Nutrient Requirements and Optimal Nutrition

The Gut Microbiota Differ in Exclusively Breastfed and Formula-Fed United States Infants and are Associated with Growth Status

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A B S T R A C T

Background: Evidence regarding the effects of infant feeding type (exclusive breastfeeding compared with exclusive formula feeding) on the gut microbiota and how it impacts infant growth status is limited.
Objectives: The primary objective was to compare gut microbiota by feeding type and characterize the associations between gut microbiota and infant growth status.
Methods: Stool samples from healthy, full-term infants (4–5 mo-old) who were either exclusively breastfed (BF) or exclusively formula-fed (FF) in Denver, CO, United States were collected, and fecal 16S ribosomal ribonucleic acid gene-based profiling was conducted. Length and weight were measured at the time of stool collection. Length-for-age z-score, weight-for-age z-scores (WAZ), and weight-for-length z-scores were calculated based on the World Health Organization standards. Associations between gut microbial taxa and anthropometric z-scores were assessed by Spearman's rank correlation test.
Results: A total of 115 infants (BF $n = 54$; FF $n = 61$) were included in this study. Feeding type (BF compared with FF) was the most significant tested variable on gut microbiota composition ($P < 1 \times 10^{-6}$), followed by mode of delivery and race. Significant differences were observed in α-diversity, β-diversity, and relative abundances of individual taxa between BF and FF. BF infants had lower α-diversity than FF infants. Abundances of *Bifidobacterium* and *Lactobacillus* were greater in the breastfeeding group. FF infants had a higher relative abundance of unclassified Ruminococcaceae ($P < 0.001$), which was associated with a higher WAZ ($P < 0.001$) and length-for-age z-score ($P < 0.01$). *Lactobacillus* was inversely associated with WAZ ($P < 0.05$).
Conclusions: Feeding type is the main driver of gut microbiota differences in young infants. The gut microbiota differences based on feeding type (exclusive breast- or formula feeding) were associated with observed differences in growth status.
This trial was registered at clinicaltrials.gov as NCT02142647, NCT01693406, and NCT04137445.

Keywords: mode of feeding, gut microbiota, growth status, breastfeeding, infant formula

Introduction

Before solid foods are introduced, the liquid diet (breastmilk compared with infant formula) accounts for virtually all of the infant's dietary intake, which affects the gut microbiota [1]. The gut microbiota has been shown to associate with human health indicators and disease development, such as allergies, autoimmune diseases, and obesity [2–4]. During infancy and early childhood, the gut microbiota also plays a role in developing certain health conditions [5]. More importantly, underdeveloped or nonage-appropriate gut microbiota has been shown to negatively impact infant growth in low-source settings [6,7]. Thus, a thorough understanding of the gut microbiota and the role of feeding type in modulating the gut microbiota during infancy is critical [1].

Research to date has produced inconsistent findings regarding the effects of feeding mode on infant gut microbiota. For example, although the majority of the literature showed that breastfed (BF) infants tend to have a higher abundance of *Bifidobacterium* [8] and lower overall α-diversity [9], some reported significantly higher *Bifidobacterium* in mixed-fed infants, defined as infants fed a mixture of both breastmilk and formula, compared with exclusively BF infants [10]. A meta-analysis concluded that exclusive breastfeeding is strongly associated with a greater abundance of the family *Bifidobacteriaceae* and lower α-diversity before 6 mo compared with nonexclusive breastfeeding [11]. One limitation of the current literature is that most findings were based on breastfeeding exclusivity, namely comparing exclusively BF with nonexclusively BF infants. Nonexclusive breastfeeding includes exclusive formula feeding and feeding both infant formula and breastmilk, adding noise to the comparison and making it difficult to interpret the results. Emerging research also suggests that gut microbiota may modulate growth and weight gain early in life [12]. One animal study [13] found that gut microbiota drives bone growth in juvenile mice. A cohort study [14] identified bacterial species, such as *Ruminococcus gnavus*, whose proportional representation defined healthy and mature gut microbiota during the first year of life in Malawian infants. It remains unknown to what extent, if any, these types of growth-discriminatory taxa might have in a high-resourced Westernized setting and/or in healthy, well-nourished populations.

In the present study, the gut microbiota of exclusively BF infants and exclusively formula-fed (FF) infants at 4–5 mo of life from Denver, CO, were assessed. The 2 objectives were as follows: *1*) to compare gut microbial profiles by feeding type and *2*) to examine the potential association between gut microbial composition and infant growth status. We hypothesized that *1*) the gut microbiota composition would differ by feeding type, with BF having higher abundances of potential commensals, such as *Bifidobacterium*, and with FF being more diverse and having taxa representing more "mature" gut microbiota; and *2*) gut microbial composition would associate with infant linear and ponderal growth status (eg, *z*-scores).

Methods

Participants

This secondary analysis uses fecal samples collected from 3 healthy, full-term (gestational age ≥37 wk) infant cohorts in Denver, CO. Healthy was defined as not having significant congenital anomalies or known chronic conditions affecting feeding, growth, or developmental potential. Two cohorts had BF infants exclusively (no formula exposure since birth), and 1 had FF infants exclusively (breastmilk exposure <2 wk). Fecal samples were collected from all 3 infant cohorts at 4–5 mo of age, which was the baseline time point of 1 completed, 1 ongoing infant dietary intervention study, and the end point of a completed observational study [15]. Thus, none of the participants received any intervention at the time of stool sample collection (4–5 mo). The Colorado Multiple Institutional Review Board approved all 3 studies. Written informed consent

was obtained from the parents or legal guardians of the infants. These studies were registered at clinicaltrials.gov (NCT02142647, NCT01693406, and NCT04137445).

Stool sample collection

To collect stool samples, sterile diaper liners, gloves, and prelabeled ziplock bags were given to caregivers to place in the infant's diaper. The liner was biodegradable, which effectively collected stool, but allowed passage of urine. Caregivers were given instructions on how to place and remove the liner from the diaper once stool was produced. The soiled liner was then placed by the caregiver in zip lock bags with written collection time and date. The caregiver placed the bagged samples in home freezers (−20°C) and notified the study coordinator immediately. The study coordinator collected the samples within 24 h and transferred them to the University of Colorado Anschutz Medical Campus on ice packs. Stool samples were then collected by research personnel from the liner in a laminar flow hood and stored in sterile vials in −80°C freezers until analysis. All 3 infant cohorts followed the same sample collection procedures, and samples were sequenced in 1 batch. One cohort of BF infants used RNAlater (Qiagen) in the sterile vials to store the stool samples, whereas the other 2 cohorts used sterile vials only. Comparison of sequencing data and other variables between the 2 BF cohorts that samples were stored with or without RNAlater showed no differences, and the 2 BF cohorts were combined.

Weight and length measurements

Infant weight and length were measured in all participants by trained pediatric research personnel at the Colorado Clinical and Translational Research Center at Children's Hospital Colorado. All measurements were performed in triplicate. The length was measured in a recumbent position using an infant stadiometer accurate to 0.1 cm (Holtain Ltd.). An electronic digital balance (Sartorius Corporation) was used to obtain naked infant weight. Growth *z*-scores (the number of SDs above or below the population median) were calculated based on WHO/CDC BF infant growth standards [16]. In brief, the weight-for-age *z*-score (WAZ, weight parameter) and length-for-age *z*-score (LAZ, length parameter) are based on weight and length using the WHO/CDC growth standards controlled for age and sex [16]. Weight-for-length *z*-score (WLZ) is derived from WAZ and LAZ.

Microbiota analysis
16S amplicon library construction

Bacterial profiles were determined by broad-range amplification and sequence analysis of 16S rRNA genes following our previously described methods [17,18]. In brief, DNA was extracted from 25 to 50 mg of stool using the QIAamp Power-Fecal DNA kit (Qiagen Inc.), which employs chemical and mechanical biomass disruption. Samples were bead-beaten using a MagNA Lyser (Roche Inc.) at $10,000 \times g$; 60 s. PCR amplicons were generated using barcoded [19] primers that target ~450 basepairs of the V3V4 variable region of the 16S rRNA gene (338F: 5'ACTCCTACGGGAGGCAGCAG and 806R: 5' GGAC-TACHVGGGTWTCTAAT) [20,21]. PCR products were normalized using a SequalPrep kit (Invitrogen) and then pooled. The amplicon pool was partially lyophilized to reduce its volume and

then purified and concentrated using a DNA Clean and Concentrator Kit (Zymo). Pooled amplicons were *quantified using a Qubit Fluorometer 2.0* (Invitrogen). Illumina paired-end sequencing was performed following the manufacturer's protocol on the MiSeq platform using a 600-cycle version 3 reagent kit and version 2.4 of the MiSeq Control Software. All samples were sequenced in a single batch.

Analysis of illumina paired-end reads

Illumina MiSeq paired-end reads were aligned to human reference genome hg19 with bowtie2 and matching sequences discarded [22,23]. As previously described [17,18], demultiplexed paired reads were assembled using phrap [24,25], and pairs that did not assemble were discarded. Assembled sequences were trimmed over a moving window of 5 nucleotides until the mean quality met or exceeded 20. Trimmed sequences with >1 ambiguity or <350 nt were discarded. Potential chimeras identified with Uchime (usearch6.0.203_i86linux32) [26] using the Schloss [27] Silva reference sequences were removed from subsequent analyses. Assembled sequences were aligned and classified with SINA (1.3.0-r23838) [28] using the 418,497 bacterial sequences in Silva 115NR99 [29] as reference configured to yield the Silva taxonomy. Taxonomic assignment by SINA used the lowest common ancestor approach with default parameters. Operational taxonomic units were produced by binning sequences with identical taxonomic assignments. A single sample generated <5000 sequences and was excluded from further analysis. The remaining 114 sequence libraries had a median of 90,914 sequences/sample (interquartile range: 73, 820–106,109), and all libraries had Good's coverage values >99%. The software package Explicet (version 2.10.5) [30] was used to calculate α-diversity indices through 1000 replicate resamplings.

Statistical and data analyses

Values are presented as mean ± SD for continuous variables. *P* values of ≤0.05 were considered to be significant. The software packages R (version 4.1.0) [31] and Explicet (version 2.10.5) [30] were used to analyze and visualize data. Independent Student's *t*-test was used to compare differences in growth and other demographic data between groups. For the gut microbiota analysis, differences in overall composition (ie, β-diversity) were assessed through permutational ANOVA (PERMANOVA) with the Aitchison dissimilarity index [32,33]. PERMANOVA *P* values were inferred through 10^6 label permutations. For 2- and 3-factor PERMANOVA tests, margin *P* values are reported for each covariate. Principal coordinates analysis was carried out using Aitchison dissimilarities and the vegan wcmdscale function. Alpha-diversity indices (ie, Chao1, Shannon H, and Shannon H/Hmax) were assessed by ANOVA. Individual taxa differing between treatment groups were identified using the ANOVA-like differential expression (ALDEx2) R package [34,35]. The distribution of taxa in each sequence library was estimated through 1000 Dirichlet Monte Carlo resamplings of sequence count data. To account for the compositional nature of microbiome data, [36] sequence count data were subjected to a centered log-ratio (CLR) transformation with all features used as the denominator. Either nominal or false discovery rate (FDR)-corrected [37] *P* values are reported, as indicated in the text and figures. Effect size plots are derived from the outputs of ALDEx2 and represent the median

effect sizes, calculated as the median between-group difference in CLR values between groups divided by the largest within-group difference in CLR values [34,35]. Associations between anthropometric measures and CLR-transformed microbiota count data were assessed by nonparametric Kendall rank correlation tests.

Results

Participants

A total of 115 infants, aged 4–5 mo, were initially included in the study; 54 infants were exclusively BF, and 61 infants were exclusively FF at the time of stool collection. The BF group was 87% White ($n = 47$), 13% non-White ($n = 7$), 50% male ($n = 27$), and 50% female ($n = 27$). The FF cohort was 69% White ($n = 42$), 31% non-White ($n = 19$), 52.5% male ($n = 32$), and 47.5% female ($n = 29$). For maternal education, 73% and 74% had an associate degree or higher in BF and FF groups, without differences between groups, respectively. Three participants had a gross family income <$18,000/y ($n = 1$ in FF; $n = 2$ in BF); 80% of families' income was >$50,000/y for both BF and FF. Table 1 depicts infant demographics, maternal BMI (kg/m^2), maternal height, delivery mode, antibiotic use, and growth *z*-scores. There was a significant difference between BF and FF infants in both WAZ and WLZ. No significant difference in LAZ was observed between groups.

Dietary intakes

At the time of stool sample collection, none of the BF infants had received complementary formula or foods. Likewise, none of the FF infants had consumed human milk for >2 wk (cumulatively) since birth or any complementary foods by caregivers' reports. All BF infants took vitamin D supplements. FF infants consumed several types of infant formula, including Enfamil AR ($n = 2$), Enfamil Gentlease ($n = 14$), Enfamil Infant ($n = 17$), Gerber Goodstart Gentle ($n = 3$), Nutramigen ($n = 1$), Similac Advance ($n = 16$), or Similac Sensitive ($n = 8$). Supplementary Table 1 shows the macronutrient composition of each type of formula. No differences in the infant gut microbiota were found among formula types, degree of protein hydrolysis, or percentage of whey protein compared with casein or lactose content (PERMANOVA tests with no significant *P* values). Also, none of

TABLE 1

Characteristics of study participants

	BF (n = 54)	FF (n = 61)
Race[1]	White n = 47 (87%)	White n = 42 (69%)
Sex	Male n = 27 (50%)	Male n = 32 (52%)
Maternal BMI[1] (kg/m^2)	25.6 ± 5.5	28.0 ± 6.5
Maternal height (cm)	164 ± 7	166 ± 7
Mode of delivery	C-section $n = 15$ (28%)	C-section n = 16 (26%)
Antibiotic use (n)	1 (2%)	2 (3%)
LAZ	–0.386 ± 0.942	–0.197 ± 0.945
WAZ[1]	–0.510 ± 0.923	–0.088 ± 0.774
WLZ[1]	–0.296 ± 0.945	0.125 ± 0.796

Abbreviations: BF, breastfed infants; BMI, body mass index; FF, formula-fed infants; LAZ, length-for-age *z*-score; WAZ, weight-for-age *z*-score; WLZ, weight-for-length *z*-score.

[1] indicate variables that were statistically significant ($P < 0.05$) between groups.

the formulas on the United States market at the time of sample collection (2013–2015) contained human milk oligosaccharides, which are prebiotics in human milk that could impact gut microbiota. Thus, all FF infants were combined as 1 group for analysis purposes and treated equally.

Type of feeding and infant gut microbiota

Bacterial profiles were generated for all 115 infant fecal samples by 16S rRNA gene sequencing; 1 infant sample was excluded from further analysis because of low sequence counts (<5000). Overall, β-diversity differed significantly between BF and FF infants (PERMANOVA $P < 1 \times 10^{-6}$; Figure 1A). A principal coordinates analysis plot showed clear clustering of participants by feeding type (Figure 1B). FF infants also have greater α-diversity than BF infants (Figure 1C), as measured by richness

(Chao1; $P = 6.6e-06$), evenness (Shannon H/Hmax; $P = 0.0095$), and diversity (Shannon H; $P = 0.00034$).

A few genera (Figure 2A) and phyla (Figure 2B) had significantly different abundances between feeding types based on a stringent cutoff of FDR-corrected $P < 0.001$. BF infants had higher relative abundances of *Staphylococcus, Lactobacillus, Haemophilus,* unclassified *Bacilli, Gardeneralla,* unclassified *Gammaproteobacteria, B38, Rothia, Atopobium, Gemella,* and *Bifidobacterium.* FF infants had higher relative abundances of *Anaerostipes,* unclassified *Peptostreptococcaceae, Blautia, Flavonifractor, Erysipelotrichaceae, Coprococcus,* unclassified *Ruminococcaceae, Akkermansia,* and unclassified *Lachnospiraceae.* Two phylum-level taxa (Proteobacteria and Verrucomicrobia) also had FDR-corrected P values of <0.001, with Proteobacteria higher in BF and Verrucomicrobia higher in FF. Actinobacteria
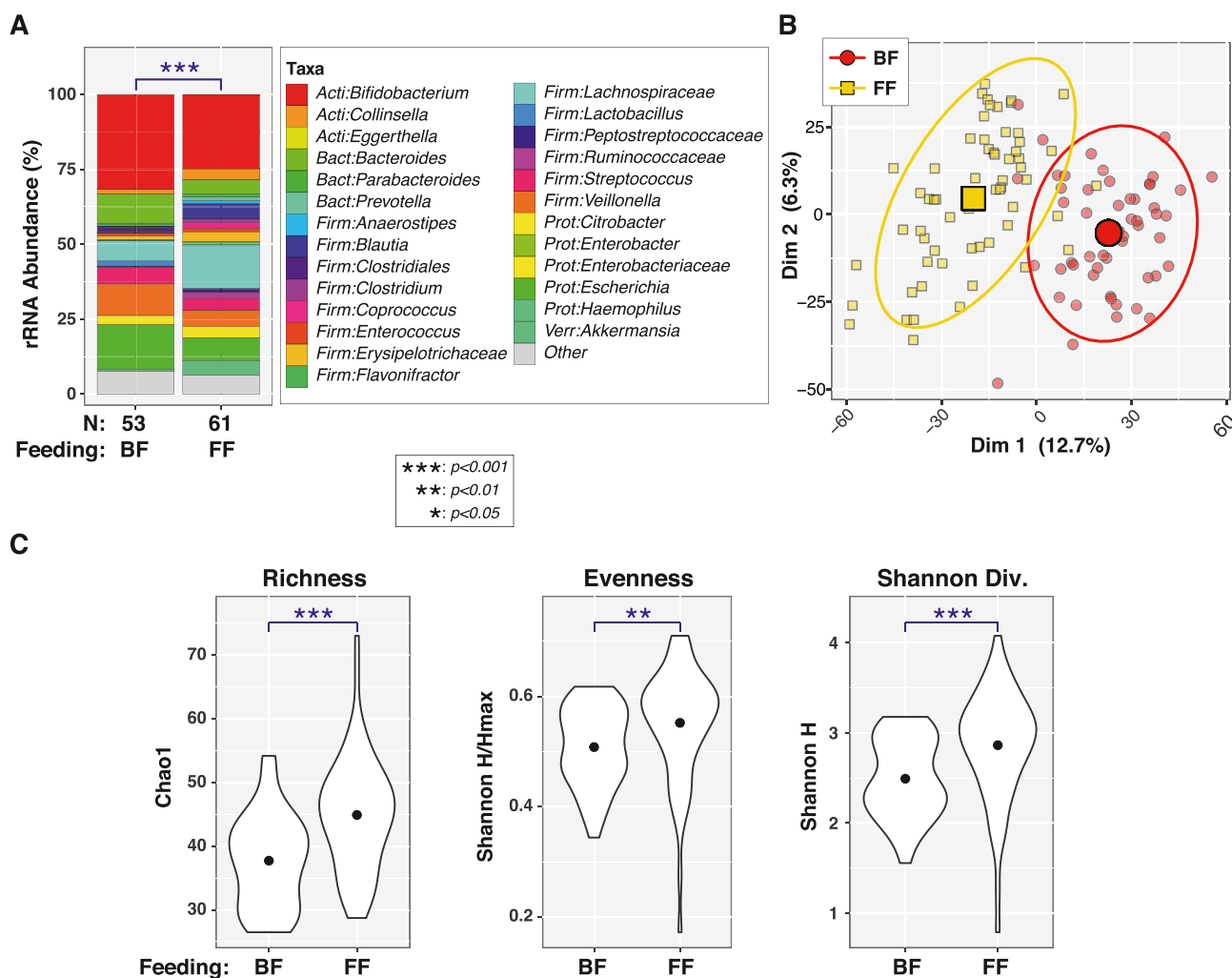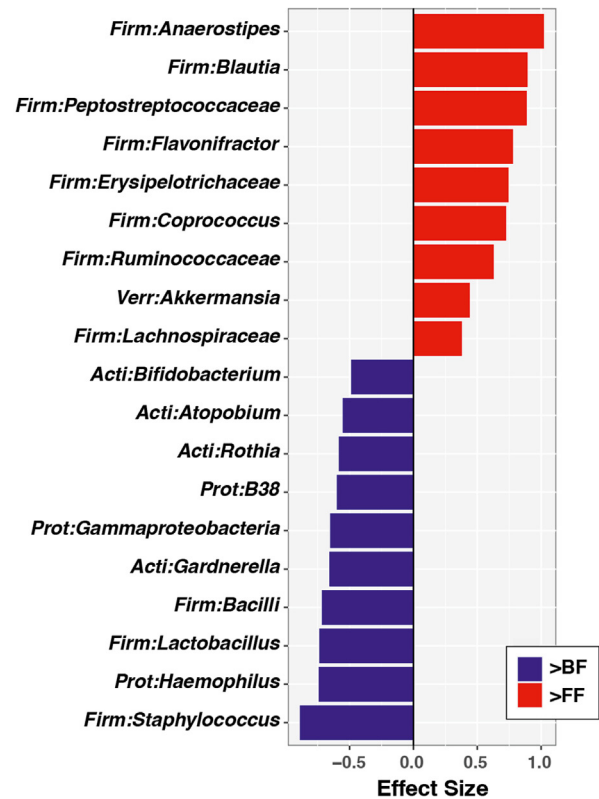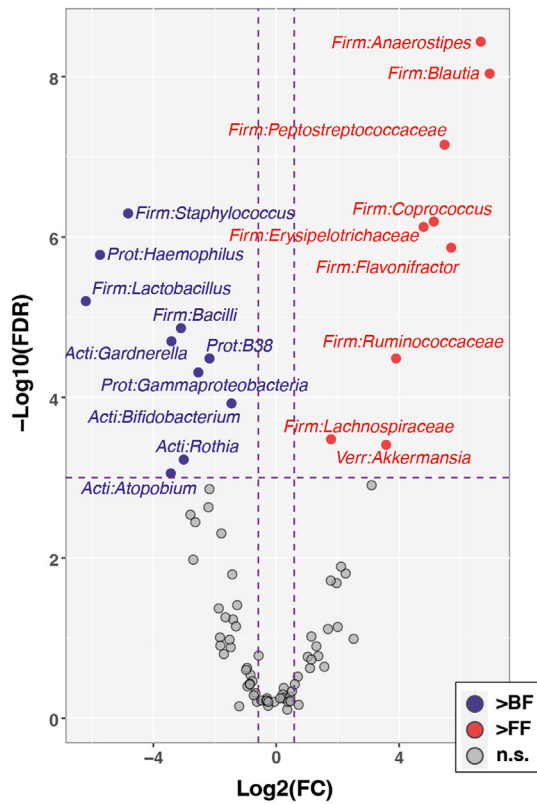

FIGURE 1. Variation in 4–5 mo-old breastfed (BF) and formula-fed (FF) infant microbiota between feeding types. (A) Bar charts with PERMANOVA results. Percent relative abundance (%RA) of genus-level taxa, stratified by feeding type. Taxa with %RA <1% were collapsed into the "Other" category. The results of PERMANOVA tests are summarized in the plot. (B) Principal coordinates plot. Individual subjects are indicated by smaller symbols (circles or squares), with group affiliations designated by symbol shapes and color coding. Mean PC values for each group along the x- and y-axes are indicated by larger shapes, whereas ellipses represent 95% CIs. (C) Alpha-diversity. Violin plots show distributions of α-diversity indices by feeding type. ANOVA test results are indicated above each plot. BF ($n = 54$), FF ($n = 61$). Taxa listed at the family level are those that cannot be identified at the genus level. ANOVA, analysis of variance; CI, confidence interval; PERMANOVA, permutational analysis of variance; rRNA, ribosomal ribonucleic acid; Dim 1 & Dim 2, Principal coordinates dimensions 1 and 2.

A. Genera (FDR<0.001)
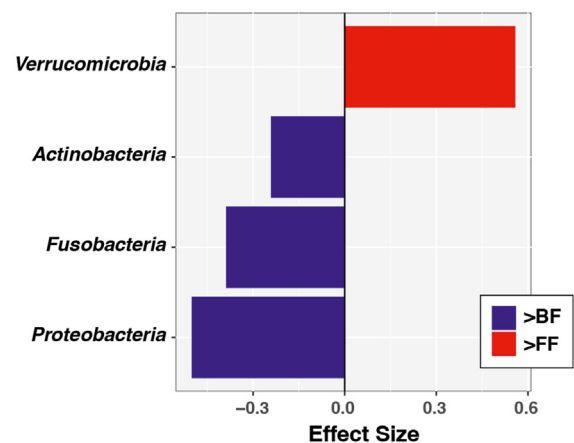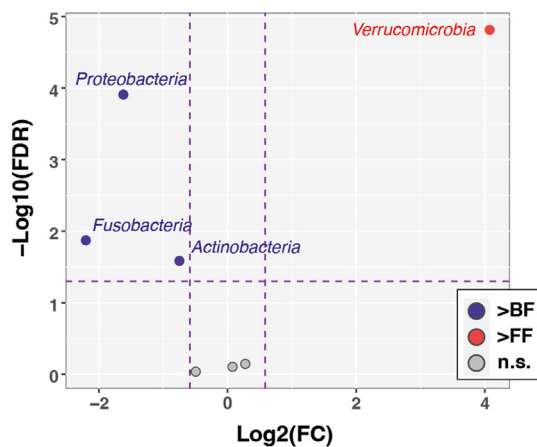

B. Phyla (FDR<0.05)

FIGURE 2. Individual taxa varying by feeding type in 4–5 mo-old breastfed (BF) and formula-fed (FF) infants. Tests were conducted at the genus (A) and phylum (B) levels. The left column shows volcano plots of fold-change (FC; Log2 transformed) compared with FDR-corrected P values (−Log10 transformed) ascertained by ALDEx2 analysis. Vertical and horizontal dashed lines represent significance cutoffs of FC ≥ 2 and FDR-corrected P value ≤ 0.001, respectively. Taxa in the upper left quadrant and colored blue were enriched in BF relative to FF infants ("">BF""). Taxa in the upper right quadrant and colored red were enriched in FF relative to BF infants ("">FF""). Taxa that did not meet the significance cutoffs are colored gray ("NS"; ie, FC <2 or FDR-corrected P value >0.001). The right column shows plots of ALDEx2-calculated effect sizes of taxa meeting FC and FDR cutoffs. BF ($n = 54$), FF ($n = 61$). Taxa listed at the family level are those that cannot be identified at the genus level. ALDEx2, ANOVA-like differential expression; FDR, false discovery rate.

and Fusobacteria were higher in BF infants at a less stringent cutoff (FDR < 0.05). Individual taxa relative abundances are in supplementary materials.

Other determinants of infant gut microbiota

Besides feeding type, maternal and infant demographic/clinical variables were also examined. Univariable PERMANOVA

TABLE 2
Variables and their associations with the gut microbiota using 3 different models

Variable	PERMANOVA *P* value[1]		
	Univariable	Adjusted for feeding type	3-variable model
Feeding type	1.00×10^{-6}	na	1.00×10^{-6}
Race	0.0011	0.038	0.064
Sex	0.290	0.157	na
Delivery mode	0.010	0.0060	0.010
Antibiotics	0.54	0.44	na
Maternal BMI[2]	0.19	0.53	na
Maternal Hgt	0.51	0.39	na
Maternal Wgt	0.0061	0.28	na
Infant LAZ	0.40	0.52	na
Infant WAZ	0.067	0.79	na
Infant WLZ	0.079	0.61	na

Abbreviations: BMI, body mass index; LAZ, length-for-age *z*-score; na, not applicable; PERMANOVA, permutational analysis of variance; WAZ, weight-for-age *z*-score; WLZ, weight-for-length *z*-score.

[1] Univariable PERMANOVA tests were used to test associations between overall microbiota composition (ie, β-diversity) and variables listed in the variable column–Aitchison dissimilarity. 10^6 permutations
[2] Included underweight, normal weight, overweight, and obese categories.

tests (Table 2) found significant or marginally significant associations between overall microbiota composition (ie, β-diversity) and feeding type ($P < 1 \times 10^{-6}$), infant race ($P = 0.001$), delivery mode ($P = 0.010$), maternal weight ($P = 0.0061$), infant WAZ ($P = 0.067$), and infant WLZ ($P = 0.079$). On the contrary, infant sex, antibiotic use, maternal BMI, maternal height, or infant LAZ were not significantly associated with gut microbiota diversity ($P > 0.05$). Only race ($P = 0.038$) and delivery mode ($P = 0.006$) remained significant when feeding type was included as a covariate in bivariable PERMANOVA tests. A 3-variable PERMANOVA test showed that feeding type ($P < 1e-06$), race ($P = 0.064$), and delivery mode ($P = 0.010$) were all independently associated with β-diversity when adjusted for the other 2 variables (Table 2). Effects of delivery mode and race were further assessed within each feeding type (Figures 3 and 4). Significant differences in β-diversity were observed between vaginal delivery and cesarean delivery groups of BF infants ($P = 0.025$), whereas the difference was marginal for FF infants ($P = 0.069$). Vaginally delivered FF infants also had greater α-diversity (richness, evenness, and Shannon diversity) than the other infants (Figure 3C). Beta diversity was different between White and non-White FF infants ($P = 0.0082$), but not BF infants ($P = 0.56$). No differentially abundant taxa were identified for either delivery mode or race by FDR-corrected *P* values (Supplementary Figure 1).

Association of gut microbiota with infant growth parameters

Associations between anthropometric measures and CLR-transformed microbiota count data were assessed by Pearson correlations. A heatmap of growth *z*-scores compared with genus-level infant gut microbiota is presented in Figure 5. There were several genera that were either positively or negatively associated with infant growth status and maternal height and weight. For example, unclassified *Ruminococcaceae* was positively associated with both WAZ ($\tau = 0.15$; $P < 0.05$) and LAZ ($\tau = 0.16$; $P < 0.05$). In contrast, *Lactobacillus* was negatively associated with WAZ ($\tau = -0.14$; $P < 0.05$) and LAZ ($\tau = -0.14$, $P < 0.05$). Maternal weight was positively associated with *Akkermensia,* and *Barnesiella* was negatively associated with WAZ WLZ.

Discussion

Findings from the present study suggest that feeding type (exclusive breastfeeding compared with exclusive formula feeding) is a major determinant of the infant gut microbial composition and diversity in 4–5 mo-old United States infants. More importantly, the gut microbiota is associated with infant growth status. In the present study, Proteobacteria was higher in BF infants, although the actual abundance was relatively low (19% in BF and 12% in FF). Previous research [38] found lower Proteobacteria abundance in BF infants compared to non-BF infants. Proteobacteria have been shown to associate with adverse health conditions, such as necrotizing enterocolitis [39] and lower hematocrit [40] in preterm infants and slower growth trajectories and smaller brain volumes in mice [41]. The higher Proteobacteria abundance in BF did not appear to negatively affect infant growth status (Figure 5). Given that the participants were healthy, term United States infants and the overall Proteobacteria abundance was low; the observed higher Proteobacteria in BF did not appear to be concerning. Consistent with previous research [42,43], the present study also found a higher abundance of *Bifidobacterium* in BF infants. Although some research showed that the differences in *Bifidobacterium* by feeding type disappeared at 3 mo [1], the present study found that *Bifidobacterium* was still more abundant in BF at 4–5 mo of age, right before solid foods are normally introduced, indicating that the impact by feeding type could be robust and persistent in young infants. Although the present study did not find associations between infant growth status and *Bifidobacterium,* emerging research suggests a positive association between *Bifidobacterium* and weight gain in preterm infants [44,45]. One recent study showed that *Bifidobacterium* species *B. infantis* treatment/supplementation promoted weight gain in malnourished Bangladeshi infants [46]. It appears that in undesirable health conditions (preterm and malnutrition), *Bifidobacterium* could promote weight gain. However, its role in growth still needs to be explored in full-term, healthy infants.

Genus *Ruminococcus* is known as 1 of the SCFA producers, which plays a critical role in energy harvesting and could affect both linear growth and body weight [47]. Emerging research also has suggested that *Ruminococcus* may directly impact infant linear growth (eg, LAZ) as a "growth-discriminating" strain that is absent or low in growth-impaired infants or immature gut microbiota [48]. In a recent study of 118 Bangladeshi children, those who received a microbiota-directed complementary food prototype were linked to 21 bacterial taxa, including *Ruminococcus,* that were positively correlated with WLZ [14]. Although the present study did not identify
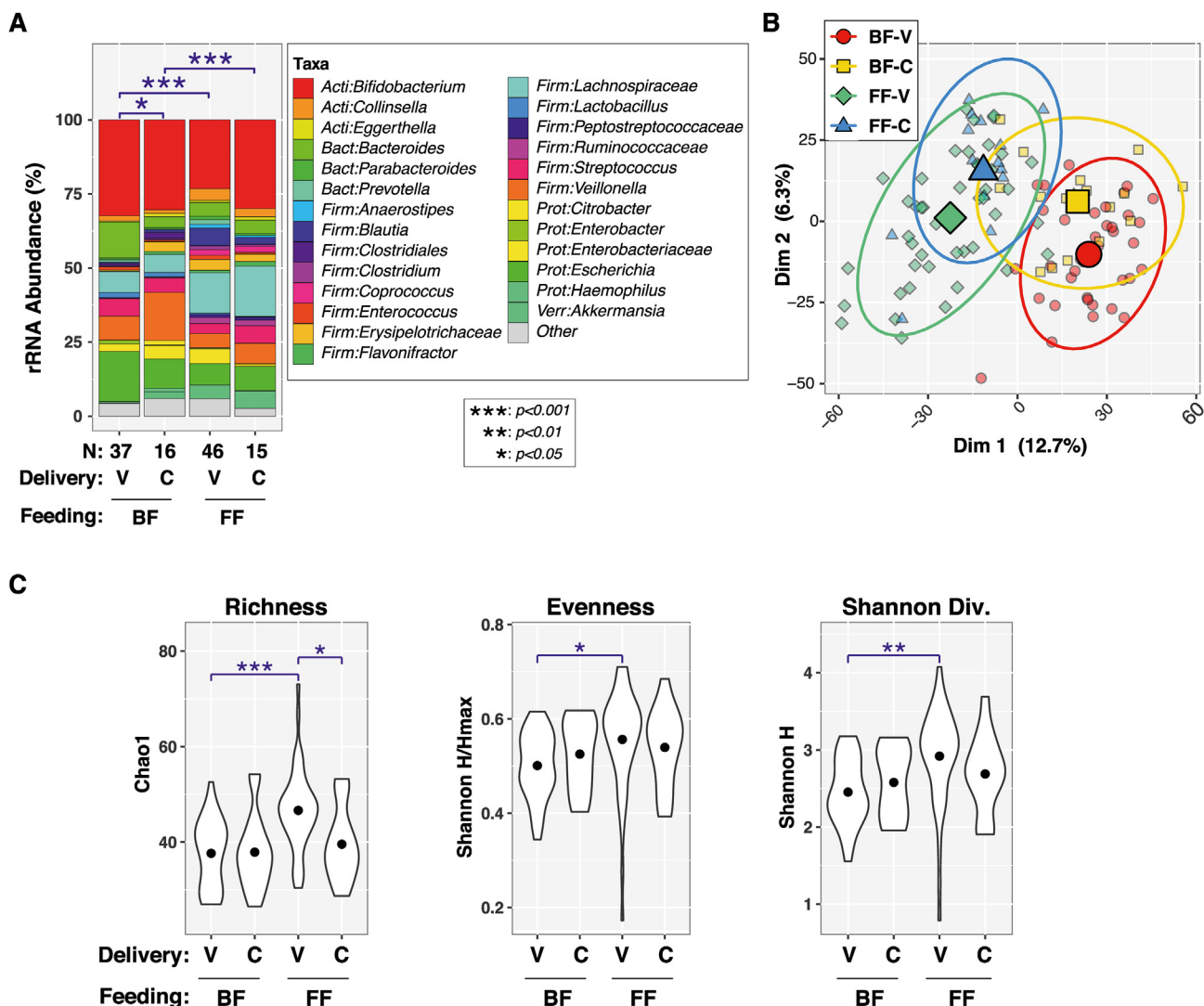
FIGURE 3. Effects of delivery mode and feeding type on α- and β-diversity in 4–5 mo-old breastfed (BF) and formula-fed (FF) infants. (A) Bar charts with PERMANOVA results. Percent relative abundance (%RA) of genus-level taxa, stratified by feeding type. Taxa with %RA <1% were collapsed into the "Other" category. The results of PERMANOVA tests are summarized above each plot for pairwise tests (blue lines/symbols). (B) Principal coordinates plot. Individual subjects are indicated by smaller symbols (circles/squares/diamonds/triangles), with group affiliations designated by symbol shapes and color coding. Mean PC values for each group along the x- and y-axes are indicated by larger shapes, whereas ellipses show 95% CI. (C) Alpha diversity. Violin plots show distributions of α-diversity indices by feeding type. Pairwise test results are indicated above each plot (blue lines/symbols). BF-V = BF infants born by vaginal delivery ($n = 39$), BF-C = BF infants born by cesarean delivery ($n = 15$), FF-V = FF infants born by vaginal delivery ($n = 45$), FF-C = FF infants born by cesarean delivery ($n = 16$). CI, confidence interval; PERMANOVA, permutational analysis of variance; rRNA, ribosomal ribonucleic acid; Dim 1 & Dim 2, Principal coordinates dimensions 1 and 2.

Ruminococcus at the genus level to associate with growth z-scores, the family level *Ruminococcaceae* was positively associated with both LAZ and WAZ.

In general, during the first year of life, BF infants are leaner than FF infants [49]. In the present study, there was a higher relative abundance of *Lactobacillus* in BF compared with FF, which was negatively associated with both WAZ and LAZ. Similar to *Bifidobacterium*, *Lactobacillus* also promotes weight gain in preterm infants [50]. Thus, the effects of these bacteria need to be considered in the context of the study population (ie, preterm compared with healthy full-term infants). Another taxon *Barnesiella* was negatively associated with both WAZ and WLZ. Human milk oligosaccharides promote the growth of *Barnesiella*, which reduces infections in animal models [51]. Although there was no statistical difference in *Barnesiella* abundance between BF

and FF, it could still partially explain the lower WAZ and WLZ in BF.

The present study showed that BF infants had lower gut microbiota α-diversity. Although most studies in developed settings [52,53] reported lower α-diversity with breastfeeding, studies of African infants [54] and Chinese infants [55] found that feeding type did not impact gut microbiota diversity, possibly because of potential environmental impact. As illustrated in Figure 3C, the higher α-diversity in FF appeared to be driven by vaginally delivered infants, and gut microbiota richness was higher in vaginally delivered infants. These findings were consistent with previous research [56].

Although recent studies suggest that varying formula sources may have differing effects on the infant microbiota [57], the present study showed no difference between formula components
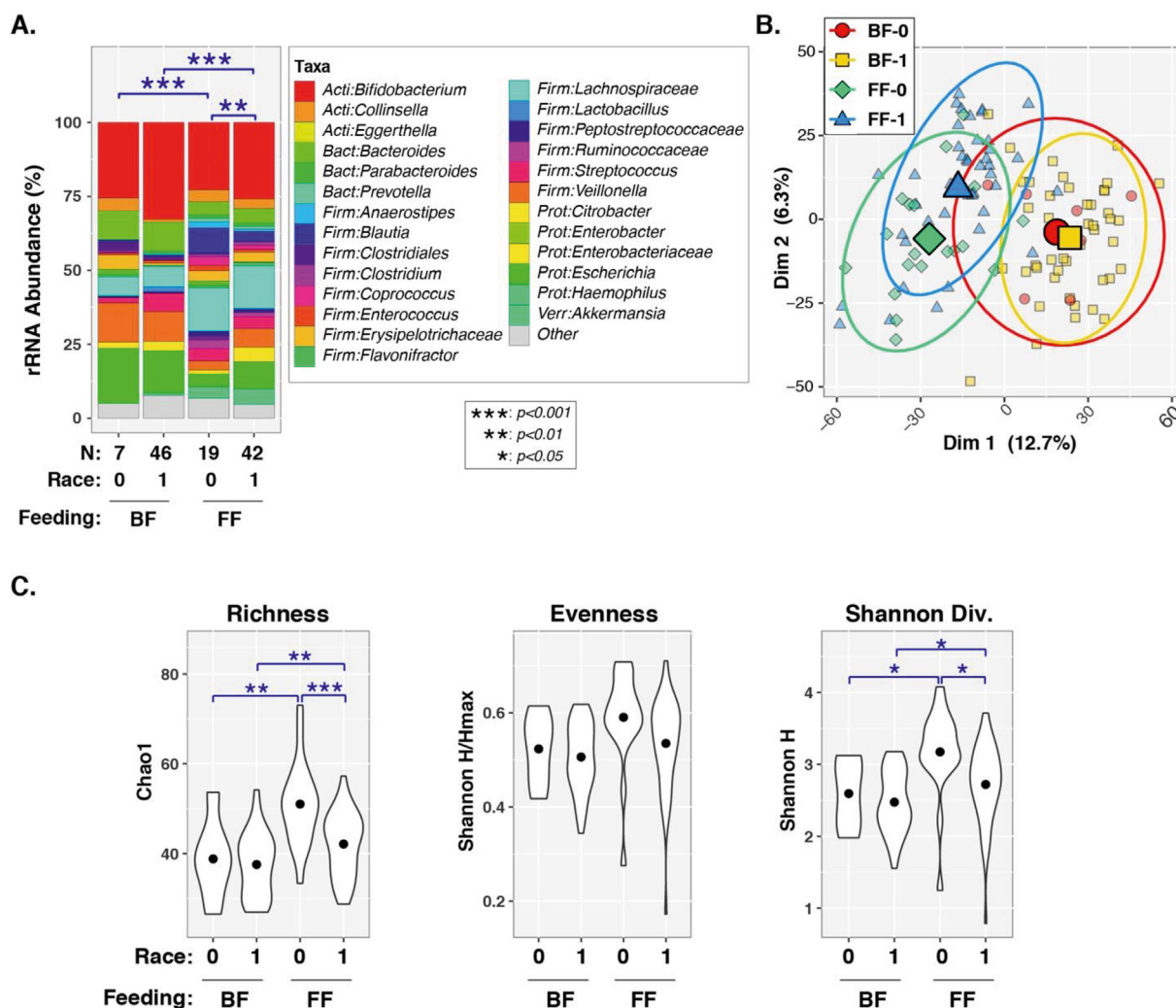
FIGURE 4. Effects of race and feeding type on α- and β-diversity in 4–5 mo-old breastfed (BF) and formula-fed (FF) infants. (A) Bar charts with PERMANOVA results. Percent relative abundance (%RA) of genus-level taxa, stratified by feeding type. Taxa with %RA <1% were collapsed into the "Other" category. The results of PERMANOVA tests are summarized above each plot for pairwise tests (blue lines/symbols). (B) Principal coordinates plot. Individual subjects are indicated by smaller symbols (circles/squares/diamonds/triangles), with group affiliations designated by symbol shapes and color coding. Mean PC values for each group along the x- and y-axes are indicated by larger shapes, whereas ellipses show 95% CI. (C) Alpha diversity. Violin plots show distributions of α-diversity indices by feeding type. Pairwise test results are indicated above each plot (blue lines/symbols). BF-0 = BF infants of non-White race ($n = 7$), BF-1 = BF infants of White race ($n = 47$), FF-0 = FF infants of non-White race ($n = 19$), FF-1 = FF infants of White race ($n = 42$). CI, confidence interval; PERMANOVA, permutational analysis of variance; rRNA, ribosomal ribonucleic acid; Dim 1 & Dim 2, Principal coordinates dimensions 1 and 2.

(protein size or percentage of whey, casein, or lactose) and the infant gut microbiota composition. This might be because of the limited sample size in some formula groups in our study (Table 2). Additionally, all infant formulas were cow milk-based instead of soy compared with cow milk-based, which might account for the limited difference in gut microbiota among FF infants [52].

One of the strengths of the present study was the inclusion of exclusively BF and FF infants. The formula feeding group was considered exclusive, although some infants received breastmilk (<2 wk within the first month of life). This allowed us to avoid possible influences of complicated mixed liquid diets and/or complementary feeding as potential confounders. Another strength is the inclusion of growth status assessment and their associations

with the gut microbiota. Some limitations of the study include the homogeneity of the study population [58]. The small sample size of each of the various infant formula subtypes was a limitation and restricted our ability to robustly characterize the impact of formula type on the gut microbiota of exclusively FF infants.

In conclusion, feeding type is a major driver of the observed gut microbiota composition in 4–5 mo-old, healthy, full-term infants. Several genera were associated with infant growth status (growth z-scores), including some previously known taxa that promote weight gain in preterm infants, although their roles in infant growth appeared different in the current cohort. Gut microbiota could be a potential target of interest in directing future feeding guidelines during infancy.
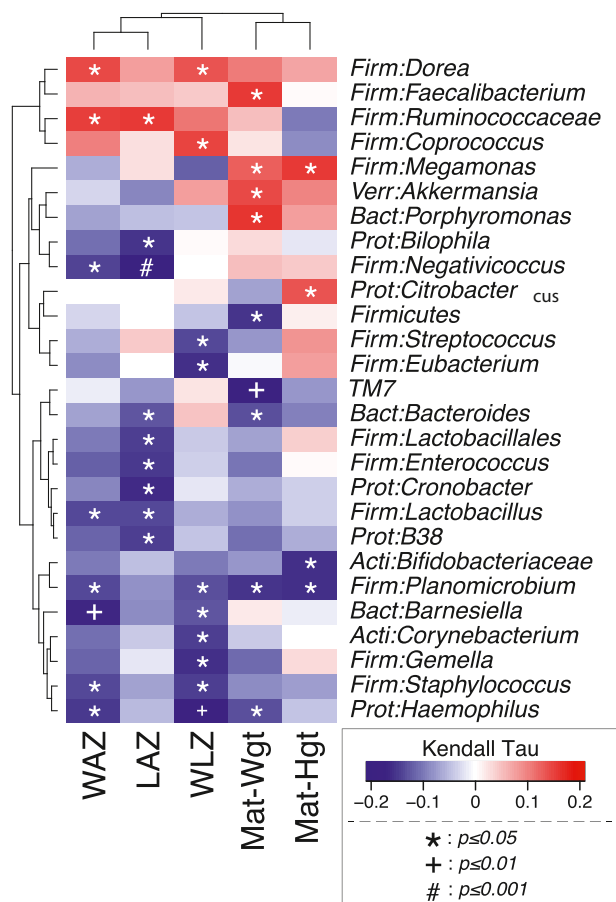
FIGURE 5. Associations between anthropometric *z*-scores and infant gut microbiota in 4–5 mo-old breastfed (BF) and formula-fed (FF) infants. Associations were assessed by Pearson correlations between anthropometric measures, and centered log-ratio (CLR) transformed microbiota count data. Darker blue indicates stronger negative Kendall rank correlation coefficients between taxa and growth parameters, whereas darker red indicates stronger positive Kendall rank correlation coefficients. LAZ = length-for-age *z*-score, WAZ = weight-for-age *z*-score, WLZ = weight-for-length *z*-score.

Author contributions

The authors' responsibilities were as follows – MT, NFK, and BEY: designed the study; MT, NFK, BEY, KND, and LMB: conducted the study; DNF, CER, and JMK: analyzed the samples; DNF, MT, and EO: analyzed the data. All authors wrote the paper, and all authors: read and approved the final manuscript.

Conflict of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.07.009.

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