Vancomycin protects against acute respiratory distress syndrome by promoting butyrate metabolism

Jianuo Chen
The First Affiliated Hospital of Wenzhou Medical University

Xi Zhang
Wenzhou Medical University

Fen Xiong
Wenzhou Medical University

Hong Zheng
Wenzhou Medical University

Wenli Zhang
Wenzhou Medical University

Yuying Shen
Wenzhou Medical University

Pengcheng Lin
The First Affiliated Hospital of Wenzhou Medical University

Hongchang Gao
Wenzhou Medical University

Yuping Li (✉ wzliyp@163.com)
The First Affiliated Hospital of Wenzhou Medical University

Research Article

Keywords:

Posted Date: December 14th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2364330/v1

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Abstract

Background

Acute respiratory distress syndrome (ARDS) represents a clinical syndrome featuring refractory hypoxemia. Several reports have identified the role of the gut microbiota in affecting the immune activity and pathological characteristics of respiratory disorders via the gut-lung axis. However, the precise metabolic mechanism remains unknown. The present work focused on investigating the processes by which gut microbiota influence ARDS and identifying potential therapeutic targets of ARDS.

Methods

The feces and serum samples of 23 ARDS patients were collected, and a lung injury model was generated by transplanting microbiota from ARDS patients into healthy C57BL/6J mice. The changes in the gut microbiota and metabolic phenotypes of the feces samples from ARDS patients and lung-injured mice were analyzed using 16sRNA sequencing technology and metabolomics based on $^1$H nuclear magnetic resonance (NMR), respectively. The effect of gut microbiota on ARDS was also explored after giving an oral vancomycin treatment to lung-injury mice. Further, enzyme-linked immunosorbent assay (ELISA) was used to evaluate the hyperinflammatory response in mice and intestinal permeability in ARDS patients. Additionally, qRT-PCR and staining were performed to analyze colonic barrier function and permeability.

Results

Firmicutes formed the vital species of microbiota that was different in lung-injury mice. Moreover, butyrate (produced by Firmicutes) was the most crucial metabolite in the feces samples of ARDS patients and lung-injury mice. Elisa and HE results showed aggravated functional disturbances in the intestinal barrier of ARDS patients and lung inflammation in the lung-injured mice. These phenomena were significantly alleviated after the oral administration of vancomycin. Besides, the utilization of butyrate in the colon of mice was increased considerably. The level of butyrate was reduced in the feces but increased in the colon.

Conclusions

Thus, vancomycin affects butyrate metabolism in the colon by influencing the gut microbiota. Modulating colonic butyrate metabolism could help treat ARDS.

Introduction
Acute respiratory distress syndrome (ARDS) represents one of the most common acute and life-threatening respiratory diseases, with a mortality rate of 30% – 60% [1, 2]. It is characterized by capillary endothelial cell or alveolar epithelial cell injuries, leading to diffuse lung injury, hyaline membrane formation, alveolar edema, and refractory hypoxemia [3]. The present treatments of ARDS include supportive therapies and drug treatments. Mechanical ventilation is the only supportive therapy that effectively improves survival. Other ventilation strategies, such as prone positioning, excessively high positive end-expiratory pressure, and conservative fluid strategies, are ineffective [4]. Drug treatment is divided into etiological and physiological treatment depending on the different physiological and pathological disorders caused by ARDS [5]. Severe infection is a frequent cause of ARDS. Due to the diversity of infectious pathogens, physicians use empirical medication to treat patients. This method results in clinical drug abuse, increasing financial pressure on patients and wasting medical resources [6]. Therefore, identifying new treatment targets for ARDS is crucial and urgent.

The gut microbiota consists of numerous microorganisms. These bacteria play an essential role in human nutrient metabolism, and the human gut provides a nutrient-rich environment for the bacteria [7]. Therefore, intestinal epithelial dysfunction, immune system dysfunction, and translocation of intestinal pathogens often lead to the occurrence or aggravation of the disease [8]. A recent study on children with cystic fibrosis demonstrated the concurrent development of gut and respiratory microbiota after birth, demonstrating a physiologically relevant link between the lung and the gut [9]. Benjamin J. Marsland proposed the “lung-gut axis” in 2015 [10]. He primarily focused on allergic airway inflammation [11]. Gut microorganisms produce a variety of metabolites that affect monocyte immune cells, such as dendritic cells [12] and macrophages [13], to influence lung pathophysiological changes. However, the impact of the composition of gut microbes or their metabolites on lung injury remains unknown.

The present study focused on identifying characteristic microbiota and ARDS metabolites using 16sRNA sequencing and metabolomic analysis based on 1H-nuclear magnetic resonance (1H-NMR). A new target for ARDS treatment could be identified by regulating gut microbiota that affects metabolic pathways.

Methods

Clinical information and sampling

For the present study, acute respiratory distress syndrome (ARDS) patients were recruited at the First Affiliated Hospital of Wenzhou Medical University (Zhejiang, China). A total of 23 hospitalized patients diagnosed with ARDS were enrolled in line with the Berlin definition within 24 h of admission to the hospital [14]. Participants provided informed consent for this experiment. Feces and blood were collected from ARDS patients and their eligible bedside relatives (henceforth considered healthy subjects). None of the participants had taken any antibiotics, probiotics, or prebiotics within one week of beginning this study. Fasting blood samples were collected in vacutainer tubes (5 mL) containing ethylene diamine tetraacetic acid (EDTA) as the chelating agent. The samples were centrifuged for 15 min at 1500 ×g. The plasma and feces samples were harvested and preserved at − 80°C till further use.
Fecal Microbiota Transplantation (Fmt)

For the study, male C57BL/6J mice (n = 45) were procured from the Vital River Laboratory Animal Technology Co. (Beijing, China) and raised in the standard SPF environment (22 ± 1°C; 55 ± 5% humidity; 12-h/12-h: light/dark cycle) at the Wenzhou Medical University for experiments. The study was performed according to the Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University. Mice were adaptively fed for a week. They were later gavaged with mixed antibiotics (100 mg/kg neomycin; 100 mg/kg metronidazole; 50 mg/kg vancomycin; 1 g/L ampicillin; 1 mg/kg amphotericin B) once daily for seven consecutive days to clean up the original microflora[15]. Thereafter, the animals were randomized into three groups: (I) control, CON, which received gavage of the supernatants containing microbiota from controls for two weeks to reconstruct the gut microbiota; (II) ARDS, which received gavage of the supernatants containing microbiota from ARDS patients for two weeks; (III) ARDS + vancomycin (ARDS + V), which received gavage of the supernatants containing microbiota from ARDS patients for two weeks and treatment of oral vancomycin (50 mg/kg) for one week. Fresh fecal samples from ARDS patients were mixed with PBS (1:8, w/v), vortexed for 30 s, and centrifuged at 4°C for 5 min at 1000 g. Afterward, the supernatant (200 µL) was intragastrically injected into ARDS and ARDS + V mice once a day for 14 days [16]. Each FMT process was conducted in the SPF environment and completed within 10 min [17, 18].

Sample Collection And Preparation

Fresh fecal pellets were obtained from the metabolic cages under SPF conditions. After that, each mouse was anesthetized using isoflurane. Blood samples were collected from eye sockets and centrifuged for 15 min at 3000 g at 4°C to yield the serum, which was stored at −80°C till further use. Colonic and lung tissues were collected carefully from the thoracic and abdominal cavities. The samples were frozen under liquid nitrogen and preserved at −80°C till further use.

Fecal pellets (0.2 g) were added into the Eppendorf (EP) tubes (1.5 mL) and diluted with 400 µL of phosphate buffer (PBS, 0.2 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH = 7.4). The mixture was thoroughly homogenized by vortexing for 5 min and centrifuged at 4°C for 15 min at 10,000 g. Thereafter, supernatants (100 µL) were carefully drawn, blended using D$_2$O (400 µL) containing 0.05 mM of sodium trimethylsilyl propionate-d$_4$ (TSP). These samples were then added to a 5-mm NMR tube for metabolomic analysis.

Approximately 0.1 g of the colon tissue samples were collected into the EP tube (1.5 mL), and cold methanol (4 mL/g) and cold water (0.85 mL/g) were added. The mixture was adequately homogenized using a flux tissue grinder (FLUKO Equipment Co., Ltd., Shanghai, China). Next, pre-chilled chloroform and water (2 mL/g) were added to the mixture under vigorous vortexing for 30 s and incubated for 15 min on ice. The samples were centrifuged at 4°C for 15 min at 10,000 g. Supernatants were carefully removed, added to the novel centrifuge tubes, and lyophilized for 48 h. Finally, D$_2$O (500 µL) containing 0.05 mM
TSP was added to redissolve the lyophilized extract powders and added into an NMR tube for measurement.

16s Rrna Gene Sequencing And Analysis

The fecal microbial DNA was isolated from the stool samples using the stool DNA isolation kit according to specific protocols (TIANGEN BIOTECH, Co., Ltd., Beijing, China). Further, the V4 region of the 16S rRNA gene was amplified using the Barcoded primer pair 515F/806R (515F: 5'-GTG CCA GCM GCC GCG GTA A-3'; 806R: 5'-GGA CTA CHV GGG TWT CTA AT-3'). Later, the PCR amplicons were sequenced on the Illumina HiSeq2500 PE250 platform (Illumina Inc., San Diego, USA) at Novogene (Novogene, Beijing, China). The sequenced data were analyzed on the QIIME software (v.1.7.0) [19]. The 97% similarity threshold was applied to the clustering operational taxonomic units (OTUs) using the UPARSE software (v.7.0.1001)[20]. Taxonomy was assigned using the mothur approach based on the SILVA SSU rRNA database[21, 22]. The alpha-diversity of the gut microbiota was determined from the QIIME software (v.1.7.0). In contrast, the beta-diversity was analyzed from the R software (v.2.15.3). The changes in the gut microbiota of the diverse groups were exhibited by the principal coordinate analysis (PCoA). In contrast, the key gut microbiota was screened using linear discriminant analysis (LDA), considering LDA score > 4.

Lung Wet/dry Weight Ratio (W/d) And Biochemical Estimations

Left lung tissues were washed to remove the blood and wiped dry using filter paper. The sample weight was determined immediately to predict its wet weight. Afterward, the sample was oven-dried for 72 h at 60°C to measure its dry weight. The W/D ratio was then determined to assess the severity of pulmonary edema. The MPO (myeloperoxidase) activity was estimated to evaluate the degree of neutrophil infiltration using the MPO activity kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The contents of cytokine, D-lactate (D-Lac), diamine oxidase (DAO), and lipopolysaccharide (LPS) were assessed using ELISA detection kits according to the manufacturer’s instructions.

Hematoxylin-eosin (H-e) And Immunohistochemical (Ihc) Staining For Lung Tissues

Tissue (0.1 g) from the right lower lung of every mouse was fixed in 4% neutral formalin. Further, the samples were cut into 5-µm thick sections and embedded in paraffin. Three random sections were then chosen from every mouse. Pulmonary morphological changes were analyzed through H-E staining. The lung histological alterations, like interstitial and alveolar edema/hemorrhage, alveolar hyperemia, inflammatory infiltration, excess alveolar expansion, and atelectasis, were evaluated from these samples [23]. For immunohistochemistry, the paraffin-embedded sections were blocked using 5% bovine serum albumin (BSA) and incubated overnight at 4°C with anti-mucin-like hormone receptor 1 (EMR1, 1:200, ProteinTech, Tokyo, Japan). After cleaning, the sections were incubated with the secondary antibodies
(anti-rabbit IgG-HRP, 1:200) for 1 h at 37°C. Positive points were exposed using the 3,3 N-Diaminobenzidine-Tetrahydrochloride (DAB) Horseradish Peroxidase Color Development Kit. Subsequently, hematoxylin was added to counterstain the sections. The sections were captured under a Nikon ECLIPSE Ti inverted microscope (Nikon Instruments Inc., Shanghai, China).

**Periodic Acid-schiff (Pas) Staining And Immunofluorescence Of The Colonic Tissues**

Colon tissues were collected, embedded in paraffin, and sliced into 5-µm sections. Some parts of the sections were stained with PAS, and the goblet cells were observed according to the instructions of the PAS staining kit (Solarbio Science & Technology, Beijing, China). The colonic sections were dewaxed with xylene for immunofluorescence and rehydrated using gradient alcohol. They were then incubated overnight at 4°C with individual primary antibodies like anti-ZO-1, anti-Occludin, and anti-Muc2 (1:50, ProteinTech, Tokyo, Japan). Sections were rinsed with PBS before incubating with the suitable secondary antibody (1:200, IgG-HRP) for 1 h at 37°C. After washing, DAPI was added to the sections. Finally, image acquisition was achieved on the Nikon ECLIPSE Ti inverted microscope, while quantification was performed on the ImageJ software S8 (version 1.47 for Windows).

**Quantitative Real-time Pcr (Qpcr) Assay For Gene Expression**

Total RNA was extracted using the TRizol reagent (Invitrogen, CA, USA). It was reverse-transcribed into cDNA by the PrimeScript RT reagent kit (Takara Bio Inc., RR037A, Tokyo, Japan) according to specific instructions. Quantitative PCR (qPCR) was performed using the StepOnePlus Real-Time PCR SYBR Green qPCR Master Mix (Takara Bio Inc., Beijing, China). The gene expression levels were analyzed by the ∆∆Ct approach using a suitable reference gene. The specific primers are listed in Table S3.

**Metabolomic Analysis**

Metabolomic analysis based on $^1$H NMR was performed at 25°C on the 600-MHz Bruker Avance III NMR spectrometer containing the 5-mm TXI probe (Bruker BioSpin GmbH, Rheinstetten, Germany). Feces of human and colon tissues were analyzed with the standard single-pulse sequence (ZGPR) underwater signal pre-saturation. Parameters used for acquisition were: relaxation delay = 4 s; acquisition time = 2.66 s/scan; spectral width = 12,000 Hz; and data points = 256 K. Additionally, the NMR spectra for mice feces were obtained by the Carr-Purcell-Meiboom-Gill (CPMG) experiment under constant receiver gain value. Major parameters included relaxation delay = 4 s; acquisition time = 2.66 s/scan; spectral width = 12,335.5 Hz; and data points = 256 K.

The baseline and the phases of the NMR spectra were manually corrected by the TopSpin 3.0 software (Bruker BioSpin GmbH, Rheinstetten, Germany). Serum spectra were obtained with reference to the lactate signal at 1.33 ppm. The metabolites were assigned on the NMR spectra by the Chenomx NMR suite 7.0.
Spectral processing was achieved on the MATLAB R2012a (MathWorks Inc.) platform. After adjusting the spectral phases, the baseline correction and integration were completed with the ‘icoshift’ procedure, followed by overall normalization. After eliminating the residual water region (4.6–5.1 ppm), the spectra (0.6–9.0 ppm) were further divided and combined in data binning (size 0.0015 ppm) to conduct multivariate analysis and quantitative analysis, respectively.

In multivariate regression, the Partial Least Squares Discriminant Analysis (PLS-DA) on the Pareto-scaled NMR data was conducted using the SIMCA 12.0 software (Umetrics, Umeå, Sweden). Additionally, PLS-DA performance was assessed by a permutation test (20 cycles), where in the goodness-of-fit was analyzed by $R^2$, whereas the model prediction ability was analyzed by $Q^2$, respectively. The PLS-DA score plot revealed different metabolic phenotypes among diverse groups. In contrast, key metabolites were screened using the variable importance in the projection (VIP) approach at the threshold of VIP > 2. For quantitative analysis, the peak areas were determined to analyze the metabolite contents.

**Data Analysis And Statistics**

The results were represented by mean ± SEM. Two-tailed Student’s t-test was used to compare two groups, while one-way ANOVA was used to compare multiple groups. $p < 0.05$ indicated statistical significance. The GraphPad Prism software was employed for statistical analysis and graph plotting.

**Results**

**Intestinal flora imbalance and impaired intestinal barrier function in ARDS cases**

In total, 23 ARDS patients and 22 normal subjects were recruited for the analysis. Table S1 shows the clinical features of the ARDS cases. The APACHE II score describing the severity-of-disease classification system had increased in the ARDS population. Further, the absolute neutrophil count (NEUT#), white blood cell count (WBC), platelet count (PLT), and the levels of lactate (LAC), D-Dimer (D-D), total bilirubin (TBIL), aspartate transamination enzyme (aspartate aminotransferase, AST), alanine transaminase (ALT), total bilirubin (TBIL), blood urea nitrogen (BUN), creatine kinase (CK), glucose (GLC), lactate dehydrogenase (LDH), interleukin-6 (IL-6), IL-10, C-reactive protein (CRP), interferon-γ (IFN-γ), ferritin (FER), erythrocyte sedimentation rate (ESR), and procalcitonin (PCT) were markedly increased. In contrast, the PaO$_2$/FiO$_2$ ratio [the ratio of arterial oxygen partial pressure (PaO$_2$ in mm of Hg) and fractional inspired oxygen (FiO$_2$ in percentage)] had reduced. Also, the absolute lymphocyte count (LYMPH) and the levels of hemoglobin (Hb), total protein (TP), albumin (ALB), CD8$^+$ T cells, and CD4$^+$ T cells had decreased. From the changes in these indices, it can be found that ARDS patients have an increased inflammatory response and reduced immunity. The changes in these indices pointed toward the increased inflammatory response and reduced immunity of the ARDS patients.
The relation between ARDS and the gut bacterial profiles in the feces was determined through 16S rRNA sequencing. The composition of the bacterial communities was significantly different among the ARDS patients and healthy individuals at the phylum level (Fig. 1A). The richness of the gut microflora was substantially higher. The species diversity was significantly lower in the ARDS patients compared to healthy individuals (Fig. 1B). The gut microflora of healthy controls and ARDS patients comprised Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria. However, the bacterial composition in the ARDS patients did not change significantly. Compared with healthy controls, the levels of Firmicutes and Proteobacteria were higher in the ARDS patients, and the content of Bacteroidetes and Actinobacteria was considerably lower (Fig. 1C).

The relative ratio of Firmicutes and Bacteroides, often used to assess gut microbial dysbiosis, was further evaluated. The proportions of these two floras had changed in the ARDS patients (Fig. 1D), although this change was not statistically significant. The gut microflora is closely related to gut barrier function. Indicators like serum D-Lac, DAO, and LPS help evaluate the damage caused to the intestinal barrier mucosa. They affect the permeability of the intestinal barrier and the translocation of bacteria in the intestinal wall [24]. The contents of serum LPS and DAO had significantly increased among the ARDS patients, and the level of D-Lac had slightly but insignificantly increased (Fig. 1E, F, G). These observations suggested that the intestinal barrier was destroyed, and endotoxin was translocated. However, intestinal permeability was not significantly different in the ARDS patients.

**Gut Microbiota Transplantation From Ards Patients Induces Lung Injury In Mice**

Collectively, microbiota might be an essential factor for ARDS development. The time points for transplanting fecal bacteria were determined through preliminary experiments (data not shown). In this study, male C57BL/6J mice were colonized with the gut microbiota of ARDS patients and healthy people. The detailed process of FMT is shown in Fig. 2A. The mice in the ARDS group (those receiving the gut microbiota of ARDS patients) exhibited evident alveolar destruction, thickening of alveolar septa, and extensive inflammatory cell infiltration (Fig. 2B). They also showed a significantly increased ratio of wet/dry lung weight (Fig. 2C); the contents of TNF-α and IL-6 within bronchoalveolar lavage fluid were higher (Fig. 2E, F). Macrophages were the primary inflammation infiltrates in the bronchoalveolar lavage fluid (Fig. 2G). Immunohistochemical staining for EMR1 exhibited a significant increase in the positive points in the ARDS group, proving a considerably elevated macrophage count in the ARDS group (Fig. 2H). The neutrophil number did not appear to increase. However, the activity of MPO was significantly increased.

**Firmicutes And Verrucomicrobia Are The Key Flora Responsible For Manifesting Lung Injury In Mice**
The gut microbiota was significantly different between the ARDS and CON groups (Fig. 3A). However, the richness of the gut microbiota did not change in the mice. Albeit, the species diversity reduced significantly (Fig. 3B). Relative to the control mice, the abundance of Verrucomicrobia was considerably elevated in the ARDS group (Fig. 3C). The microflora at the phylum level in the ARDS group mainly consisted of Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria. LDA estimated the impact of each microbiota species and identified their significant differences among the groups. The Firmicutes and Verrucomicrobia were the major microbiota that differed between the ARDS and CON groups with an LDA Score > 4 (Fig. 3D). The Firmicute abundance remarkably decreased in the ARDS group compared with the CON group (Fig. 3E); the Verrucomicrobia abundance was significantly higher (Fig. 3F).

**Butyrate Is The Characteristic Metabolite In Lung Injury**

A total of 21 metabolites were identified from the feces of mice and patients using NMR-based metabolomes. These metabolites were mainly involved in energy, amino acid, and fatty acid metabolism (Fig. 4A,B). The metabolic phenotypes in ARDS cases were notably separated from the healthy controls. The PLS-DA results exhibited excellent model performances (R2X = 0.47, Q2 = 0.606) for distinguishing between the HCON and HARDS groups (Fig. 4C). The VIP plots detected various key metabolites like valine, acetate, butyrate, propionate, phenylalanine, and glycine, with a VIP value > 2 (Fig. 4D). The metabolites were screened after considering FDR < 0.05 (Table. S2), and a metabolic pathway map was established according to the KEGG signaling pathway. The short-chain fatty acid (SCFA) metabolism (acetate, butyrate, propionate) within the feces of ARDS patients had reduced. In contrast, the amino acid and energy metabolism had significantly increased (Fig. 4G). Similarly, a significant difference was observed between the CON and ARDS groups, with excellent model performance parameters (R2X = 0.458 and Q2 = 0.246) for distinguishing between the HCON and HARDS groups (Fig. 4E). When the projected VIP value > 2, the VIP plots detected various key metabolites like valine, acetate, butyrate, propionate, and taurine (Fig. 4F). A metabolic pathway map based on the KEGG signaling pathway was established after considering FDR = 0.05 (Table. S2), which showed lower levels of butyrate and taurine in the ARDS group (Fig. 4H).

**Lung injury in mice is alleviated after vancomycin administration**

One of the lung-injured mice groups (ARDS + V) was administered vancomycin, a narrow-spectrum antibiotic, by gavage. The detailed process of vancomycin treatment in lung-injury mice is shown in Fig. 5A. After two weeks of vancomycin treatment, the ARDS + vancomycin (ARDS + V) group exhibited intact alveoli and poorer inflammatory cells. Inflammatory infiltration was significantly reduced (Fig. 5B). In addition, significant changes were observed in the lung injury scores (Fig. 5C) and wet/dry lung weight ratio (Fig. 5D). The MPO activity (Fig. 5E) was reduced, and the serum TNF-α and IL-6 contents decreased (Fig. 5E, and 5F, G). The serum butyrate content did not change significantly, indicating that butyrate had not entered the blood to cause lung injury (Fig. 5I). LPS often causes lung injury by activating the TLR4
receptor of macrophages. The LPS content was not significantly different in the NORM compared with CON groups in the serum, but the LPS content in the ARDS group was higher. After vancomycin administration, the LPS content in the ARDS + V group decreased (Fig. 5J).

**Oral vancomycin improves intestinal function and protects the intestinal barrier**

Goblet cells play a crucial role in intestinal immunity by secreting hyperglycosylated mucin to form a mucus layer attached to the epithelium to isolate and resist the colonization and invasion of intestinal microorganisms [25]. The morphological number of goblet cells in the colon was evaluated by PAS staining. The ARDS group showed shorter intestinal villi, shallower colonic crypts, and decreased goblet cells relative to the CON group. In contrast, after the oral vancomycin treatment, the ARDS + V group displayed longer intestinal villi, deeper crypts, intact mucosa, and increased goblet cells (Fig. 6A-B). Mucin 2 (Muc2), the most crucial mucin in the gastrointestinal tract, was significantly decreased in the colon of the ARDS group but increased after oral vancomycin treatment (Fig. 6C). This observation conformed to the alterations in the Muc2 mRNA level (Fig. 6D), indicating that the intestinal mucosal barrier of the mice was damaged. Occludin and Zonula occludens-1 (ZO-1) are transmembrane proteins and markers of cytoplasmic attachment [26]. The ARDS group showed reduced ZO-1 and occludin protein expression in immunofluorescence staining. The expression dramatically elevated following oral vancomycin treatment. The mRNA levels of occludin decreased in the ARDS group and increased after oral vancomycin treatment (Fig. 6E-H).

**Vancomycin protects the colon from bacterial invasion by enhancing colonic butyrate metabolism**

Earlier experiments showed that butyrate does not enter the blood through the damaged intestinal barrier. Hence, vancomycin was suspected of altering the intestinal barrier function by affecting colonic butyrate metabolism. A total of 24 metabolites were identified from the colon of mice through 1H-NMR metabolomics (Fig. 7A). Interestingly, the metabolic phenotypes were significantly different between the three groups. In addition, the permutation test showed excellent model performances (R2X = 0.468, Q2 = 0.317) for distinguishing between the three groups (Fig. 7B). Based on the KEGG signaling pathway, butyrate metabolism (butyrate and 3-hydroxy-3-methylglutarate) was significantly elevated in the ARDS + V group, but acetoacetate content did not change significantly. After oral vancomycin treatment, 3-hydroxybutyrate showed an increasing trend but without statistical significance in the colon (probably due to the small sample size). While the glucose level increased significantly in the colon, lactate, the product of glycolysis, had significantly decreased. The relative quantity of metabolites of the butyrate metabolic pathway increased in the colon of mice after oral vancomycin treatment, suggesting enhanced colonic utilization of butyrate. In contrast, the availability of glucose, a butyrate-replacing cellular fuel, was reduced in the colon of ARDS + V mice (Fig. 7C).
Discussion

The reasonable symbiosis of host microorganisms is an integral part of maintaining human health. Once the balance of this cycle is disturbed, host diseases occur [7]. The current study sheds novel insights into host-microbe interactions during lung inflammation. Firstly, the intestinal flora of ARDS patients was significantly different from that of healthy people. The richness of the intestinal flora was increased considerably, while the species diversity was significantly decreased in ARDS patients. Additionally, the colon function of ARDS patients was evidently damaged due to intestinal barrier disruption and endotoxin translocation. The intestinal permeability had not changed significantly, consistent with the intestinal changes in other acute and critical illnesses. Previous studies on the brain-gut axis have shown that gut microbiota transplantation affects the host symptom performance. After receiving gut microbiota from ARDS patients, the healthy mice developed lung-injury-related inflammatory factors and showed an increased wet-to-dry lung weight ratio. Macrophages and neutrophils were the most altered inflammatory cells in ARDS lungs. EMR1 is a marker of mature mouse macrophages [27]. MPO is a functional marker of neutrophils, and changes in its level and activity represent the active status of neutrophil polymorphonuclear leukocytes (PMNs) [28]. The central inflammatory cells in the lung injury mice were macrophages, like that in the ARDS patients. Further, the number of neutrophils did not change, but their activity increased.

At the phylum level, Firmicutes and Verrucobacterium contributed to the differences in the fecal microbial communities between the ARDS and CON mice. Relative to the CON group, the abundance of Firmicutes in the ARDS group remarkably declined, while that of Verrucobacterium increased significantly. The Firmicutes mainly affect the physiological and pathological processes of the body by producing butyrate, the primary fuel of the colon [29]. Many studies have verified the anti-inflammatory activity of butyrate [30, 31]. Subsequently, the relative content of fecal metabolites was quantified in the ARDS patients by $^1$H-NMR. The metabolism of three main SCFAs (butyrate, acetate, propionate) had decreased within the feces of the ARDS patients. Butyrate was identified as the characteristic metabolite of ARDS in lung-injury mice. The decrease in the relative abundance of Firmicutes reduced the butyrate in lung injury, which was consistent with the current experimental results. *Akkermansia muciniphila* is an anaerobic, Gram-negative Verrucobacterium. The mucin-degrading bacteria in the mucus layer account for 1%-4% of the healthy human fecal microbiota. They are closely related to intestinal barrier function due to their ability to degrade mucins produced by the goblet cells in the intestinal mucosal barrier [32]. Thus, butyrate and mucin were suspected to be important targets of lung injury.

Subsequently, the relative content of fecal metabolites in the patients and mice was quantified by $^1$H-NMR. Butyrate was the only metabolite commonly reduced in the feces of patients and mice. Hence, it was clear that butyrate was the key metabolite of ARDS.

Butyrate enhances the gut mucosal barrier by direct induction of epithelial tight junction proteins [33]. Furthermore, butyrate-induced IL-22 secretion in ILC3 cells further augments this effect [33]. By interacting with GPCRs 43 and 41, butyrate inhibits the pro-inflammatory cytokine secretion by neutrophils [34].
Butyrate acts directly on dendritic cells (DCs) and macrophages by GPCRs. It also modulates T cell activity by up-regulating the Foxp3 T cells and suppressing IFN-α-producing T cells [35]. Furthermore, butyrate acts as an HDAC inhibitor and inhibits the NF-KB pathway to exert anti-inflammatory effects [36].

In the next set of experiments, the Firmicute population was killed by oral vancomycin to reduce butyrate in the gut further, thereby expecting aggravated lung damage. However, unexpectedly, all indicators of lung injury in the mice improved. The serum butyrate did not change significantly. Thus, butyrate did not directly enter the blood via damage to the intestinal barrier to affect other body organs. Now, gut microbes are the largest bacterial reservoir within the human body, suggesting that LPS in the serum may have originated from the gut. Thus, the increased LPS in the serum of the ARDS group reached the blood through the damaged intestinal barrier. In a classic model, LPS is usually administered to mice by intraperitoneal injection or intratracheal instillation to induce acute lung injury. Therefore, lung injury in FMT mice could have been caused by the increased LPS in the blood. The colonic goblet cells, mucins, and gut-associated tight junction proteins had decreased in the ARDS mice. In contrast, after oral vancomycin treatment, the goblet cells and muc2 increased, and tight junction proteins increased, indicating improvement in intestinal barrier function and reduction in intestinal permeability. The butyrate metabolism in the colon was enhanced, and the glucose metabolism was weakened after oral vancomycin treatment. Possibly, the goblet cells utilized the excess glucose to produce the highly glycosylated mucin 2 (muc2), which forms a mucus layer and resists colonization and invasion by gut microbes [37]. The measurement of butyrate metabolism and glucose metabolism was different. However, in the colitis mouse model created through gavage with 4% dextran sodium sulfate (DSS) for seven days, butyrate was abnormally oxidized, and the glucose oxidation rate was significantly increased [38]. The β-hydroxybutyrate content declined while lactate levels were elevated in the DSS colitis group, which was consistent with the current experimental results.

This study was the first to establish a novel lung injury model by transplanting microbiota from ARDS patients into healthy C57BL/6J mice and illustrating the pathogenic mechanism of the ARDS model. Most existing studies used fecal microbiota transplantation as a treatment. The study used 16S rRNA sequencing technology and 1H-NMR to analyze the microbiota and metabolic phenotype characteristics of the fecal samples from ARDS patients and mice with lung injury, providing a multidisciplinary approach to investigate ARDS. Many previous experiments administered high doses of butyrate to mice to protect against lung injury. However, the current study explored the mechanism of physiological butyrate concentrations. Vancomycin was innovatively identified to enhance colonic butyrate metabolism to protect against ARDS. Thus, gut microbiota potentially contributes to the symptomatic presentation of lung injury. Modulating colonic butyrate metabolism can be a valuable treatment option for ARDS. Nevertheless, the method and efficiency of modulating colonic butyrate metabolism must be evaluated carefully. The present findings provide a reference for analyzing the relationship between the lungs and the gut. However, in the complex intestinal microecological environment of clinical patients, the role of butyrate metabolism in improving ARDS needs further research.
Conclusion

In this study, we established a new model of lung injury and verified that intestinal flora plays an important role in the occurrence and development of ARDS. In addition, we found that vancomycin effectively protects against ARDS by modulating colonic butyrate metabolism, indicating that the butyrate metabolism could be an effective target for ARDS therapy.

Abbreviations

ARDS: Acute respiratory distress syndrome; NMR: Nuclear magnetic resonance; Gly, glycine; Pyr, pyruvate; Phe, phenylalanine; Ile, isoleucine; Leu, leucine; Val, valine; Ala, alanine; CoA, coenzyme A; Ace, acetate; But, butyrate; Pro, propionate; TCA, tricarboxylic acid; Tau, taurine; PLS-DA, Partial least squares discrimination analysis; VIP: Variable importance in the projection; FDR: False discovery rate; APACHE II: Acute physiology and chronic health evaluation II scores; LAC, lactate; D-D, D-Dimer; WBC, white blood cell; NEUT#, absolute neutrophil count; LYMPH, absolute lymphocyte count; Hb, hemoglobin; PLT, platelets; TBIL, total bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TP, total protein; ALB, albumin; BUN, blood urea nitrogen; SCR, serum creatinine; GLC, glucose; CK, creatine kinase; LDH, lactate dehydrogenase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; PCT, procalcitonin; IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; CD4: Cluster of differentiation 4; CD8: Cluster of differentiation; MPO, myeloperoxidase; EMR1, mouse EGF-like module-containing mucin-like hormone receptor-like 1.

Declarations

The authors declare no competing conflicts of interest.

Authors’ contributions

Contributions: (I) Conception and design: YP Li, HC Gao; (II) Administrative support: YP Li, HC Gao, Hong Zheng; (III) Clinical sample collection: JN Chen, PC Lin; (IV) Data analysis and interpretation: JN Chen, Xi Zhang; (V) Animal experiments and statistical analysis of data: JN Chen, Yuying Shen, WL Zhang; (VI) Manuscript writing: JN Chen, Xi Zhang; (VII) Final approval of manuscript: All authors.

Acknowledgments

We would like to thank all participants for collecting the data for this clinical study.

Funding

This work was supported by the Project of National Natural Science Foundation of China (No. 81970066).

Availability of data and materials
All the relevant raw data and materials are freely available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

This study received approval from the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (No. 2020-111) and was conducted following the Declaration of Helsinki (as revised in 2013). Informed consent was obtained for experimentation with human subjects.

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Figures
Intestinal flora imbalance and impaired intestinal barrier function in ARDS patients.

(A) Analysis of differences in fecal microbial communities between ARDS patients and healthy individuals which as assessed by the mean relative abundance of PCoA. PCoA, principal co-ordinates analysis. (B) Changes in microflora richness and diversity. (C) phylum-level species relative abundance
bar stacked chart (D) The ratio of Firmicutes to Bacteroides in feces. (E)(F)(G) Elisa kits measure diamine oxidase (DAO), D-lactic acid (D-Lac) and lipopolysaccharide (LPS) levels in serum. HCON: healthy control human; HARDS: ARDS patients.

Figure 2

Gut microbiota transplantation from ARDS patients induces lung injury in mice.

(A) Flow chart of animal experimental flora transplantation. (B) Histopathological analyses of lung tissue (H&E staining; x200). (C) Wet/dry weight ratio of the lungs. (D) MPO activity in lung tissue was detected using commercial kits. The levels of TNF-α (E) and IL-6 (F) in BALF were examined using ELISA. (G) Differential cell counts in the BALF. (H) The expression and distribution of EMR1 were analyzed using immunohistochemistry (x400). WBC, white blood cell; Neu, neutrophils; Lym, lymphocyte count; Mon,
macrophages; Eos, eosinophils; Bas, basophils; MPO, myeloperoxidase. CON: mice transplanted with gut microflora of healthy control humans; ARDS: mice transplanted with gut microflora of ARDS patients.

Figure 3

Firmicutes and Verrucomicrobia are the key flora responsible for the manifestations of lung injury in mice.

(A) Analysis of differences in fecal microbial communities between ARDS group mice and CON group mice which as assessed by the mean relative abundance of principal co-ordinates analysis. PCoA, principal co-ordinates analysis. (B) Changes in microflora richness and diversity. (C) phylum-level species relative abundance bar stacked chart. (D) The distribution map of LDA values of different species. the color represents the corresponding group, and the length of the histogram represents the contribution of the different species (the LDA Score). The figure shows that the abundance of different groups is significantly different when the LDA Score > 4. (E) Quantitative map of relative abundance of Firmicutes. (F) Quantitative map of relative abundance of Verrucomicrobiota. CON: mice transplanted with gut microflora of healthy control humans; ARDS: mice transplanted with gut microflora of ARDS patients.
Figure 4

Butyrate is the characteristic metabolite in lung injury.

(A) Typical $^1$H NMR spectrum obtained from the feces of humans. (B) Typical $^1$H NMR spectrum obtained from the feces of mice; (C) Score scatter plot of PLS-DA from the feces of human. (D) the human feces metabolites were screened by the variable importance in the projection (VIP) scores. (E) Score scatter plot
of PLS-DA from the feces of mice. (F) the mice feces metabolites were screened by the variable importance in the projection (VIP) scores. (G) Metabolic pathway changes between ARDS patient and healthy people. (H) Metabolic pathway changes between ARDS group mice and CON group mice; the red or blue shades represent significantly increased and decreased levels of metabolites. 1 n-butyrate; 2 leucine; 33 isoleucine; 4 valine; 5 propionate; *ethanol; 6 lactate; 7 acetate; 8 proline; 9 Glutamate; 10 succinate; 11 trimethylamine; 12 lysine; # methanol; 13 taurine; 14 glycine; 15 glucose; 16 fumarate; 17 homovanillate; 18 tyrosine; 19 phenylalanine; 20 nicotinate; 21 galactose. Abbreviations: Gly, glycine; Pyr, pyruvate; Phe, phenylalanine; Ile, isoleucine; Leu, leucine; Val, valine; Ala, alanine; CoA, coenzyme A; Ace, acetate; But, butyrate; Pro, propionate; TCA, tricarboxylic acid; Tau, taurine. HCON: healthy control human; HARDS: ARDS patients; CON: mice transplanted with gut microflora of healthy control humans; ARDS: mice transplanted with gut microflora of ARDS patients; PLS-DA, Partial least squares discrimination analysis.
Figure 5

Lung injury in mice was alleviated after vancomycin administration. (A) Flow chart of animal experimental flora transplantation. (B) Histopathological analyses of lung tissue (H&E staining; x200). (C) Severity of lung injury evaluated using histological scoring. (D) Wet/dry weight ratio of the lungs. (E) MPO activity in lung tissue was detected using commercial kits. The levels of TNF-α (F) and IL-6 (G) in serum examined using ELISA. (H) The levels of butyrate in feces examined using NMR. The levels of
butyrate (I) and LPS (J) in serum examined using ELISA. MPO, myeloperoxidase; LPS, Lipopolysaccharide. The same letters suggested no statistical significance, $P > 0.05$; while different letters indicated the statistical significance, $P < 0.05$. NORM: mice without any treatment at all; CON: mice transplanted with gut microflora of healthy control humans; ARDS: mice transplanted with gut microflora of ARDS patients; ARDS+V: ARDS patient gut microflora transplant tract microflora and given oral vancomycin-treated mice.

**Figure 6**

Oral vancomycin can improve intestinal function and protect the intestinal barrier.

PAS staining(A) and quantitative plot of goblet cells(E) in colon tissue of mice; Muc2 immunofluorescence staining(B) and muc2 changes in mRNA levels(F) in colon tissue of mice; Occludin immunofluorescence staining (C) and occludin changes in mRNA levels(G); ZO-1 immunofluorescence staining (D) quantitative plot of goblet cells(E) in colon tissue of mice.
Figure 7

(A) D Typical $^1$H NMR spectrum obtained from the colon of mice. (B) Score scatter plot of PLS-DA from the feces of humans; (C) Butyrate metabolism in the colon. The same letters suggested no statistical significance, $P > 0.05$; while different letters indicated the statistical significance, $P < 0.05$. 1 Leucine; 2 Valine; 3 Isoleucine; 4 propionate; 5 Alanine; 6 butyrate; 7 2-Hydroxybutyrate; 8 Acetate; 9 Acetaminophen; 10 Butanone; 11 Glutamate; 12 Succinate; 13 3-Hydroxy-3-methylglutarate; 14 Glutamine; 15 Aspartate;
16 Choline; 17 taurine; 18 Acetoacetate; 19 Glycine; 20 Glucose; 21 Creatine; 22 Myo-Inositol; 23 Lactate; 24 3-Hydroxybutyrate; CON: mice transplanted with gut microflora of healthy control humans; ARDS: mice transplanted with gut microflora of ARDS patients; ARDS+V: ARDS patient gut microflora transplant tract microflora and given oral vancomycin-treated mice; PLS-DA, Partial least squares discrimination analysis.

Vancomycin protects the colon from bacterial invasion by enhancing colonic butyrate metabolism.

**Supplementary Files**

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