Change of Intestinal Microbiota in Mice Model of Bronchopulmonary Dysplasia

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Research Article

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Abstract

Background: Gut microbiota has been proposed to be related to the pathogenesis of pulmonary diseases such as asthma and lung cancer, according to the gut-lung axis. However, little is known about the relationship between broncho-pulmonary dysplasia (BPD) and gut microbiota. This study was designed to investigate the changes of gut microbiota in neonatal mice with BPD.

Methods: BPD model was induced through exposure to high concentration of oxygen. HE staining was utilized to determine the modeling efficiency. Stool samples were collected from the distal colon for the sequencing of V3-V4 regions of 16S rRNA, in order to analyze the gut microbiota diversity.

Results: BPD models were established in this study. Alpha diversity indicated that there were no statistical differences in the abundance of gut microbiota between model group and control group. On day 14, there were statistical differences in the genetic diversity between two groups (p<0.05). Beta diversity analysis showed that there were statistical differences in the gut microbiota on day 14 (R=0.368, p=0.021). Line discriminant analysis (LDA) showed that there were 22 markers with statistical differences on day 14 (p<0.05), while those on day 7 and 21 were 3 and 4, respectively. Functional prediction analysis showed that the top 3 metabolic pathways were signal transduction (P_{FDR}=0.037), glycan biosynthesis and metabolism (P_{FDR}=0.032), and metabolism of terpenoids and polyketides (P_{FDR}=0.049).

Conclusions: BPD mice showed disorder of gut microbiota, which may involve with specific metabolic pathways in the early stage. In the presence of intestinal maturity in mice, the differences of the gut microbiota between the two groups would gradually disappear.

Background

Broncho-pulmonary dysplasia (BPD) is a chronic pulmonary disease that is commonly reported in neonates after long-term oxygen inhalation or mechanical ventilation [1]. Some children with BPD may present persistent lung function deterioration until reaching the adulthood [2, 3]. These patients show a higher risk for asthmatic symptoms in school age [4] and increased risks for chronic obstructive pulmonary disease (COPD) in their adulthood [5]. To date, there are still no ideal treatment options for treating children with BPD. Some scholars proposed that there might be association between gut microbiota and pathogenesis of pulmonary diseases based on gut-lung axis [6]. For instance, the abundance of *Proteobacteria* among the gut microbiota in asthmatic patients is relatively higher, while that of *Firmicutes*, *Actinobacteria*, and *Saccharibacteria* showed decline [7]. It has been well acknowledged that alternation of *Streptococcus*, *Haemophilus*, and *Moraxella* has been associated with the severity of airway inflammation [8]. In patients with tuberculosis, there was relative increase in the abundance of *Bacteroides*, *Parabacteroides*, *Fusobacterium* and *Lachnoclostridium* in gut microbiota, and decline of *Blautia*, *Roseburia* and *Bifidobacterium* [9]. In addition, among pulmonary cancer patients, the abundance of *Firmicutes* and *Actinobacteria* showed decline to some extent [10]. On this basis, we speculated that there might be changes in the gut microbiota among BPD children, which may provide a

new treatment target for BPD. In this study, a BPD mice model was established, and then we determined the specific changes of gut microbiota diversity.

Materials And Methods

Animals

Pregnant mice of specific-pathogen free (SPF) were purchased from Sipeifu Biotech (Beijing, China, approval No.: SCXK-2019-0010). Experiments were implemented in the Experimental Animal Center of Qingdao University and the Medical Research Center of Affiliated Hospital of Qingdao University.

Induction of BPD and grouping

Neonatal mice were randomly divided into control group (n=15) and BPD group (n=15). For the induction of BPD, neonatal mice were subject to high concentration of oxygen (80±5%) for 3 weeks. In addition, the carbon dioxide generated by the mice was removed using the absorption agent. In control group, the animals were exposed to oxygen at a concentration of 21%. All the animals in both groups were kept under a temperature of 20±2°C, in a humidity of 55±5%. They were all free access to water and food, in a light cycle of 12 h/12 h. The mice for breeding these animals were exchanged per day.

Experimental procedure

Five mice that were randomly selected were sacrificed on day 7, 14 and 21 after birth using cervical dislocation, and then the pulmonary tissues and stool samples were obtained. Pulmonary samples were embedded using paraffin, followed by HE staining to determine the presence of pulmonary alveolar fusion, inflammatory infiltration, pulmonary septum thickening, pulmonary tissue disorder, in order to validate the modeling.

To prevent the environmental pollution, the stool samples were obtained under sterilized conditions from the distal colon, followed by storing at -80°C for analysis. Stool samples were subject to sequencing of V3-V4 regions of 16s rRNA [11] based on Illumina HiSeq platform provided by BMKCloud (Beijing, China), in order to obtain the raw sequencing data of the gut microbiota.

In this section, we analyzed the gut microbiota in both groups at different stages. Ace index and Phylogenetic diversity index were obtained under a similarity of 97%. Then diversity index dilution curve was established. These data could reflect the abundance and diversity between BPD model group and control group. Afterwards, PCoA data from each group were obtained, in order to validate the significance of the sample similarity. Then the biomarkers at different stages were obtained, in order to further analyze the significances at the phylum level. Finally, we tried to identify different metabolic pathways between two groups.

Statistical analysis

Data were presented as mean ± standard deviation. Alpha and Beta analyses were conducted by QIIME2 analysis platform. We conducted the LDA between groups with the Lefse data analysis toolkit of Python language, and the analysis of variance (ANOVA) with the vegan toolkit of R language. The metabolic pathway analysis was conducted using Picrust2 software. On the Student's t-test, 95 % confidence intervals were used. $p< 0.05$ was considered to be statistically significant. P_{FDR} was *p*-value using false discovery rate [12]. The analyses were drawn using GraphPad Prism 9.

Results

Morphological changes of lung in different groups

HE staining indicated that the morphology of pulmonary alveoli in control group was regular on day 7 with even sizes (**Fig. 1a**). On day 14, the structure of pulmonary alveoli was normal, and there was narrowing in the alveolar septum in control (**Fig. 1b**). On day 21, there was increase in number of pulmonary alveoli, together with narrowing of alveolar septum. There were no aberrant changes in terminal bronchus in control group (**Fig. 1c**). For the HE staining in BPD model group, part of pulmonary alveoli showed fusion on day 7, combined with infiltration of inflammatory cells (**Fig. 1a**). On day 14, the number of pulmonary alveoli showed decrease and the structure was not regular. There was massive interstitial cell hyperplasia, together with thickening in alveolar septum (**Fig. 1b**). On day 21, pulmonary alveoli were no longer available, and there was obvious dilatation in terminal bronchus. In addition, structural disorder was noticed in pulmonary tissues, indicating block in pulmonary development (**Fig. 1c**).

Alpha diversity

Multi-sample Shannon curves indicated that the data volume was adequate for the sequencing, and the sample traits would not increase with the elevation of sequencing volume (**Fig. 2a**). Alpha diversity was analyzed to evaluated overall differences between the gut microbiota in model group and control group. The ACE index showed no statistical differences in richness of gut microbiota between control group and model group on day 7, 14, and 21, respectively ($p>0.05$, **Fig. 2b**). *PD_whole_tree index* evaluated the diversity of gut microbiota, homogeneity and evolution. There were no statistical differences between control group and model group on day 7 and 21 ($p>0.05$). The difference between the two groups was statistically significant on day 14 ($p<0.05$, **Fig. 2c**). This implied that there were no changes in gut microbiota richness in BPD mice compared with control, however, the genetic diversity showed statistical differences between the two groups.

Beta diversity

In this section, Beta diversity analysis was performed based on un-weighted unifrac distance. PCoA plot showed that there was no obvious separation between two groups on day 7 and day 21, respectively. In contrast, there was significant separation of PC1 between two groups on day 14 (**Fig. 3a-3c**). Analysis of similarities indicated that there was no significant difference in gut microbiota between two groups on

day 7 (R=-0.028, p=0.628), while the difference was statistically significant between two groups on day 14 (R=0.368, p=0.021). On day 21, there was no difference in gut microbiota between two groups (R=0.188, p=0.079, **Table 1**).

Difference analysis

LDA analysis was performed to investigate the biomarkers, and all biomarkers had LDA scores of higher than 4. There were three biomarkers with statistical differences at all biological levels on day 7, all of which were in the control group (p<0.05). On day 14, there were statistical differences in 22 biomarkers at each biological level, among which 16 were enriched in BPD group and 6 were enriched in control group (p<0.05, **Fig. 4a and 4b**). On day 21, there were statistical differences in four biomarkers at all biological levels, all of which were in BPD group (**Fig. 4c**).

On day 14, the relative abundance of intestinal microbiota showed that the proportion of *Firmicutes*, *Bacteroidetes* and *Proteobacteria* in phylum level was higher than 80% (**Fig. 5**). Analysis of variance indicated that the relative richness of *Bacteroidetes* in model group was significantly lower than that of control group (11.6% vs. 54.8%, P_{FDR}<0.01), while the relative richness of *Proteobacteria* in model group was significantly higher than that of control group (29.8% vs. 5.1%, P_{FDR} <0.05). This was consistent with the LDA results. The relative richness of *Cyanobacteria*, *Acidobacteria*, *Chloroflexi*, *Rokubacteria*, *Epsilonbacteraeot*, *Nitrospirae* and *Gemmatimonadetes* in model group was significantly higher than that of control (all P_{FDR}<0.05, **Table 2**).

Functional prediction

A total of 17 KEGG metabolic pathways associated with intestinal microbiota were significantly different between the two groups. Three of them were enriched in the model group and 14 were enriched in the control group. Signal transduction (P_{FDR}=0.037), glycan biosynthesis and metabolism (P_{FDR}=0.032), metabolism of terpenoids and polyketides (P_{FDR}=0.049) are the top three metabolic pathways with statistically significant differences (**Fig. 6**).

Discussion

Patients with intestinal diseases may present symptoms in respiratory system [13], while those with respiratory diseases may accompany by intestinal symptoms [14-16]. Thus, there is a close interaction between respiratory and intestinal diseases [17, 18], which is defined as gut-lung axis. To our best knowledge, the microflora in gut and lung are rather complicated, which play important roles in the pathogenesis of pulmonary and intestinal diseases through the gut-lung axis [19-21]. In previous studies, disturbance of microorganism was closely associated with the progression and prognosis of lung cancer [22, 23]. In addition, coronavirus infection would alternate intestinal permeability and subsequent bacterial translocation through interacting with ACE2 receptor, which then led to deterioration of systemic inflammation [24-26]. Bacterial load of pulmonary microorganism and enrichment of gut microbiota

would contribute to prediction of critically ill patients [27]. As is known to all, microorganisms are crucial for the development of immune system and metabolic balance in hosts [6, 28, 29], However, there is a lack of studies on changes of gut microbiota in neonates with BPD.

Our study was designed to investigate the changes of gut microbiota in BPD model. On day 7, the anti-oxidant system in the pulmonary tissues was immature, and the anti-infection and immune system development were not well developed. There would be blockage in alveolarization in lung-term exposure of high concentration of oxygen [30]. There was massive generation of TNF-α, IL-6, IL-8 and MCP-1 in lung tissues [31] and inflammatory reactions in lung tissues. On day 14, HE staining showed serious injuries in lung tissues than before, and the differences in gut microbiota between two groups were statistically significant (p<0.05). KEGG pathway analysis indicated that there were statistical differences in signal transduction, glycan biosynthesis and metabolism, as well as metabolism of terpenoids and polyketides between two groups. This indicated that BPD rats showed disorder in the gut microbiota associated with regulation of signal transduction and metabolism, which was in line with the different findings in gut microbiota and metabolomics between COPD patients and normal individuals [32]. Nevertheless, the exact mechanisms are still unclear. The hyperoxic environment would induce damages to alveolar septum [33] and there might be persistent inflammatory response in lung tissues [34], which further aggravated lung injury. On day 21, the pulmonary alveoli was no longer available in the pulmonary tissues of BPD model, and the structure in pulmonary tissues was not regular. In control group, the development of pulmonary tissues was normal. There was no statistical difference in gut microbiota between two groups. The composition of gut microbiota was affected by diet, age, development, genetics, and antibiotics [35, 36]. In cases of any changes of diet, there would be rapid spontaneous remodeling for gut microbiota [37]. In our study, mice were randomly divided into different groups after birth, without exposure to any antibiotics. All the animals were free access to a diet and water on day 12-14, followed by termination of lactation. On this basis, we hypothesized that with the increase of age, there would be gradual maturity for gut development on day 21. In a previous study, there would be a gut microbiota balance in mice since termination of lactation [38]. After spontaneous intake of diet, the uptake of fiber showed increase, which promoted the stability of gut microbiota [39] and reduction of differences in gut microbiota between two groups.

Our data showed that there were no statistical differences in gut microbiota on day 7 in model group compared with that of control group. With the deterioration of lung injury, it may trigger imbalance of gut microbiota in model group. We then discussed the potential signaling pathways involved in this process. Microorganisms could regulate immune reactions in intestinal and pulmonary tissues through modulating NLRP3 inflammatory bodies, which then affect intestinal and pulmonary disorders [40]. Short chain fatty acid generated by gut microbiota would regulate immune balance in lung tissues through inhibiting histone deacetylase, which played protective roles in children susceptible to asthma [41]. According to the previous study, probiotics would facilitate to the prevention and treatment of respiratory infection and nervous system diseases through recovering the gut microbiota [42, 43]. Breast milk may involve in prevention of BPD through affecting formation of microorganisms and regulating inflammatory reactions [44]. As previously described, microorganism would affect the progress of certain

diseases rather than improving their severity [45]. Besides, gut microbiota was closely associated with the severity of influenza [46]. Changes of microorganism at early stage would affect the lung response in male mice responding to environmental changes [47]. The effects of fiber uptake on gut microbiota were associated with the sex of mice [48]. Therefore, further studies are required to further investigate whether early-stage interference to gut microbiota would affect the pathogenesis of BPD.

There are some limitations in this study. There was no grouping based on gender in this study. Although the changes of pulmonary tissues in BPD mice and control mice were similar, there might be differences in microorganism formation and microbiota between neonatal mice and neonates [49]. In addition, there were differences in the interference of gut microbiota, which required further validation in clinical practices.

Conclusion

A BPD model was established in neonatal mice through exposure of hyperoxic environment. Then we analyzed the difference of gut microbiota diversity between natural mice and BPD model mice. Our data proved that there was alternation of gut microbiota in BPD mice, which may be related to the signal transduction and metabolic signaling pathways in the early stage. Specifically, the proportion of *Bacteroidetes* and *Proteobacteria* showed significant changes. In the presence of neonatal maturity, the gut microbiota gradually stabilized.

Abbreviations

broncho-pulmonary dysplasia (BPD); line discriminant analysis (LDA); chronic obstructive pulmonary disease (COPD); specific-pathogen free (SPF); analysis of variance (ANOVA).

Declarations

Ethics approval and consent to participate

The study protocols were approved by the Ethical Committee of Affiliated Hospital of Qingdao University (approval No.: QYFYWZLL26150) and there's no written informed consent was obtained from a parent or guardian for participants under 18 years old.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contributions

FTQ and LL wrote the manuscript and performed the experiments; JR performed the experiments; SAH analyzed the data; GRZ drew the pictures; CFJ collected the data; QZH and LDY conceived the study and critically revised the manuscript and designed the experiments.

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Tables

Table 1 Analysis of similarities

Variable	Day 7	Day 14	Day 21
Sample similarity within groups			
All	0.212±0.049	0.298±0.035	0.236±0.043
Control	0.243±0.044	0.348±0.065	0.265±0.034
BPD	0.181±0.031	0.248±0.038	0207±0.031
Sample similarity within groups	0.204±0.042	0.341±0.035	0.246±0.028
R value	-0.028	0.368	0.188
p value	0.628	0.021	0.079

BPD: bronchopulmonary dysplasia

Table 2 ANOVA for species with difference between two groups on day 14

Variable	Control	BPD	Variation	P_{FDR}
Cyanobacteria	6.20E-04±1.08E-04	6.03E-03±9.29E-04	Increase	0.00E+00
Bacteroidetes	5.48E-01±7.26E-02	1.16E-01±4.85E-02	Decrease	3.83E-03
Acidobacteria	1.93E-03±5.58E-04	7.60E-03±1.01E-03	Increase	7.33E-03
Chloroflexi	3.45E-04±7.75E-05	1.21E-03±1.76E-04	Increase	8.61E-03
Rokubacteria	6.74E-05±2.17E-05	4.23E-04±7.94E-05	Increase	8.61E-03
Epsilonbacteraeota	2.16E-03±6.32E-04	1.78E-02±3.81E-03	Increase	1.15E-02
Proteobacteria	5.14E-02±1.02E-02	2.98E-01±6.34E-02	Increase	1.18E-02
Nitrospirae	1.33E-04±5.54E-05	4.60E-04±7.33E-05	Increase	1.71E-02
Gemmatimonadetes	1.35E-04±5.26E-05	6.63E-04±1.43E-04	Increase	1.75E-02

ANOVA: analysis of variance; BPD: bronchopulmonary dysplasia

Figures

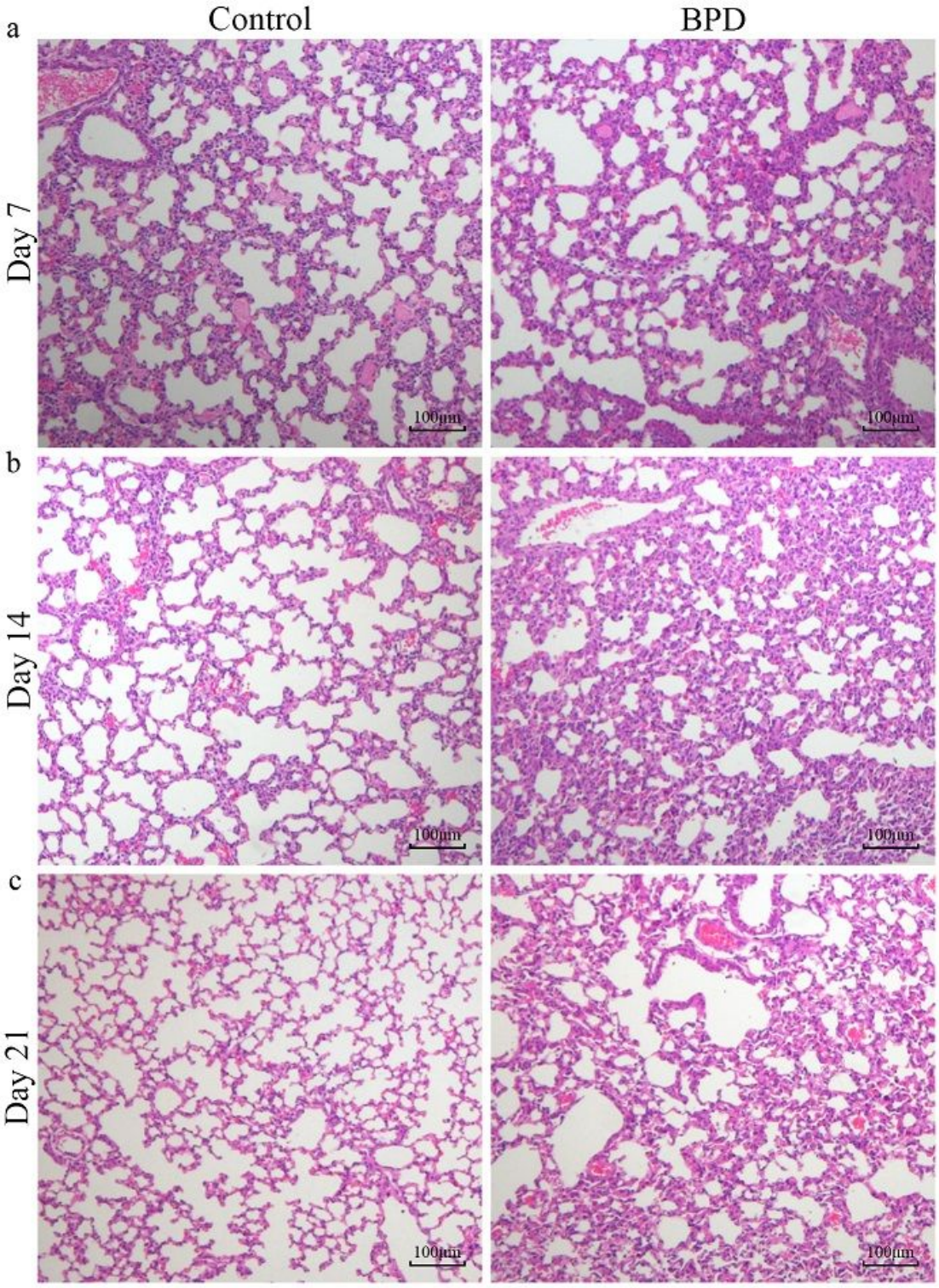

Figure 1

HE staining of lung tissue under a magnification of ×100. a-c: HE staining of lung tissue on day 7, 14 and 21. BPD: bronchopulmonary dysplasia.

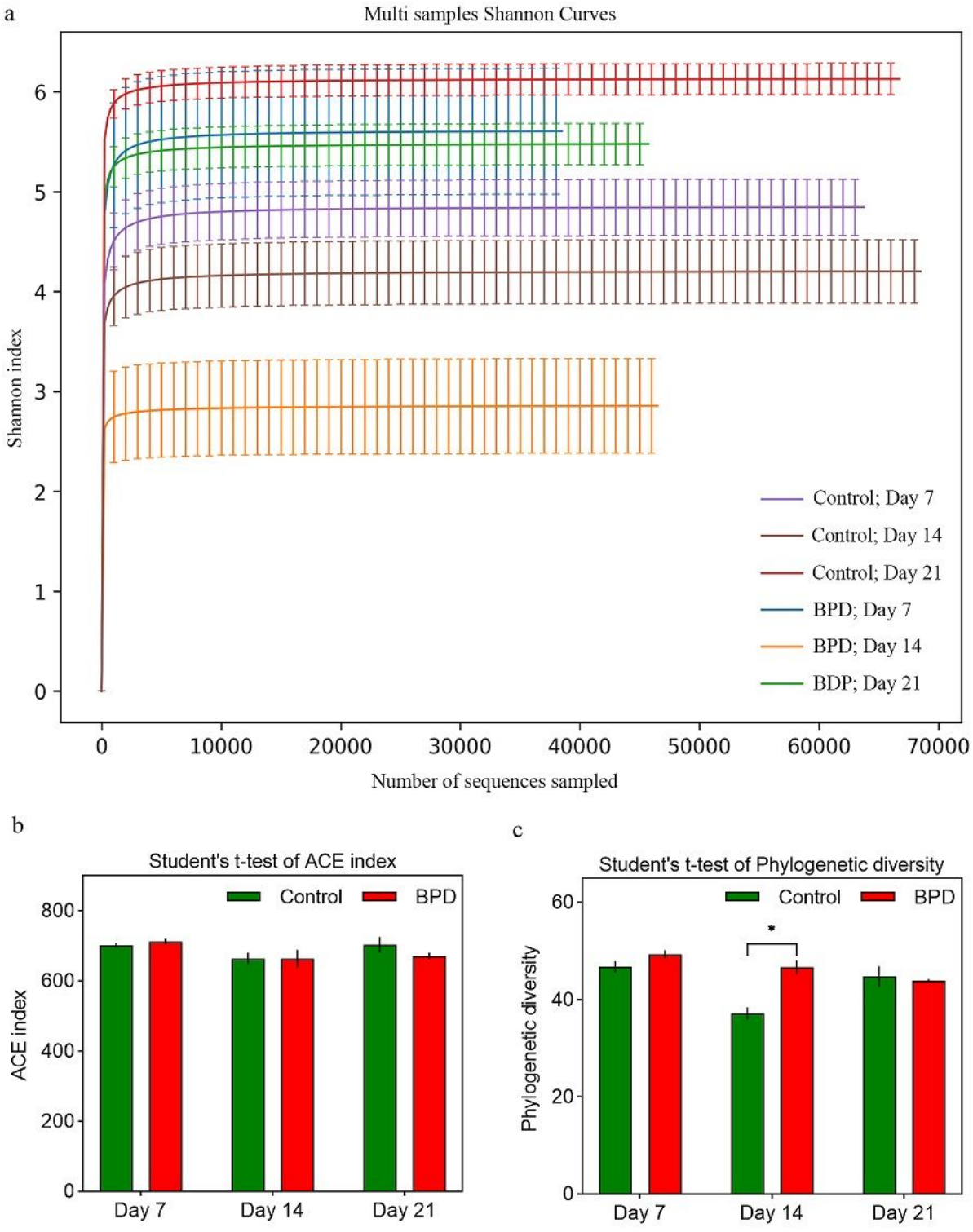

Figure 2

Alpha diversity reflected richness and diversity of bacterial communities. a: Shannon index curve of samples at different time. Curve was flat, and amount of sequencing tended to be saturated, which could reflect the biological diversity of the samples. b, c: Student' s t-test was used to test significance based on ACE index and PD_whole_tree index. *p<0.05; BPD: bronchopulmonary dysplasia.

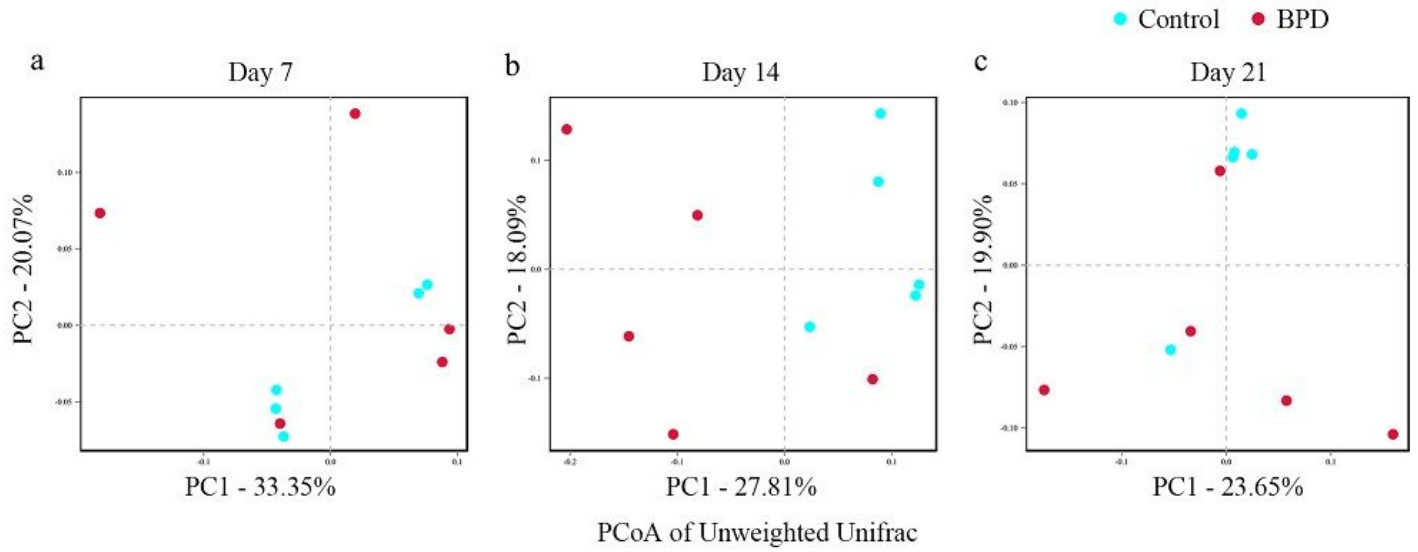

Figure 3

PCoA analysis based on un-weight Unifrac distance between groups. a-c: PCoA analysis on day 7, 14 and 21. PCoA: principal coordinates analysis; BPD: bronchopulmonary dysplasia.

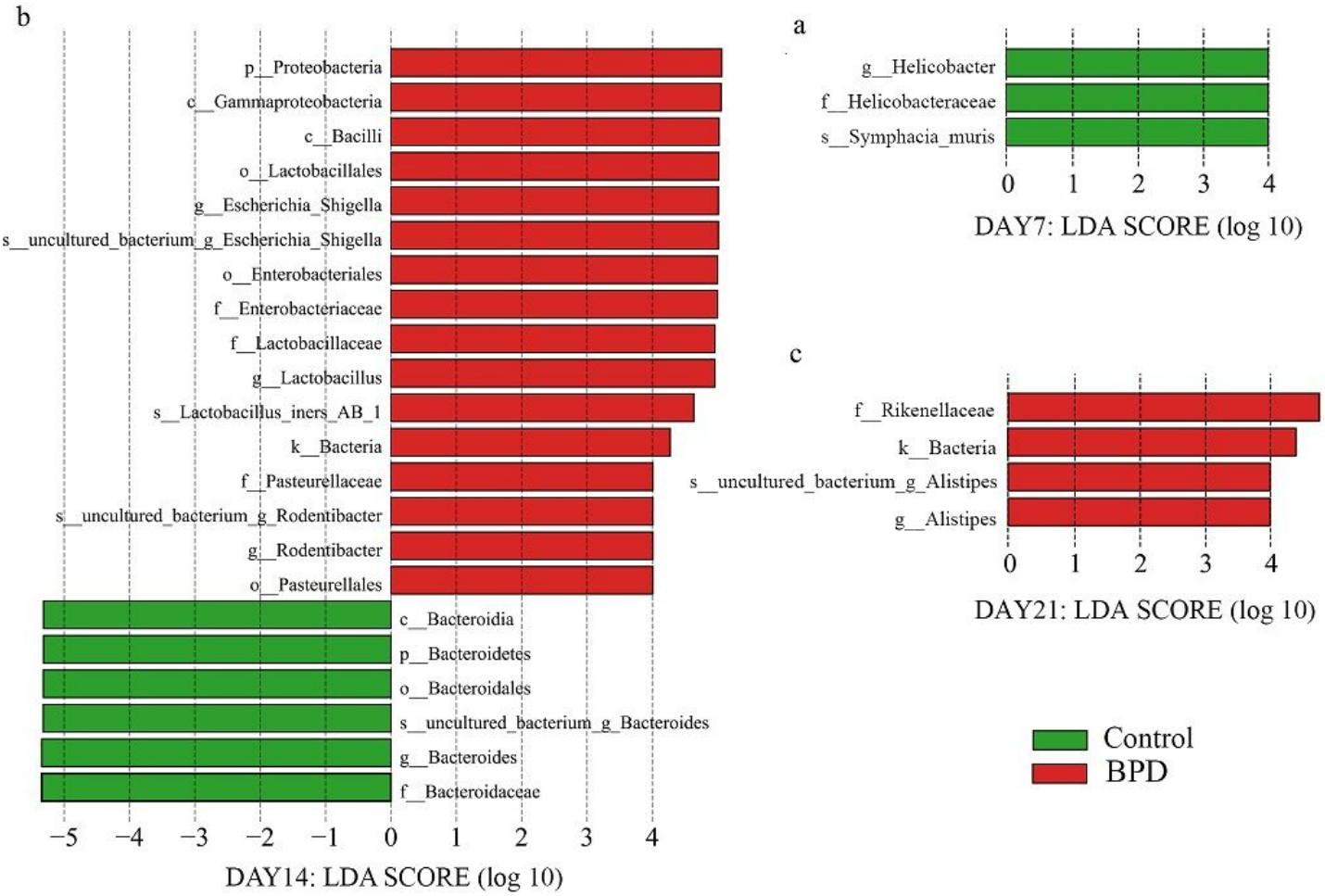

Figure 4

LDA Effect Size showed the different microbiota from the kingdom level to the species level between groups (LDA score > 4 and p < 0.05). a: Three biomarkers were significantly different in the control group on day 7; b:Twenty-two biomarkers were significantly different between groups on day 14; c: Four biomarkers were significantly different in the BPD model group on day 21; LDA: linear discriminant analysis; BPD: bronchopulmonary dysplasia.

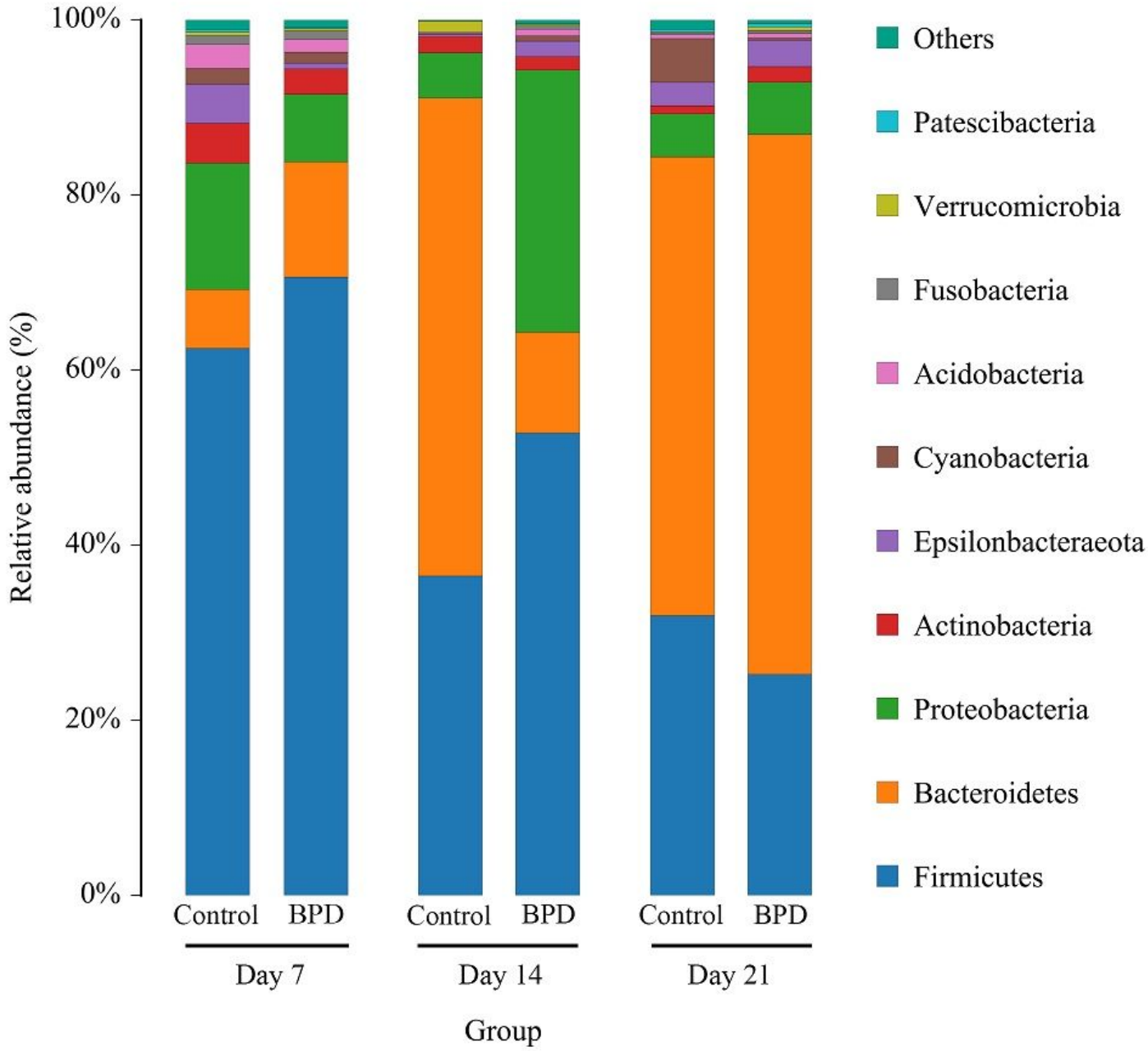

Figure 5

The relative abundance of intestinal microbiota at the phylum level in different periods. Only 10 groups of microbiota with the highest relative content were shown in the picture, and other intestinal microbiota were classified as Others. BPD: bronchopulmonary dysplasia.

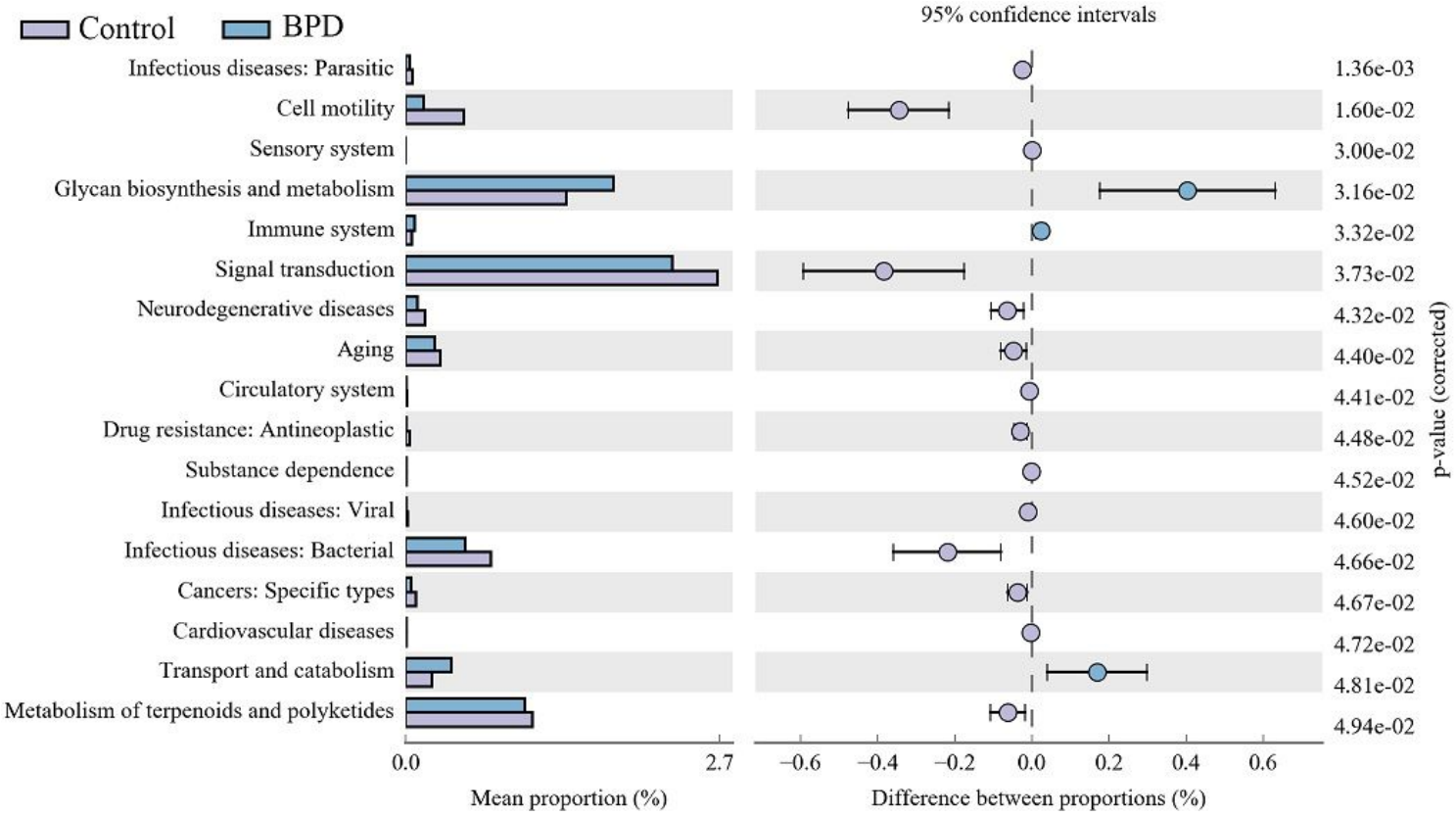

Figure 6

KEGG pathway analysis of the intestinal microbiota. Three pathways were enriched in BPD model group, and fourteen pathways were enriched in control group. BPD: bronchopulmonary dysplasia.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AuthorChecklistFull.pdf