EVALUATION OF GUT MICROBIOTA 16S RRNA SEQUENCING IN SILICO FOR SPECIES LEVELS AND CO-OCCURRENCE IN CARBAPENEM-RESISTANT ENTEROBACTERIACEAE CARRIERS

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Abstract. Recently, there has been a focus on the connection between the composition of gut microbiota and the development of Carbapenem-resistant Enterobacteriaceae (CRE). The objective of our work was to assess the available data on 16srRNA sequences of gut bacteria in patients admitted to the ICU. We used the ALDEx2 R package (v.1.22.0) to examine the association between these sequences and the presence and simultaneous occurrence of several bacterial species, in comparison to a control group. Out of the 60 stool samples, 24 individuals carrying CRE and 26 non-carrier groups have a higher abundance of Enterococcus durans compared to the control group. The abundance of Erysipelatoclostridium ramosum, Eubacterium limosum, Klebsiella pneumoniae, Parabacteroides distasonis, Proteus mirabilis, Bacteroides thetaiotaomicron, and Pseudomonas aeruginosa was higher in the group of individuals carrying CRE compared to non-carrier group. The CRE carrier group had a distinct profile of gut microbiota compared to the control group. These findings could contribute to advancements in the detection and treatment of antibiotic resistance patients.
Keywords: *dysbiosis, gut microbiome, phylogenetic diversity, antibiotic resistance, CRE-colonization*

Introduction

Because anaerobic infections are typically polymicrobial, drugs that have a major influence on anaerobic flora disrupt gut homeostasis the most. The diversity and richness of the gut microbiota are two critical elements of a resilient, healthy gut. *Faecalibacterium prausnitzii*, *Bifidobacterium*, and *Akkermansia muciniphila* have all been shown to increase butyrate production, which helps to maintain healthy gut mucosal barriers. *A. muciniphila* is a mucolytic bacterium that has been identified as a biomarker for positive immune responses (Reunanen et al., 2015). The stages of microbiome degeneration associated with unhealthy aging are most likely to begin with a decrease in the abundance of specific keystone species, followed by a complete loss of the surrounding bacterial community structure, allowing pathobionts to proliferate (Biagi et al., 2010; Ghosh et al., 2022). Moreover, the gut microbiota of adults is rather steady, whereas that of the elderly over 80 is marked by a reduction in microbial diversity (Yatsunenko et al., 2012). According to Yang et al.'s (2020) findings, the commensal of *B. longum* was the most abundant in the age group between 20 and 80 years old.

Antibiotic resistance is becoming more of a problem. All known antibiotic resistance genes exist, and very resistant infections are becoming increasingly widespread. Gram-negative bacteria, especially Carbapenemase-producing Enterobacteriaceae (CRE), have achieved the largest spectrum of resistance due to numerous structural changes and antibiotic breakdown enzymes (Codjoe and Donkor, 2018). Antibiotic gene transfer is more likely to occur between closely related bacteria that coexist in the same environment and are present in the gut undetected as subdominants of the original niche

(Ruppé et al., 2019). These "resistomes" seen in opportunistic *Enterobacteriaceae* infections, such as *Klebsiella pneumoniae*, originate from other species of the same Proteobacteria phylum (Lupo et al., 2012).

In many trials, antibiotic delivery to the gut microbiota resulted in bacterial population simplification rather than eradication (Dethlefsen et al., 2008; Robinson and Young, 2010). According to Odamaki et al. (2016) the richness of *Enterobacteriaceae* generated an endotoxin challenge for the weakened intestinal barrier, resulting in increased inflammatory responses. Also, elderly patients with *Clostridium difficile* infections treated with broad-spectrum antibiotics have shown a high abundance of Proteobacteria species and a decrease in *Clostridiales Incertae Sedis* XI, *Ruminococcaceae*, *Lachnospiraceae*, and *B. longum* (Lagier, 2016). These findings were confirmed previously in Vincent et al. (2013) *Enterococcaceae* family was enriched in severe intestinal diversity depleted cases. *Enterococci* and *C. difficile* can take advantage of the intestinal ecosystem's diminished biodiversity to expand their population in a competitive interaction against the commensals (Donskey et al., 2000). In order to learn about the molecules produced in the human gut microbiome that may affect the choice of bacteria that are associated with either symbiosis or dysbiosis (Baron et al., 2018), it is required to determine the microbiota cluster and dynamics. For example, the ribosome defence mechanisms are often used by bacteria in the human gut microbiome to resist tetracycline (Gibson et al., 2015). The IgA protease produced by the gut bacterium *Clostridium ramosum* as a virulence factor that cleaves both IgA1 and IgA2 is a notable example of adaptation to its preferred habitat and immune system evasion (Fujiyama et al., 1985). In addition, these IgA secretory cells might play a protective role against *E. coli* O55 infections (Raskova Kafkova et al., 2021).

In this work, we test the hypothesis that patients in intensive care units receiving antibiotics have more distinct bacterial species structures than controls. Infections produced by the most common possible pathogens discovered, such as *P. distasonis* in the Bacteroidetes phylum have been documented rather infrequently. Infections produced by the most common possible pathogenic species, in the Proteobacteria phylum were *K. pneumonia P. mirabilis* and *P. aeruginosa*, in the Firmicutes phylum *E. durans* all have been frequently reported.

Materials and methods

In silico analysis of microbiomics data

We performed a further analysis to investigate the bacterial species makeup of the intestinal microbiota in CRE-carrier and non-carrier compared to controls Out of a total of 60 fecal samples, 24 CRE-carrier, 26 CRE non-carrier, and 10 samples were taken from controls who had not had any antibiotic treatment for at least 6 months and had no previous gastrointestinal illnesses. 22 CRE-carrier and 23 non-carrier CRE were administered broad-spectrum antibiotics, specifically meropenem and piperacillin-tazobactam. 23 CRE-carrier and 24 non-carrier have been administered the narrow spectrum antibiotics, vancomycin and colistin.). Sindi et al. (2022) detailed the study design, sequencing, and taxonomic identification of fecal specimens (n = 60; one per patient) ranging in age from 20 to 90 years.

OTU table was corrected for gene copy number (GCN) differences at the genus level using data from rrnDB, a database of ribosomal RNA operons (Stoddard et al., 2015). ALDEx2 R package (v.1.22.0) (Fernandes et al., 2014) was used to examine the

presence of any dysbiotic species between the examined conditions. Differentially abundant taxa were defined as those having an |effect size| > 1 and adjusted p-value ≤ 0.05 for the expected Benjamini–Hochberg-corrected p value of Welch's t-test (we.eBH) and/or expected Benjamini–Hochberg-corrected p value of Wilcoxon test (wi.eBH). Correlation analyses were performed using Spearman's test on GCN-corrected absolute abundance values. Relationships having |rho| >= 0.5 and p-value < 0.05 were deemed significant. All analyses were performed in R (v.4.0.4), while data were handled using phyloseq R package (v.1.34.0) (McMurdie and Holmes, 2013). Visualizations were created using ggplot2 (v.3.3.5), ggVennDiagram (v.1.2.0) (Gao et al., 2021) and UpSetR (v.1.4.0) package (Conway et al., 2017).

Results

A differential abundance analysis comparing CRE-carrier, non-carrier, and control samples in a pairwise fashion showed multiple cases of microbial species with unregulated abundance. To begin, 12 species were discovered in a dysbiosis state in CRE-non-carrier and control cases, but more than double that number was discovered in CRE-carrier and control instances (*Fig. 1A-B; Table 1*). There was no difference between CRE-carrier and CRE-non-carrier microbial populations at the species level (*Fig. 1C*). Enterococcus durans, in particular, is the only species whose abundance has been reported to increase under both CRE-carrier and non-carrier cases, suggesting its levels are regulated by another factor (*Fig. 1D; Table 1*). Ten species with lower abundance, on the other hand, are shared by the two comparisons (*Fig. 1E; Table 1*).
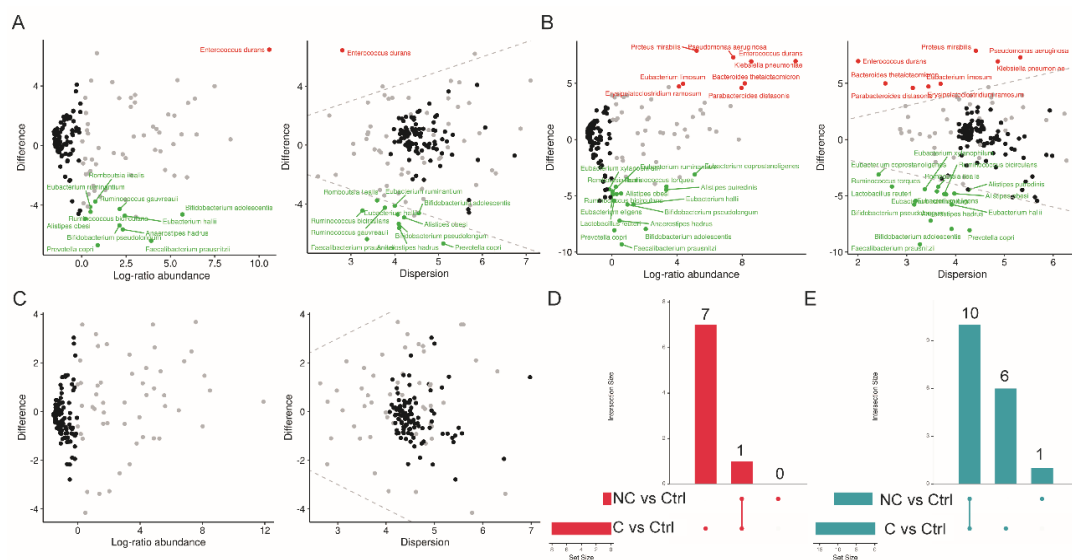

Figure 1. NC for non-carriers and C for carriers, red marks species with increased and green with decreased abundance

When the abundance of species was compared between treatment and control groups, 19 significant species were found, 14 of which had decreased abundance and 5 of which had increased abundance after treatment (*Fig. 2A; Table 2*). Compared to the control, *E. durans* was found to be differentially abundant in treated versus control sample, indicating a treatment-driven alteration in the species abundance (*Fig. 2B*). The same may be said for

another set of ten species that have decreased abundance in comparison to control samples (*Fig. 2C*). *Bacteroides thetaiotaomicron*, *Erysipelatoclostridium ramosum*, *Parabacteroides distasonis*, *Proteus mirabilis*, *Alistipes putredinis*, *Eubacterium coprostanoligenes*, *Eubacterium xylanophilum*, *and Lactobacillus reuteri* are also microorganisms whose numbers seem to be affected by the pharmaceutical treatment prescribed and not by the CRE-carrier status of their bearers (*Fig. 2B–C*). Additionally, there are species that are unique to a particular comparison and whose population shift can be traced to a single source (*Fig. 2B–C*). We discovered that the overall lower abundance of the two mucolytic bacteria normally present in the healthy gut of *Ruminococcus gnavus* among non-carriers and *R. torques* in carriers could be explained by sample type.

Table 1. *Differentially abundant species between sample types. Red marks species with increased and green with decreased abundance. Across comparisons common species are recorded in bold*

Non-carrier vs control	*Alistipes obesi*	*Eubacterium ruminantium*
	Anaerostipes hadrus	*Faecalibacterium prausnitzii*
	Bifidobacterium adolescentis	*Prevotella copri*
	Bifidobacterium pseudolongum	*Romboutsia ilealis*
	Enterococcus durans	*Ruminococcus bicirculans*
	Eubacterium hallii	*Ruminococcus gauvreauii*
CRE-carrier vs control	*Alistipes obesi*	*Eubacterium ruminantium*
	Alistipes putredinis	*Eubacterium xylanophilum*
	Anaerostipes hadrus	*Faecalibacterium prausnitzii*
	Bacteroides thetaiotaomicron	*Klebsiella pneumoniae*
	Bifidobacterium adolescentis	*Lactobacillus reuteri*
	Bifidobacterium pseudolongum	*Parabacteroides distasonis*
	Enterococcus durans	*Prevotella copri*
	Erysipelatoclostridium ramosum	*Proteus mirabilis*
	Eubacterium coprostanoligenes	*Pseudomonas aeruginosa*
	Eubacterium eligens	*Romboutsia ilealis*
	Eubacterium hallii	*Ruminococcus bicirculans*
	Eubacterium limosum	*Ruminococcus torques*

Table 2. *Differentially abundant species between treatment and control counterparts. Red marks species with increased and green with decreased abundance*

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Treatment vs control	*Alistipes obesi*	*Eubacterium ruminantium*
	Alistipes putredinis	*Eubacterium xylanophilum*
	Anaerostipes hadrus	*Faecalibacterium prausnitzii*
	Bacteroides thetaiotaomicron	*Lactobacillus reuteri*
	Bifidobacterium adolescentis	*Parabacteroides distasonis*
	Bifidobacterium pseudolongum	*Prevotella copri*
	Enterococcus durans	*Proteus mirabilis*
	Erysipelatoclostridium ramosum	*Romboutsia ilealis*
	Eubacterium coprostanoligenes	*Ruminococcus bicirculans*
	Eubacterium hallii	
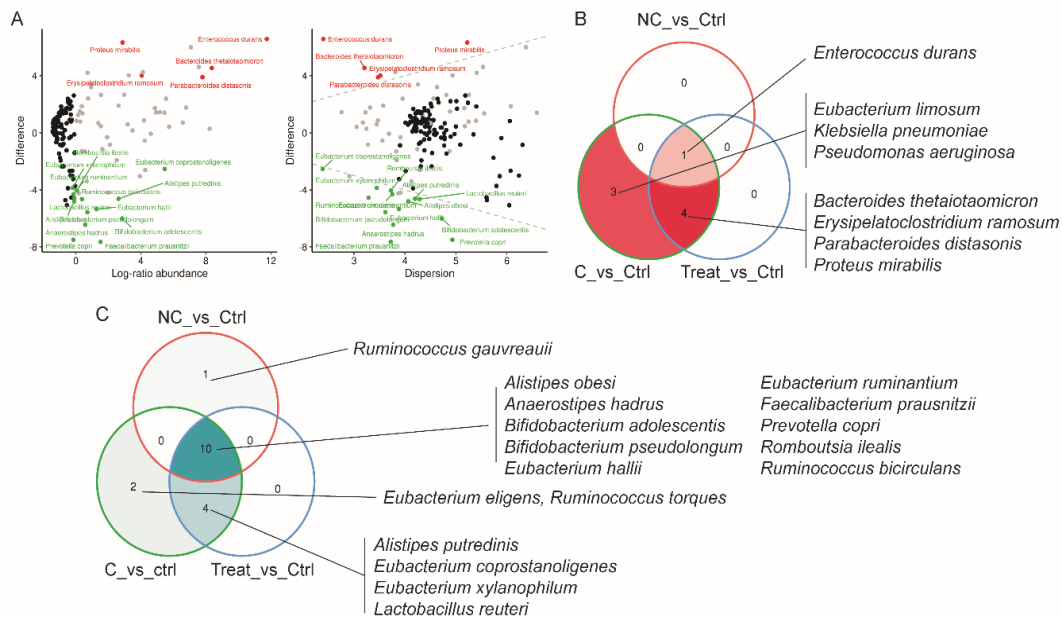
Figure 2. NC for non-carriers and c for carriers, red marks species with increased and green with decreased abundance

Microbial communities have been shown to be affected by age. We correlated absolute species abundance with the age of the three groups to see if aging influenced our data. Except for one, all significant connections are established for control samples in both per sample type and per treatment correlations (*Tables 3–4*). However, the abundance of *Prevotella stercorea* appears to be connected to the aging variable between the CRE-carrier cases (*Table 3*), a relationship that is not shown when all treated samples are evaluated together (*Table 4*). Based on healthy human gut metagenomes, *P. stercorea* is likely the second most prevalent and relatively abundant species after *Prevotella copri* (Yeoh et al., 2022). *Prevotella* strains are typically classified as commensal bacteria due to their vast frequency associated with plant-based diets and their infrequent involvement in illnesses (Precup and Vodnar, 2019). New research, however, has linked *Prevotella* abundance and specific strains to inflammatory diseases, implying that at least some strains have pathobiontic properties. Their ability to break down mucin, for example, may contribute to the disruption of mucosal barrier function (Wright et al., 2000).

Discussion

The gut microbiota, as one of the most microbe-rich habitats in the human body, is an ideal ecology for demonstrating microbiomics; therefore, it is important to invest in sequence analysis infrastructure. Human infections occur in complex habitats in which the pathogen interacts with the host and a symbiotic microbial population. Many human infections are polymicrobial, which can result in increased severity due to pathogen collaboration or decreased morbidity when one pathogen affects the fitness of another (Frisan, 2021).

According to Karakonstantis et al. (2020), the most problematic three bacterial species in carbapenem resistance cases were *K. pneumoniae*, *P. aeruginosa*, and

Acinetobacter baumannii. In comparison to our study, we found a high abundance of bacterial species clusters in CRE-carriers of *E. ramosum*, *E. limosum*, *K. pneumoniae*, *P. distasonis*, *P. mirabilis*, *B. thetaiomicron*, and *P. aeruginosa*. The significant abundance of *E. durance* in both CRE-carrier and non-carrier groups was due to antibiotic treatments; according to Sjölund et al. (2003), drug-resistant bacteria thrive quickly in the gut by disrupting the delicate microbial ecosystem and persisting for years after exposure. Furthermore, *Enterococcus* spp. have been found to be capable of harboring and transmitting resistance genes and virulence factors (Sharma et al., 2014). The most essential factor in the outbreak of hospital vancomycin-resistant enterococci is the colonization of the excretory system, which almost precedes bacteremia and is the main reservoir from which germs disseminate in the hospital environment (Krawczyk et al., 2021).

Table 3. *Spearman's correlation between patients' age and species absolute abundance collected per sample type. All results have a p-value < 0.05*

Sample type	Species	Rho
Control	*Alistipes inops*	-0.8
	Alistipes onderdonkii	0.68
	Bacteroides caecimuris	-0.7
	Bacteroides cellulosilyticus	0.7
	Bacteroides eggerthii	0.87
	Bacteroides thetaiotaomicron	-0.7
	Butyrivibrio crossotus	0.72
	Cloacibacillus porcorum	0.81
	Clostridium perfringens	0.94
	Corynebacterium renale	0.79
	Desulfovibrio piger	0.76
	Eubacterium coprostanoligenes	0.78
	Eubacterium hallii	-0.8
	Eubacterium limosum	0.83
	Eubacterium siraeum	0.64
	Faecalibacterium prausnitzii	-0.8
	Gordonibacter pamelaeae	0.7
	Klebsiella pneumoniae	0.89
	Leuconostoc lactis	-0.7
	Leuconostoc mesenteroides	-0.7
	Mycoplasma salivarium	-0.7
	Oxalobacter formigenes	0.64
CRE-carrier	*Prevotella stercorea*	-0.5
Control	*Prevotella stercorea*	0.7
	Proteiniphilum bacterium	0.76
	Streptococcus urinalis	0.79
	Weissella cibaria	0.85

Table 4. *Spearman's correlation between patients' age and species absolute abundance collected per treatment status. All results have a p-value < 0.05*

Sample type	Species	Rho
Control	*Alistipes inops*	-0.8
	Alistipes onderdonkii	0.68
	Bacteroides caecimuris	-0.7
	Bacteroides cellulosilyticus	0.7
	Bacteroides eggerthii	0.87
	Bacteroides thetaiotaomicron	-0.7
	Butyrivibrio crossotus	0.72
	Cloacibacillus porcorum	0.81
	Clostridium perfringens	0.94
	Corynebacterium renale	0.79
	Desulfovibrio piger	0.76
	Eubacterium coprostanoligenes	0.78
	Eubacterium hallii	-0.8
	Eubacterium limosum	0.83
	Eubacterium siraeum	0.64
	Faecalibacterium prausnitzii	-0.8
	Gordonibacter pamelaeae	0.7
	Klebsiella pneumoniae	0.89
	Leuconostoc lactis	-0.7
	Leuconostoc mesenteroides	-0.7
	Mycoplasma salivarium	-0.7
	Oxalobacter formigenes	0.64
	Prevotella stercorea	0.7
	Proteiniphilum bacterium	0.76
	Streptococcus urinalis	0.79
	Weissella cibaria	0.85

K. pneumoniae and *P. mirabilis* were two of the most well-known polymicrobial infections in the human lower gastrointestinal tract, urinary infection, bloodstream, and nosocomial settings (Paczosa and Mecsas, 2016; Kallel et al., 2020; Gómez et al., 2021). In addition to these two species, it was discovered in Kotaskova et al. (2019) that *P. aeruginosa* and *Morganella morganii* in urinary tract infections, generating polymicrobial biofilms. Interestingly, Juarez et al. (2020) established a competitive interaction between the two species in which *P. mirabilis* secretes an antibacterial substance (ammonia) in vitro against the gram-negative *K. pneumoniae*. More research on the infection's virulence factors is required. Examining common components in highly immunogenic polysaccharides and identifying various cross-reactions are important steps in developing a vaccine that protects against both opportunistic infections (Palusiak, 2022).

E. limosum is the type species in the Firmicutes phylum; it is an obligatory anaerobic rod, difficult to cultivate, and cannot be consistently separated from other related species (Liderot et al., 2010). In bacteremia-leaky gut instances, for example, Lee et al. (2012) encountered polymicrobial infections of the three genus *Paraeggerthella*, *Eggerthella*, and *Eubacterium*.

Bacteroides species, on the other hand, are important clinical pathogens in most anaerobic illnesses, including abscess forms and bacteremia, due to their ability to thrive and adapt in the human gut environment through a variety of mechanisms, such as being resistant to carbapenem (Wexler, 2007). Interestingly, Béchon et al. (2022) showed that gut microbiota members could alter various bile acids for biofilm growth in competition and cohabitation. As in the case of *P. aeruginosa* (taurine-conjugated bile acids) and *B. thetaiotaomicron* isolates, extracellular DNase BT3563 is produced. In the same phylum, *P. distasonis*, a potential pathogen, has been described infrequently in the human gut microbiota (Yang et al., 2020), despite our results that it was abundant in the CRE-carrier group. There is no established consensus on *P. distasonis* multidrug resistance function in regulating the pathogenicity of the human gut microbiota due to selection pressures such as the acquisition of new resistance genes that code for energy-dependent tetracycline efflux (Ezeji et al., 2021). Similarly, whereas *E. ramosum* is rarely a pathogen, it has been previously isolated as a component of polymicrobial-mediated diseases associated with severe underlying disease (Milosavljevic et al., 2021). Also, Iadsee et al. (2023) recently demonstrated that the opportunistic *E. ramosum* was prevalent in colorectal cancer cases above the age of 50, hinting that it could be a useful marker.

Conclusion

Future research should focus on bacterial species and strains in the human gut microbiota, especially under antibiotic treatment. which could lead to new insights on biomarker bacterium identification. Our data suggest that the presence of multiple factors, ranging from age to antibiotic treatment, in both groups compared to controls resulted in significant physiological adaptation, while the precise influence of these polymicrobial habitats is unknown. The gut microbial communities of the individuals studied in the CRE carrier cases shared an 8-species pool of possible pathogens, with E. durance dominating. *K. pneumoniae*, *P. aeruginosa*, and *P. mirabilis*, the top three potentially dangerous species, have all been linked to human illnesses.

Conflict of interests. None declared.

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