Clinical Science (2020) **134** 289–302
https://doi.org/10.1042/CS20191253

PORTLAND PRESS

Research Article

Short-chain fatty acids accompanying changes in the gut microbiome contribute to the development of hypertension in patients with preeclampsia

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Preeclampsia (PE) is regarded as a pregnancy-associated hypertension disorder that is related to excessive inflammatory responses. Although the gut microbiota (GM) and short-chain fatty acids (SCFAs) have been related to hypertension, their effects on PE remain unknown. We determined the GM abundance and faecal SCFA levels by 16S ribosomal RNA (rRNA) sequencing and gas chromatography, respectively, using faecal samples from 27 patients with severe PE and 36 healthy, pregnant control subjects. We found that patients with PE had significantly decreased GM diversity and altered GM abundance. At the phylum level, patients with PE exhibited decreased abundance of Firmicutes albeit increased abundance of Proteobacteria; at the genus level, patients with PE had lower abundance of *Blautia*, *Eubacterium_rectale*, *Eubacterium_hallii*, *Streptococcus*, *Bifidobacterium*, *Collinsella*, *Alistipes*, and *Subdoligranulum*, albeit higher abundance of *Enterobacter* and *Escherichia_Shigella*. The faecal levels of butyric and valeric acids were significantly decreased in patients with PE and significantly correlated with the above-mentioned differential GM abundance. We predicted significantly increased abundance of the lipopolysaccharide (LPS)-synthesis pathway and significantly decreased abundance of the G protein-coupled receptor (GPCR) pathway in patients with PE, based on phylogenetic reconstruction of unobserved states (PICRUSt). Finally, we evaluated the effects of oral butyrate on LPS-induced hypertension in pregnant rats. We found that butyrate significantly reduced the blood pressure (BP) in these rats. In summary, we provide the first evidence linking GM dysbiosis and reduced faecal SCFA to PE and demonstrate that butyrate can directly regulate BP *in vivo*, suggesting its potential as a therapeutic agent for PE.

Introduction

Preeclampsia (PE) is diagnosed as new-onset hypertension with a systolic blood pressure (BP) over 140 mm Hg and/or a diastolic BP over 90 mm Hg after 20 weeks of gestation, combined with proteinuria or new onset of thrombocytopaenia, renal insufficiency, impaired liver function, pulmonary oedema, or cerebral or visual symptoms [1]. PE affects 3–5% of all pregnant women and remains a leading cause of maternal and perinatal morbidity and mortality worldwide [2,3]. The pathogenesis of PE is different from that of primary hypertension [4]. Placental insufficiency is generally thought to play a central role in the pathogenesis of PE, although it cannot explain all occurrences of the disease [5].

Recently, it was reported that components of the gut microbiota (GM) are related to hypertension [6]. A shift in the gut microbial genera is associated with the pathophysiological and immune statuses of high

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Received: 09 December 2019
Revised: 17 January 2020
Accepted: 21 January 2020

Accepted Manuscript Online: 21 January 2020
Version of Record published: 30 January 2020

Clinical Science (2020) **134** 289–302
https://doi.org/10.1042/CS20191253

BP [7]. Treating rats exhibiting Ang-II-induced hypertension with minocycline can increase the microbial diversity, restore the Bacteroidetes population, and attenuate hypertension [8]. Faecal transplantation from hypertensive individuals to germ-free mice also resulted in elevated BP [9].

Short-chain fatty acids (SCFAs) are derived from the fermentation or degradation of carbohydrates and proteins by the GM, which are crucial for intestinal health [10,11]. The most abundant SCFA in the gut constitute acetic acid, propionic acid, and butyric acid, whereas isobutyric acid, valeric acid, and isovaleric acid are present in lower amounts [12]. SCFAs can modulate vasodilatation in both rodents and humans [13,14] and can regulate the BP, mainly through G protein-coupled receptor (GPCR) [15,16].

Considering that the new onset of hypertension after 20 weeks of gestation constitutes the most important feature of PE, it is rational to question whether GM and SCFA also participate in PE. However, to date, the roles of the GM and SCFA in PE pathogenesis have not been well-studied. In the present study, we tested the hypothesis that dysbiosis of the GM and associated SCFA contribute to the development of PE. We compared the faecal GM abundance and SCFA levels between women with severe PE and healthy pregnant women in the third trimester, calculated correlations between the GM abundance and SCFA levels, and observed the effect of butyrate treatment in a lipopolysaccharide (LPS)-induced rat model of pregnant hypertension (PH). Our study revealed several new members of the GM and SCFA that are related to PE, suggesting novel candidates for treating hypertension during pregnancy.

Materials and methods

Study population and sample collection

The study was approved by the Ethics Committee of Ren Ji Hospital (School of Medicine, Shanghai Jiao Tong University). All experiments were conducted in compliance with the Helsinki Declaration and all subjects provided written informed consent. For the present study, patients with PE and healthy pregnant control (HPC) subjects were recruited from the out- and in-patients at the Department of Obstetrics in Ren Ji Hospital (School of Medicine, Shanghai Jiao Tong University). The maternal age, gestational weeks when the faecal samples were collected, gravidity, parity, and body-mass index (BMI) before pregnancy were matched between the two groups. All patients with PE met the criteria for diagnosis with severe PE with at least one of the following symptoms: an elevated systolic BP of $\geq$160 mm Hg or a diastolic BP of $\geq$110 mm Hg, proteinuria of $\geq$3 g/24 h (without urinary tract infection), abnormal liver or kidney function, or thrombocytopaenia. In our study, patients with chronic hypertension or gestational diabetes were not excluded from the PE group, whereas women with any pregnancy complications by the time of faecal samples collection were excluded from the HPC group. Other exclusion criteria for both groups included multiple pregnancies or intake of antibiotics within 1 month before sample collection. The subjects were recommended to ingest a normal diet at least 3 days before their faecal samples were collected. Two individual fresh stool samples were aseptically collected for each patient and stored within 4 h at –80°C (Thermo Scientific, U.S.A.) until they were analysed using 16S ribosomal RNA (rRNA) sequencing or gas chromatography.

DNA preparation and sequencing

Patient faecal samples were collected, and the microbial deoxyribonucleic acid (DNA) was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, U.S.A.) according to manufacturer recommended protocols. The DNA quality was checked via 1% agarose gel electrophoresis. The V3–V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with the primers 338F(5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) in a polymerase chain reaction (PCR) (GeneAmp 9700, Applied Biosystems, U.S.A.). The PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.A.) and quantified using QuantiFluor™-ST (Promega, U.S.A.). According to the standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China), the purified amplicons were pooled in equimolar amounts and subjects to paired-end sequencing (2 × 300) using the Illumina MiSeq platform (Illumina, San Diego, U.S.A.). Raw fastq files were quality-filtered using Trimmomatic and merged with FLASH. Operational taxonomic unit (OTU) was clustered with a 97% similarity cutoff using UPARSE (version 7.1 http://drive5.com/uparse/) using a novel 'greedy' algorithm that performs chimera filtering and OTU clustering simultaneously. The RDP Classifier algorithm (http://rdp.cme.msu.edu/) was used to analyse the 16S rRNA gene sequences. By comparison with the Silva (SSU123) 16S rRNA database, 70% confidence thresholds were determined.

Metabolomic analysis of SCFA in faeces

Faecal samples were diluted in acidified water spiked with stable, isotope-labelled SCFA standards and then extracted with diethyl ether. The ether layer was immediately analysed by GC-2010 plus gas-chromatography with a hydrogen ion detector (Shimadzu, Japan), using a DB-FFAP column (Agilent, U.S.A.). Quantitation was performed after calibration with internal standards and generating standard curves, which were prepared using LabSolutions software (Shimadzu, Japan). All levels are expressed in mM.

Animal experiments

All animal experiments were conducted following the guidelines of the Institutional Animal Care and Use Committee of Ren Ji Hospital (School of Medicine, Shanghai Jiao Tong University), and all animal protocols were approved by the same committee. Sprague–Dawley (SD) rats (female, 7–8 weeks old, weighing 190–200 g) were purchased from the Shanghai Laboratory Animal Research Center (Shanghai, China). Rats were raised in a light-, humidity-, and temperature-controlled room with free access to food and water under specific pathogen-free (SPF) conditions at the Department of Animal Facility in Ren Ji Hospital (School of Medicine, Shanghai Jiao Tong University); all animal work was performed in this animal laboratory. After acclimating to that environment for 1 week, female rats were randomly housed overnight with fertile male rats at a 2:1 ratio, beginning at 5 p.m. Gestational day (GD) 0 was confirmed by the presence of vaginal spermatozoa. We were unable to replicate the ultra-low-dose LPS-induced PE rat model of Xue et al. [17]. Instead, we modified their protocol by injecting 0.5 μg/kg LPS (*Escherichia coli* serotype 0111:B4, Sigma–Aldrich) or phosphate-buffered saline (PBS) vehicle control on GDs 5 and 10, respectively, through the tail veins of the rats. Following the LPS or PBS injections, the pregnant rats were separated into different cages, which were still maintained under the SPF conditions at the Department of Animal Facility in Ren Ji Hospital (School of Medicine, Shanghai Jiao Tong University).

The BPs of the female rats were monitored daily between 8:30 and 11:30 a.m. by tail-cuff plethysmography (BP-2010, Softron, Japan) from 3 days before mating (calculated as the baseline BP) and during the whole pregnancy. When measuring BPs, we placed the rats in a 38°C heater to preheat them and keep them calm. The average values of three measurements were taken and recorded as the BP values. In addition, 24-h protein-urine samples were collected from female rats on GDs 0 and 19. On the day of urine collection, the rats were fasted but were free to drink water in the standard rat metabolic cages. The concentration of protein urine was determined using the BCA reagent (Sangon Biotech, China). On GD 20, the pregnant rats were killed by cervical dislocation under anaesthesia with pentobarbital (0.3%, 1 ml.100 g^{-1}, Merck, Germany) and the foetuses were collected and weighed. Butyrate solution (1 $mM.kg^{-1}.day^{-1}$, B5887-5G, Sigma–Aldrich) or PBS was administered by gavage once daily from GDs 4 to 19.

Statistical analysis

All data analysis was performed using the R software package (V.3.2.1) and the free online Majorbio I-Sanger Cloud Platform (www.i-sanger.com). Only the 20 most abundant bacteria at the genus level were analysed. The data for women in the PE and HPC groups are presented as the means ± standard deviation, median (quartile spacing), or number (percentage). Spearman's correlation was performed for correlation-heatmap. The Mann–Whitney U test was used to study the continuous variable and non-normally distributed data were tested by Wilcoxon's rank-sum test. For the categorical variable data, we performed chi-squared test analysis. For the animal experiments, the difference in BP between the two groups was evaluated by using a repeated-measures analysis.

Results

Study subjects

We recruited 63 pregnant women in the third trimester, including 27 patients with PE and 36 women with uncomplicated pregnancies among out-patients and in-patients in the Department of Obstetrics of Ren Ji Hospital (School of Medicine, Shanghai Jiao Tong University) from August 2017 to October 2018. No significant differences were observed in terms of the maternal age, gestational weeks at the time of faecal sample collection, gravidity, parity, or BMI prior to pregnancy between the two groups. In the PE group, 48% of patients exhibited at least one comorbidity of chronic hypertension or gestational diabetes whereas none were reported in the control group. The PE group also recorded earlier gestational weeks on delivery, lighter birth weight, and more adverse perinatal outcomes such as induced premature birth < 35 weeks, foetal growth restriction, and perinatal death than the HPC group (Table 1).

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Table 1 Baseline characteristics of HPC vs PE women

	HPC (*n*=36)	PE (*n*=27)	*P*-value
Maternal age (*years*)	30.4 ± 4.1	31.7 ± 4.9	0.265
Gravidity (*N*)	2.0 (1.0, 3.0)	2.0 (1.0, 3.3)	0.426
Parity (*N*)	1.0 (1.0, 2.0)	1 .0 (1.0, 1.3)	0.168
Pre-pregnancy BMI (kg/m^2)	22.5 (20.1, 25.7)	24.2 (22.5, 28.6)	0.113
Gestational week of faecal collection (*weeks*)	30.9 ± 2.5	29.9 ± 3.8	0.203
In-vitro fertilisation [number (%)]	3 (8)	1 (4)	0.456
BP at faecal collection (*mmHg*)			
Systolic	110.9 ± 12.6	160.4 ± 15.8	0.000
Diastolic	65.0 ± 9.0	100.2 ± 14.8	0.000
24-h proteinuria collection (*g*)	Not available	4.5 (2.0, 11.4)	-
Pregnancy comorbidities of chronic hypertension or Gestational diabetes mellitus [number (%)]	0	13 (48)	0.000
Pregnancy outcome [number (%)]			
Gestational weeks on delivery(*weeks*)	39.1 ± 2.5	31.7 ± 3.1	0.000
Placental abruption	0	1 (4)	0.429
Postpartum haemorrhage	0	1 (4)	0.429
Induced preterm birth <35 weeks	1 (3)	17 (63)	0.000
Birth weight (*g*)	3280.1 ± 561.5	1354.3 ± 665.1	0.000
Foetal growth restriction	1 (3)	16 (48)	0.000
Perinatal death	0	5 (19)	0.011

The GM diversity changes significantly in the PE group

To evaluate the sequencing data, we performed rarefaction-curve analysis with 97% similarity in terms of OTUs. The curve in each group was almost saturated, indicating that sufficient sequencing data were obtained to reflect nearly all microbial diversity in the samples (Figure 1A). A Venn diagram showed that, although the PE and HPC groups shared 772 identical bacteria in their faecal microbiomes, each group also contained some unique bacteria. The PE group had fewer unique bacteria than the healthy controls (100 versus 227; Figure 1B), indicating that the GM diversity changed in patients with PE. Principal coordinate analysis (PCoA) was performed using Bray–Curtis-distance analysis to cluster the 63 samples at the OTU levels. We found that PCoA clearly separated patients in the PE and HPC groups (Figure 1C), which indicated that the two groups had significantly different gut environments. Faecal biomass DNA contents were used to evaluate the GM load; however, no significant difference was found between the PE and HPC groups (Figure 1D). The Shannon and Sobs indexes of OTU levels were used to evaluate the microbial α diversity [18], which were found to decrease significantly in the PE group ($P<0.001$, Figure 1E,F). Collectively, these results showed that the GM diversity changed significantly in patients with PE.

GM abundance changes significantly in patients with PE

We performed linear discriminant analysis effect size (LEfSe) analysis and linear discriminant analysis (LDA) to distinguish the PE and HPC groups by identifying microbiota biomarkers at different taxonomic levels and estimating the effect size of each differentially abundant microbiota [19]. We found that the GM abundance from phylum to genus levels differed significantly between the two groups. Specifically, p_Firmicutes, c_Clostridia, o_Clostridiales, o_Bifidobacteriales, f_Lachnospiraceae, f_Ruminococcaceae, f_Streptococcaceae, f_Bifidobacteriaceae, g_*Blautia*, g_*Streptococcus*, g_*Eubacterium_rectale*, g_*Eubacterium_hallii*, and g_*Bifidobacterium* were more abundant in the HPC group, whereas p_Proteobacteria, c_Gammaproteobacteria, o_Enterobacteriales, f_Enterobacteriaceae, f_Veillonellaceae, and g_*Escherichia_Shigella* were more abundant in the PE group (Figure 2A). Figure 2B shows the GM according to differences in abundance between the two groups. The larger the LDA score, the greater the effect of the microbiota had on the difference of the GM abundance between the two groups. The Firmicutes/Bacteroidetes (F/B) ratio decreased slightly within the PE group, but showed no significant difference between the groups (Figure 2C).

When comparing the relative abundance of the GM at the phylum level, we found that the Firmicutes abundance decreased significantly and that Proteobacteria abundance increased significantly in the PE group (Figure 2D). At the genus level, *Blautia*, *Eubacterium_rectale*, *Eubacterium_hallii*, *Streptococcus*, and *Subdoligranulum* (which

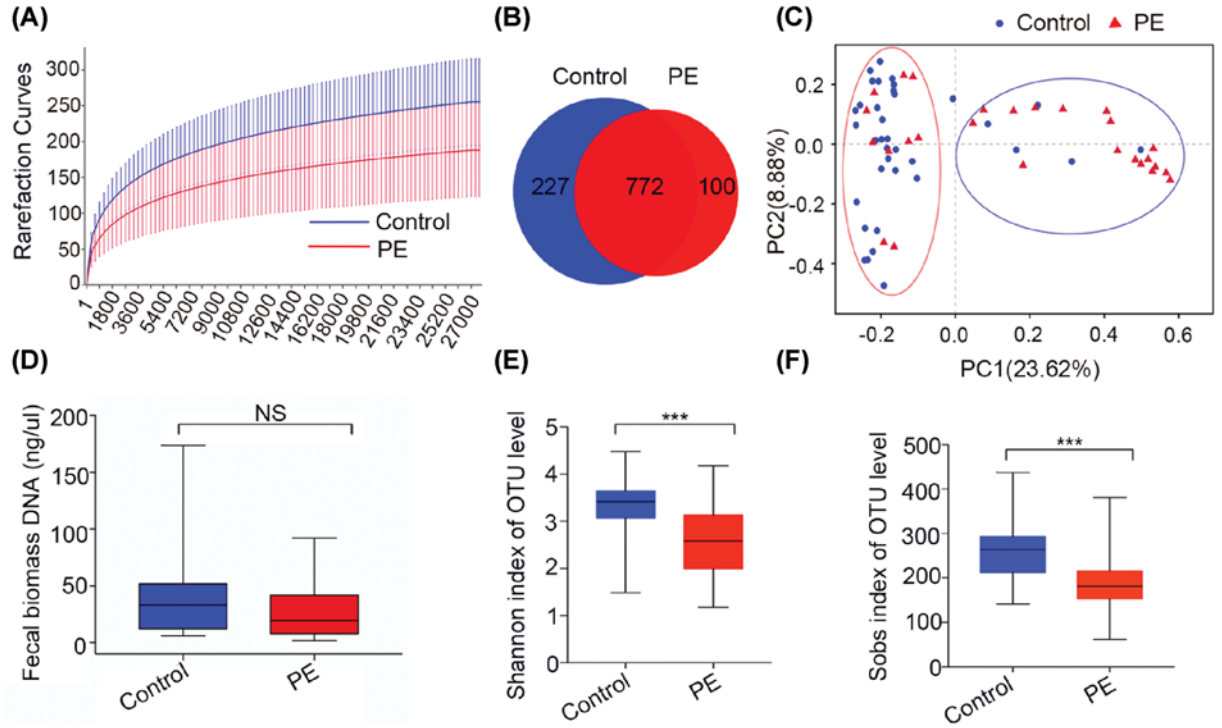

Figure 1. Changes in faecal microbial diversities between the PE group (*n*=27) and the HPC group (*n*=36)

(**A**) Rarefaction curves for the OTU levels. (**B**) The number of OTU present in the HPC only (blue), PE patients only (red), or overlap between the two groups in a Venn diagram. (**C**) PCoA was performed by using Bray–Curtis analysis method to calculate the distance between the PE and HPC groups in faecal samples. The percentages shown on each axis explain the proportion of each dimension. (**D**) Faecal biomasses were measured between both groups. NS, not significant. (**E**,**F**) Comparison of the microbial α diversity using the Shannon index and Sobs index, based on the OTU levels in both groups. Wilcoxon's test was performed. ***$P<0.001$.

belong to the Firmicutes phylum); *Bifidobacterium* and *Collinsella* (Actinobacteria phylum); and *Alistipes* (Bacteroidetes phylum) were more abundant in the HPC group. In contrast, *Escherichia_Shigella* and *Enterobacter* (which belong to the Proteobacteria phylum) were more abundant in the PE group (Figure 2E). Moreover, we found that the significant change in GM abundance in the PE group at both the phylum and genus levels were retained even when we excluded all patients with comorbidities of chronic hypertension or gestation diabetes (Supplementary Figure S1).

Faecal SCFA levels differ significantly between the PE and HPC groups

For each subject enrolled in the present study, we also analysed the SCFA levels in their faecal samples. We found that acetic acid, propionic acid, and butyric acid were the most abundant SCFA, whereas isobutyric acid, valeric acid, and isovaleric acid only accounted for a small portion of all SCFA (Figure 3A). In our study, the faecal levels of butyric acid and valeric acid were significantly lower in patients with PE (Figure 3B). After we excluded all the patients with PE plus comorbidities of chronic hypertension or gestational diabetes, we found that the levels of faecal valeric acid still differed significantly between the PE and HPC group ($P<0.001$), whereas the difference of butyric acid between the groups had lost significance and still decreased in patients with PE ($P=0.05$, Supplementary Figure S2).

Correlations between differential GM and SCFA levels

We generated correlation heatmaps related to differential GM and SCFA levels, using data from all patients in the PE and HPC groups. We found that the faecal levels of butyric acid, acetic acid, propionic acid, and valeric acid positively correlated with phylum Firmicutes but negatively correlated with phylum Proteobacteria (Figure 4A). At the genus level, *Blautia*, *Eubacterium_rectale*, *Eubacterium_hallii*, *Subdoligranulum*, *Streptococcus*, *Collinsella*, and *Alistipes*, which belong to the Firmicutes, Actinobacteria, or Bacteroidetes phyla, were positively correlated with faecal SCFA levels, especially with valeric acid and butyric acid. Genus *Escherichia_Shigella* (which belongs to phylum

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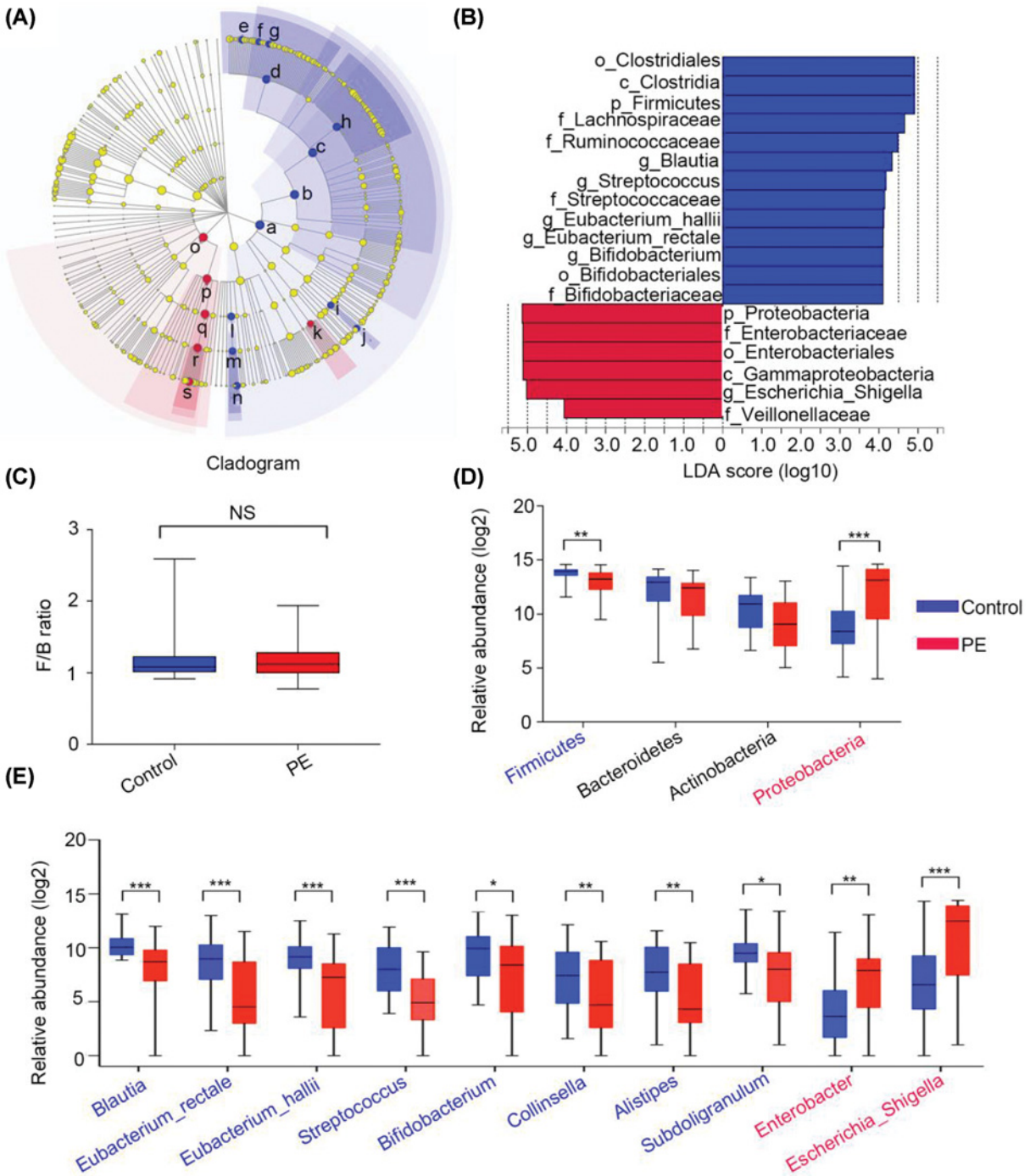

Figure 2. Distinguishing microbiota biomarkers between the PE and HPC groups

(**A**) LEfSe analysis was used to distinguish the differential microbiome between two groups. The different-coloured nodes represent microbial populations that were significantly enriched in the corresponding groups and that showed significant differences between the groups. The yellow nodes indicate microbial groups that showed no significant differences between two groups. The circles going from the inside to the outside represent the phylum, class, order, family, and genus. a: Firmicutes, b: Clostridia, c: Clostridiales, d: Lachnospiraceae, e: *Blautia,* f: *Eubacterium_hallii*, g: *Eubacterium_rectale*, h: Ruminococcaceae, i: Streptococcaceae, j: *Streptococcus*, k: Veillonellaceae, l: Bifidobacteriales, m: Bifidobacteriaceae, n: *Bifidobacterium*, o: Proteobacteria, p: Gammaproteobacteria, q: Enterobacteriaceae, r: Enterobacteriaceae, s: *Escherichia_Shigella*. (**B**) LDA was performed, and only the microbiota with LDA scores of >4 are shown. (**C**) The F/B ratio was calculated using the Mann–Whitney U test. NS, not significant. (**D**) At the phylum level, the four most abundant bacterial communities were compared using Wilcoxon's rank-sum test. Microbiota enriched in PE are indicated with red text, whereas those enriched in HPC are indicated with blue text. $^{**}P<0.01$, $^{***}P<0.001$. (**E**) At the genus level, the most abundant bacterial communities were compared using Wilcoxon's rank-sum test. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

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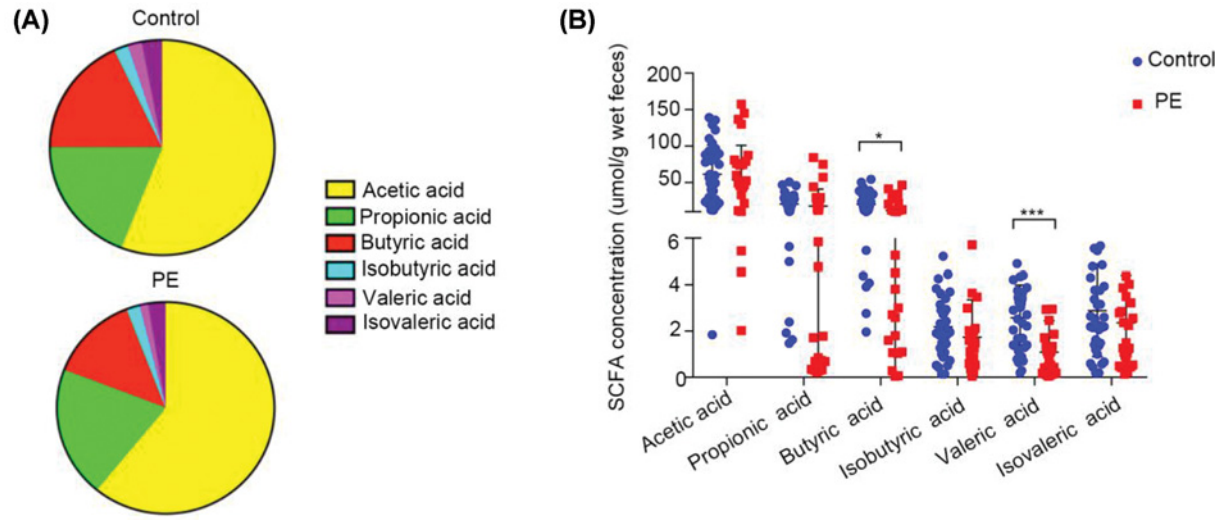

Figure 3. Significant changes of SCFA in patients with PE

(**A**) Mean proportional values of the indicated SCFA, as depicted in a pie chart. (**B**) SCFA were compared between the PE and HPC groups using Wilcoxon's rank-sum test. *$P<0.05$, ***$P<0.001$.

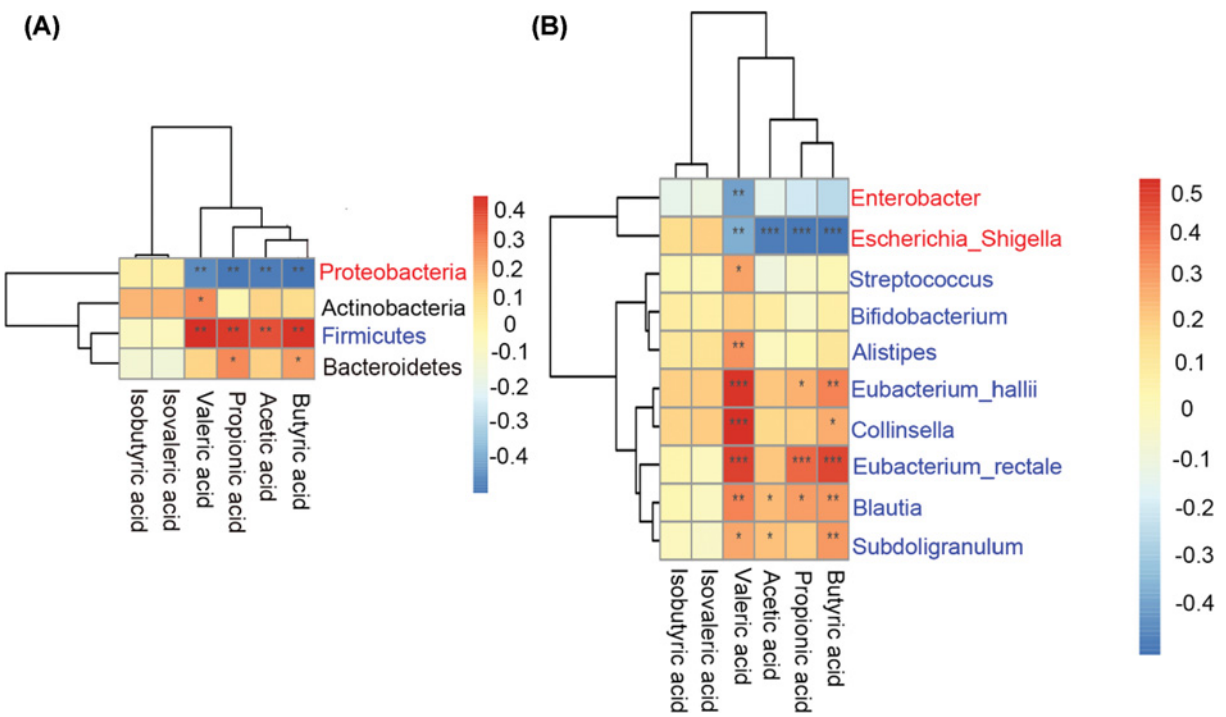

Figure 4. Differentially abundant GM correlate with butyric acid and valeric acid levels

(**A**,**B**) Spearman-correlation heatmap analysis was performed at the phylum and genus levels. Microbiota and SCFA enriched in the PE are indicated with red text, whereas those enriched in the HPC are indicated with blue text. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Proteobacteria) was negatively correlated with the faecal levels of butyric acid, acetic acid, propionic acid, and valeric acid. Our data showed that *Enterobacter* (which also belongs to phylum Proteobacteria) was negatively correlated with valeric acid only (Figure 4B). These results revealed changes in faecal SCFA levels that were in accordance with changes in the GM abundance of the PE group.

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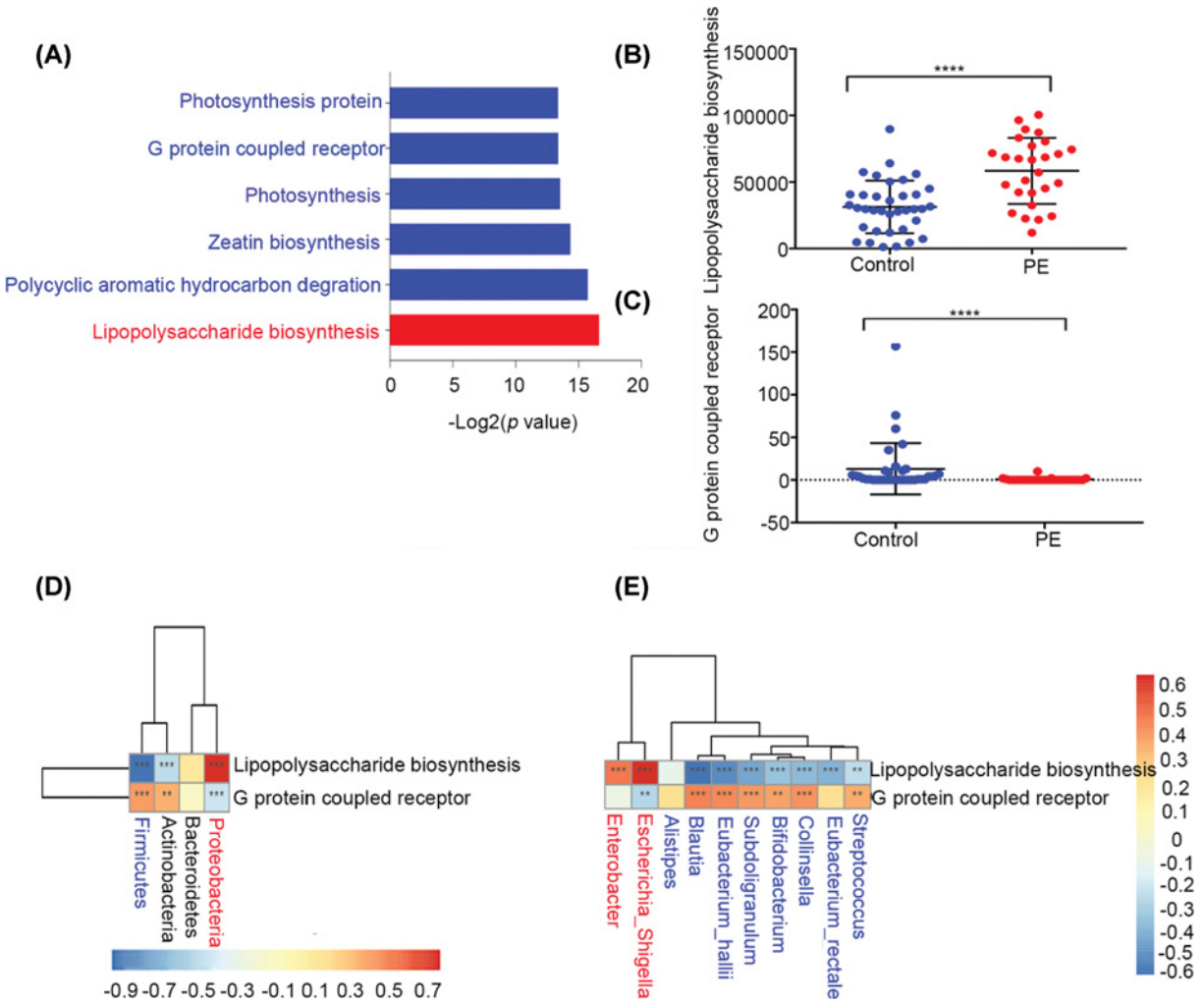

Figure 5. LPS biosynthesis and GPCR pathways are altered in the PE group

(**A**) The KEGG pathway (level 3) was significantly different between the PE and HPC groups. $P<0.0001$; enriched pathway in the PE or HPC group indicated with red or blue text, respectively. (**B**,**C**) LPS-biosynthesis and GPCR pathways differed significantly between the PE and HPC groups, as determined by Wilcoxon's rank-sum test. ****$P<0.0001$. (**D**,**E**) Spearman-correlation heatmap analysis was performed between differential GM and significant pathway at the phylum and genus levels. The GM enriched in the PE group are indicated with red text, whereas those enriched in the HPC group are indicated with blue text. **$P<0.01$, ***$P<0.001$.

Functional predictions and changes in the GM in the PE group

We predicted functional composition profiles based on 16S rRNA sequencing data by performing phylogenetic reconstruction of unobserved states (PICRUSt) analysis of all samples in the PE and HPC groups. We also analysed Kyoto Encyclopedia of Genes and Genomes (KEGG) level 3 categories and found that changes in the abundance of LPS biosynthesis and GPCR pathways were the most relevant among all KEGG pathways (Figure 5A). The abundance of the LPS-biosynthesis pathway increased significantly in the PE group ($P<0.0001$), whereas that of the GPCR pathway decreased significantly ($P<0.0001$; Figure 5B,C). Then, we correlated these two pathways with differentially abundant GM between the two groups. We found that the LPS-biosynthesis pathway was negatively correlated with phylum Firmicutes (and its subordinate genera *Blautia*, *Eubacterium_rectale*, *Eubacterium_hallii*, *Streptococcus*, and *Subdoligranulum*) and Actinobacteria (and its subordinate genera *Bifidobacterium* and *Collinsella*), but positively correlated with phylum Proteobacteria (and its subordinate genera *Escherichia_Shigella* and *Enterobacter*). In contrast, the GPCR pathway was positively correlated with phylum Firmicutes (and its subordinate genera *Blautia*, *Eubacterium_hallii*, *Streptococcus*, and *Subdoligranulum*) and Actinobacteria (and its subordinate genera *Bifidobacterium* and *Collinsella*), but negatively correlated with phylum Proteobacteria (and its subordinate genera

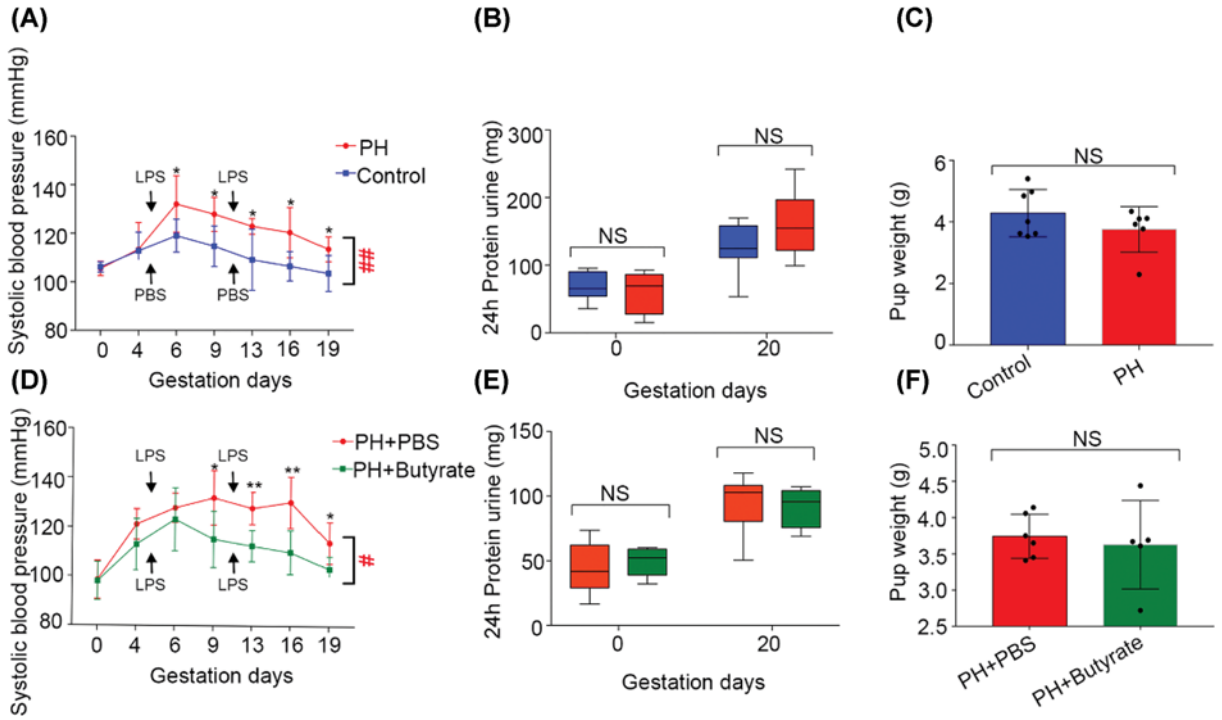

Figure 6. Butyrate decreases BP in PH rats

(**A**) LPS was administered to rats via the tail vein on the fifth and tenth days of gestation. The BP trend in PH (n=6) and control rats (n=7) differed significantly, as determined by repeated-measures analysis. $^{\#\#}P<0.01$. In particular, BP increased significantly in PH rats beginning at GD 6 as compared with that in control rats. $^{*}P<0.05$. (**B**) The 24-h protein urine levels did not significantly differ between PH and control rats, either on the first or last gestation day. NS, not significant. (**C**) No significant difference was found in the average pup weight for each pregnant rat between the PH group and control rats. (**D**) Oral butyrate or PBS was administration from GD 4 to GD 19. The BP trends in PBS-treated rats (n=6) and butyrate-treated (n=5) PH rats were significantly different, as determined by repeated-measures analysis. $^{\#}P<0.05$. BP values decreased significantly beginning at GD 9 between PH rats administered with butyrate or PBS. $^{*}P<0.05$, $^{**}P<0.01$. (**E**,**F**) The 24-h protein urine levels and the average pup weights of pregnant rats did not significantly differ between butyrate-and PBS-treated PH rats.

Escherichia_Shigella) (Figure 5D,E). These results suggested that the abundance of the LPS biosynthesis and GPCR pathways was correlated with changes in the GM abundance in patients with PE.

Butyrate treatment significantly decreases the BP in PH-model rats

As we were unable to replicate the LPS-induced PE rat model of Xue et al. [17], we modified their protocol by injecting rats with LPS on GDs 5 and 10 to generate a PH rat model. The BPs measured in our PH rat model differed significantly ($P<0.01$) between LPS- and PBS-treated rats. Specifically, the BPs of the LPS-treated rats were significantly elevated from GD 6 until the day of killing, compared with those in PBS-treated rats (Figure 6A). However, no significant differences were observed between the two groups with respect to 24-h protein urine samples on GD 19 (Figure 6B) and average pup weights (Figure 6C). We did not find any foetal absorptions in either of the two groups.

We treated the PH rats with a daily gavage of butyrate from GD 4 until they were killed. Overall, the BPs between the butyrate- and PBS-treated PH rats differed significantly ($P<0.05$), with the systolic BPs of the butyrate-treated PH rats decreasing significantly from GD 9 and remaining within the normal range until killing (Figure 6D). However, the 24-h protein urine and average pup weight did not significantly change following butyrate administration in the PH rat model (Figure 6E,F). And we did not observe obvious foetal absorption in either the PH group or butyrate-treated PH group.

Discussion

In the present study, we found that both the diversity and abundance of faecal GM decreased in patients with PE in addition to the SCFA levels, indicating that GM dysbiosis as well as SCFA disturbances are involved in the disease. We

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found significant correlations between faecal GM abundance and SCFA levels, which suggested that the differential GM and SCFA might contribute cooperatively to PE. Our findings that butyrate restored normal BP levels in a rat model of LPS-induced PH demonstrated that butyrate could directly down-regulate BP *in vivo*. We also found that the abundance of the LPS biosynthesis and GPCR pathways was correlated with differential GM abundance, suggesting that these two pathways may be involved in GM dysbiosis-related PE.

Analysis of the GM at mainly the phylum and genus levels identified numerous genera belonging to phylum Firmicutes or Actinobacteria that were significantly less abundant in the PE group, whereas the abundance of other genera belonging to phylum Proteobacteria increased significantly in patients with PE. Among the GM genera that showed decreased abundance in patients with PE, *Blautia*, *Eubacterium_hallii*, *Subdoligranulum*, and *Collinsella* were also reported to exhibit decreased relative abundance in patients with hypertension or diabetes [20–25]. Therefore, chronic hypertension and gestational diabetes may constitute confounding factors of our results. However, in view of our findings that changes in the GM were consistent regardless of the presence or absence of comorbidities of chronic hypertension or gestational diabetes in patients with PE, we confirmed that changes in GM contributed to PE independently.

The abundance of *Blautia*, *Eubacterium_rectale*, and *Bifidobacterium* was also negatively correlated with systolic BP [26,27]. The PE-enriched genera *Escherichia_Shigella* and *Enterobacter*, both of which comprise Gram-negative bacteria that can produce a large amount of LPS, were previously reported to cause excessive inflammatory responses [28,29]. Combined with these previous reports, our current findings of disturbances in the relative abundance of the GM in patients with PE suggested that GM dysbiosis may be important for PE pathogenesis.

Two recent studies were also performed to compare GM composition and abundance between PE and HPC groups during late pregnancy [30,31]. However, our results were notably different from those obtained in these previous studies. Specifically, Liu et al. [30] did not find significant changes in GM diversity or in the abundance of the four predominant microbes at the phylum level in patients with PE. Lv et al. [31] found a different GM abundance at the genus level in patients with PE, when compared with our study. These discrepancies may reflect that following consideration: the average BP and protein urine of the PE patients in our study were notably higher than those in the previous two studies, implying that the patients in our study had more severe PE. Thus, the between-group differences in the faecal GM composition in our study might represent more typical changes in patients with severe PE.

SCFAs comprise metabolites generated from carbohydrates and proteins by the GM that play important roles in host physiology and health. They are reported to regulate metabolism and stabilise BP mainly through different GPCR [32,33], although few reports have described faecal SCFA levels in patients with PE [34]. In the present study, we found for the first time that faecal butyric acid and valeric acid levels were decreased significantly in patients with PE, especially the latter. Moreover, although the significance of the between-group difference of butyric acid was lost after we excluded the patients with comorbidities of chronic hypertension or gestational diabetes, that of valeric acid remained.

To date, few studies have been conducted to investigate the effects of butyric acid on BP. Butyric acid was reported to down-regulate LPS-induced inflammation by modulating the function of macrophages and inhibiting histone deacetylation [35–38]. In addition, LPS may induce Toll-like receptor 4-mediated immune inflammation responses and was related to PE pathogenesis [39]. Notably, although the significance of the decrease in butyric acid was reduced after excluding the patients with comorbidities in the present study, the trend of reduction in patients with PE was retained, suggesting that decreased faecal butyric acid levels might contribute to PE development through an LPS-related mechanism. However, further research is needed to clarify the role(s) of butyric acid in PE.

Although, we identified a significantly robust reduction in valeric acid in patients with PE, little is currently known regarding this SCFA. Specifically, it has been reported to inhibit histone deacetylation [40] and in patients with ischaemic stroke, valeric acid was positively correlated with C-reactive protein, an inflammatory marker [41]. Recently, it was reported that valeric acid could suppress autoimmunity by enhancing IL-10 production and suppressing Th17 cells [42]. Because the proportion of valeric acid was low among all faecal SCFA, its role in PE pathogenesis requires further investigation.

We found consistent correlations between SCFA and differential GM in patients with PE. The phylum Firmicutes and its subordinate genera were related to increased SCFA, whereas phylum Proteobacteria and its subordinate genus *Escherichia_Shigella* were related to reduced SCFA. All SCFAs showed synchronous correlations with the differential GM, suggesting that the differential GM and the SCFA they produced might work synchronously in the pathogenesis of PE. Among the SCFA, valeric acid, and butyric acid showed the strongest correlations with differential GM. Therefore, they may constitute the main SCFA that contribute to PE pathogenesis. Our study is the first to measure faecal GM abundance and SCFA levels simultaneously, and to find strong correlations between these measures.

Notably, the reduced gut genera *Blautia*, *Eubacterium_rectale*, *Eubacterium_hallii*, *Collinsella*, and *Subdoligranulum* found in patients with PE are mainly butyric acid-producing bacteria [43–45]. This finding suggests that the reduced butyric acid in these patients might be caused by a shortage of these faecal bacteria. Moreover, valeric acid appeared to be more closely related to the GM than butyric acid, i.e., valeric acid was positively correlated with more genera belonging to phylum Firmicutes and negatively correlated with more genera belonging to phylum Proteobacteria, suggesting that the faecal valeric acid level might serve as a sensitive indicator of GM dysbiosis.

Results from the PH rat model and clinical findings in the present study provided evidence that butyrate participates in BP regulation. We developed a rat model of LPS-induced PH and found that butyrate gavage administration normalised the PH rats' BPs. This result suggested that butyrate directly affects BP regulation; thus, playing an important role in PE development. Since we did not include a butyrate-treated normal pregnant rat group in the present study, we could not exclude butyrate's effects on normal BP. However, a previous report had shown that butyrate treatment attenuates angiotensin II-induced hypertension in C57BL6 mice but does not affect BP in healthy control mice [46]. Those results complement our study's findings to indicate that butyrate affects hypertension, but not normal BP.

Based on 16S rRNA metagenomic predictions generated using PICRUSt software, we found that LPS-synthesis and GPCR pathways may have been disturbed in the PE group. The LPS-synthesis pathway increased significantly in the PE group, which was negatively correlated with phylum Firmicutes (and many of its subordinate genera) and positively correlated with phylum Proteobacteria (and two of its subordinates, including *Escherichia_Shigella*). The genus *Escherichia_Shigella* comprises Gram-negative bacteria that produce large amounts of LPS [47,48]. Considering that LPS has been found to participate in PE pathogenesis [49,50], our new findings related to increased LPS-producing bacteria, correlations with the LPS-synthesis pathway, and the differential GM in patients with PE suggested that increased LPS synthesis induced by GM changes is associated with PE.

We also found, through the PICRUSt analysis, that the abundance of GPCR pathways was significantly lower in patients with PE. The GPCR pathway was positively correlated with phylum Firmicutes and many of its subordinate genera, which are known to produce butyric acid, and negatively correlated with phylum Proteobacteria and genus *Escherichia_Shigella*. It is widely accepted that most common function of the GPCR family is to provide anti-inflammatory signals. Insufficient signalling through GPCR can lead to diabetes, cardiovascular disease, and inflammatory bowel disease [51]. It has been reported that SCFA regulate BP and inhibit chronic inflammation, mainly through GPCR pathways [16]. We found that changes in the abundance of the GPCR-pathway were synchronous with changes in the abundance of faecal GM and SCFA in patients with PE. These data suggested that deficiencies in GM and SCFA may affect PE pathogenesis by regulating GPCR pathways.

The strengths of the present study were as follows. **First**, we reported for the first time that some new GM dysbiosis and significant faecal butyric acid and valeric acid reduction existed in patients with PE. **Second**, faecal SCFA levels were correlated with altered GM abundance in patients with PE, which suggested that GM dysbiosis and SCFA may contribute cooperatively and causatively to PE development. **Third**, our *in vivo* study provided new evidence that butyrate could directly regulate BP in an LPS-induced PH rat model. **Finally**, we found that LPS synthesis and the GPCR pathway may be involved in GM dysbiosis-related PE, which provides clues for more in-depth investigations in this field. **Notably**, we observed for the first time that valeric acid exhibited a greater correlation with GM abundance than that of butyric acid did. As a member of the SCFA family that receives little attention, valeric acid might serve as a new sensitive indicator of GM dysbiosis, which merits further study.

The limitations of the present study were as follows. **First**, although the maternal age, gestational week, gravidity, parity, and other pregnancy complications (excluding PE) were matched when we recruited the patients, the effects of medication, diet, and environmental and endocrine factors on our results cannot be eliminated completely. To minimise these effects, we asked all subjects to follow their normal diets for at least 3 days before we collected their faecal samples, and we excluded patients who had received antibiotics within 1 month prior to collecting faecal samples. **Second**, although our rat model of LPS-induced PH demonstrated the PH phenotype, this rat model still does not fully represent PE. Furthermore, a GM dysbiosis-induced PE rat model would be more efficacious than the LPS-induced model. **Third**, performing PICRUSt analysis to predict the function of the 16S rRNA sequenced GM only provided some preliminary results; more accurate GM function might be determined by metagenomics analysis.

Conclusion

In summary, we observed, for the first time, a link between GM dysbiosis and simultaneously reduced faecal SCFA with PE, which suggests that GM and SCFA might cooperatively contribute to PE. Our findings provide evidence that butyrate can directly regulate high BP *in vivo*, suggesting its application as a new candidate molecule for treating PE and hypertension. In the present study, we were unable to build a GM dysbiosis-induced PE rat model owing to the

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shortage of germ-free rats in our laboratory; however, further research should be conducted to elucidate the effects of dysbiosis of GM and SCFA on the pathogenesis of PE in this rat model, in order to demonstrate their contribution to the development of this intractable disease during pregnancy.

Clinical perspectives

- PE is associated with PH and damage to multiple organs; whereas GM and SCFA have been related to hypertension, but their effects on PE remain unknown.
- We discovered that GM dysbiosis and reduction in SCFA levels occurred synchronously in patients with PE, suggesting that GM and SCFA might cooperatively contribute to PE.
- Our results showing that oral butyrate reduced BP levels in a rat model of PH demonstrated that butyrate could directly down-regulate BP *in vivo*, thus suggesting butyrate as a new therapeutic agent for PE.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 81270714]; the Key Discipline Project of Shanghai Municipal Commission of Health and Family Planning [grant number 15GWZK0701]; the Shanghai Municipal Commission of Health and Family Planning [grant number 2017ZZ02016]; and the Shanghai Municipal Key Clinical Specialty.

Author Contribution

Yanling Chang analysed the data and wrote the manuscript. Yunyan Chen conceived the study and collected samples. Lei Chen performed the microbiota analysis. Qiong Zhou and Chuan Wang revised the manuscript. Wen Di and Yu Zhang provided financial support. Yu Zhang designed and supervised the study. All authors read and approved the final manuscript.

Acknowledgements

We thank the faculty of the Department of Obstetrics of Ren Ji Hospital (School of Medicine, Shanghai Jiao Tong University) for their help in recruiting patients with PE and normal pregnant women for our study. We also thank Dr. Wei Zhang of the Department of Epidemiology, Shanghai Cancer Institute for her valuable help in data analysis.

Abbreviations

BMI, body-mass index; BP, blood pressure; DNA, deoxyribonucleic acid; GD, gestational day; GM, gut microbiota; GPCR, G protein-coupled receptor; HPC, healthy pregnant control; KEGG, Kyoto Encyclopedia of Genes and Genomes; LDA, linear discriminant analysis; LPS, lipopolysaccharide; OTU, operational taxonomic unit; PBS, phosphate-buffered saline; PCoA, principal coordinate analysis; PCR, polymerase chain reaction; PE, preeclampsia; PH, pregnant hypertension; PICRUSt, phylogenetic reconstruction of unobserved states; rRNA, ribosomal RNA; SCFA, short-chain fatty acid; SPF, specific pathogen-free.

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