Journal of Developmental Origins of Health and Disease

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Association between extrauterine growth restriction and changes of intestinal flora in Chinese preterm infants

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Abstract

The aim of the study was to investigate any association between extrauterine growth restriction (EUGR) and intestinal flora of <30-week-old preterm infants. A total of 59 preterm infants were assigned to EUGR ($n = 23$) and non-EUGR ($n = 36$) groups. Intestinal bacteria were compared by using high-throughput sequencing of bacterial rRNA. The total abundance of bacteria in 344 genera (7568 *v.* 13,760; $P < 0.0001$) and 456 species (10,032 *v.* 18,240; $P < 0.0001$) was significantly decreased in the EUGR group compared with the non-EUGR group. After application of a multivariate logistic model and adjusting for potential confounding factors, as well as false-discovery rate corrections, we found four bacterial genera with higher and one bacterial genus with lower abundance in the EUGR group compared with the control group. In addition, the EUGR group showed significantly increased abundances of six species (*Streptococcus parasanguinis*, *Bacterium* RB5FF6, two *Klebsiella* species and *Microbacterium*), but decreased frequencies of three species (one *Acinetobacter* species, *Endosymbiont*_of_*Sphenophorus_lev* and one *Enterobacter*_species) compared with the non-EUGR group. Taken together, there were significant changes in the intestinal microflora of preterm infants with EUGR compared to preterm infants without EUGR.

Introduction

Extrauterine growth restriction (EUGR) is defined as growth values less than the 10th percentile of intrauterine growth expectation in preterm neonates at the time of discharge.[1] Although enteral nutrient supply for preterm or low birth weight neonates might support their growth at a rate similar to those of normal intrauterine growth within the first 2–3 months after birth, EUGR frequently prevails in very low birth weight (VLBW) (<1500 g) and extremely low birth weight (<1000 g) infants,[2,3] indicating that low birth weight is a major risk factor for EUGR.[4,5] In the follow-up, preterm infants with EUGR without interventions for 6–12 months after birth were found to suffer from slower cognitive and communicative development, limitations of movement (fetal head, sitting and walking), and 5.5–6.5% of them presented with transient disorders in spatial orientation and body position transformation.[6] Since the physical and mental development of preterm infants is significantly affected by EUGR in both the short-term and long-term, it is important to provide adequate and effective nutritional support for preterm infants with EUGR at three stages, including the first few weeks after birth (acute stage), the development stage and the post-discharge stage.[7–11]

While recent studies have mainly focused on the short- and long-term effects of metabolic functions and neurodevelopment of preterm infants with EUGR, previous studies have shown that the incidence of EUGR is related to factors such as birth weight, gestational age (GA) and the time required for catch-up growth.[12] Although it remains unclear whether there is a relationship between the incidence of EUGR and neonatal necrotizing enterocolitis, both are common in very preterm infants.[13–18] Moreover, the intake of adequate nutrients in neonates plays a vital role in the development of cell signal transduction of the gastrointestinal system, nervous system and the immune system. Defects in energy accumulation and deficiency of microelements greatly affect normal growth and development, at both the cellular and histological levels. Several factors affect preterm neonates' intake of nutrients, including gastrointestinal dysfunction, endocrine abnormalities and other complications, such as respiratory problems, abnormal immunological functions, central nervous system damage and difficulties in coordinating sucking and swallowing.[3,19,20] Enteral support to meet the nutritional needs of premature neonates at the critical postnatal period should decrease the risk of

Original Article

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Cite this article: Zhang J, Dai Y, Fan S, Zhang K, Shuai C, Bian X, Hui L, Wu Z, Zhang J, Guo Z, Deng F, Guo M. (2019) Association between extrauterine growth restriction and changes of intestinal flora in Chinese preterm infants. *Journal of Developmental Origins of Health and Disease* **10**: 513–521. doi: 10.1017/S2040174419000084

Received: 3 January 2018
Revised: 23 October 2018
Accepted: 8 January 2019
First published online: 5 March 2019

Key words:
classification of bacteria; extrauterine growth restriction (EUGR); gastrointestinal bacteria; high-throughput sequencing; preterm infant

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contracting various conditions.[21,22] However, although previous studies have suggested that the intestinal flora affects the incidence of ulcerative colitis, asthma and inflammatory bowel disease,[23] few studies have been carried out on the intestinal flora of preterm infants. Understanding alterations in the intestinal flora of preterm infants may yield appropriate treatment options, and thereby promote the growth and development of preterm infants with EUGR.

There are approximately 100 trillion live microorganisms present in the human intestine, of which 99% are bacteria of 500–1000 species. The tremendous diversity of bacteria is roughly divided into three categories, including probiotics, pathogenic bacteria and neutral bacteria. Significant differences in the intestinal flora have been found between premature and term birth infants, along with different postnatal growth and development.[24] The purpose of the current study was to investigate the classification of intestinal bacteria in preterm neonates with EUGR and compare it with that of preterm neonates without EUGR.

Patients and methods

Subjects

Our study was performed in accordance with the Declaration of Helsinki with regard to ethical principles for research involving human subjects. The study protocols were approved by the Research Ethics Committee of the East Campus of Shanghai Sixth People's Hospital Affiliated to Shanghai Jiaotong University. Written, informed consent was obtained. A single-center cohort of 59 premature babies (<1000 g and/or <30 weeks GA) born in the Children's Hospital of Fudan University between January 1, 2016 and December 1, 2017 was prospectively included in the current study. Infants were excluded if they had severe apnea, a congenital malformation and/or required surgery.

EUGR has been diagnosed as a weight 10% lower than that of infants in the same GA when they were discharged from hospital. The discharge standard has been that the infant is >36 weeks old, weighting more than 2 kg without any underlying disease needing hospital care and can be feed orally. As a result, a total of 23 preterm infants with definitely diagnosed EUGR and 36 preterm infants without EUGR were recruited as the study group (EUGR group) and the control group (non-EUGR), respectively.

Birth weight recovery time has been defined as the period of physiological weight loss after birth (generally no more than 10%) and the return to birth weight (approximately 7–10 days). Weight increasing time refers to the growth phase after the end of weight loss.

Stool sample preparation

Fresh stool samples were collected at the point when infants were discharged from hospital and immediately preserved in liquid nitrogen, after which they were transported to our laboratory in a mobile refrigerator and frozen at −80°C until DNA extraction

Table 1. Baseline characteristics of preterm infants in the EUGR and non-EUGR (control) groups

	EUGR (preterm) $n=23$	Control (preterm) $n=36$	P
Gender	Male 19	Male 25	> 0.05
Intrauterine growth restriction	2	4	> 0.05
Gestational age (week)	$28^{+5}\pm3$	$28^{+1}\pm4$	> 0.05
Birth weight (g)	1065 ± 52	1032 ± 41	> 0.05
Delivery mode	C-S 5	C-S 9	> 0.05
Birth weight recovery (days)	12 ± 8	11 ± 6	> 0.05
Start of breast feeding (days after birth)	1 ± 0.5	1 ± 1	> 0.05
Weight increase (d)	7 ± 6	7.5 ± 5.5	> 0.05
Antibiotics administration (d)	21 ± 10	14 ± 9.5	< 0.05
Age of stool sampling (weeks)	38 ± 6 days	38 ± 2 days	> 0.05

EUGR, extrauterine growth restriction.

Table 2. Characteristics of nurturing form and food composition for preterm infants in the EUGR and non-EUGR groups

	EUGR (preterm) $n=23$	Control (preterm) $n=36$	P-value
Parenteral nutrition (days)	22 ± 14	17 ± 10	< 0.05
Human milk (n, %)	14 (60.9%)	30 (83.3%)	< 0.05
Preterm formula (n, %)	3 (13.0%)	3 (8.3%)	< 0.05
Human milk and preterm formula (n, %)	1 (4.4%)	3 (8.3%)	> 0.05
Human milk plus thickener (n, %)	5 (21.7%)	0 (0%)	< 0.05

EUGR, extrauterine growth restriction.

https://doi.org/10.1017/S2040174419000084 Published online by Cambridge University Press

within 12 weeks. A total of 200 mg of stool sample was used for DNA extraction each time.

DNA extraction

Total community genomic DNA extraction was performed using an E.Z.N.A. Soil DNA Kit (Omega Bio-tek, USA) following the manufacturer's instruction. The level of DNA was measured using a Qubit 2.0 fluorometer (Life Technologies, USA) to ensure that adequate amounts of high-quality genomic DNA had been extracted. The extracted total DNA was preserved at −80°C.

16S rRNA gene amplification by PCR

Our target was the V3–V4 hypervariable region of the bacterial 16S rRNA gene. Polymerase chain reaction (PCR) was started immediately after the DNA was extracted. The 16S rRNA V3–V4 amplicon was amplified using KAPA HiFi HotStart ReadyMix (2 ×) (TaKaRa Bio Inc., Japan). Two universal bacterial 16S rRNA gene amplicon PCR primers (PAGE purified) were used: the amplicon PCR forward primer (CCTACGGGNGGCWGCAG) and the amplicon PCR reverse primer (GACTACHVGGGTATCTAATCC).

The reaction was set up as follows: Microbial DNA (10 ng/μl) 2 μl; amplicon PCR forward primer (10 μM) 1 μl; amplicon PCR reverse primer (10 μM) 1 μl; 2 × KAPA HiFi HotStart ReadyMix 15 μl (total 30 μl). The plate was sealed and PCR was performed in a thermal instrument (Applied Biosystems 9700, USA) using the following program: one cycle of denaturing at 95°C for 3 min, five cycles of denaturing at 95°C for 30 s, annealing at 45°C for 30 s, elongation at 72°C for 30 s, then 20 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s

Table 3. Abundance of bacteria in terms of genus and species in preterm infants with and without EUGR

	Abundance				
	Control		EUGR		Total
	All *N* (% of total)	Positive *N* (% of all *N*)	All *N* (% of total)	Positive *N* (% of all *N*)	*N*, %
Genus (344)	13760 (64.5)	1352 (9.8)	7568 (35.5)*	756 (10.0)	21328 (100.0)
Species (456)	18240 (64.5)	1404 (7.7)	10032 (35.5)*	821 (8.2)	28272 (100.0)

EUGR, extrauterine growth restriction.
*$P < 0.001$, the abundance number of EUGR compared to number of control

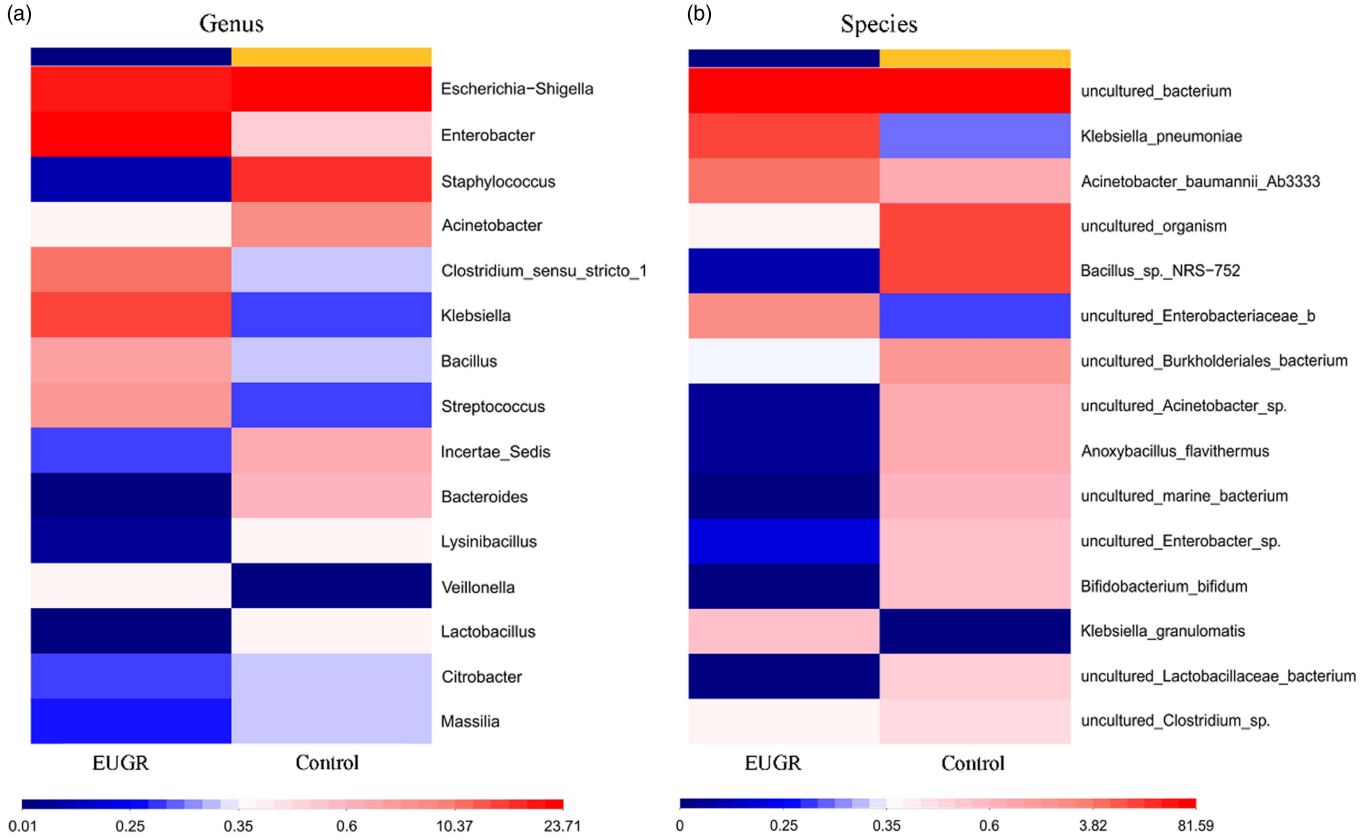

Fig. 1. Heatmaps of (a) bacterial genera and (b) species in stool samples of EUGR (left sides) and control (right sides) infants. The red color indicates enhanced abundance (percentage) of the genera and species.

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with a final extension at 72°C for 5 min. The PCR products were checked using electrophoresis in 1% (w/v) agarose gel in Tris, boric acid, ethylenediaminetetraacetic acid (TBE) buffer stained with ethidium bromide and visualized under UV light.

16S gene library construction, quantification and high-throughput sequencing

AMPure XP beads were used to purify the free primers and primer dimer species in the amplicon products. Samples were delivered to Sangon BioTech (Shanghai) for library construction using a universal Illumina adapter and index. Before sequencing, the DNA concentration of each PCR product was determined using a Qubit® 2.0 Green double-stranded DNA assay and the quality was confirmed using a bioanalyzer (Agilent 2100, USA). Depending on coverage needs, all libraries were pooled for one run. The amplicons from each reaction mixture were pooled in equimolar ratios based on their concentrations. High-throughput sequencing was performed at Sangon Biotech in Shanghai using the MiSeq PE300 Sequencing System (Illumina, San Diego, CA, USA).

Statistical and bioinformatics analysis

After sequencing, data were collected as follows: The two short Illumina readings were assembled by PEAR software (version 0.9.6) according to the overlap, and the FASTQ files were processed to generate individual FASTA and QUAL files, which could then be analyzed by standard methods.

Sequences containing ambiguous bases or sequences longer than 480 base pairs (bp) were dislodged, with an allowance of a maximum homopolymer length if 6 bp.[25] Sequences shorter than 200 bp were removed and any ambiguous base calls in the adapter and barcode sequences were deleted from the dataset.

The pre-cluster and chimeras.uchime commands of Mothur[26] were used to detect and remove chimera sequences. Distance matrices were established using the dist.seqs command, with the operational taxonomic units (OTUs) defined by using the furthest neighbor clustering algorithm at a phylogenetic similarity of 93–97%. Good's coverage estimator was used to confirm the completeness of sampling. The rarefaction curves of OTUs, Good's coverage and other richness and diversity indices of the bacterial community (i.e., ACE, Chao1, Shannon and Simpson) were estimated using the Mothur software.[26]

Taxonomic classifications were conducted using the online Ribosomal Database Project (RDP) classifier, with a confidence threshold of 80%.[27] A one-sample Kolmogorov–Smirnov (K–S) test was used to test the normality of the data. A general linear model (for normally distributed data) and a generalized linear model (for non-normally distributed data) were used to quantify the effects of host age and host species on the relative abundance of the top 5 phyla.

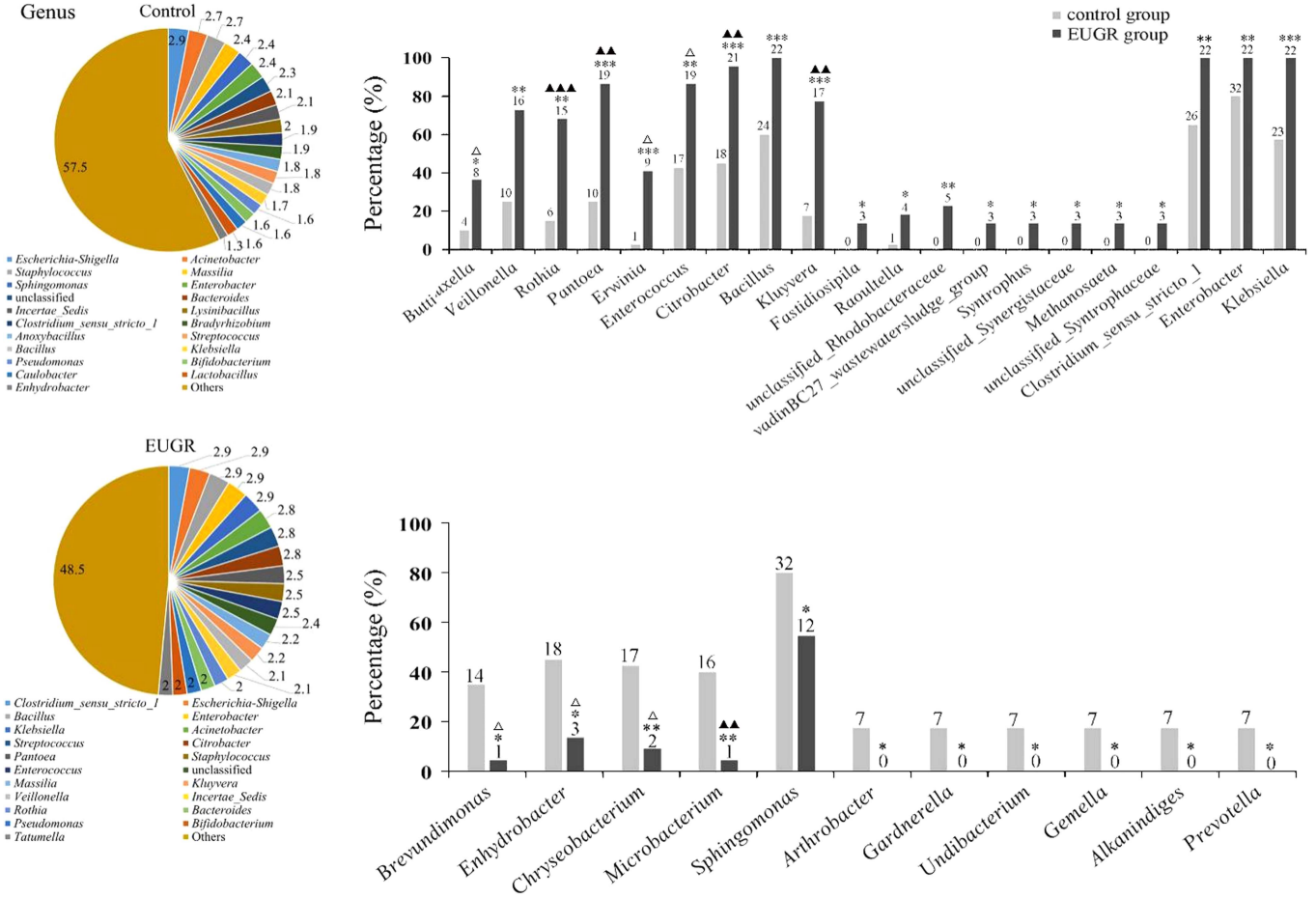

Fig. 2. Different bacterial genera occurrence in the EUGR and control groups. Genera with higher (upper panel) and lower (lower panel) abundance in the EUGR group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (a nominal association with EUGR and Genera); $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$, $^{\triangle\triangle\triangle}P<0.001$ (after adjusting for confounders), $^{\blacktriangle}P<0.05$, $^{\blacktriangle\blacktriangle}P<0.01$, $^{\blacktriangle\blacktriangle\blacktriangle}P<0.001$ (after adjusting for confounders and FDR analysis).

https://doi.org/10.1017/S2040174419000084 Published online by Cambridge University Press

An independent sample *t*-test (for normally distributed data) or a Mann–Whitney *U*-test (for non-normally distributed data) was used to compare data between groups with the same classification.

A multivariate logistic model was employed to compare the positive rates of bacterial genera between the EUGR and control groups after adjusting for potential confounding factors, including birth weight, birth weight recovery days, start time of breast feeding, weight increase, parenteral nutrition days, antibiotics administration days and human milk or human milk plus thickener. A false-discovery rate (FDR) correction for multiple testing was applied for comparisons of bacterial genera to minimize type I errors, and only a corrected *P*-value of <0.05 was considered to be significant.

The Bray–Curtis similarity index was used as a metric of similarity between the bacterial communities based on the abundance of OTUs between samples. A heatmap analysis was conducted to compare the overall bacterial composition associated with the species and the age of the hosts. Venn diagrams and statistical clustering were used to determine the shared OTUs by all group members that were defined as core microbiome. The heatmap figures and Venn diagrams were produced using R1.

Results

Characteristics of preterm infants

A total of 59 premature infants (EUGR group, $n = 23$; non-EUGR group, $n = 36$) were eligible for our study. As shown in Table 1, no significant difference was found between the EUGR and non-EUGR control group in terms of GA, birth weight, birth weight recovery time, feeding age and weight increase time. However, preterm infants in the EUGR group had a significantly longer time of parenteral nutrition absorption and antibiotics administration (22 ± 14 *v.* 17 ± 10 days, $P < 0.05$; 21 ± 10 *v.* 14 ± 9.5 days, $P < 0.05$, respectively).

In addition, the nurturing form and food composition for preterm infants in the two groups were also significantly different, as shown in Table 2. Significantly increased percentages of preterm infants with EUGR were nurtured with preterm formula and starch-based thickener, compared with the non-EUGR (13.0 *v.* 8.3%, $P < 0.05$; 21.7 *v.* 0%, $P < 0.05$, respectively), in contrast to significantly decreased percentages of human milk and carob-based thickener (60.9 *v.* 83.3%, $P < 0.05$; 60.9 *v.* 83.3%, $P < 0.05$, respectively).

Table 3 summarizes and compares the abundance of 344 genera and 456 species of bacteria identified using high-throughput sequencing of all stool samples in the two groups. The total abundance of bacteria and the frequency of bacteria in terms of genus (7568 *v.* 13,760, $P < 0.000$), and species (10,032 *v.* 18,240, $P < 0.000$) were significantly decreased in the EUGR group compared with the non-EUGR group, while positive percentages of bacterial strains in the different groups were not different (10.0 *v.* 9.8%, $P = 0.746$ and 8.2 *v.* 7.7%, $P = 0.187$).

Comparison of gastrointestinal bacterial abundance in terms of genus

Figure 1 shows heatmaps of bacterial genera (left side) and species (right side) abundances in stool samples of EUGR and control infant groups.

As shown in Fig. 2, there were 20 and 11 bacteria genera with significantly increased (upper panel) and decreased (lower panel) abundance in the EUGR group compared with the control group, respectively.

Comparison of gastrointestinal bacterial abundance in terms of species

As shown in Fig. 3, preterm infants with EUGR had significantly increased species of *Streptococcus*, *Bacterium*_RB5FF6 and *Staphylococcus* compared with the non-EUGR group. The abundance of *Enterococcus* was different due to the presence of different subgroups. *Lactis* was significantly decreased, while *Faecium* significantly increased in the EUGR group compared with the non-EUGR control group.

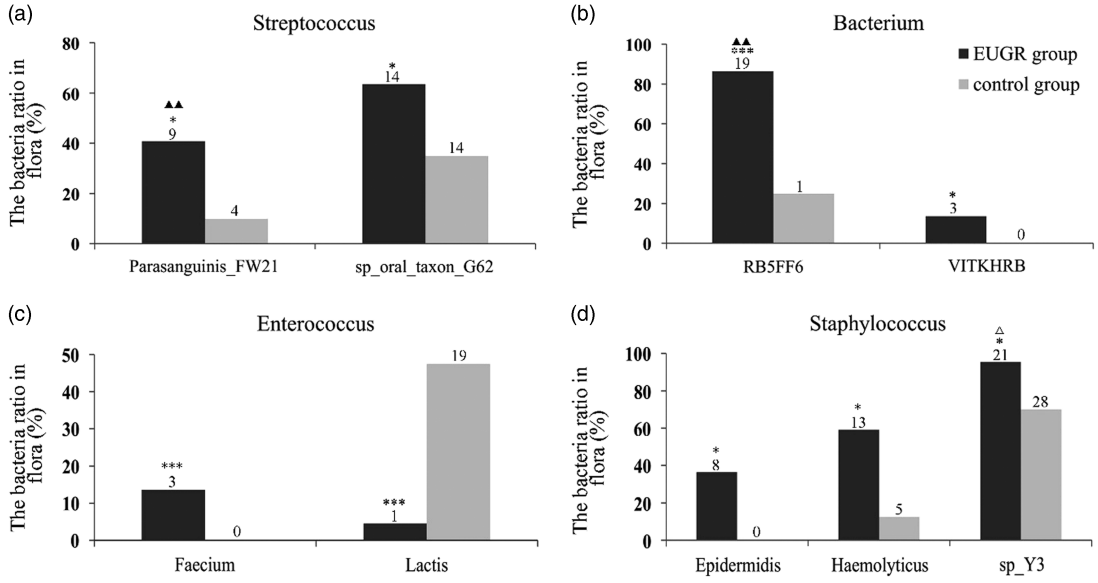

Fig. 3. Comparison of *Streptococcus*, *Bacterium*, *Enterococcus* and *Staphylococcus* ratio in flora between the two groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (a nominal association with EUGR and genera); $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$, $^{\triangle\triangle\triangle}P < 0.001$ (after adjusting for confounders), $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$ (after adjusting for confounders and FDR analysis).

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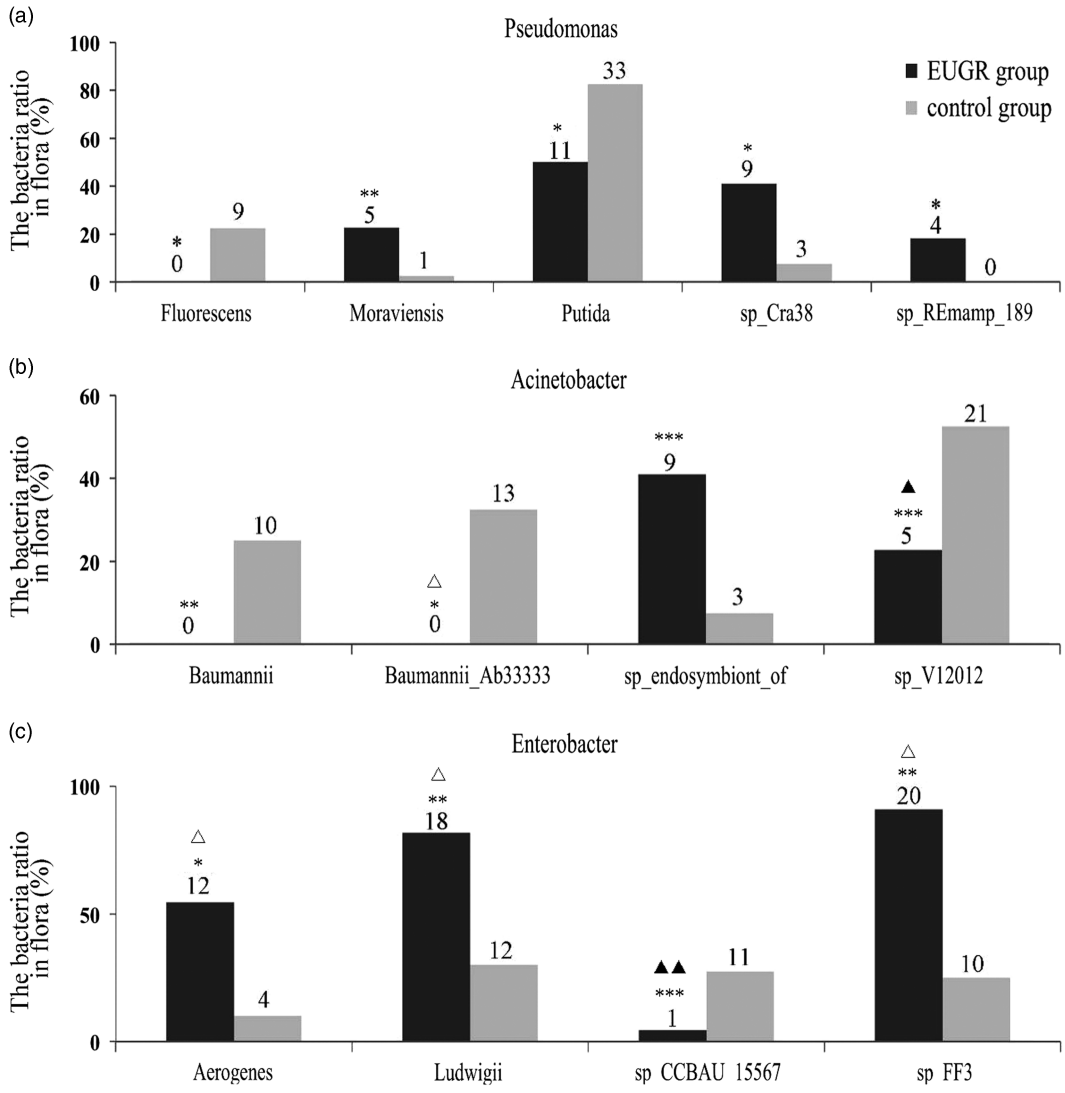

Fig. 4. Comparison of *Pseudomonas*, *Acinetobacter* and *Enterobacter* ratios in flora between the two groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (a nominal association with EUGR and genera); $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$, $^{\triangle\triangle\triangle}P < 0.001$ (after adjusting for confounders), $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$ (after adjusting for confounders and FDR analysis).

In addition, as shown in Fig. 4, some species of *Pseudomonas* (*Fluorescens* and *Putida*) significantly decreased, while others (*Moraviensis*, sp_Cra38 and sp_REmamp_189) significantly increased in the EUGR group compared with the non-EUGR group. The *baumannii* species of *Acinetobacter* significantly decreased, in contrast to the significantly increased Sp_ Endosymbiont of *Acinetobacter* in the EUGR group. Similarly, preterm infants with EUGR showed significantly increased *Aerogenes*, *Ludwigii* and sp_FF3, in contrast to significantly decreased sp_CCBAU_15567 species of *Enterobacter*, compared with non-EUGR preterm infants.

Moreover, as shown in Fig. 5, there were significant changes in eight *Klebsiella* species, with seven species (*Granulomatis*, *Michiganenis*, *Pneumoniae*, sp_enrichment_culture, sp_ok1_1_9_ 814, sp_SCAUS856 and *Variicola*) significantly increased and one species (sp_enrichment_culture1) significantly decreased in the EUGR group. Furthermore, there were 11 other species that showed either significantly increased (*Eubacterium_budayi*, *Marine_bacterium*_BPYW9, *Microbacterium*_sp_TSWCW12, *Propionibacterium_avidum*_44067, *Rothia_ mucilaginosa*_ M508 and *Unidentified marine_bacterioplan*) or decreased (*Bacillus*_sp_3B1332, *Bifidobacterium_animalis*, *Carnobacterium_maltaromaticum*_ LM, Endosymbiont_of_*Sphenophorus_lev* and *Pantoea_agglomerans*) abundance in the EUGR group compared with the control group.

Comparison of gastrointestinal bacterial abundance in terms of genus and species with multivariate logistic analysis and FDR correction

After using a multivariate logistic model to compare the positive rates of bacterial genera and species between the EUGR and control groups, after adjusting for potential confounding factors including birth weight, birth weight recovery days, start times of breast feeding, weight increase, parenteral nutrition days, antibiotics administration days and human milk or human milk plus thickener and FDR correction, we found five genera with significantly increased or decreased abundance in the EUGR compared with the control group. In addition, the abundance of nine species differed significantly between the two groups, including three *Klebsiella* and one *Enterobacter* species, *Streptococcus parasanguinis*, *Bacterium RB5FF6* and *Endosymbiont*_of_*Sphenophorus_lev*, which occurred

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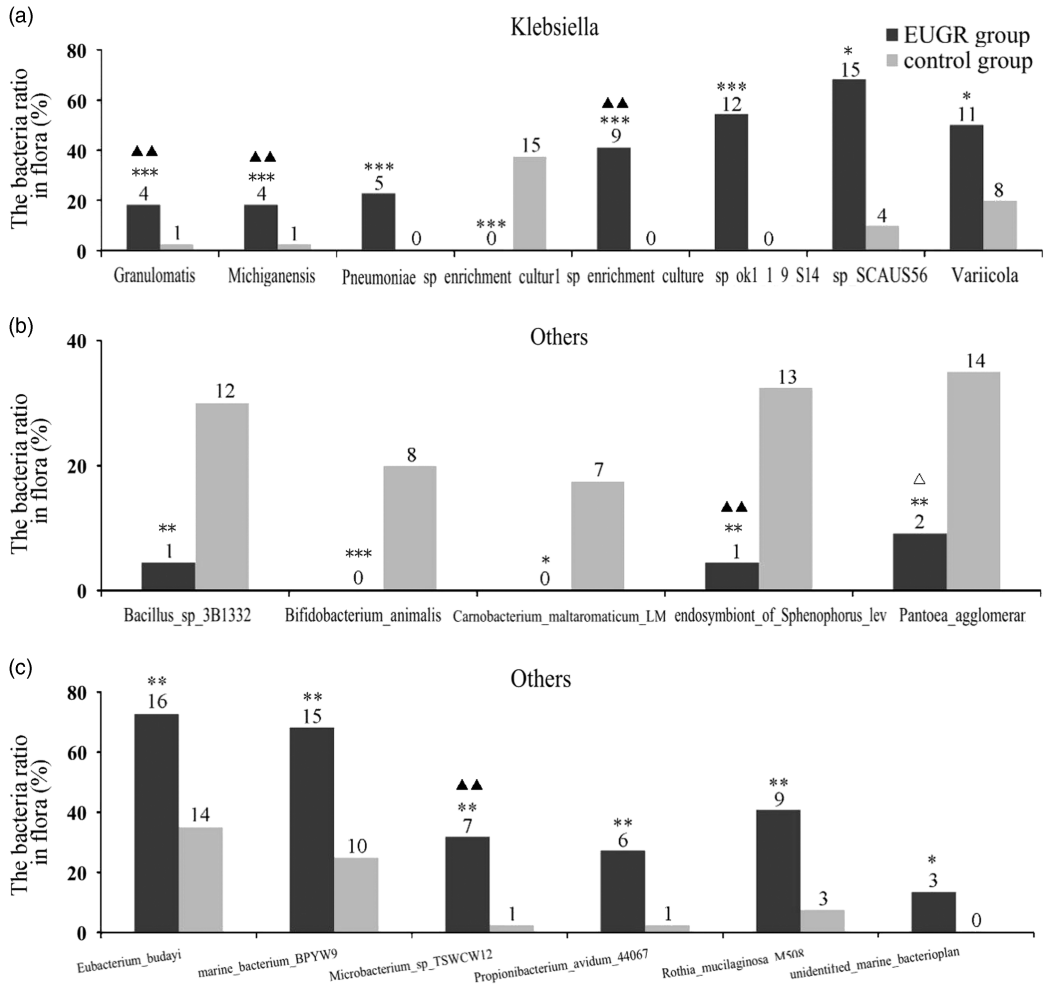

Fig. 5. Comparison of Klebsiella and other species with significantly different ratios in flora between the two groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (a nominal association with EUGR and genera); $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$, $^{\triangle\triangle\triangle}P < 0.001$ (after adjusting for confounders), $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$ (after adjusting for confounders and FDR analysis).

more abundantly and one *Microbacterium* and one *Acinetobacter* species, which occurred less abundantly in the EGFR group samples (Table 4, Figs 2–5).

Discussion

The incidence rate of EUGR is around 63% in our hospital. The main risk factors include premature birth and low birth weight, long time use of antibiotics, not breast-fed and contracting hospital infections more than once. In the current study, we found significant differences in the intestinal bacterial species between premature infants with and without EUGR. First, we observed that although having similar baseline characteristics, different feeding patterns, longer times of parenteral nutrition and antibiotic administration occurred in EUGR preterm infants, which resulted in significant differences in the total abundance of bacterial genus and species compared with the non-EUGR control group. There were significant differences in five genera and nine species (Table 4) after adjusting for confounding factors and FDR correction.

Interestingly, especially in terms of bacterial species, preterm infants with EUGR had significantly increased *Staphylococcus* compared with the non-EUGR group. In addition, significantly increased *Enterococcus faecium* was found in the EUGR infants without adjusting analysis, which is in line with a previous study that found four phases of microbiota development in newborns and the *Enterococcus* phase was a characteristic of prematurity.[28] However, after adjustments for cofounding factors and FDR corrections these differences disappeared.

Previous studies have also demonstrated that the growth of microflora could be affected by the birth state of the neonate, infections and exposure to antibiotics, which may result in irreversible alteration of bacterial species.[29,30] In addition, preterm infants might develop a unique neonatal intensive care unit flora, due to prolonged exposure.[31] In the present study, before applying the adjustment analysis, *Lactobacillus*, *Pseudomonas fluorescens* and *Pseudomonas putida* were significantly decreased in the EUGR group. Especially delayed development of a *Lactobacilli*-rich microbiota has been associated with poor health in preterm infants.[32] The decreased *Lactobacillus* could have been caused by antibiotic administration to premature infants with EUGR or by EUGR itself, suggesting that the immunity and resistance of EUGR infants could be strengthened by supplementary *Lactobacillus*, and the low birth weight of EUGR neonates could be increased by promoting the absorption of protein, monosaccharides and other mineral nutrients, such as calcium and magnesium.[33] However, after correction analysis, we identified that these genera changes were all caused by confounding factors such antibiotic usage, human milk or lower birth weight.

Previous studies have shown that autoimmune and allergic diseases in the fetus and newborn were caused by immune system

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Table 4. Multivariate logistic analysis for comparison of bacterial genera and species between the EUGR and control groups after adjusting for confounding factors and false-discovery rate (FDR) corrections

Positive stool culture	Control ($n = 40$, %)	EUGR ($n = 22$, %)	*P*-value
Genera			
Rothia	6 (15.0)	15 (68.2)	<0.001
Pantoea	10 (25.0)	19 (86.4)	0.002
Citrobacter	18 (45.0)	21 (95.5)	0.009
Kluyvera	7 (17.5.0)	17 (77.3)	0.009
Microbacterium	16 (40.0)	1 (4.5)	0.009
Species			
Endosymbiont_of_Sphenophorus_lev	5 (12.5)	11 (50)	0.006
Bacterium_RB5FF6	13 (32.5)	19 (86.4)	0.006
Enterobacter_sp_CCBAU_15567	5 (12.5)	13 (59.1)	0.004
Streptococcus_parasanguinis_FW21	8 (20.0)	11 (50)	0.002
Klebsiella_sp_enrichment_cultur1	4 (10.0)	15 (68.2)	0.001
Klebsiella_granulomatis	10 (25.0)	20 (90.9)	0.001
Klebsiella_michiganensis	4 (10.0)	12 (54.5)	0.010
Microbacterium_sp_TSWCW12	16 (40.0)	1 (4.5)	0.009
Acinetobacter_sp_V12012	19 (47.5)	1 (4.5)	0.011

EUGR, extrauterine growth restriction.

disorders, which resulted from intestinal flora maladjustment.[34] This finding suggests that intestinal microflora disorders or abnormalities could affect the immunological functions of preterm infants, since the disappearance of some bacterial species is associated with a shortage of some immunocytes involved in the regulation of immune responses. Therefore, the alteration of intestinal bacterial species at the initial stage of the neonate is very likely to affect the development of the cellular and immune system by influencing the recognition of various antigens, which first appear at the mucosal barrier and then throughout the whole body. In the current study, we report for the first time, that preterm infants with EUGR had a significantly different abundance of intestinal bacterial genus and species compared with the non-EUGR premature infants, by analyzing high-throughput sequencing of the bacterial rRNA library from the total community genomic stool DNA. As suggested by other studies, the maladjustment of bacterial species in EUGR preterm infants is likely to result in susceptibility to food allergies through Toll-like receptor 4 signaling and promote T(H)1/T(H)2 immune deviation.[7,35]

In addition, previous studies have demonstrated an increased presence of bio indicators such as C-reactive protein (CRP), human growth factor (HGF), interleukin-8 (IL-8), monocyte chemotactic protein 1 (MCP-1) and tumor necrosis factor α (TNF-α) in EUGR neonates, suggesting early changes in intestinal metabolism, nutrition absorption and immunological functions.[36] A concerning finding in the present study is the increasing prevalence over time of *Streptococcus*, *Bacterium_RB5FF6*, as well as *Enterobacter* and *Klebsiella* species in EUGR infants compared to healthy infants. It has been reported that *Enterococcaceae* were also composed of potential pathogens that have been frequently associated with late-onset sepsis in VLBW infants, and infrequently associated with necrotizing enterocolitis (*Clostridiaceae*).[37] However, it remains to be established what causes these changes in intestinal microflora in EUGR neonates. It is not known whether they are due to decreased immune functions and/or decreased metabolic functions of the EUGR neonates themselves. A prospective study with completely identical baseline characteristics in the affected and the control groups will be needed to investigate the possible causes of intestinal microflora changes in premature infants.

In summary, the current study has shown obvious changes in intestinal microflora between premature neonates with EUGR and without EUGR, with a significantly increased abundance of *Klebsiella* and *Streptococcus* strains as well as an *Enterobacter* species, while abundances of *Microbacterium* and *Acinetobacter* were significantly decreased in EUGR compared with non-EUGR preterm infants. However, we cannot unequivocally specify whether the abundance difference of these bacterial genera and species directly affects the growth of EUGR infants.

In conclusion, there was a significant difference in gut microbiota between EUGR and control children at discharge from the hospital, which might be attributed to different feeding or the use of antibiotics during hospitalization. Whether these different microorganisms in EUGR patients will be maintained and have an effect on their childhood growth and development will be the focus of a future study.

Acknowledgments. This study was supported by the National Natural Scientific Foundation [grant number: 81200456] as well as the Project of Chinese and Canadian Newborn Collaboration Network and the Shanghai University of Medicine & Health Science hundred teacher plan and Science and Technology Commission livelihood projects in Pudong District (PKJ2017-Y09).

https://doi.org/10.1017/S2040174419000084 Published online by Cambridge University Press

Ethical Standards. ***Ethics approval and consent to participate.*** Our study was performed in accordance with the Declaration of Helsinki with regard to ethical principles for research involving human subjects. The protocols for our study were approved by the Research Ethics Committee of Children's Hospital of Fudan University. Written, informed consent was obtained.

Data availability. The datasets supporting the conclusions of this article are included in the article.

Author contributions. J.Z. and Y.D. were responsible for the conception and design of the study. All authors contributed to the acquisition and analysis of data. Furthermore, J.Z., Y.D., X.B. and L.H. were in charge of statistical analysis. J.Z. and L.H. drafted the manuscript; J.Z., X.B., Z.W. and J.Z. revised the draft, and J.Z. approved the final version of the manuscript.

Conflicts of interest. The authors declare no conflicts of interest.

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