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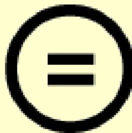

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의학박사 학위논문

중증 환자에서 카바페넴 내성 장내세균 집락화와 연관된 장내 미생물 총 조성 변화

Gut microbiota alterations in critically ill patients with carbapenem-resistant *Enterobacteriaceae* colonization

울 산 대 학 교 대 학 원
의 학 과
백 문 성

중증 환자에서 카바페넴 내성 장내세균 집락화와 연관된 장내 미생물 총 조성 변화

지 도 교 수 허 진 원

이 논문을 의학박사 학위 논문으로 제출함

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2023 년 8 월

감사의 글

박사 학위 논문이 완성될 수 있도록 아낌없는 지도와 격려를 해 주신 허진원 교수님과 권미나 교수님께 깊은 감사의 말씀을 올립니다.
저의 논문 심사를 맡아 주시고 진심 어린 조언을 해 주신 심사위원님들, 그리고 논문을 완성하는데 큰 도움을 주신 서울아산병원 융합의학과 김승일 박사님께 감사를 드립니다.
끝으로, 묵묵히 응원해준 아내 박혜영과 그 누구보다 애타게 아빠의 박사논문을 기다려온 사랑하는 나의 아들 수현이, 아울러 양가 부모님들께 감사의 마음을 전합니다.

요약

배경: 카바페넴 내성 장내세균 (Carbapenem-resistant *Enterobacteriaceae*, CRE)은 카바페넴을 포함한 대부분의 항생제에 광범위한 내성을 갖기 때문에, 중환자에서 높은 이환율과 사망률과 관련이 있다. 광범위한 항생제 사용과 중환자에서 미생물 군집 불균형이 CRE 집락화에 대한 위험 요인으로 작용한다. 그러므로, 본 연구에서는 중환자의 CRE 집락화에 따른 장내 미생물 군집의 변화를 측정하여 비교하고자 한다.

연구방법: 패혈성 쇼크 또는 호흡 부전으로 진단받고 중환자실에 입실한 41 명의 분변 샘플을 수집하였다. CRE 양성 9 명과 CRE 음성 32 명의 장내 미생물에 대해서 16S rRNA 유전자 염기서열 분석과 분변 단쇄 지방산의 정량 분석을 통해 비교하였다. CRE 집락화와 대사 경로 사이의 연관성을 확인하기 위해 박테리아 대사 풍부도 (bacterial metabolic abundance) 분석을 수행하였다.

결과: CRE 양성 환자는 CRE 음성 환자에 비해 프로테오박테리아문 (Proteobacteria)이 유의하게 증가했고 의간균문 (Bacteroidetes)은 감소한 것으로 나타났다. CRE 양성 환자에서 선형판별분석 (Linear discriminant analysis, LDA) 상 상위 2 LDA 점수를 보이는 것은 에르비니아속, 시트로박터속, 클레브시엘라속, 크로노박터속, 클루이베라속, 디스고노모나스속, 판토에아속, 알리스티페스속이었다. 알파 다양성 (Alpha diversity) 지수는 CRE 양성 환자에서 저하되었고, 베타 다양성 (Beta diversity) 분석은 CRE 집락화에 따라 뚜렷한 차이가 있음을 보여주었다. CRE 양성 환자에서 이소부틸산 (isobutyric acid)과 발레르산 (valeric acid)과 같은 측쇄지방산 (branched short-chain fatty acids)이 저하되어 있었다. 또한, PICRUSt 로 예측한 두 군 간 대사 경로 비교에서 ATP-binding cassette transporters, phosphotransferase systems, sphingolipid metabolism, other glycan degradation, microbial metabolism 가 유의한 차이가 있음을 확인하였다.

결론: CRE 양성 중환자는 CRE 음성 중환자에 비해 뚜렷하게 다른 장내 미생물 구성을 보였고, 측쇄지방산이 저하되어 있다. 또한, 항생제 유출펌프 (efflux pump)와 연관된 대사경로의 증가 소견을 보였다. CRE 환자에서 프리바이오틱스 또는 프로바이오틱스 투여로 장내 미생물 군집 불균형을 개선할 수 있는 지 추가 연구가 필요하다.

차　례

국문요약 ··· i
도표목차 ··· iii
영문요약 ··· iv
서론 ··· 1
연구방법 ··· 3
결과 ··· 9
고찰 ··· 25
결론 ··· 33
참고문헌 ··· 34

도표목차

표 1 ······ 9

표 2 ······ 11

표 3 ······ 12

표 4 ······ 14

그림 1 ······ 15

그림 2 ······ 19

그림 3 ······ 20

그림 4 ······ 22

그림 5 ······ 23

Abstract

BACKGROUND: Carbapenem-resistant *Enterobacteriaceae* (CRE) are an emerging concern for global health and are associated with high morbidity and mortality in critically ill patients. Risk factors for CRE colonization include broad-spectrum antibiotic use and microbiota dysbiosis in critically ill patients. Therefore, we evaluated the alteration of the intestinal microbiota associated with CRE colonization in critically ill patients.

METHODS: Fecal samples of 41 patients who were diagnosed with septic shock or respiratory failure were collected after their admission to the intensive care unit (ICU). The gut microbiota profile determined using 16S rRNA gene sequencing and quantitative measurement of fecal short-chain fatty acids were evaluated in CRE-positive (n=9) and CRE negative (n=32) patients. The analysis of bacterial metabolic abundance to identify an association between CRE acquisition and metabolic pathway was performed.

RESULTS: CRE carriers showed a higher proportion of the phyla Proteobacteria and lower abundance of the phyla Bacteroidetes as compared to the CRE non-carriers. Linear discriminant analysis (LDA) with linear discriminant effect size showed that the genera *Erwinia*, *Citrobacter*, *Klebsiella*, *Cronobacter*, *Kluyvera*, *Dysgonomonas*, *Pantoea*, and *Alistipes* had an upper 2 LDA score in CRE carriers. CRE carriers showed lower phylogenetic diversity, and beta-diversity analysis demonstrated that the two groups were clustered significantly apart. CRE carriers had lower levels of branched short-chain fatty acids such as isobutyric acid and valeric acid. Furthermore, the PICRUSt-predicted metabolic pathways

revealed significant differences in five features, including ATP-binding cassette transporters, phosphotransferase systems, sphingolipid metabolism, other glycan degradation, and microbial metabolism, in diverse environments between the two groups.

CONCLUSIONS: Critically ill patients with CRE have a distinctive gut microbiota composition and community structure, altered branched short-chain fatty acid production and changes in the metabolic pathways. Further studies are needed to determine whether prebiotics and/or probiotics improves microbiota dysbiosis in patients with CRE.

Introduction

Nosocomial multidrug-resistant organism (MDRO)-induced infections constitute a major problem in intensive care units (ICU) worldwide (1). Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), multidrug resistant *Acinetobacter baumannii* (MRAB), and extended-spectrum β-lactamases producing *Enterobacteriaceae* (ESBL-E) are the organisms that commonly cause nosocomial infections (2). However, carbapenem-resistant *Enterobacteriaceae* (CRE) has recently emerged as a global challenge because of the high CRE-associated morbidity and mortality rates (3). CRE infections are difficult to treat because of extensive resistance patterns and the limited effective antimicrobial therapeutic options (4). Several factors, including prior antimicrobial exposure, longer ICU stay, or residence in a long-term care facility, contribute to the occurrence of CRE (5). The presence of CRE colonization at ICU admission is associated with higher CRE infection-associated 30-day and 90-day mortality risks (6). Furthermore, the prevention of CRE transmission incurs a substantial economic burden. In a surgical ICU, the financial costs accrued from CRE outbreaks in 3 years was approximately €1,300,000 (7). Therefore, decreasing the risk for CRE colonization and ensuring active surveillance for the detection of CRE carriers is essential in the ICU (5).

The identification of gut microbiota such as next-generation sequencing or 16S rRNA profiling has substantially improved the understanding and insight into the composition and

function of the gut microbiota (8, 9, 10). In the gut microbiome, the abundant phyla are Firmicutes (60–75%), Bacteroidetes (30–40%), Actinobacteria, and Proteobacteria (11). The normal microbiota confers protection against the enteric pathogens, including MDROs, and decrease the colonization pressure (12). However, the use of antibiotics or ICU-specific therapies, such as selective digestive decontamination, can result in a state of disequilibrium of the microbiota (8). This dysbiosis of microbiota in critically ill patients is associated with a poor prognosis and/or an increased risk for infection. Furthermore, short-chain fatty acids, which are produced by the bacterial fermentation of undigested carbohydrates (13), play a crucial role as the major energy source, ensure the maintenance of the barrier function in the colonic epithelium, and help regulate the immune system (14).

We hypothesized that the gut microbiota, the related short-chain fatty acids, or multiple metabolic pathways differed between CRE carriers and CRE non-carriers. In this study, we aimed to determine the differences in the intestinal microbiota according to the identification of the CRE status of critically ill patients. Furthermore, immunosuppression is associated with increased risks of CRE colonization (15). There is a possibility that immunocompromised status may influence the CRE colonization. In addition, we evaluated the dysbiosis of microbiota in critically ill patients according to immunocompromised status.

Methods

Study design and eligible patients

This retrospective study was conducted at Asan Medical Center, which is a tertiary hospital with 2,800 beds in the Republic of Korea. Between October 1, 2016, and August 31, 2021, adult critically ill patients (age >18 years) who were diagnosed with septic shock or respiratory failure were eligible for inclusion. Patients who were admitted to the ICU with the abovementioned diagnoses were requested to provide a stool sample after obtaining written informed consent for participation in the study. This study was approved by the Institutional Review Board (IRB) of Asan Medical Center (IRB 2011-0001), and was conducted in accordance with the Declaration of Helsinki as well as the local regulations governing clinical studies.

Data collection and definitions

The following data were retrospectively obtained from the electronic medical records of the participants: demographic data, including age, sex, body mass index, smoking status (current smoker, ex-smoker, and never smoker), comorbidities (hypertension, diabetes, chronic lung disease, chronic kidney disease, chronic liver disease, cardiovascular disease, neurologic disorder, solid tumor, and hematologic malignancy), and gastrointestinal symptoms. Additionally, clinical data at hospitalization, such as hospitalization history and medication history (chemotherapy or immunosuppressant use, antibiotic therapy, and digestive therapy)

within the preceding 3 months were obtained. Furthermore, data pertaining to the ICU admission, including the interval between hospitalization to ICU admission, route of ICU admission (emergency department, ward, and other hospital), cause of ICU admission (septic shock or respiratory failure), severity scores (Acute Physiology and Chronic Health Evaluation [APACHE II] and Sequential Organ Dysfunction Assessment [SOFA] scores) at ICU admission, mechanical ventilation, renal replacement therapy, length of ICU stay, duration of mechanical ventilation, length of hospital stay, and the in-hospital mortality rate, were collected.

At the time of fecal sampling, the following variables were collected: interval from hospitalization to sampling, interval from ICU admission to sampling, diet (nil per os and enteral nutrition), and medications (e.g., opioid, sedatives, norepinephrine, vasopressin, probiotics, H2 blocker, proton-pump inhibitor, antibiotics, and antifungal agents) within the preceding 7 days. Moreover, infection-related variables, including bacteremia, *Clostridium difficile* infection (CDI), aspergillosis, and the MDROs (ESBL-E, MRAB, VRE, MRSA, and CRE) that were identified within 1 month, were collected.

CRE are defined as *Enterobacteriaceae* that are resistant to any carbapenem antibiotics (i.e., ertapenem, meropenem, doripenem, or imipenem), and include both non-carbapenemase-producing *Enterobacteriaceae* (non-CP-CRE) and carbapenemase-producing *Enterobacteriaceae* (CP-CRE) (4). We defined CRE carriers as participants in whom CRE was detected in any type of specimen, including blood, sputum, urine, stool, or rectal swab,

regardless of the presence of CRE-related symptoms (4). Based on the Sepsis-3 definition, septic shock was defined based on the requirement of vasopressors to maintain a mean arterial pressure of 65 mmHg or higher and lactic acid 2 mmol/L or higher in patients with sepsis (16). Respiratory failure included gas-exchange dysfunction due to a respiratory system failure, which was characterized as hypoxemic ($PaO2$ <60 mmHg) and/or hypercapnic ($PaCO2$ >45 mmHg) respiratory failure (17). The diagnosis of CDI was established in patients with diarrhea based on a positive result of the stool test for toxigenic *C. difficile* or its toxins, or from colonoscopic/histopathologic findings of pseudomembranous colitis (18).

Immunocompromised status was identified based on a diagnosis of malignancy, a diagnosis of human immunodeficiency virus or acquired immune deficiency syndrome, organ transplantation within 3 years, prescribed corticosteroids or oral immunosuppressants for ≥30 days during the last year and prescribed non-oral immunosuppressants at least once during the last year (19).

Microbiome analysis

Fecal samples of participants (n=48) were collected, and we excluded fecal samples (n=7) that did not meet the analytical quality. Stool samples were transported to the laboratory and stored at −80°C. Total DNA was extracted from the feces by using QIAamp Fast DNA stool mini kits (Qiagen) in accordance with the manufacturer's instructions. Primers 341F and 805R that target bacterial 16S rRNA gene were used for bacterial PCR amplification, and the

amplified products were purified and sequenced by Chunlab (Seoul, Republic of Korea) with an Illumina Miseq Sequencing System (Illumina). The processing of raw reads started with a quality check and the filtering of low-quality reads (<Q25) using Trimmomatic ver. 0.32 (20). After a quality control pass, the paired-end sequence data were merged using the VSEARCH version 2.13.4 and default parameters. Nonspecific amplicons that did not encode 16S rRNA were detected by the "nhmmer" program in the HMMER package ver. 3.2.1 (21). We used the EzBioCloud 16S rRNA database for taxonomic assignment using precise pairwise alignment (22). After chimeric filtering, reads that were not identified to the species level (with <97% similarity) in the EzBioCloud database were compiled. Operational taxonomic units with single reads (singletons) were omitted from further analysis. The alpha and beta diversities were estimated for ascertaining the difference in samples. A taxonomic cladogram was generated using linear discriminant effect size (LEfSe) with a threshold of 2 on the logarithmic LDA score (23).

Alpha and beta diversity

Alpha-diversity was used to assess gut microbial diversity within fecal samples (8). Several indices were used to estimate the within-sample microbial richness and diversity as following: Chao1 and Abundance-based coverage Estimator (ACE) for richness, and Shannon and Simpson for evenness. Beta-diversity, quantified by calculating Bray-Curtis dissimilarities, was used to assess gut microbiota diversity across samples.

Quantitative measurement of short-chain fatty acids

All reagents and solvents for metabolite analysis were purchased from Sigma. Freeze-dried feces (10 mg) were homogenized vigorously with 400 μL internal standard solution [1 mM propionic acid (C3)-d6, 100 μM butyric acid (C4)-d7, 100 μM of valeric acid (C5)-d4, 100 μM of Hexanoic acid (C6)-d5 in water] and centrifuged with 13,200 rpm for 10 min (22). After centrifugation, the supernatant was filtered out. Then, 20 μL 20 mM AABD-SH, 20 μL 20 mM TPP, and 20 μL DPDS in dichloromethane were added to the filtrate. The solution was incubated for 10 min at RT with vortexing and thereafter vacuum-dried. The sample was reconstituted with 80 μL methanol prior to the LC-MS/MS analysis in an LC-MS/MS system equipped with a 1290 HPLC (Agilent Technologies, Denmark) Qtrap 5500 (ABSciex) and a reverse-phase column (Pursuit 5 C18 150 × 2.0 mm; Agilent Technologies). The extracted ion chromatogram (EIC), which corresponded to the specific transition for each metabolite, was used for quantitation. The area under the curve of each EIC was normalized to the internal standard. The peak area ratio of each metabolite was normalized to the internal standard using the feces weight in a sample, and the value obtained was then used for the comparison.

Prediction of metabolic pathway

Kyoto Encyclopedia of Genes and Genome (KEGG) pathway is a collection of pathway maps representing the molecular interaction, reaction and relation networks including metabolism, genetic information processing, environmental information processing, cellular

processes, organismal systems, human diseases, and drug development. To predict bacterial metabolic abundance and functional gene profiles defined by the Kyoto Encyclopedia of Genes and Genome (KEGG) pathway, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) with the bacterial 16S rRNA gene sequences dataset was used (10). The statistically significant KEGG pathway of each group was identified by the LEfSe (LDA scores > 2).

Statistical analysis

Statistical analyses were performed by using Prism software (GraphPad, La Jolla) and R ver. 4.1.2 (R Project for Statistical Computing) with the Fisher's exact test and Mann–Whitney U test. Categorical variables are expressed as the number (percentage) whereas continuous variables are expressed as the median [interquartile range (IQR)]. Data are presented as the mean ± SD; $p < 0.05$ was considered statistically significant. Univariate and multivariate analyses of the risk factors associated with CRE colonization were performed. Candidate variables for inclusion in the multivariate regression model were variables with $p < 0.1$ in the univariate analysis and clinical parameters. Calibrations of the models were evaluated using the Hosmer–Lemeshow goodness-of-fit test. We obtained the adjusted odds ratio (OR) along with 95% confidence interval (CI) and to define the variables that were independently associated with CRE colonization.

Results

Characteristics of the study population

Between October 1, 2016, and August 31, 2021, data from 41 critically ill patients were included in the analysis. This cohort included 9 CRE carriers (22%) and 32 non-carriers (78%). CRE was detected in one blood, two sputum, and six stool specimens. Among the CRE carriers, the pathogens identified were seven cases with *Klebsiella pneumoniae*, one with *Escherichia coli*, and one with *Klebsiella oxytoca*. The cohort included five non-CP-CRE and four CP-CRE cases. All CP-CRE cases had the KPC enzyme. Baseline characteristics, including demographic data, are summarized in Table 1. The mean age of the participants in this cohort was 64 years (IQR 54–68.5) and 70.7% (n=29) was male. The proportions of patients with a history of hospitalization and of antibiotic therapy within 3 months were 31.7% (n=13) and 34.1% (n=14), respectively.

Table 1. Baseline characteristics of the participants

Variables	Total (n=41)	Carriers (n=9)	Non-carriers (n=32)	*p*-value
Age, years	64 (54– 68.5)	64 (55–69)	64.5 (54–69)	0.801
Male	29 (70.7)	6 (66.7)	23 (71.9)	1.000
Body mass index, kg/m^2	22.7 (19.3–24.5)	23.0 (21.0–26.3)	22.7 (18.6–24.4)	0.244
Smoking status				1.000
Current smoker	7 (17.1)	1 (11.1)	6 (18.8)	
Ex-smoker	15 (36.6)	3 (33.3)	12 (37.5)	
Never smoker	19 (46.3)	5 (55.6)	14 (43.8)	
Comorbidities				

Hypertension	6 (14.6)	0 (0.0)	6 (18.8)	0.309
Diabetes	10 (24.4)	3 (33.3)	7 (21.9)	0.662
Chronic lung disease	12 (29.3)	3 (33.3)	9 (28.1)	1.000
Chronic kidney disease	5 (12.2)	2 (22.2)	3 (9.4)	0.299
Chronic liver disease	7 (17.1)	3 (33.3)	4 (12.5)	0.165
Cardiovascular disease	4 (9.8)	0 (0.0)	4 (12.5)	0.559
Neurologic disorder	2 (4.9)	0 (0.0)	2 (6.3)	1.000
Solid tumor	14 (34.1)	2 (22.2)	12 (37.5)	0.692
Hematologic malignancy	6 (14.6)	3 (33.3)	3 (9.4)	0.107
Gastrointestinal symptoms	24 (58.5)	4 (44.4)	20 (62.5)	0.450
Hospitalization history*	13 (31.7)	6 (66.7)	7 (21.9)	0.018
Chemotherapy or immunosuppressant use*	16 (30.9)	4 (44.4)	12 (37.50	0.717
Antibiotic therapy*	14 (34.1)	5 (55.6)	9 (28.1)	0.231
Digestive therapy*	5 (12.2)	2 (22.2)	3 (9.4)	0.299

Values are presented as mean (IQR) or number (percentage).

*within 3months

CRE: carbapenem-resistant *Enterobacteriaceae*.

The causes of ICU admission were septic shock in 46.3% (n=19) and respiratory failure in 53.7% (n=22) of the cohort (Table 2). The median SOFA score at ICU admission was 12 (7–15), and mechanical ventilation was used in 90.2% (n=37) of the cohort. The ICU and in-hospital mortalities were 22.0% (n=9) and 36.6% (n=15), respectively. Gastrointestinal symptoms were not different between CRE positive and CRE negative group (44.4% vs. 62.5%, p=0.450). The hospital stay before ICU admission was longer in the CRE positive than in the CRE negative group.

Table 2. Clinical course of the patients

Variables	Total (n=41)	Carriers (n=9)	Non-carriers (n=32)	*p*-value
Hospitalization to ICU, days	2 (0–15)	15 (4–22.5)	1 (0–8)	0.008
ICU admission				0.022
Via emergency department	14 (34.1)	0 (0.0)	14 (43.8)	
Via ward	21 (51.2)	8 (88.9)	13 (40.6)	
From other hospital	6 (14.6)	1 (11.1)	5 (15.6)	
Cause of ICU admission				0.712
Respiratory failure	22 (53.7)	4 (44.4)	18 (56.3)	
Septic shock	19 (46.3)	5 (55.6)	14 (43.8)	
Cause of septic shock				0.577
Pneumonia	8 (42.1)	1 (20.0)	7 (50.0)	
Gastrointestinal	2 (10.5)	1 (20.0)	1 (7.1)	
Urinary tract infection	1 (5.3)	0 (0.0)	1 (7.1)	
Bacteremia	4 (21.1)	2 (40.0)	2 (14.3)	
Hepatobiliary	4 (21.1)	1 (20.0)	3 (21.4)	
APACHE II*	26 (19.5–34.5)	31 (22–39)	25 (19–32.5)	0.196
SOFA score*	12 (7–15)	13 (7.5–15)	12 (7–15.5)	0.752
MV	37 (90.2)	7 (77.8)	30 (93.8)	0.204
Renal replacement therapy	22 (53.7)	5 (55.6)	17 (53.1)	1.000
Length of hospital stay, days	53 (24.5–107)	60 (33.5–91.5)	50 (22–129)	0.422
Length of ICU stay, days	15 (8.5–44.5)	17 (8.5–38)	14.5 (8–45)	0.862
Duration of MV, days	12 (7–38.5)	12 (9–27)	11.5 (7–42)	0.938
ICU mortality	9 (22.0)	2 (22.2)	7 (21.9)	1.000
In-hospital mortality	15 (36.6)	4 (44.4)	11 (34.4)	0.701

Values are presented as mean (IQR) or number (percentage).

*at ICU admission

CRE: carbapenem-resistant *Enterobacteriaceae*; ICU: intensive care unit; APACHE: Acute Physiology and Chronic Health Evaluation; SOFA: Sequential Organ Dysfunction Assessment;

MV: mechanical ventilation.

The details of medications and infection status at fecal sampling are presented in Table 3. The interval from hospitalization to fecal sampling date was longer in CRE carriers (26 days [IQR 22–43] vs. 8.5 days [IQR 3.5–20], p=0.006). At the time of fecal sampling, 68.3% (n=28) were receiving enteral feeding, and H2 blockers or proton-pump inhibitors were administered in 92.7% (n=38) of the cohort. Beta-lactam antibiotics were given in 97.6% of the participants. Carbapenem antimicrobials were administered in 53.7% of the participants, and there was no significant difference between CRE positive and negative groups (66.7% vs. 50.0%, p=0.466). Bacteremia and CDI were noted in 43.9% (n=18) and 12.2% (n=5) of the cohort, respectively. The MDROs constituted 7.3% (n=3) of MRSA and 12.2% (n=5) of VRE infections.

Table 3. Medications and co-infection at fecal sampling according to CRE carrier

Variables	**Total (n=41)**	**Carriers (n=9)**	**Non-carriers (n=32)**	***p*-value**
Hospitalization to sample, days	15 (5.5–25.5)	26 (22–43)	8.5 (3.5–20)	0.006
Diet				0.228
Nil per os	13 (31.7)	1 (11.1)	12 (37.5)	
Enteral nutrition	28 (68.3)	8 (88.9)	20 (62.5)	
Medications				
Opioid	25 (61.0)	5 (55.6)	20 (62.5)	0.717
Sedatives	18 (43.9)	3 (33.3)	15 (46.9)	0.706
Norepinephrine	18 (43.9)	6 (66.7)	12 (37.5)	0.147
Vasopressin	6 (14.6)	1 (11.1)	5 (15.6)	1.000
Probiotics	5 (12.2)	0 (0.0)	5 (15.6)	0.568

H_2 blocker or PPI	38 (92.7)	9 (100.0)	29 (90.6)	1.000
Carbapenem	22 (53.7)	6 (66.7)	16 (50.0)	0.466
Quinolone	18 (43.9)	3 (33.3)	15 (46.9)	0.706
Beta-lactam	40 (97.6)	9 (100.0)	31 (96.9)	1.000
Piperacillin/tazobactam	17 (41.5)	2 (22.2)	15 (46.9)	0.262
Antifungal agent	11 (26.8)	3 (33.3)	8 (25.0)	0.680
Bacteremia	18 (43.9)	5 (55.6)	13 (40.6)	0.471
C. difficile infection	5 (12.2)	2 (22.2)	3 (9.4)	0.299
IPA	5 (12.2)	1 (11.1)	4 (12.5)	1.000
MDROs				
ESBL-E	11 (26.8)	2 (22.2)	9 (28.1)	1.000
MRAB	4 (4.0)	0 (0.0)	4 (3.1)	0.559
MRSA	3 (7.3)	0 (0.0)	3 (9.4)	0.340
VRE	5 (12.2)	2 (22.2)	3 (9.4)	0.299

Values are presented as mean (IQR) or number (percentage).

CRE: carbapenem-resistant *Enterobacteriaceae*; ICU: intensive care unit; PPI: proton-pump inhibitor; IPA: invasive pulmonary aspergillosis; MDRO: multidrug-resistant organism; ESBL-E: extended-spectrum β-lactamases producing *Enterobacteriaceae*; MRAB: multidrug-resistant *Acinetobacter baumannii*; MRSA: methicillin-resistant *Staphylococcus aureus*; VRE: vancomycin-resistant *Enterococcus*.

Risk factors associated with CRE colonization

Table 4 shows the risk factors associated with CRE colonization. The univariate and multivariate analyses revealed that hospitalization within 3 months was a significant risk factor for CRE colonization (OR 7.143 [95% CI: 1.414–36.080], p=0.017 and OR 7.607 [95% CI:1.058–54.722], p=0.044, respectively).

Table 4. Univariate and multivariate analyses of the risk factors associated with CRE colonization

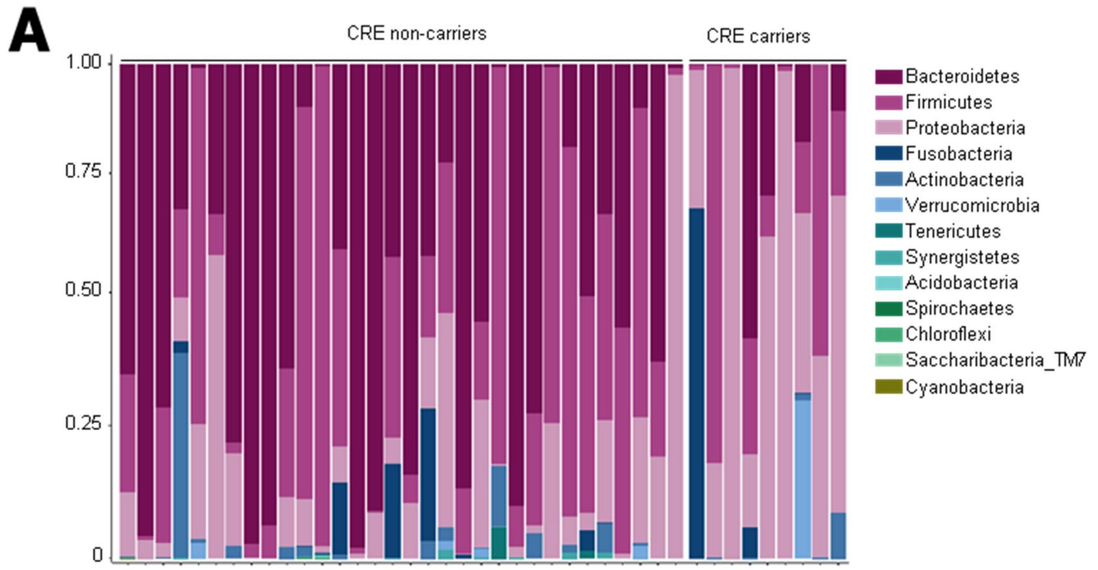
Variables	Univariate analysis	*p*-	Multivariate analysis	*p*-
	OR (95% CI)	value	OR (95% CI)	value
Age	0.989 (0.941–1.040)	0.675	1.009 (0.938–1.084)	0.816
Male (vs. female)	0.783 (0.160–3.821)	0.762		
Body mass index	1.106 (0.915–1.336)	0.297		
Diabetes	1.786 (0.354–9.020)	0.483		
Chronic lung disease	1.278 (0.262–6.239)	0.762		
Chronic kidney disease	2.762 (0.385–19.813)	0.312		
Solid tumor	0.476 (0.085–2.677)	0.400		
Hematologic malignancy	4.833 (0.779–30.005)	0.091	7.607 (1.058–54.722)	1.274
Immunocompromised state	1.103 (0.249–4.878)	0.897		
Hospitalization history	7.143 (1.414–36.080)	0.017	7.607 (1.058–54.722)	0.044
Chemotherapy or immunosuppressant use	1.333 (0.298–5.957)	0.706		
Antibiotic therapy	3.194 (0.696–14.664)	0.135		
Digestive therapy	2.762 (0.385–19.813)	0.312		
Hospitalization to ICU	1.057 (0.998–1.120)	0.058	1.065 (0.994–1.140)	0.074
Mechanical ventilation	0.233 (0.028–1.955)	0.180		
Carbapenem	2.000 (0.425–9.418)	0.381		
Bacteremia	1.827 (0.411–8.123)	0.429		
C. difficile infection	2.762 (0.385–19.813)	0.312		
ESBL-E	0.730 (0.127–4.203)	0.725		

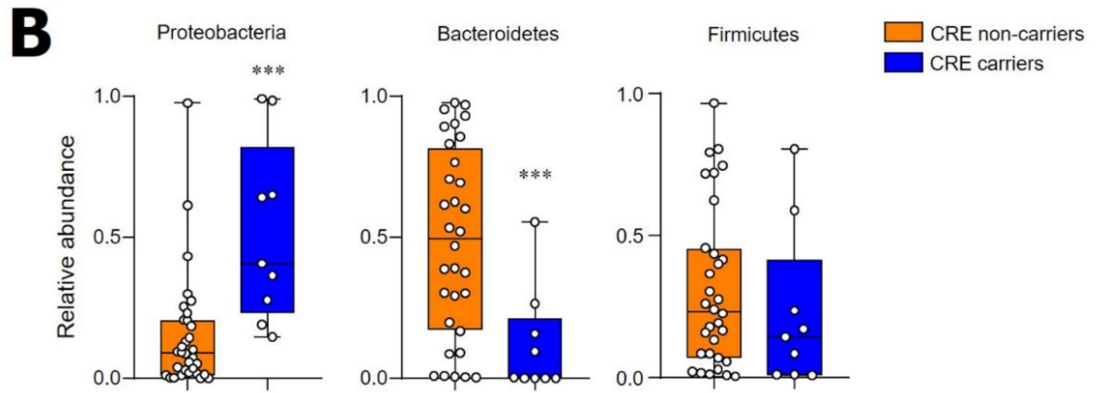
OR, odds ratio; CI, confidence interval; CRE: carbapenem-resistant *Enterobacteriaceae*; ICU: intensive care unit; ESBL-E: extended-spectrum β-lactamases producing *Enterobacteriaceae*. The clinical variables entered into the model were age, hematologic malignancy, hospitalization history (<3 months), and interval from hospitalization to ICU admission.

CRE carriers showed gut microbiota composition and metabolic pathway alterations

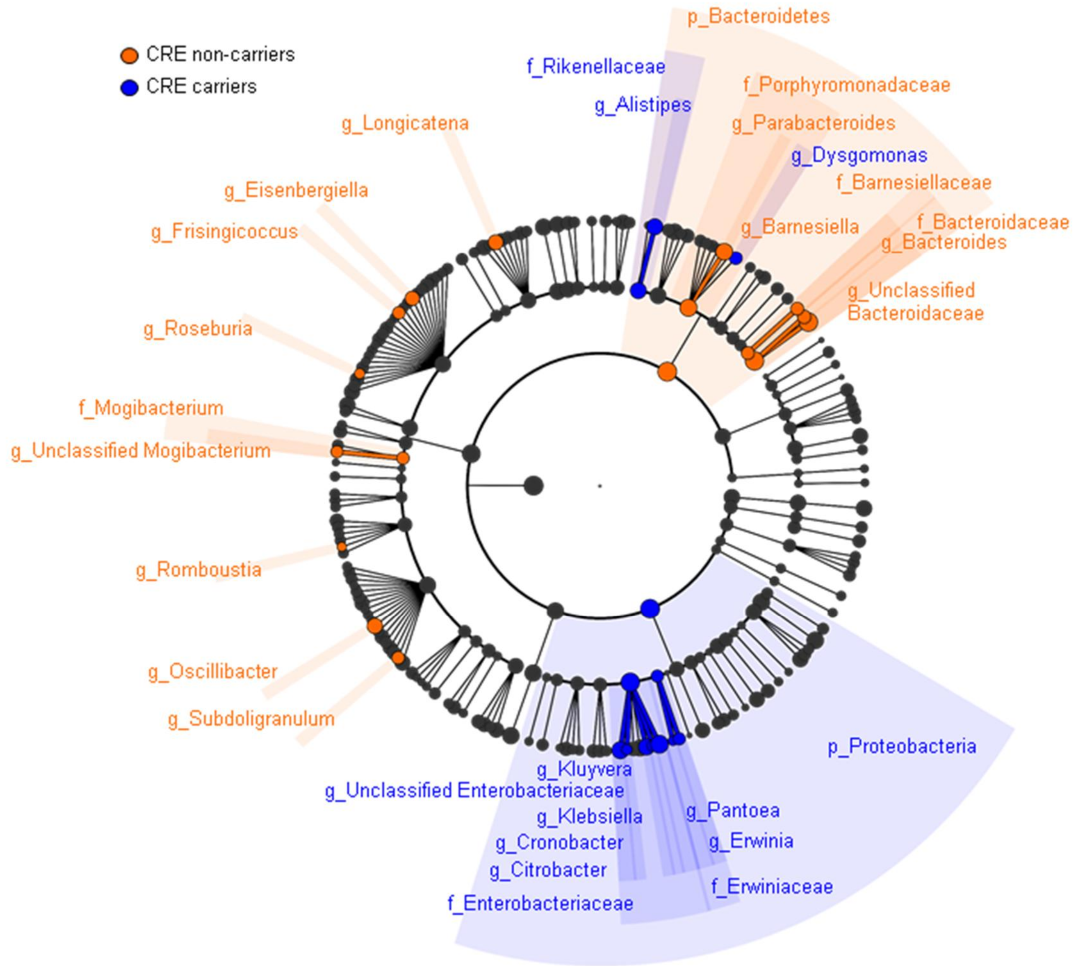
We first addressed the changes of gut microbiota composition from CRE carriers (Figure 1A). Of note, the relative abundance of the phyla in CRE carriers comprised 52% Proteobacteria, 23% Firmicutes, and 12% Bacteroidetes. In CRE non-carriers, the relative abundance of the phyla comprised 48% Bacteroidetes, 31% Firmicutes, and 15% Proteobacteria. Compared to CRE non-carriers, the gut microbiota from CRE carriers showed a higher proportion of Proteobacteria and lower proportion of Bacteroidetes ($p < 0.001$; Figure 1B). Additionally, linear discriminant analysis (LDA) with LEfSe revealed that several bacterial genera were noticeably altered in the gut microbiota of CRE carriers compared with those in non-carriers (Figure 1C). In CRE carriers, the genera *Erwinia*, *Citrobacter*, *Klebsiella*, *Cronobacter*, *Kluyvera*, *Dysgonomonas*, *Pantoea*, and *Alistipes* showed upper 2 LDA scores (Figure 1D) whereas the genera *Barnesiella*, *Subdoligranulum*, *Eisenbergiella*, *Frisingicoccus*, *Longicatena*, *Roseburia*, *Oscillibacter*, *Parabacteroides*, *Romboutsia*, and *Bacteroides* showed lower 2 LDA scores.

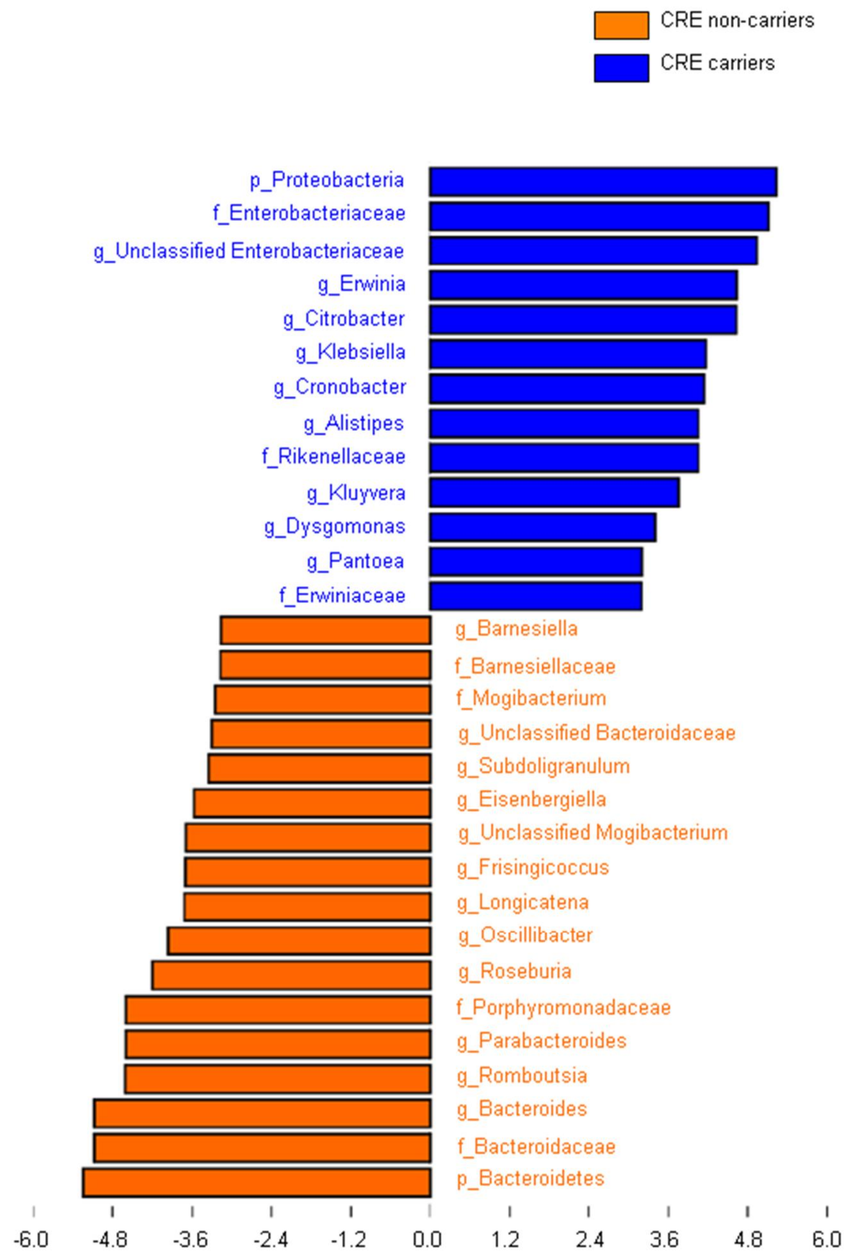
Figure 1. Carbapenem-resistant *Enterobacteriaceae* (CRE) influences gut microbiota composition and community structure. (A) Microbiota composition of feces from CRE non-carriers or carriers at the phylum level. (B) Relative abundance of the phyla, Proteobacteria, Bacteroidetes, and Firmicutes. (C) Taxonomic cladogram from linear discriminant effect size (LEfSe) analysis. Dot size is proportional to taxon abundance. (D) Linear discriminant analysis (LDA) scores obtained from LEfSe analysis of fecal microbiome. An LDA effect size of more than 2 was used as a threshold for the LEfSe analysis. Statistical analyses were performed using the Mann–Whitney *U* test. $^{***}p < 0.001$.


A
CRE non-carriers
CRE carriers
1.00
0.75
0.50
0.25
0
Bacteroidetes
Firmicutes
Proteobacteria
Fusobacteria
Actinobacteria
Verrucomicrobia
Tenericutes
Synergistetes
Acidobacteria
Spirochaetes
Chloroflexi
Saccharibacteria_TM7
Cyanobacteria


B
Proteobacteria
Bacteroidetes
Firmicutes
CRE non-carriers
CRE carriers
Relative abundance
1.0
0.5
0.0
***
***

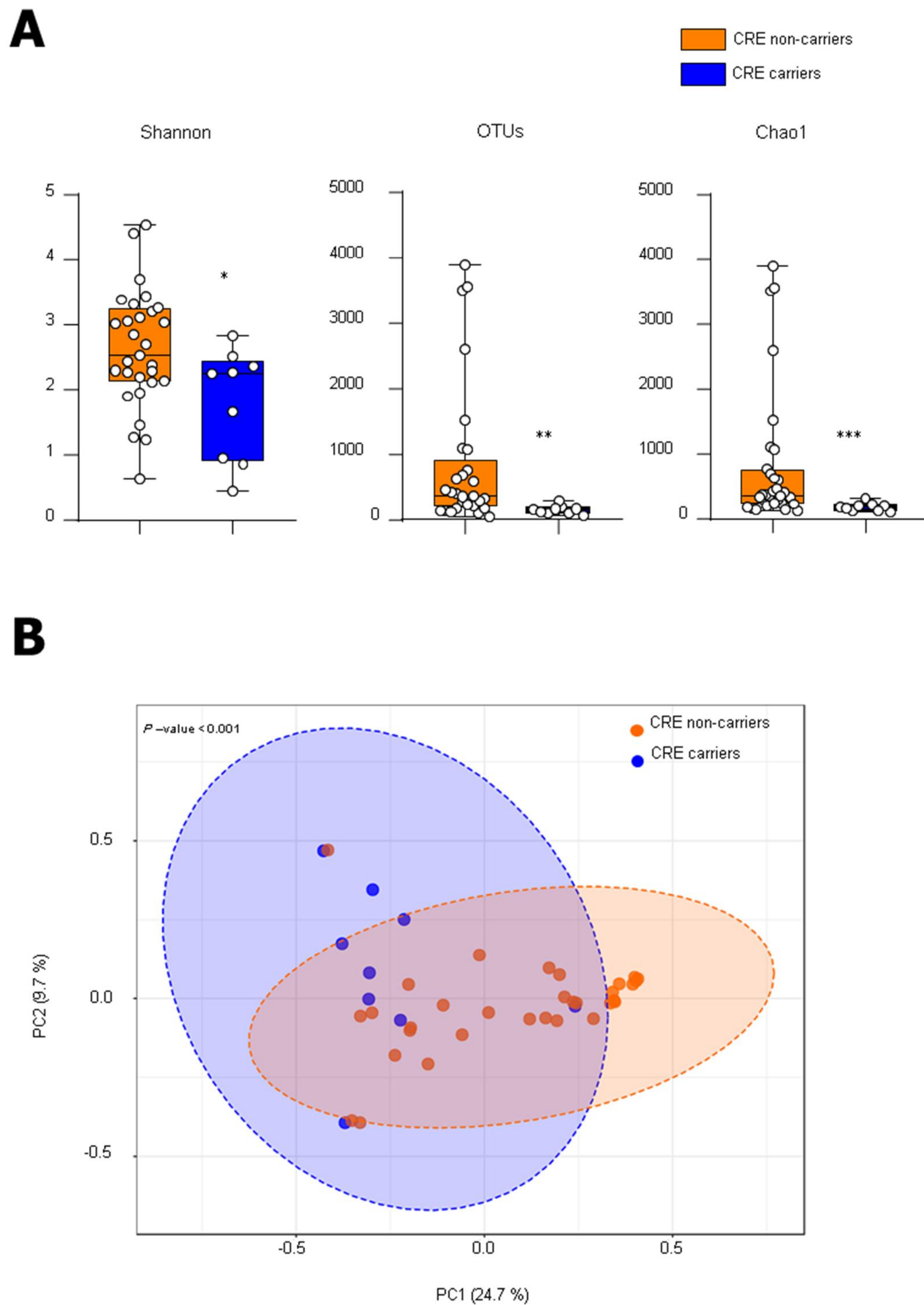
C





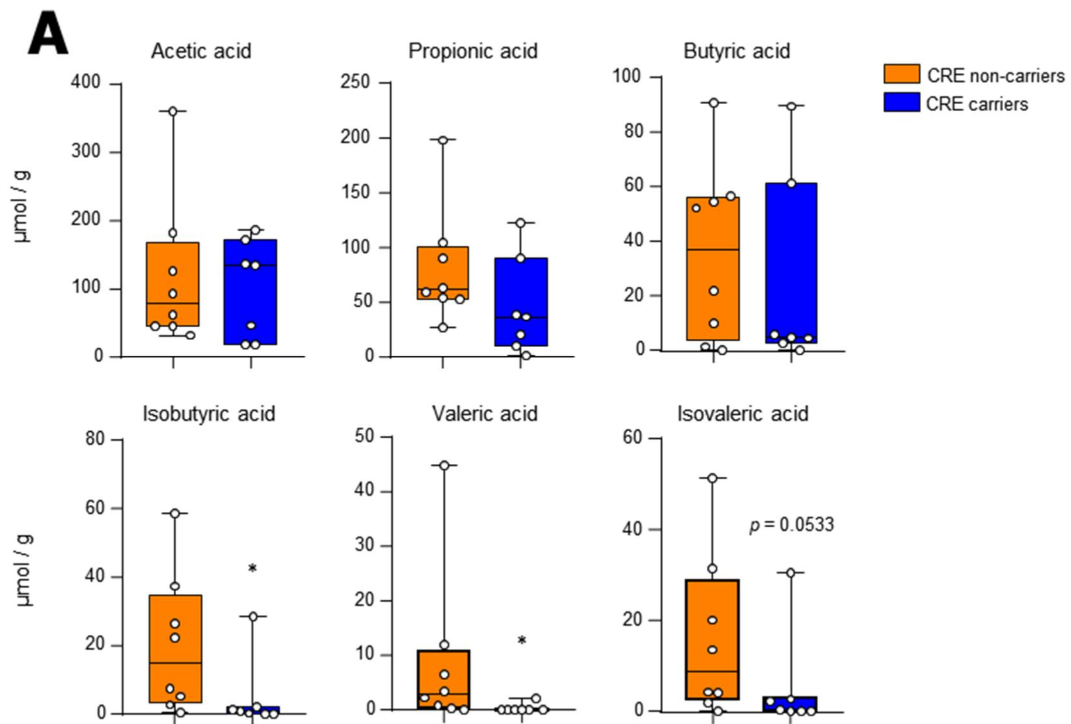
The alpha diversity index (Shannon, OTUs, and Chao1) were lower in CRE carriers compared to the CRE non-carriers (Figure 2A). Furthermore, the beta diversity analysis (Bray–Curtis distances) demonstrated that the two groups were clustered significantly apart (p < 0.001; Figure 2B).

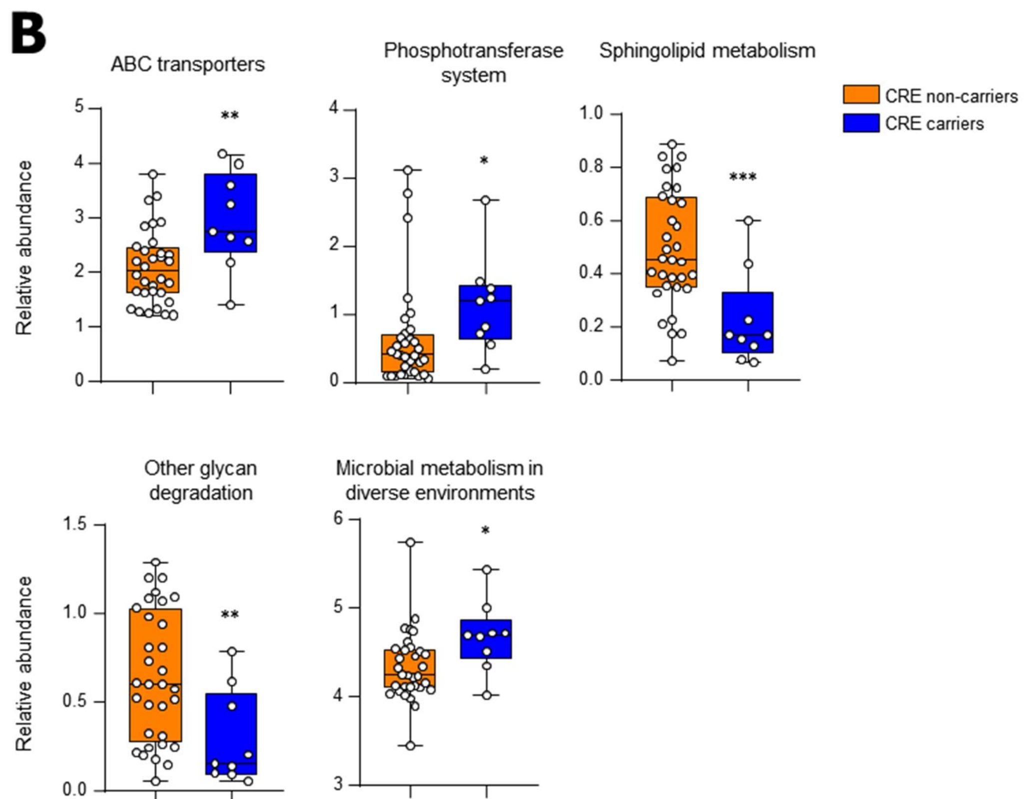
Figure 2. The community structure of the gut microbiome is associated with CRE colonization. (A) Alpha-diversity indices (Shannon, Observed OTUs, and Chao1). (B) Beta-diversity analysis based on the Bray–Curtis distance. Statistical analyses were performed using the Mann–Whitney *U* test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

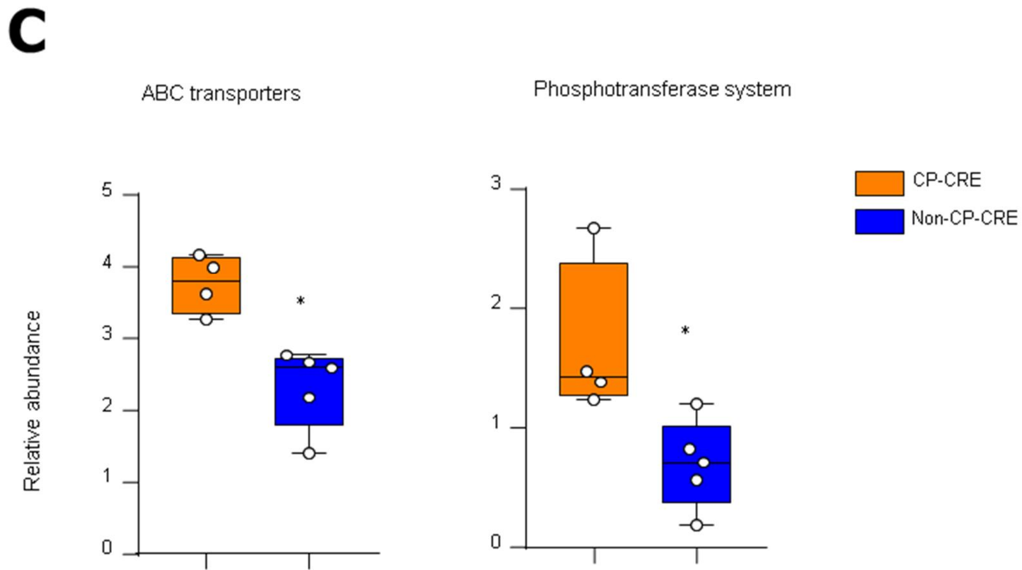


The short-chain fatty acid levels of fecal extracts were examined to identify which metabolites were associated with CRE carriers and non-carriers. Among the short-chain fatty acids, the levels of isobutyric acid and valeric acid were lower in CRE carriers ($p < 0.05$; Figure 3A). PICRUSt-predicted metabolic pathways showed five significant differences in the features between CRE carriers and non-carriers and these included: the KEGG pathway for ATP-binding cassette (ABC) transporters, phosphotransferase systems, sphingolipid metabolism, other glycan degradation, and microbial metabolism in diverse environments (Figure 3B). Among CRE carriers, patients with CP-CRE had significantly increased KEGG pathways for ABC transporters and phosphotransferase systems than those with non-CP-CRE ($p < 0.05$; Figure 3C).

Figure 3. CRE changes associated with the levels of short-chain fatty acids and functional metabolic profile. (A) Quantification of acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids derived from the fecal extract. (B) Predicted functional profiling of microbial communities based on 16S rRNA sequences. The relative abundances of level 3 KEGG pathway (LDA scores more than 2 are shown); ABC transporters (KO 02010), phosphotransferase system (KO 02060), sphingolipid metabolism (KO 00600), other glycan degradation (KO 00511), and microbial metabolism in diverse environments (KO 01120). (C) Predicted functional profiling of microbial communities between CP-CRE and non-CP-CRE. Statistical analyses were performed using the Mann–Whitney U test. *p < 0.05, **p < 0.01, *** p < 0.001.


A
Acetic acid
Propionic acid
Butyric acid
CRE non-carriers
CRE carriers
μmol / g
Isobutyric acid
Valeric acid
Isovaleric acid
p = 0.0533
*


B
ABC transporters
Phosphotransferase system
Sphingolipid metabolism
CRE non-carriers
CRE carriers
Relative abundance
Other glycan degradation
Microbial metabolism in diverse environments
**
*
***



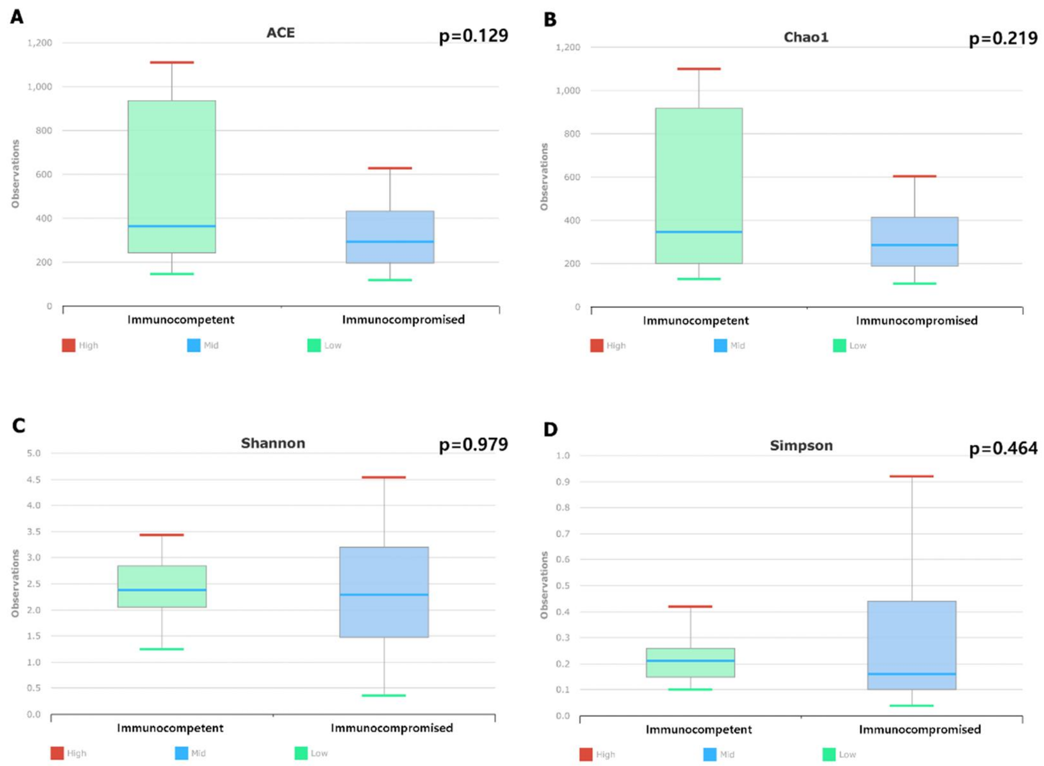
Gut microbiota composition according to immunocompromised status

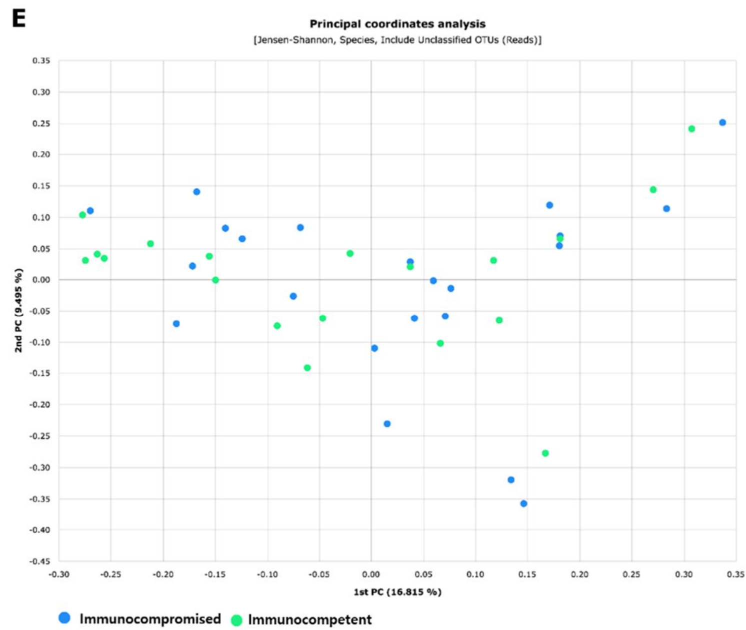
Further, we identified the changes of gut microbiota composition according to immunocompromised status at the phylum and genus levels (Figure 4). There were no differences in the relative abundance of the phyla Proteobacteria (p=0.100) and Firmicutes (p=0.333) according to immunocompromised status.

Figure 4. Gut microbiota composition and community structure according to immunocompromised status. (A) Microbiota composition of feces at the phylum level. (B) Microbiota composition of feces at the genus level. (C) Relative abundance of the phylum Bacteroidetes. (D) Relative abundance of the phylum Firmicutes.

There were no significant differences in the alpha diversity indices (ACE, Chao1, Shannon, and Simpson) according to immunocompromised status (Figure 5). Furthermore, the beta diversity analysis demonstrated that the two groups were not clustered apart.

Figure 5. The community structure of the gut microbiome according to immunocompromised status (A) ACE (B) Chao1 (C) Shannon (D) Simpson (E) beta-diversity index


A
ACE
p=0.129
Observations
Immunocompetent
Immunocompromised
High
Mid
Low
B
Chao1
p=0.219
C
Shannon
p=0.979
D
Simpson
p=0.464


E
Principal coordinates analysis
[Jensen-Shannon, Species, Include Unclassified OTUs (Reads)]
2nd PC (9.495 %)
1st PC (16.815 %)
Immunocompromised
Immunocompetent

Discussion

In this study, we compared the gut microbiome in critically ill patients according to the CRE carrier status and found that CRE carriers showed higher and lower proportions of the Proteobacteria and Bacteroidetes phyla, respectively. The alpha-diversity indices were lower in CRE carriers as compared with those in CRE non-carriers. Of note, the two groups were clustered significantly in the beta-diversity analysis. Furthermore, we observed that levels of short-chain fatty acids, such as isobutyric acid and valeric acid, had lower in CRE carriers. In terms of metabolic pathways, CRE carriers showed distinctive features as compared with CRE non-carriers and these included increased ABC transporters and decreased sphingolipid metabolism.

Recent studies demonstrated that CRE carriers had significantly higher number of Proteobacteria and lower prevalence of Firmicutes and Bacteroidetes than CRE non-carriers and healthy controls (25, 26). The results of our study revealed findings that are aligned with those previous reports. Proteobacteria dominance is associated with increased inflammation (27, 28), which can be attributed to CRE (29). Another notable finding is the change of the alpha- and beta-diversity indices in the two groups. The findings of this study are consistent with the results of a previous study (25), wherein the alpha-diversity indices of gut microbiota were lower in patients who were CRE carriers as compared to that in non-carriers.

Alpha-diversity index represents the microbial richness that refers the diversity of bacterial communities found in a particular ecosystem (8). Microbial richness decreases when the total

number of taxa within a given sample is low. Microbial diversity refers to how dominant a bacterial community is over another. Diversity decreases when a specific bacterial taxon overwhelmed other bacteria. We presented this using various methods such as Shannon, OTUs, Chao1, etc. Shannon index estimates the bacterial richness and diversity (8). Our data evidenced that bacterial richness and diversity were lower in CRE carriers than those in non-carriers, and the bacterial composition was different between two groups. Although our study results did not show the differences in mortality between the two groups, it is known that survival rate decreases in patients with reduced diversity (30). Yin and colleagues compared microbiome data base between ICU patients and healthy volunteers. They identified that decreased microbial diversity and reduced symbiotic bacteria in ICU patients, and poor outcome may be due to the decrease in symbiosis.

Intestinal dysbiosis has various effects such as changes in colonic mucus intestinal integrity and the production of metabolites (31). Hydrophobic mucus is covered in the intestinal wall and protect the intestine from the bacteria like a barrier (32). In critically ill patients with decreased mucus production and hydrophobicity, enterocytes injury and cell apoptosis can occur. Therefore, recovery of intestinal dysbiosis in CRE carriers may be beneficial. Lee et al. reported alterations of gut microbiota in CRE carriers during fecal microbiota transplantation (FMT) (33): the Shannon diversity index of gut microbiota in recipients significantly increased ($p < 0.05$), and 90% of CRE carriers were decolonized after FMT. Therefore, the restoration of microbiota balance may reduce CRE colonization (15).

In critically ill patients, alterations of the gut microbiota can affect patient outcomes. Garcia et al. demonstrated that the abundance of the *Enterococcaceae* family was associated with an increased risk of infection and death in the ICU (34). We further evaluated the microbiome taxonomy abundance at the genus level. We observed that the genera *Erwinia*, *Citrobacter*, *Klebsiella*, *Cronobacter*, and *Pantoea* were abundant in CRE carriers. These results resemble those in the report of Korach-Rechtman et al and revealed an increased abundance of *Enterobacter*, *Erwinia*, *Pantoea*, and *Klebsiella* (25). Notably, although phylum Bacteroidetes is lower, genus *Alistipes* is higher in CRE carriers. Some strains of *Alistipes* have antimicrobial resistance to beta-lactam antibiotics (35), and these may be associated with the abundance in CRE positive patients. The abovementioned authors suggested that these potentially virulent resident species become a predisposing factor, and consequently lead to host infection. A previous animal study showed that intestinal *Enterobacteriaceae* colonization was promoted by antibiotic treatment (36). Therefore, antibiotic therapy may accelerate these alterations of microbiota composition or induce changes in colonization resistance due to the decreased diversity. Furthermore, we observed that the *Barnesiella* and *Bacterioides* genera were lower in critically ill patients with CRE carriers. A previous study demonstrated that oxygen-tolerant bacteria, such as VRE, were suppressed by commensal anaerobic bacteria (37). In particular, the genus *Barnesiella*, which belongs to the phylum Bacteroidetes, was associated with protection against VRE domination. Similar to the findings of Ubeda et al., the *Barnesiella* species was less abundant in CRE carriers than in non-carriers. Therefore, this species is

expected to be involved in restriction of proliferation of CRE as well as VRE. There were no significant differences in demographic data, medications, ICU stay or mortality in critically ill patients according to CRE colonization. Therefore, this implies that these alterations of the gut microbiota were originated from the difference of CRE acquisition in critically ill patients.

Short-chain fatty acids are involved in the colonization resistance of antibiotic-resistant pathogens such as VRE or CRE (38). According to Sorbara et al., triggering short-chain fatty acid–mediated intracellular acidification is associated with in inhibiting the overgrowth of antibiotic-resistant *Enterobacteriaceae* (39). Furthermore, level of short-chain fatty acid was negatively correlated with *E. coli* within the microbiota in a patient with allogeneic hematopoietic stem cell transplantation. In line with this, previous studies showed that critically ill patients had a decrease in fecal short-chain fatty acids (40, 41). Of note, our results revealed that the major three short-chain fatty acids did not differ between the two groups. It is interesting to note, however, that valeric acid and branched short-chain fatty acid levels, including those of isobutyric acid and isovaleric acid, are reduced in CRE carriers compared with CRE non-carriers. The decreased levels of short-chain fatty acids are associated with infection as follows: isobutyric acid in patients diagnosed with infection upon ICU admission (41) and valeric acid in patients with recurrent CDI (42). These short-chain fatty acids are mainly produced by the phyla Firmicutes and Bacteroidetes (43). Therefore, low levels of short-chain fatty acids in CRE carriers might be attributable to the changes in the phyla. Furthermore, valeric acid, isobutyric acid, and isovaleric acid are produced by the amino acids

proline and hydroxyproline and valine and leucine, respectively (44, 45). Therefore, amino acid supplementation could affect the microbiome dysbiosis (46).

The KEGG pathway analysis revealed that CRE carriers have a distinctive metabolic pathway, with an abundance of ABC transporters, which are one of the superfamily of integral membrane proteins and are involved in the translocation of various ATP-using substrates (47). Drug resistance against anticancer or antimicrobial agents can be caused by activation of transmembrane proteins that efflux substances from the cells (48). Although enzymatic production is the main mechanism of CP-CRE, efflux pumps constitute an important mechanism of non-CP-CRE (49). Furthermore, we identified that ABC transporters are more abundant in patients with CP-CRE than those with non-CP-CRE. Therefore, these results suggest that ABC transporters play a role in the efflux of carbapenem in the CRE. Another notable finding in the KEGG pathway analysis is that sphingolipid metabolism is significantly decreased in CRE carriers compared with non-carriers. Sphingolipids are structural membrane components, and dysregulated sphingolipid homeostasis is associated with various pathophysiologic conditions, such as inflammatory bowel disease, cancer, or neurodegenerative diseases (50). Brown et al. reported that the levels of sphingolipids produced by *Bacteroides* spp. are decreased in the stool of the subjects with inflammatory bowel disease, and that sphingolipid-deficiency is associated with intestinal inflammation and barrier dysfunction (51). Therefore, the sphingolipid signaling pathways may play an essential role in the pathogenesis of inflammatory bowel disease. Furthermore, An et al. suggested that

a sphingolipid-mediated mechanism is utilized by the *Bacteroides* spp. to survive in the stressful intestinal environment (52). Therefore, the lower proportion of *Bacteroides* in CRE carriers could potentially result in decreased sphingolipid metabolism.

We attempted to identify risk factors for CRE colonization. Although the small number of study population, the univariate and multivariate analyses showed that hospitalization within 3 months was a significant risk factor for CRE colonization. Inevitably, patients with a history of repeated hospitalizations or long-term care facility residents are more likely to be exposed to MDROs. According to Yi et al., risk factors for CRE colonization are underlying comorbid conditions, prior antimicrobial exposure, indwelling catheters or device, prior use of healthcare facilities, prior hospitalization, length of hospital or ICU stay, exposure to long-term care facility, or co-colonization of MDROs (5). Although there are unavoidable factors associated with CRE acquisition, there are modifiable factors such as indwelling catheter or prior antimicrobial exposure. Therefore, if possible, efforts should be made to reduce the use of unnecessary antibiotics and to remove unnecessary catheters or devices.

Bacterial translocation concomitant with intestinal barrier dysfunction plays crucial roles in development of sepsis in immunocompromised hosts (53). Altered microbiome and increased intestinal permeability is associated with development of sepsis. It is known that immunocompromised state is associated with dysbiosis and change in the mucosal barrier permeability (54). However, our study did not reveal the difference of gut microbiota composition or diversity according to immunocompromised status in critically ill patients.

This result is presumed to have occurred because the interval from ICU admission to fecal sampling was very variable. Therefore, well-designed further study is needed for this issue.

We extensively evaluated the gut microbiome and the related metabolic pathways of critically ill patients with CRE. Nonetheless, there are several limitations of this study that warrant mention. First, due to the retrospective study design, detailed data of the diet were limited and were not controlled for between two groups. The diet is closely associated with the gut microbiota (14, 55), however, the majority of the patients received commercial enteral nutrition and parenteral nutrition. Nonetheless, there was a greater tendency for enteral nutrition in CRE carriers than in non-carriers. Second, since no healthy controls were enrolled, we analyzed the microbiome in critically ill patients with or without CRE. The ICU environment and medications might be associated with the similar proportion of Firmicutes between two groups (56, 57). Third, the interval between hospitalization and fecal sampling date was significantly longer in CRE carriers than in CRE non-carriers. CRE colonization is associated with longer hospitalization, ICU stay, and antibiotic usage (15). Most participants were exposed to a broad spectrum of antibiotics, and the number of CRE positive groups was small, so there was no difference in carbapenem usage between the two groups. Fourth, we cannot rule out the possibility that co-infection or co-colonization may have affected the gut microbiota. In the ICU setting, patients with CRE acquisition have multiple co-colonization with other MDROs (58). Therefore, it was difficult to recruit only CRE carriers who did not have other MDROs in our cohort. Fifth, given the relatively small number of study population,

it is difficult to generalize the results of our study. However, our study showed the alterations of levels of short-chain fatty acids and metabolic pathways according to CRE carriage. We also showed the significant differences of KEGG pathways related to efflux systems between CP-CRE and non-CP-CRE though we need to validate on large samples. Our study may suggest the role of metabolites in the management of CRE colonization.

Conclusion

In critically ill patients, CRE carriers demonstrated distinctive features of the gut microbiota and diversity indices. Furthermore, the levels of branched short-chain fatty acids were lower, and several changes in metabolic pathways, such as higher levels of ABC transporters and decreased sphingolipid metabolism, were observed in CRE carriers. Further research is needed to ascertain whether prebiotics and/or probiotics could suppress the pressure of CRE colonization.

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