ORIGINAL RESEARCH

Probiotic Combination CBLEB Alleviates *Streptococcus pneumoniae* Infection Through Immune Regulation in Immunocompromised Rats

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Background: *Streptococcus pneumoniae* (*SP*) is the most common cause of bacterial pneumonia, especially for people with immature or compromised immune systems. In addition to vaccination and antibiotics, immune regulation through microbial intervention has emerged in recent anti-*SP* infection research. This study investigated the therapeutic effect of a combination of live *Bifidobacterium, Lactobacillus, Enterococcus*, and *Bacillus* (CBLEB), a widely used probiotic drug, on *SP* infection in rats.

Methods: An immunocompromised *SP*-infection rat model was established by intraperitoneal injection of cyclophosphamide and nasal administration of *SP* strain ATCC49619. Samples from *SP*-infected, *SP*-infected and CBLEB-treated, and healthy rats were collected to determine blood indicators, serum cytokines, gut microbiota, faecal and serum metabolomes, lung- and colon-gene transcriptions, and histopathological features.

Results: CBLEB treatment alleviated weight loss, inflammation, organ damage, increase in basophil percentage, red cell distribution width, and RANTES levels and decrease in total protein and albumin levels of immunocompromised *SP*-infection rats. Furthermore, CBLEB treatment alleviated dysbiosis in gut microbiota, including altered microbial composition and the aberrant abundance of opportunistic pathogenic bacterial taxa such as *Eggerthellaceae*, and disorders in gut and serum metabolism, including altered metabolomic profiles and differentially enriched metabolites such as 2,4-di-tert-butylphenol in faeces and L-tyrosine in serum. The transcriptome analysis results indicated that the underlying mechanism by which CBLEB fights *SP* infection is mainly attributed to its regulation of immune-related pathways such as TLR and NLR signalling in the lungs and infection-, inflammation- or metabolism-related pathways such as TCR signalling in the colon.

Conclusion: The present study shows a potential value of CBLEB in the treatment of *SP* infection.

Keywords: *Streptococcus pneumonia* infection, probiotics, microbiota, metabolism, transcriptome

Introduction

Pneumonia is the single greatest infectious cause of death worldwide and is associated with a tremendous economic burden.[1] According to the World Health Organization's report, pneumonia accounted for 15% of all deaths among children under 5 years old, killing 808,694 children in 2017.[2] *Streptococcus pneumoniae* (*SP*) is one of the major human pathogens and the most common cause of bacterial pneumonia, septicaemia and meningitis.[3,4] However, unlike some major bacterial respiratory pathogens, such as *Mycobacterium tuberculosis, SP* is also a ubiquitous human commensal of the upper respiratory tract, specifically the nasopharynx.[5] Colonization with different pneumococcal strains occurs in children up to 2 years of age, with the prevalence of nasopharyngeal carriage has been estimated at 27–65% among infants.[6] Because the *SP* strains causing

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invasive infections are surrounded by a polysaccharide capsule layer that inhibits innate and adaptive immune responses to infection,[7] such asymptomatic colonization in people with an immature or compromised immune system can progress to mild disease (ie, sinusitis and otitis media) and occasionally to pneumonia, sepsis, etc.[8]

Vaccination and the use of antibiotics are the most conventional methods of prevention and treatment of *SP* infection. However, antibody-based immunity induced by protein-polysaccharide conjugate pneumococcal vaccines (PCVs) is highly serotype specific, protecting against only certain serotype strains whose capsular polysaccharide types are included in the vaccines,[9] and PCVs are less effective at preventing pneumonia than septicaemia and meningitis.[10,11] The excessive use of antibiotics has led to a serious situation of high-level antimicrobial resistance worldwide,[12] and the antibiotics themselves can also have an impact on health (ie, disrupting the normal human microbiota, leading to secondary infections), especially for immunocompromised individuals, elderly individuals, and children under 2 years of age. Thus, new strategies to fight against pneumococci, such as targeting enhancement of the immunity of high-risk groups, are also required.

The human microbiota is important for the development and maintenance of immunity, and it also helps the host resist pathogen invasion. Therefore, appropriate microbiota regulation is considered a potential solution to prevent and treat *SP* infection. In recent years, oral and nasal administration of some lactic acid bacteria, such as *Lacticaseibacillus rhamnosus* CRL1505[13] and *Lacticaseibacillus casei* CRL431,[14] has been reported to improve T cell-mediated immunity against *SP* infection. The probiotic drug combination of live *Bifidobacterium infantis* CGMCC0460.1, *Lactobacillus acidophilus* CGMCC0460.2, *Enterococcus faecalis* CGMCC0460.3 and *Bacillus cereus* CGMCC0460.4 (CBLEB), is widely used in China. It has been reported that taking CBLEB has a therapeutic effect on immunodeficiency.[15] The present study aimed to investigate the effect of CBLEB against pneumococcal infection in immunocompromised rats and the underlying mechanism.

Materials and Methods

Microorganisms

The probiotic drug CBLEB was purchased from Hangzhou Yuanda Biopharmaceutical Co., Ltd. The probiotic powders were dissolved in sterile saline to prepare a CBLEB solution (*B. infantis*, 4.7×10^9 colony-forming units (CFU)/kg; *L. acidophilus*, 4.7×10^9 CFU/kg; *E. faecalis*, 4.7×10^8 CFU/kg; and *B. cereus*, 4.7×10^7 CFU/kg). *S. pneumoniae* ATCC49619 (serotype 19) was purchased from the American Type Culture Collection (ATCC). The pathogen was incubated at 37 °C in an atmosphere of 5% CO_2 and harvested by centrifugation at 3600 ×g for 10 min at 4 °C. Then, the concentration of the pathogen was adjusted to the dose used in the nasal administration.

Animal Experimental Design and Sample Collection

Specific pathogen-free male Sprague-Dawley rats (6–8 wks old, 200–300 g) were randomly divided into three groups, the healthy control (HC), *SP* infection (*SP*) and probiotic treatment (*SP*+CBLEB) groups, and each group consisted of ten rats. All animals were supplied with balanced rodent food and water and maintained with a controlled light/dark cycle. The entire experiment lasted for 30 days.

The immunocompromised rat model representing a group with high risk of *SP* infection was established by intraperitoneal injection of cyclophosphamide (40 mg/kg), and the rats were subsequently infected with *SP*. Briefly, the rats in the *SP* and *SP*+CBLEB groups received cyclophosphamide on days 12, 13, 14, and 21 and nasal administration of *SP* (1.0×10^8 CFU per rat) on day 22, while the rats in the HC group received the same dose of saline. Additionally, the rats in the *SP*+CBLEB group were gavaged with CBLEB solution once daily for 28 days, while the rats in the HC and *SP* infection groups were administered normal saline. On day 30, rats were anaesthetized with 400 mg/kg chloral hydrate by intraperitoneal injection. Spleen and lung samples were fixed in 10% paraformaldehyde for 24 h, embedded in paraffin, cut into 3-μm sections and stained with haematoxylin and eosin (H&E). Blood and faecal samples were collected and immediately used or stored at −80 °C until use. All experiments were approved by the Institutional Animal Care and Research Advisory Committee at Zhejiang University.

Quantitative Detection of SP Invasion

SP invasive infection was verified by using real-time PCR (RT-PCR), as previously described.[16] Briefly, DNA was extracted from lung samples with DNeasy Blood & Tissue Kits (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The burden of *SP* was estimated from the number of *ply* gene copies per gram of lung sample. Quantitative PCR was performed with a ViiA7 RT-PCR system (Applied Biosystems, Waltham, Massachusetts, USA) and using SYBR Premix Ex Taq™ (RR420A; TaKaRa, Tokyo, Japan). The reaction mixture (10 μL) contained 5 μL of TB Green, 0.4 μL (1 μM) of each primer (forward: TGCAGAGCGTCCTTTGGTCTAT; reverse: CTCTTACTCGTGGTTTCCAACTTGA), 0.2 μL of ROX reference dye, 2 μL of DNA template, and 2 μL of PCR-grade water.[16] The reaction was hot-started at 95 °C for 30s, followed by 40 cycles of 95 °C for 5 s, 57 °C for 30s and 72 °C for 30s, and the subsequent melt curve was assessed from 60 °C to 95 °C at 0.2 °C/read. Plasmids harbouring the PCR-amplified *ply* gene were used as standards to produce a calibration curve.

Histology

Spleen index = spleen weight/body weight × 100.

Thymus index = thymus weight/body weight × 100.

Spleen and lung sections were scanned with a section scanner after H&E staining, and the images were used to evaluate the degree of tissue damage, inflammation, and necrosis. Pulmonary histological damage was quantified using the American Thoracic Society 2010 Lung Injury Scoring System.[17] The spleen pathological severity score was graded from 0 to 3 points as described previously.[18]

Haematological Tests

Routine blood indexes, such as white blood cell, neutrophil, lymphocyte, monocyte, eosinophil and basophil counts, were measured with an XN-2000 automatic haematology analyser (Sysmex, Tokyo, Japan). Liver and kidney function indicators, including total protein, albumin, globulin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, indirect bilirubin, g-glutamyltransferase (GGT), creatinine, urea nitrogen and uric acid levels, were measured with an automatic biochemical analyser (Hitachi 7600–210; Tokyo, Japan).

Cytokine Assay

Serum cytokine concentrations were measured by using a Bio-Plex Pro™ rat cytokine 23-plex assay kit (Bio-Rad Laboratories, Hercules, USA), including interleukin-1α (IL-1α), IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p70), IL-13, IL-17A, IL-18, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1a (MIP-1α), MIP-3α, tumour necrosis factor-a (TNF-α), interferon-g (IFN-γ), vascular endothelial growth factor (VEGF), growth-regulated a protein (GRO/KC), and regulated upon activation normal T cell expressed and secreted protein (RANTES).

Gut Microbiota Analysis

DNA was extracted from faecal samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, CA, USA). The V3-V4 region of the 16S rDNA gene was amplified using the primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT). PCR amplification, library preparation, and DNA sequencing were conducted as described previously on the Illumina MiSeq platform (Illumina, San Diego, CA, USA).[5] The raw reads were trimmed, filtered and then merged using FLASH v1.2.11. Quality control was conducted using FastQC v0.11.8. Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 software was used for the operational taxonomic unit (OTU) assignment, clustering, identification against the Greengenes database and the NCBI 16S Microbial database, and subsequent statistical analysis of microbial diversity and differential enrichment.

Metabolomic Analysis

Serum (200 μL) and faecal (20 mg) samples were pretreated before gas chromatography-mass spectrometry (GC-MS) analysis as described previously.[19] Briefly, samples were added to 800 μL of prechilled methanol before homogenization, centrifugation and filtration. The supernatant was subsequently dried with nitrogen, methoxymated and trimethylsilylated with 20 μL of heptadecanoic acid (1 μg/μL) as an internal standard. The prepared samples were then assayed with an Agilent 7890A-5975C GC-MS system (Agilent, CA, USA) using an Agilent J&W Hp-5 MS column. Raw GC-MS data were processed using Agilent Qualitative Analysis software (vB.07.00). Metabolite identification was based on the results of a programmed comparison with the National Institute of Standards and Technology (NIST) 17 mass spectral library using a matching score higher than 80 as the threshold. The identified compounds were then manually screened to remove the derivatization reagent to obtain the final result. Data were normalized to the internal standard prior to multivariate analysis. Orthogonal partial least squares discriminant analysis (OPLS-DA) was performed to visualize metabolic differences between groups using Umetrics SIMCA software v14.1.

Transcriptomic Analysis

Total RNA was extracted from lung and colon samples using an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). RNA quantification, library preparation and sequencing were conducted as described previously.[15] Prior to alignment, raw reads were trimmed using Trimmomatic to generate clean data. The paired-end clean reads were then aligned to a reference genome (Rnor_6.0, GCA_000001895.4) using HISTA2 v2.0.5. Each gene's expression value (fragments per kilobase of transcripts per million mapped fragments, FPKM) was calculated using FeatureCounts v1.5.0-p3. The R package "DESeq2" was used to analyse differential expression between two groups, and "ClusterProfiler" package was used to determine the gene annotation, functional classification, and pathway enrichment. The significant transcriptome results were verified by RT-PCR. The mRNA of representative genes was reverse transcribed and then measured in triplicate. Gene transcription was transformed into relative expression based on internal control, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences are provided in Supplemental Table S1.

Statistics

The Shapiro–Wilk test was used to determine whether the data were normally distributed. The Wilcoxon test was used to compare any two data sets that were not normally distributed; otherwise, one-way ANOVA followed by the Student-Newman-Keuls method was used. Differentially abundant metabolites were selected according to the variable importance in the projection (VIP) values obtained from the OPLS-DA model and the P-values from the Wilcoxon test or one-way ANOVA. Spearman's rank test was used to analyse correlations between every two significant factors. P-values of the hypergeometric test were used to determine significance in gene set enrichment analysis. P-values were adjusted for the false discovery rate using the Benjamini-Hochberg method where necessary (*$P_{adj} < 0.05$; **$P_{adj} < 0.01$; ***$P_{adj} < 0.001$).

Results

CBLEB Treatment Alleviates Weight Loss, Inflammation and Organ Damage in Immunocompromised SP-Infection Rats

The number of *SP* in the lung of rats was quantified. The *SP* burden in the lung samples of the *SP* and *SP*+CBLEB groups increased significantly, indicating that *SP* invasive infection occurred (Supplemental Figure S1). The changes in body weight of rats in each group were measured during the study (Figure 1A). In the First 11 days (before cyclophosphamide injection), the weight of the rats in each group increased, and there were no significant differences among these groups. From 12 to 21 days, compared with the rats in the HC group, the rats in the *SP* group (injected with cyclophosphamide, gavaged with normal saline) had significant weight loss, and this weight loss trend slowed in the *SP*+CBLEB group (injected with cyclophosphamide, gavaged with CBLEB). On the 22nd day, the body weight of rats in all groups decreased whether *SP* or saline was nasally administered. This outcome may be because anaesthesia
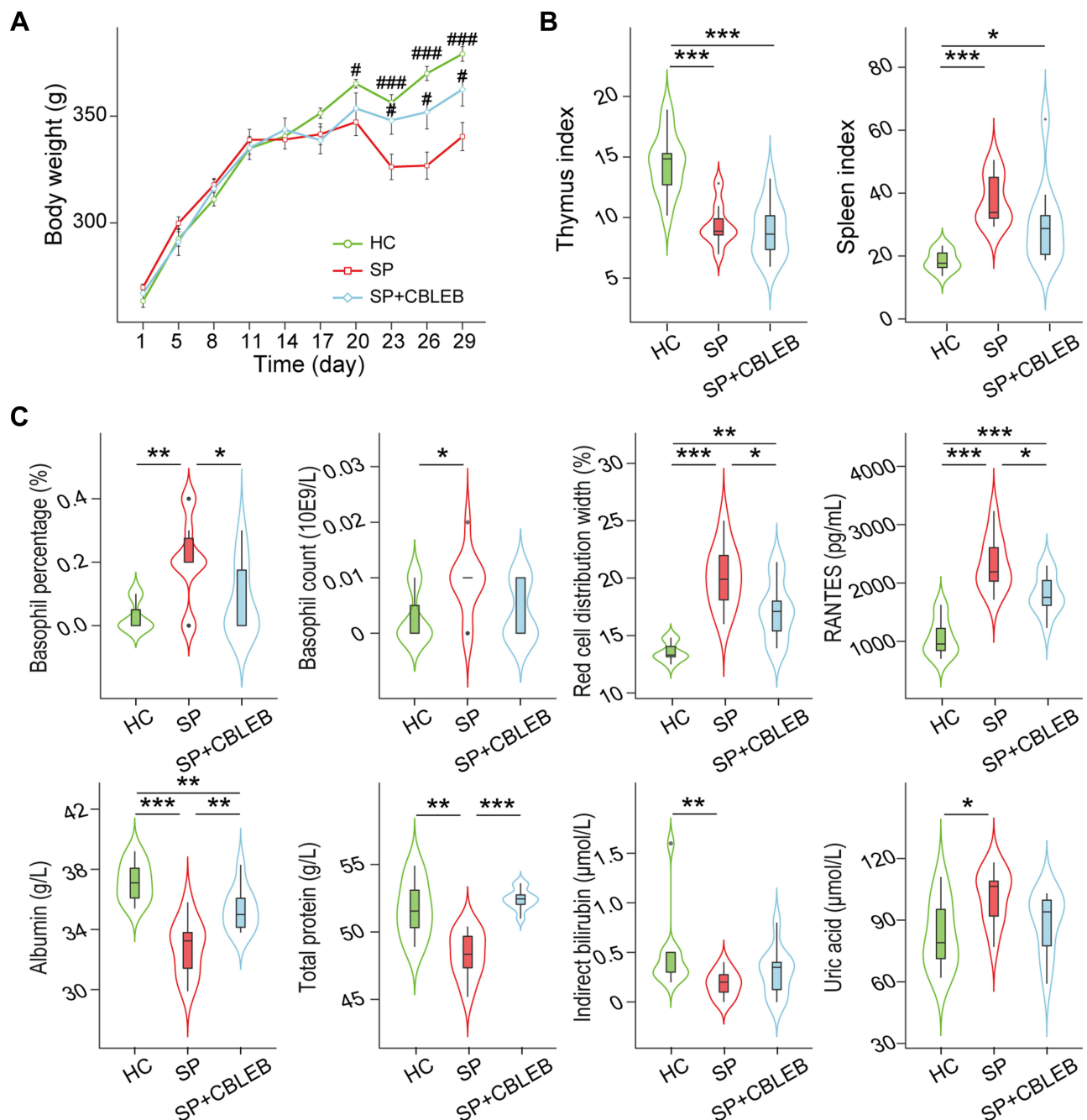
Figure I CBLEB treatment alleviates *SP* infection-induced weight loss, abnormal blood indicator levels and immune dysfunction. (**A**) Weight plot. (**B**) Thymus and spleen indexes. (**C**) Basophil counts and percentages, red cell distribution width, total protein, albumin, indirect bilirubin, urea acid, and RANTES levels in blood samples. #$P < 0.05$; ###$P < 0.001$ versus the *SP* group in weight plot; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

administration affected the normal diet of the rats. Among the groups of rats, the weight loss of rats in the *SP* group was the most significant, reflecting the detrimental effects of *SP* infection on health.

Administration of CBLEB partially alleviated abnormality of blood indicator levels and immune dysfunction in immunocompromised *SP*-infection rats. First, CBLEB administration significantly alleviated the elevation in blood basophil percentage and red cell distribution width (RDW) and serum RANTES levels, as well as the decline in blood total protein (TP) and albumin (ALB) levels. Second, in the *SP*+CBLEB group, the decrease in blood indirect bilirubin (IB) levels and increase in basophil count and uric acid levels in the *SP* group became insignificant when compared with those in the HC group (Figure 1C).
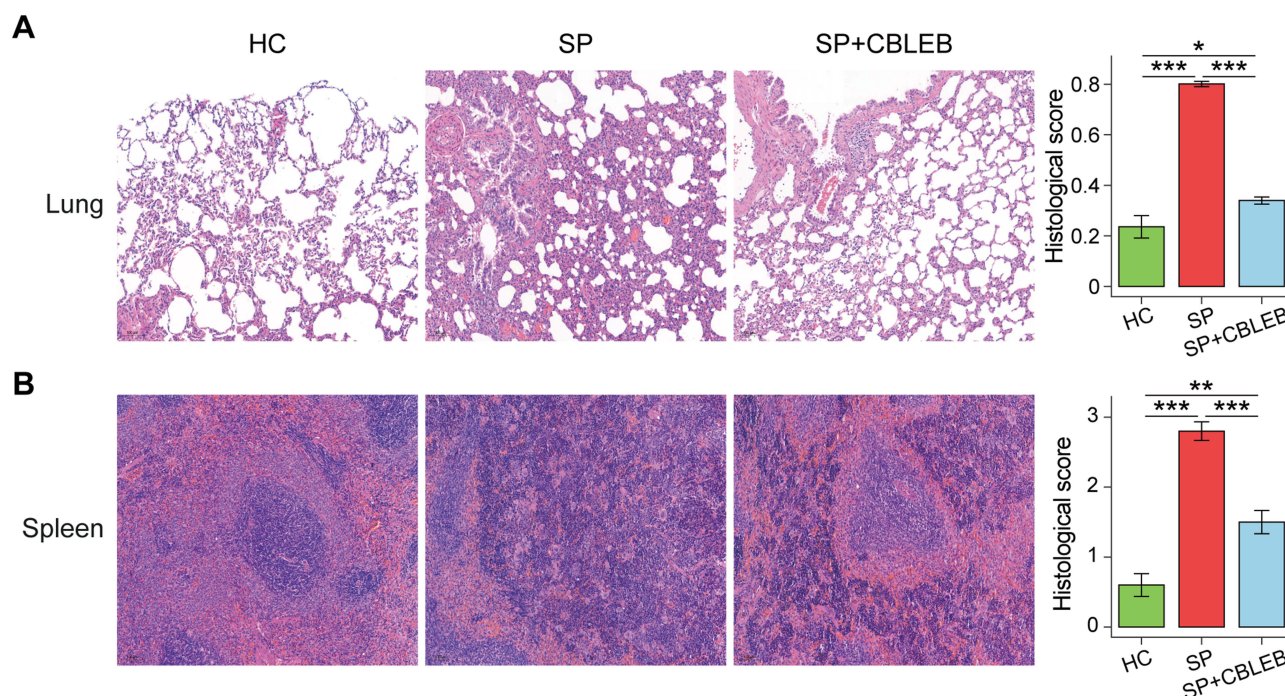
Figure 2 CBLEB treatment partially alleviated *SP* infection-induced organ damage. (**A**) Representative images of lung samples stained by H&E and the corresponding histological scores. (**B**) Representative images of spleen samples stained by H&E and the corresponding histological scores. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

CBLEB administration also partially alleviated organ damage in immunocompromised *SP*-infection rats. First, the spleen index was significantly increased, while the thymus index was decreased in both the *SP* and *SP*+CBLEB groups compared with those in the HC group (Figure 1B). CBLEB administration also tended to alleviate this increase in the spleen index ($P = 0.065$ in *SP* vs *SP* + CBLEB). Second, as shown in the photomicrographs of H&E-stained tissue sections, CBLEB administration significantly ameliorated pulmonary pathological changes, such as inflammatory cell infiltration, alveolar cavity collapse, alveolar wall and interstitial thickening, and fibrinoid exudation in the bronchial cavity in immunocompromised *SP*-infection rats (Figure 2A), as well as changes in the spleen, including enlarged red pulp area, decreased white pulp area, histiocyte proliferation, hyperaemia, haemosiderin deposition, and increased multinucleated giant cell (MGC) numbers (Figure 2B). Furthermore, CBLEB administration significantly lowered the histological scores in the lung and spleen (Figure 2A and B).

CBLEB Treatment Rescues Dysbiosis of the Gut Microbiota in Immunocompromised SP-Infection Rats

We obtained a total of 2,632,886 reads from 30 faecal samples from the three groups by 16S rDNA amplicon sequencing. Alpha diversity analyses showed that no significant differences in the Shannon and Chao1 indexes were present among the HC, *SP*, and *SP*+CBLEB groups, indicating similar community diversity in these groups (Figure 3A).

For beta diversity, a principal coordinates analysis (PCoA) plot based on the unweighted UniFrac distance demonstrated that the overall microbial compositions of the three groups varied (Figure 3B). This result was confirmed by analysis of similarity (ANOSIM) (R = 0.47, $P = 0.001$).

Then, we further analysed alterations in the relative abundance of bacterial taxa in the three groups. Compared with that of HC rats, the gut microbiota of rats in the *SP* group was significantly altered (Figure 3C). First, the bacterial taxa from the phylum Firmicutes, such as *Streptococcaceae, Fournierella, Monoglobus* and *Eubacterium ventriosum* group, were significantly enriched in the *SP* group, whereas *Peptostreptococcaceae, Jeotgalicoccus, Tuzzerella, Eubacterium, Romboutsia* and an unclassified *Peptostreptococcaceae* genus were significantly depleted. Among them, *Streptococcaceae, Peptostreptococcaceae, Romboutsia* and the unclassified genus
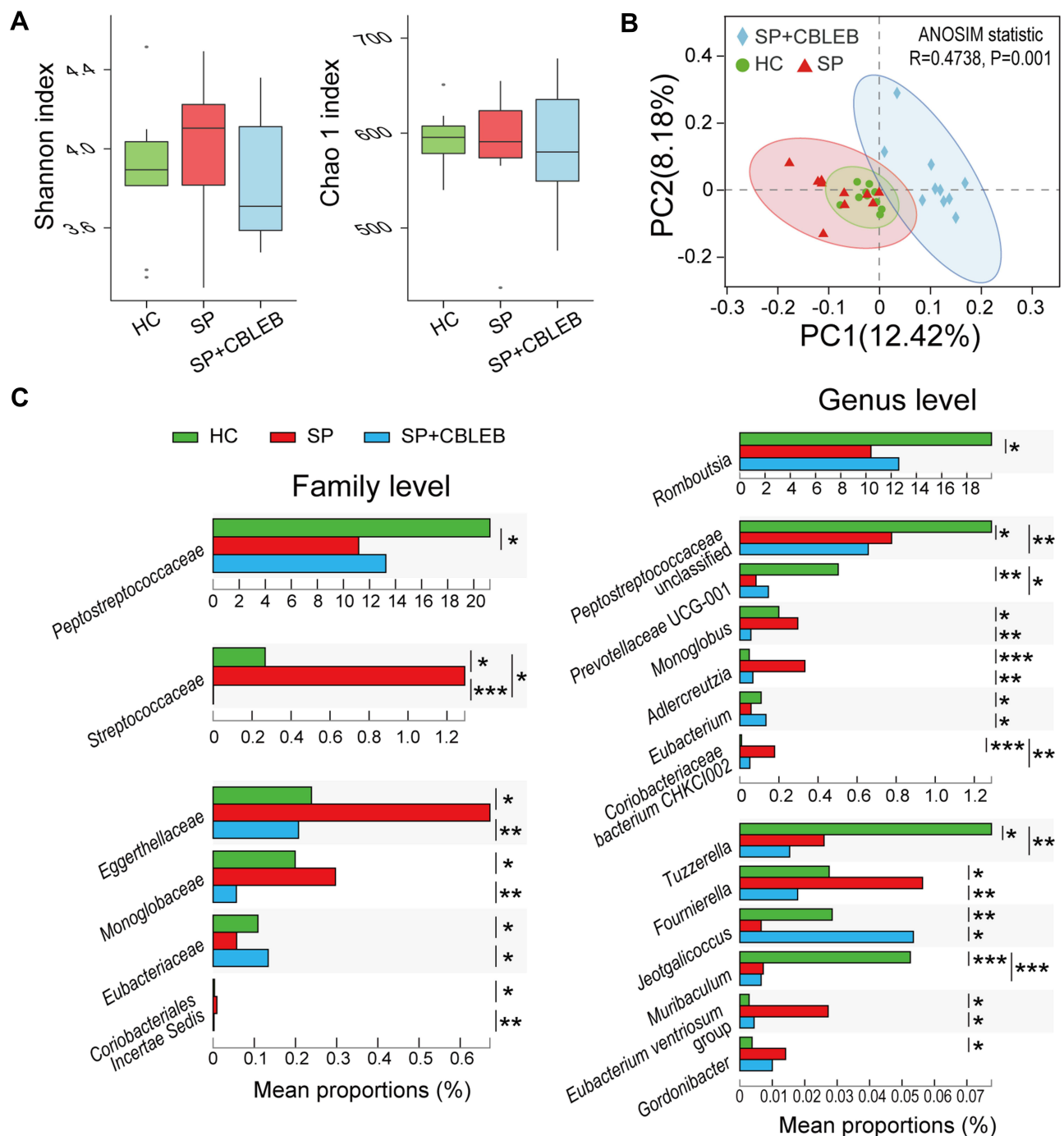
Figure 3 CBLEB treatment alleviates *SP* infection-induced dysbiosis of the gut microbiota. (**A**) Box plot of flora diversity and species richness estimated based on the Shannon indexes and Chao1 indexes. (**B**) Two-dimensional PCoA plot based on the unweighted UniFrac matrix confirmed by ANOSIM. (**C**) Alterations in the relative abundance of bacterial taxa in the *SP*, *SP*+CBLEB, and HC groups at the family and genus levels. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Peptostreptococcaceae were the most predominant taxa whose abundance was altered by *SP* (relative abundances were more than 1% in at least one group), suggesting their special role in the gut microbiota changes associated with *SP* infection. Second, the bacterial taxa from the phylum Actinobacteria, such as *Eggerthellaceae, Adlercreutzia, Gordonibacter* and *Coriobacteriaceae bacterium* CHKCI002, were significantly enriched in the SP group, whereas the bacterial taxa from the phylum Bacteroidetes, such as *Muribaculum* and *Prevotellaceae* UCG-001, were significantly depleted.

Compared with *SP* infection only, administration of CBLEB significantly alleviated the enrichment of *Streptococcaceae, Eggerthellaceae, Monoglobaceae*, Coriobacteriales Incertae Sedis, *Monoglobus, Adlercreutzia, Fournierella* and *Eubacterium ventriosum* group and the depletion of *Eubacteriaceae, Eubacterium* and *Jeotgalicoccus* in immunocompromised rats (Figure 3C). At the same time, compared with those in the HC group, the relative abundances of *Peptostreptococcaceae* and *Romboutsia* in the *SP*+CBLEB group did not change significantly, which indirectly implied the regulatory effect of CBLEB on the gut microbiota.

CBLEB Treatment Alleviates Disorder of Gut Metabolism in Immunocompromised SP-Infection Rats

To investigate the regulatory effect of CBLEB on the gut metabolism of *SP*-infected rats, we analysed faecal samples using GC-MS. In total, we identified 81 metabolites from the three groups. A OPLS-DA plot showed that the metabolic profiles of rats in the HC, *SP*, and *SP*+CBLEB groups were clearly separated from each other (Figure 4A), indicating significant differences in their faecal metabolomic profiles. The VIP values of 12 metabolites (arachidonic acid, D-ribose, D-fructose, pantothenic acid, heptanoic acid, L-methionine, 1-linolenoylglycerol, L-sorbose, 5-hydroxyhexanoic acid, m-cresol, 2-propenoic acid, and phosphoric acid) were greater than 1.5, suggesting their important contribution to distinguishing the metabolic profiles of the three groups in this OPLS-DA model (Figure 4B).

According to the comparison of each metabolite, the levels of 11 metabolites (2,4-di-tert-butylphenol, D-glucose, D-lyxose, 1-hexadecanol, 1-octadecanol, 1-pentadecanol, D-fructose, glyceric acid, m-cresol, L-alanine, and L-threonine) were higher, while the levels of (Z,Z)-9,12-octadecadienoic acid, 1-monolinolein, and 2-propenoic acid were lower in the *SP* group than in the HC group. Upon treatment with CBLEB, the changes in 7 of these metabolites (2,4-di-tert-butylphenol, D-lyxose, 1-octadecanol, glyceric acid, m-cresol, (Z,Z)-9,12-octadecadienoic acid, and 2-propenoic acid) became nonsignificant (Figure 4C), reflecting the regulatory effect of CBLEB on gut metabolism disorder.

CBLEB Treatment Alleviates Serum Metabolism Disorder in Immunocompromised SP-Infection Rats

Next, we investigated the regulatory effect of CBLEB on serum metabolism. A total of 60 metabolites were identified from the HC, *SP*, and *SP*+CBLEB groups. An OPLS-DA plot showed that the metabolic profiles of the rats in the three groups were also clearly separated from each other (Figure 5A), indicating significant differences in their serum metabolomic profiles. The VIP values of 6 metabolites (L-proline, D-glucose, malic acid, L-hydroxyproline, D-arabinose, and 2,3,4-trihydroxybutyric acid) were greater than 1.5, suggesting their important contribution to distinguishing the metabolic profiles of the three groups in this OPLS-DA model (Figure 5B).

During the comparison of each metabolite among the groups, we found that L-lysine and L-tyrosine were enriched, whereas malic acid, L-glutamic acid, L-proline, D-glucose, 2,3,4-trihydroxybutyric acid, and glyceric acid were depleted in the *SP* group compared with those in the HC group (Figure 5C). Upon treatment with CBLEB, the enrichment of L-tyrosine was significantly reversed in immunocompromised *SP*-infection rats, and differential serum levels of L-lysine, malic acid and D-glucose between the *SP* and HC groups were no longer observed, indicating the regulatory effect of CBLEB on serum metabolism disorder.

CBLEB Treatment Partially Reversed Transcriptional Regulatory Changes in the Lungs and Colons of Immunocompromised SP-Infection Rats

Compared with that in the lung samples of rats in the HC group, the transcription of 597 genes was increased and that of 399 genes was decreased in the lung samples of rats in the *SP* group ($P_{adj} < 0.05$). Mapping these genes to the KEGG pathway database revealed 21 upregulated pathways and 6 downregulated pathways. Among them, the upregulation of 11 pathways, which mainly relate to the immune response (Toll-like receptor (TLR) signalling, natural killer (NK) cell-mediated cytotoxicity, NOD-like receptor (NLR) signalling, haematopoietic cell lineage, and chemokine signalling), infection (malaria, measles, influenza A, and Epstein-Barr virus infection), and immune signalling (cytokine-cytokine receptor interaction, viral protein interaction with cytokines and cytokine receptors), was relieved by CBLEB treatment
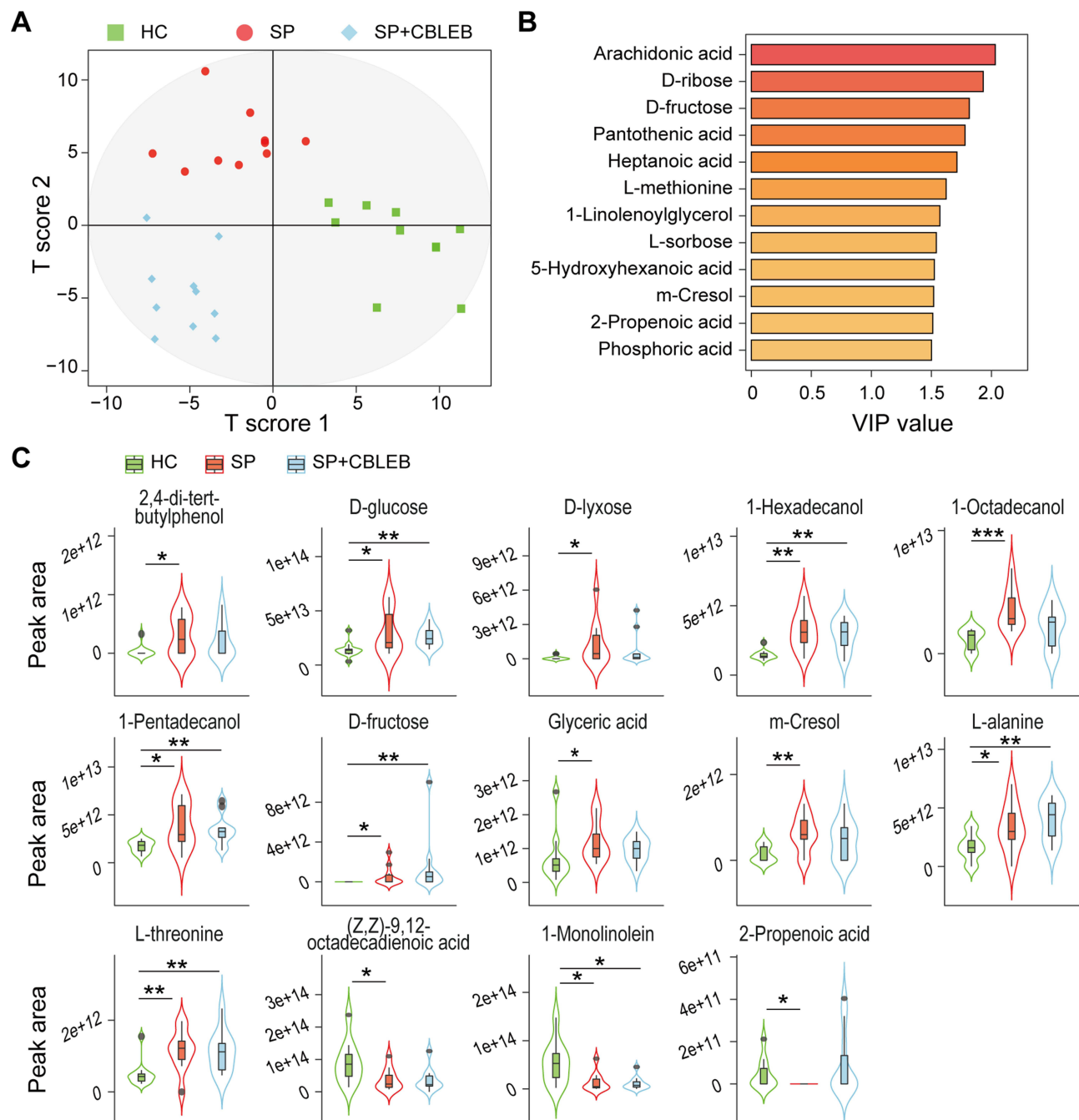
Figure 4 CBLEB alleviates *SP* infection-induced gut metabolism disorder. (**A**) OPLS-DA plot illustrating clear separation of gut metabolic profiles of the *SP, SP*+CBLEB, and HC groups. (**B**) VIP values of 12 metabolites with the highest contribution to the separation of the three groups in the OPLS-DA model. (**C**) Levels of fourteen differentially distributed metabolites in the three groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

(Figure 6A). To validate these transcriptome results, we conducted RT-PCR analysis of representative genes involve in above immune response pathways, including *Tlr2, Tlr9, Cxcl10, Ccl5, Tnf, Il-1b, Il-6, Cd80, Cd48* and *Nod2*. We found that their expression was similar to that in the transcriptome analysis (Supplemental Figure S2A and 2C). Moreover, when comparing the *SP*+CBLEB and HC groups, the changes in the upregulated pathways, including homologous recombination, Fanconi anaemia, p53 signalling, transcriptional misregulation in cancer and hepatitis C, and the down-regulated pathways, including oxidative phosphorylation, metabolism of xenobiotics by cytochrome P450, and hepato-cellular carcinoma, caused by *SP* infection were no longer significant. These results indicate that the regulation of
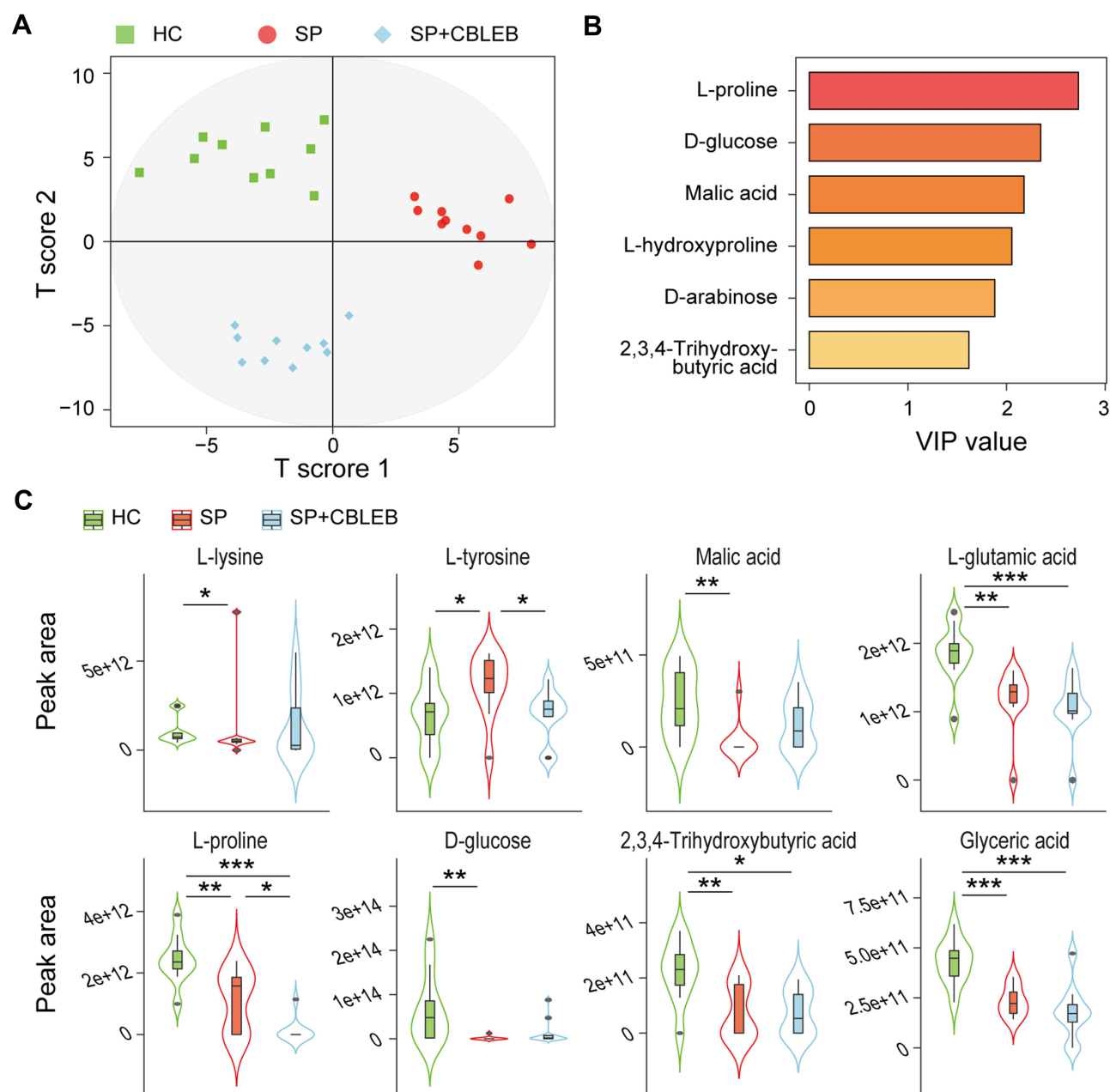
Figure 5 CBLEB alleviates *SP* infection-induced serum metabolism dysbiosis. (**A**) OPLS-DA plot illustrating clear separation of serum metabolic profiles of the *SP, SP+CBLEB*, and HC groups. (**B**) VIP values of 6 metabolites with the highest contribution to the separation of the three groups in the OPLS-DA model. (**C**) Levels of eight differentially distributed metabolites in the three groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

pathways such as immunity, infection, and metabolism in the lung may be involved in the mechanism of CBLEB activity against *SP* infection.

In the colon, the transcription of 210 genes was upregulated, while that of 360 genes was downregulated in the *SP* group compared with the HC group ($P_{adj} < 0.05$). Mapping these genes to the KEGG database revealed 23 downregulated pathways and 5 upregulated pathways. Among them, the upregulation of 3 pathways, axon guidance, African trypanosomiasis and *Staphylococcus aureus* infection, as well as the downregulation of 3 pathways, phenylalanine metabolism, renin-angiotensin system and chemical carcinogenesis, were reversed by CBLEB treatment (Figure 6B). To validate these results, we conducted RT-PCR analysis of representative genes involve in above pathways, including *Pak2* and *Pak4* in axon guidance, *Krt14* in *Staphylococcus aureus* infection, *Hbb* in African trypanosomiasis, *Aldh3a1* in phenylalanine
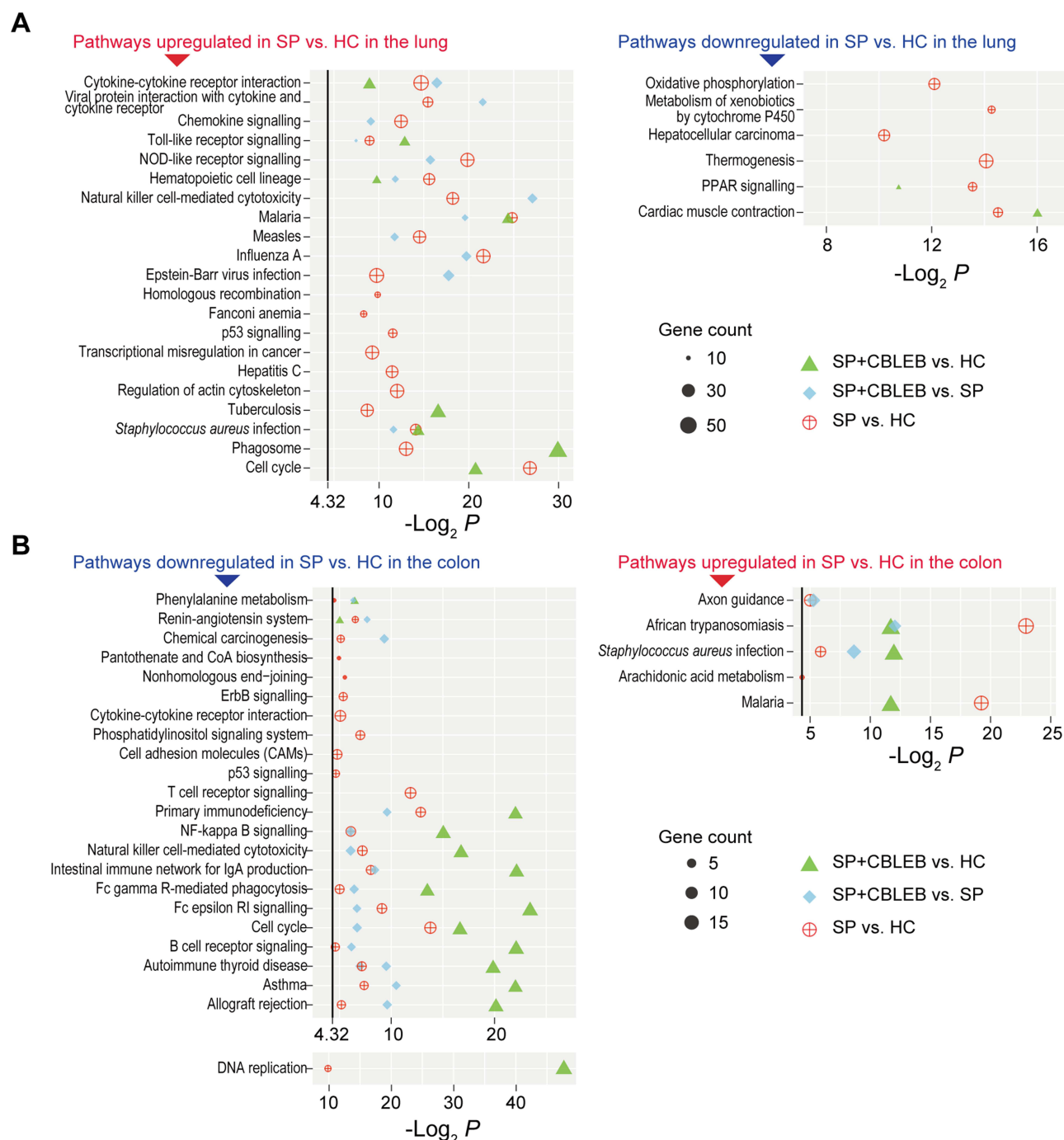
Figure 6 Pathways altered by *SP* infection and whose alterations were alleviated by CBLEB in the lung and colon. (**A**) Pathways upregulated or downregulated by *SP* infection and those whose alterations were alleviated by CBLEB in the lung. (**B**) Pathways upregulated or downregulated by *SP* infection and those whose alterations were alleviated by CBLEB in the colon. n = 3 in all groups; black vertical lines represent $-\mathrm{Log}_2 P = 4.32$ corresponding to $P = 0.05$; plots with $P \geq 0.05$ are not displayed.

metabolism and chemical carcinogenesis, and *Mcpt1* in renin-angiotensin system. We found that their expression was similar to that in the transcriptome analysis ([Supplemental Figure S2B](#) and [2D](#)). Moreover, when comparing the *SP* +CBLEB and HC groups, the upregulation of pathways, including pantothenate and CoA biosynthesis, nonhomologous end-joining, T cell receptor (TCR) signalling, ErbB signalling, cytokine-cytokine receptor interaction, phosphatidylinositol signalling system, cell adhesion molecules, and p53 signalling, and the downregulation of pathways, including arachidonic acid metabolism, caused by *SP* infection became nonsignificant. These results indicate that these pathways in the colon may be involved in the mechanism of CBLEB activity against *SP* infection.

The Gut Microbiota and Metabolites Involved in CBLEB Treatment are Closely Correlated with Improved Health

First, we analysed the correlations between the relative abundances of gut microbes and the levels of metabolites involved in CBLEB treatment using Spearman's rank correlation test. Both the absolute value of the correlation coefficient r > 0.4 and $P < 0.01$ were used as the screening threshold to identify extremely significant results.[20] *Adlercreutzia* and *Gordonibacter* were positively correlated with 1-octadecanol and m-cresol, while *Peptostreptococcaceae, Eubacterium* and *Romboutsia* were negatively correlated with glyceric acid (Figure 7A). Additionally, *Adlercreutzia* was also found to be negatively correlated with 2-propenoic acid.

Second, some gut microbes and metabolites may affect host health and diseases. Therefore, we analysed the correlations between gut microbes/metabolites and CBLEB treatment-improved serum indicators. We found that gut bacterial taxa, including *Eggerthellaceae, Adlercreutzia, Gordonibacter, Streptococcaceae, Fournierella* and *Monoglobus*, as well as gut metabolites, including 1-octadecanol, D-lyxose, glyceric acid and m-cresol, were negatively correlated with at least one of the following markers: serum malic acid, TP, ALB, and IB. Additionally, gut *Streptococcaceae* abundance was negatively correlated with serum basophil count and percentage and RDW; gut
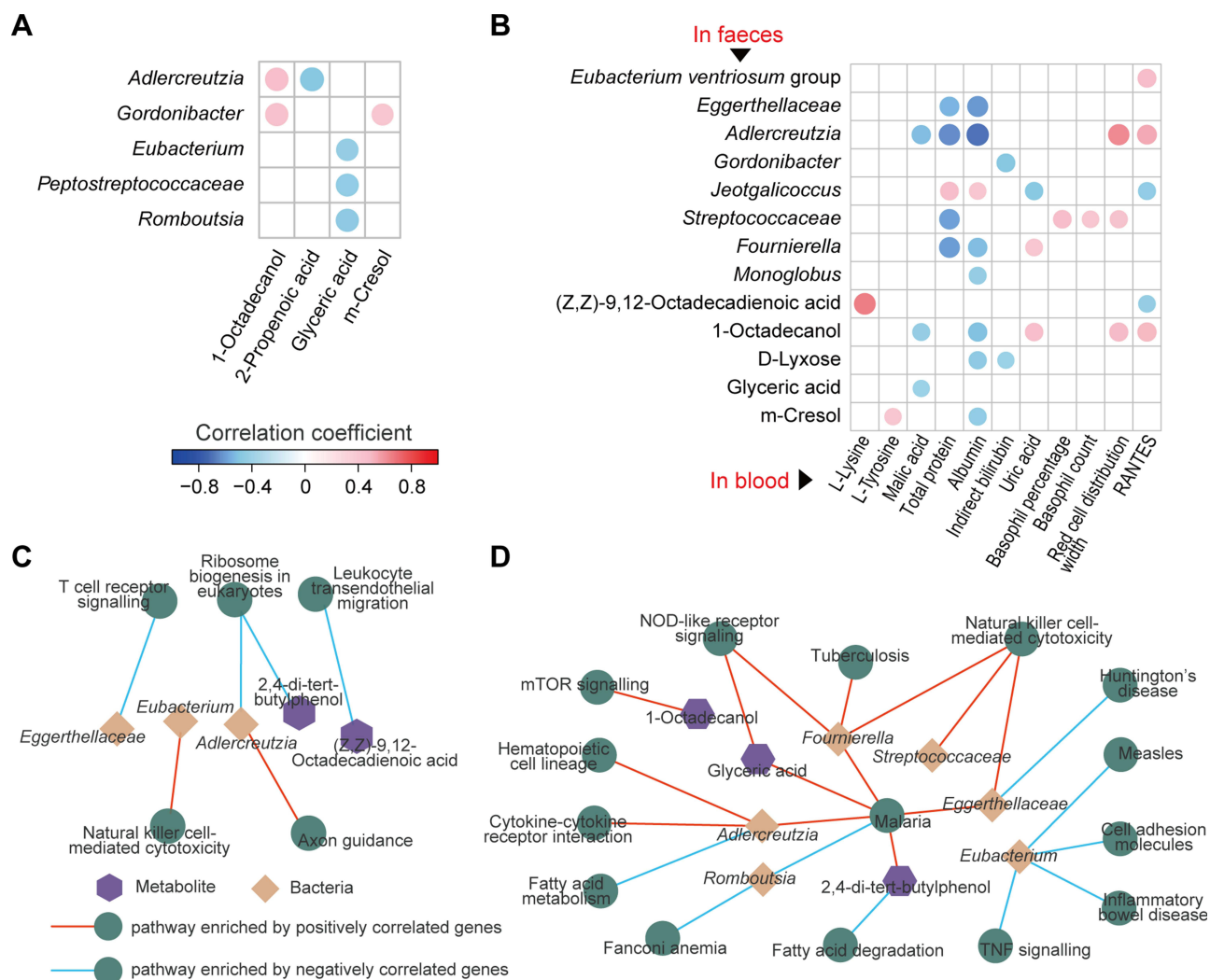

Figure 7 Associations among faecal bacteria, faecal and serum metabolites, and gut and lung pathways influenced by CBLEB. (**A**) Correlation of CBLEB-influenced faecal bacteria with faecal metabolites ($P < 0.01$). (**B**) Correlation of CBLEB-influenced faecal bacteria and metabolites with blood metabolites, liver and kidney function indicators, and cytokines ($P < 0.01$). (**C** and **D**) Pathways enriched by genes that were positively or negatively correlated with individual faecal bacteria or metabolites according to the KEGG database in the colon (**C**) and lung (**D**) ($P < 0.001$).

Adlercreutzia and 1-octadecanol were positively correlated with serum RDW and RANTES; and gut *Jeotgalicoccus* was positively correlated with serum TP and ALB and negatively correlated with uric acid and RANTES (Figure 7B).

The Gut Microbiota and Metabolites are Closely Correlated with the Pathways Involved in CBLEB Treatment

To reveal how gut microbes or metabolites affect the gut-lung axis during CBLEB treatment, we mapped the differentially expressed genes in the lung and colon that were significantly correlated with gut microbes and/or metabolites involved in CBLEB treatment to the KEGG pathway database.

In the colon, differential genes negatively correlated with *Eggerthellaceae* were significantly enriched in the TCR signalling pathway; those negatively correlated with *Adlercreutzia* or 2.4-di-tert-butylphenol were significantly enriched in ribosome biogenesis in eukaryotes; and those negatively correlated with (Z,Z)-9,12-octadecadienoic acid were significantly enriched in leukocyte transendothelial migration. Furthermore, the differential colon-expressed genes positively correlated with *Eubacterium* were enriched in NK cell-mediated cytotoxicity, and those positively correlated with *Adlercreutzia* were enriched in axon guidance (Figure 7C).

In the lung, differential genes negatively correlated with *Eubacterium* were significantly enriched in the measles, inflammatory bowel disease, cell adhesion molecules and TNF signalling pathways, those negatively correlated with *Romboutsia* were significantly enriched in Fanconi anaemia and malaria, those negatively correlated with *Eggerthellaceae* were significantly enriched in Huntington's disease, those negatively correlated with *Adlercreutzia* were significantly enriched in fatty acid metabolism, and those negatively correlated with 2.4-di-tert-butylphenol were significantly enriched in fatty acid degradation. Moreover, the differential lung-expressed genes positively correlated with *Fournierella, Streptococcaceae*, or *Eggerthellaceae* were significantly enriched in NK cell-mediated cytotoxicity; those positively correlated with *Adlercreutzia, Fournierella, Eggerthellaceae*, 2.4-di-tert-butylphenol or glyceric acid were significantly enriched in malaria; those positively correlated with *Fournierella* or glyceric acid were significantly enriched in NLR signalling; those positively correlated with *Adlercreutzia* were significantly enriched in cytokine-cytokine receptor interaction and haematopoietic cell lineage; those positively correlated with *Fournierella* were significantly enriched in tuberculosis; and those positively correlated with 1-octadecanol were significantly enriched in mTOR signalling (Figure 7D).

Discussion

S. pneumoniae is the leading cause of bacterial pneumonia, the single greatest infectious cause of death worldwide.[3] Microbiota regulation by probiotic administration has been reported to have great application potential in the preventing and treating *SP* infections. For example, heat-killed *Lactiplantibacillus pentosus* b240 protects mice from *SP* infection by augmenting the innate immune response;[21] dietary supplementation with *L. casei* CRL431[22] and *L. rhamnosus* CRL1505[23] is effective in modulating *SP* infection-induced coagulation activation, the inflammatory process, and lung damage in malnourished mice; and oral administration of a fermented milk drink containing *L. rhamnosus* GG (LGG), *Bifidobacterium* sp. B420, *L. acidophilus* 145, and *Streptococcus thermophiles* reduces the *SP* burden in the upper respiratory tract.[24] However, there have been no reports on the use of any probiotic drug in the clinical prevention and treatment of *SP* infection. The probiotic drug CBLEB is popular in China as a gut microbiota regulator and is used by nearly 10 million people every year. A previous study reported that taking CBLEB has a therapeutic effect on immunodeficiency.[15] In this study, we found that CBLEB can prevent weight loss in immunocompromised rats with *SP* infection, reduce lung inflammation and injury, and alleviate gut microbiota dysbiosis and gut and serum metabolism disorders. Its mechanism is related to the regulation of pathways involved in immunity, infection, and metabolism.

The gut microbiota plays an important role in host defence against pneumococcal pneumonia. The gastrointestinal tract is the primary interaction site between the host immune system and microbes, and it is well known that the gut microbiota is critical in establishing immune balance.[25,26] In a mouse model, the use of broad-spectrum antibiotics after *SP* infection causes the depletion of the gut microbiota, increases bacterial loads in the lungs and blood, augments inflammation, accelerates organ failure and even results in death. Faecal microbiota transplantation to gut microbiota-

depleted mice leads to normalization of pulmonary bacterial loads and TNF-α and IL-10 levels after *SP* infection.[27] We found that CBLEB administration alleviated the enrichment of *Streptococcaceae* and the depletion of *Eggerthellaceae, Adlercreutzia, Eubacterium, Fournierella*, and *Romboutsia* in the gut of immunocompromised *SP*-infection rats. Some of these microbes have been reported to have important physiological or pathological functions. For example, members of the genus *Eubacterium* have been recognized as beneficial microbes since they produce butyrate and contribute to the homeostasis of bile acid and cholesterol, and alterations in *Eubacterium* spp. have been linked with various human diseases.[28] Correspondingly, we found that the colon-expressed genes that positively correlated with *Eubacterium* were significantly enriched in the NK cell-mediated cytotoxicity pathway, while the lung-expressed genes that negatively correlated with this bacterial taxon were enriched in the measles, inflammatory bowel disease, cell adhesion molecule and TNF signalling pathways. These findings suggest that it is possible to infer the physiological and pathological functions of gut microbes through their correlated signalling pathways in the host. For example, we also found that colon-expressed genes positively correlated with *Fournierella* were enriched in the NK cell-mediated cytotoxicity and NLR signalling pathways; and the lung-expressed genes positively correlated with *Adlercreutzia* were enriched in the malaria, cytokine-cytokine receptor interaction and haematopoietic cell lineage pathways. These correlations imply the important roles of *Fournierella* and *Adlercreutzia* in the regulation of *SP* infection.

Some components and metabolites of gut microbes, such as lipopolysaccharides and short-chain fatty acids, which can be transported via the circulatory system, are important means of communication between the gut microbiota and the lungs.[29] However, there are no reports on faecal metabolome alterations during *SP* infection. We found that CBLEB alleviates the faecal enrichment of 1-octadecanol, 2-propenoic acid, 2.4-di-tert-butylphenol, D-lyxose, glyceric acid and m-cresol, as well as the depletion of (Z,Z)-9,12-octadecadienoic acid in immunocompromised *SP*-infection rats. Among these metabolites, 1-octadecanol, glyceric acid and (Z,Z)-9,12-octadecadienoic acid are commonly found in animals, plants and microbes and can also be used in cosmetics, medicines or food additives;[30–32] D-lyxose, a rare pentose found in bacterial glycolipids, is not usually utilized by microbes and is a precursor to antitumour and immunostimulatory α-galactosylceramide agents;[33] and 2-propenoic acid, 2.4-di-tert-butylphenol and m-cresol are compounds with low toxicity and antioxidant functions.[34–36] Nevertheless, the effects of these compounds on the lung and gut are not fully understood. In this work, we found that colon-expressed genes that negatively correlated with 2.4-di-tert-butylphenol were enriched in the ribosome biogenesis in eukaryotes pathway, as well as those negatively correlated with (Z,Z)-9,12-octadecadienoic acid were enriched in leukocyte transendothelial migration. Additionally, the differential lung-expressed genes that negatively correlated with 2.4-di-tert-butylphenol were enriched in fatty acid degradation, while those positively correlated genes were enriched in the malaria pathway; the differential lung-expressed genes positively correlated with 1-octadecanol were enriched in mTOR signalling, and those positively correlated with glyceric acid were enriched in the malaria and NLR signalling pathways. These findings elucidate how CBLEB-regulated gut metabolites function in the prevention and treatment of *SP* infection.

We also found that CBLEB alleviated the enrichment of L-tyrosine and L-lysine and the depletion of D-glucose and malic acid in the serum of immunocompromised *SP*-infection rats. Among these metabolites, the depletion of glucose in serum was observed in both a mouse model and patients challenged with *SP* infection, which may be due to liver metabolism disorder and excessive glucose consumption by the pathogen.[37] In contrast, aberrant tyrosine, lysine and malic acid levels have not been reported in *SP* infection, which may be mainly due to the scarcity of serum metabolomic research on these patients or models. In serum samples from tuberculosis patients, enrichment of lysine and tyrosine has also been observed, and the reason may be related to liver damage.[38] Importantly, malic acid is an intermediate metabolite of the citric acid cycle, and changes in its content reflect the regulation of the tricarboxylic acid (TCA) cycle by CBLEB treatment.[39] Therefore, the changes in serum metabolism also indicate the positive regulatory effect of CBLEB on *SP* infection. Since serum components directly reflect and affect health, the rehabilitation effect of CBLEB on *SP* infection-related dysbiosis of serum metabolism is one of the important manifestations of its therapeutic role and mechanism.

Our results also showed that the most likely mechanism of CBLEB combatting *SP* infection is its regulation of immune-related pathways in the host's lungs. First, CBLEB administration alleviates the upregulation of pathways involved in innate immunity, including TLR signalling, NLR signalling, and NK cell-mediated cytotoxicity. TLR2

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recognizes lipoteichoic acid and peptidoglycan and thereby plays a protective role in *SP* infection; streptolysin can bind to TLR4 to induce an inflammatory response, and NOD2 mainly recognizes the bacterial muramyldipeptide, which can induce the production of inflammatory cytokines such as TNF-α and IL-16 through the NF-κB pathway and can also induce caspase-related cell apoptosis.[40] It has been reported that intestinal microbes (*Lactobacillus reuteri, E. faecalis, Lactobacillus crispatus* and *Clostridium orbiscindens*) promote resistance to lung infection through NOD2 and GM-CSF.[41] NK cells are involved in the control of microbial infections and protect the host directly through the production of cytotoxic effectors. During *SP*-triggered lung infection, NK cells are one of the major cell types responsible for IFN-γ production.[42] Second, the upregulation of pathways involved in both innate and adaptive immunity, including the chemokine signalling and haematopoietic cell lineage pathways, was also alleviated by CBLEB treatment. *SP* strains causing invasive infections are surrounded by a polysaccharide capsule layer that inhibits innate and adaptive immune responses to infection.[7] Chemokines and their signalling pathways play a critical role in regulating innate and adaptive immune responses and are involved in many physiological and pathological processes, such as *SP* infection.[43] The haematopoietic cell lineage bears the hallmark characteristics of both the innate and adaptive immune systems. Haematopoietic tissues accelerate both the release of mature granulocytes and the production of new granulocytes to support and reinforce the ongoing recruitment of neutrophils in tissues infected by pathogens.[44] Additionally, CBLEB alleviated the upregulation of pathways related to infection (malaria, measles, influenza A, and Epstein-Barr virus infection) and immune signalling (cytokine-cytokine receptor interaction, viral protein interaction with cytokines and cytokine receptors), which also reflects that the improvement in immune-related pathways is an important mechanism of CBLEB activity.

In the colon, we found that partial restoration of those infection-, inflammation- and metabolism-related pathways is the main mechanism by which CBLEB combats *SP* infection. First, CBLEB alleviates the upregulation of the axon guidance, arachidonic acid metabolism, African trypanosomiasis and *S. aureus* infection pathways. The expression of genes related to the axon guidance pathway, the critical step in neural circuit development, has been reported to be upregulated in the gastrointestinal tract of neonatal goats with diarrhoea.[45] Arachidonic acid may be released from the phospholipids of the cell membrane during phospholipid hydrolysis and is important for the proper function of the immune system, such as promoting allergies and inflammation.[46] Second, CBLEB alleviates the downregulation of some important life activity-related pathways, including phenylalanine metabolism, pantothenate and CoA biosynthesis, renin-angiotensin system, nonhomologous end-joining, ErbB signalling, TCR signalling, phosphatidylinositol signalling, cell adhesion molecules, and cytokine-cytokine receptor interaction. Defects in the phenylalanine metabolism pathway are the cause of three well-known disorders: phenylketonuria, albinism, and alkaptonuria;[47] coenzyme A (CoA) is an essential cofactor for cell growth and involved in many metabolic reactions, such as phospholipid synthesis, fatty acid synthesis and degradation, and TCA cycle function;[48] the renin-angiotensin system is involved in the regulation of smooth muscle of the blood vessels and bowel, the absorption and secretion of glucose, amino acids, fluids and electrolytes, the permeability of the gut mucosa, and gut inflammation;[49] nonhomologous end-joining is the predominant DNA repair pathway required to detect, process, and ligate DNA double-stranded breaks throughout the cell cycle, and defects in its function may result in severe combined immunodeficiency;[50] disruption of the ErbB signalling network, an essential component for mucosal protection and/or the adaptive response to external injury in the gut, will result in major defects in gut epithelial development and in the reparative response to gut injury;[51] TCR activation promotes a number of signalling cascades that ultimately determine cell fate through regulating cytokine production, cell survival, proliferation, and differentiation; various phosphoinositides are involved in many signalling pathways, such as the PI3K-Akt pathway that mediates cell proliferation, survival, and metabolism;[52] and cell adhesion molecules play a critical role in many biological processes, such as haemostasis, the immune response, inflammation, embryogenesis, and neuronal tissue development.[53] Therefore, the regulation of these pathways in the intestine is an important part of the mechanism by which CBLEB combats *SP* infection.

In conclusion, CBLEB administration reduces the pathogen load in the lungs, ameliorates weight loss and lung inflammatory injury, and improves systemic health in *SP*-infected rats. Its mechanism of action is related to the role of

CBLEB in remodelling the gut microbiota, improving faecal and serum metabolism, and thereby regulating the host's immune response.

Data Sharing Statement

The original contributions presented in the study are publicly available. This data can be found in NCBI Sequence Read Archive (SRA) database with BioProject ID PRJNA771216.

Ethical Statement

All experiments were approved by the Institutional Animal Care and Research Advisory Committee at Zhejiang University and followed the Laboratory animal-Guidelines for ethical review of animal welfare (GB/T 35892-2018).

Acknowledgments

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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