A Comparison of Two Probiotic Strains of Bifidobacteria in Premature Infants

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Objective To determine the impact of 2 probiotic bifidobacteria on the fecal microbiota of premature infants fed either human milk or formula.

Study design In the first of two phase 1 clinical trials, 12 premature infants receiving formula feedings were assigned randomly to receive either *Bifidobacterium longum* ssp *infantis* or *Bifidobacterium animalis* ssp *lactis* in increasing doses during a 5-week period. In the second, 9 premature infants receiving their mother's milk received each of the two bifidobacteria for 2 weeks separated by a 1-week washout period. Serial stool specimens from each infant were analyzed by terminal restriction fragment-length polymorphism and quantitative polymerase chain reaction for bacterial composition.

Results Among the formula-fed infants, there was a greater increase in fecal bifidobacteria among infants receiving *B infantis* (Binf) than those receiving *B lactis* (Blac). This difference was most marked at a dose of 1.4×10^9 colony-forming units twice daily ($P < .05$). Bacterial diversity improved over dose/time in those infants receiving Binf. Among the human milk-fed infants, greater increases in fecal bifidobacteria and decreases in γ-*Proteobacteria* followed the administration of Binf than Blac. The *B longum* group (which includes Binf but not Blac) was the dominant bifidobacteria among the human milk-fed infants, regardless of the probiotic administered.

Conclusions Binf was more effective at colonizing the fecal microbiota than Blac in both formula-fed and human milk-fed premature infants. The combination of human milk plus Binf resulted in the greatest fecal levels of bifidobacteria. *(J Pediatr 2013;163:1585-91)*.

Necrotizing enterocolitis (NEC) is a common and devastating disease of premature infants. Multiple clinical trials have demonstrated a decrease in the risk of NEC with the oral administration of probiotic microorganisms.[1,2] Probiotics are dietary supplements containing live bacteria that are intended to improve intestinal health. In the US, the Food and Drug Administration considers probiotics in the category of supplements "generally regarded as safe" but has not approved administration to treat or prevent disease. The routine administration of probiotics to premature infants has not been recommended because of concerns about safety, efficacy, and many unanswered questions such as optimal dosage, optimal organism or combination of organisms, and the purity, composition, and oversight of available probiotic products.[3]

To begin to address some of these questions, we performed an open dose-escalation trial and a cross-over trial of 2 strains of bifidobacteria, *Bifidobacterium longum* ssp *infantis* (American Type Culture Collection strain 15697) and *Bifidobacterium animalis* ssp *lactis* (University of California, Davis [UC Davis] strain 316), in premature infants. These 2 strains were chosen for their genetic diversity. The *B infantis* (Binf) strain has encoded in its genome the enzymes necessary to digest and use the human milk oligosaccharides (HMOs). This strain has evolved the capacity to thrive in the presence of oligosaccharides produced specifically by the mother to shape the intestinal microbiota of her infant.[4,5] The *B lactis* (Blac) strain is a member of a species that is popularly used as a probiotic and able to consume lactose. However, Blac does not contain the enzymes necessary to digest HMOs in its genome and, in fact, is unable to thrive in an environment in which HMOs are the sole carbon source (**Figure 1**; available at www.jpeds.com).[6] Our primary outcome was changes in composition and diversity of the intestinal microbiota with changes in dosage and types of

Bac-TRFLP	Bacilli-specific terminal restriction fragment-length polymorphism	H+Blac	Human milk plus Blac
		HMO	Human milk oligosaccharides
		NEC	Necrotizing enterocolitis
Bif-TRFLP	Bifidobacteria-specific terminal restriction fragment-length polymorphism	PBS	Phosphate-buffered saline
		PCoA	Principal coordinate analysis
		qPCR	Quantitative polymerase chain reaction
Binf	*B infantis*		
Blac	*B lactis*	rRNA	Ribosomal RNA
cfu	Colony-forming units	TRFLP	Terminal restriction fragment-length polymorphism
F+Binf	Formula plus Binf		
F+Blac	Formula plus Blac	UC Davis	University of California, Davis
H+Binf	Human milk plus Binf	WO	Washout

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Supported by the *Eunice Kennedy Shriver* National Institute of Child Health and Development, the National Institute of Allergies and Infectious Diseases, the National Institute of Diabetes and Digestive and Kidney Diseases (R01-HD059127), and the National Center for Advancing Translational Sciences, National Institutes of Health (UL1 TR000002). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The authors declare no conflicts of interest.

Registered with ClinicalTrials.gov: NCT00810160.

 http://dx.doi.org/10.1016/j.jpeds.2013.07.017

probiotics in infants receiving either formula or mother's own milk. We hypothesized that both strains would be detectable in the feces of premature infants in a dose-dependent manner, that both bifidobacterial strains would colonize the formula-fed infants similarly (ie, the Binf strain would have no advantage in the absence of human milk), and that infants receiving human milk plus Binf (H+Binf) would have more fecal bifidobacteria, fewer γ-*Proteobacteria*, and greater diversity than the other groups of premature infants.

Methods

This 2-trial study was approved by the Institutional Review Board at the UC Davis and performed at the UC Davis Children's Hospital in Sacramento, California, from June 2009 to July 2012. Written informed consent was obtained from the infants' parents. In the first trial, 12 formula-fed premature infants (birth weight <1500 g, gestational age <33 weeks) were assigned randomly to receive increasing doses of either Binf (ie, formula plus Binf [F+Binf]) or Blac (ie, formula plus Blac [F+Blac]) for 5 weeks. The dosage schedule was based on the range of published doses in this population at the time of study design (**Table I**; available at www.jpeds.com). The study group was not blinded, although specimens were labeled with randomly generated numbers to blind the laboratory analysis. In the second trial, an additional 9 premature infants (birth weight <1500 g, gestational age <33 weeks) receiving their mother's own milk were assigned randomly to receive Binf (4×10^9 organisms twice daily) for 2 weeks followed by a 1-week washout (WO) period (no probiotics) and then Blac (same dose) for 2 weeks (Group H+Binf/Blac) or the alternative (Blac first and then Binf, Group H+Blac/Binf).

To avoid the challenges of using over-the-counter probiotic products (eg, unknown composition and viability[7]) the 2 strains of bifidobacteria were grown by a food-grade commercial facility (Culture Systems, Inc, Mishawaka, Indiana) specifically for this study and stored at −80°C. Purity and number of viable bacteria per gram of both probiotics were confirmed by the investigators every 6 months by culture. The probiotic doses were prepared each day by the UC Davis investigational pharmacy by dissolving the freeze-dried powder in water and administered twice daily through a feeding tube if present or directly into the infant's mouth if there was no feeding tube.

Stool specimens were collected from the formula-fed infants at baseline and then weekly for 5 weeks. Specimens from the human milk-fed infants were collected at baseline, after the first course of probiotics, after the WO period, and after the second course of probiotics. The stool specimens were collected from a soiled diaper, placed in a sterile container, refrigerated overnight, then diluted 1:1 with phosphate-buffered saline (PBS), homogenized, transported on dry ice, and stored at −80°C.

Details regarding DNA extraction, analysis of the fecal microbiota by terminal restriction fragment-length polymorphism (TRFLP) and quantitative polymerase chain reaction (qPCR), and statistical analysis of microbial composition and diversity with references are presented in the supplementary methods document (**Appendix** and **Table II**; available at www.jpeds.com).

Results

Descriptive statistics for the four groups are summarized in **Table III**. There were no side effects attributed to probiotic administration in any of the infants. There was one case of NEC during the period of study participation (Stage 2A based on established criteria)[8] in patient 45, who received human milk plus Blac (H+Blac). Clinical details for individual patients are presented in **Table IV** (available at www.jpeds.com).

The 2 approaches to analysis of the microbiota are complementary. TRFLP provides broad semiquantitative information about the percentage of the total bacterial population in a given group (in this case class/genus and phylum), and qPCR provides more precise quantification of total bacteria and group based on the primers chosen (in this case genus *Bifidobacterium*). For the percentage of bifidobacteria in this study, the correlation between TRFLP and qPCR was strong in the first trial ($R^2 = 31\%$) but weaker in the second trial ($R^2 = 9.8\%$).

Formula-Fed Infants: Dose-Escalation Trial

Figure 2, A (available at www.jpeds.com) is the Bray-Curtis principal component analysis (PCoA) of the TRFLP data

Table III. Demographics

	F+Binf (n = 6)	F+Blac (n = 6)	H+Binf/Blac (n = 4)	H+Blac/Binf (n = 5)
Sex (F)	5	2	1	2
Birth weight, mean (SD)	805 (238)	847 (305)	1016 (316)	812 (162)
Gestational age at birth, mean (SD)	25 (2)	26 (2)	27 (3)	26 (2)
Corrected gestational age at first dose, mean (SD)	31 (3)	31 (1)	31 (3)	30 (2)
Cesarean delivery, no.	5	6	3	4
Apgar score at 1 min, median (range)	7 (1-8)	5 (1-7)	4 (1-8)	5 (4-5)
Apgar score at 5 min, median (range)	7 (5-9)	7 (2-8)	6 (3-9)	8 (7-9)
Hispanic	1	1	2	0
Black	2	0	0	0
Multiples	1 surviving twin	1 set of twins	0	2 surviving twins
Days of antibiotics (at or before onset of study), mean (SD)	16 (14)	16 (16)	8 (7)	12 (8)
Days of antibiotics (during study period)	4 (4)	4 (9)	0	2 (2)
Number of infants with NEC during study period	0	0	0	1