Correlation between the growth and development of extremely preterm infants in intensive care units and the intestinal microecology

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Research

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Abstract

Objective: To study the association between the growth and development of extremely preterm infants at the neonatal intensive care unit (NICU) and their intestinal microflora

Methods: Extremely low gestational age infants admitted to the NICU were included in this study. The subjects were divided into the extrauterine growth retardation (EUGR) group and normal growth group, and the growth and development were evaluated at 2 and 4 weeks after birth. Meanwhile, the stool samples were taken to perform high throughput 16S rRNA sequencing of the intestinal microflora.

Results: A total of 22 infants were included. There was no significant difference in the alpha diversity indexes the two groups at 2 weeks or 4 weeks after birth. The beta diversity analysis showed that the principal components of the intestinal microflora were similar between the two groups. LEfSe analysis showed that after 2 weeks of birth, the items with an absolute LDA higher than 4 included Streptococcaceae, Streptococcus, Bacteroidetes, Bacteroidales and Stenotrophomonas in the EUGR group and Enterococcaceae and Enterococcus in the control group. At the 4th week after birth, the items with an absolute LDA higher than 3 in the EUGR group included Clostriaceae, Eubacteriaceae and Eubacterium. The microbial community composition comparison showed significant differences in the principle components of Enterococcus and Streptococcus on the family and genus levels.

Conclusion: The diversity and richness of the intestinal microflora in preterm infants at the NICU are significantly insufficient, and the establishment of intestinal homeostasis is obviously delayed, which may cause growth retardation.

Introduction

The establishment of the neonatal intensive care units (NICU) and the extensive development in advanced life support for preterm infants have generally improved their survival rate. In 2019, the World Health Organization (WHO) reported the incidence of preterm infants to be about 10.6%, with an average of 14.8 million premature births per year, among which more than 1.1 million happen in China, with an incidence of 6.9%[1]. Due to their immature immune system and physiological characteristics, preterm infants often receive many forms of support, including respiratory and nutritional support, at the NICU after birth and have a higher incidence of antibiotic treatment, as well as other problems, such as different degrees of feeding intolerance, growth retardation and challenges in the establishment of the intestinal microflora [2]. Healthy intestinal microecology plays an indispensable role in the neonatal intestinal development, maintaining the integrity of the intestinal mucosa and nutritional status of the host[3]. Therefore, maintaining the colonization of the normal intestinal microflora is undoubtedly a key factor contributing to the overall health of newborns. Extrauterine growth retardation (EUGR) does not only affect the growth and development of infants, but it also affects the occurrence and development of diseases, which leads to prolonged hospitalization period and long-term development backwardness, finally affecting the brain development and the occurrence of metabolic diseases in adulthood. In this work, we monitored the extremely preterm infants with a gestational age of less than 32 weeks at the NICU of our hospital, investigated the correlation between growth and development and the intestinal microecology and analyzed the possible causes of the effects in order to find a new way to improve the extrauterine growth and development of extremely preterm infants.

Materials And Methods

1.1 Study design

Extremely preterm infants with a gestational age of less than 32 weeks who were admitted to the NICU of Beijing Friendship Hospital affiliated to the Capital Medical University from January to December 2018 were included in this study. A total of 22 extremely preterm infants were included and divided into the EUGR group and normal growth group. The extrauterine growth and development were evaluated at 2 and 4 weeks after birth. Meanwhile, stool samples were taken and stored at -80 ℃ to perform high throughput 16S rRNA sequencing of the intestinal microflora, and the correlation between the extrauterine growth

and development of extremely preterm infants and the intestinal microecology was analyzed. This study was approved by the Ethics Committee of Beijing Friendship Hospital affiliated to the Capital Medical University.The infants' parents (or respsonsible relative) gave written informed consent.

The inclusion criteria were as follows: (1) Admission to the NICU immediately after birth; (2) A gestational age of 28~32 weeks, single pregnancy; (3) Hospitalization time > 28 days; (4) Antibiotic treatment after birth.

Growth and development were evaluated according to the Fenton growth chart for preterm infants (2013) as follows: The infants with a body weight less than the 10th percentile of the corresponding gestational age were considered to have extrauterine growth retardation, and those between the 10th and 90th percentiles were considered to have normal growth and development.

The exclusion criteria were as follows: (1) Severe congenital malformations and congenital genetic metabolic diseases; (2) Discharged automatically with unclear outcomes after discharge; (3) Incomplete data; (4) Received no microecological preparations during the collection of stool samples.

1.4 Stool sample collection

The stool samples of preterm infants were collected, strictly following the principle of aseptic operation, on the 14th and 28th day after birth using a disposable sterile stool container and preserved at -80 ℃. Next, they were sent to Allwegene Technology Inc. to perform DNA extraction, sequencing and bioinformatic analysis.

1.5 Bioinformatic analysis

The FLASH software was used to merge the sequencing data, and the chimeras were filtered using the VSearch software. Sequences with a similarity greater than 97% were defined as one operational taxonomic unit (OTU), and the representative sequences were accordingly selected. BLAST was used for sequence alignment, and the QIIME software was used to analyze the α diversity, which represents the species richness in the sample, along with the composition of the samples, on the phylum, class, order, family and genus levels.

1.6 Statistical methods

The SPSS 20.0 software was used for data analysis. Count data, such as the gestational age (stratification), gender, delivery mode and feeding mode, were compared using the Pearson's χ2 test. When the sample size was too small, the Fisher exact probability method was used for intergroup comparison. A $p < 0.05$ was considered to be statistically significant.

Results

2.1 Basic information of the subjects

Among the 22 enrolled preterm infants, there were 14 males and 8 females. Regarding the gestational age, there were 8 cases with 28 weeks <= gestational age < 30 weeks, 6 cases with 30 weeks <= gestational age < 31 weeks and 8 cases with 31 weeks <= gestational age < 32 weeks. There was no significant difference in the gender ratio, delivery mode, feeding mode or gestational age stratification between the EUGR and control groups (Table 1).

2.2 Sequencing results and species accumulation curve

DNA extraction and PCR were successfully performed in all the stool samples, and the MiSeq libraries were prepared and sequenced. The species accumulation curve showed that the number of OTUs rapidly increased with the increase of the stool samples at the beginning, then slowly increased and entered a stationary phase, which proved that the number of samples in this study was sufficient to reflect the species richness in the community.(Fig.1).

2.3 OTU analysis

On the 14th day after birth, 8 infants had growth retardation, and a total of 1288 OTUs were obtained. Among the obtained OTUs, 331 were specific to the control group, 159 were specific to the EUGR group, and 798 were shared by the two groups. On the 28th day after birth, 10 infants developed growth retardation, and a total of 1177 OTUs were obtained. These OTUs included 238 ones specific to the control group, 226 ones specific to the EUGR group, and 713 OTUs shared by the two groups (Fig.2).

2.4 Alpha diversity analysis

Both the species richness (observed_species) and Shannon index (shannon) showed a stable pattern of the curve, which indicated that the sequencing depth was sufficient to reflect the vast majority of the microbial diversity in the samples, and an increased amount of data could result in limited new species. At the 2nd week after birth, there was an obvious difference between the Shannon index of the EUGR group and that of the control group, but this difference was insignificant. Meanwhile, there was no significant difference in any of the other indexes such as chao1, observed_species and PD_whole_tree between the two groups at 2 weeks or 4 weeks after birth (Fig. 3).

2.5 Beta diversity analysis

The principal component analysis (PCA) and non-metric multidimensional scaling (NMDS) analysis based on the OTU abundance in the stool samples showed that after 2 or 4 weeks of birth, the two groups had similar principal components of the intestinal microflora, but they could be roughly separated. This finding suggested that there was a certain difference in the structure of intestinal microflora between the two groups (Fig. 4).

2.6 LeFSe analysis

We performed the linear discriminant analysis effect size (LefSe) analysis, which is mainly used to identify species with a significantly different abundance among different groups. Based on the obtained species, an inter-group difference analysis was carried out, followed by a linear discriminant analysis (LDA) to estimate the effect of each species on the difference. At the 2nd week after birth, the items with an absolute LDA higher than 4 in the EUGR group included Streptococcaceae, Streptococcus, Bacteroidetes, Bacteroidales and Stenotrophomonas, while those in the control group included Enterococcaceae and Enterococcus. On the other hand, at the 4th week after birth, items with an absolute LDA higher than 3 in the EUGR group included Clostriaceae, Eubacteriaceae and Eubacterium (Fig.5).

2.7 Comparison of the community composition

On the 14th day after birth, there was a significant difference between the community principal components of the infants in the EUGR group and those of the normal control group on the family and genus levels. On the family level, the principal components in the control group were Enterococcaceae (53.95%) and Enterobacteriaceae (24.96%), while the Enterococcaceae content in the EUGR group significantly decreased to 23.45%, and there was no significant difference in the Enterobacteriaceae content (24.05%). Streptococcaceae accounted for 11.33% in the EUGR group and 2.58% in the control group, with no significant difference. On the genus level, the principal component of the control group was Enterococcus (53.92%), which accounted only for 26.44% in the EUGR group. The proportion of Streptococcus significantly differed between the two groups, accounting for 2.57% in the control group and 11.33% in the EUGR group. On the 28th day after birth, there was no significant difference between the community composition of the infants in the EUGR and control groups.

Discussion

The intestinal microflora undergoes special dynamic changes in the neonatal period, and its colonization and composition are not only affected by the body weight, gestational age, delivery mode, first milk and feeding mode, but they are also related to the living environment and drug use (such as antibiotics)[4, 5]. In this study, a total of 22 extremely preterm infants with a

gestational age of less than 32 weeks were included. There were 8 infants with a growth retardation at 2 weeks after birth and 10 infants with growth retardation at 4 weeks after birth (10/22), and the incidence of EUGR was about 36%-45%. In Tokyo area of Japan, the incidence of EUGR was reported to be 8.4% [6]. In 2016, Griffin et al. [7] analyzed the data of the California Perinatal Quality Care Collaborative from 2005 to 2012 and found that the incidence of EUGR at discharge of very low birth weight infants (VLBWIs) was 52.7% and 44.4% in the 1000-1249 g and 1250-1500 g groups, respectively. In 2017, Park et al.[8] reported that at 40 weeks of corrected gestational age, the incidence of EUGR was 58.4% by the weight and 50.5% by the height. In China, the incidence of EUGR was higher. In 2015, the Neonatal Physician Branch of Chinese Physician Association, Neonatal Health Professional Committee conducted a multicenter survey of 572 VLBWIs in 15 hospitals across the country. Their results showed that the incidence of EUGR at discharge was 80.9%, and the cases with a weight < 3rd percentile accounted for 63.6%. The high incidence of EUGR reveals the challenging nature of the nutritional status of VLBWIs in China during hospitalization[9]. The lower the gestational age, the lower the birth weight and the higher the incidence of EUGR[10]. In this study, the incidence of EUGR is significantly lower, which may be due to the small sample size or better NICU preterm infant management. All the included extremely preterm infants were admitted to the NICU immediately after birth, which excludes the influence of the delivery mode, feeding mode and antibiotic factors. This study detected the intestinal microflora composition of the two groups by performing 16s rRNA high-throughput sequencing technique, and found the intestinal microecology of the infants in the two groups to be significantly different at 2 weeks after birth. The OTU number of the infants in the control group was significantly higher than that in the EUGR group. The colonization of the intestinal microflora in the EUGR group was delayed, the diversity was decreased, and the difference was significantly reduced at the 4th week. The first 2 weeks after birth represent the period of moving from the unstable to stable state in extremely preterm infants, during which the infants face great challenges. After birth, the nutrient demands of the infants directly change from maternal nutrient supply into active nutrition absorption. However, since a perfect intestinal microbial system cannot be established in preterm infants, and due to the invasive operation of the NICU in the early stage of birth, the NICU environment has a particularly special treatment of critically ill patients, which includes a more strict disinfection and sterilization system and a massive use of broad-spectrum antibiotics, resulting in its special composition of environmental microorganisms. Therefore, preterm infants at the NICU have particular intestinal bacterial colonization model, species types and diversity. It is very easy to develop feeding intolerance and growth retardation in such conditions[11].

We performed a comparison between the intestinal microflora composition of the two groups and found that Enterococcus was the dominant intestinal microflora of the two groups. However, the proportion of Enterococcus in the EUGR group was significantly lower than that in the control group, and the proportion of pathogenic bacteria, such as Streptococcus, significantly increased. The LEfSe analysis showed that at the 2nd week after birth, Streptococcaceae and Streptococcus in the EUGR group and Enterococcaceae and Enterococcus in the control group were the most significantly different bacterial species between the two groups. Although the contents of Bacteroidetes, Bacteroidales and *Stenotrophomonas maltophilia* were also significantly different between the two groups, their proportions in the community composition of the two groups were low, which has no clear clinical significance. Streptococcus is a common pathogen for early-onset neonatal infection. Group B streptococcus (GBS) is the most common cause of early septicemia and meningitis in neonates. The mortality of early-onset GBS infection in full-term infants is 2%-3%, while it is about 20% in preterm infants and as high as 30% in preterm infants with a gestational age of less than 33 weeks[12]. An analysis of 104186 extremely preterm infants admitted to 312 NICUs in the United States from 1997 to 2011 showed that the rates of early-onset and late-onset GBS infection were 10.2% and 11.8%, respectively, such that early-onset infection increased the risk of death [13]. In China, the conducted studies showed that the incidence of neonatal infection in GBS-positive pregnant women was 29.8%, which was significantly higher than that in GBS-negative pregnant women (13.2%). Our results are basically consistent with previous studies. Schwiertz et al. [14] analyzed the stool microbial diversity of 29 preterm infants at the NICU and 15 full-term infants within 4 weeks of birth and found that it took 10 days for preterm infants to reach homeostasis. Jacquot et al.[15] showed that almost no Bifidobacterium was detected in preterm infants within 8 weeks after birth. Our results suggest that the proportion of Enterococcus in infants with EUGR is lower than that in the control group, while the proportion of *Escherichia coli* and *Klebsiella pneumoniae* are higher, which indicates that the change in the intestinal microflora composition is closely associated with growth and development [16]. However, there was no significant difference in the intestinal structure between

the two groups at 4 weeks after birth, when stability was reached. Our results show that the intestinal stability of preterm infants is significantly delayed, which may be related to the special environment of the NICU after birth.

EUGR represents a risk factor for neurodystrophy. A follow-up analysis conducted on 242 cases of VLBWI and 233 infants with a normal birth weight found that the neurological development score of the VLBWIs at the age of 20 was lower than that of infants with a normal birth weight, and they had learning difficulties at school age[17]. In addition, the effect of intestinal microflora on newborns is not limited to the intestinal tract, since it maintains a bidirectional interaction with the central nervous system (CNS) through the gut-brain axis. Metabolites from the intestinal microflora disorders destroy the blood-brain barrier (BBB), producing harmful components, which can enter the brain more easily and cause brain damage[18]. The intestinal colonization of Bifidobacterium can weaken the hypothalamic-pituitary-adrenal (HPA) axis response, and this inhibitory effect occurs in the early stage of life, which indicates that the original microbial exposure is necessary to inhibit the neural regulation of the HPA axis[19]. A recent study conducted by Bercik et al.[20] showed that after transferring the feces of the donor mice to the recipient mice, the recipient mice showed a similar behavioral phenotype to the donor mice, suggesting that the intestinal microbes can communicate with the brain through certain mechanisms.

To sum up, the diversity and richness of the intestinal microflora in preterm infants at the NICU are significantly insufficient, the establishment of intestinal homeostasis is obviously delayed, and there is a higher incidence of growth retardation. Early detection of the intestinal microflora and early intervention can improve the growth and development of preterm infants. The question of whether the early intervention, such as the supplement of specific probiotics, can prevent growth retardation will be the major topic of our following studies. Probiotics have a high strain specificity in the intestinal tract, and different probiotics have different functions; questions such as the possibility of bacteremia caused by translocation, the colonization rate and time (short-term or long-term) and suitable strains and doses put forward high requirements for the safety of probiotics. There is a long way to go in the research and development direction of probiotics that are suitable for preterm infants.

Declarations

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Contributorship statement

Ying-xue Ding: design the study, analysize all data of whole study.

Shou-ni Wang:collected clinical samples

Hong Cui:analysize some data of clinical samples

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Tables

Table1.Clinical characteristics of the subjects

Group		Gender		Delivery		Feeding		Gestation age		
		Boy	Girl	Natural	Caesarean	Breast milk	Formular	GA<30w	30w≤GA<31w	31w≤GA<32w
2w	Control	10	4	8	2	8	6	6	5	3
	EUGR	4	4	6	6	4	4	2	1	5
		p=0.386		*p*=0.204		*p*=1.000		*p*=0.649		
4w	Control	9	3	7	5	6	6	5	4	3
	EUGR	4	6	4	6	6	4	4	2	4
		p=0.092		*p*=0.392		*p*=0.691		*p*=1.000		

Figures

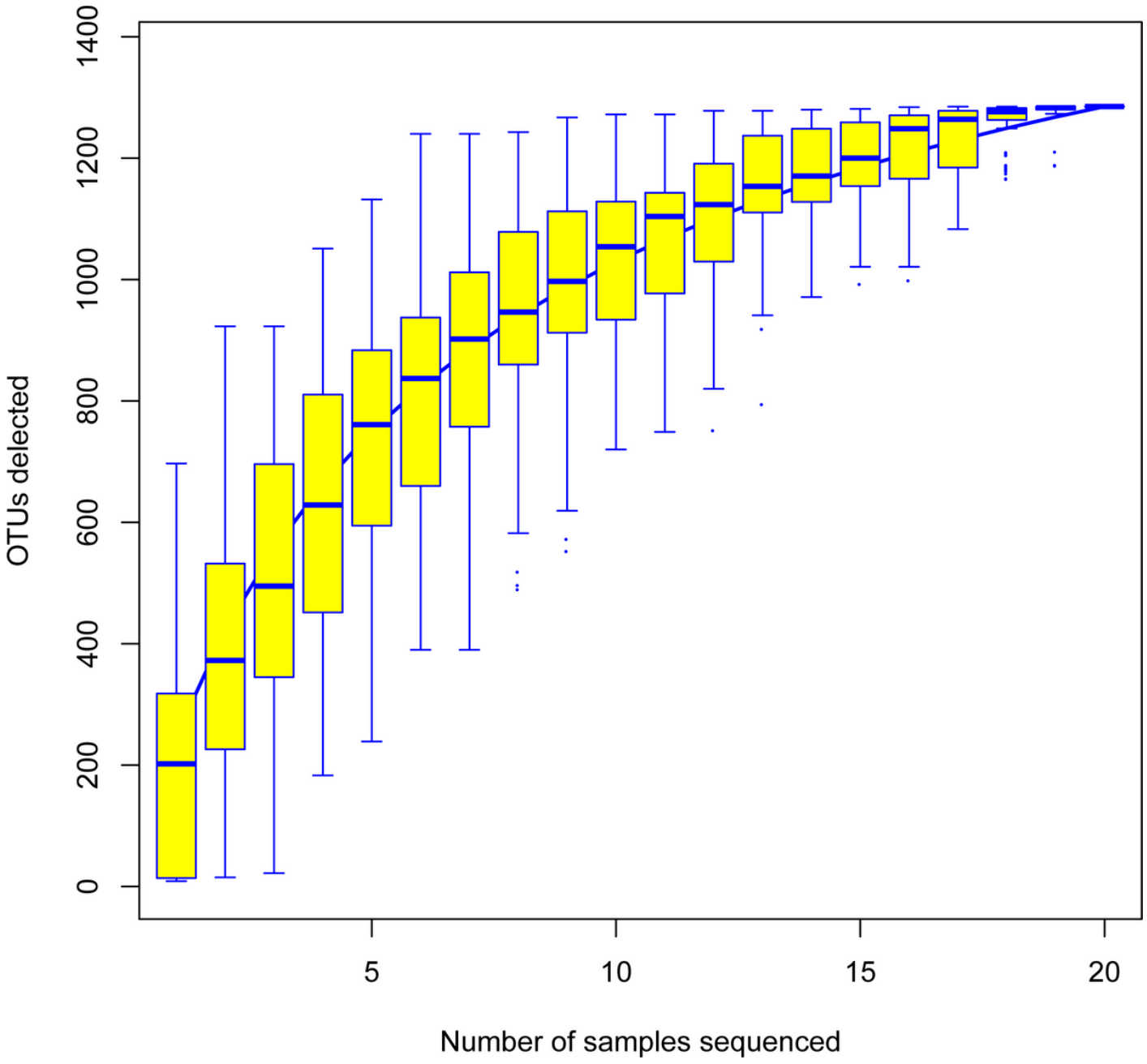

Figure 1

Species accumulation curve.

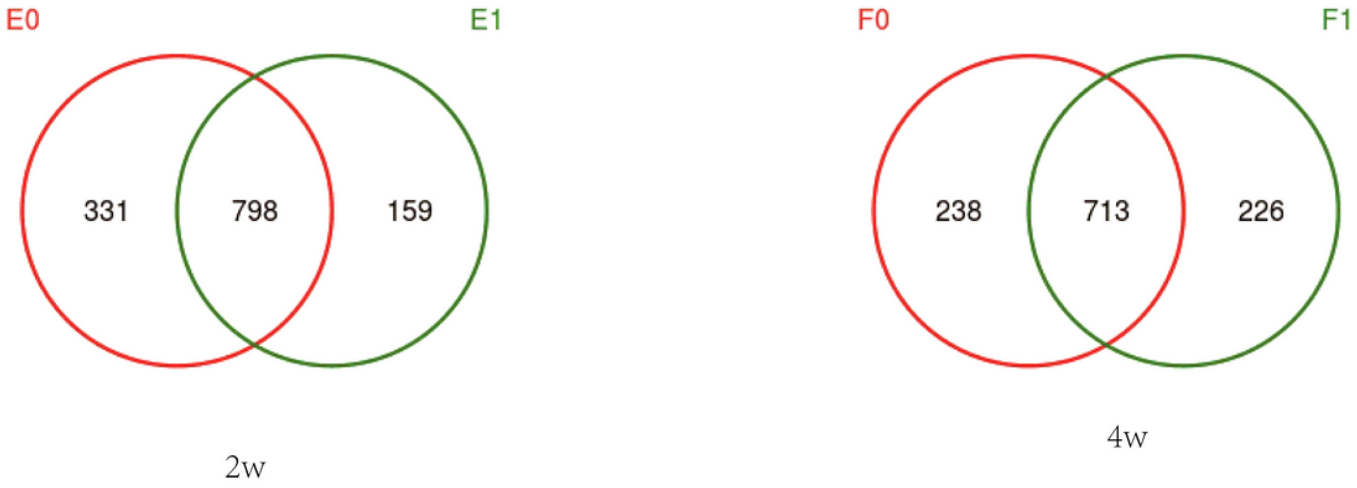

Figure 2

Venn plot. E0: control group, 2 weeks after birth; E1: EUGR group, 2 weeks after birth; F0: control group, 4 weeks after birth; F1: EUGR group, 4 weeks after birth. The overlapping areas of the circles with different colors indicate the shared OTUs, while the non-overlapping areas are OTUs specific to one group.

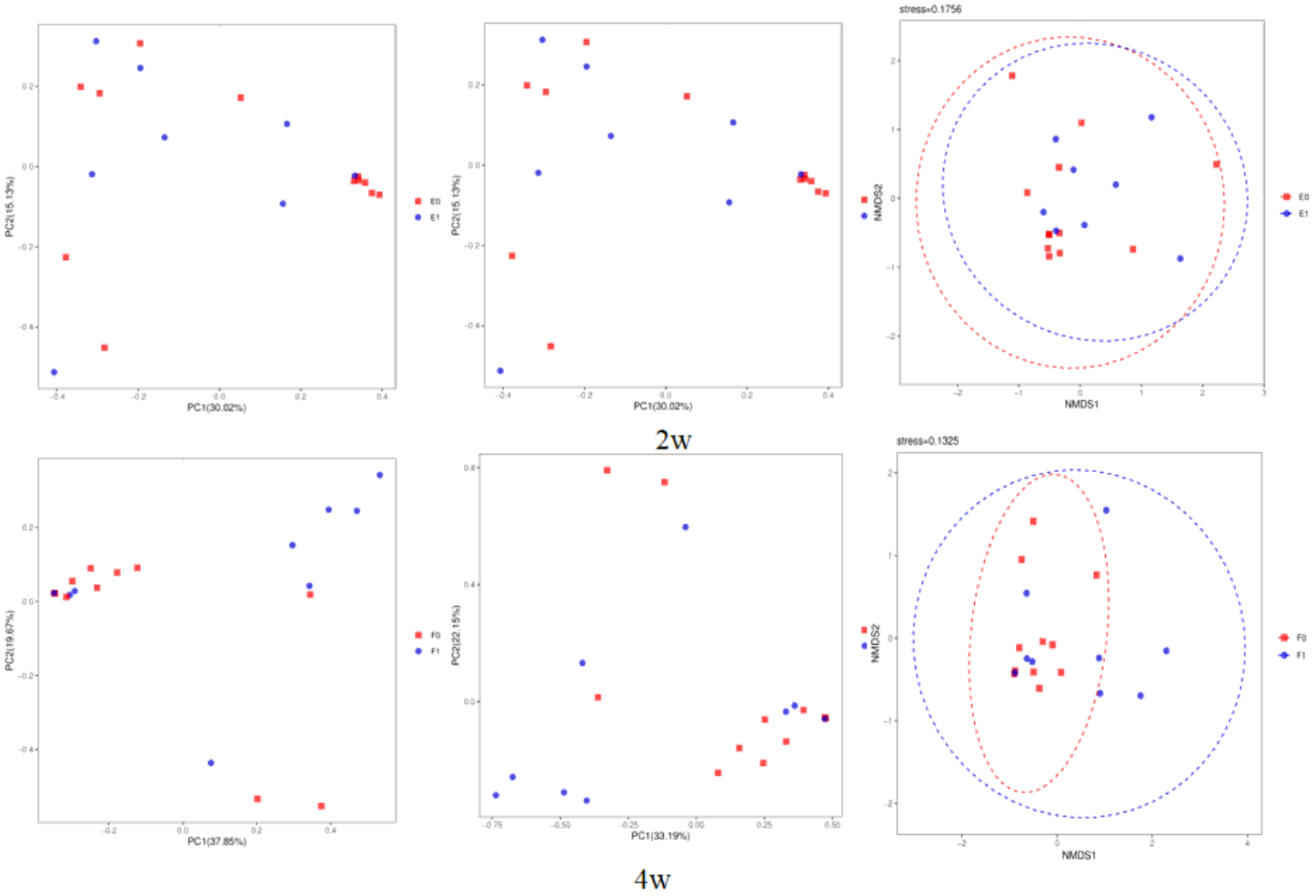

Figure 3

Alpha diversity analysis of the two groups at different postnatal stages, including the indexes of chao1, observed_species, PD_whole_tree and shannon. E0: control group, 2 weeks after birth; E1: EUGR group, 2 weeks after birth; F0: control group, 4 weeks after birth; F1: EUGR group, 4 weeks after birth.

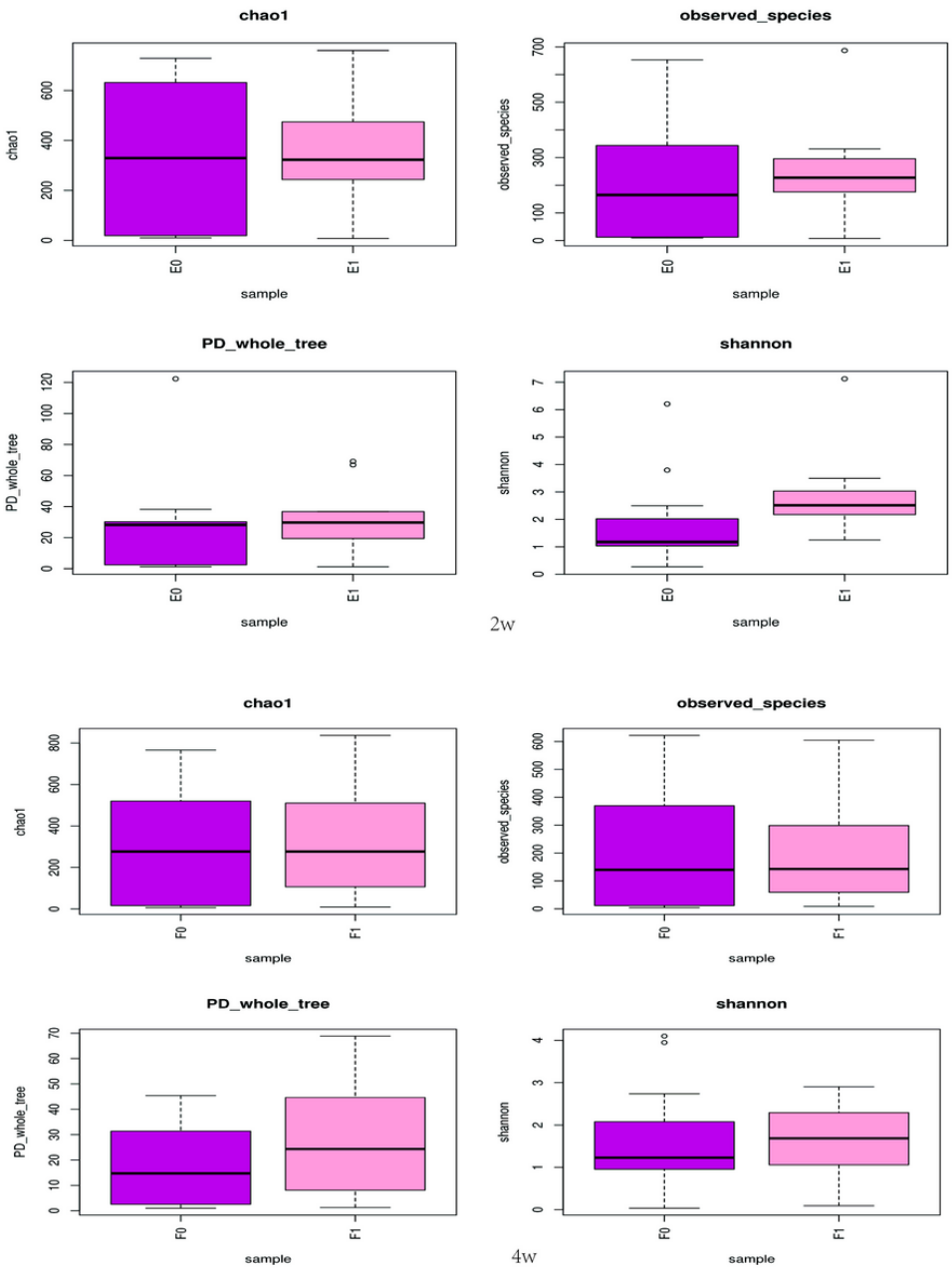

Figure 4

PCA and NMDS analysis at the 2nd and 4th weeks after birth. E0: control group, 2 weeks after birth; E1: EUGR group, 2 weeks after birth; F0: control group, 4 weeks after birth; F1: EUGR group, 4 weeks after birth.

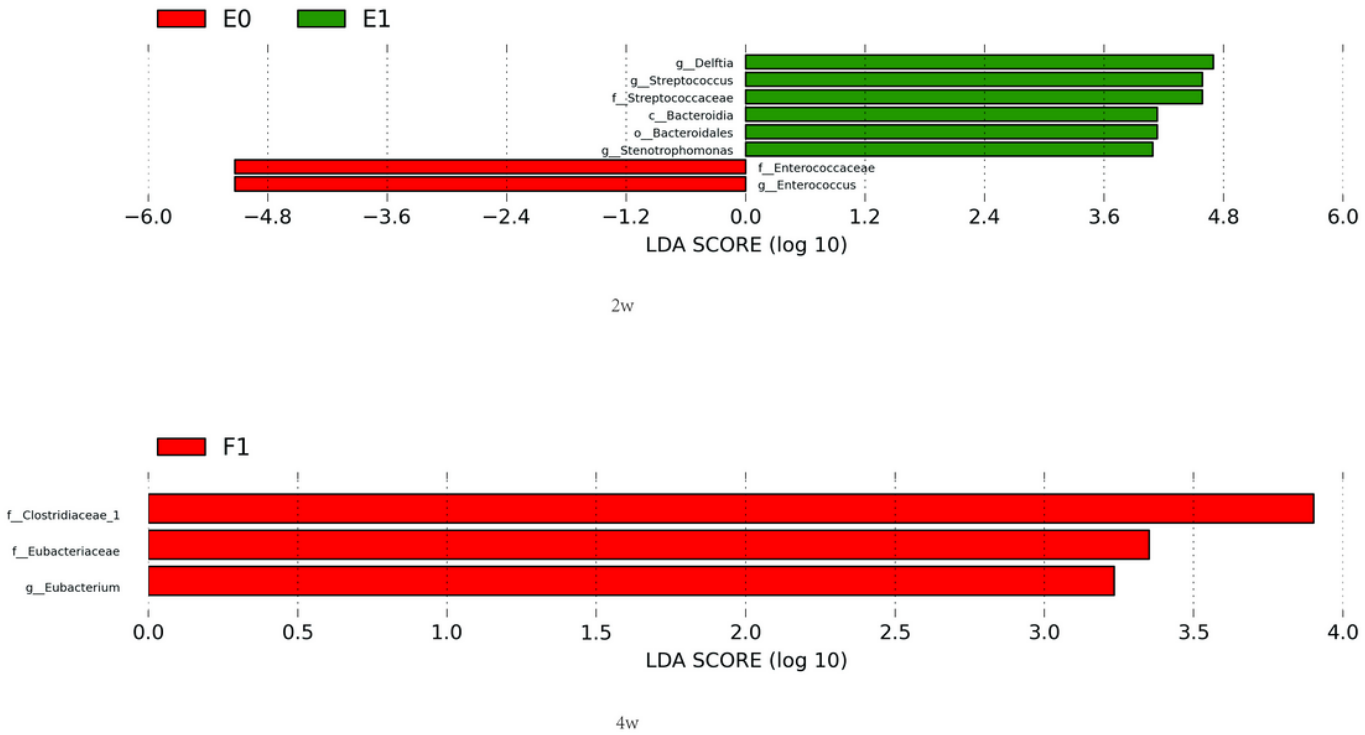

Figure 5

LEfSe analysis of the two groups at different postnatal stages. E0: control group, 2 weeks after birth; E1: EUGR group, 2 weeks after birth; F1: EUGR group, 4 weeks after birth.