mean weight and height were comparable among the study groups (Table II). There were no withdrawals attributable to any adverse effects related to the study products or their administration regimen reported by the parents (Figure).

During the first 2 months of life, 27 of the 94 infants (29%) were classified as excessive criers, significantly less frequently among the prebiotic and probiotic groups than in the placebo group (19% vs 19% vs 47%, respectively; $P = .02$) (Table III). The frequency of stools (>3 stools/d) tended to be higher in the prebiotic than in the probiotic and placebo groups at the age of 1 month (71% vs 50% vs 57%, respectively; $P = .08$), but there was no difference between the groups in consistency of stool. The frequency of perinatal antibiotic treatment (42% vs 22%, respectively; $P = .07$) and the number of vaginal deliveries (81% vs 63%, respectively; $P = .07$) tended to be higher in excessive criers than among contented infants. Other possible factors influencing gut microbiota colonization, such as postnatal treatment in a neonatal intensive care unit, duration of breastfeeding, and antibiotic treatment during the first 2 months of life, were comparable between excessive criers and contented infants (Table III).

In analysis of the gut microbiota composition by use of FISH, a significant difference between the study groups was detected in the proportion of *Clostridium histolyticum*–type bacteria to total bacterial count. This proportion was significantly lower in the probiotic group compared with the prebiotic and the placebo group (8.9% vs 10.5% vs 13.9%, respectively; $P = .047$). There were no statistically significant differences in gut microbiota composition among the study groups in qPCR analyses (Table IV; available at www.jpeds.com).

Finally, we compared the gut microbiota composition between excessive criers and contented infants by FISH and qPCR at the age of 1 month. The proportion of *Lactobacillus-Lactococcus-Enterococcus* group to total bacterial count was

Table II. Clinical characteristic of the study subjects

Characteristic	Prebiotics (n = 31)	Probiotics (n = 31)	Placebo (n = 32)
Male	16 (52)	19 (61)	23 (72)
Vaginal delivery	20 (65)	24 (77)	20 (63)
Apgar score at 5 min	8 (2)	8 (1)	8 (1)
Maternal peripartal antibiotic treatment	6 (19)	10 (32)	10 (31)
Antibiotic treatment during the first 2 mo of age	20 (65)	18 (58)	17 (53)
Exclusively breast-fed, mo*	1.3 (1.9)	1.6 (2.2)	1.9 (2.1)
Total duration of breast-feeding, mo†	5.5 (3.3)	7.2 (4.4)	5.9 (4.5)
Weight at age of 1 y, g‡	9873 (1083)	9717 (1415)	10 107 (1633)
Length at age of 1 y, cm‡	75 (2)	75 (3)	76 (3)

Results are given as mean (SD) or as number (%) of subjects.
All clinical characteristics were comparable among the study groups ($P > .05$).
*n = 24 for prebiotic group, n = 22 for probiotic group, and n = 25 for placebo group.
†n = 23 for prebiotic group, n = 21 for probiotic group, and n = 23 for placebo group.
‡n = 22 for prebiotic group, n = 21 for probiotic group, and n = 23 for placebo group.

Table III. Clinical characteristics of contented infants and excessive criers

	Contented (n = 67)	Excessive crier (n = 27)	P
Study group			.02
Prebiotic	25 (37)	6 (22)	
Probiotic	25 (37)	6 (22)	
Placebo	17 (25)	15 (56)	
Male	38 (57)	20 (74)	.16
Vaginal delivery	42 (63)	22 (81)	.07
Gestational age at birth, wk	35 (0.1)	35 (0.3)	.92
Birth weight, g	2380 (60)	2424 (76)	.68
Birth length, cm	47 (0.3)	47 (0.5)	.86
Apgar score at 5 min	8 (0.2)	9 (0.2)	.29
Maternal perinatal antibiotic treatment	15 (22)	11 (41)	.07
Postnatal treatment in NICU	52 (78)	18 (67)	.28
Antibiotic treatment during the first 2 mo of life	41 (61)	14 (52)	.40
Exclusively breast-fed over 2 mo*	20 (42)	5 (22)	.09
Total duration of breast-feeding over 2 mo†	39 (85)	15 (71)	.21

NICU, neonatal intensive care unit.
Results are given as mean (SD) or as numbers (%) of subjects.
*n = 48 for contented infants and n = 23 for excessive criers.
†n = 46 for contented infants and n = 21 for excessive criers.

Table V. The mean (SD) proportion of different bacterium count to total bacterial count by FISH at the age of 1 month in content infants and excessive criers

	Contented (n = 45)	Excessive crier (n = 21)	*P*-value
Bifidobacterium	23.03 (21.54)	23.46 (22.07)	.38
Bacteroides-Prevotella	11.85 (10.99)	10.60 (5.97)	.58
Clostridium Histolyticum	10.36 (5.01)	13.23 (6.61)	.11
Lactobacillus-Enterococcus	10.51 (4.23)	14.52 (5.5)	.005
Akkermansia muciniphila	9.39 (3.79)	10.33 (4.53)	.33

higher in excessive criers than in contented infants (14.5% vs 10.5%, respectively; $P = .005$) (Table V). Moreover, the former tended to have an increased percentage of *Clostridium histolyticum* in their stools compared with the latter group (13.2% vs 10.4%, respectively; $P = .11$) (Table V). The species composition of *Bifidobacterium* also differed between these 2 groups: the number of *Bifidobacterium infantis* by qPCR was found to be decreased among excessive criers compared with contented infants (1.3×10^7 vs 2.5×10^8, respectively; $P = .035$). There were no other statistically significant differences in gut microbiota composition between these two groups.

Discussion

During the first weeks of life, irritability and crying of a preterm infant coincide with diverse maturational processes that take place in the immature gastrointestinal tract in response to massive antigen challenges by microbial colonization and food intake. Consequently, infant crying has been related to cow's milk allergy and deviating compositional development of the gut microbiota,[4,12,13] and the principal attempts to control irritability in full-term infants have focused on various dietary supplementations such as prebiotics and probiotics.[4,14-16] In the present study, specific prebiotics and probiotics, administered during the first 2 months of life to infants born preterm, not only were well tolerated, also in terms of normal growth, but also concomitantly provided relief to their crying and fussing. The clinical benefit was paralleled by a lowering of the relative number of pathogenic bacteria, *C histolyticum,* in stools.

Our results support earlier demonstrations in term infants on deviations in gut microbiota composition in colicky infants[4,13,17,18-20] and clinical benefit from gut microbiota modification with specific probiotics.[4,14] We extend these data in 3 respects: to infants born preterm, to specific microbial species, and to interventions with both probiotics and prebiotics.

In contrast to these previous studies, we chose to use questionnaires and interviews rather than cry diaries to identify those infants who cry excessively. Because our participant families were encountering extra stress due to the prematurity of their infant, we selected the former methods in the hope of enhancing adherence to the study regimen. Indeed, we consider our classification of infants into contented and excessive criers reliable, because both parents and our experienced study nurse, blinded to the randomization and in close contact with the families from the outset and throughout the follow-up period, participated in the process. In the same vein, we found that irritable preterm infants had higher numbers of *Lactobacillus-Lactococcus-Enterococcus* group than did the contented infants. Increased numbers of both *Enterococcus* and *Lactobacillus*, 2 of the first families colonizing the intestine, have been related to prematurity per se, thus suggesting that the preterm infant irritability found here reflects a less mature gut microbiota colonization process compared with contented infants.[21] Our molecular assays did not allow characterization of the *Lactobacillus-Lactococcus-Enterococcus* group of bacteria in more detail, to specify whether the mentioned difference was related to specific lactobacilli, enterococci, or both. Future research is thus needed to clarify this issue.

In the case of *Bifidobacterium* microbiota, typifying the microbiota of the healthy, term breast-fed infant, we have previously shown that different species may exert distinct effects on infant crying.[20] Our present findings suggest that delayed colonization by *B infantis* is linked to the risk of irritability in preterm infants. This species has been found to secrete bioactive factors capable of normalizing permeability defects in the intestinal mucosa.[22,23] Early *B infantis* colonization has also been associated with appropriate development of immune tolerance.[22,24] Furthermore, according to 1 recent probiotic study, supplementation of *B infantis* in untreated celiac patients significantly alleviated gastrointestinal symptoms, including constipation, indigestion, and reflux.[25] In light of the aforesaid and our findings here, we would consider a decreased number of *B infantis* and further delayed gut microbiota colonization combined with immature gut barrier function to be potential risk factors for the development of crying and irritability in preterm infants.

In this preterm population, we demonstrated a relative abundance of *C histolyticum* in the placebo group, suggesting that *L rhamnosus* GG promotes gut health through a reduction of *C histolyticum* colonization via a variety of mechanisms such as competitive exclusion.[26,27] Beyond the incompletely developed immune exclusion functions in preterm infants, it is suggested that high numbers of *Clostridia spp* might be associated with degradation of antigen-specific immunoglobulin A in the gut, which further impairs the immature gut barrier function.[28,29] Furthermore, *C histolyticum* has been shown to evoke proinflammatory cytokines such as tumor necrosis factor-α, which might already be overexpressed in antigen challenges in preterm infants.[30,31] Thus, imbalance between proinflammatory and anti-inflammatory cytokines might be one of the mechanisms behind infant crying, the symptom associated with increased amounts of calprotectin, a gut inflammatory marker.[13,31] Insufficient gut barrier function and aberrant immune responses, typical features of premature infants, could lead to an increased risk of oral tolerance failure and further excessive irritability and crying.

Notwithstanding differences in gut microbiota in the probiotic and prebiotic groups, the clinical outcomes appeared similar. Earlier studies using supplementation of formula

with polydextrose/galacto-oligosaccharide have been demonstrated to lessen abdominal discomfort, soften stools, and increase defecation frequency compared with placebo[11,32-34] but not fussiness or gassiness in term infants.[15,16] In our study, stool frequency tended to be higher in the prebiotic than in the probiotic or placebo groups, although the consistency of stools was comparable between the groups. Frequent defecation may lessen abdominal distention and flatulence, and thus constitute one of the possible reasons why the prebiotic intervention reduced infant irritability in our study.

In conclusion, breast-feeding is the first choice of nutrition for all infants, especially preterm, because it constitutes a key factor in the metabolic, immunological, and microbiological programming of the infant's health. However, the composition of breast milk varies and reflects maternal health status and diet and, consequently, influences the compositional development of the gut microbiota in the offspring.[2] The results presented here suggest that redirecting the deviating colonization process in the preterm infant gut microbiota by prebiotic and probiotic supplementation may offer a safe and well-tolerated means to optimize preterm infant well-being and to facilitate the development of novel preventive and therapeutic options against infant irritability and excessive crying. ■

We would like to thank the families participating in our study, Jaakko Matomäki, MSc, for statistical consultation, Robert MacGilleon, MA, for language review of the manuscript, and Ulla-Maija Eriksson, RN, and Anne Yrjänä, RN, for help with the follow-up of participants.

Submitted for publication Mar 4, 2013; last revision received Apr 15, 2013; accepted May 14, 2013.

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The Nature and Origin of Fluid in the Fetal Lamb Lung

Adams FH, Fujiwara T, Rowshan G. J Pediatr 1963;63:881-8

Fifty years ago, it must have been such an exciting time for researchers in the field of lung biology! Surfactant, a novel surface active material in the lung, had just been discovered. Avery and Mead's observation[1] that preterm infants afflicted with hyaline membrane disease lacked surfactant had spurred excitement around a potential remedy for this deadly disease. Yet, vexing questions remained: Where is surfactant made? How does it reach amniotic fluid? What kind of fluid fills fetal lungs, and where does it come from?

Fifty years ago, Adams et al also made the astute observation that certain characteristics of fetal lung fluid point to an active secretory process. They directed our attention to its low pH, low protein, low bicarbonate, and high chloride content; they also used the Gibbs-Donnan equation to make a strong case for an active secretory process, pointing to a big disparity in sodium and chloride ratios between the tracheal fluid and plasma. Years later, a Na-K-2Cl cotransport mechanism was found to be the primary source of fetal lung fluid secretion, a process that is readily suppressed by diuretics such as bumetanide.

Yet, the question of how the fetus reverses this process at birth and clears a large amount of lung fluid had to wait until 1994 for a definitive answer, when Hummer et al[2] showed that mice pups lacking a key epithelial sodium channel (ENaC) died within the first day or 2 of life, all from an inability to clear lung fluid. The subsequent cloning of ENaC allowed studies of its regulation by key endogenous hormones such as catecholamines and glucocorticoids. We now have a better understanding of how the alveolar surface fluid layer is regulated, balancing secretory forces (to which Adams et al eluded 50 years ago) and fluid absorption by ENaC and its related ion channels. Therapeutic interventions based on translation of this knowledge are currently in use or under investigation for conditions such as high-altitude pulmonary edema, cystic fibrosis, and respiratory distress in the newborn. The calf lung extract first introduced by Dr Fujiwara as a surfactant is still in use today. We have come a long way!

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http://dx.doi.org/10.1016/j.jpeds.2013.04.007

References

1. Avery ME, Mead J. Surface properties in relation to atelectasis and hyaline membrane disease. AMA J Dis Child 1959;97:517.
2. Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A, et al. Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. Nat Genet 1996;12:325-8.

Table I. Sequences of the probes used in FISH[9]

Probe	Target	Sequence from 5′ to 3′	Hybridization temperature
EUB 338	Total bacteria	GCT GCC TCC CGT AGG AGT	50°C
Bif 164	*Bifidobacterium*	CAT CCG GCA TTA CCA CCC	50°C
Bac 303	*Bacteroides-Prevotella*	CCA ATG TGG GGG ACC TT	45°C
Chis 150	*Clostridium histolyticum*	TTA TGC GGT ATT AAT CTY CCT TT	50°C
Lab 158	*Lactobacillus-Enterococcus*	GGT ATT AGC AYC TGT TTC CA	45°C
Muc 1437	*Akkermancia muciniphila*	CCT TGC GGT TGG CTT CAG AT	50°C

Y represents a (C/T) wobble nucleotide.

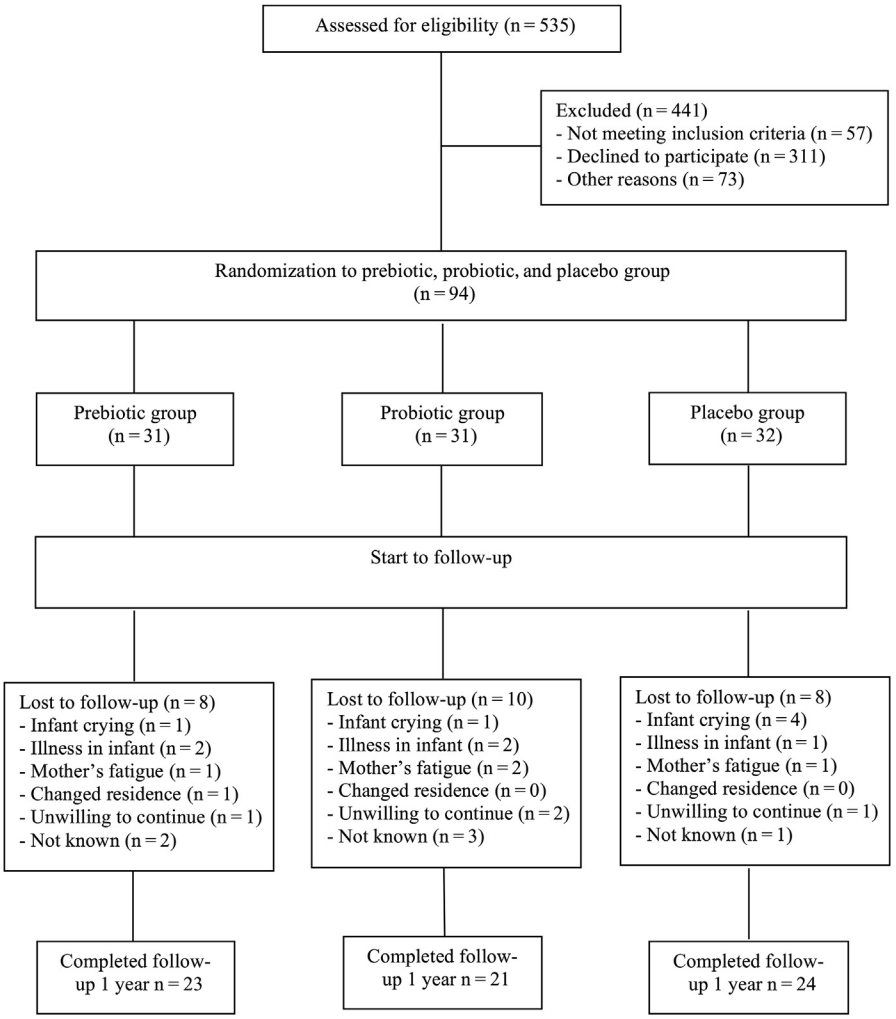

Figure. Flow chart of the procedure (N = 94).

Table IV. Proportion of different bacterium count to total bacterial count by FISH at the age of 1 month in placebo, probiotic, and prebiotic groups

Bacterium	Prebiotic (n = 25)	Probiotic (n = 19)	Placebo (n = 22)	*P*
Bifidobacterium	18.26 (18.24)	30.56 (26.76)	22.78 (19.50)	.17
Bacteroides-Prevotella	14.46 (13.87)	9.75 (5.96)	9.50 (4.89)	.59
C histolyticum	10.46 (5.15)	8.90 (2.92)	13.88 (6.81)	.047
Lactobacillus-Enterococcus	11.67 (5.51)	10.83 (4.48)	12.37 (4.74)	.64
Akkermansia muciniphila	9.73 (3.86)	8.66 (2.80)	10.41 (4.90)	.61

Results are given as mean (SD).