The Nasal Microbiome of Predicting Bronchopulmonary Dysplasia in Preterm Infants

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Abstract

Bronchopulmonary dysplasia (BPD) is chronic lung disease of prematurity and associated with substantial long-term disabilities. To characterize and compare the nasal swabs microbiome of early stage in premature infants and determine whether microbial diversity or composition in the airway associated with BPD disease. We performed a prospective observational cohort design. Preterm neonates less than 32 weeks of gestation were recruited from NICU, Children's Hospital, Zhejiang University School of Medicine from 2019 to 2020. Sterile foam swabs were collected from anterior nares at 1 and 3 weeks of postnatal age. We used PCR amplification and 16S rDNA sequencing. Neonatal demographic data including gestational age, birth weight, medication administration history were recorded. A total of 98 nasal swabs samples were collected from 54 preterm infants, 13 developed BPD infants and 41 control infants were finally involved in the study. Birth weights ranged from 700 to 2,050 g. Gestational age ranged from 25 $^{2/7}$to 31 $^{6/7}$. We found increased in the expression of *Prevotella, Marinomonas, Enterobacteriaceae, Weissella, Selenomonas, Oribacterium, Nubsella* and *Antricoccus* in BPD group at two time points. *Prevotella* was correlated with the severity of BPD (Spearman r=0.361, *P*=0.000). Given possible roles for noninvasive upper airway microbiota in BPD pathobiology, the nasal microbiome in BPD is a compelling area of research to continue to expand.

Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease caused by mechanical ventilation and oxygen therapy and is the most common complication that affects premature infants[1]. The disease leads to longstanding consequences involving adverse effects on pulmonary function and neurodevelopmental outcome. BPD rates are reported 40–55% in surviving extremely low gestational age neonates over the last few decades[2]. Despite the new advances in the field of neonatology, the incidence of BPD has largely been unchanged due to increased survival of extremely premature infants. There is still no ideal management for BPD disease and the only method to alleviate severity and improve the prognosis is early prevention. Therefore, early recognition of susceptible BPD is absolutely essential for timely intervention. Recent studies have demonstrated early airway microbiome may serve a role in modulating the infant's future susceptibility to severe BPD development[3],suggesting another underlying pathway related to abnormal lung development [4–7].

The airway microbiome can be identified early after birth and evolves over time with increasing bacterial loads and diversity[8]. Lal et al. were surprised to find that the airway microbiome of the neonates delivered by vaginal or cesarean section were similar, which indicated that the microbial DNA in the airway may be obtained through the placenta[9]. They described the composition of the airway microbiota by analyzing the airway secretions on the first day of birth: *Firmicutes* and *Proteobacteria* were dominant and *Actinobacteria, Bacteroidetes, Tenericutes, Fusobacterium, Cyanobacteria*, and *Verrucomicrobia* were observed[9]. In BPD patients, as the disease progresses, the microbial community turnover increases, the relative abundance of *Proteobacteria* and *Firmicutes* changes, and *Lactobacillus* decreases[6]. Some

important factors affect the composition and colonization of the pulmonary microbiota including prenatal and postnatal exposure to antibiotics, sepsis, environmental microbiome, method of delivery, feeding and nutrition [10,11]. Study has also been reported the crosstalk between the lung and the intestine, which proposes a concept of the gut-lung axis, and the concomitant intestinal microbiota development also affects lung microbiota, resulting in pulmonary diseases [12].

Pathogen detection requires sampling of lower airway secretions, which remains a challenge in non-expectorating patients. Currently, bronchoalveolar lavage (BAL) is considered as the gold standard; however, it cannot be performed and not suitable for every premature infant, so we explore a non-invasive nasal swab. The nasal cavities represent a highly accessible airway microbial community that recently was confirmed to have a pivotal role in human health and, to date, few studies focused on the microbiome of the nostrils of neonates[13,14]. Nasal cavity communicates with the outside world and is exposed to a variety of exogenous and endogenous microbes. It may play important roles in protecting against nasal colonization as well as invasive disease[15]. Previous studies have shown that there was a large amount of overlap between the nasal microbiota and the respiratory microbiota[15,16], so the nasal microbiota could, to some extent, reflect the characteristics of the respiratory microbiota. Therefore, studying the composition and characteristic of nasal microbiota may open a window for exploring respiratory microbiota in preterm infants. The aim of this study is to: (1) characterize and compare the nasal swabs microbiome in premature infants and BPD infants (2) determine whether BPD disease is correlated with any microbial function in the airway.

Results

Clinical and sampling information for all infants

After applying inclusion and exclusion criteria, a total of 98 nasal swabs samples were collected from 54 preterm infants, 13 developed BPD infants and 41 control infants were finally involved in this study in the NICU at Children's Hospital, Zhejiang University School of Medicine, from 2019 to 2020 (Table 1). Six premature infants were transferred to the other units before collecting the second time specimens, so we didn't get second nasal swabs. Gestational age ranged from 25 $^{2/7}$to 31 $^{6/7}$. Birth weights ranged from 700 to 2,050 g. There are no significant differences between two groups in terms of gender, delivery mode, feeding, PDA, NEC, sepsis and antibiotics exposure ($P > 0.05$). Although the BPD group has a lower gestational age, it is still appropriate for the control to compare the nasal microbiome.

Microbial community characterization.

Eight of the samples had inadequate biomass for DNA sequencing and was excluded. A total of 5,581,298 high quality reads were obtained from the 94 samples, with a mean read count per sample of 59,376 (range 14,783 to 76,132). As shown in Figure 1A, *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* were dominant phylums and shown in Figure 1B, *Muribaculaceae, Escherichina, Staphylococcus* and *Corynebacterium* were dominant genus in all group. There was no significant

difference between BPD group and control group both at first week and third week. PCoA was performed to study the similarities or differences in sample community composition. NMDS analysis was performed using the weighted UniFrac distance algorithm, and two coordinate axes that could reflect the differences between samples to the greatest extent were selected for graphical display by dimension reduction of the multidimensional data. Based on the beta diversity, including PCoA and NMDS, no significant differences were detected in comparisons ($P > 0.05$, Figure 2A, 2B). To evaluate which bacterial genera were involved in these observed temporal differences, we examined the relative abundances of the prevalent taxa. Some of the abundant genera changed significantly in relative abundance at first and third week (Kruskal–Wallis test, all P-values < 0.05) (Supplementary Figure 1A, 1B). We found increased in the expression of *Prevotella, Marinomonas, Enterobacteriaceae, Weissella, Selenomonas, Oribacterium, Nubsella* and *Antricoccus* in BPD group at both time points. *Prevotella* (phylum *Bacteroidetes*) was shown in Figure 3. We also found that *Prevotella* was correlated with the severity of BPD (Spearman r=0.361, P=0.000).

Reduced Coumarins And Mannosylglycerate Biosynthesis Associated With Bpd

To infer metabolic pathways associated with the nasal taxa identified as differentially abundant based on BPD, we used PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) and STAMP (STatistical Analysis of Metagenomic Profiles)[17] to map microbial genes to metabolic databases to infer microbial functions differentially expressed by BPD. Coumarins and mannosylglycerate biosynthesis were the metabolic pathway that was differentially abundant (Kruskal-Wallis, all P <0.05) across the study groups. Compared to control group, coumarins and mannosylglycerate biosynthesis were reduced in BPD (Figure 4A, 4B).

Discussion

There is no study that have analyzed the preterm infant's nasal microbiome in BPD populations that provide mechanistic explanations for microbiome change during BPD, and its impact on host-microbiome interaction. The present study of the nasal microbiome in BPD, we found no difference in nasal microbial composition between preterm infants with BPD and controls. Our study identified *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria* were dominant phylums and *Muribaculaceae, Escherichina, Staphylococcus, Corynebacterium* were dominant genus in BPD infants relative to controls. We found increased in the expression of *Prevotella* in BPD group at both time points. *Prevotella* was correlated with the severity of BPD. Metagenomic prediction identified reduced *coumarins* and *mannosylglycerate* biosynthesis as the metabolic pathway differentially associated with BPD.

In comparison to the gut microbiome, nasal microbiome has remained under studied. The nasal cavity is a part of the mucosal system of the upper respiratory tract, and its microbial composition can reflect the entire respiratory tract composition of microorganisms [15,18]. Several studies revealed how the microbiota developed in regions of the respiratory tract in newborns and during early life[14,19]. The nasal microbiota is of particular concern as the nostrils may harbor pathogens, which can cause severe respiratory

diseases[20]. Nasal microbiota compositions characterized by *Moraxella*, *Streptococcus*, or *Haemophilus* have been reported to be associated with upper respiratory infection[21]. Our study found that the expression of *Prevotella* was higher in the BPD group and it was correlated with the severity of BPD disease. Interestingly, bacterial *Prevotella* have been found to be prevalent commensal colonizers at mucosal sites; being the predominant genus in the respiratory system[22,23]. In light of the abundant *Prevotella* colonization and low pathogenicity it is likely that humans have co-evolved with *Prevotella.* However, emerging studies have linked increased *Prevotella* abundance and specific strains to inflammatory disorders, suggesting that at least some strains exhibit pathobiontic properties. Increased *Prevotella* abundance is associated with augmented Th17 mediated mucosal inflammation[24]. *Prevotella*-high profile being associated with enhanced “subclinical” lung inflammation, that is notable for enhanced expression of inflammatory cytokines and elevated Th-17 lymphocytes[25]. Our results support the hypothesis of persistent lung inflammation after mechanical ventilation and/or lung infection, especially as *Prevotella* is a potential pathogen in respiratory disease in early period. Contrast to previous data from young adults born extremely preterm exhibit significant dysbiosis, which is characterized by a significant reduction in the relative abundance of genus *Prevotella*[26]. However, to clearly examine this, “*Prevotella*” animal model should be assessed in future studies.

Coumarins are found in many bacteria, with promising pharmacological activities, including antioxidant, antimicrobial, and anti-inflammatory efficacies. The beneficial effects of coumarins include antimicrobial [2728]. The antioxidant and anti-inflammatory activities of coumarins have been well-acknowledged *in vitro* and *in vivo* studies[29,30]. In our study, we found that *coumarins* biosynthesis decreased in BPD infants. It may suggest BPD infants have lower anti-inflammatory activities in early period. Moreover, coumarins can effectively reduce tissue edema-associated inflammation through suppressing both lipoxygenase and cyclooxygenase enzymatic activities and prostaglandin synthesis and release[31]. Another metabolic pathway that is reduced is the mannosylglycerate biosynthesis pathway. The synthesis process is the conversion of GDP mannose and d-glycerate or d-3-phosphoglycerate to mannosylglycerate. The compatible solute mannosylglycerate has properties in terms of protein stabilization and protection under heat and freeze-drying stresses as well as against protein aggregation. Due to these characteristics, it possesses large potential for clinical applications[32]. The synthesis of mannosylglycerate in BPD group decreased, but the mechanism is not clear.

On the one hand, a limitation of this study is the small number of infants. Despite the high number of samples, a larger study population is needed to detect additional differences between subjects. On the other hand, although BPD is a disease of lower airways, our data are based on nasal airway samples. However, lower airway sampling is both ethically and technically challenging in prematurity. Importantly, analysis of microbiota during BPD in a higher number of infants is needed to understand the role of *Prevotella*. Also, the causal and mechanistic pathways between *Prevotella* infections and the microbiota coumarins and mannosylglycerate biosynthesis metabolic pathways remain unclear. It needs to be assessed in translational approaches or using animal models. Given possible roles for noninvasive upper

airway microbiota in BPD pathobiology, monitoring and investigation of BPD infants, the nasal microbiome in BPD is a compelling area of research to continue to expand.

Materials And Methods

Recruited infants and sample collection

The study was performed as a prospective observational cohort design, and was approved by the ethics committee of the Children's Hospital, Zhejiang University School of Medicine(2018-IRB-090-A2). Informed consent was obtained from at least one guardian of each patient and all procedures were conducted according to the guidelines. Preterm neonates less than 32 weeks of gestation were recruited from neonatal intensive care unit (NICU), Children's Hospital, Zhejiang University School of Medicine from 2019 to 2020. Exclusion criteria were major congenital anomalies of the lung or airway, known infection, or pneumonia. Sterile foam swabs were collected from anterior nares. Swabs were collected at 1 and 3 weeks of postnatal age. The first swab was collected by 5-7 days after birth following written informed consent from parents. The second swab was collected by 15-21 days of age. Swab tips were snapped off into sterile 1.5-ml polyethylene tubes, transferred immediately to −80°C freezer for storage. All infants were followed up until 36-week postmenstrual age, when the physiological definition of BPD. We used National Institute of Child Health and Human Development (NICHD) 2019 revision to define severity of BPD [1]. All infants were stratified into the following two groups: developed BPD (BPD group) or did not develop BPD (control group). Neonatal demographic data including gestational age, birth weight, gender, delivery mode, medication administration history, infants' diet type (human milk vs. formula) and significant events during NICU course, were extracted from the electronic medical records.

DNA extractions

DNA was extracted from swabs using the E.Z.N.A. ®Stool DNA Kit (D4015, Omega, Inc., USA) according to manufacturer's instructions. The total DNA was eluted in 50 μL of Elution buffer and stored at -80 °C until measurement.

PCR amplification and 16S rDNA sequencing

The V3-V4 region of the bacterial small-subunit (16S) rRNA gene was amplified with primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3')[33]. PCR amplification was performed in a total volume of 25 μL reaction mixture containing 25 ng of template DNA, 12.5 μL PCR Premix, 2.5 μL of each primer. The PCR conditions is initial denaturation at 98 ℃ for 30 seconds; 32cycles of denaturation at 98 ℃ for 10 seconds, annealing at 54 ℃ for 30 seconds, and extension at 72 ℃ for 45 seconds; and then final extension at 72 ℃ for 10 minutes. The PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing and the size and quantity of the amplicon library were assessed on Agilent 2100 Bioanalyzer (Agilent, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. Samples were sequenced on an Illumina

NovaSeq platform according to the manufacturer's recommendations (LC-Bio Technology Co., Ltd, Hang Zhou, China).

Data analysis

Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH Quality filtering on the raw reads were performed under specific filtering conditions to obtain the high-quality clean tags according to the fqtrim(v0.94). Chimeric sequences were filtered using Vsearch software (v2.3.4). After dereplication using DADA2, we obtained feature table and feature sequence. Principal coordinate analysis (PCoA) analysis was displayed by QIIME2 and ggplot2 package. Nonmetric multidimensional scaling (NMDS) analysis was performed with the vegan package and displayed with the ggplot2 package in R software. The figures were drawn by R (v3.5.2).

Declarations

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Contributors Yanping Xu, Yeqing Huang, Zhen Shen and Liping Shi had full access to all of the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. Study concept and design: Yanping Xu and Liping Shi. Acquisition, analysis or data interpretation: Yanping Xu and Yeqing Huang. Drafting of the manuscript: Yanping Xu. Critical revision of the manuscript: all authors. Statistical Analysis: Yanping Xu and Zhen Shen. Obtained funding: Yanping Xu. Study supervision: Liping Shi. All authors reviewed the manuscript.

Competing interests The author(s) declare no competing interests.

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Data availability statement Data are available in a public, open access repository. Sequence data have been deposited to the NCBI Sequence Read Archive and are available under accession number PRJNA782204.

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Tables

Table 1. Demographics of infants enrolled in the study

	BPD (n=13)	Control (n=41)	*P*
Birth weight in g, median (range)	1160 (700-1950)	1400 (840-2050)	0.053
Gestational age in weeks, median (range)	29 $^{1/7}$ (25 $^{2/7}$-31 $^{5/7}$)	30 $^{4/7}$ (25 $^{2/7}$-31 $^{6/7}$)	0.007*
Male gender, *n* (%)	6 (46.2)	18 (43.9)	0.887
Cesarean section, n (%)	9 (69.2)	28 (68.3)	0.949
Breast milk, n (%)	11 (84.6)	35 (85.4)	0.821
Rupture of membranes>18h, n (%)	2 (15.4)	7 (17.1)	0.887
NEC	0 (0.0)	4 (9.8)	0.242
PDA	9 (69.2)	19 (46.3)	0.150
Sepsis	1 (7.7)	3 (7.3)	0.964
Perinatal maternal antibiotic exposure, n (%)	11 (84.6)	25 (61.0)	0.115
Postnatal antibiotic exposure, -1w, median (range)	5 (0-8)	5 (0-7)	0.789
Postnatal antibiotic exposure, -3w, median (range)	5 (0-13)	6 (0-16)	0.604
Severity of BPD			
Mild	5	0	-
Moderate	7	0	-
Severe	1	0	-

BPD, bronchopulmonary dysplasia; NEC, Necrotizing enterocolitis; PDA, patent ductus arteriosus * Results significant with $P<0.05$

Figures

Figure 1A

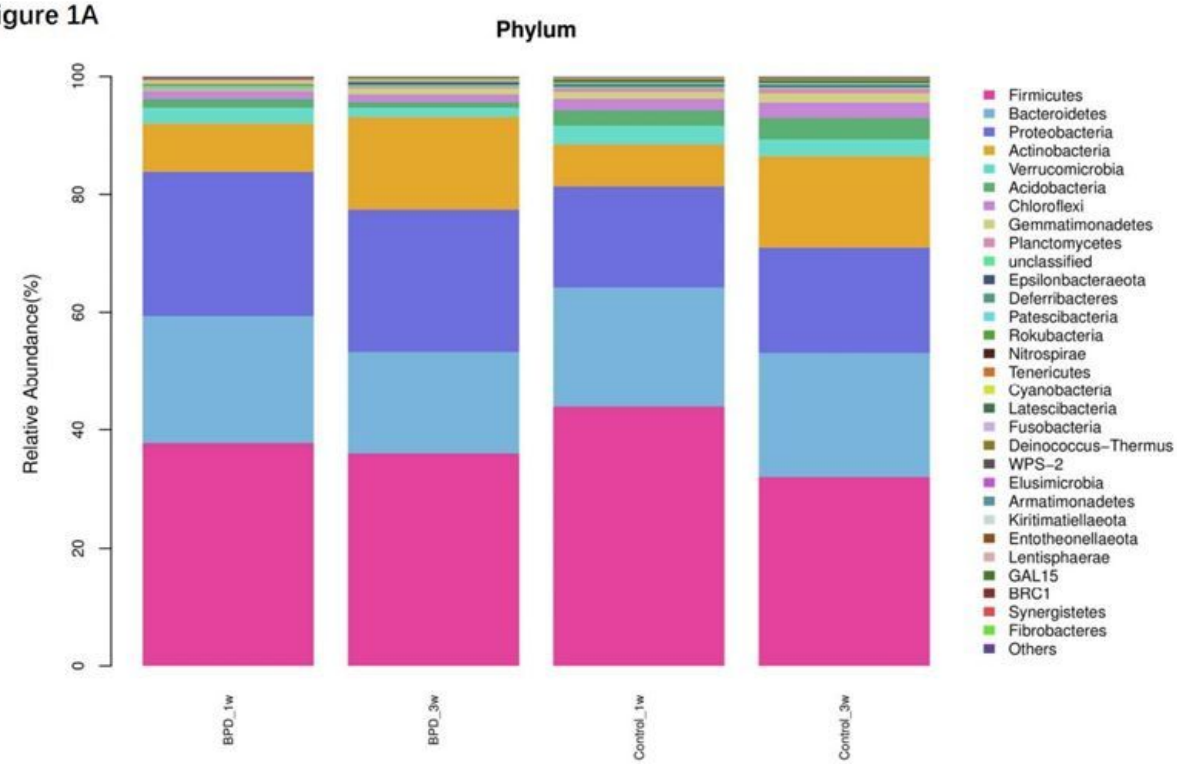

Figure 1B

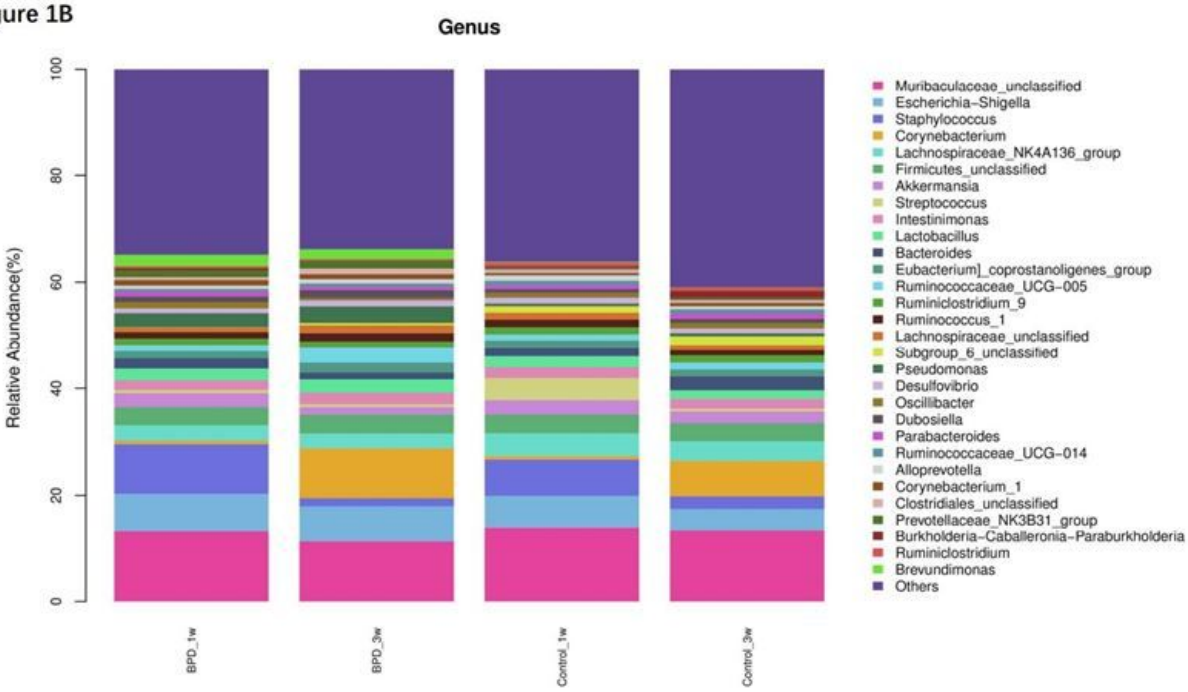

Figure 1

Gut microbiota composition and abundance in nasal swabs of preterm infants analyzed by 16S rDNA sequencing. (A) Composition of microbiota at the phylum level; (B) Composition of microbiota at the genus level.

Figure 2A

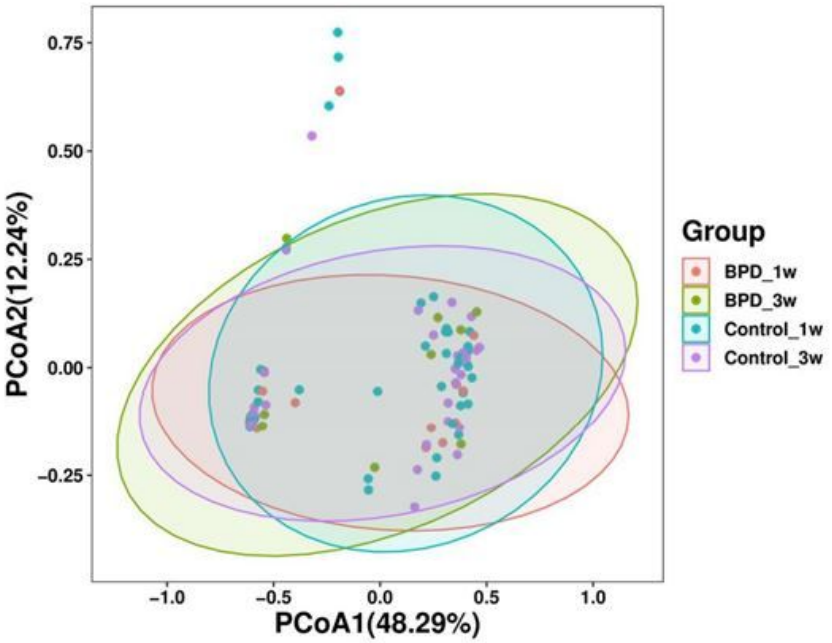

Figure 2B

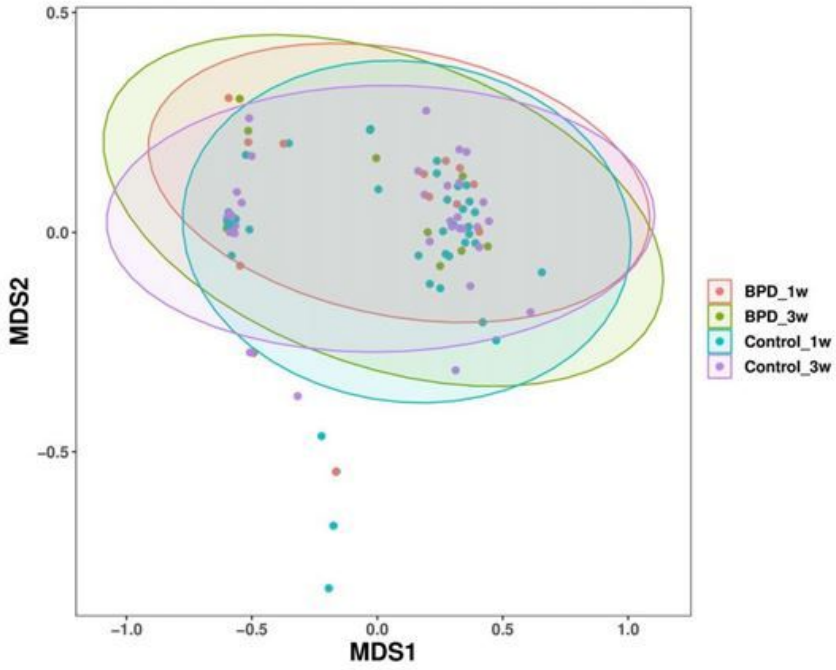

Figure 2

Beta diversity, each point in the figure represents a sample, and the points of the same color come from the same group. The closer the distance between the two points, the smaller the difference in community composition between the two points. The figure (A) shows the PCoA analysis results based on Weighted UniFrac, p values on the graph are derived from ANOSIM's calculations, p>0.05. (B) Stress is an indicator reflecting the advantages and disadvantages of NMDS analysis results, Stress>0.05.

Figure 3

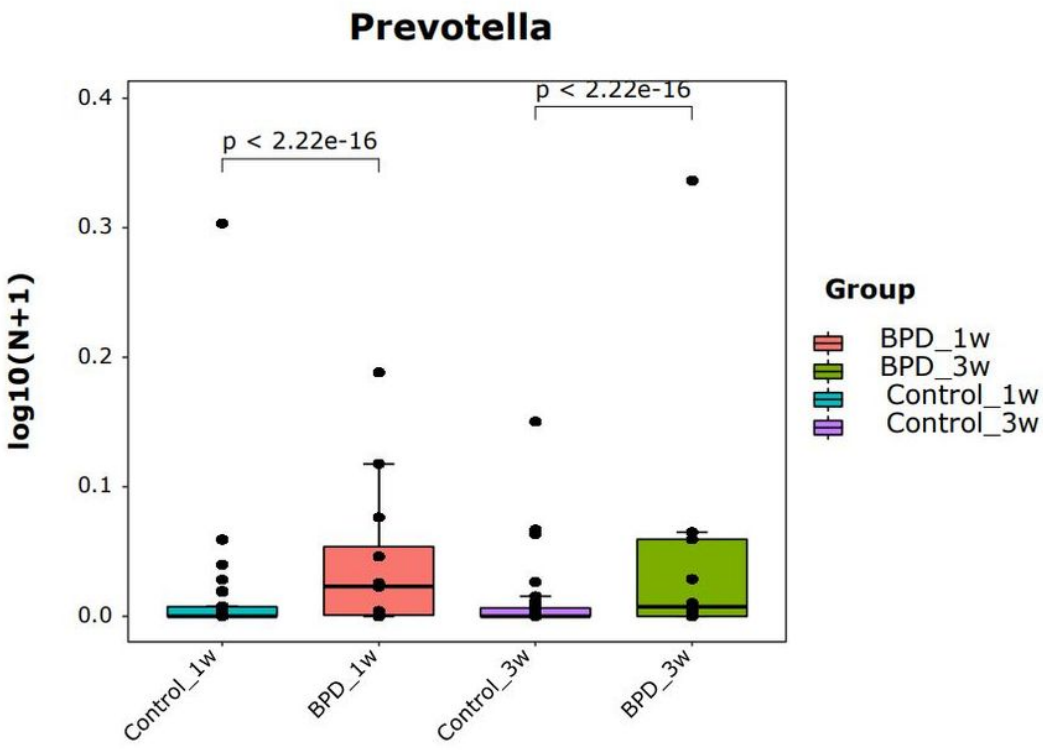

Figure 3

Increased in the expression of *Prevotella* in BPD group at both time points ($p<0.05$).

Figure 4A

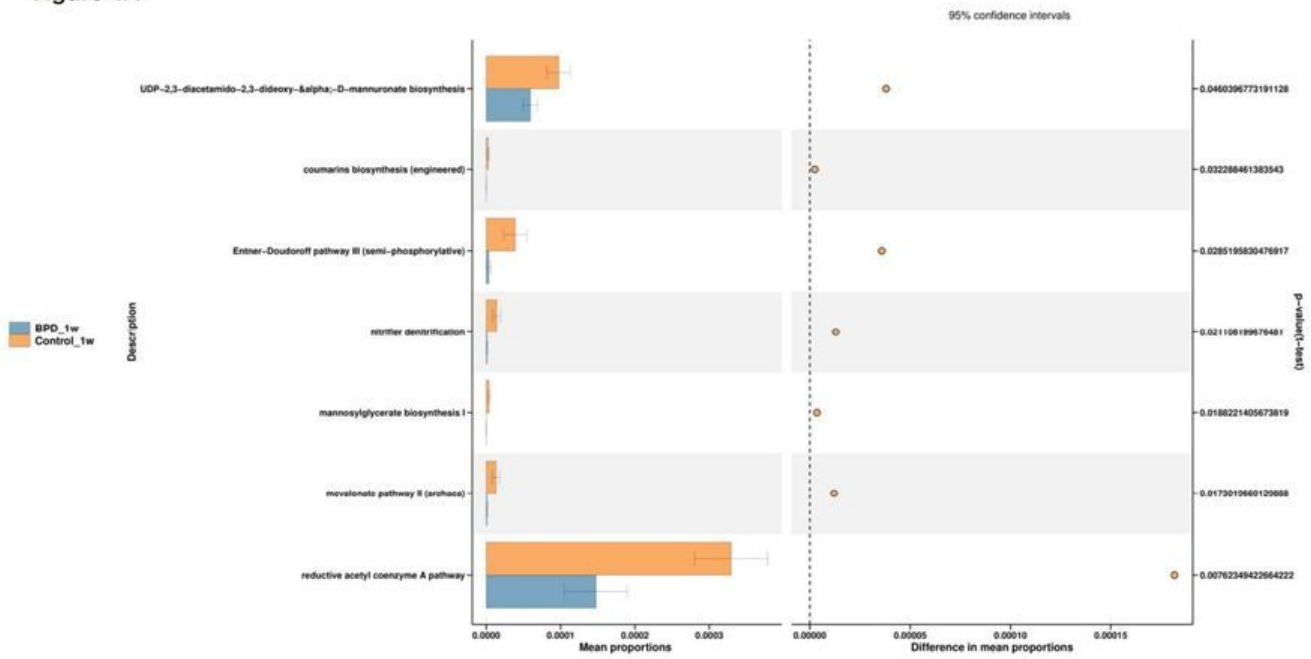

Figure 4B

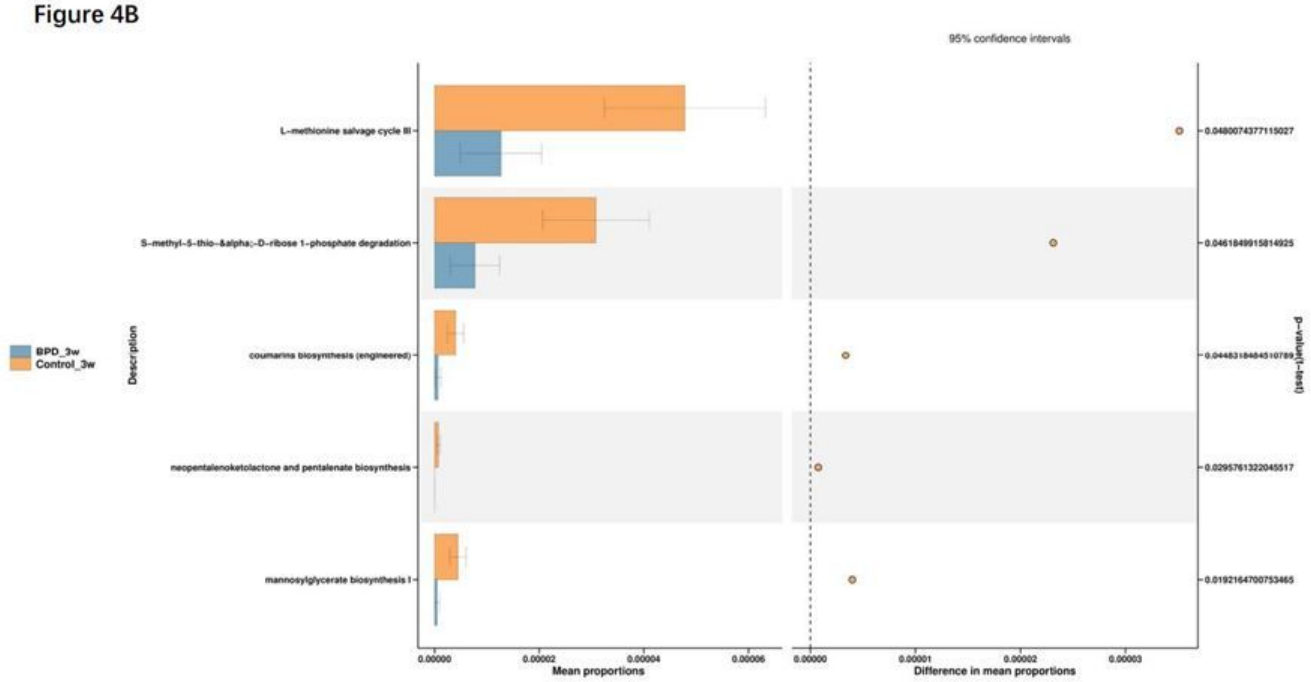

Figure 4

Reduced coumarins and mannosylglycerate biosynthesis associated with BPD. In the figure above, the COG database function annotation results of different grouping samples are compared, and the functions with significant differences between groups are screened out, where blue represents BPD group and orange represents control group. The horizontal bar chart on the left represents the percentages of all metabolic pathways enriched in the abundance of this metabolic pathway in the two groups of samples, and corrected p values are on the right.

Supplementary Files

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

- SupplementalFigurelegend.docx
- SupplementaryFigure.pdf