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Alterations in Gut Microbiota and Serum Metabolites in Children with *Mycoplasma pneumoniae* Pneumonia

Shu Wang[1,*], Chengzhong Liu[2,*], Ruipei Ding[2], Shumei Wang[2], Yousheng Ye[1], Maozhang He[2]

[1]Department of Geriatrics, The First People's Hospital of Hefei, Hefei, 230061, People's Republic of China; [2]Department of Microbiology, School of Basic Medical Sciences, Anhui Medical University, Hefei, 230032, People's Republic of China

*These authors contributed equally to this work

Correspondence: Maozhang He; Yousheng Ye, Email jnzd_hemaozhang@hotmail.com; ye.ys@qq.com

Background: Over the past years, there has been a significant increase in the incidence of *Mycoplasma pneumoniae* (MP) infections, particularly among pediatric patients, nationwide. An emerging body of research has established a link between dysbiosis of the host microbiome and the metabolic functioning of the host, which contributes to the development of respiratory diseases.

Methods: A total of 25 children were included in the study, comprising 15 pneumonia patients and 10 healthy children. Stool samples were collected from all participants to analyze the 16S ribosomal RNA (16S rRNA) gene, while serum samples were prepared for untargeted metabolomics to qualitatively and quantitatively assess short-chain fatty acids.

Results: The gut microbial composition of individuals with *Mycoplasma pneumoniae* pneumonia (MPP) exhibited significant differences compared to healthy children. Notably, diseased children demonstrated higher microbial diversity and an enrichment of opportunistic pathogens, such as *Erysipelatoclostridium* and *Eggerthella*. Analysis revealed elevated levels of two specific short-chain fatty acids, namely acetic acid and isobutyric acid, in the MPP group, suggesting their potential as biomarkers for predicting MP infection. Metabolomic signature analysis identified a significant increase in major classes of glycerophospholipids in the MPP group. Moreover, we identified a total of 750 significant correlations between gut microbiota and circulating serum metabolites. MPP enriched genera *Erysipelatoclostridium* and *Eggerthella*, exhibited negative associations with indole-3-butyric acid. Additionally, *Eggerthella* showed a positive correlation with inflammatory metabolites LPC (18:0).

Discussion: Collectively, these findings provide novel insights into the selection of potential biomarkers and the pathogenesis of MPP in children based on the gut microbiota and systemic circulating metabolites.

Keywords: *Mycoplasma pneumoniae*, gut microbiota, serum metabolomics, short-chain fatty acids, inflammation

Introduction

Mycoplasma pneumoniae (MP) is one of the primary pathogens responsible for both upper and lower respiratory tract infections in humans. It can transmit between individuals through respiratory droplets containing microorganisms, which are dispersed via coughing or sneezing. The incubation period for MP can vary from one to three weeks, and infections are prevalent in both children and adults globally.[1,2] The proportion of MP in pneumonia cases increases progressively with age. It has become the most common pathogen in community-acquired pneumonia among children, accounting for 10% to 40% of community-acquired pneumonia (CAP) cases.[3–5] In adults in Europe, the incidence of endemic pneumonia ranges from 4% to 8%, which can escalate to 20–70% during epidemics.[6] The prevalence of MP exhibits seasonality, indicating that while MP infections occur throughout the year in Beijing, there is a peak during the pneumonia season from October to January, where MP accounts for 27.78% of CAP cases, reflecting a pattern of annual epidemic peaks.[7] Macrolide antibiotics, as the first-line treatment for MP infections, have seen a rise in the prevalence of macrolide-resistant *Mycoplasma pneumoniae* (MRMP) due to their extensive use.[8] There is a significant variation in the

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prevalence of MRMP across different countries and regions globally. In Western Europe and North America, the prevalence of MRMP is below 30%,[9] whereas it is considerably higher in Asian countries, with rates in our country reaching up to 90%.[10]

MP promotes the occurrence of inflammatory responses by inducing proliferation of B lymphocytes and T lymphocytes, secretion of major histocompatibility complex proteins, and release of various cytokines such as interleukins, interferons, tumor necrosis factor, and colony-stimulating factors. This leads to increased airway obstruction and heightened airway hyperresponsiveness, exacerbating asthma.[11] Apart from directly causing damage to the lungs, MP can mimic host cell components, activate the host's immune response, form immune complexes with corresponding antigens, produce neutrophil chemotactic factors, and activate complement. This results in extensive infiltration of leukocytes at the site of injury, release of lysosomal hydrolases, and subsequent destructive damage and lesions in multiple organs such as the liver, kidneys, brain, smooth muscles, and lungs.[12] In children, MP is the most common etiology of pediatric encephalitis. However, up to now, detection, prevention, and treatment of *Mycoplasma pneumoniae* face a series of challenges. In terms of detection, there are various complex methods available for *Mycoplasma pneumoniae* detection.[13] While nucleic acid testing offers high sensitivity and specificity, it is associated with complex procedures and high costs. Serological testing and cell culture methods, on the other hand, have lower sensitivity and specificity. Early diagnosis is also a challenge as the symptoms of *Mycoplasma pneumoniae* infection resemble those of other respiratory pathogens, leading to potential misdiagnosis or underdiagnosis. Moreover, there is a growing concern regarding antibiotic resistance in *Mycoplasma pneumoniae*, with varying degrees of resistance observed against commonly used antibiotics, which further complicates treatment.[14] Accordingly, rapid and accurate diagnostic methods, as well as novel treatment targets are required.

Human-associated microbiota refers to the community of bacteria, fungi, protozoa, archaea, and viruses that reside within the human body. Each individual's microbiota is unique, and its diversity and metabolic products exhibit dynamic changes throughout different stages of life.[15] Extensive research on the gut microbiota over the years has revealed its essential connection to human health, including conditions such as obesity and diabetes,[15] hypertension,[16] musculoskeletal function,[17] mental disorders,[18] inflammatory bowel diseases,[19] and even aging.[17] As is known to all, pulmonary diseases are often accompanied by gastrointestinal symptoms and vice versa, the gut/respiratory microbiota and its metabolites are bidirectional communication between the gut and lung is called the gut-lung axis.[20] The gut-lung axis has been demonstrated play a pivotal role in respiratory diseases and is often associated with chronic gastrointestinal disorders. This connection highlights the interplay between gut microbiota and lung health, suggesting that disruptions in respiratory function, such as pathogens infection, may influence gut conditions and vice versa. Understanding this axis could lead to new therapeutic approaches for managing both respiratory and gastrointestinal diseases. Research has found that certain serum inflammatory factors and pro-inflammatory cytokines were significantly elevated in children with MP infection, and there was an increased abundance of fatty acid metabolic pathways in the gut, suggesting a potential link between the gut-lung axis and gut microbiota metabolites.[11]

Moreover, the metabolome undergoes alterations as the ultimate response of a biological system to stimuli, such as pathogens infection, thereby theoretically providing a more accurate depiction of the phenotype. Consequently, this enables the evaluation of cellular states and processes at a functional level.[21] A recent study indicated that MP infection markedly altered the metabolites involved in glycerophospholipid and sphingolipid metabolism, and established a correlation with extrapulmonary complications. Therefore, these metabolic disorders can indirectly indicate the presence of pulmonary inflammation and contribute to the development of various severe extrapulmonary complications.[22] To date, there is a lack of research investigating the interplay between MP infection, gut microbiota, and host metabolism. Furthermore, there is limited research exploring the potential of targeting the interactions between microbiota and metabolism for MP infection-related diagnostics and therapeutic interventions. In this study, we combined 16S rRNA gene sequencing, non-targeted metabolomics to select the most discriminant microbial and metabolic biomarkers. We aim to investigate the intestinal microbiota in children with *Mycoplasma pneumoniae* pneumonia (MPP) and explore the relationship between MP infection, the microbiota, and associated metabolites. By maintaining the balance of the intestinal microbiota, our goal is to deepen the understanding of the mechanisms

underlying MP infection and provide a certain basis for new therapeutic measures and the prevention of respiratory diseases in children caused by MP and potential damage to other organs outside the lungs.

Materials and Methods

Study Design and Participants

A total of participants (ages ranged from three to five years; sex: female) were recruited and assessed in the First People's Hospital of Hefei, Anhui. Briefly, children with cough and low-grade fever were screened for infection of *Mycoplasma pneumoniae* using serum antibody tests. *Mycoplasma pneumoniae*-specific immunoglobulin M (IgM) titer that great than 1:32 was regarded as MP infection (Table S1).

Serum Sample Collection and Pretreatment

Elbow vein blood samples (5 mL) were collected from MP patients before antibiotic usage and MP-negative volunteers during the fasting state between 6 a.m. and 8 a.m. using vacuum negative-pressure blood collection vessels. The blood samples were immediately centrifuged for 15 minutes (3500 rpm, 4 °C), and the serum was aliquoted (0.5 mL) and stored at −80 °C until analysis.[23] Then, all samples were thawed on ice before short-chain fatty acids (SCFAs) and untargeted metabolomics profiling.[24] Untargeted metabolomics of sera samples was performed similarly to previously described methods with slight modification.[25] In brief, eighty microliters of sample were mixed with 320 μL of acetonitrile by vortexing for 60 seconds. Then, the sample was centrifuged at 15,000 rpm for 10 min (4°C) to precipitate the protein. Fifty microliters of a 25% (by volume) acetonitrile aqueous solution were used to reconstitute the sample before the liquid chromatography–mass spectrometry (LC-MS) analysis. Quality control (QC) samples were prepared by pooling the same volume of each sample to evaluate the reproducibility of the analysis. The pretreatment of the QC samples paralleled and was the same as that of the study samples. The QC samples were evenly inserted in each set of the analysis running sequence to monitor the stability of the large-scale analysis.[26]

Quantification the Concentration of Serum Short-Chain Fatty Acids

All of the 11 SCFA standards were obtained from ZZ Standards Co., LTD. (Shanghai, China). Methanol (Optima LC-MS), acetonitrile (Optima LC-MS), ammonium acetate, and isopropanol (Optima LC-MS) were purchased from Thermo-Fisher Scientific (FairLawn, NJ, USA). Ultrapure water was purchased from Millipore (MA, USA). An ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) system (Vanquish™ Flex UHPLC-TSQ Altis™, Thermo Scientific Corp., Germany) was used to quantitate SCFA in Novogene Co., Ltd. (Beijing, China). Separation was performed on a Waters ACQUITY UPLC BEH C18 column (2.1×100mm, 1.7μm) which was maintained at 40°C. The mobile phase, consisting of 10 mm ammonium acetate in water (solvent A) and acetonitrile: isopropanol (1:1) (solvent B), was delivered at a flow rate of 0.30 mL/min. The solvent gradient was set as follows: initial 25% B, 2.5 min; 25–30% B, 3 min; 30–35% B, 3.5 min; 35–38% B, 4 min; 38–40% B, 4.5 min; 40–45% B, 5 min; 45–50% B, 5.5 min; 50–55% B, 6.5 min; 55–58% B, 7 min; 58–70% B, 7.5 min; 70–100% B, 7.8 min; 100–25% B, 10.1min; 25% B, 12 min. The mass spectrometer was operated in negative multiple reaction mode (MRM) mode. Parameters were as follows: IonSpray Voltage (−4500V), Sheath Gas (35psi), Ion Source Temp (550°C), Auxiliary Gas (50psi), Collision Gas (55 psi). Next, LC-MS was used to detect the concentration series of standard solution. The ratio of concentration of standard to internal standard as abscissa, and the ratio of peak area of standard to internal standard as ordinate to investigate the linearity of standard solution. The correlation coefficient (r) > 0.99 of each metabolite were the necessary condition. The limit of quantification (LOQ) was determined by the method of signal-to-noise ratio (S/N), which is comparing the signal measured by the standard solution concentration with the blank matrix. Generally, when the S/N = 10:1, the corresponding concentration is the LOQ. SCFAs in samples were identified by comparing both MS spectra and retention times with those of standard compounds by calibration curve.

Untargeted LC-MS/MS Analysis, Data Processing and Metabolite Identification

The raw data files generated by UHPLC-MS/MS were processed using the Compound Discoverer 3.3 (CD3.3, ThermoFisher) to perform peak alignment, peak picking, and quantitation for each metabolite. The main parameters

were set as follows: peak area was corrected with the first QC, actual mass tolerance, 5 ppm; signal intensity tolerance, 30%; and minimum intensity, and other parameters. After that, peak intensities were normalized to the total spectral intensity. The normalized data was used to predict the molecular formula based on additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud (https://www.mzcloud.org/), mzVault and MassList database to obtain the accurate qualitative and relative quantitative results. Statistical analyses were performed using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS release 6.6), When data were not normally distributed, standardize according to the formula: sample raw quantitation value / (The sum of sample metabolite quantitation value / The sum of QC1 sample metabolite quantitation value) to obtain relative peak areas; And compounds whose CVs of relative peak areas in QC samples were greater than 30% were discarded, and finally the metabolites' identification and relative quantification results were obtained. These metabolites were identified using the KEGG database (https://www.genome.jp/kegg/pathway.html), HMDB database (https://hmdb.ca/metabolites) and LIPIDMaps database (http://www.lipidmaps.org/).

Stool Collection and 16S rRNA Gene Sequencing Analysis

Stool samples were collected before antibiotics administration by the participants' parents, who received detailed instructions on the proper collection and storage procedures. Following collection, the samples were promptly frozen at −80°C. DNA extraction was performed with QIAamp DNA mini kit (Qiagen). DNA library preparation and 16S ribosomal RNA gene sequencing were performed by NovoGene, Tianjin, China. The extracted paired DNA was utilized for amplification and sequencing of the 16S rRNA gene, following the established protocol of the Earth Microbiome Project, as an integral part of the Qiagen AllPrep DNA/RNA Mini Kit. The amplification of the 16S rRNA genes, specifically targeting the V3-V4 region using specific primers (341F [CCTAYGGGRBGCASCAG] and 806R [GGACTACNNGGGTATCTAAT]), was carried out using a one-step, single-indexed PCR approach with duplicate amplification. This process was performed in batches, incorporating appropriate negative controls. Subsequently, the Illumina MiSeq platform (Illumina, San Diego, USA) was employed for paired-end sequencing (2 × 250bp). The resulting sequencing data were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) pipeline. Taxonomic classification was performed against the Silva-132 database.

Statistical Analysis

Principal components analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) were performed to elucidate the metabolic changes between MP patients and controls using R package ropls. Mann–Whitney *U*-test was applied to calculate the statistical significance, metabolites with VIP > 1 and P-value< 0.05 and fold change ≥ 2 or FC ≤ 0.5 were considered to be differential metabolites. Volcano plots were used to filter metabolites of interest which based on log2 (Fold Change) and -log10 (p-value) of metabolites by ggplot2 in R language. For clustering heat maps, the data were normalized using z-scores of the intensity areas of differential metabolites and were plotted by Pheatmap package in R language. The functions of these metabolites and metabolic pathways were studied using the KEGG database. The metabolic pathways enrichment of differential metabolites was performed, when ratio was satisfied by x/n > y/N, metabolic pathway were considered as enrichment, when P-value of metabolic pathway < 0.05, metabolic pathway were considered as statistically significant enrichment. With respect to metagenomic data, linear discriminant analysis Effect Size (LEfSe)[27] analysis was applied on the relative abundance of species, gene families, and pathways to identify disease-associated biomarkers. Features with Linear discriminant analysis (LDA) score > 2.0 and p-value < 0.05 were considered as statistically significant. In addition, correlation analysis between gut microbiota and metabolites was performed using Spearman rank correlation analysis. All statistical tests were two-tailed, and the differences reached the statistical significance was set at $p < 0.05$.

Results

The Overall Landscape of Participants and Data Outputs

In the present study, a total of 25 participants were recruited. Feces and serum specimens were collected from the participants, with the feces specimens being utilized for 16S rRNA amplicon sequencing and the serum specimens for

untargeted metabolomic profiling and short-chain fatty acids determination (Figure 1a). Consequently, a 16S rRNA microbial data set consisting of 2,344,155 filtered high-quality sequences was generated, with an average of 90,160 ± 18,758 sequences per individual sample. A sum of 4623 amplicon sequence variants (ASVs, equivalent to bacteria at the species level) were identified in the gut communities based on 100% sequence similarity. Untargeted metabolomics analysis based on ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) was performed to analyze the serum samples. The QC samples in the PCA plot were observed to cluster tightly together, confirming the reliability of the present study (Figure S1a and b). The partial least squares discriminant analysis revealed significantly clear separations between the MPP and MP-negative (MPN) groups without overfitting (Figure S1c and d). After peak alignment, peak picking, and deconvolution of the untargeted metabolomics data, metabolic features with a relative standard deviation (RSD%) greater than 30% in QC samples were excluded, and only those peaks present (non-zero value) in more than 50% of the total samples were included in the data analysis. Ultimately, 756 and 494 metabolites in the positive and negative ion modes, respectively, were reserved based on searches of mzCloud, mzVault, and MassList or confirmed with authentic standards from Novogene company. These variables were used for the subsequent multivariate and univariate analyses.

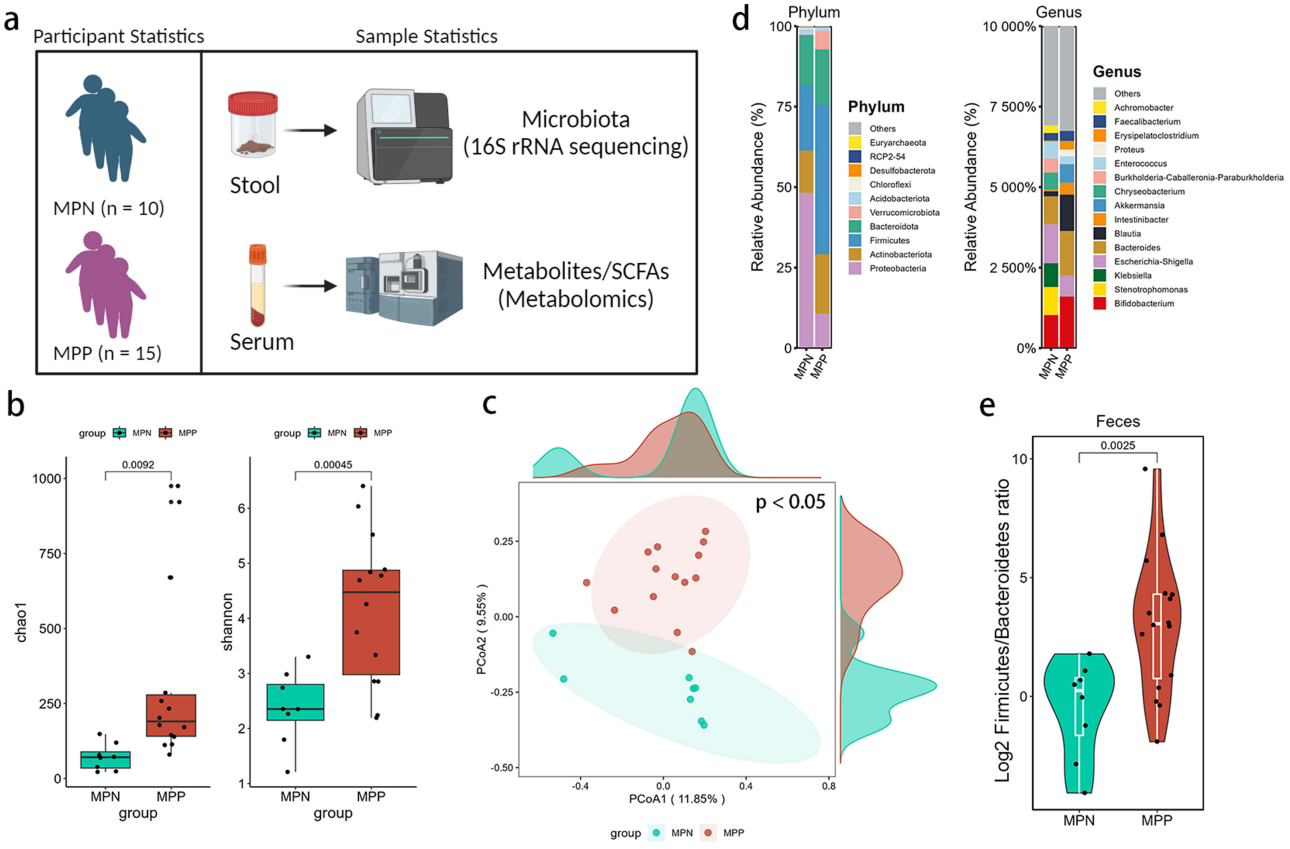

Figure 1 MP infection is associated with significant changes in the gut microbiota. (**a**) The study workflow begins with the collection of biological samples, including serum and stool samples, from participants grouped into two categories: the MP infection group and the normal control group. Following collection, microbial DNA is extracted from the stool samples, and the 16S rRNA gene is amplified using specific primers for sequencing. High-throughput sequencing is performed to characterize the gut bacterial communities, and bioinformatics tools are utilized to analyze the resulting data, focusing on the diversity and composition of the microbiota. Concurrently, metabolomic analysis is conducted on serum samples to provide insights into the metabolic changes associated with MP infection. This comprehensive workflow enables a detailed examination of the interactions between MP infection, gut microbiota, and metabolic profiles. (**b**) Box and whisker plots show the alpha diversity indices (richness and diversity) of the bacterial communities at the amplicon sequence variant (ASV) level in the MPP group and the MPN group. The reduced richness and diversity in the MPP group suggest that MP infection is associated with a less diverse gut microbiota. (**c**) Principal component analysis (PCA) based on the Bray-Curtis dissimilarity metric, which measures the compositional differences between the gut microbial communities, reveals a significant separation between the MPP and MPN groups. This indicates that MP infection is linked to distinct changes in the overall gut microbial composition. (**d**) The relative abundance of bacterial phyla and genera differs between the MPP and MPN groups, suggesting specific taxonomic shifts in the gut microbiota due to MP infection. (**e**) The ratio of Firmicutes compared to Bacteroidota, a commonly used indicator of gut dysbiosis, is significantly higher in the MPP group compared to the MPN group, further highlighting the perturbation of the gut microbiome in response to MP infection.

Microbial Dysbiosis in the Stool of MPP Patients

To investigate the effect of *Mycoplasma pneumoniae* (MP) infection on gut microbial composition, 16S rRNA gene sequencing was performed on fecal samples. Alpha diversity indices analysis indicated that MP infection increased the gut microbial richness and diversity based on Chao1 and Shannon parameters (Figure 1b). Principal coordinate analysis (PCoA) and PERMANOVA test based on Bray-Curtis distance revealed a significantly distinct bacterial community structure between MP patients and healthy volunteers ($P < 0.05$) (Figure 1c). Comparison of the relative abundances of the bacterial communities between MPP and MPN individuals showed notable changes at the phylum and genus levels. Specifically, MPP patients exhibited a higher relative abundance of Firmicutes and Verrucomicrobiota than MPN participants. Furthermore, MP infection was found to increase the relative abundance of several bacterial genera, including *Erysipelatoclostridium, Intestinibacter, Blautia*, and *Proteus* (Figure 1d, Figure S2). The Firmicutes/Bacteroidetes (F/B) ratio, which reflects the relative abundance of these two major bacterial phyla in the gut microbiome, has been suggested as a potential indicator of overall gut health and association with various health conditions. Accordingly, a significantly higher F/B ratio was observed in the MPP group compared to the MPN group (Figure 1e). Additionally, LEfSe analysis identified significant differences in microbial abundances between the two groups, with a total of 48 genera and species enriched in the MP group, such as *Erysipelatoclostridium*, Ruminococcus gnavus group, *Blautia*, and *Eggerthella*, while *Streptococcus peroris* and *Actinomyces* were decreased in MP patients (Figure 2). These findings suggest gut microbial dysbiosis in MPP patients.

Effects of MP Infection on the SCFAs in the Serum

To determine the effect of *Mycoplasma pneumoniae* infection on SCFAs levels that might be caused by altered gut microbial composition, the concentrations of eleven SCFAs (including acetic acid, propionic acid, butyric acid, and valeric acid) were measured in the serum of study participants. The results showed that the levels of acetic acid and isobutyric acid were significantly increased in the MPP group compared to the MPN group ($p<0.05$, Figure 3a), while no significant differences were observed for the other SCFAs. Notably, the diagnostic performance of using acetic acid and isobutyric acid as potential biomarkers for MP infection was evaluated. The area under the receiver operating characteristic curve (AUC) values reached 77.1% and 75.7%, respectively (Figure 3b and c), suggesting that these MP infection-related SCFA biomarkers could be utilized as potential markers for MP infection.

Changed Metabolic Landscape in Serum of MP Patients

Serum metabolites serve as a crucial link between the gut microbiome and its host by regulating metabolism. Principal component analysis (PCA) revealed a significant separation in the serum metabolite profiles between the MPP and MPN groups (Figure 4a). Volcano plot analysis identified a total of 257 significantly different metabolites, with 140 upregulated and 117 downregulated metabolites in the MPP group compared to the MPN group (Figure 4b). Among the top 25 upregulated and downregulated metabolites, a significant increase in serum glycerophospholipids of certain major classes was observed in the MPP group, such as LPC O (17:0), LPC O (18:0), Lysopc (14:0), LysoPE (18:0), PC (40:7), and PC (18:0_22:6), indicating an alteration in glycerophospholipid metabolism. Conversely, steroid biosynthesis-related metabolites, including 17α-Hydroxypregnenolone, 17α-Hydroxyprogesterone, Corticosterone, Dehydroepiandrosterone (DHEA), Testosterone sulfate, and Androsterone glucuronide, showed a significant decrease. Additionally, the levels of Indole-3-butyric acid, Indole-3-lactic acid, Bilirubin, and Taurocholic acid were notably reduced (Figure 4c).

To investigate the underlying metabolic pathways in patients with MP infection, we performed KEGG pathway analysis on the selected differentially abundant metabolites and identified the top 20 significantly altered metabolic pathways. These pathways included Glycerophospholipid metabolism, Steroid hormone biosynthesis, Primary bile acid biosynthesis, beta-Alanine metabolism, and Taurine and hypotaurine metabolism (Figure 4d). These results reveal the specific metabolic pathways that are enriched or depleted in the MPP group compared to the MPN group (Figure S3a and b), providing insights into the potential functional implications of the observed serum metabolomic changes during MP infection.

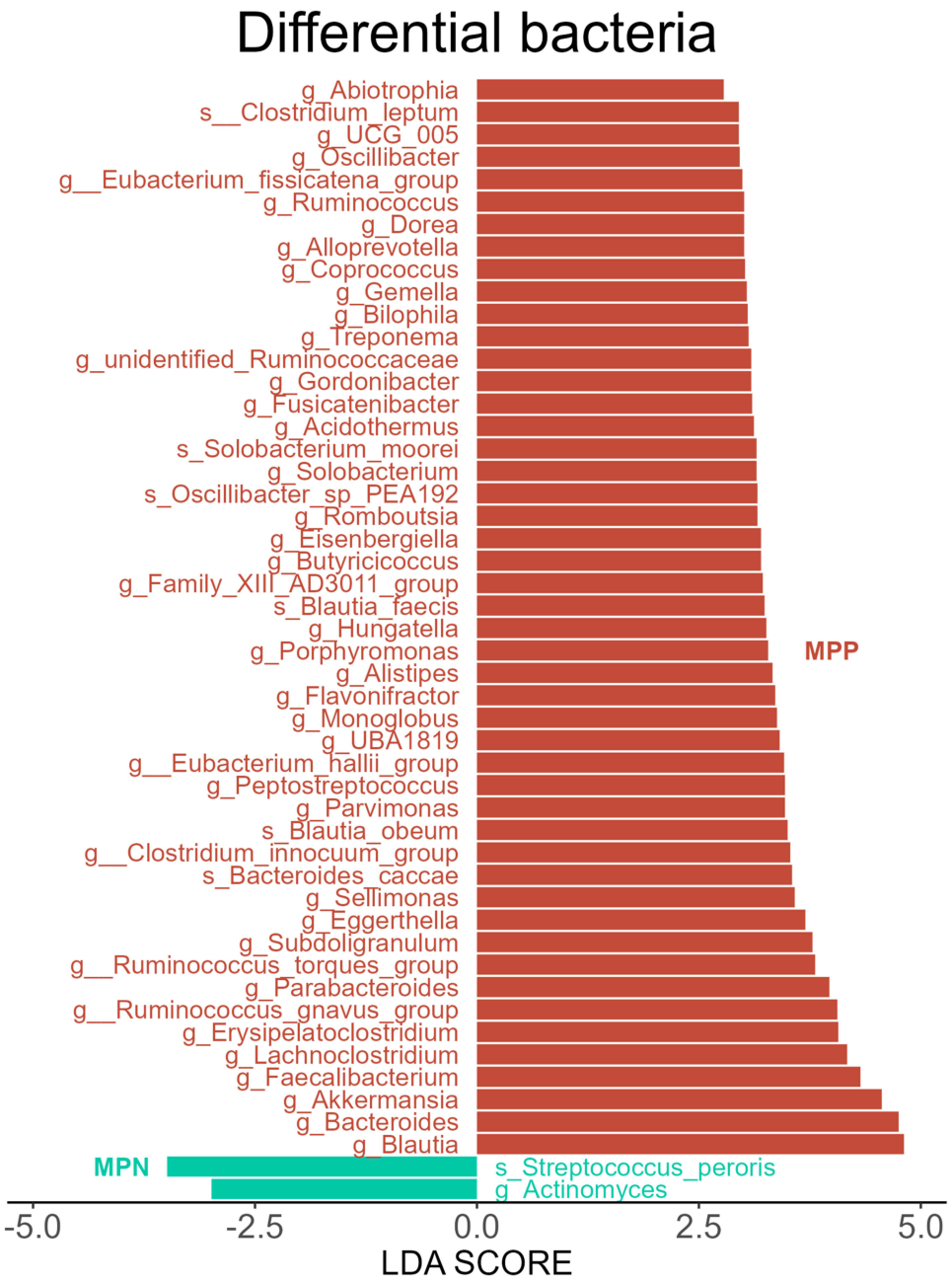

Figure 2 Identification of differentially abundant gut microbiota taxa between MP-infected and uninfected individuals. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) analysis reveals the specific gut microbiota signatures associated with MP infection, highlighting the bacterial genera and species that are either increased or decreased in the gut of MP-infected individuals compared to the uninfected controls (Positive LDA scores represent taxa that are enriched in the MPP group, while negative LDA scores indicate taxa that are more abundant in the MPN (uninfected) group.). The identified differentially abundant taxa provide insights into the microbial community changes that may occur during MP infection.

Substantial Correlations Linked Gut Microbiota and Serum Metabolites

Spearman correlation analyses were performed to investigate the associations between gut bacteria and serum metabolites. At the genus level, a total of 488 and 262 associations were detected between 42 genera and 110 metabolites in positive ion mode, and 40 genera and 91 metabolites in negative ion mode, respectively (Figure 5a). For instance, *Erysipelatoclostridium* was found to be negatively correlated with metabolites in ESI^- mode, such as LPC (34:3), Indole-3-butyric acid, L-Glutamic acid, and Indole-3-acetic acid (Figure 5b). Additionally, *Eggerthella* had positive correlations with LPC (18:0) and CRA (24:0), while being negatively associated with Indole-3-butyric acid and Kynurenic acid

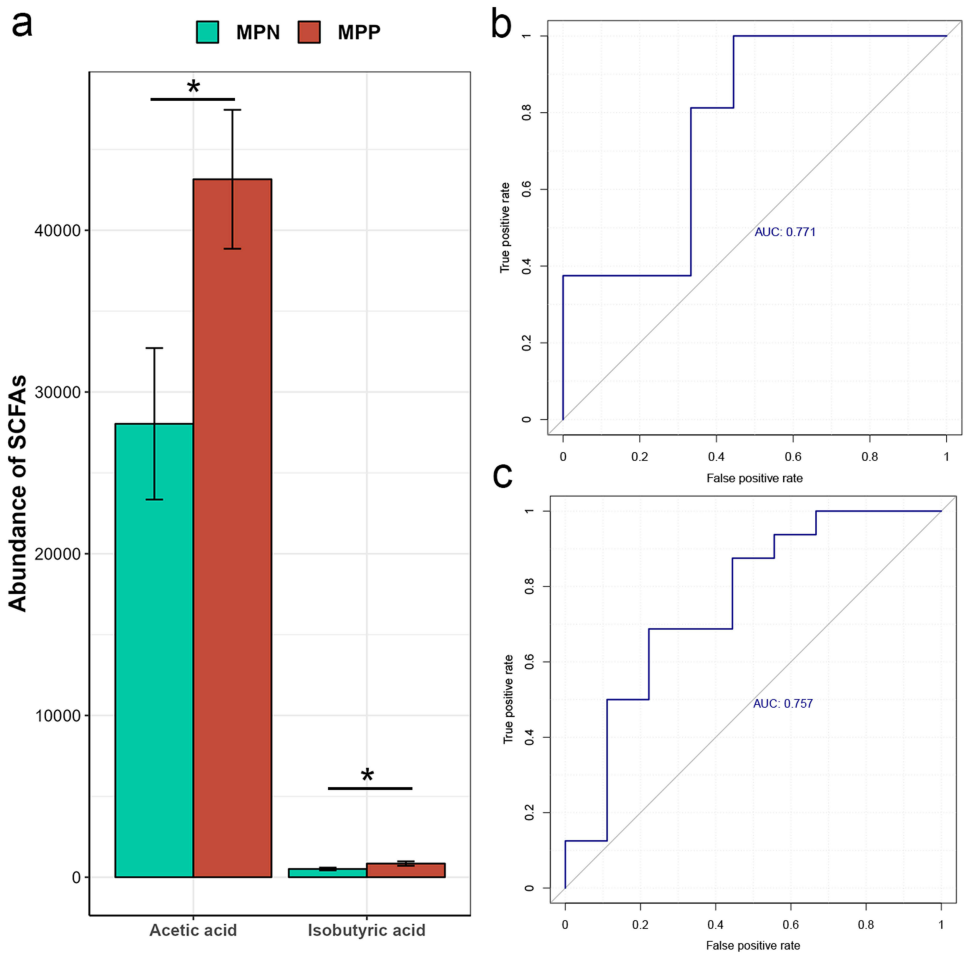

Figure 3 MP infection is associated with altered serum short-chain fatty acid (SCFA) levels. (**a**) Bar plots showing the concentrations of two specific SCFAs, acetic acid and isobutyric acid, in the serum samples of MPP individuals and MPN controls. The concentrations of both acetic acid and isobutyric acid were significantly higher in the MPP group compared to the MPN group (asterisk above the bar chart indicates statistical significance, "*", $P < 0.05$), indicating that MP infection is associated with changes in the levels of these serum SCFAs. (**b** and **c**) Receiver Operating Characteristic (ROC) curves were used to evaluate the diagnostic performance of using serum acetic acid (panel b) and isobutyric acid (panel c) levels as potential biomarkers for MP infection. The Area Under the Curve (AUC) values for both acetic acid and isobutyric acid were greater than 0.7, suggesting that these SCFA levels have reasonable accuracy in discriminating between MP-infected and non-infected individuals.

(Figure 5c). Furthermore, 1-Palmitoyl-Sn-Glycero-3-Phosphocholine (LPC (16:0)), a pro-inflammatory metabolite enriched in MP patients, was found to be significantly positively correlated with the genera *Abiotrophia* and *Actinomyces* (Figure S4).

Discussion

It is acknowledged that mycoplasmal pneumoniae is one of the most predominant pathogens for community-acquired pneumonia worldwide, especially in children and adolescents.[28,29] In addition to causing upper respiratory tract infections, MP can also cause bronchitis, pneumonia, as well as potentially fatal extra-pulmonary complications. The majority of studies have disclosed the effect of MP infection on the microbial composition of respiratory tract.[30–32] While previous studies have revealed changes in the composition of gut flora in patients with MP infection, comprehensive investigations into the associations between gut bacteria and the metabolome in individuals infected with MP are currently limited. The primary aim of this work is to discern the alterations of gut microbiota, as well as serum metabolites and SCFAs, and to unravel the inter-relationships among these molecules in order to shed light on the complex dynamics of host-microbiota interactions upon MP infection.

Interestingly, it was characterized that the levels of acetic acid and isobutyric acid in the sera of MP patients were higher compared to the MPN group, which regarded as one of the distinctive findings in MP infections compared to

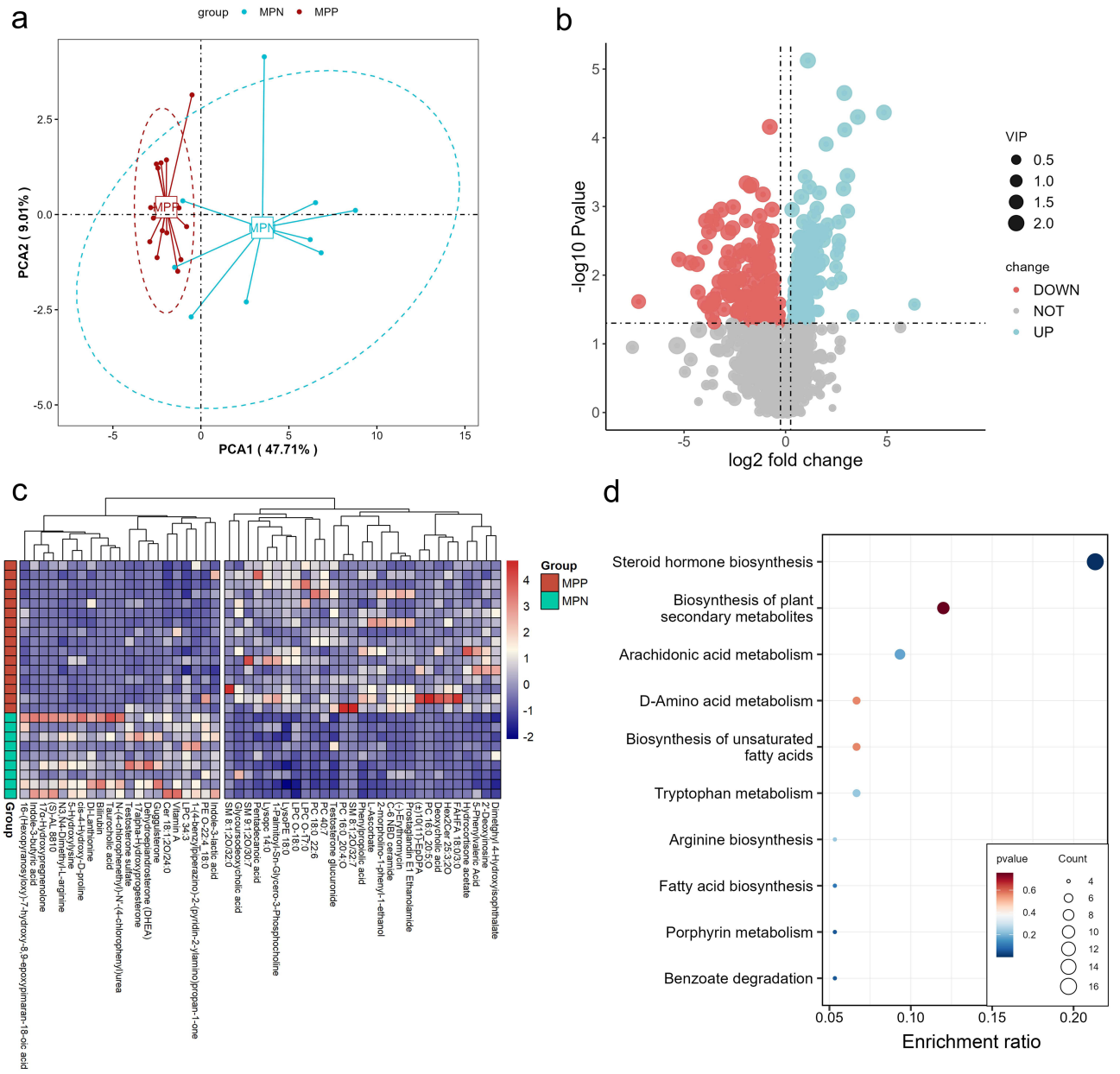

Figure 4 Altered serum metabolomic profile associated with MP infection. (**a**) Principal Coordinate Analysis (PCoA) score plot shows the separation of serum metabolite profiles between the MPP and MPN groups. The clear clustering of the two groups indicates that the overall serum metabolite composition is substantially different between the MPP and MPN individuals. (**b**) Volcano plot showed the metabolites with increased levels in the MPP group compared to the MPN group are shown in wine yellow, while those with decreased levels are shown in cyan. The thresholds used to identify the significantly altered metabolites were: False Discovery Rate (FDR) < 0.05, log2 fold change (FC) > 0.25 or < −0.25, and Variable Importance in Projection (VIP) value > 1.0. (**c**) Hierarchical clustering heatmap visualizes the relative abundance of the significantly different serum metabolites between the MPP and MPN groups. The heatmap clearly demonstrates the distinct metabolomic profiles associated with MP infection. (**d**) Bubble plot shows the results of a pathway enrichment analysis based on the significantly altered serum metabolites. The color of the bubbles represents the p-value, and the size of the bubbles indicates the number of metabolites mapped to each KEGG pathway.

pediatric pneumonia caused by other pathogens. However, while extensive research has consistently demonstrated the beneficial effects of SCFAs on the host, some studies have also reported associations between elevated levels of certain SCFA members and various diseases, such as non-alcoholic fatty liver disease, adverse metabolic health, ulcerative colitis, and brain injury. Previous research considered excess butyrate in the context of liver cancer is hazardous, because they found it obstructs the immune system from functioning.[33–35] Therefore, the present study suggests that the increases in acetic acid and isobutyric acid levels observed in MP infections may be a consequence of the infection, and these elevated SCFA components may also play a role in exacerbating the severity of the diseases caused by MP infection.

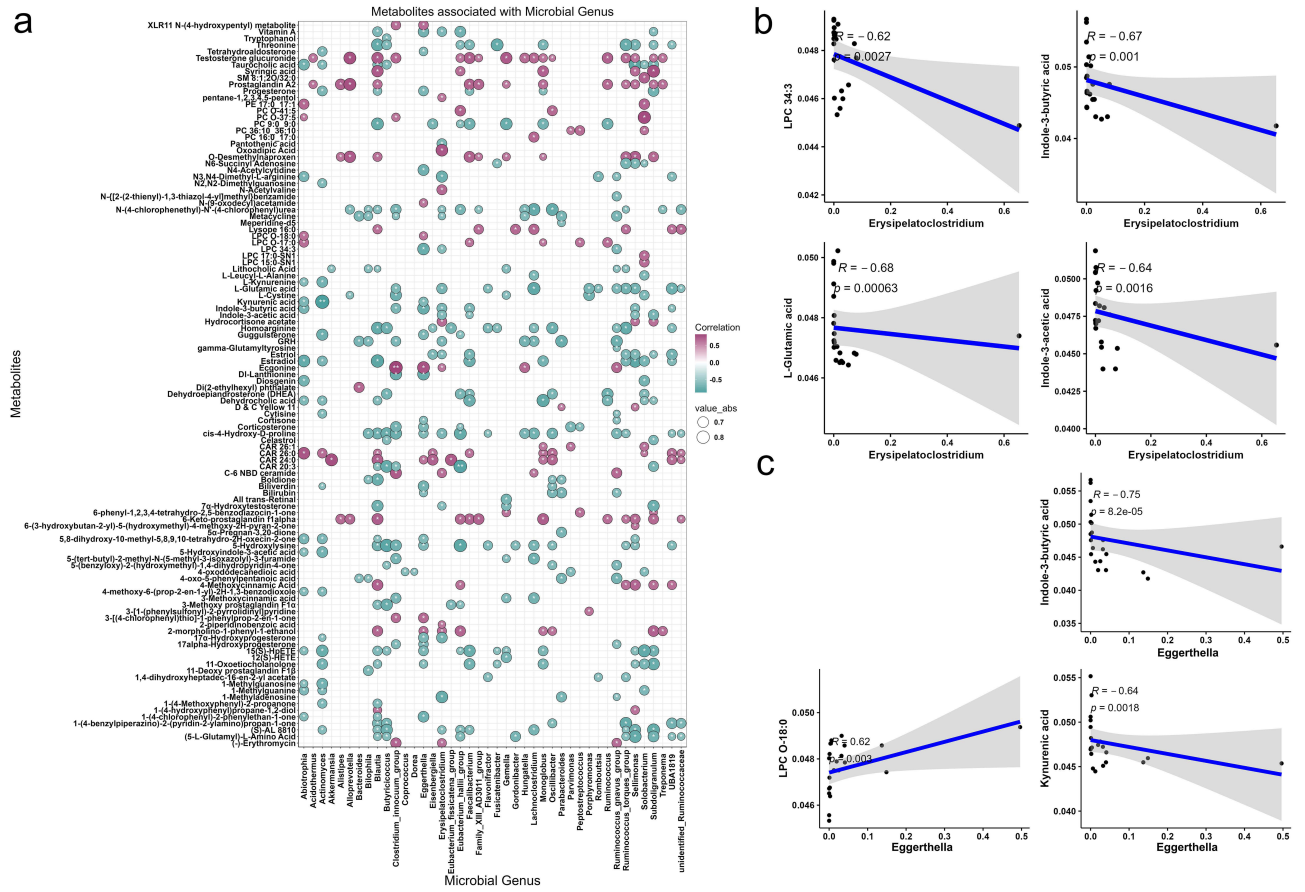

Figure 5 Correlations between differential serum metabolites and gut microbiota of the participants in MPP and MPN groups. (**a**) The correlations between gut microbiota at the genus level and serum metabolites analyzed in positive ionization mode (ESI^+) were calculated. The absolute correlation coefficient (|r|) is represented by the size of the circle, and the adjusted p-values are indicated by asterisks ("*", $P < 0.05$; "**", $P < 0.01$) showing the statistical significance of the correlations. (**b**) Scatter plots representing the relationships between the genus *Erysipelatoclostridium* and the metabolites LPC 34:3, Indole-3-butyric acid, L-Glutamic acid, and Indole-3-acetic acid, respectively, as analyzed using Spearman rank sum test. (**c**) The relationships between *Eggerthella* and LPC 18:0, Indole-3-butyric acid, Kynurenic acid was analyzed using a scatter plot and spearman rank sum test.

Further mechanistic investigations through additional experimentation are warranted to provide more substantive evidence for this observation.

Notably, it has been documented that MP infection is accompanied with respiratory tract damage and inflammation injury induced by the cellular components including metabolites and toxin released by MP. However, whether MP infection can provoke the proinflammatory metabolites in serum is still in infancy. Lysophosphatidylcholine (LPCs) is reported as a bioactive lipid molecule that has been shown to play tremendous roles. LPCs have been reported to exhibit pro-inflammatory, anti-thrombotic, and cytotoxic properties. Interestingly, polyunsaturated LPC species, such as LPC 22:4 and LPC 22:6, have been found to possess anti-inflammatory effects, while the saturated LPC 16:0 and LPC 18:0 has been shown to induce inflammation in vivo. However, it remains unclear whether these anti-inflammatory effects are due to the direct actions on target cells or the formation of other metabolites from these precursors.[36,37] LPCs is a class of phospholipids involved in various physiological processes.[38,39] Previous studies have found that LPCs can activate immune cells such as macrophages and neutrophils, leading to the production of pro-inflammatory cytokines and chemokines.[37] Additionally, LPC 18:0 has been shown to stimulate the production of reactive oxygen species (ROS), which can contribute to airway inflammatory injury.[40,41] Furthermore, elevated levels of both saturated LPC (16:0) and LPC (18:0) have been demonstrated to cause an increase in intracellular LPC species, concomitant with the induction of apoptosis, accumulation of lipid droplets, and endoplasmic reticulum and mitochondrial stress, ultimately contributing to cell and tissue damage.[42] The available evidence suggests that LPC 18:0 is involved in the regulation of inflammation and may contribute to the development and progression of various inflammatory and injured conditions. The current

work identified that the abundance of LPC 18:0 was increased in patients infected with *Mycoplasma pneumoniae*. The elevated level of LPC 18:0 was first identified in the blood of MP patients and we thought it may signify the manifestation of inflammation, which could contribute to the exacerbation of the infection. The correlations between microbial species and this particular serum metabolite may have implications for children's health against MP infection. However, further research is needed to fully understand the mechanisms underlying these associations and their potential impact on MP infection.

Erysipelatoclostridium is part of the human gut commensal microbiota; however, certain strains produce IgA1 and IgA2 proteases, potentially increasing host susceptibility to opportunistic bacterial translocation across the intestinal mucosa.[43] This risk is particularly pronounced in children under five and immunocompromised individuals, who may, albeit rarely, develop invasive infections.[44] In this study, we investigate the gut microbial composition in children around five years old infected with MP. Our results indicate a significant increase in the relative abundance of *Erysipelatoclostridium* among MP-infected children. Additionally, the genus *Eggerthella*, known for its pathogenic potential, was also enriched in these children. Notably, *Eggerthella lenta*, the type species, can induce intestinal Th17 activation by disrupting the inhibition of the Th17 transcription factor Rorγt through mechanisms that are independent of cells and antigens. *E. lenta* has been associated with inflammatory bowel disease (IBD) and exacerbation of colitis. Furthermore, we observed a significant reduction in Indole-3-acetic acid (IAA), an aryl hydrocarbon receptor (AHR) ligand derived from the microbiota, in children infected with MP.[45] Previous studies have highlighted the protective role of IAA in reducing inflammation and free radical generation.[46,47] Impaired production of tryptophan-derived AHR ligands by gut microbiota has been linked to the pathogenesis of metabolic syndrome.[48,49] Probiotics such as *L. reuteri* have been shown to synthesize IAA.[21] The observed negative correlation between Erysipelatoclostridium and IAA raises the hypothesis that Erysipelatoclostridium may directly inhibit the growth of specific IAA-producing bacteria, thus mediating beneficial effects. This suggests that supplementing with targeted probiotics could enhance IAA levels and help mitigate inflammation associated with MP infection. Nevertheless, it is noteworthy that the alterations observed in the intestinal bacterial flora composition associated with *Mycoplasma pneumoniae* infection appear to be distinct from the changes reported in other types of pediatric pneumonia. The ratio of Firmicutes to Bacteroidetes in the MPP group was significantly higher compared to the MPN group, which contrasts with the characteristic lower ratio observed in children with pneumonia due to SARS-CoV-2 infection. Additionally, children with MPP exhibited a greater relative abundance of Firmicutes and Verrucomicrobia, including increased counts of genera such as *Clostridium, Ruminococcus, Blautia*, and *Eggerthella*. In contrast, there were reduced numbers of *Streptococcus peroris* and Actinobacteria. In children with COVID-19, there was a significant increase in *Streptococcus* counts and a marked decrease in Actinobacteria and Verrucomicrobia.[50,51] Analysis of the gut microbiota structure in pneumonia caused by *Enterococcus faecalis* revealed a decreasing trend in the proportions of *Bacillus* and *Ruminococcus* and a noticeable increase in *Bacillus* spp.[52] The ratio of Firmicutes and Bacteroidetes decreased in children infected with *Streptococcus pneumoniae*, while Proteobacteria showed a higher abundance as the dominant phylum. There was also a significant increase in the abundance of *Ralstonia, Sphingomonas, Ochrobactrum*, and *Streptococcus* spp., whereas *Coprococcus, Prevotella, Butyricicoccus, Ruminococcus, Rikenella, Dehalobacterium*, and Parabacteroides showed a decline.[53] This suggests that the differences in host immune responses and metabolic perturbations elicited by distinct pathogenic microorganisms may lead to divergent shifts in the intestinal microbiome composition. Therefore, it can be emphasized that the specific diagnostic and treatment approaches for pneumonia should be tailored to the causative pathogen, as the infection by different microbes can result in varied impacts on the host's immune system and metabolism, thereby manifesting in distinct alterations of the gut microbial community.

In conclusion, these results indicate that MP infection caused an aberrant gut microbiota, and differentially expressed metabolites in MP patients compared to controls using 16S rRNA amplicon sequencing and untargeted metabolomics. A significant impact of MPP on lipid metabolism, especially pathways involving glycerophospholipid metabolism were identified and may contribute to the pathogenesis of MPP. Furthermore, we identified numerous interrelationships between perturbed microbial species and serum metabolites, potentially influencing the disease severity caused by MP infection. However, the challenge of mitigating opportunistic pathogen and harmful metabolites to defense or prevent MP infection remains a significant obstacle. These findings hold significant implications for the advancement of therapeutic

interventions and the progress of clinical applications targeting MP infection based on gut microbiota and serum metabolites. In addition, this study has some limitations. First, the sample size of collected MP patient samples was relatively small, as obtaining parental consent for pediatric patients proved to be challenging. Consequently, our current research was unable to stratify patients based on the severity of their condition. Secondly, there is a paucity of existing research, both domestically and internationally, on the gut microbiome and serum metabolome of MP patients. Additionally, the sampling conducted at a single geographical location may limit the generalizability of the findings. Such a focused approach can introduce potential confounding factors, including variations in dietary habits among the participants. These dietary differences may influence the composition of the gut microbiota and, consequently, the results presented in this study. Therefore, it is essential to consider these limitations when interpreting the findings, as they may affect the overall conclusions drawn about the relationship between MP infection and gut microbiota changes.

Data Sharing Statement

All 16S rRNA gene sequencing data were submitted to the GSA database with accession number: subCRA026249. Another data or tables can be obtained from the corresponding author upon reasonable request.

Ethics Statement

The study protocols were all approved by the Biomedical Ethics Committee of Anhui Medical University (No 2023H019). Parents or legal guardians of the participants were informed about the purpose of the study, in accordance with the Declaration of Helsinki. Written consent to participate was obtained from the parents or legal guardians of all minor participants (defined as individuals under the age of 16) included in this study.

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.

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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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