CLINICAL RESEARCH ARTICLE

Multi-strain probiotics for extremely preterm infants: a randomized controlled trial

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BACKGROUND: Effects of probiotics on intestinal microbiota and feeding tolerance remain unclear in extremely low-birth-weight (ELBW) infants.
METHODS: ELBW infants were randomly assigned to receive probiotics or no intervention. Stool samples were collected prior to, 2 and 4 weeks after initiation, and 2 weeks after probiotics cessation for infants in the probiotics group, and at matched postnatal age time points for infants in the control group.
RESULTS: Of the 102 infants assessed for eligibility, sixty-two were included. Infants who received probiotics reached full enteral feeds sooner (Mean difference (MD) −1.8; 95% CI:−3.7 to −0.01 day), had a tendency toward lower incidence of hematochezia before hospital discharge (22.6% vs 3.2%; $P = 0.053$), and were less likely to require extensively hydrolyzed- or amino acids-based formulas to alleviate signs of cow's milk protein intolerance in the first 6 months of life (19.4% vs 51.6%; $P = 0.008$). Infants on probiotics were more likely to receive wide-spectrum antibiotics (64.5% vs 32.2%; $P = 0.01$). Multi-strain probiotics resulted in significant increase in fecal *Bifidobacterium* ($P < 0.001$) and *Lactobacillus* ($P = 0.005$), and marked reduction in fecal *candida* abundance ($P = 0.04$).
CONCLUSION: Probiotics sustained intestinal *Bifidobacterium* and reduced time to achieve full enteral feeds in extremely preterm infants. Probiotics might improve tolerance for cow's milk protein supplements.
CLINICAL TRIAL REGISTRATION: This trial has been registered at www.clinicaltrials.gov (identifier NCT03422562).

IMPACT:

- Probiotics may help extremely preterm infants achieve full enteral feeds sooner.
- Probiotics may improve tolerance for cow's milk protein supplements.
- Multi-strain probiotics can sustain intestinal *Bifidobacterium* and *Lactobacillus* until hospital discharge.

INTRODUCTION
Extremely preterm infants are at high risk for intestinal microbiome perturbations due to high rates of Cesarean birth, prolonged stay in neonatal intensive care unit (NICU), and exposure to multiple antibiotics.[1] This state is characterized by overgrowth of pathogenic bacteria such as *Enterobacter* and *Pseudomonas* species at the expense of commensal and beneficial bacteria such as *Bifidobacterium* species.[2] Current evidence suggests that disruption and delayed microbiome maturation trajectory play an important role in the development of necrotizing enterocolitis (NEC)[3,4] and the pathogenesis of cow's milk protein intolerance.[5–7] NEC is a leading cause of mortality and morbidity in preterm infants, particularly extremely low birth weight (ELBW; less than 1000 g) infants. Cow's milk protein intolerance may result in frequent interruption of feeding plans and often prolonged time on parenteral nutrition during hospital stay.[8]

Probiotics are frequently proposed as beneficial supplements for preterm infants. Nonetheless, the evidence for benefits have been largely generated from small randomized controlled trials (RCTs).[9] A recent Cochrane systematic review indicated that probiotic supplementation in very low birth weight infants (VLBW; less than 1500 g) might reduce the risk of NEC, death, and serious infection.[9] However, only a few trials provided data for ELBW infants, and these trials did not show any benefit.[9] There are several gaps in current knowledge about the effect of probiotics on intestinal microbiota, the fate after administration of a specific probiotic strain to a given infant, and the degree of influence on the pre-existing and developing microbiome. Therefore, it is essential that gut microbial data are obtained and correlated with clinical data for the probiotic used, particularly in ELBW infants.

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Although many NICUs across the world have implemented routine probiotic supplementation, the low certainty of the evidence, limited benefits in ELBW infants who are at the highest risk for NEC, and the fear from probiotic infection[10,11] have resulted in significant skepticism. There are several gaps in current knowledge about the effect of probiotics on intestinal microbiota, the fate after administration of a specific probiotic strain to a given infant, and the degree of influence on the pre-existing and developing microbiome. Therefore, it is essential that gut microbial data are obtained and correlated with clinical data for the probiotic used, particularly in ELBW infants. A recent network meta-analysis of 45 RCTs suggested a combined use of Lactobacillus, Bifidobacterium, and prebiotics to achieve optimal effects on preterm infants' health.[12] However, due to a lack of cumulative effects between Bifidobacterium, Lactobacillus and prebiotics, and insufficient data for extremely preterm infants, the authors indicated the need for more RCTs to identify the benefits and optimal dosage in ELBW infants.

The aim of our RCT was to examine the effects of the combined use of Bifidobacterium, Lactobacillus, and prebiotics on intestinal microbiota and to study the association between probiotics-altered gut microbiota and feeding outcomes in ELBW infants.

METHODS
Study design and participants
This study was an open-label, parallel, RCT conducted between October 2017 and December 2019 in the level III neonatal intensive care unit (NICU) of Foothills Medical Centre in Calgary, Alberta, Canada. The trial was approved by the Conjoint Research Ethics Board of the University of Calgary and registered at www.clinicaltrials.gov (identifier NCT03422562). Written informed consent was obtained from parents prenatally or within 24 h after birth.

Infants born less than $29^{0/7}$ weeks gestation and weighing ≤1000 g were eligible for the study. Infants were excluded if they had major congenital anomalies, antenatal concern related to the fetal gastrointestinal tract, hypoxic-ischemic injury, and intestinal perforation before enrollment.

The primary outcome of the study was the changes in fecal microbiota after probiotic supplementation. The secondary outcomes included: (i) incidence of feeding intolerance defined by interruption in enteral feeding, unrelated to a clinical procedure or diagnosis of NEC, that lasted for equal to or more than 24 h, and (ii) time required to reach full enteral feeds defined by reaching 120 mL/kg per day. Our definition of feeding intolerance did not differentiate between causes related to immaturity of gastrointestinal functions and cow's milk protein intolerance/allergy (CMPA). Therefore we elected to report the incidence of CMPA separately. CMPA defined by occurrence of recurrent vomiting, significant abdominal distension, or presence of frank blood in stool in the absence of clinical or radiological evidence of NEC that improved after elimination of cow's milk protein products from mother and infant's diets. A registered dietitian counseled lactating mothers to avoid products that contain cow's milk protein. Extensively hydrolyzed protein (Nutramigen® or Pregestimil®) or amino acid-based formula (Neocate®) were used to fortify expressed human milk or as a sole formula in exclusively formula-fed infants with suspected CMPA.

Randomization
A biostatistician, who was independent of the study investigators, conducted the randomization sequence using SAS statistical software (SAS Institute Inc., Cary, NC). Randomization was stratified to <750 g and 750–1000 g. Allocation was 1:1 with random block size of 4. The sequence code was kept in sequential numbered sealed opaque envelopes. Infants from multiple births who met the inclusion criteria were included as one unit and randomized to the same arm of the study. Once the informed consent was obtained from a parent, the research assistant or a study investigator opened the numbered sealed envelope to reveal the study group.

Study intervention
The probiotic supplement used was Florababy (Appendix 1). Infants in the study group received one probiotic sachet per day once the enteral feeding started, and after informed consent was obtained. Probiotic sachets were mixed with mother's own milk (MOM) or donor human milk (DHM) by the infant's registered nurse at the bedside and were administered via naso- or oro-gastric feeding tube. Probiotics were held if the infant was placed nil per os, and restarted when the most responsible physician recommended resuming feeding. The study group received probiotics until 37 weeks corrected gestational age (CGA) or hospital discharge, whichever occurred earlier. Probiotics (Florababy®) are used routinely for preterm infants weighing ≥1000 g in our NICU. Given the ongoing concern about the commercially available probiotics preparation and potential contamination,[13] we designed the study in a way that infants in the control group receive no placebo to minimize the exposure to the manufactured products. We intended to evaluate the product as a whole before expanding its use for ELBW infants. All infants in the study received oral immune therapy by administering 0.1–0.2 mL of MOM every 4 h as soon as it was available. Actual feeds of MOM or DHM were initiated within 12 h of life based on standardized feeding tables specific to birth weight.

Fecal sample collection. Nurses collected stool samples at four time points in NICUs: prior to, 2 weeks and 4 weeks after probiotic administration was commenced, and 2 weeks after cessation. Stool samples for the control group were collected at matched postnatal age time points. Infants discharged home prior to the 4th collection time point had their stool sample collected and stored in a home fridge by their parents. A courier transported home-collected samples in a cold chain from the participant's home to Alberta Precision Laboratories. Microbial analyses are summarized in Appendix 2. Species-specific primer was developed to identify that Bifidobacterium and Lactobacillus in the probiotic group were the same as in the probiotics sachets.

Statistical analyses. Assuming an alpha value of 0.05 and a power of 0.80, a sample size of 60 was calculated to detect a 0.70 effect size for a two-tailed Wilcoxon–Mann–Whitney test when comparing relative abundance of fecal probiotics microbial species between the 2 groups after a conservative assumption of a background logistic distribution.

Descriptive statistics including means, medians, and standard deviations (SD) were used to describe the study population. Continuous variables were analyzed using independent, two-sample t tests or Wilcoxon rank-sum test as appropriate. Categorical variables were evaluated using χ^2 and Fisher's exact tests. All statistical tests were two-tailed, based on intention-to-treat principle, and the level of significance was set at 0.05. For continuous outcomes with outliers, we used robust regression analysis to examine the effect of probiotics on these outcomes. Changes in outcome measures over time (T1, T2, T3, and T4), the main effect of randomization groups, and randomization group (probiotics) by time (T) interactions were investigated on an intention-to-treat basis using linear mixed-effects regression models (LMA) accounting for correlations arising from repeated measures. LMA models allow using all data available from each participant under the assumption of missing at random. Whether changes in outcomes over time differed by randomization groups were evaluated by examining the interaction effects of group (probiotics) by time (probiotics × time point). R version 4.0.5 (R Development Core Team, Vienna, Austria) R and STATA16 (Stata Corporation, College Station, TX) were used.

RESULTS
Study population
One hundred and two infants were assessed for eligibility. Forty of them were excluded for parental refusal to participate ($n = 38$) and not meeting the inclusion criteria ($n = 2$). A total of 62 infants were included in the trial. Three participants randomized into the probiotics group developed spontaneous intestinal perforation (SIP) before the use of probiotics but after the first stool sample collection, therefore they did not receive the probiotic treatment. Of note, there were no changes to the statistical significance when these infants were excluded therefore we kept them for the analysis. Two infants in probiotics group died due to severe lung disease that led to respiratory failure. Death of these two infants occurred after time point 3 at 42 and 46 days of life. Figure 1 shows the participants flow diagram.
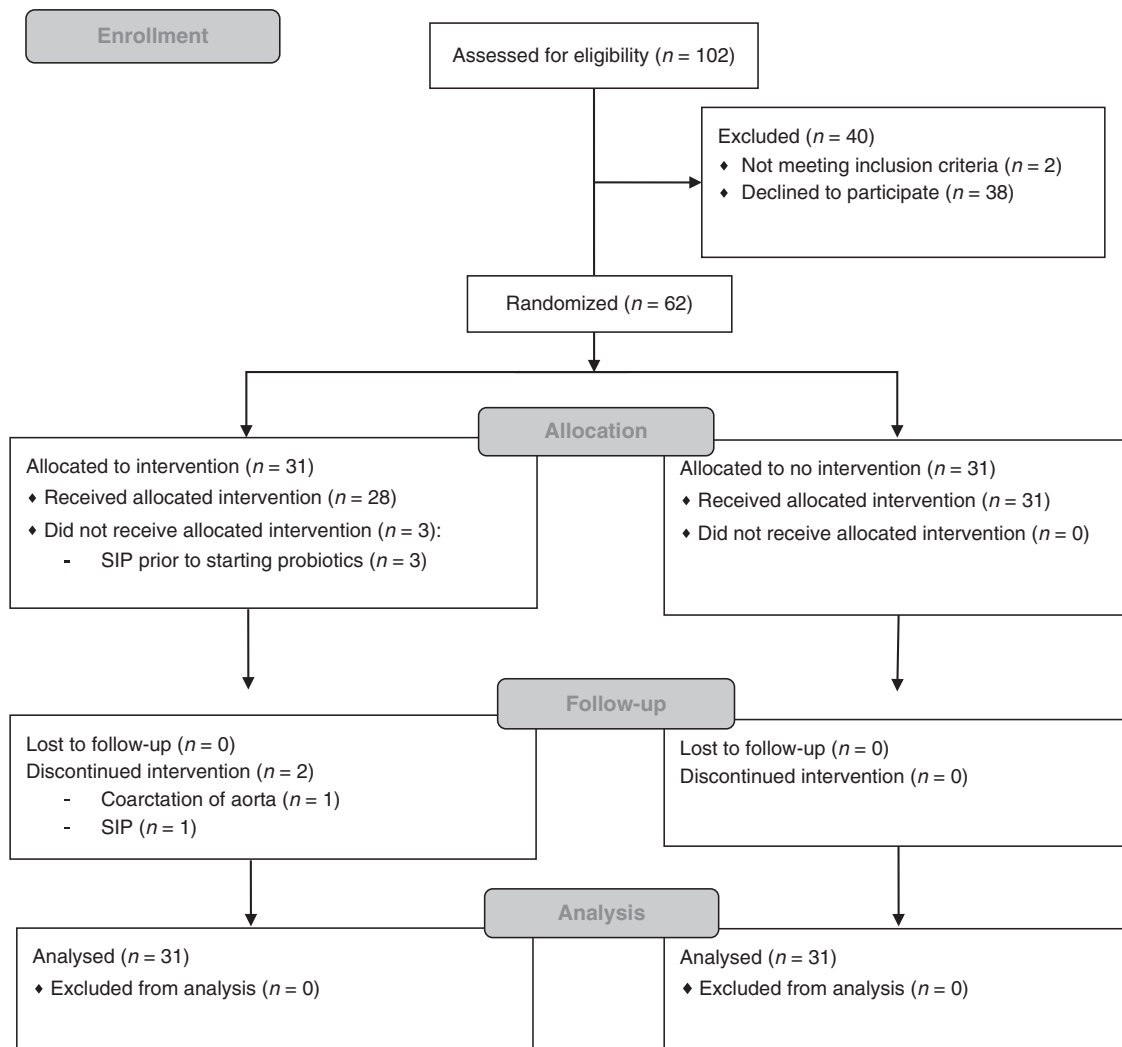
Fig. 1 Study flow diagram. CONSORT flow diagram of study participants. SIP spontaneous intestinal perforation.

Clinical outcomes

Maternal and neonatal baseline characteristics are summarized in Table 1. Clinical outcomes of the study are summarized in Table 2. Major neonatal morbidities including culture-proven sepsis were similar between the two groups. The organisms identified by blood culture in the control group were Coagulase-negative *Staphylococcus* ($n = 2$) and *Escherichia coli* ($n = 1$). The organisms identified in the probiotics group were Coagulase-negative *Staphylococcus* ($n = 2$), *E. coli* ($n = 3$) and *Staphylococcus aureus* ($n = 3$). There was no sepsis with any of the probiotics strains. Total antimicrobial days of therapy (DOT) was higher in the probiotics group. Twenty (64.5%) infants in the probiotics group received wide-spectrum antibiotics compared to 10 (32.2%) in the control group. Proportion of infants that received each anti-microbial drug is presented in supplementary Table S3.

Duration of parenteral nutrition, total duration of central venous access, and length of hospital stay were similar between the two groups, however, outliers were common for duration of parenteral nutrition, duration of central venous access, and time to full enteral feeds. Using robust regression methods revealed similar durations for parenteral nutrition (MD 0.4; 95% CI: −4.6 to 3.8 day) and central vascular catheter days (MD 24.4; 95%CI: −59.0 to 10.2 days per 1000 patient-days) but less time to full enteral feeds in the probiotics group (MD −1.8; 95% CI: −3.7 to −0.01 day).

More infants in the control group required elimination of cow's milk protein-based fortifiers from mother and infant's diet due to

suspected CMPA between birth and 6 months CGA (Table 2). The post-discharge use of extensively hydrolyzed protein- or amino acids-based formula was directed by pediatricians who were neither affiliated with our NICU nor aware of the study.

Intestinal microbiome

Species richness and diversity. Before hospital discharge, 216 fecal samples were collected and subjected to 16S rRNA gene sequencing. Number of fecal samples at each time point, and postnatal age when the samples are collected are summarized in Supplementary Table S4.

Figure 2 shows the changes in relative abundance of genera over the study period. *Bifidobacterium* was dominant in the probiotics group with high relative abundance during supplementation (T2–T3) and at 2 weeks after cessation of probiotics (T4) (Supplementary Fig. S1). *Lactobacillus* was detected in a fewer number of infants in both the probiotics and control groups with a higher relative abundance during supplementation (T2–T3) in the probiotics group compared with control group (Supplementary Fig. S1). The effects of probiotic supplementation, postnatal age (time points), and the interaction between probiotics and time points are summarized in Supplementary Table S5. Postnatal age has significant effect on *Bifidobacterium*, *Staphylococcus* and *Enterobacteria*, particularly after 2–3 weeks of age (after T2). Significant interactions are noted between probiotics and time point for *Bifidobacterium* and *Lactobacillus* only.

Table 1. Baseline characteristics of study participants.

	Control group (n = 31)	Probiotics group (n = 31)	P value
Gestational age (week), mean (SD)	25.6 (1.3)	25.8 (1.5)	0.53
Birth weight (g), mean (SD)	751 (132)	763 (209)	0.79
Pregnancy induced hypertension, n (%)	8 (25.8)	9 (29.0)	0.78
Gestational diabetes, n (%)	0 (0.0)	2 (6.5)	0.49
Membranes ruptured, n (%)			0.34
<24 h	23 (74.2)	23 (74.2)	
24 h to 7 days	6 (19.4)	3 (9.7)	
>7 days	2 (6.5)	5 (16.1)	
Antenatal steroids, n (%)	30 (96.8)	28 (90.3)	0.30
Chorioamnionitis, n (%)	5 (16.1)	3 (9.7)	0.71
Maternal antibiotics within 24 h from birth, n (%)	19 (61.3)	24 (77.4)	0.17
Maternal antibiotics in 24 h excluding pre-operation for Cesarean section, n (%)	14 (45.2)	11 (35.5)	0.44
Cesarean section, n (%)	25 (80.7)	23 (74.2)	0.54
Primiparous mother, n (%)	18 (58.1)	25 (80.1)	0.06
Male sex, n (%)	21 (67.7)	16 (51.6)	0.20
Multiple birth, n (%)	8 (25.8)	10 (32.3)	0.58
APGAR score at 5 min	7 (5, 7)	6 (5, 7)	0.13
Small for gestational age, n (%)	4 (12.9)	8 (25.8)	0.34
Any use of antibiotics, n (%)	31 (97)	28 (90.4)	0.61
Initial empiric antibiotics*, n (%)	26 (83.9)	23 (74.2)	0.35
Duration of initial empiric antibiotics (day), median (IQR)	2 (2, 2)	2 (0, 5)	0.60

*First 48 h of life.

A significant increase was observed in species richness and diversity over time in both groups (Supplementary Fig. S2). There were no significant effect of probiotics on Shannon or Chao1 indices as indicated by the lack of interactions between probiotics and time points (probiotics x time point) when the linear mixed-effect models used to adjust for gestational age, Cesarean birth, early use of antibiotics, and type of feed (Fig. 3). Of note, both early use of antibiotics and type of feed, particularly formula, have significant influence on alpha diversity indices. There was significant difference in beta diversity after probiotic supplementation (Supplementary Fig. S2). The effect of probiotics on beta diversity continued after probiotics cessation.

Fungal species. Probiotic supplementation resulted in a transient increase in fungal alpha diversity (Shannon) at time point 4 (Supplementary Fig. S3A). These effects disappeared after probiotics cessation. In contrast, fungal beta diversity (beta dispersion) decreased during probiotic supplementation (Supplementary Fig. S3B). Beta dispersion continued to be lower in the probiotic group after probiotics cessation. Figure 2b shows the relative abundance of fungal phyla and genera in control and probiotics groups. Probiotic supplementation resulted in a significant decrease in heterogeneity of fungal microbiome with marked reduction in *candida sp* abundance ($P = 0.04$).

DISCUSSION
Probiotic supplementation for ELBW infants in our study led to less time required to establish full enteral feeds and a lower requirement for extensively hydrolyzed- or amino acids-based formulas to alleviate signs of cow's milk protein intolerance. Probiotic supplementation also increased *Bifidobacterium* and sustained *Lactobacillus*. Postnatal age, early empiric antibiotic use after birth, and type of milk feeding appear to have more effect on microbial diversity than probiotics.

Feed intolerance is commonly encountered in ELBW infants and often leads to disruption of the enteral feeding plan and delayed time to attain full enteral feeds.[8] Our finding of lower incidence of feeding intolerance with probiotic supplementation is consistent with that in the literature. In a systematic review and meta-analysis by Athalye-Jape et al., probiotics reduced the time to full enteral feeds in preterm infants with a mean difference of −1.54 days (95% CI −2.75, −0.32).[14] A more recent prospective study nested within a RCT investigating single (*Bifidobacterium breve*) versus multi-strain (*Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum*) probiotic supplementation showed significant reduction in the time to full feeds and any hemorrhagic gastric residuals in extremely preterm infants who received single- or multi-strain probiotics compared with infants who received placebo.[15] Improved gastric emptying and gut motility may explain the benefits of probiotics (*Limosilactobacillus* formerly *Lactobacillus Reuteri*) on feed tolerance, particularly in formula-fed preterm infants.[16,17] It is important to note that comparison between studies reporting feeding intolerance, particularly in relation to probiotic supplementation, is challenging given the lack of consensus on the definition.[18]

Infants who received probiotics in our study had lower rates of cow's milk protein intolerance, or suspected CMPA, and were less likely to require extensively hydrolyzed protein fortification during the first 6 months of life. In our study, the main reason to change the infants' feeds to extensively hydrolyzed or amino acid-based formula was the presence of blood in the stool in the absence of NEC or other causes such as anal fissure. Improvement of hematochezia with cow's milk protein elimination was used by health care providers to make the diagnosis of CMPA. Diagnosis of CMPA in preterm infants, particularly during NICU stay, remains challenging and is largely based on improvement of gastro-intestinal signs after exclusion of cow's milk protein from mother and infant's diets.[19,20] Cow's milk protein challenge; the gold

Table 2. Neonatal outcomes of study participants.

	Control group (n = 31)	Probiotics group (n = 31)	P value
Use of surfactant, n (%)	18 (58.1)	21 (67.7)	0.43
Severe intraventricular hemorrhage (≥ grade 3), n (%)	2 (6.5)	2 (6.5)	1.00
Spontaneous intestinal perforation, n (%)	0 (0.0)	4 (12.9)	0.11
Necrotizing enterocolitis, n (%)	0 (0.0)	2 (6.5)	0.15
Culture-proven late-onset sepsis, n (%)	3 (9.7)	8 (25.8)	0.18
Patent ductus arteriosus required treatment, n (%)	21 (67.7)	17 (54.8)	0.30
Total antibiotics DOT per 1000 patient-day, median (IQR)	97 (37, 129)	136 (71, 256)	0.027
Antimicrobial use in infants with negative-culture sepsis, n (%)	23 (74.2)	18 (58.1%)	0.18
Bronchopulmonary dysplasia, n (%)	27 (87.1)	26 (83.9)	0.75
Severe retinopathy of prematurity (≥ stage 3), n (%)	4 (12.9)	6 (19.4)	0.73
Length of hospital stay, median (IQR)	96 (81, 112)	107.5 (91, 126)	0.78
Duration of central vascular catheter per 1000 patient-days, median (IQR)	129 (95, 177)	108 (90, 258)	0.96
Nutrition			
Duration of parenteral nutrition (day), median (IQR)			
- All infants	17 (13, 20)	20 (11, 34)	0.24
- Infants without SIP	14 (12, 19)	14.5 (11, 25)	0.73
Duration of DHM (day), median (IQR)	3 (1, 17)	3 (2, 5)	0.41
Cumulative MOM intake in week 1 (mL/kg per week), median (IQR)	95 (20, 137)	44 (6, 90)	0.07
Time to full enteral feeds, median (IQR)	13 (12, 17)	12 (11, 19)	0.03
Use of any breastmilk at discharge, n (%)	8 (25.8)	9 (29.0)	0.78
Total duration of *nil per Os* in infants with feeding intolerance (days), median (IQR)	4 (2, 6)	3 (2, 6)	0.84
Extensively hydrolyzed protein or amino acid-based fortifier/formula, n (%)			
- Birth to hospital discharge	7 (22.6)	2 (6.4)	0.07
- Birth to 6 months CGA	16 (51.6)	6 (19.4)	0.008
Hematochezia excluding necrotizing enterocolitis (protein-induced enterocolitis), n (%)			
- Birth to hospital discharge	7 (22.6)	1 (3.2)	0.053
Growth			
Growth velocity between day 7 of life and 36 weeks CGA (g/kg per day), mean (SD)	18.5 (1.7)	17.4 (2.7)	0.08
Change in weight z-score between birth and 40 weeks CGA (Δ z-score), mean (SD)	−1.2 (0.7)	−1.2 (1.1)	0.88
Weight at 40 weeks CGA (g), mean(SD)	3516 (74)	3484 (78)	0.12
Head circumference at 40 weeks CGA (cm), mean (SD)	33.0 (1.3)	32.4 (2.5)	0.30
Length at 40 weeks CGA (cm), mean (SD)	48.1 (1.2)	48.0 (1.5)	0.62
Weight at 40 weeks CGA (z-score), mean (SD)	−1.6 (1.0)	−1.7 (1.6)	0.66
Head circumference at 40 weeks CGA (z-score), mean (SD)	−1.4 (−0.9)	−1.8 (1.8)	0.30
Length at 40 weeks CGA (z-score), mean (SD)	−2.5 (1.1)	−2.5 (1.2)	0.96
Extra-uterine growth restriction defined by loss of >1.28 z-score between birth and 40 weeks CGA, n (%)	0 (0%)	1 (3.45)	1.0
Weight <10th percentile at 40 weeks CGA, n (%)	17 (54.8)	15 (48.4)	0.61

DOT days of therapy, *DHM* donor human milk, *MOM* mother's own milk, *SIP* spontaneous intestinal perforation, *CGA* corrected gestational age.

standard for the diagnosis of CMPA, is rarely undertaken in preterm infants.[20] Data on probiotics for prevention of CMPA in preterm infants is scarce. A systematic review and meta-analysis reported that probiotic supplementation to mothers and term-born infants in the first 6–12 months of life is effective in reducing food hypersensitivity.[21] However, postnatal only supplementation failed to show similar benefit.[21] A sub-study of the ProPrems (probiotics to reduce incidence of late-onset sepsis in very preterm infants) trial revealed a similar incidence of food allergy including CMPA between infants who received probiotics (*B. infantis, B. bifidum*, and *Streptococcus thermophilus*) versus placebo.[22] Evidence suggests that certain commensal bacterial species (clostridia) and their metabolites, particularly short-chain fatty acids (butyrate), may positively modulate intestinal permeability and immune tolerance mechanisms.[23,24] Available data suggest that intestinal dysbiosis might be associated with CMPA in infants with IgE- and non-IgE-mediated CMPA.[7,25] Canani et al. indicated higher microbial diversity and significant decrease in *Bifidobacterium* with IgE-mediated CMPA.[26] Of note, extensively hydrolyzed casein formula containing *Lactobacillus rhamnosus* in the former study resulted in significant enrichment in fecal butyrate-producing bacteria.[26] In another study by Canani *et al.* in infants affected by non-IgE-mediated CMPA, microbial dysbiosis was characterized with an enrichment in *Bacteroides* and *Alistipes*.[7] Identification of specific bacteria that may contribute to the development of cow's milk protein intolerance or allergy could
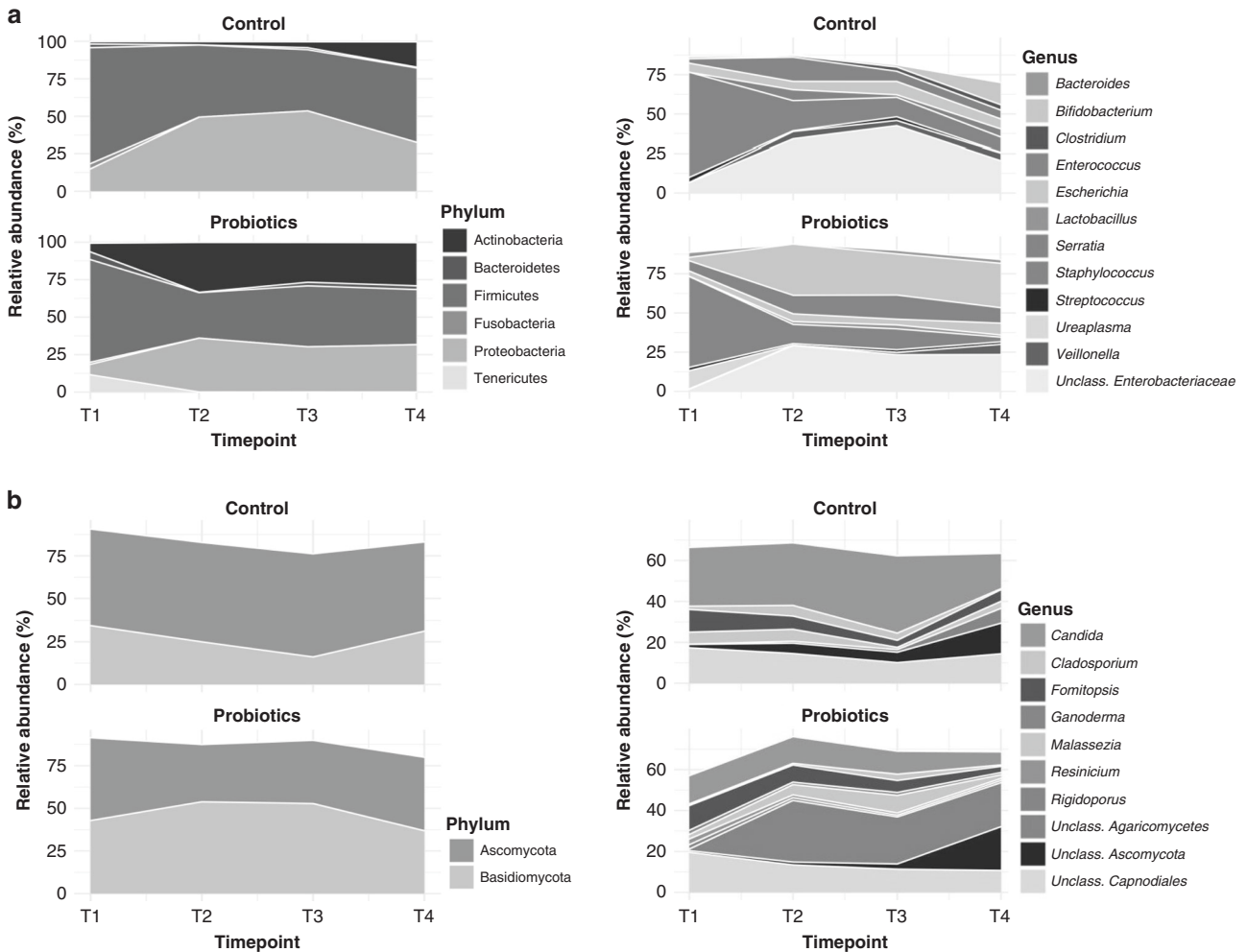
Fig. 2 Relative abundance of bacterial and fungal phylum and genera. a Relative abundance of bacterial phylum and genera in control and probiotics groups. **b** Relative abundance of fungal phylum and genera in control and probiotics groups. Temporal abundance plots include the most abundant taxa.

open the door for future prevention or treatment of these conditions.

Exposure to widespread antibiotics was more common in infants who received probiotics although the rate of sepsis was similar between the two groups. Esaiassen et al. reported similar findings in extremely preterm infants receiving probiotics. However, the infants in their control group were more mature.[27]

Probiotic supplementation resulted in significant increase in the relative abundance of *Bifidobacterium*. A recent cohort study by Alcone-Giner et al. revealed a similar association between probiotics (*Bifidobacterium* and *Lactobacillus*) supplementation and increased *Bifidobacterium* abundance that is comparable to natural gut colonization of full-term infants.[28] It is noteworthy to mention that *Bifidobacterium* became dominant within 2 weeks of probiotic supplementation. The rapid increase of *Bifidobacterium* is not surprising given that all preterm infants in our study were on breastmilk during the first 4 weeks of life; which provides the necessary human milk oligosaccharides for the growth of *Bifidobacterium*.[29] Nevertheless, the increase was more pronounced in infants who received probiotics. *Bifidobacterium* continued to be dominant after stopping probiotics in the study group. Yousuf *et al.* indicated that *Bifidobacterium* could persist at high abundance up to 5 months after stopping probiotic supplementation.[30]

The difference in the *Lactobacillus* abundance observed after 4 weeks of probiotic supplementation was driven by the decline in the control group while maintaining stable, albeit very low,

relative abundance in the probiotics group. The lack of association between probiotics exposure and the abundance of *Lactobacillus* is described in several studies in preterm infants and could be multifactorial.[30–32] Stool analysis may underestimate *Lactobacillus* abundance as *Lactobacillus* colonizes the small intestine and attaches to colonic mucosa.[33] Watkins et al. reported late increases in relative *Lactobacillus* abundance after 31 weeks CGA using another product (Infloran®) that contains 1 billion CFU of bifidobacteria and 1 billion CFU of *Lactobacillus*.[32] Whether a higher *Lactobacillus* dose can enhance genus colonization or confer clinical benefit is yet to be determined.

Probiotic supplementation in our study did not affect alpha diversity measures. In contrast, beta diversity analysis showed a marked shift in bacterial community during and two weeks after probiotics cessation. Other studies in preterm infants described similar findings. Yousuf et al. found no effect of probiotic supplementation on alpha diversity in a small cohort of preterm infants.[30] In their study, microbial communities from probiotic-exposed infants at term equivalent age were found to cluster more with full-term compared with those from unexposed participants.[30] Of note, postnatal age as reflected by the time point of stool collection, early use of empiric antibiotics, and feeding formula had important impact on alpha diversity. These findings are consistent with other studies in preterm infants.[30,34]

Supplementation with multi-strain probiotics modified intestinal mycobiome with a marked anti-*Candida* effect. This agrees
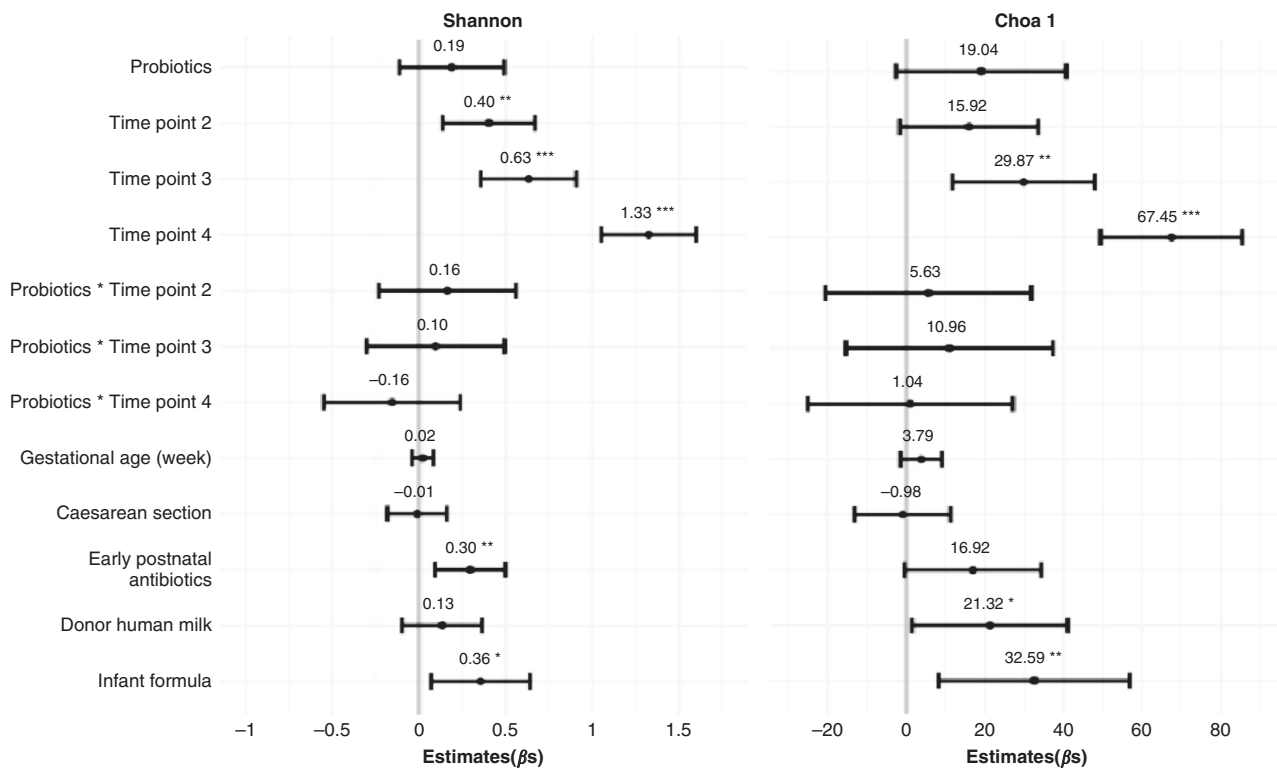
Fig. 3 Effect of probiotics supplementation on bacterial diversity and richness. Linear mixed-effect model analysis for the effect of probiotics supplementation, time, gestational age, Cesarean section, early postnatal antibiotics and type of feeding on diversity (Shannon) and richness (Chao1) measures of alpha diversities.

with previous studies that primarily involved older preterm infants.[35,36] A systematic review and meta-analysis in preterm infants indicated that probiotics can reduce the risk of *Candida* colonization in preterm neonates in NICUs and may prevent invasive fungal sepsis.[35] Similar effects are also reported in a single-strain probiotics. In a RCT in VLBW infants, Manzoni et al revealed, that oral supplementation with *Lactobacillus casei* subspecies *rhamnosus* prevents enteric colonization by *Candida* species.[36] Our findings add to the growing body of evidence on the role of probiotics in stabilizing not only the bacterial but also the intestinal fungal microbiome of preterm infants.

Strengths of our study include the randomized controlled design, longitudinal collection of stool samples, and the inclusion of ELBW infants who are at the highest risk for intestinal dysbiosis and complications. Nonetheless, our study has some limitations that include the lack of a placebo control to account for the prebiotic effect of maltodextrin and ascorbic acid, and the limited ability to explore the effects of probiotics on common morbidities such as NEC due to small sample size. Furthermore, the diagnosis of cow's milk protein intolerance or allergy is made based on clinical signs and health care providers may have used different criteria to define these conditions.

CONCLUSION
Multi-strain probiotic supplementation for ELBW infants during hospitalization in NICU may result in improved feed tolerance and lower the incidence of suspected cow's milk protein intolerance. Although the signs of CMPA, particularly the hematochezia, improved shortly after utilizing the cow's milk protein elimination diet in the NICU, a challenge with cow's milk protein-based diet before discharge would have improved the accuracy of our CMPA diagnosis. Probiotic supplementation results in a significant increase in fecal *Bifidobacterium* and, to less extent, *Lactobacillus*. Further research is needed to

identify whether higher doses or different ratios between *Bifidobacterium* and *Lactobacillus* can induce favorable microbiome composition and explore functional roles of probiotic supplementation on the intestinal immune development.

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AUTHOR CONTRIBUTIONS
B.A. conceptualized, designed, supervised the study, and drafted the initial protocol and the manuscript. J.S. supervised the recruitment, collected the data, performed the microbial analysis, and critically reviewed the manuscript. A.S. interpreted the data and critically reviewed the manuscript. S.M. and T.F. conducted the statistical analyses and critically reviewed the manuscript. D.D. supervised the drug supplementation and distribution, and critically reviewed the manuscript. M.A. reviewed the protocol, supervised the microbial analyses, interpreted the data, and critically reviewed the manuscript. H.A. designed the study, and critically reviewed the manuscript.

COMPETING INTERESTS
The authors declare no competing interests.

CONSENT STATEMENT
Consent from parents was obtained.

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