ORIGINAL RESEARCH

CURRENT DEVELOPMENTS IN NUTRITION

Maternal and Pediatric Nutrition

Feeding Practice and Delivery Mode Are Determinants of Vitamin K in the Infant Gut: An Exploratory Analysis

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ABSTRACT

Background: Infants have low stores of vitamin K at birth. Dietary intake of phylloquinone (PK) differs dramatically by infant feeding practice, but the contribution of microbially produced vitamin K (menaquinones) to infant vitamin K status is not well understood.

Objectives: The objective of this study was to investigate determinants of infant fecal vitamin K profiles in mother-infant dyads at 6 wk postpartum.

Methods: Fecal and breast milk samples were collected from a subsample of breastfeeding ($n = 23$) or formula-feeding ($n = 23$) mother and infant dyads, delivered vaginally ($n = 26$) or by cesarean section (CS) ($n = 20$) in the Synergistic Theory and Research on Nutrition and Growth (STRONG) Kids 2 cohort. Vitamin K concentrations in breast milk and feces were analyzed by LC/MS and/or HPLC. Fecal bacterial metagenomes were analyzed to derive taxonomy and vitamin K biosynthetic genes. Multivariate linear modeling was used to assess effects of delivery and feeding modes on infant fecal vitamin K.

Results: Breast milk contained 1.3 ± 0.2 ng/mL PK, and formula was reported to contain 52 ng/mL PK. Fecal PK was 38-times higher ($P < 0.001$) in formula-fed than breastfed infants. Infant fecal menaquinones (MKn) MK6, MK7, MK12, and MK13 were higher ($P < 0.001$) in formula-fed than breastfed infants, whereas MK8 predominated in breastfed and was 5-times higher than formula-fed infants. Total MKn were greater ($P < 0.001$) in vaginally delivered than CS infants. Relative abundances of 33 bacterial species were affected by feeding mode, 2 by delivery mode, and 4 by both ($P < 0.05$). Bacterial gene content of 5/12 vitamin K biosynthetic genes were greater ($P < 0.05$) in breastfed compared with formula-fed infants, and 1 differed by delivery mode.

Conclusions: Feeding practice and delivery mode influence bacterial vitamin K production in the infant gut. High concentrations of unmetabolized PK in feces of formula-fed infants suggests formula PK content exceeds the absorptive capacity of the infant gut. *Curr Dev Nutr* 2022;6:nzac019.

Keywords: vitamin K, phylloquinone, menaquinone, infant gut microbiota, infant nutrition

Manuscript received January 10, 2022. Initial review completed January 29, 2022. Revision accepted February 2, 2022. Published online February 12, 2022.
Supported by the USDA Agricultural Research Service under Cooperative Agreement No. 58-8050-9-004, NIH R01DK107561 (SMD), The National Dairy Council (SMD), and The Gerber Foundation (SMD). The National Dairy Council and the Gerber Foundation had no input into study design or implementation or analysis and interpretation of the data. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the USDA.
Author disclosures: SMD has received grant funding and receives honoraria for service on a Scientific Advisory Board for the National Dairy Council. SLB is a Deputy Editor on *Current Developments in Nutrition* and played no role in the journal's evaluation of the manuscript. All other authors report no conflicts of interest.
Supplemental Figures 1–2 and Supplemental Tables 1–6 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/cdn/.

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Abbreviations used: CS, cesarean section; LLOD, lower limit of detection; MKn, menaquinone-n; ND, nondetectable; PK, phylloquinone; RDA, redundancy analysis; STRONG, Synergistic Theory and Research on Nutrition and Growth; VKDB, vitamin K deficiency bleeding.

Introduction

Infants have low vitamin K stores due to poor placental transfer of vitamin K, low concentrations of vitamin K in breast milk, and possibly insufficient vitamin K from gut microbiota (1). As vitamin K is an essential nutrient required for blood clotting, newborns are at risk of a rare vitamin K deficiency bleeding (VKDB) disorder that occurs during the first 6 mo of life (2). Prophylactic administration of vitamin K at birth has successfully reduced incidence of VKDB (3).

The Adequate Intake (AI) for vitamin K in infants is based on the mean vitamin K intake of healthy breastfed infants (4), and does not take into account vitamin K prophylaxis at birth. All infant formula are fortified with vitamin K so infant vitamin K intakes can differ drastically by feeding method (3). The daily intake of exclusively breastfed infants averages 1–2 μg/d phylloquinone (PK), whereas formula-fed infants have an average intake of around 50 μg/d PK (5, 6). Although there is no reported toxicity associated with PK, the health implications of supraphysiological concentrations of PK in the infant are unknown. In preterm infants who received both vitamin K prophylaxis and PK supplementation through parenteral nutrition feeds, the presence of vitamin K 2,3-epoxide in serum and excretion of a less-metabolized vitamin K catabolite in urine were suggestive of a possible overload of both

vitamin K recycling and metabolic pathways (7). These observations raise the following questions. In the context of prophylaxis, what are the nutritional vitamin K requirements of the infant? Additionally, what (if any) role do the gut microbiota have in influencing these requirements?

Both feeding practice and route of delivery influence the infant gut microbiota composition (8–10). It has been previously demonstrated that bacterially produced vitamin K forms (collectively termed menaquinones, MKn, where n denotes the side chain length) produced by the infant gut microbiota differ by feeding practice (11). To our knowledge, the effect of delivery mode [cesarean section (CS) or vaginal] or any maternal transfer of MKn-producing bacteria to the infant has not been explored, nor has the effect of feeding practice or delivery mode on the infant gut bacterial MKn biosynthetic gene content. We recently demonstrated in mice that dietary PK and MKn can be partially remodeled to MKn by gut microbes (12). As such, dietary vitamin K may also influence the infant gut microbiota and its endogenous production of vitamin K. Therefore, the objective of this study was to characterize the fecal microbiome and fecal vitamin K of mother-infant dyads at 6 wk postpartum to identify determinants of the infant gut vitamin K profile. We hypothesized that the total vitamin K content and forms present in infant feces would differ by source of nutrition (breast milk or infant formula). We further hypothesized that the infant gut bacterial vitamin K biosynthesis potential would differ by delivery mode.

Methods

Selection and description of participants

Study participants were drawn from the Synergistic Theory Research Obesity and Nutrition Group (STRONG) Kids 2 birth cohort study, designed to examine multilevel predictors of weight trajectories and dietary habits over the first 7 y of life ($n = 450$) (13). Participants were living in East Central Illinois and samples were collected between 2017 and 2018. The STRONG Kids 2 study was approved by the University of Illinois Institutional Review Board (#13,448). The goals of the STRONG Kids 2 study were explained in person and parents or caregivers completed online informed consent forms for themselves and their child prior to participation. The participants did not directly benefit from study participation but did authorize all future uses of their biological samples in published research. The STRONG Kids Principal Investigators (including SMD) are responsible for the security of the identifiable data for the cohort. Original data are held in a secure data repository, which is maintained by the Biostatistics, Epidemiology and Research Design Core at the Interdisciplinary Health Sciences Institute, University of Illinois at Urbana-Champaign. Data is requested by participant ID, with no personally identifiable information. The Tufts researchers only had access to deidentified demographic data by participant ID. Study participants were not directly informed of the results of this study.

For the purpose of this study, mother-infant dyads who were either exclusively breastfeeding ($n = 23$) or formula-feeding ($n = 23$) were matched for demographic characteristics (**Table 1**; **Supplemental Figure 1**). In addition, 10 of the mothers in each feeding group had delivered by CS and 13 delivered vaginally. The number of subjects was determined using a power analysis of previous fecal metagenomic findings from our laboratory (14). Attempts were made to reduce the introduction of selection bias relative to the full cohort, with the exception that 43% of the participants were delivered by CS, compared with 25.6% in the full cohort (13). All infants were born in hospitals where vitamin K prophylaxis is standard of care. At 6 wk postpartum, breastfeeding mothers provided a milk sample, and freshly voided stool samples from the mothers and infants were collected in the home as previously described (13). Samples for vitamin K analysis were stored at −80°C prior to shipment on dry ice to Tufts University, and then stored again at −80°C until analysis.

Determination of vitamin K in feces and breast milk

Maternal feces were freeze-dried prior to vitamin K analysis, and infant feces were corrected for % dry matter after analysis to compare all fecal vitamin K concentrations on a dry fecal weight basis. All HPLC-grade solvents (Fisher Scientific Inc.) were used for vitamin K extraction and chromatography procedures. Vitamin K in feces and breast milk was quantified by LC-MS as previously described with the following lower limits of detection (LLOD): 1 pmol/g MK10; 5 pmol/g MK5, MK7, MK8, MK9, MK11, MK12, MK13; 10 pmol/g MK6; 30 pmol/g PK and MK4 (15). As low concentrations of PK and MK4 were detected in breast milk (but no longer-chain MKn), these samples were analyzed by HPLC for more sensitive measures (LLOD 0.1 pmol/g for both PK and MK4) as described previously (16).

Shotgun metagenomic sequencing and sequence analysis

DNA extraction.

DNA was extracted from ∼200 mg of feces by the QIAamp Fast DNA Stool Mini Kit (Qiagen) in combination with bead beating on the FastPrep-24 System (MP Biomedicals) as previously described (17). DNA concentration was measured on a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) and its quality was assessed on a 1% agarose gel.

Construction of shotgun libraries and sequencing.

Library contraction and sequencing were carried out at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign (UIUC). The shotgun genomic DNA libraries were constructed from 200 ng of DNA after sonication with a Covaris ME220 (Covaris) to an average fragment size of 300 bp with the Hyper Library Preparation Kit from Kapa Biosystems (Roche). To prevent index switching, the libraries were constructed using unique dual indexed adaptors from Illumina. Individually barcoded libraries were amplified with 5 cycles of PCR and run on a Fragment Analyzer (Agilent) to confirm the absence of free primers and primer dimers and to confirm the presence of DNA of the expected size range. Libraries were pooled in equimolar concentration and size selected on a 2% agarose gel for fragments 220 bp to 280 bp in length. The pool was further quantitated by qPCR on a BioRad CFX Connect Real-Time System (Bio-Rad Laboratories, Inc.). The pooled barcoded shotgun libraries were loaded on a NovaSeq lane for cluster formation and sequencing at a length of 150 nt from each side of the DNA fragments. The fastq read files were generated and demultiplexed with the bcl2fastq v2.20 Conversion Software (Illumina).

TABLE 1 Demographics of the mother-infant dyads from the STRONG Kids 2 cohort included in this study[1]

					P value		
	BF + VD	BF + CS	FF + VD	FF + CS	Feeding	Delivery	Interaction
Infants							
n	13	10	13	10	—	—	—
Sex (male)	6 (46.2%)	5 (50%)	6 (46.2%)	6 (60%)	—	—	—
Gestational age at birth delivery, wk	39.5 ± 0.39	40.2 ± 0.39	39.3 ± 0.29	39.5 ± 0.4	0.231	0.192	0.441
Weight-for-length *z*-score							
At birth	−0.12 ± 0.42	0.09 ± 0.65	−0.64 ± 0.33	−1.04 ± 0.47	0.066	0.831	0.497
At 6 wk	0.76 ± 0.33	0.48 ± 0.25	0.03 ± 0.23	−0.02 ± 0.42	0.049[2]	0.594	0.707
Antibiotic intake 2 wk prior to sample collection, *n*	1	0	0	2	—	—	—
Race							
Non-Hispanic/Latino white	11 (84.6%)	8 (80%)	7 (53.8%)	7 (70%)	—	—	—
Non-Hispanic/Latino nonwhite	1 (7.7%)	1 (10%)	3 (23.1%)	1 (10%)	—	—	—
Hispanic/Latino	1 (7.7%)	0	2 (15.4%)	1 (10%)	—	—	—
Not reported	0	1 (10%)	1 (7.7%)	1 (10%)	—	—	—
Formula brand							
Enfamil	—	—	11 (84.6%)	9 (90%)	—	—	—
Parent's choice	—	—	1 (7.7%)	1 (10%)	—	—	—
Similac Soy Isomil	—	—	1 (7.7%)	0	—	—	—
Mothers							
Age	32.1 ± 1.0	31.4 ± 1.3	28.1 ± 1.6	26.4 ± 2.0	0.005[2]	0.466	0.824
Prepregnancy BMI	27.05 ± 1.52	29.67 ± 2.14	27.85 ± 2.17	35.46 ± 3.13	0.131	0.022[2]	0.252
Race							
Non-Hispanic/Latino white	12 (92.3%)	7 (70%)	9 (69.2%)	7 (70%)	—	—	—
Non-Hispanic/Latino nonwhite	1 (7.7%)	2 (20%)	2 (15.4%)	2 (20%)	—	—	—
Hispanic/Latino	0	0	1 (7.7%)	0	—	—	—
Not reported	0	1 (10%)	1 (7.7%)	1 (10%)	—	—	—

[1] Data presented as *n* (%) or mean ± SEM, unless otherwise specified. BF, breastfed; CS, cesarean section; FF, formula-fed; STRONG, Synergistic Theory and Research on Nutrition and Growth; VD, vaginally delivered.
[2] Values indicate statistically significant differences ($P < 0.05$).

Sequence processing and metagenomics analysis.
Quality control was performed before and after the trimming steps with FastQC and MultiQC with default parameters (18, 19). Adapter sequences and low-quality reads were trimmed on both reads with Trimmomatic (20). Trimmed paired reads were merged with vsearch (version 2.8.0) (21), and sequences generated from the human genome were removed with KneadData (https://github.com/biobakery/kneaddata). The remaining reads were aligned to the National Center for Biotechnology Information non-redundant database with DIAMOND (22). The functional annotations were determined with MEGAN6 (23) using the KEGG database (24). Taxonomic profiling was performed with MetaPhlan2 (version e7761e78f362) (25, 26). The Shannon index was calculated using the following equation: $H' = -\sum_{i=1}^{S} p_i \ln(p_i)$, where p_i is the proportion of the i^{th} species in the community and s is the number of species (27).

Statistical analyses

Demographic data, α-diversity (numbers of observed species and Shannon indices), and KEGG Orthology (KO) reads assigned to vitamin K biosynthesis pathways were analyzed using the PROC MIXED procedure of SAS (version 9.4; SAS Institute). The statistical model included feeding, delivery, and the interaction between feeding and delivery. Data are presented as mean ± SEM. A P value of < 0.05 was considered statistically significant.

Redundancy analysis (RDA) was used to evaluate the effect of feeding and delivery modes on infant fecal bacterial composition. The relative abundance of bacterial species from each sample was log-transformed prior to RDA, which was performed in CANOCO for Windows 5 (Microcomputer Power). Statistical significance was assessed using the Monte Carlo permutation test with 500 random permutations. Key bacterial species responding to feeding and delivery modes were confirmed further by the BDM.2way command from the asbio package of R (28). Data are reported as median (25–75 percentiles) and significance was evaluated at $\alpha = 0.05$.

All vitamin K concentration data are presented as geometric mean ± SEM. Nondetectable (ND) vitamin K concentration values were replaced with the LLOD. Data were assessed for normality and, if required, were natural log (ln)-transformed for normality prior to parametric testing. For fecal vitamin K analysis, statistical significance was evaluated at $\alpha = 0.0045$ (Bonferonni adjustment for 11 vitamin K outcomes: PK, MK5-MK13, and total MKn). For all other analyses, statistical significance was evaluated at $\alpha = 0.05$. Pearson correlations were used to test the associations between mother and infant fecal vitamin K concentrations, and within breastfeeding dyads, the association between breast milk PK concentration and infant fecal PK concentrations. Within infants and mothers separately, Welch 2 sample t-tests were used to compare fecal vitamin K concentrations (and % total MKn) by feeding mode and by delivery mode. To assess the effects of both delivery mode and feeding mode together, multivariate linear models were used. Within each model, an interaction term between feeding mode and delivery mode was tested; however, no interaction terms were significant and, therefore, the term was dropped from the models. All analyses were conducted in R Version 3.6.3 (02/29/2020).

Results

Vitamin K content of infant food source

Breast milk from mothers within breastfed dyads contained 1.3 ± 0.2 ng/mL (2.9 ± 0.5 pmol/mL) PK and 0.4 ± 0.1 ng/mL (0.9 ± 0.2 pmol/mL) MK4. The predominant formula brand used by formula-feeding dyads was Enfamil Lipil (Mead Johnson), which is reported by the USDA food database to contain 5.2 μg PK/100 g reconstituted formula (29), expressed in equivalent units is 52 ng/mL (115 pmol/mL) PK of reconstituted formula.

Infant fecal vitamin K analysis by feeding mode

Within breastfed dyads, PK concentrations of maternal breast milk and infant feces were correlated ($r = 0.519$, $P = 0.013$). Between infant feeding groups, fecal PK was higher in formula-fed compared with breastfed infants ($P < 0.001$, **Figure 1**A and **Supplemental Table 1**). Bacterially produced MKn in infant feces also differed by feeding mode: MK6, MK7, MK12, and MK13 were higher in formula-fed infants compared with breastfed infants (all $P < 0.001$), and MK8 was significantly higher in breastfed compared with formula-fed infants ($P < 0.001$, Figure 1A and Supplemental Table 1). When bacterial MKn were compared as a % of the total fecal MKn, all the same relations held, with % MK9 additionally emerging as higher in formula-fed compared with breastfed infants (**Supplemental Table 2**). Among mothers, there were no differences in fecal vitamin K forms between those who breastfed or formula-fed, and fecal vitamin K concentrations were not correlated within infant-mother dyads (Supplemental Table 1).

Infant fecal vitamin K analysis by delivery mode

Fecal MK9–MK11 and total MKn were higher in infants delivered vaginally compared with CS (all $P < 0.004$, Figure 1B and **Supplemental Table 3**). When compared as a percentage of total MKn, only MK10 was higher in vaginally delivered infants compared with CS (**Supplemental Table 4**). Among mothers, fecal MK8 was higher in those who delivered by CS compared with vaginal delivery ($P = 0.004$, Supplemental Table 3). However, fecal vitamin K concentrations were not correlated within infant-mother delivery dyads (Supplemental Table 3).

Multivariate infant fecal vitamin K analysis

Multivariate analyses of infant fecal vitamin K content in which the model contained both feeding and delivery mode were largely consistent with univariate analyses (**Table 2**). Fecal PK, MK6, MK7, MK9, MK12, and MK13 were greater in formula-fed infants (all $P < 0.002$), and MK8 was higher in breastfed infants ($P < 0.001$). Infant fecal MK9 was also independently influenced by delivery mode, such that vaginally delivered infants had greater MK9 fecal content than CS infants. Fecal MK10 and total MKn were also greater in vaginally delivered compared with CS infants (both $P < 0.001$), however, differences in fecal MK11 by delivery mode (in contrast to univariate analyses) did not retain significance in the multivariate model. No significant interactions between feeding and delivery mode were detected. All the same multivariate relations held when compared on a relative basis as a percentage of total MKn (**Supplemental Table 5**).

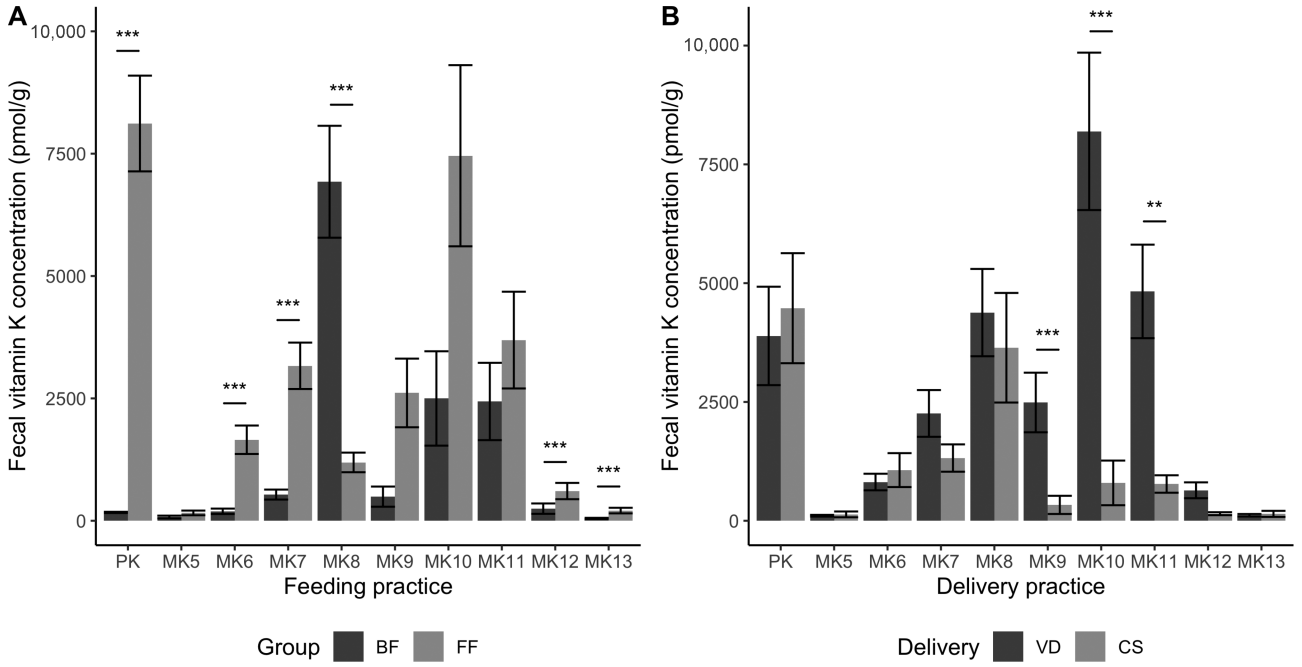

FIGURE 1 Bar charts of infant fecal vitamin K content (geometric mean ± SEM, pmol/g dry weight) at 6 weeks postpartum by A) feeding method and B) delivery method. BF, breastfed; CS, cesarean section; FF, formula-fed; PK, phylloquinone; MKn, menaquinone-n; VD, vaginally delivered.$^{**}P < 0.01$, $^{***}P < 0.001$.

Microbiome and metagenome results

To assess the bacteria present in infant feces, metagenomic shotgun sequencing was performed on the DNA extracted from 46 fecal samples. After performing quality control, merging the paired reads, and removing human sequence reads, over 1.57 billion sequences (range = 10,980,603–49,565,363 reads per sample) were used for further analysis. MetaPhlan2 assigned reads into 220 bacterial species. Compared with formula-fed infants, the numbers of observed species were lower (42.6 ± 1.86 compared with 17.0 ± 1.04; $P < 0.0001$) in breastfed infants regardless of delivery mode. Similarly, Shannon indices, which represent the α-diversity of the bacterial community, were also lower in breastfed (1.06 ± 0.09) than formula-fed (1.87 ± 0.11) infants ($P < 0.0001$). Delivery mode had no impact on observed species (CS compared with vaginally delivered: 30.0 ± 3.39 compared with 29.7 ± 2.91) or Shannon indices (CS compared with vaginally delivered: 1.50 ± 0.15 compared with 1.44 ± 0.12).

The impact of feeding and delivery modes on the overall infant fecal microbiota composition were evaluated using RDA. RDA was

TABLE 2 Vitamin K concentrations (pmol/g) in infant feces at 6 wk postpartum by infant feeding and delivery mode[1]

	Group				Multivariate linear models *P* value	
Vitamin K form	BF + VD (n = 13)	BF + CS (n = 10)	FF + VD (n = 13)	FF + CS (n = 10)	Feeding mode	Delivery mode
	(pmol/g dry weight)					
PK	170 ± 20.9	155 ± 8.2	5260 ± 1470	8010 ± 1230	<0.001	0.450
MK5	48.2 ± 18.1	34.3 ± 62.8	114 ± 27.6	60.7 ± 107	0.017	0.110
MK6	85.9 ± 56.4	136 ± 104	1310 ± 221	936 ± 617	<0.001	0.842
MK7	403 ± 94.9	359 ± 206	2630 ± 693	1570 ± 433	<0.001	0.277
MK8	5500 ± 1330	2730 ± 2040	864 ± 208	798 ± 388	<0.001	0.277
MK9	323 ± 343	56.1 ± 16.5	1690 ± 1030	195 ± 373	0.001	<0.001
MK10	838 ± 1550	26.5 ± 155	4070 ± 2550	196 ± 907	0.011	<0.001
MK11	1200 ± 1310	445 ± 295	2490 ± 1430	333 ± 219	0.579	0.006
MK12	79.6 ± 180	42.8 ± 27.9	434 ± 269	173 ± 43.6	<0.001	0.071
MK13	40.4 ± 18.1	27.4 ± 9.6	101 ± 45.5	127 ± 119	<0.001	0.790
Total MK5-MK13	13,400 ± 3340	6084 ± 2190	24,800 ± 4550	7160 ± 1420	0.073	<0.001

[1]Geometric means ± SEM. Nondetectable values were replaced with the lower limit of detection (LLOD) to avoid transformation of zero. LLODs: 1 pmol/g MK10; 5 pmol/g MK5, MK7, MK8, MK9, MK11, MK12, MK13; 10 pmol/g MK6; 30 pmol/g PK and MK4. Data was natural long (ln)-transformed for normality prior to testing using parametric linear models. Feeding x Delivery mode interaction was tested, but not significant in any model, so was dropped. BF, breastfed; CS, cesarean section; FF, formula-fed; PK, phylloquinone; MKn, menaquinone-n; VD, vaginally delivered.

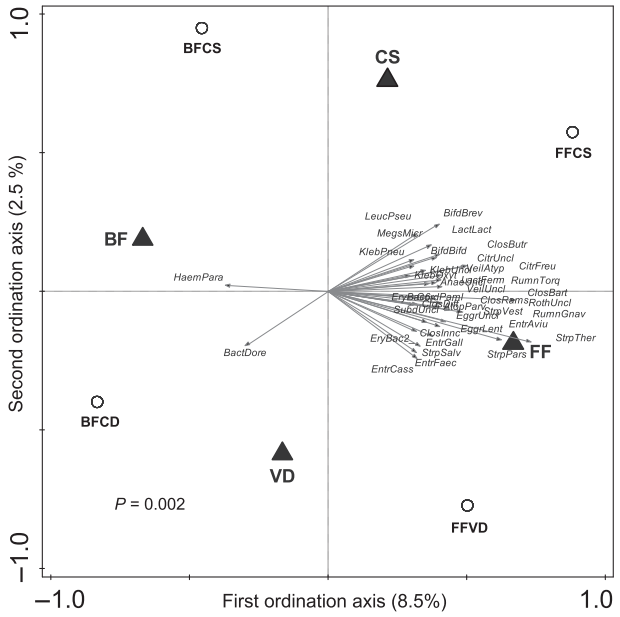

FIGURE 2 Triplot of RDA based on species relative abundance of infant gut microbiota at 6 wk postpartum relative to feeding and delivery mode. Environmental variables (feeding: BF compared with FF, and delivery: VD compared with CS) indicated by triangles. The center of the samples from each group indicated by circles. Species with ≥8.5% of the variability in their values explained by the first axis are indicated by arrows. *P* value was assessed by Monte Carlo permutation test. BF, breastfed; CS, cesarean section; FF, formula-fed; VD, vaginally delivered.

performed on the relative abundance of bacterial species. The RDA triplot (**Figure 2**) showed that the breastfed and formula-fed infants were distinguished at the first constrained axis (explaining 8.5% of the total variability), whereas the vaginally delivered and CS infants were separated at the second constrained axis (explaining 2.5% of the total variability), indicating that both feeding and delivery modes impacted the fecal microbiota composition (Monte Carlo permutation test, $P = 0.002$). Of 220 species identified by MetaPhlan2, 39 bacterial species contributing to ≥8.5% of the total variability were identified as the key species (indicated by gray arrows in Figure 2 and also listed in **Table 3**) responding to the feeding and delivery modes, which were further confirmed by univariate statistical analysis (Table 3). Among the 39 key bacterial species, 33 were affected by feeding mode. Of those 33 species, 32 were found in greater relative abundance in feces of formula-fed compared with breastfed infants, and only 1 species, *Haemophilus parainfluenzae*8 was greater in breastfed compared with formula-fed infants (though the relative abundance was very low, Table 3). Two bacterial species differed by delivery route: the relative abundance of *Bifidobacterium breve* was higher in CS compared with vaginally delivered infants, and *Bacteroides dorei* was lower in CS than vaginally delivered infants. Four bacterial species, *Leuconostoc pseudomesenteroides*, *Megasphaera micronuciformis*, *Citrobacter freundii,* and *Citrobacter (unclassified)*, were influenced by both feeding and delivery modes (Table 3). The mean relative abundances of all bacterial species detected in infant feces with relative abundances over 0.05% are shown in **Supplemental Figure 2**.

Bacterial gene content in menaquinone biosynthesis and utilization pathways also differed in the feces of infants by feeding and delivery mode (**Supplemental Table 6**). The bacterial gene content of 5 of 12 menaquinone genes (*MenD*, *MenE*, *MenI*, *wrbA*, and *qprB*) were greater in the feces of breastfed compared with formula-fed infants, and *qprB* was also greater in the feces of cesarean-delivered compared with vaginally delivered infants (Supplemental Table 6).

Discussion

To our knowledge, this study is the first to examine the fecal vitamin K content of infants in conjunction with the fecal microbial composition and the early life factors of feeding and mode of delivery. PK, the predominant dietary form of vitamin K, was higher in feces of formula-fed compared with breastfed infants, and feeding practice was also predictive of differences in 6 of the 9 bacterially produced MKn forms. Although to a lesser extent, mode of delivery was also predictive of differences in infant fecal vitamin K. An earlier study characterized the fecal menaquinone status of newborns (30), stating that the gut bacterial production of vitamin K may have important implications for infant vitamin K status, yet 40 y later this remains unclear.

The PK content of breast milk samples reported here is consistent with concentrations reported in previous literature (31), as is the absence of any bacterial MKn in breast milk (32) [MK4, unlike other MKn, is a metabolite of dietary PK formed in tissues (33)]. Though the PK content of breast milk is low, PK concentrations in maternal breast milk samples and infant feces were correlated within breastfed dyads. This result was expected as the maternal diet influences the PK content of breast milk (34, 35), and thus maternal diet is relevant to vitamin K status of breastfed infants. It has been previously established that the PK content of formula is magnitudes higher than that of breast milk (30), and the difference in the fecal PK content of infants by feeding practice reported herein reflects this difference in dietary PK exposure. The reported PK content of the infant formula is 40-fold higher than the PK content of breast milk in this cohort, and the fecal PK content of formula-fed infants exceeded that of breastfed infants by 38-fold. Fortification targets for the vitamin K content of formula were based on early measurements of vitamin K content of cow milk (36), and was originally set at 60 μg/L. These concentrations are now known to have been overestimated by the original assay (5, 37), yet these concentrations (and higher) persist in infant formula.

Unlike other fat-soluble vitamins that are stored in the body, vitamin K is rapidly excreted. Vitamin K is catabolized to 5-carbon and 7-carbon aglycones (38), and these urinary metabolites have been characterized in infants (7). Though there is no known toxicity associated with the current concentrations of vitamin K in infant formula, the high concentrations of unmetabolized PK in feces of formula-fed infants (which also exceeded the adult fecal PK content of the mothers) suggests that the current PK content of infant formula may exceed the absorptive and/or metabolic capability of the infant gut, a speculation which should be addressed in future studies.

As different bacteria produce MKn of different lengths (39, 40), the vitamin K profile varies with microbial composition (41). In the current study, infant fecal vitamin K composition and overall fecal microbial community composition differed significantly by feeding mode.

TABLE 3 Relative abundances of bacterial species that differed by feeding method and delivery mode in infant feces at 6 wk postpartum[1]

					P value		
	BF + VD	BF + CS	FF + VD	FF + CS	Feeding	Delivery	Interaction
Actinobacteria							
Atopobium parvulum	0 (0–0)	0 (0–0)	0 (0–0.004)	0.001 (0–0.029)	<0.001[2]	0.110	0.433
Bifidobacterium bifidum	0 (0–0)	0 (0–0)	0 (0–0)	0.643 (0–19.5)	0.052[2]	0.088	0.359
Bifidobacterium breve	0 (0–0)	0.071 (0–60.9)	0 (0–14.5)	2.87 (0.207–21.4)	0.095	*0.002*	0.095
Eggerthella lenta	0 (0–0)	0 (0–0)	0 (0–0.016)	0.004 (0–0.012)	0.001[2]	0.896	0.911
Eggerthella unclassified	0 (0–0)	0 (0–0.896)	0.376 (0.051–0.755)	0.149 (0.01–0.280)	0.02[2]	0.682	0.070
Gordonibacter pamelaeae	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)	0.034[2]	0.771	0.771
Rothia unclassified	0 (0–0)	0 (0–0)	0 (0–0.003)	0 (0–0.006)	0.004[2]	0.577	0.821
Bacteroidetes							
Bacteroides dorei	0 (0–0.019)	0 (0–0)	0 (0–0)	0 (0–0)	0.362	0.024[2]	0.362
Firmicutes							
Anaerostipes unclassified	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0.365)	0.005[2]	0.194	0.194
Clostridium bartlettii	0 (0–0)	0 (0–0)	0.032 (0–0.101)	0.142 (0.025–0.407)	<0.001[2]	0.253	0.253
Clostridium butyricum	0 (0–0)	0 (0–0)	0 (0–0.002)	0.001 (0–0.842)	<0.001[2]	0.451	0.451
Clostridium difficile	0 (0–0)	0 (0–0)	0 (0–0.045)	0.010 (0–0.352)	0.009[2]	0.977	0.493
Clostridium innocuum	0 (0–0)	0 (0–0)	0 (0–0.115)	0 (0–0.137)	0.003[2]	0.693	0.746
Clostridium ramosum	0 (0–0)	0 (0–0)	0 (0–0.009)	0 (0–0.094)	0.008[2]	0.916	0.389
Enterococcus avium	0 (0–0)	0 (0–0)	0 (0–0.087)	0.012 (0–0.078)	<0.001[2]	0.396	0.396
Enterococcus casseliflavus	0 (0–0)	0 (0–0)	0 (0–0.084)	0 (0–0.005)	0.001[2]	0.358	0.358
Enterococcus faecium	0 (0–0)	0 (0–0)	0 (0–0.207)	0 (0–0.004)	0.002[2]	0.571	0.571
Enterococcus gallinarum	0 (0–0)	0 (0–0)	0 (0–0.165)	0 (0–0.065)	0.001[2]	0.424	0.424
Erysipelotrichaceae bacterium 2_2_44A	0 (0–0)	0 (0–0)	0 (0–0.014)	0 (0–0.018)	0.019[2]	0.746	0.611
Erysipelotrichaceae bacterium 6_1_45	0 (0–0)	0 (0–0)	0 (0–0.003)	0 (0–0.009)	0.029[2]	0.263	0.687
Lactobacillus fermentum	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0.053)	0.005[2]	0.194	0.194
Lactococcus lactis	0 (0–0)	0 (0–0)	0 (0–0)	0.001 (0–0.280)	0.001[2]	0.146	0.146
Leuconostoc pseudomesenteroides	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0.021)	0.005[2]	0.005[2]	0.005[2]
Megasphaera micronuciformis	0 (0–0)	0 (0–0)	0 (0–0)	0.008 (0–0.023)	0.003[2]	0.021[2]	0.021[2]
Ruminococcus gnavus	0 (0–0)	0 (0–0)	0.027 (0–2.06)	0.97 (0–13.7)	0.001[2]	0.954	0.949
Ruminococcus torques	0 (0–0)	0 (0–0)	0.003 (0–0.027)	0.069 (0.014–0.265)	<0.001[2]	0.358	0.164
Streptococcus parasanguinis	0 (0–0)	0 (0–0.011)	0.073 (0.027–0.185)	0.084 (0.017–0.111)	<0.001[2]	0.604	0.604
Streptococcus salivarius	0.024 (0.008–0.041)	0.018 (0–0.035)	0.113 (0.035–0.534)	0.085 (0.017–0.237)	0.017[2]	0.348	0.943
Streptococcus thermophilus	0 (0–0)	0 (0–0)	0.103 (0.031–0.485)	0.281 (0.056–0.566)	<0.001[2]	0.899	0.899
Streptococcus vestibularis	0 (0–0)	0 (0–0)	0 (0–0.066)	0.008 (0–0.124)	0.003[2]	0.519	0.884
Subdoligranulum unclassified	0 (0–0)	0 (0–0)	0.004 (0–0.154)	0.061 (0.001–0.878)	0.001[2]	0.465	0.726
Veillonella atypica	0 (0–0.080)	0 (0–0)	0.01 (0.001–0.281)	0.213 (0.100–0.520)	0.001[2]	0.759	0.186
Veillonella unclassified	0.003 (0–0.026)	0.016 (0.002–0.603)	0.503 (0.022–3.42)	0.641 (0.291–0.880)	0.019[2]	0.468	0.361
Proteobacteria							
Citrobacter freundii	0 (0–0)	0 (0–0)	0 (0–0.007)	0.051 (0.003–0.141)	<0.001[2]	0.023[2]	0.023[2]
Citrobacter unclassified	0 (0–0)	0 (0–0)	0 (0–0.011)	0.041 (0.006–0.099)	<0.001[2]	0.026[2]	0.026[2]
Haemophilusparainfluenzae	0.007 (0 –0.072)	0 (0–0.111)	0 (0–0.003)	0 (0–0)	0.011[2]	0.115	0.666
Klebsiella oxytoca	0 (0–0)	0 (0–0.009)	0.028 (0.003–0.113)	0 (0–0.224)	0.007[2]	0.814	0.071
Klebsiella pneumoniae	0 (0–0)	0 (0–0)	0.002 (0–0.158)	0.261 (0.029–0.869)	0.005[2]	0.209	0.563
Klebsiella unclassified	0 (0–0)	0 (0–0.003)	0.022 (0–0.040)	0.007 (0.001–0.153)	0.001[2]	0.220	0.256

[1]Data are presented as median (25–75 percentiles). Key bacterial species were identified by redundancy analysis (RDA) and confirmed by univariate analysis. BF, breastfed; CS, cesarean section; FF, formula-fed; VD, vaginally delivered.

[2]Values indicate statistically significant differences ($P < 0.05$).

Differences in gut microbial composition and α-diversity by infant feeding practice is consistent with the literature (8, 42), and may, in part, explain some of the differences in fecal MKn content by feeding practice. *Eggerthella lenta* (43) and *Gordinobacter pamelaeae* (44) both produce MK6 as their main respiratory quinone, and some *Veillonella* sp. (40) produce MK7. These taxa were higher in formula-fed compared with breastfed infants, and formula-fed infants also had higher corresponding fecal MK6 and MK7 content. However, multiple species that differed by feeding practice belonged to genera that are not known to produce MKn, such as *Bifidobacterium* (39, 40), *Clostridium* (39), *Lactobacillus* (39), and *Ruminococcus* (40). This is not unexpected, as the compositional differences between breast milk and formula are known to have effects on the gut microbiota (8, 10) irrespective of vitamin K.

Infant feeding practice has direct effects on the gut bacterial composition by providing substrates for bacterial metabolism (45). The effects of feeding practice on the fecal vitamin K content may therefore, in part, be a result of differences in substrate availability for de novo MKn synthesis. MKn are produced and used by bacteria as electron carriers in anaerobic respiration (46). Here, infant fecal bacterial MKn gene content varied by feeding mode, with MKn gene content of MKn pathways generally being higher in breastfed compared with formula-fed infants. We recently discovered in mice that dietary vitamin K forms can be partially remodeled by gut microbes to bacterial MKn (12), therefore, feeding practice may influence MKn production in the infant gut through provision of substrate for both de novo vitamin K synthesis and remodeling.

Historically, it was assumed the gut was sterile at birth and, thus, contributed no vitamin K (47). Although the absolute sterility of the gut at birth is contested (48), after birth the microbiome develops rapidly during infancy and early childhood (49). Here, infant fecal bacterial composition differed by delivery mode, consistent with previous literature (9). Vertical transfer of gut bacteria from the mother and normal colonization has been reported to be disrupted in cesarean-delivered infants (50), therefore, it should similarly be considered that mode of delivery might then also affect the infant gut bacterial capacity to produce vitamin K. Total MKn was lower in infants delivered by CS compared with infants delivered vaginally. Transmission of bacteria from the mother may also occur postnatally (51, 52). We did not observe any relation between the vitamin K content of maternal and infant feces at 6 wk postpartum, suggesting that any transfer of vitamin K or vitamin K-producing bacteria from the mother is limited to a smaller window surrounding delivery.

This study has several strengths. This cohort contained mother-infant dyads who practiced exclusive breastfeeding or formula-feeding practices, which allowed us to clearly delineate the effect of feeding on the infant gut microbiota and associated vitamin K production. Furthermore, this cohort will be followed throughout childhood, allowing for future research on the same cohort and potential long-term outcomes that may be related to vitamin K. This study also has several limitations. We did not have direct measures of maternal or infant vitamin K status. Vitamin K status is currently evaluated using plasma PK, and MKn are not typically found in circulation (53). Secondly, bacterial data were assessed on a relative abundance basis and bacterial load was not assessed. Measures of absolute bacterial abundance may have enhanced the comparisons to vitamin K concentrations. Lastly, we oversampled cesarean-delivered infants in this analysis [relative to the full STRONG kids 2 cohort (43% compared with 25.6%)] in order to have a sufficient number of subjects to test for route of delivery. This may have introduced some bias and reduced generalizability to the US population, which had a CS rate of 31.7% in 2019 (54).

In conclusion, we report that feeding mode and delivery mode are independently predictive of infant fecal vitamin K content. High concentrations of unmetabolized PK in feces of formula-fed infants suggests formula PK content exceeds the absorptive capacity of the infant gut. Further investigation into the role of gut bacterially produced MKn in early life nutrition and health is needed to understand how these early life practices influence the vitamin K status of the infant, either directly through the vitamin K content of the infant diet, or indirectly through modulation of the gut microbiome.

Acknowledgments

The authors' responsibilities were as follows—JLE, SMD, and SLB: designed the research; JLE, MW, XF, and CJF: conducted data analysis; JLE: prepared the initial draft of the manuscript; all authors: reviewed and contributed; SMD and SLB: had primary responsibility for final content; and all authors: read and approved the final manuscript.

Data Availability

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval.

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