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Gut Microbes and Circulating Cytokines in Preterm Infants with Growth Failure

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A B S T R A C T

Background: Growth failure (GF) is a multifactorial problem in preterm infants. The intestinal microbiome and inflammation may contribute to GF.

Objectives: This study's objective was to compare the gut microbiome and plasma cytokines in preterm infants with and without GF.

Methods: This was a prospective cohort study of infants with birth weights of <1750 g. Infants with a weight or length *z*-score change from birth to discharge or death that was less than or equal to −0.8 (GF group) were compared with infants without GF [control (CON) group]. The primary outcome was the gut microbiome (at weeks 1–4 of age), assessed by 16S rRNA gene sequencing using Deseq2. Secondary outcomes included inferred metagenomic function and plasma cytokines. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States determined metagenomic function, which was compared using ANOVA. Cytokines were measured by 2-multiplexed immunometric assays and compared using Wilcoxon tests and linear mixed models.

Results: GF ($n = 14$) and CON group ($n = 13$) had similar median (IQR) birth weight (1380 [780–1578] g vs. 1275 [1013–1580] g) and gestational age (29 [25–31] weeks vs. 30 [29–32] weeks). Compared with the CON group, the GF group had a greater abundance of *Escherichia/Shigella* in weeks 2 and 3, *Staphylococcus* in week 4, and *Veillonella* in weeks 3 and 4 (P-adjusted < 0.001 for all). Plasma cytokine concentrations did not differ significantly between the cohorts. When all time points are combined, fewer microbes were involved in TCA cycle activity in the GF group compared with the CON group ($P = 0.023$).

Conclusions: In this study, when compared with CON infants, GF infants had a distinct microbial signature with increased *Escherichia/Shigella* and Firmicutes and fewer microbes associated with energy production at later weeks of hospitalization. These findings may suggest a mechanism for aberrant growth.

Keywords: growth failure, microbiome, cytokines, premature infants, tricarboxylic acid cycle

Introduction

Worldwide, 15 million babies are born premature [1]. Fifty percent of very low birth weight infants have growth failure (GF) [2, 3]. Traditionally, in preterm infants, GF is defined as a decline in weight or length *z*-scores over time [4]. In severe cases of GF, an infant may develop microcephaly. In preterm infants, GF is associated with increased mortality, sepsis, retinopathy of prematurity, chronic lung disease, necrotizing enterocolitis, prolonged hospitalization, and poor neurodevelopment [5–8]. The etiology of GF is complex. Unfortunately, improved nutritional practices have failed to completely eradicate this problem. This suggests that nonnutritional factors may play a role in GF.

Gut microbiota–derived metabolites act locally and systemically and influence the host's physiology and disease susceptibility. Bacteria and microbiota-derived metabolites increase intestinal permeability, facilitating their passage into the systemic circulation. These metabolites alter growth by promoting a proinflammatory milieu and perturbing energy metabolism [9]. In a study of 58 extremely low birth weight infants, when compared to infants without GF, infants with poor growth

Abbreviations used: CON, control; GF, growth failure; GIR, glucose infusion rate; NPO, nil per os; PiCRUSt2, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; UCLA, University of California Los Angeles.

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[weight <10th percentile (*z*-score: −1.28) at hospital discharge] had a lower α-diversity and a predominance of the gram negative *Enterobacteriaceae* family and related members at the genus level (*Citrobacter, Enterobacter, Serratia, and Klebsiella*) [10]. In this study, infants with poor growth demonstrated increased fatty acid metabolites, which have been described in the fasting state and malnourished children [11].

The role of the gut microbiome and its relationship to growth in preterm infants warrants further exploration. In this pilot prospective cohort study, we hypothesized that in comparison to premature infants without GF, premature infants with GF would have a higher abundance of intestinal gram negative bacteria, increased circulating cytokine concentrations, and decreased microbiota-related energy production, uptake, and storage.

Methods

Patient population

This prospective cohort study was conducted at the University of California Los Angeles (UCLA) neonatal intensive care units (UCLA Ronald Reagan Hospital/UCLA Mattel Children's Hospital, Los Angeles, CA and UCLA Santa Monica Hospital, Santa Monica, CA). The UCLA Institutional Review Board granted ethical approval for this study. Verbal informed consent was obtained from parents/legal guardians. This study targeted male and female preterm infants at risk of GF. Inclusion criteria for this study included infants with a birth weight <1750 g, <14 d of age, and who were predicted to require parenteral nutrition for ≥2 wk. Subjects were divided into 2 groups, the GF group and the control group (CON). GF infants had a weight or length *z*-score difference from birth to discharge of less than or equal to −0.8 [4]. In contrast, CON infants had a weight or length *z*-score difference from birth to discharge of more than −0.8. The exclusion criteria included infants who were unlikely to survive and infants with congenital anomalies and congenital infections.

Diet composition

Parenteral nutrition was prescribed shortly after birth and consisted of dextrose, amino acids (Premasol; Baxter Healthcare Corp), and intravenous lipid emulsions (Intralipid; Fresenius Kabi). Each parenteral nutrition prescription was administered over 24 h. In general, parenteral amino acids were initiated at ∼2 g/kg birth weight/d and advanced by 0.5–1 g/kg/d to a goal of 3.5–4 g/kg/d. Intravenous lipids were initiated at 0.5–1 g/kg/d within the first 24–48 h of age and were advanced by 0.5–1 g/kg/d to a goal of 3 g/kg/d. Glucose infusion rates (GIRs) were initiated at 6–8 mg/kg birth weight/min and advanced by 1–2 mg/kg/min to a goal of 11–14 mg/kg/min. If mother's milk was not available or was insufficient, infants ≤34 wk received donor milk. Donor breast milk was prescribed until the infant was 34 wk postmenstrual age. Thereafter, the infant was transitioned to a premature formula if the mother's milk was not available. Human milk and premature formula were routinely fortified to provide additional calories and nutrients. All infants received enteral iron (∼2–4 mg/kg/d) and vitamin D (∼200–400 IU/d).

Clinical information

Weight, length, and head circumference *z*-scores at birth and ∼1 wk of age, 2 wk of age, 4 wk of age, 36 wk postmenstrual age, and hospital discharge were collected from the electronic medical record. Per routine clinical practice, weight was measured daily, and length and head circumference were measured every Monday. The *z*-scores were calculated using the Olsen growth curve [12]. Full feeds were defined as 100 mL/kg/d of enteral nutrition or ad libitum feeding, whichever occurred first. Early onset sepsis was defined as a positive blood culture before 72 h of age and antibiotics for ≥5 d. Late onset sepsis was defined as a positive blood culture after 72 h of age and antibiotics for ≥5 d. Necrotizing enterocolitis was defined by Bell's stage II or greater [13]. Bronchopulmonary dysplasia was defined as the need for supplemental oxygen at 36 wk postmenstrual age [14]. Retinopathy of prematurity was defined as any type of staging.

Microbial collection and sample analysis

Stool samples were collected weekly while the infants were on parenteral nutrition and 1 wk after parenteral nutrition was discontinued. Stool samples were collected Monday to Friday during daylight, business hours. Stool samples were stored at −80°C for future analysis. The DNA extraction, library preparation, and sequencing of the V4 (515F/806R) region of the 16S rRNA gene on a MiSeq sequencer (Illumina Inc) were performed using standard procedures with quality control. In addition to negative controls from the DNA extraction and PCR steps used to identify contaminant sequences, independent aliquots of a bacterial mock community were processed together with samples to evaluate the extraction, amplification, and relative abundance of bacteria included. Exact sequence inference and chimera removal were performed using the DADA2 software program (version 1.16.0). Amplicon sequence variants that did not have sum counts of >50 and were not present in ≥10% of samples were eliminated.

Cytokine collection and sample analysis

Weekly blood samples were collected while the infant was on parenteral nutrition and 1 wk after parenteral nutrition was discontinued. Blood samples were collected at the same time as a routine laboratory draw. Blood samples were not collected at all time points in all infants due to limitations in the amount of blood that can be taken for research purposes. All plasma samples were stored at −80°C for future analysis. Multiplexed immunometric assays (R&D Systems High Sensitivity Human Inflammation Kit) were used to determine plasma concentrations of IL-1β, IL-8, IL-6, IL-10, and TNF-α. The lowest limits of detection for IL-1β, IL-8, IL-6, IL-10, and TNF-α were 0.38, 0.71, 0.88, 0.61, and 0.73 pg/mL, respectively. The highest limits of detection for IL-1β, IL-8, IL-6, IL-10, and TNF-α were 1550, 2900, 3600, 2500, and 3000 pg/mL, respectively. There was no cross-reactivity seen for any species with the plasma cytokines.

Data analysis

This study's primary outcome was the difference in microbial abundance (measured as amplicon sequence variant counts) between the GF and CON groups obtained at postnatal ages 1–4 wk. Secondary outcomes included the difference in plasma

cytokine concentrations and metagenomic function between the GF group and the CON group at the ages of weeks 1–4.

Clinical

Subject characteristics were calculated using percentage or median (IQR) for categorical and continuous variables. The GF group and CON group were compared using the Fisher test and nonparametric Wilcoxon rank sum test for categorical and continuous variables, respectively. Changes in weight, length, and head circumference *z*-scores from birth to hospital discharge were calculated. Differences in *z*-score changes were compared between the GF group and the CON group. Weight, length, and head circumference *z*-scores over time (postmenstrual age weeks) were compared between the groups using mixed models for repeated measures. The slopes were calculated using linear regression models. GIRs, parenteral nutrition calories, enteral nutrition calories, total calories, and parenteral amino acid and lipid allotment were compared over time using mixed models for repeated measures. The slopes were calculated using linear regression models. The *z*-score differences from birth to discharge or death and nutrition parameters were also compared between cohorts at each week of age using nonparametric Wilcoxon rank sum tests.

Cytokines

Cytokine concentrations from weeks 1 to 4 of age were compared pairwise and longitudinally using the Wilcoxon rank sum test and linear mixed models accounting for repeated measurements.

Microbial

Microbial samples from the ages of weeks 1–4 were studied. Samples after week 4 were deemed "week >4." Statistical analyses were performed using the "phyloseq" (version 1.32.0) and "Deseq2" (version 1.28.1) packages in the R statistical software (version 4.0.3) for normalization of the data and comparison of groups. Deseq2 was used to calculate the microbial differential abundance between the study weeks (week 1 used for reference), the GF group and CON group, infants born via cesarean section and vaginal delivery, infants ≥7 d and <7 d of antibiotics, and infants with ≥7 d and <7 d of nil per os (NPO) days. Changes were noted using log2 fold-change. Benjamini-Hochberg false discovery rate method was used to adjust *P* values for multiple comparisons. A *P* value of <0.01 (false discovery rate) was considered statistically significant. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PiCRUSt2) (https://github.com/picrust/picrust2) was used to estimate metagenome function measures from the 16S rRNA gene data. These measures were compared between groups and across time points using ANOVA to evaluate differences between the groups over time. The Welch *t* test was used to calculate post hoc differences when comparing functional features of the GF group to the CON group, and infants treated with antibiotics for ≥7 d and infants treated with antibiotics for <7 d. Shannon α-diversity was calculated using the plot richness function in the phyloseq R package (version 1.32.0), and the Welch *t* test was used to compare the 2 groups. Unifrac β-diversity was calculated using the distance function in the phyloseq R package, and Welch *t* test was used to compare the 2 groups.

Results

There were 14 infants in the GF group and 13 infants in the CON group. The total number of samples is depicted in Supplemental Table 1. Subject characteristics are described in Table 1. There were no significant differences in median gestational age ($P = 0.08$) and birth weight ($P = 0.70$). The majority (74%, $n = 30$) of infants were delivered via cesarean section. Compared with the CON group, the GF group had more total NPO days ($P = 0.02$) and had more late onset sepsis episodes ($P = 0.02$). As a result, the GF group received more days of antibiotics compared to the CON group ($P = 0.007$). Parenteral nutrition days positively correlated with the number of NPO days ($r = 0.81$; $P < 0.001$). NPO days positively correlated with total antibiotic days ($r^2 = 0.57$; $P = 0.001$).

As expected, the weight *z*-score decline was greater in the GF group than in the CON group [GF group: −1.3 (−1.4, −1.2); CON group: −0.22 (−0.48, 0.036); $P = 0.01$]. In the GF group, 60% had a weight *z*-score decline of more than −1.2, and 14% had a *z*-score decline of more than −2. The length *z*-score change followed a similar pattern [GF group: −0.88 (−1.5, −0.37); CON group: 0.28 (−0.19, 0.60); $P = 0.01$]. In the GF group, 29% had a length *z*-score decline of more than −1.2, and 7% had a *z*-score decline of more than −2. There also was a significant difference

Table 1
Clinical characteristics of premature infants without ($n = 13$) and with GF ($n = 14$)

Cohort characteristics[1]	CON ($n = 13$)	GF ($n = 14$)	*P* value
Maternal age (y)	37 (33–41)	27 (24–35)	0.003
Gravida	2 (1–5)	2 (1–3)	0.21
Para	2 (1–3)	2 (1–2)	0.65
Gestational age (wk)	30 (29–32)	29 (25–31)	0.08
Male sex	38 (5)	42 (8)	0.81
Birth weight (g)	1275 (1013–1580)	1380 (780–1578)	0.70
Change in weight *z*-score	−0.22 (−0.48 to −0.036)	−1.28 (−1.4 to −1.2)	<0.001
Change in length *z*-score	0.28 (−0.19 to 0.60)	−0.88 (−1.5 to −0.37)	0.001
Change in head circumference *z*-score	0.23 (−0.14 to 0.38)	−0.82 (−1.3 o 0.13)	0.01
Small for gestational age	15 (2)	14 (2)	0.93
Cesarean section	84 (11)	64 (9)	0.23
Parenteral nutrition days	11 (8–19)	19 (9–37)	0.11
First feed – human milk	100%	100%	1
Received acid blockers	15 (2)	21 (3)	0.69
Necrotizing enterocolitis	8 (1)	14 (2)	0.58
Late onset sepsis	0 (0)	36 (5)	0.02
Antibiotic days	2 (0–12)	21 (7–34)	0.007
Nil per os days	1 (1–7)	9 (2–19)	0.02
Days until full enteral feeds	19 (14–23)	28 (12–40)	0.15
Bronchopulmonary dysplasia	4 (1)	29 (4)	0.15
Retinopathy of prematurity	10 (1)	43 (6)	0.03
Death	0 (0)	7 (1)	0.34
Length of stay (d)	58 (47–81)	54 (45–110)	0.58

[1] Data are presented as medians (IQRs) for continuous variables and % (*n*) for categorical variables. Change in *z*-score parameters was from birth to discharge. All subjects had complete data in all categories except bronchopulmonary dysplasia and retinopathy of prematurity ($n = 26$) due to the death of a patient. CON, control; GF, growth failure.

in the change in head circumference *z*-score [GF group: −0.83 (−1.3, 0.13); CON group: 0.23 (−0.13, 0.38); $P = 0.01$]. Weight and length *z*-score changes over postmenstrual age (wk) were significantly different between the GF group and CON group (weight estimate: −0.26; GF group: −0.075 compared with CON group: −0.049 *z*-score units/postmenstrual age week; $P = 0.01$ and length estimate: −0.27; GF group: −0.047 compared with CON group: −0.029 *z*-score units/postmenstrual age week; $P = 0.01$). The head circumference *z*-score change over postmenstrual age was not significantly different (estimate: −0.097; GF group: 0.015 compared with CON group; −0.0065 *z*-score units/postmenstrual age week; $P = 0.31$).

The nutritional intake is depicted in (Figure 1). Compared with the GF group, the CON group received fewer calories from parenteral nutrition over time (estimate: −7.91; GF group: 2.3 kcal·study wk·kg/d compared with CON group: −13.5 kcal·study wk·kg/d; $P < 0.001$) and more calories from enteral nutrition over time (estimate: 9.19; GF group: 9.53 kcal·study wk·kg/d compared with CON group: 28.2 kcal·study wk·kg/d; $P < 0.001$). Parenteral nutrition doses over time for amino acids (estimate: −0.24; GF group: −0.185 g·study wk·kg/d compared with CON group: −0.585 g·study wk·kg/d; $P = 0.03$), GIR (estimate: −0.95, GF group: 0.311 mg·study wk·kg^{-1}·min^{-1} compared with CON group: 2.3 mg·study wk·kg^{-1}·min^{-1}, $P < 0.001$), and intravenous lipid emulsions (estimate: −0.24, GF group: 0.144 g·study week·kg/d compared with CON group: −0.331 g·study week·kg/d; $P = 0.03$) were greater in the GF group compared with the CON group (Figure 1). However, over time, total calories from both parenteral and enteral nutrition were not different between the 2 groups (estimate 1.18, GF 11.8 compared with CON 14.7 kcal·study week·kg/d, $P = 0.46$).

Figure 2A and B show the abundance of microbes at the phyla and genus levels in all subjects. Compared with week 1, there was an increase at weeks 2, 3, and 4 in the abundance of specific bacteria at the genus level, namely, *Staphylococcus haemolitycus* (*P*-adjusted < 0.001), *Veillonella* (*P*-adjusted < 0.001), *Enterococcus* (*P*-adjusted < 0.001), and *Finegoldia* (*P*-adjusted < 0.001). Compared with week 1, there was an increase at week 3 in the abundance of *Klebsiella* and *Escherichia/Shigella* ($P < 0.001$ both). Compared with week 1, only 1 genus, *Haemophilus,* was less abundant in weeks 2 and 4 (*P*-adjusted < 0.001 both, compared with week 1). One amplicon sequence variant associated with the genus *Staphylococcus* was less abundant in week 3 compared with week 1. Overall, there was a paucity of *Bifidobacterium*.

The microbial communities differed between the GF group and CON group at the genus and phylum levels (Figure 2C–F). Overall, there are significantly more of the gram-negative rod genera, *Escherichia/Shigella* (phyla Proteobacteria) and *Veillonella* (phyla Firmicutes) in GF infants compared to CON infants (Figure 2C and D), *P*-adjusted < 0.001 for both)). (Figure 2 E and F) show microbial abundance differences between groups across time. At week 1, compared with the CON group, the GF group had significantly less *Escherichia/Shigella*, *Staphylococcus*, and *Haemophilus* (all *P*-adjusted < 0.001). However, by weeks 2 and 3, the GF group had a greater abundance of *Escherichia/Shigella* (*P*-adjusted < 0.001 both compared to CON). In addition, the GF group compared with the CON group demonstrated an increase in *Veillonella* at weeks 2, 3, and 4 (*P*-adjusted < 0.001) and *Staphylococcus haemolitycus* at week 4 (*P*-adjusted < 0.001). *Haemophilus* was the only genus that was significantly reduced in the GF group compared with the CON group at week 4 (*P*-adjusted < 0.001). Once again, both groups demonstrated a lack of *Bifidobacterium*. When comparing the Shannon α-diversity between cohorts at each week of age, there were no significant differences across time. However, overall, when all time points are combined, the GF group had greater α-diversity and β-diversity than the CON group (CON 1.3 compared with GF 1.8, $P = 0.01$; CON 0.3 compared with GF 0.4, $P = 0.01$).

Figure 3A shows microbial differences in infants who received antibiotics for ≥7 d and those who received antibiotics for <7 d. At the genus level, those who received antibiotics for

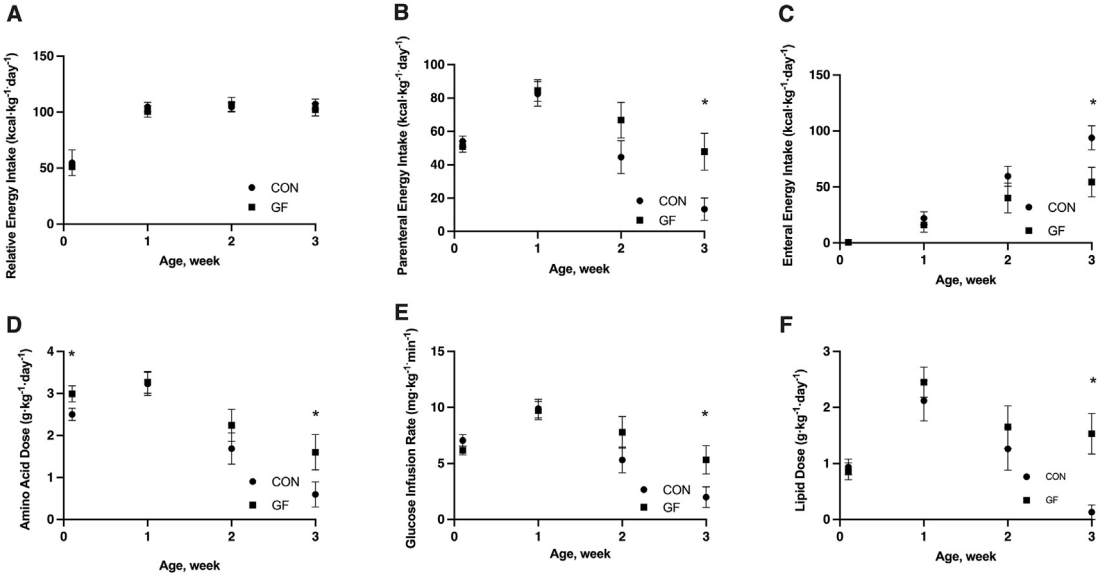

Figure 1. Mean ± SE dose of (A) relative total energy intake, (B) parenteral energy intake, (C) enteral energy intake, (D) amino acid dose, (E) glucose infusion rate, and (F) lipid dose from initial nutrition to week 3 of hospitalization for preterm infants without GF (CON) (weeks 0–1, $n = 13$; weeks 2–3, $n = 12$) and infants with GF (GF) ($n = 14$ for each week). CON, control; GF, growth failure.
[1]*CON and GF means are significantly different at that time; $P < 0.05$.

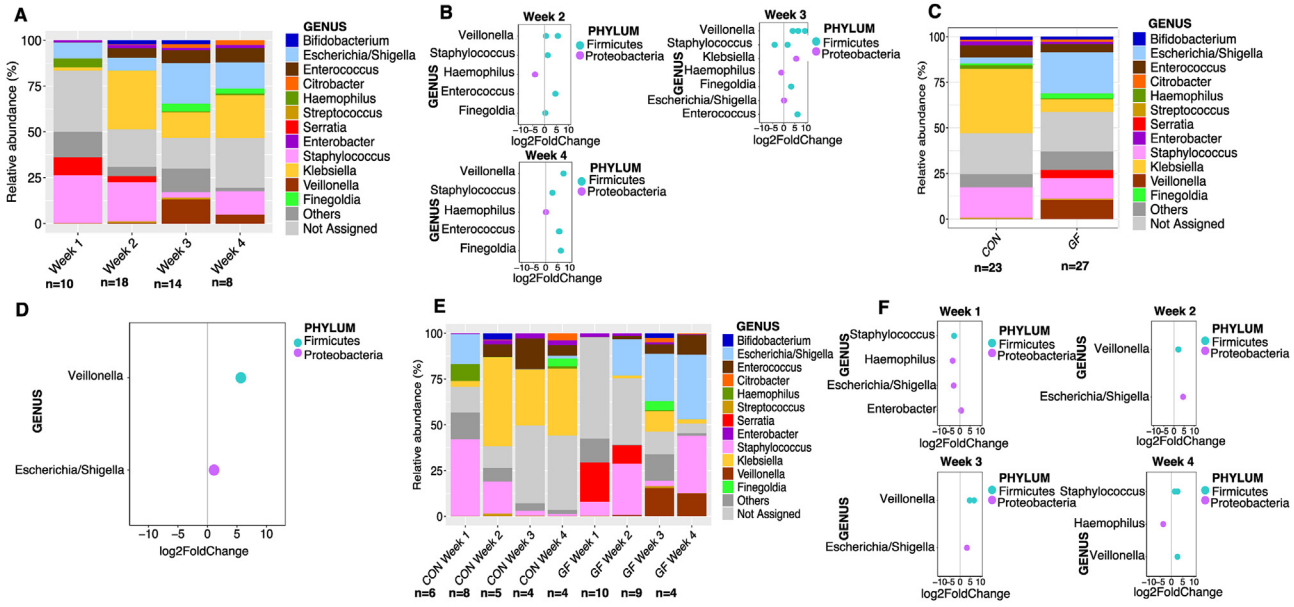

Figure 2. (A) Relative abundance of microbes from weeks 1 to 4 for all premature infants. The sample size is shown by week (week 1, $n = 10$; week 2, $n = 18$; week 3, $n = 14$; week 4, $n = 8$). (B) The scatter plot represents the log2 fold-change of the genera that are significantly different over time (P-adjusted < 0.001). (C) Relative abundance of microbes across all time points in premature infants without GF (CON) ($n = 23$) and premature infants with GF ($n = 27$). (D) The scatter plot represents the log2 fold-change of the genera that are significantly (P-adjusted < 0.001) different. The left side of the scatter plot represents the genera that are less in the GF group compared to the CON group. The right side represents the genera that are increased in the GF group than in the CON group. (E) Relative abundance of microbes from weeks 1 to 4 for the CON and GF groups. The sample size is shown by week and cohort (week 1 CON, $n = 6$; week 1 GF, $n = 4$; week 2 CON, $n = 8$; week 2 GF, $n = 10$; week 3 CON, $n = 5$; week 3 GF, $n = 9$; week 4 CON, $n = 4$; week 4 GF, $n = 4$). (F) The scatter plot represents the log2 fold-change of the genera that are significantly different (P-adjusted < 0.001) over time. The left side of the scatter plot represents the genera that are less in the GF group compared to the CON group. The right side represents the genera that are increased in the GF group than in the CON group. CON, control; GF, growth failure. [1]The "not assigned" category refers to the amplicon sequence variants not assigned to any specific genus in DADA2. "Other" includes genera representing <2% of the overall relative composition in <10% of the samples.

≥7 d had increased *Escherichia/Shigella* (P-adjusted < 0.01) and decreased *Enterobacteriaceae* (P-adjusted < 0.01) and *Enterobacter* (P-adjusted < 0.01). (Figure 3B) shows microbial differences in infants born via cesarean section compared with vaginal delivery. Infants born by vaginal delivery had significantly higher abundance of *Veillonella* (P-adjusted < 0.001) and *Haemophilus* (P-adjusted < 0.001), whereas infants born by cesarean section had significantly more *Serratia* (P-adjusted < 0.001). (Figure 3C) shows microbial differences between infants who had ≥7 NPO days compared to those who had <7 d. Those who had ≥7 NPO days had significantly less of the phyla Firmicutes (*Veillonella* and *Finegoldia;* both P-adjusted < 0.01), and Proteobacteria (*Enterobacteriaceae* and *Enterobacter*; P-adjusted < 0.01).

Plasma concentrations of IL-1β, IL-10, IL-6, IL-8, and TNF-α over time did not significantly differ between the GF group and the CON group ($P = 0.06$, $P = 0.47$, $P = 0.36$, $P = 0.09$, $P = 0.49$, respectively, Table 2). TNF-α concentrations were greater in the GF group compared with the CON group at week 2; however, this difference was not statistically significant and only represented a trend ($P = 0.05$).

PiCRUSt2 was used to infer the microbial gene content and aggregated relative abundance based on the 16S rRNA gene data to estimate metagenomic functional roles within metabolic pathways. The GF cohort had a higher number of sequences assigned to the TCA cycle at week 1 compared to week 3 ($P <$ 0.01, (Figure 4A)). At week 2, the GF cohort had a higher proportion of sequences assigned to the TCA cycle compared to week 3 and week >4 ($P < 0.01$, (Figure 4A)). There was an increase in the cysteine and methionine pathways in the GF cohort in week 3 compared to week 2 ($P < 0.01$, (Figure 4B)). In contrast, the CON group also had a decrease in the histidine metabolism and an increase in the pentose phosphate pathway in week 1 compared with week 3 (both $P < 0.01$, (Figures 4C and D)). Lastly, the CON group revealed a temporal increase in the proportion of sequences related to the fructose and mannose metabolism pathways in weeks 3 and 4 compared with week 1 (both $P < 0.01$, (Figure 4E)). There were no significant differences between the 2 cohorts or over time with respect to the fatty acid metabolism pathway.

When PiCRUSt2 analysis was performed, the proportion of sequences assigned to the pathogenic *Escherichia coli*–related infection pathway was significantly increased in the GF group ($n = 24$) compared with the CON group ($n = 23$) ((Figure 5A and B); $P < 0.001$). Similar results were observed when comparing subjects who received antibiotics for ≥7 d to subjects who received antibiotics for <7 d (Figure 5C and D). Furthermore, when all time points were combined, the TCA cycle activity was noted to be significantly reduced in the GF group when compared to the CON group ($P = 0.023$).

Discussion

In this pilot study, premature infants with GF were found to have a gut microbial signature characterized by an increasing

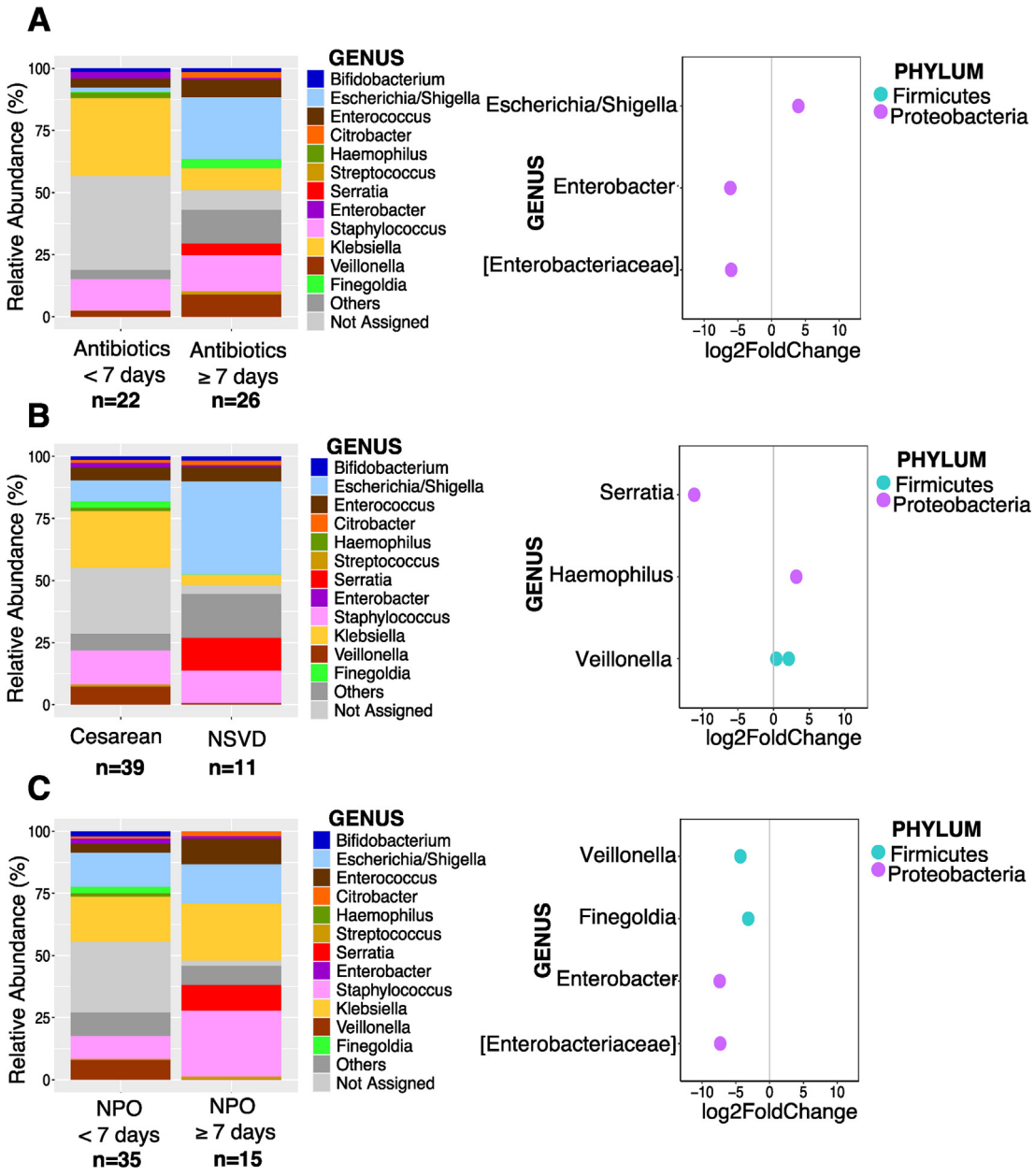

Figure 3. (A) The left panel represents the relative abundance of microbes in premature infants who received <7 d of antibiotics (n = 22) compared with infants who received ≥7 d of antibiotics (n = 26). The right panel represents the log2 fold-change in bacterial sequences. The left side represents sequences less present in infants who received antibiotics for ≥7 d compared with infants who received antibiotics for <7 d. The right side shows the bacterial sequences that are increased in infants who received antibiotics for ≥7 d compared with infants who received antibiotics for <7 d. (B) The left panel represents the relative abundance of microbes in premature infants delivered via normal spontaneous vaginal delivery (NSVD) (n = 11) or by cesarean section (n = 39). The right panel represents the log2 fold-change in bacterial sequences. The left side represents sequences increased in cesarean section cohort, whereas the right side of the graph shows the bacterial sequences that increased in the normal spontaneous vaginal delivery cohort. (C) The left panel represents the relative abundance of microbes in premature infants who were nil per os (NPO) <7 d (n = 35) compared with infants who were ≥7 d NPO (n = 15). The right panel represents the log2 fold-change in bacterial sequences, and the left side represent sequences less present in infants who were NPO ≥7 d compared with those who were NPO <7 d. The right side of the graph shows the bacterial sequences that were increased in infants who were NPO ≥7 d compared with those who were NPO <7 d. [1]The "not assigned" category refers to the amplicon sequence variants not assigned to any specific genus in DADA. "Other" includes genera representing <2% of the overall relative composition in <10% of the samples.

abundance of Firmicutes, *Staphylococcus, Escherichia/Shigella*, and *Serratia.* At week 2, plasma TNF-α showed a trend toward a higher value in the GF group compared with the CON group, but this was not statistically significant. In a metagenomic analysis, we inferred that GF was associated with decreased TCA cycle activity, possibly indicating limited energy metabolism. In contrast, infants with appropriate growth demonstrated increased fructose–mannose metabolism, which can fuel de novo

Table 2
Plasma cytokine concentrations for premature infants without and with GF at weeks 1 ($n = 31$) and 2 ($n = 24$)

Cytokines,[1] pg/mL	Week 1			Week 2		
	GF ($n = 15$)	CON ($n = 16$)	*P* value	GF ($n = 13$)	CON ($n = 11$)	*P* value
IL-1β	1.21 (0.47–1.58)	0.70 (0.56–1.47)	0.49	1.58 (1.0–14.7)	0.86 (0.47–2.07)	0.21
IL-6	14.1 (6.4–20.2)	7.34 (3.65–12.3)	0.19	6.05 (4.70–35.2)	5.73 (3.78–9.57)	0.27
IL-8	39.4 (26.9–64.2)	31.3 (18.5–62.9)	0.35	79.2 (43.4–149)	35.7 (27.6–104.0)	0.09
IL-10	0.94 (0.65–1.29)	1.21 (0.85–1.64)	0.29	1.94 (1.25–3.75)	1.37 (0.94–2.05)	0.09
TNF-α	12.7 (10.9–16.4)	14.3 (11.6–20.3)	0.29	17.8 (15.4–24.2)	16.0 (13.5–18.0)	0.05

[1] Data are represented as medians (IQRs). CON, control; GF, growth failure.

lipogenesis. These findings suggest that gut microbiota may play an important role in growth in preterm infants by altering energy and nutrient metabolism and systemic inflammation.

Malnutrition and prematurity have been associated with intestinal dysbiosis. However, there is a paucity of investigations focused on the gut microbiome in preterm infants displaying GF. In a study of very low birth weight infants, *Staphylococcus, Klebsiella*, and *Enterococcus* were negatively associated with weight gain [15, 16]. In another study examining extremely low birth weight infants, infants with GF had a lower α-diversity and increased *Staphylococcaea, Enterobacteriaceae, Citrobacter, Enterobacter, Serratia,* and *Klebsiella* compared with infants without GF [10]. However, the investigators in this study examined only extremely low birth weight infants and defined GF as a weight less than the third percentile at hospital discharge or at 40 wk postmenstrual age [10]. In contrast, in our study, the GF cohort had an increase in α- and β-diversity compared to the CON group. However, decreased diversity may reflect stability. Term breastfed infants have been found to have decreased α-diversity and richness compared to formula-fed infants [17, 18]. It remains unclear what the significance of diversity is in preterm infants with and without GF. Moreover, in our study, we opted to define GF as a decline in weight or length *z*-score less than or equal to −0.8 because previous literature has found a drop of ≤0.8 to be expected in a large international cohort of preterm infants [4, 19]. Also, changes in *z*-scores are predictive of neurodevelopmental impairment [20–22]. Because the definition of GF is not universally agreed upon and varies among studies, it is difficult to compare studies and draw any firm conclusions [4, 10, 19].

Our metagenomic analysis inferred that GF was associated with reduced TCA cycle activity without a change in FA metabolism. In contrast, the CON group was associated with an increase in the fructose–mannose pathway. A study that investigated preterm infants and studies examining malnourished children found increased fatty acid metabolism with GF and in the starvation state [10]. Our findings support a downregulation of oxidative energy metabolism in preterm infants with GF. In studies examining malnutrition in infancy and childhood, early loss of *Bifidobacterium* led to deficiencies in energy harvesting, immune response, and vitamin synthesis [23]. In our study, there was a low abundance of *Bifidobacterium* colonization in all infants. The minimal quantities of this genus have been well-documented in preterm infants [10, 16].

The CON group had a temporally related increase in bacteria involved with the fructose–mannose pathway. The fructose–mannose pathway fuels de novo lipogenesis under normal circumstances [24]. It is possible this pathway may go awry under pathological states. The lack of increase in fructose metabolism in the GF group may suggest inadequate fueling of de novo lipogenesis. However, an increase in dietary fructose can increase hepatic de novo lipogenesis and has been associated with nonalcoholic fatty liver disease [25]. Further studies investigating long-term growth, body composition, and metabolic function in preterm infants with and without GF are indicated.

A number of factors influence the microbiome of a newborn, including mode of delivery, antibiotics, fasting, and feeding type [26]. In term, healthy infants born vaginally and fed breastmilk, *Klebsiella* and *Escherichia* are the predominant genera during the first week of life, likely secondary to placental and vaginal microbiota [27, 28]. Over time, these bacteria diminish and *Bifidobacterium* predominates [27]. In contrast, very low birth weight infants are more likely to be delivered via cesarean section due to breech positioning, maternal indications, and fetal distress. In this study, 74% of subjects were delivered via cesarean section (84% in the CON group and 64% in the GF group), which is consistent with US data [29]. In term infants, mode of delivery appears to alter the microbiota in the first 6 wk, but long-term changes are controversial [30, 31]. In our study, combining cohorts and all time points, infants born by vaginal delivery had a significantly higher abundance of *Veillonella* whereas infants born via cesarean section had significantly more *Serratia.* However, our study is limited by sample size, lack of samples immediately after birth and beyond the first of month of life, and the inability to account for various confounders.

Antibiotics are commonly prescribed to preterm infants. In fact, in our study, 88% of infants received ≥48 h of antibiotics. The GF cohort had a median of 21 antibiotic days compared to 2 d in the CON cohort. In our microbial analysis, the GF group and infants who received ≥7 d of antibiotics had demonstrated an increase in *Escherichia/Shigella* compared with the CON group and infants who received <7 d of antibiotics. This was confirmed in our PiCRUSt2 analysis. In our PiCRUSt2 analysis, we found an increased proportion of sequences involved in the pathogenic *E. coli* pathway in the cohort of preterm infants who received ≥7 d of antibiotics. Fifteen of the 22 subjects who received antibiotics for ≥7 d also had GF. However, the GF group also had more NPO days and received more parenteral calories and less enteral calories compared with the CON group. It is well known that extremely preterm infants who develop sepsis or are critically ill have increased energy consumption, are more likely to be NPO, and receive prolonged courses of parenteral nutrition and antibiotics. Due to a limited sample size, we could not disentangle these highly related variables. However, the role of antibiotics, particularly in the absence of a confirmed infection, and their impact on growth in preterm infants remains unclear. Given the mounting evidence that antibiotics alter the gut microbiome and

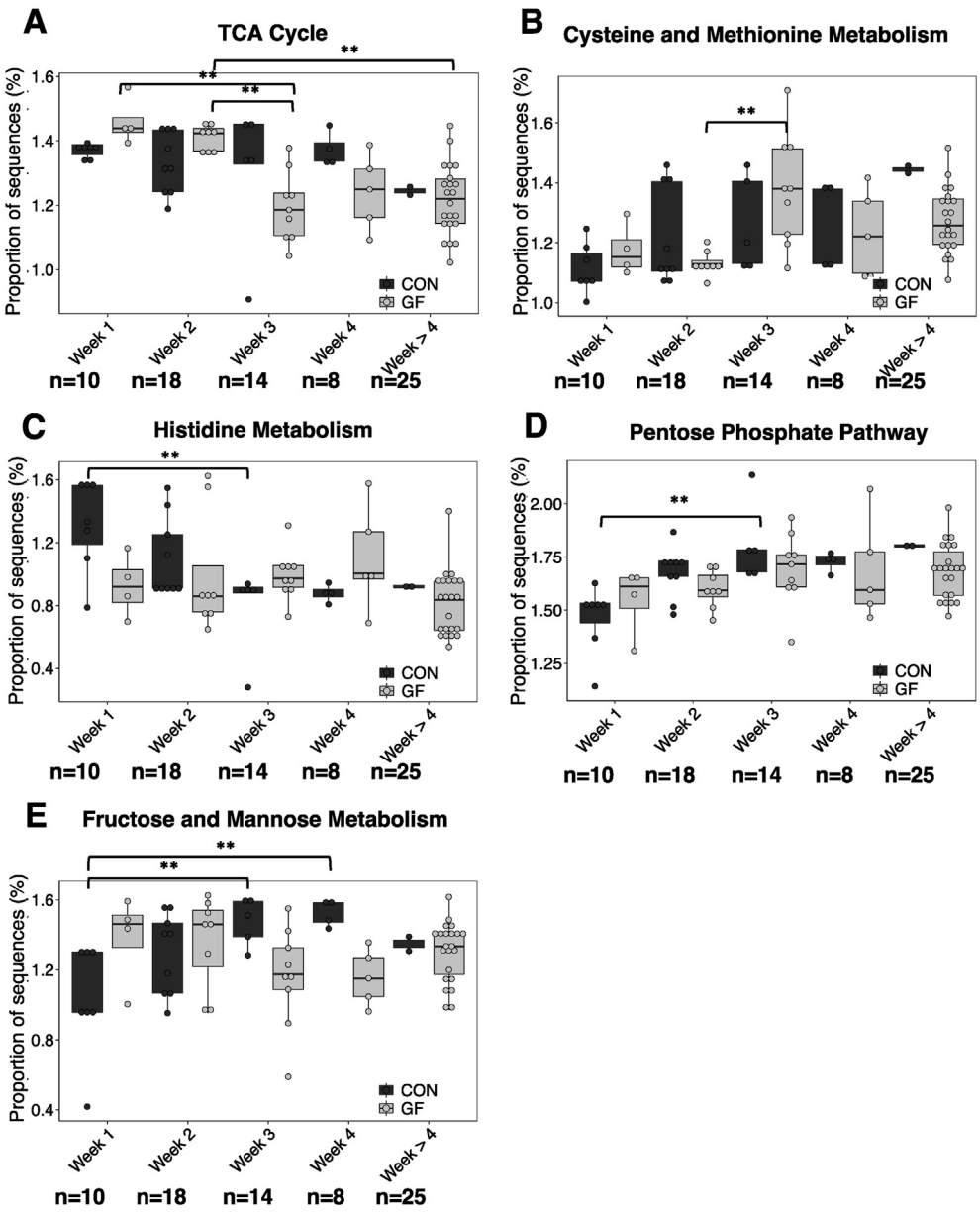

Figure 4. Box plots representing the median (IQR) proportion of sequences of microbes in premature infants without GF (CON) and premature infants with GF from weeks 1 to >4 (week 1, $n = 10$; week 2, $n = 18$; week 3, $n = 14$; week 4, $n = 8$; week >4, $n = 25$) involved in (A) the TCA cycle, (B) cysteine and methionine metabolism, (C) histidine metabolism, (D) pentose phosphate metabolism, and (E) fructose and mannose metabolism. CON, control; GF, growth failure. [1]Week >4 represents samples after week 4. [2]*Significantly different medians between groups; $P < 0.05$.

have long-term health sequelae, clinicians should be vigilant when prescribing antibiotics in the absence of infection [32, 33].

Short courses of antibiotics (<3 d) are associated with decreased *Bifidobacterium* for 3 wk in preterm neonates [34]. Greater than 3 d of antibiotics is associated with less *Bifidobacterium* for ≤6 wk in premature infants. Both cohorts in this study had a paucity of *Bifidobacterium*. Intrapartum and early antibiotic use during infancy is associated with a dysbiotic gut and childhood obesity [34–37]. Obesity has been associated with increased Firmicutes and Bacteroidetes, which increases circulating LPS concentrations causing chronic low-grade inflammation [38]. This inflammation alters liver and adipose tissue metabolism, eventually leading to organ dysfunction [38]. Another study found that an increasing Firmicutes to Bacteroidetes ratio was correlated with a higher body mass index in adults [39]. Firmicutes have been found to be a more effective energy source than Bacteroidetes [40].

In our study, we observed an increase in the abundance of the Firmicutes phyla (*Staphylococcus* and *Veillonella*) in the GF group than in the CON group. The study by Younge et al. [10] noted an association between catch-up growth and 2 genera, *Bifidobacterium* and *Veillonella*. It remains unclear if this microbial environment conveys a future risk of obesity and metabolic syndrome. It is well known that a subset of preterm infants develop childhood and adulthood obesity with excessive amounts of visceral adiposity [41]. It is unknown if a subgroup consists of preterm infants with preceding GF and an altered microbiome dominated by the Firmicutes phyla.

Despite similar nutritional calories, the GF cohort developed stunting. In our study, like the study by Younge et al. [10], the GF group and CON group had similar total caloric intakes. However, the GF group received more parenteral nutrition calories and less enteral nutrition calories than the CON group. Also, the GF group had more fasting days than the CON group. Prolonged parenteral nutrition is

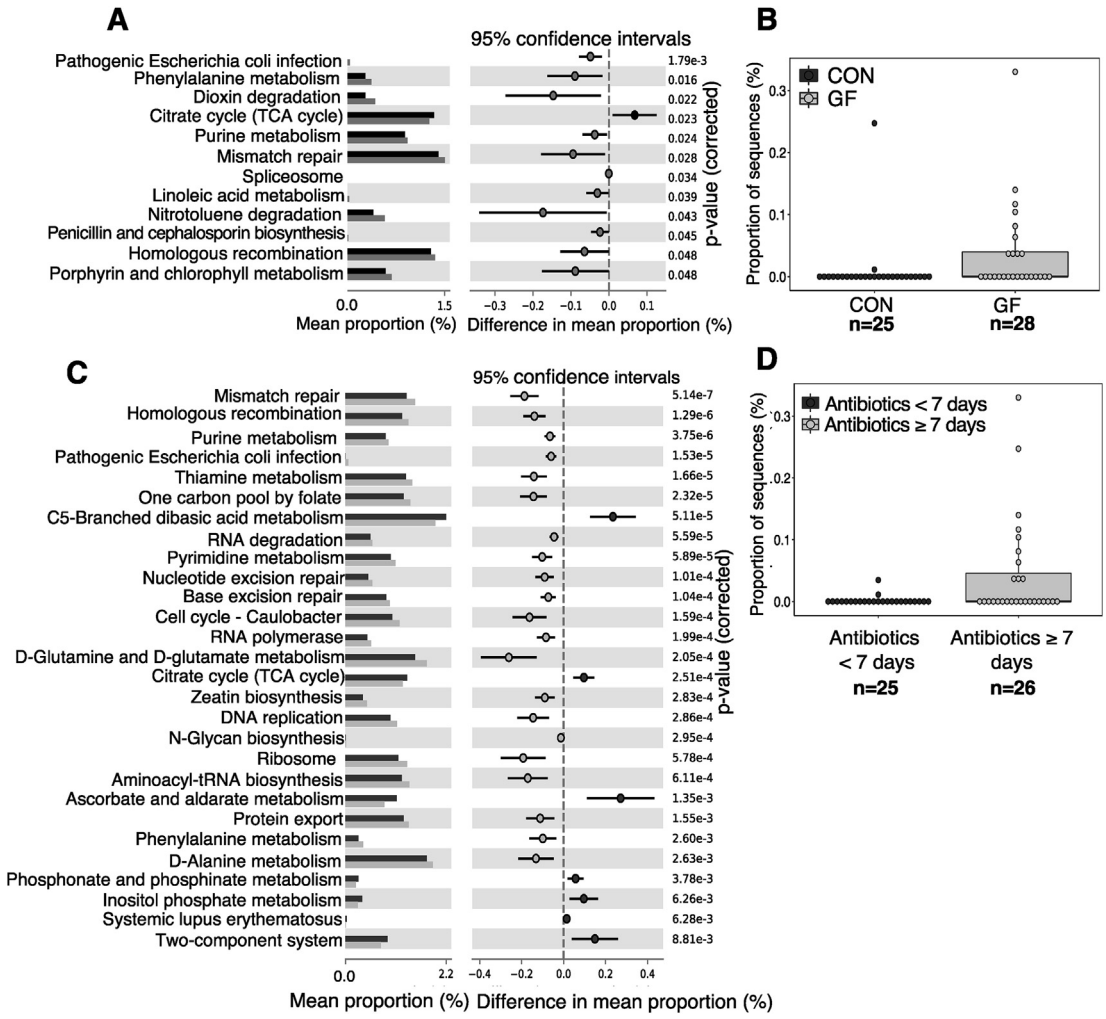

Figure 5. (A) Bar graph showing more abundant functional features in premature infants with ($n = 28$) and without ($n = 25$) GF. The difference between effect sizes for each feature is indicated by a dot. (B) A boxplot representing proportion of sequences of microbes involved in pathogenic *Escherichia coli* infection for the GF and CON groups. (C) Bar graph showing more abundant functional features in premature infants with ≥7 d of antibiotics ($n = 26$) and infants with <7 d of antibiotics ($n = 25$). The difference between effect sizes for each feature is indicated by a dot. All the represented pathways have a $P < 0.05$. (D) A boxplot representing proportion of sequences of microbes involved in pathogenic *Escherichia coli* infection for infants who received antibiotics ≥7 d and <7 d. CON, control; GF, growth failure.

associated with parenteral nutrition associated cholestasis. In piglets, parenteral nutrition is associated with less lean mass, hepatic steatosis, insulin resistance, and elevated fasting plasma glucose and insulin [42]. In contrast, enteral nutrition enhances intestinal adaptation, gut and systemic hormone secretion, and bile acid secretion. In summary, it remains unknown how prolonged parenteral nutrition, fasting, antibiotics, and GF in premature infants set the stage for child and adult-onset insulin resistance, sarcopenia, and liver disease, including nonalcoholic fatty liver disease.

Cytokines, although important in the developing intestine and for detection of infection, can lead not only to excessive weight gain but also stunted growth. IL-1β and TNF-α have been found to diminish IGF-I mRNA response to growth hormone [43]. In another study, rats injected with TNF-α and IL-6 demonstrated a downregulation of the growth hormone receptor [44]. Stunted children have been noted to have chronic small bowel inflammation and enteric dysfunction [38]. In a murine model, fecal transplants from undernourished children from Malawi caused inflammatory changes in the mouse small bowel and decreased lean body mass and weight gain compared with transplants from healthy children [45, 46]. In our study, week 2 TNF-α concentrations trended toward higher values in the GF cohort compared to the CON cohort, but this was not statistically significant ($P = 0.05$).

There is some evidence that modifying the microbiome may lead to improved growth. Term children 6 mo to 2 y of age with severe malnutrition were found to have a more "immature" microbiota compared to their appropriate growth counterparts [47]. The investigators developed a complementary food diet with the goal of "driving" microbial development. This diet was developed using malnourished mice that demonstrated successful weight gain [48]. This "growth-promoting" diet was then evaluated in piglets and malnourished children. Stunted children demonstrated increased weight and arm circumference *z*-scores and circulating growth hormone, leptin, and osteoblast differentiation protein concentrations after receiving the diet that was supplemented with chickpea, peanut, soy, and banana. In developing countries, where acute malnutrition is common, another microbiome-directed dietary intervention (rice, chickpea, lentils, soybeans, and peanuts) in 12- to 18-mo-old malnourished children significantly improved growth [49]. These findings suggest that

intestinal bacteria may play a functional role in nutrition and growth during critical periods of development. Once the microbiota changes are unequivocally defined in preterm infants with GF, similar microbiota-driven targeted dietary interventions rather than traditional parenteral and enteral nutritional support may help overcome the problem of postnatal GF.

Our study has limitations. It is a small single-site study and was not powered to examine specific differences in the microbiome. There are missing stool and plasma samples. Preterm infants, particularly when receiving limited enteral nutrition, have delayed stool passage. As a result, we could not collect stool samples for each time point for each subject. Blood collections were limited by the subjects' size and anemia status. We also made inferences about microbial function using PiCRUSt2 software and did not directly investigate the metabolome. Lastly, we were unable to control for the mode of delivery, antibiotics and NPO days, and the type of feeding (mother's own milk compared with donor milk) due to the small sample size. Despite these limitations, our study had a homogenous group of premature infants with similar gestational age, birth weight, and total caloric intake. All infants received human milk at the time of stool collection. Hence, we believe our results are hypothesis-generating. Well-powered future studies are required to account for these confounders.

In this pilot study of dysbiotic premature infants, GF was associated with a unique microbial signature characterized by increased Firmicutes, *Staphylococcus*, *Escherichia/Shigella*, and *Serratia*. The microbiota was also associated with a decrease in the TCA cycle genes in infants with GF and an increase in the fructose–mannose pathway genes in infants without GF. Our findings suggest that further research may be warranted to explore how the gut microbiome and metabolome contribute to the growth and body composition in preterm infants.

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Disclosures

KLC has served as an advisor for Fresenius Kabi, Mead Johnson Nutrition, Baxter, and Prolacta and has served as an institutional principal investigator, with no salary funding, for a consortium database sponsored by Mead Johnson. All other authors report no conflicts of interest.

Data availability statement

The data that supports the findings of this study are available from the corresponding author upon reasonable request and may require institutional data agreements.

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We acknowledge Anahit Nersiseyan and Sparsha Govardhan's assistance in collecting the plasma and fecal samples for the study. The authors' responsibilities were as follows – KMS, SUD, and KLC: designed the research; KMS and GDV: conducted the research and analyzed the data; KMS, GDV, SUD, and KLC: wrote the manuscript; KLC: had primary responsibility for the final content; and all authors: read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2022.10.005.

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