JAMA Pediatrics | Original Investigation

Association of Exposure to Formula in the Hospital and Subsequent Infant Feeding Practices With Gut Microbiota and Risk of Overweight in the First Year of Life

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IMPORTANCE The effect of neonatal and infant feeding practices on childhood obesity is unclear. The gut microbiome is strongly influenced by feeding practices and has been linked to obesity.

OBJECTIVE To characterize the association between breastfeeding, microbiota, and risk of overweight during infancy, accounting for the type and timing of supplementary feeding.

DESIGN, SETTING, AND PARTICIPANTS In this study of a subset of 1087 infants from the prospective CHILD pregnancy cohort, mothers were recruited between January 1, 2009, and December 31, 2012. Statistical analysis was performed from February 1 to December 20, 2017.

MAIN OUTCOMES AND MEASURES Feeding was reported by mothers and documented from hospital records. Fecal microbiota at 3 to 4 months (from 996 infants) and/or 12 months (from 821 infants) were characterized by 16S ribosomal RNA sequencing. Infants with a weight for length exceeding the 85th percentile were considered to be at risk for overweight.

RESULTS There were 1087 infants in the study (507 girls and 580 boys); at 3 months, 579 of 1077 (53.8%) were exclusively breastfed according to maternal report. Infants who were exclusively formula fed at 3 months had an increased risk of overweight in covariate-adjusted models (53 of 159 [33.3%] vs 74 of 386 [19.2%]; adjusted odds ratio, 2.04; 95% CI, 1.25-3.32). This association was attenuated (adjusted odds ratio, 1.33; 95% CI, 0.79-2.24) after further adjustment for microbiota features characteristic of formula feeding at 3 to 4 months, including higher overall richness and enrichment of *Lachnospiraceae*. A total of 179 of 579 infants who were exclusively breastfed (30.9%) received formula as neonates; this brief supplementation was associated with lower relative abundance of *Bifidobacteriaceae* and higher relative abundance of *Enterobacteriaceae* at 3 to 4 months but did not influence the risk of overweight. At 12 months, microbiota profiles differed significantly according to feeding practices at 6 months; among partially breastfed infants, formula supplementation was associated with a profile similar to that of nonbreastfed infants (higher diversity and enrichment of *Bacteroidaceae*), whereas the introduction of complementary foods without formula was associated with a profile more similar to that of exclusively breastfed infants (lower diversity and enrichment of *Bifidobacteriaceae* and *Veillonellaceae*). Microbiota profiles at 3 months were more strongly associated with risk of overweight than were microbiota profiles at 12 months.

CONCLUSIONS AND RELEVANCE Breastfeeding may be protective against overweight, and gut microbiota may contribute to this effect. Formula feeding appears to stimulate changes in microbiota that are associated with overweight, whereas other complementary foods do not. Subtle microbiota differences emerge after brief exposure to formula in the hospital. These results identify important areas for future research and distinguish early infancy as a critical period when transient gut dysbiosis may lead to increased risk of overweight.

JAMA Pediatr. 2018;172(7):e181161. doi:10.1001/jamapediatrics.2018.1161
Published online June 4, 2018. Corrected on July 2, 2018.

Supplemental content

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Obesity originates early in life,[1] and breastfeeding appears to be protective against obesity.[2] Hypothesized mechanisms for this protection include the promotion of self-regulation in breastfed infants and the lower protein content of breast milk compared with infant formula.[3] Another potential mechanism involves modification of the developing gut microbiota, which contributes to nutrient acquisition, energy regulation, and fat storage.[4] Microbiota shifts have been associated, albeit inconsistently,[5] with obesity in adults, including lower diversity, enrichment of *Ruminococcus gnavus*,[6] and a higher ratio of Firmicutes to Bacteroidetes.[7] Microbiota transplant experiments in mice suggest that these associations are causal,[8] and studies of children[9-12] suggest that they originate early in life, although few studies have been conducted for infants. Breastfeeding is among the most influential factors shaping the infant gut microbiome because breast milk contains prebiotic oligosaccharides and probiotic microorganisms, including bifidobacteria.[13]

Despite this evidence, we do not fully understand how infant feeding practices affect the developing microbiota and influence weight gain. Studies often do not differentiate between partially breastfed infants receiving formula vs those receiving complementary foods, yet these forms of nutrition clearly provide very different substrates for microbiota. The definition of *exclusive breastfeeding* also varies, and few studies have accessed hospital records to confirm exclusivity in the neonatal period. To address these knowledge gaps, we characterized these specific infant feeding practices in the Canadian Healthy Infant Longitudinal Development (CHILD) birth cohort and examined their association with gut microbiota and risk of overweight in the first year of life.

Key Points

Question How do infant feeding practices influence gut microbiota and risk of overweight?

Findings Among 1087 infants from the Canadian Healthy Infant Longitudinal Development (CHILD) cohort, earlier cessation of breastfeeding and supplementation with formula (more so than complementary foods) were associated with a dose-dependent increase in risk of overweight by age 12 months; this association was partially explained by specific gut microbiota features at 3 to 4 months. Subtle but significant microbiota differences were observed after brief exposure to formula limited to the birth hospital stay, but these differences were not associated with overweight.

Meaning Breastfeeding may contribute to protection against overweight by modifying the gut microbiota, particularly during early infancy.

Methods

Study Design

We accessed data from the CHILD birth cohort (http://childstudy.ca) of 3495 families across 4 sites in Canada.[14] Women were recruited between January 1, 2009, and December 31, 2012, and remained eligible if they delivered a healthy, full-term infant. This study included 1087 infants enrolled in the general cohort at the Manitoba, Edmonton, and Vancouver sites. This subset is a representative selection of infants with fecal samples analyzed at 3 to 4 months (from 996 infants) and/or 12 months (from 821 infants), of which 730 infants had samples analyzed at both times (eFigure 1 in the Supplement). The rates of breastfeeding, overweight, and other demographics in this subset were similar to those of the general cohort (eTable 1 in the Supplement). The Human Research Ethics Boards at McMaster University, University of Manitoba, University of Alberta, University of Toronto, and University of British Columbia approved this study. Parents provided written consent at the time of enrollment.

Overweight

At 12 months of age (mean [SD] age, 12.4 [1.3] months), infants were weighed and measured by CHILD Study staff. Age- and sex-specific weight for length *z* (WFL*z*) scores were calculated according to World Health Organization standards.[15] A WFL*z* score greater than the 97th percentile was considered overweight, and a WFL*z* score greater than the 85th percentile was considered at risk for overweight[16]; these 2 groups were combined into a composite outcome for logistic regression analyses.

Infant Feeding

Mothers completed questionnaires at 3, 6, and 12 months post partum, reporting on breastfeeding and the introduction of formula and complementary foods. At 3 months, breastfeeding status was classified as exclusive (breast milk only), partial (breast milk and formula), or none (formula only). Using hospital data, we further classified infants as exclusively breastfed after hospital discharge if they briefly received formula in the hospital but were exclusively breastfed after hospital discharge. At 6 months, feeding was defined as exclusively breastfed (breast milk only), partially breastfed with formula (breast milk and formula, with or without complementary foods), partially breastfed without formula (breast milk and complementary foods), or not breastfed (formula with or without complementary foods). The duration of breastfeeding was determined from the earliest report of cessation of breastfeeding. For microbiota analyses, breastfeeding status was determined on the date of collection of the fecal sample. In this study, *breastfeeding* refers to feeding the infant breast milk, whether at the breast or from a bottle.

Covariates

Mode of birth, parity, gestational diabetes, infant sex, birth weight, and hospital-administered antibiotics to the mother or neonate were documented from hospital records.[17] Oral antibiotic use was reported by parents. As described previously,[18] the quality of the maternal diet was estimated using the Healthy Eating Index,[19] and the maternal prepregnancy body mass index was self-reported and validated against medical records. Data on maternal race/ethnicity, smoking status, educational level, and pet ownership were self-reported during pregnancy.

Fecal Microbiota Analysis

Fecal samples were collected at a home visit (3-4 months; mean [SD], 3.7 [1.0] months) and a clinic visit (12 months; mean [SD], 12.3 [1.2] months); DNA was extracted using the QIAamp DNA

Table 1. Crude and Adjusted Association of Infant Feeding Practices With Infant Weight Status at 12 Months

Breastfeeding Exposure	Prevalence of Overweight, No. (%)	Crude OR (95% CI) (n = 1020)	Adjusted OR (95% CI) With Multiple Imputation of Missing Data (N = 1087)[a]
Breastfeeding at 3 mo			
None (formula only)	53/159 (33.3)	2.11 (1.39-3.19)	2.02 (1.18-3.45)
Partial (breast milk and formula)	84/304 (27.6)	1.61 (1.13-2.30)	1.63 (1.09-2.44)
Exclusive after hospital discharge	35/171 (20.5)	1.09 (0.68-1.69)	1.13 (0.68-1.89)
Exclusive (breast milk only)	74/386 (19.2)	1 [Reference]	1 [Reference]
Breastfeeding at 6 mo		(n = 1001)	
None (formula with or without food)	77/249 (30.9)	2.11 (1.33-3.42)	1.59 (0.92-2.74)
Partial with formula (breast and formula with or without food)	81/296 (27.4)	1.77 (1.13-2.85)	1.43 (0.87-2.37)
Partial without formula (breast milk and food)	55/279 (19.7)	1.16 (0.71-1.90)	0.96 (0.57-1.64)
Exclusive (breast milk only)	31/177 (17.5)	1 [Reference]	1 [Reference]
Breastfeeding duration		(n = 978)	
<6 mo[b]	68/219 (31.1)	2.02 (1.39-2.93)	1.64 (1.06-2.52)
6 to <12 mo	85/309 (27.5)	1.70 (1.21-2.41)	1.47 (0.99-2.18)
≥12 mo	82/450 (18.2)	1 [Reference]	1 [Reference]

Abbreviation: OR, odds ratio.

[a] Adjusted for maternal body mass index, smoking, postsecondary education, race/ethnicity, cesarean delivery, dog in household, infant sex, any oral antibiotics between 0 and 12 mo, and study site.

[b] Excludes infants who were never breastfed. Breastfeeding refers to breast milk feeding regardless of feeding mode (at the breast or from a bottle).

Stool Mini Kit (Qiagen); and the 16S ribosomal RNA gene, hypervariable region V4, was amplified and sequenced by Illumina MiSeq (eAppendix in the Supplement). Using QIIME, version 1.8.0,[20] reads were assembled, demultiplexed, filtered against the Greengenes reference database, version 13.8,[21] and clustered at 97% similarity. After filtering, a total of 265 095 597 reads were retained (median, 235 623 per sample [range, 13 134-833 392]), representing 939 unique operational taxonomic units. For subsequent analyses, data were rarefied to 13 000 sequences per sample and summarized at the family taxonomic level.

Statistical Analysis

Statistical analysis was performed from February 1 to December 20, 2017. Covariates were tabulated against feeding and overweight and compared by use of the χ^2 test. Multivariable regression was used to investigate associations between feeding and overweight. Models were adjusted for suspected confounders selected a priori or identified in univariate analyses, grouped as maternal body mass index, other maternal factors (educational level, smoking status, ethnicity, and study site), and microbiota-related factors (cesarean delivery, dog ownership, infant sex, and antibiotics). Sensitivity analyses were conducted to adjust for birth weight, exclude never-breastfed infants, and evaluate continuous WFL*z* scores as an alternative outcome. Results are presented as crude odds ratios (ORs) and adjusted ORs (aORs) or differences in WFL*z* scores (SDs with 95% CIs). Multiple imputation (20 imputed data sets) was performed for all covariates using the R package mice.[22] Microbiota alpha diversity was assessed using the abundance-based coverage estimator and Chao1 indices of species richness and the Simpson and Shannon indices of diversity. Microbiota measures were compared between feeding or weight status groups by use of nonparametric Kruskal-Wallis tests and post hoc Dunn tests with false discovery rate (FDR) correction for multiple comparisons. Microbiota community structures were compared by permutational analysis of variance (PERMANOVA) on UniFrac[23] distance matrices and visualized by principal coordinate analysis. Microbiota composition and diversity (classified in quartiles) were further investigated in multivariable logistic regression models to evaluate their influence on the association between breastfeeding and risk of overweight. All analyses were performed in R, version 3.3.3 (R Development Core Team). $P < .05$ (2-sided) after FDR correction was considered significant.

Results

Study Population

Most mothers were white (817 of 1078 [75.8%]) and delivered vaginally (790 of 1064 [74.2%]); 408 of 1025 mothers (39.8%) were overweight or obese (eTable 1 in the Supplement). The breastfeeding initiation rate was 95.5% (1032 of 1081) (eTable 2 in the Supplement). At 3 months, 53.8% of infants (579 of 1077) were exclusively breastfed, including 37.1% (400 of 1077) who were exclusively breastfed since birth and 16.6% (179 of 1077) who briefly received formula in the hospital. The remaining infants were partially breastfed (323 of 1077 [30.0%]) or not breastfed (175 of 1077 [16.2%]). By 6 months, the rate of exclusive breastfeeding had decreased to 17.6% (183 of 1040), and partial breastfeeding had increased to 54.6% (593 of 1087), including 28.2% (307 of 1087) who received formula with or without food and 26.3% (286 of 1087) who received food but not formula. At 12 months, 42.2% of infants (459 of 1087) were still breastfeeding; the mean (SD) WFL*z* score was 0.29 (1.08), and 22.9% of infants (249 of 1087) were overweight or at risk for overweight.

Infant Feeding and Risk of Overweight

Breastfeeding was associated with a lower risk of overweight at 12 months, with dose responses observed according to breastfeeding exclusivity and duration (**Table 1**). Among infants who

were exclusively breastfed at 3 months, 19.2% (74 of 386) were overweight or at risk of overweight by 12 months compared with 27.6% of infants (84 of 304) who were partially breastfed (OR, 1.61; 95% CI, 1.13-2.30) and 33.3% of infants (53 of 159) who were not breastfed (ie, exclusively formula fed) (OR, 2.11; 95% CI, 1.39-3.19). There was no increase in risk of overweight among exclusively breastfed infants who briefly received formula in the hospital (35 of 171 [20.5%] at risk; OR, 1.09; 95% CI, 0.68-1.69). These associations were largely unaffected by adjustment for maternal body mass index, education, smoking, and other potential confounders (eTable 3 in the Supplement) (partial breastfeeding: aOR, 1.63; 95% CI, 1.09-2.44; exclusive formula feeding: aOR, 2.02; 95% CI, 1.18-3.45; exclusive breastfeeding after hospital discharge: aOR, 1.13; 95% CI, 0.68-1.89) (Table 1).

At 6 months, partial breastfeeding supplemented with formula was associated with an increased risk of overweight when adjusting individually for maternal body mass index (aOR, 1.60; 95% CI, 1.01-2.59), other maternal factors (aOR, 1.65; 95% CI, 1.03-2.68), or microbiota-related factors (aOR, 1.64; 95% CI, 1.02-2.70), although statistical significance was lost in the fully adjusted model (aOR, 1.43; 95% CI, 0.87-2.37) (Table 1). In contrast, partial breastfeeding without formula (ie, with foods only) was not associated with risk of overweight (aOR, 0.96; 95% CI, 0.57-1.64). Earlier cessation of breastfeeding was associated with an increased risk of overweight (before 6 months: aOR, 1.64; 95% CI, 1.06-2.52; between 6 and 12 months: aOR, 1.47; 95% CI, 0.99-2.18 compared with 12 months or longer). Sensitivity analyses using the WFL*z* score as a continuous outcome, adjusting for infant birth weight or excluding infants who never received breast milk, followed similar patterns of association (eTable 4 in the Supplement).

Infant Feeding and Gut Microbiota

As expected, breastfeeding was strongly associated with the richness, diversity, and composition of gut microbiota at 3 to 4 months, with clear dose responses according to exclusivity (**Figure 1** and eTables 5 and 6 in the Supplement). The richness and diversity of microbiota were highest in infants who were not breastfed, lower in partially breastfed infants, and lowest in exclusively breastfed infants (Figure 1A). The community structure of microbiota also differed significantly (overall $P = .001$, pseudo F, 10.9 [unweighted UniFrac]; $P = .001$, pseudo F, 12.4 [weighted UniFrac], determined by use of PERMANOVA; eTable 6 in the Supplement), with principal coordinate analysis (Figure 1D and eFigure 2A and B in the Supplement) showing clear separation between the exclusively breastfed and nonbreastfed groups. The group that briefly received formula in the hospital overlapped almost completely with the exclusively breastfed group ($P = .24$, pseudo F, 0.24, determined by use of pairwise PERMANOVA) (Figure 1D and eTable 6 in the Supplement), indicating similar microbiota community structures.

Nearly all phyla and families demonstrated disproportional abundances across breastfeeding groups, and significant dose responses were observed with particular taxa (Figure 1B and C and eTable 5 in the Supplement). Increasing exclusivity of breastfeeding was associated with increasing relative abundance of *Bifidobacteriaceae* and *Enterobacteriaceae* and decreasing relative abundance of *Lachnospiraceae*, *Veillonellaceae*, and *Ruminococcaceae*. Although most taxa were similarly abundant between infants who were exclusively breastfed from birth and those exclusively breastfed after hospital discharge, the relative abundance of *Bifidobacteriaceae* was significantly lower after brief exposure to formula in the hospital (median, 4.3% vs 8.3% of total microbiota; FDR $P = .03$) and the relative abundance of *Enterobacteriaceae* was higher (29.8% vs 24.5% of total microbiota; FDR $P = .05$) (Figure 1C and eTable 5 in the Supplement).

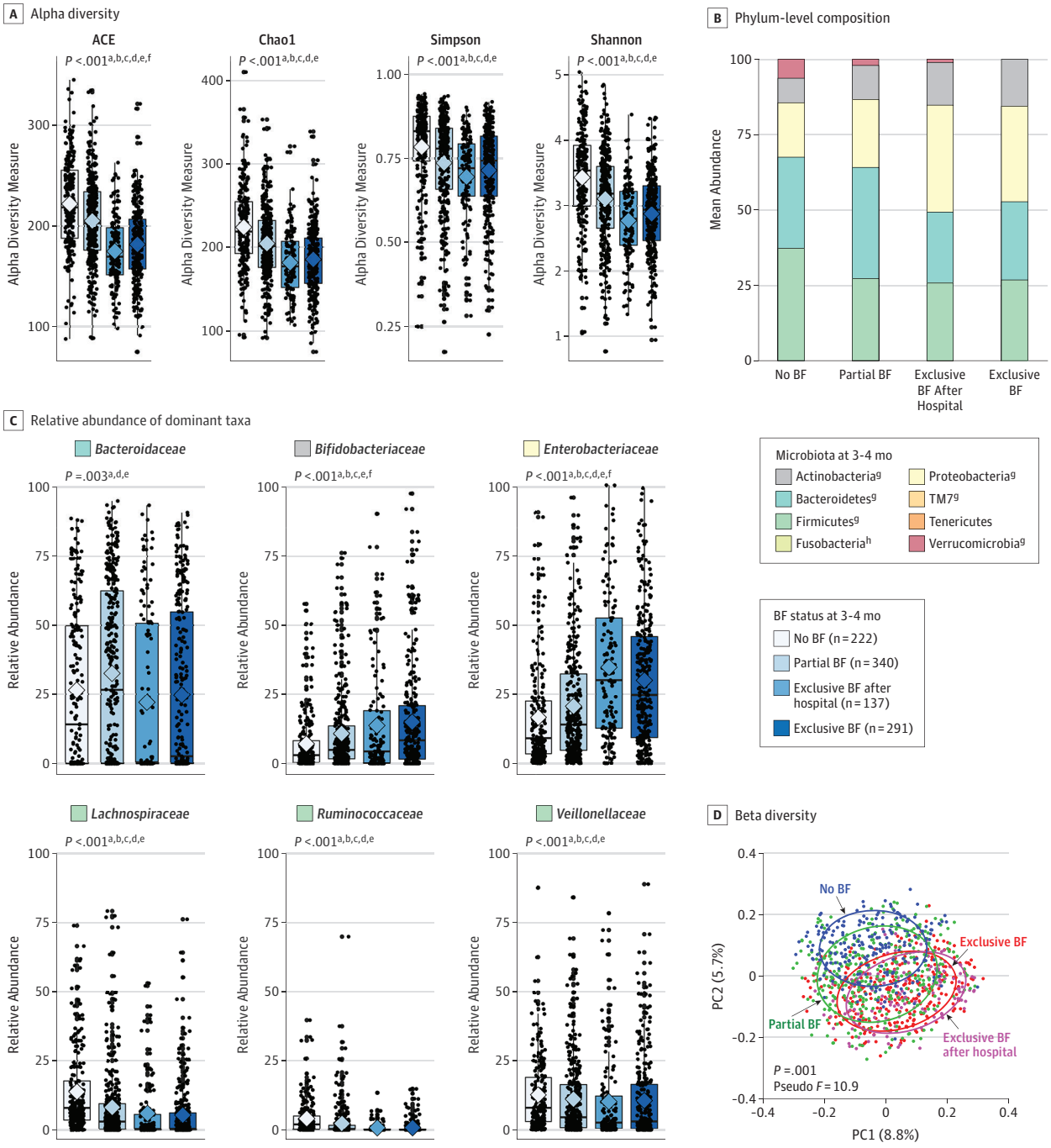
Twelve-month microbiota profiles were more homogeneous overall, but significant differences were still detectable according to dietary exposures at 6 months (**Figure 2**A-D, eFigure 2C and 2D, and eTables 6 and 7 in the Supplement). Richness was significantly higher among formula-fed infants (whether or not they were also receiving breast milk) compared with breastfed infants (whether or not they were receiving complementary foods) (Figure 2A). The relative abundances of Actinobacteria and Proteobacteria were highest in exclusively breastfed infants and lowest in nonbreastfed infants (Figure 2B). Several differences were observed between the partial breastfeeding groups, including significantly higher relative abundance of *Bifidobacteriaceae* and *Veillonellaceae* in those receiving complementary foods without formula (Figure 2C). Overall, the microbiota of partially breastfed infants who did not receive formula were similar to the microbiota of exclusively breastfed infants (no significant differences by 12 months; $P = .78$, pseudo $F = 0.40$, determined by use of pairwise PERMANOVA), whereas the microbiota of those who received formula were more similar to the microbiota of nonbreastfed infants (Figure 2D and eTable 6 in the Supplement).

The duration of breastfeeding was also associated with gut microbiota at 12 months (eFigure 3 and eTable 8 in the Supplement). Richness and diversity were lowest among infants who were still breastfeeding at 12 months and highest among those who had weaned before 6 months. *Bifidobacteriaceae*, *Veillonellaceae*, and Proteobacteria were enriched among infants who were still breastfeeding and depleted among infants who had never been breastfed. In contrast, *Lachnospiraceae*, *Ruminococcaceae*, and *Porphyromonadaceae* were enriched among infants who were not breastfeeding at 12 months.

Gut Microbiota and Overweight

Infants who were overweight or at risk of overweight at 12 months had significantly higher richness of microbiota by 3 to 4 months of age (**Figure 3**A); significant differences in composition were also detected (Figure 3B and C and eTable 9 in the Supplement). The strongest association was the enrichment of *Lachnospiraceae* among infants who subsequently became overweight (median relative abundance, 5.9% of total microbiota) or at risk for overweight (median relative abundance, 4.7% of total microbiota) by 12 months compared with normal-weight infants (median relative abundance, 1.9% of total microbiota; FDR $P = .01$). We also observed significantly higher relative abundance of *Coriobacteriaceae*, *Erysipelotrichaceae*, and *Ruminococcaceae*

Figure 1. Infant Gut Microbiota at 3 to 4 Months According to Breastfeeding (BF) Status



A, Alpha diversity evaluated by richness (abundance-based coverage estimator [ACE] and Chao1) and diversity (Simpson and Shannon). Median estimates are compared across feeding groups using the Kruskal-Wallis test (nonparametric analysis of variance) and Dunn post hoc tests for multiple comparisons. Boxes indicate interquartile range, lines indicate medians, diamonds indicate means, and whiskers represent range. B, Mean phylum-level composition. C, Relative abundance of dominant taxa across feeding groups. Breastfeeding (BF) status is assessed at the time of sample collection. Breastfeeding refers to breast milk feeding regardless of feeding mode (at the breast or from a bottle). D, Principal coordinate analysis (PC1 and PC2) based on unweighted UniFrac distances, with community structure differences tested by permutational analysis of variance with 999 permuations.

P values represent false discovery rate–corrected *P* values testing for overall differences across the 4 feeding groups. Significant pairwise comparisons:

[a] No BF/partial BF;

[b] No BF/exclusive BF after hospital;

[c] No BF/exclusive BF;

[d] Partial BF/exclusive BF after hospital;

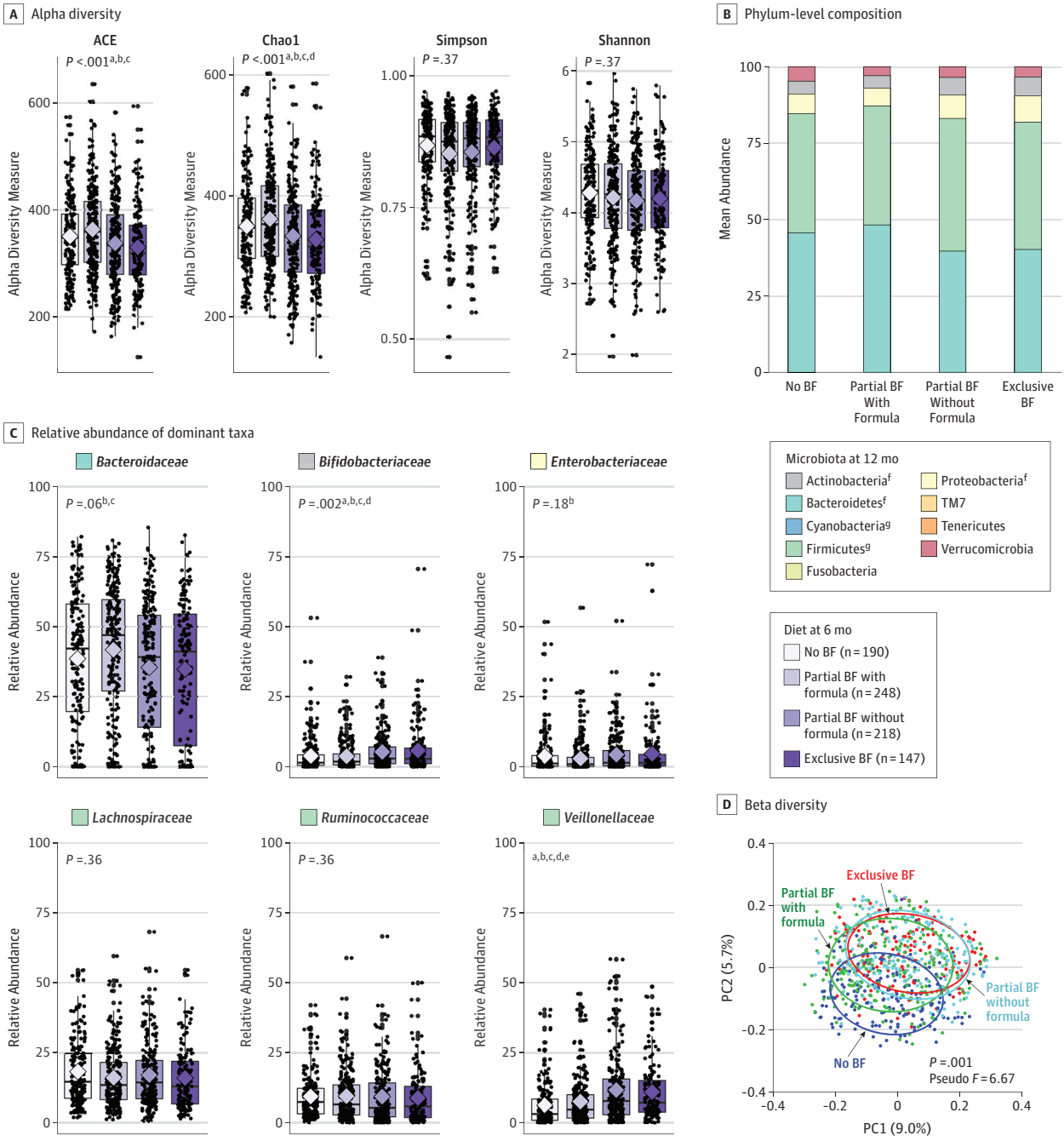
[e] Partial BF/exclusive BF;

[f] Exclusive BF after hospital/exclusive BF.

[g] $P < .001$.

[h] $P < .05$.

Figure 2. Infant Gut Microbiota at 12 Months According to Diet at 6 Months



A, Alpha diversity evaluated by richness (abundance-based coverage estimator [ACE] and Chao1) and diversity (Simpson and Shannon). Median estimates are compared across feeding groups using the Kruskal-Wallis test and Dunn post hoc tests for multiple comparisons. Boxes indicate interquartile range, lines indicate medians, diamonds indicate means, and whiskers represent range. B, Mean phylum-level composition. C, Relative abundance of dominant taxa across feeding groups. Breastfeeding (BF) refers to breast milk feeding regardless of feeding mode (at the breast or from a bottle). D, Principal coordinate analysis (PC1 and PC2) based on unweighted UniFrac distances, with community structure differences tested by permutational analysis of variance with 999 permutations.

P values represent false discovery rate–corrected *P* values testing for overall differences across the 4 feeding groups. Significant pairwise comparisons:

[a] No BF/exclusive BF;

[b] Partial BF with formula/partial BF without formula;

[c] Partial BF with formula/exclusive BF; no significant differences observed between partial BF without formula and exclusive BF;

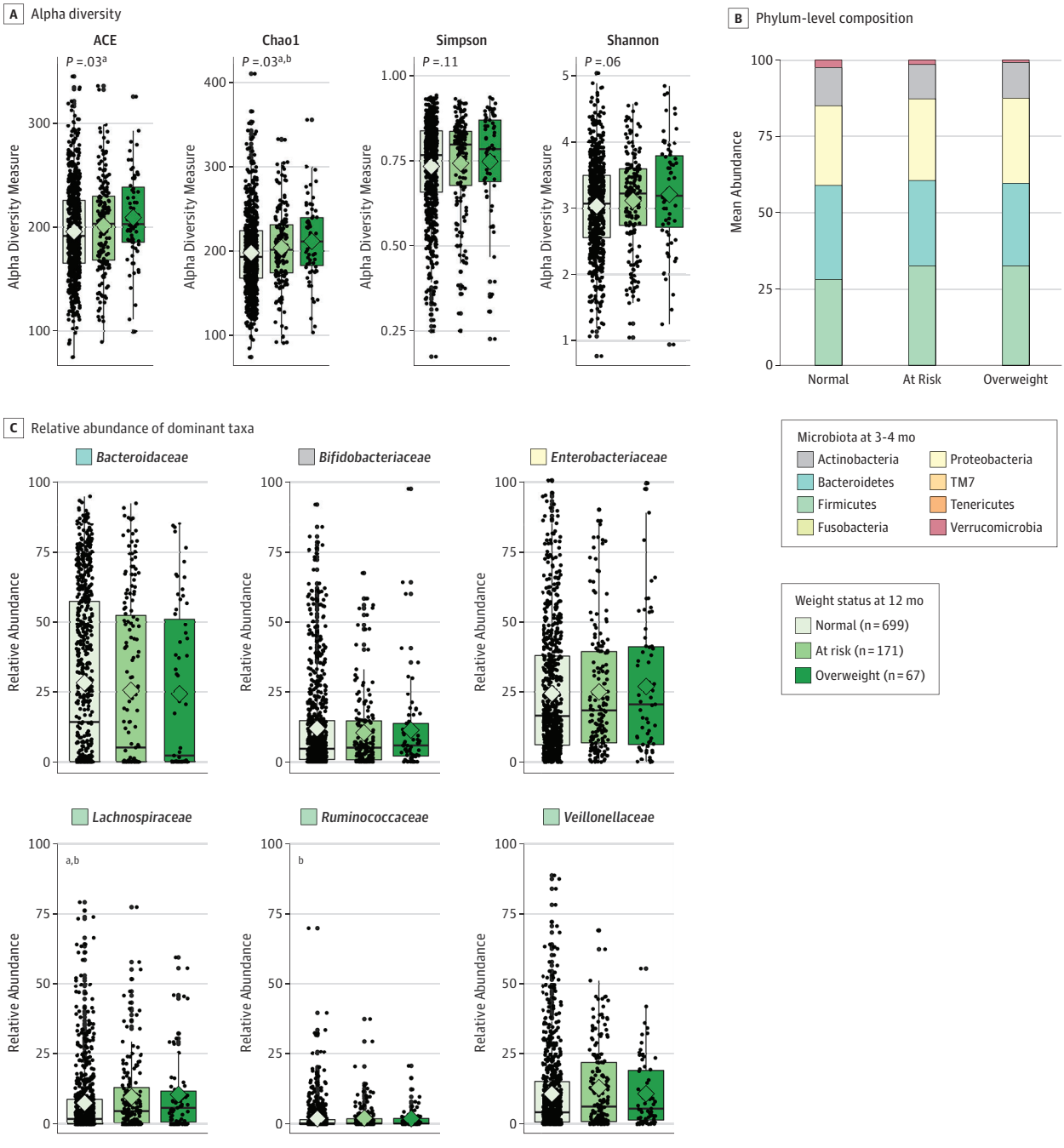
[d] No BF/partial BF without formula;

[e] No BF/partial BF with formula.

[f] $P < .01$.

[g] $P < .05$.

Figure 3. Infant Gut Microbiota Characterization at 3 Months According to Infant Weight Status at 12 Months



A, Alpha diversity evaluated by richness (abundance-based coverage estimator [ACE] and Chao1) and diversity (Simpson and Shannon). Median estimates are compared across weight status using the Kruskal-Wallis test and Dunn post hoc tests for multiple comparisons. Boxes indicate interquartile range, lines indicate medians, diamonds indicate means, and whiskers represent range. B, Mean phylum-level composition. C, Relative abundance of dominant taxa across weight status groups. Breastfeeding refers to breast milk feeding regardless of feeding mode (at the breast or from a bottle).

Significant pairwise comparisons:

[a] Normal/overweight;

[b] Normal/at risk.

at 3 to 4 months among infants who became overweight. The Firmicutes to Bacteroidetes ratio was highest in infants who became overweight at 1 year, although this difference was not significant. By 12 months, few differences in microbiota were observed according to weight status (eFigure 4 and eTable 9 in the Supplement).

To further explore the association of weight status at 12 months with the composition and diversity of gut micro-

Table 2. Association of Infant Feeding and Key Microbiota Measures at 3 and 12 Months With Weight Status at 12 Months

Breastfeeding and Microbiota Exposure	OR (95% CI) for Overweight or at Risk of Overweight (WFL*z* score >85th Percentile) at 12 mo					
		Mutually Adjusted				
	Adjusted for Covariates Plus Feeding or Microbiota (Individually)[a]	For Covariates, Feeding, and Chao1	For Covariates, Feeding, and Shannon	For Covariates, Feeding, and *Lachnospiraceae*	For Covariates, Feeding, and F/B Ratio	For Covariates, Feeding, and Selected Microbiota Measures[b]
Breastfeeding status at 3 mo (n = 795)						
None (formula only)	1.79 (1.09-2.93)	1.56 (0.93-2.59)	1.63 (0.98-2.70)	1.47 (0.87-2.45)	1.77 (1.07-2.91)	1.33 (0.79-2.24)
Partial (breast milk and formula)	1.49 (0.98-2.26)	1.37 (0.90-2.09)	1.41 (0.93-2.16)	1.37 (0.90-2.09)	1.52 (1.00-2.32)	1.28 (0.83-2.97)
Exclusive after hospital discharge	1.00 (0.58-1.69)	1.02 (0.59-1.73)	1.02 (0.59-1.73)	1.00 (0.58-1.69)	0.93 (0.53-1.58)	1.02 (0.59-1.73)
Exclusive (breast milk only)	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
Microbiota measures at 3 mo (n = 795)						
Chao1 (per quartile increase)	1.25 (1.08-1.46)	1.20 (0.59-1.73)	NA	NA	NA	1.16 (0.99-1.37)
Shannon (per quartile increase)	1.18 (1.02-1.38)	NA	1.13 (0.97-1.32)	NA	NA	NA
High *Lachnospiraceae* (above median)[c]	1.82 (1.29-2.57)	NA	NA	1.66 (1.16-2.39)	NA	1.58 (1.10-2.28)
F/B ratio (per quartile increase)	1.17 (1.00-1.38)	NA	NA	NA	1.20 (1.02-1.42)	NA
Breastfeeding duration at 12 mo (n = 695)						
<6 mo	1.99 (1.23-3.22)	1.97 (1.21-3.18)	1.95 (1.20-3.15)	1.98 (1.22-3.20)	2.02 (1.25-3.27)	1.96 (1.21-3.16)
6 to <12 mo	1.59 (1.02-2.48)	1.53 (0.98-2.39)	1.57 (1.00-2.45)	1.57 (1.00-2.45)	1.60 (1.02-2.50)	1.52 (0.97-2.38)
≥12 mo	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
Microbiota measures at 12 mo (n = 695)						
Chao1 (per quartile increase)	1.15 (0.97-1.36)	1.13 (0.96-1.35)	NA	NA	NA	1.13 (0.95-1.34)
Shannon (per quartile increase)	1.18 (1.00-1.40)	NA	1.17 (0.99-1.39)	NA	NA	NA
High *Lachnospiraceae* (above median)[c]	1.27 (0.87-1.85)	NA	NA	1.24 (0.85-1.81)	NA	1.21 (0.83-1.78)
F/B ratio (per quartile increase)	1.06 (0.90-1.26)	NA	NA	NA	1.08 (0.91-1.28)	NA

Abbreviations: F/B ratio, Firmicutes to Bacteroidetes ratio; NA, not applicable; OR, odds ratio; WFL*z*, weight for length *z*.

[a] Adjusted for maternal race/ethnicity, educational level, body mass index, smoking, cesarean delivery, dogs in household, infant sex, antibiotic exposure between 0 and 12 mo, and study site.

[b] The final model is adjusted for Chao1 and *Lachnospiraceae* because these were the strongest individual microbiota variables associated with risk of overweight; Shannon and F/B ratio were omitted to avoid multicollinearity because Shannon and Chao1 are highly correlated with each other (as 2 measures of alpha diversity), as are the F/B ratio and *Lachnospiraceae* relative abundance (*Lachnospiraceae* is a family in the Firmicutes phylum). Breastfeeding refers to breast milk feeding, regardless of feeding mode (at the breast or from a bottle). There were 795 infants for the 3-mo analyses and 695 infants for the 12-mo analyses.

[c] High relative abundance of *Lachnospiraceae*.

biota, we classified candidate microbiota measures in quartiles and conducted logistic regression analyses (eFigure 5 in the Supplement). At 3 to 4 months, higher relative abundance of *Lachnospiraceae* (above vs below median) were associated with an 89% increase in risk of overweight by 12 months (OR, 1.89; 95% CI, 1.40-2.56). Each quartile increase in the Firmicutes to Bacteroidetes ratio was associated with a 12% increase in the risk of overweight (OR, 1.12; 95% CI, 0.98-1.28). The richness of gut microbiota was also positively associated with the risk of overweight by 12 months (OR, 1.24 per quartile increase; 95% CI, 1.09-1.42 per quartile increase), as was the diversity of gut microbiota (OR, 1.21 per quartile increase; 95% CI, 1.06-1.38 per quartile increase). No comparable associations were detected for microbiota measures at 12 months.

Contribution of Gut Microbiota to Association of Infant Feeding Practices and Overweight

To examine whether gut microbiota contribute to the increased risk of overweight associated with formula feeding and shorter duration of breastfeeding, we tested these associations in mutually adjusted models. Adjustment for richness of microbiota, diversity of microbiota, or relative abundance of *Lachnospiraceae* substantially attenuated the effect estimate for cessation of breastfeeding before 3 months (**Table 2**). Simultaneous adjustment for richness of microbiota and *Lachnospiraceae* attenuated this estimate from 2.04 (95% CI, 1.25-3.32) to 1.33 (95% CI, 0.79-2.24). In contrast, associations between infant feeding and weight status were largely unaffected by adjustment for concurrent microbiota measures at 12 months.

Discussion

Our findings demonstrate a strong inverse and dose-dependent association between breastfeeding and the risk of overweight in the first year of life that is partially explained by gut microbiota. Although the effect of breast milk on the development of the gut microbiome is well known,[24-27] our

findings address important nuances that, to our knowledge, have not been explored in previous studies, identifying differences according to the type and timing of supplemental feeding. We also report novel longitudinal associations between the composition of gut microbiota at 3 to 4 months of age and weight status at 12 months of age.

Similar to previous studies,[28,29] we found a 63% increased risk of overweight among infants who were partially vs exclusively breastfed at 3 months and a 102% increased risk among exclusively formula-fed infants. As others have reported,[25,27,30] we detected significantly lower bacterial richness and diversity in breastfed infants, accompanied by enrichment of several taxa (eg, *Bifidobacteriaceae*, *Pasteurellaceae*, and *Enterobacteriaceae*) and depletion of others (eg, *Bacteroidaceae* and *Lachnospiraceae*), with dose effects according to the degree of breastfeeding exclusivity. These findings are consistent with evidence that human milk oligosaccharides function as selective substrates for particular groups of microorganisms, including *Bifidobacteriaceae*.[31-34]

Building on previous studies of adults,[35,36] children,[9-12] and infants,[37-42] our study provides new evidence linking gut microbiota with the risk of overweight in the first year of life. Prior research of infants has reported reduced relative abundance of Bifidobacteria and enrichment of streptococci and *Bacteroides fragilis* to be associated with overweight later in childhood.[37-42] Although we did not observe these particular trends, perhaps owing to cohort differences in age, geography, or feeding practices (eg, extremely high rates of initiation of breastfeeding in the CHILD Study), we identified several novel associations. Although few associations were detected between microbiota and overweight measured concurrently at 12 months, several microbiota features associated with overweight were identified at 3 to 4 months. For example, while *Lachnospiraceae* were similarly abundant in normal-weight and overweight infants at 12 months, they were significantly enriched among overweight infants at 3 to 4 months. *Lachnospiraceae* has been associated with maternal obesity and is enriched in meconium from neonates born to mothers with diabetes.[43] In our study, enrichment of *Lachnospiraceae* was associated with exposure to formula in a dose-dependent manner, along with the richness and diversity of microbiota; adjustment for these microbiota features partially explained the association between exposure to formula and the risk of overweight.

Taken together, our results suggest that the transient perturbation of microbiota in early infancy (related to feeding practices or other exposures) may influence weight gain and body composition, which may ultimately influence the risk of metabolic disease risk later in life.[44] This hypothesis (eFigure 6 in the Supplement) is consistent with studies of mice showing that the disruption of gut microbiota limited to early life has permanent metabolic effects, including elevated adiposity, despite "recovery" of the microbiota.[45] Other important mechanisms linking gut microbiota and obesity include microbial metabolites influencing levels of and sensitivity to the satiety hormone leptin.[46,47]

To our knowledge, this is the first study to evaluate the potential association of brief exposure to formula during the neonatal period as it pertains to the development of microbiota and the risk of overweight. These are clinically important questions since many neonates receive formula in the hospital, often without medical indication,[48] yet the effect of this brief intervention on the developing microbiota (and related clinical outcomes) is not known. In our cohort, 179 of 579 infants (30.9%) reported by their mothers as exclusively breastfed actually received some formula in the hospital. Overall, we found no difference in the risk of overweight among these infants. However, while their microbiota profiles at 3 to 4 months were clearly more similar to those of exclusively breastfed than partially or nonbreastfed infants, some significant differences were detected. The richness and diversity of the microbiota were lower, as was the relative abundance of *Bifidobacteriaceae*, suggesting that even brief exposure to formula may disrupt normal colonization of the infant gut. We have likely underestimated this disruption, since our first sample was not collected until 3 to 4 months after hospital discharge. It is possible that the reason for formula supplementation contributed to the observed microbiota differences, but this possibility could not be directly examined in our study because we did not systematically document reasons for supplementation.

Multiple studies have investigated the effects of breast milk on the gut microbiome[24-26,34,49,50]; however, many of these studies did not distinguish between partial breastfeeding mixed with formula vs mixed with foods. We found that breastfed infants supplemented with formula were more similar to nonbreastfed infants, whereas breastfed infants given complementary foods (without formula) were more similar to exclusively breastfed infants. These differences might explain why mixed feeding with (but not without) formula was associated with an increased risk of overweight, although more research is needed to characterize these complex associations.

Strengths and Limitations

The strengths of our study include the detailed description of infant feeding practices, repeated analysis of microbiota, and adjustment for multiple confounders. However, we lacked information about the reasons for supplementation and did not address the mode of breast milk feeding, type of formula, quantity of breast milk or formula intake, or breast milk composition. Finally, a limitation of 16S ribosomal RNA analysis is that it cannot quantify or accurately resolve individual bacterial species.

Conclusions

Our findings indicate that breastfeeding is protective against overweight and suggest that the gut microbiota contribute to this effect. Formula feeding was associated with higher microbiota diversity and enrichment of *Lachnospiraceae* at 3 to 4 months, and these microbiota features partially explained the increased risk of overweight among nonbreastfed infants. Subtle but statistically significant differences in the microbiota were observed after brief exposure to formula in the hospital, although the clinical implications of these changes are unclear. Together, these results identify important areas for future research and emphasize the importance of early infancy as a critical period during which transient gut dysbiosis is associated with the subsequent risk of overweight.

ARTICLE INFORMATION

Accepted for Publication: April 5, 2018.

Published Online: June 4, 2018. doi:10.1001/jamapediatrics.2018.1161

Correction: This article was corrected on July 2, 2018, to fix an error in a color key in all 3 figures.

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Statistical analysis: Forbes, Azad, Tun, Scott.
Obtained funding: Sears, Becker, Mandhane, Turvey, Subbarao, Scott, Kozyrskyj.
Administrative, technical, or material support: Tun, Konya, Guttman, Field, Lefebvre, Sears, Mandhane, Turvey, Moraes, Scott, Kozyrskyj.
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Conflict of Interest Disclosures: Dr Azad reported holding a Canada Research Chair in the Developmental Origins of Chronic Disease. Dr Sears reported holding the AstraZeneca endowed chair in respiratory epidemiology. No other disclosures were reported.

Funding/Support: The Canadian Institutes of Health Research (CIHR) and the Allergy, Genes and Environment Network of Centres of Excellence provided core funding for the Canadian Healthy Infant Longitudinal Development (CHILD) Study. This research was specifically funded by CIHR Microbiome Initiative team grant 227312 and the Children's Hospital Foundation of Manitoba. Additional funding was provided by Health Canada, Environment Canada, Canada Mortgage and Housing Corporation, the Sick Children's Hospital Foundation, Don & Debbie Morrison, the Silver Thread Foundation, the Childhood Asthma Foundation, Research Manitoba, and the Government of Manitoba (Healthy Child Manitoba Office).

Role of the Funder/Sponsor: The funding sources had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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Additional Contributions: We thank all the families who took part in this study and the entire CHILD team, which includes interviewers, nurses, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, and receptionists. We also acknowledge the generosity of ALK-Abello, Mississauga, Ontario, Canada, in supplying all allergens for the study, and Lincoln Diagnostics Inc, Decatur, Illinois, for supplying the Duotip-Test II devices and skin testing kits.

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