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Restoration of Disturbed Gut Microbiota in Mice Infected with Multidrug-Resistant *Staphylococcus aureus* and Treated with Cefazolin is Facilitated by the Application of an Iodine-glycine Complex

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ABSTRACT

Antibiotic therapy frequently disrupts the gut microbiota, compromising treatment outcomes and increasing adverse effects. Restoration of the intestinal microbiota is crucial, especially in cases of multidrug-resistant infections. This study evaluated the efficacy of an iodine-glycine complex (CC-195) in restoring the gut microbiota of mice infected with multidrug-resistant *Staphylococcus aureus* and treated with cefazolin. Mice were categorized into five groups, including controls, monotherapies, and combination therapy. Microbiota composition was analyzed using metagenomic sequencing on the Ion Torrent PGM platform. Taxonomic classification was performed using Kaiju software, and diversity analyses were conducted with R-based bioinformatics packages, including phyloseq and vegan. *S. aureus* infection and cefazolin monotherapy reduced microbial diversity and promoted dysbiosis, while CC-195, especially in combination with cefazolin, restored beneficial taxa and reduced opportunistic pathogens. These findings underline the potential of iodine-based adjunctive therapies for managing antibiotic-induced microbiome disturbances and combating antibiotic resistance.

Key words: Gut Microbiota, Dysbacteriosis, Antibiotic Resistance, Iodine Complex, Metagenomics.

INTRODUCTION

The gut microbiota plays a fundamental role in maintaining host health by regulating intestinal mucosal integrity, supporting immune functions, and supplying essential metabolites such as vitamins and enzymes (Li et al. 2020). However, antibiotic therapy, which is indispensable for treating bacterial infections, often disrupts gut microbial ecosystems, leading to dysbiosis and compromised drug metabolism, which may in turn reduce treatment efficacy and increase adverse effects (Pant et al. 2022). Therefore, restoring the gut microbiota during or after antibiotic treatment is a critical therapeutic objective. Supplementary drugs, known as xenobiotics, can aid the rapid restoration of the natural gut microbiome and its functionality.

Antibiotic resistance remains a growing global challenge. According to the World Health Organization (WHO), antimicrobial resistance (AMR) is one of the top ten threats to global health, with drug-resistant infections projected to cause up to 10 million deaths annually by 2050 if no effective countermeasures are taken (WHO 2023; AMR 2024). An analysis of data collected by the WHO from 49 countries has highlighted the global spread of multidrug-resistant (MDR) pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Acinetobacter spp*., particularly in healthcare settings (WHO 2017). Compounding this problem, the pace of new antibiotic development remains insufficient to catch up with the rapid evolution of MDR. Whereas the development of a new antibiotic may take up to a decade and billions of dollars, microbial resistance to it can emerge within a year of clinical use (WHO 2022; Murray et al. 2022).

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Given these limitations, alternative approaches are urgently needed. One such candidate is iodine, a broad-spectrum antimicrobial agent with a well-documented efficacy against bacteria, fungi, and viruses. Notably, there are no confirmed reports of acquired resistance to iodine in pathogens (Mulani et al. 2019). Although the use of molecular iodine has historically been limited owing to its toxicity, stable iodine complexes with amino acids and other organic compounds allow for systemic administration in safer, controlled forms (Ilin et al. 2017).

Research at the Scientific Center for Anti-Infectious Drugs (SCAID, Almaty, Kazakhstan) has focused on the development of iodine-based compounds with therapeutic potential. Several such complexes incorporating triiodide and/or iodide ions into organic carriers have shown promise in modulating gut microbiota and supporting their recovery during antibiotic treatment (Ilin et al. 2017; Reva et al. 2020; Korotetskiy et al. 2021a; Korotetskiy et al. 2021b; Bukeyeva et al. 2023). One of these, a glycine-triiodide complex designated CC-195, was synthesized in this study.

A structurally related compound (KS-25) has been previously registered in the CCDC database (https://www.ccdc.cam.ac.uk/) under accession number 1036607 (https://www.ccdc.cam.ac.uk/structures/Search?Ccdcid=1036607&DatabaseToSearch=Published). This study aimed to evaluate the effect of CC-195 on the gut microbiota of laboratory mice infected with a drug-resistant *Staphylococcus aureus* strain (SCAID OTT1-2022) with and without concurrent cefazolin therapy. By comparing microbiota composition across untreated, antibiotic-treated, iodine-treated, and combination therapy groups, this study sought to elucidate the potential of iodine-based therapeutics as adjuncts to traditional antibiotic regimens.

MATERIALS AND METHODS

Antimicrobials used in this study

CC-195 is a complex of crystalline iodine with hydrofluoric acid and glycine and was developed at the Scientific Center for Anti-Infective Drugs in Kazakhstan (SCAID). This complex has a broad spectrum of antimicrobial activity. Cefazolin was used to prepare the injection solution by dissolving it in sterile distilled water to a concentration of 25.0mg/kg. (Santo, Kazakhstan). Cefazolin belongs to the first generation of cephalosporin antibiotics and has broad-spectrum antimicrobial activity against Gram-positive and Gram-negative microorganisms.

Laboratory animals and experimental infection procedure

White outbred laboratory mice (both female and male, 4 weeks old, purchased from the Scientific and Practical Center for Sanitary and Epidemiological Expertise and Monitoring, Almaty, Kazakhstan) were used in this study. The mice were acclimatized to the conditions in the animal facility for one week before the beginning of the experiments. All mice were fed a standard rodent chow diet and maintained under a 12-hour light and dark cycle. The protocol was approved by the Committee of Institutional Animal Care and Use in the Scientific Center for Anti-Infectious Drugs (SCAID), Almaty (ID: #21/2 from 08.03.2020). Bacterial pneumonia was provoked by infection with multidrug-resistant *S. aureus* SCAID OTT1-2022, which was isolated from a hospital environment in Almaty (Kazakhstan). The whole genome sequence of the strain was obtained and deposited at NCBI under the accession number CP102945. *S. aureus* SCAID OTT1-2022 was injected oropharyngeally at a concentration of 1.5×10^9 CFU/ml per animal. To prepare the concentration of *S. aureus* SCAID OTT1-2022 at 1.5×10^9 CFU/ml, the strain was first cultured on nutrient agar, then suspended in sterile PBS and adjusted to match a 6 McFarland standard (~1.5×10^9 CFU/ml), as confirmed by spectrophotometry and viable count plating. This approach aligns with established protocols for bacterial inoculum standardization (Andrews 2001). Bacterial pneumonia was treated for five days, starting on the third day after the confirmation of infection. The presence of infection was confirmed using PCR. Briefly, PCR amplification was performed according to the following protocol: 10x buffer (Invitrogen, USA) containing 2mM of a mixture of dNTPs (Invitrogen, USA), 20.0pM forward and reverse primers, $MgCl_2$ (Invitrogen, USA), and 5 U/µL Taq DNA polymerase (Invitrogen, USA) was mixed with 2.0µl of DNA extracted from a 200µL aliquot of shredded lung tissue. The following primers were used: STPYF, 5'ACGGTCTTGCTGTCACTTATA3' and STPYR2, 5'TACACATATGTTCTTCCCTAATAA3' (Luo et al. 2021). The target locus was amplified by 35 cycles of melting at 94°C for 30s, annealing at 62°C for 30s, and elongation at 72°C for 45s after preheating at 94°C for 5min. The animals were divided into five experimental groups (n=6 in each group): 1) intact negative control mice; 2) infected and untreated mice serving as the positive control; 3) infected animals treated with 100.0mg/kg CC-195 daily; 4) infected animals treated with 25.0mg/kg cefazolin daily; and 5) animals under the combinatorial treatment regimen with both drugs: CC-195 (100.0mg/kg) + cefazolin (25.0mg/kg). The 100.0mg/kg dose of CC-195 was selected based on *in vitro* MIC data and preliminary PK/PD modeling. Specifically, the dose was extrapolated to achieve plasma concentrations exceeding the MIC of *S. aureus* SCAID OTT1-2022, considering the estimated bioavailability, distribution volume, and clearance *in vivo*. The drugs were administered once a day. During joint therapy, CC-195 was first administered *per os*, and the antibiotic was administered intramuscularly after 30min. The therapy was performed for 5 days. After completion of the study, the animals were euthanized using isoflurane inhalation (≥5% with an exposure time of at least one minute after breath-holding stops), according to (IR #17 2021). Samples from the intestines (colon and small intestine) were collected five days after the treatment. The samples were used for total DNA extraction and sequencing.

Sample collection

Samples were obtained separately from each animal group. Mouse intestines were placed into cuvettes with 10.0ml saline buffer containing 0.1% Tween 80 and were triple washed using a syringe. The obtained suspensions were centrifuged for 10min at 4°C using 500rpm rotation to remove fecal matter and epithelial cells. The supernatant was centrifuged again for 10min at 4°C and 16,000rpm to obtain bacterial cells in pellets. The pellets were resuspended in 1.0ml saline buffer (0.9% NaCl solution).

DNA extraction

DNA was extracted from 30 samples using the PureLink Genomic DNA Mini Kit (Invitrogen, USA) following the manufacturer's protocol. The quality and quantity of the resulting DNA were determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) at optical wavelengths of 260 and 280nm, respectively. DNA samples were stored frozen (−20°C) until use.

Metagenome sequencing

The DNA library was sequenced using the Ion Torrent PGM sequencing platform. Briefly, a DNA library was obtained using the Ion Xpress Plus Fragment Library Kit (Life Technologies, USA). The degree and quality of DNA fragmentation in the library were evaluated using the Bioanalyzer 2100 capillary electrophoresis system (Agilent Technologies). DNA read barcoding for multiplex sequencing was performed using the Xpress Barcode Adapters Kit (Life Technologies). Sequencing of the consequent library was performed on a 318-chip using the Ion PGM Hi-Q View Sequencing Kit. At each intermediate stage, the quality and quantity of the DNA library were monitored.

Bioinformatics

Taxonomic analysis was carried out using Kaiju (Menzel et al. 2016) integrated within KBase (Arkin et al. 2018), set to operate in the Greedy mode. The parameters used were as follows: (1) a minimum match length of 15 nucleotides, (2) a greedy minimum Bitscore threshold of 65, (3) a maximum allowance of three mismatches, and (4) a Greedy maximum e-value threshold set at 0.01 to exclude low-complexity sequences. Taxonomic identification was conducted using the NCBI BLAST non-redundant reference database, excluding eukaryotic sequences. The resulting classifications obtained from Kaiju were graphically visualized using stacked bar plots. R (v.4.4.3) (R 2025) was used to calculate alpha and beta diversity indices using the vegan (Oksanen et al. 2025), phyloseq (McMurdie 2013), microbiome (Lahti 2017), ggplot2 (Wickham 2016), and other auxiliary libraries packages.

Raw count data were normalized using total sum scaling (TSS) by dividing each taxon count by the total number of reads per sample, yielding relative abundance, and normalization was conducted using the transform_sample_counts of the phyloseq package.

To visually assess the similarity of microbial communities between samples, hierarchical clustering was performed using the average pooling method (UPGMA). Distances were calculated from the normalized Bray-Curtis matrix using the *vegdist* function from the vegan package and visualized using *hclust* (Murtagh et al. 2014). The *ggplot* function from the ggplot2 package was applied to generate a heatmap by mapping group identifiers to the x-axis, taxon names to the y-axis, and abundance values to a color gradient. After using this tool/service, the authors reviewed and edited the content as needed, and took full responsibility for the content of the published article.

RESULTS

Collection samples

The Ion Torrent PGM (Life Technologies) sequencing platform was used to generate single-end reads from bacterial DNA extracted from stool samples of laboratory mice (Table 1). All the samples from each group were pooled and processed together.

Taxonomic diversity

DNA reads were analyzed using the Kaiju program integrated within the KBase platform (Menzel et al. 2016; Arkin et al. 2018). Taxonomic classification was performed at the genus level based on the NCBI non-redundant protein database using default parameters. Fig. 1 shows the representation of different operational taxonomic units (OTUs) in the samples, as revealed by Kaiju.

Rarefaction curves were generated in R using OTU counts in the sample, as shown in Fig. 2. The rarefaction curves reflect the dependence of the number of detected OTUs on the sample volume (number of generated reads) for each of the five experimental groups and the level of disclosure of taxonomic diversity of the samples. In all the groups, the curves reached a plateau, indicating that the sequencing depth was sufficient to reveal the microbial diversity of the sampled microbiomes. It should be noted that in the Ab group, the curve reached the plateau early, reflecting a relatively limited diversity of this microbiome.

Several alpha diversity indices, such as Chao1 taxonomic richness, Simpson dominance, and Shannon diversity coefficients, were calculated using R. These metrics provided a quantitative evaluation of the skewness of the abundances of individual OTUs in the samples (Fig. 3).

The negative control (NC) group was characterized by the highest species richness and diversity, reflecting the healthy and balanced gut microbial community of the mice. Animals infected with the MDR *S. aureus* SCAID OTT1-2022 were considered the positive control (PC) group. The gut microbiome of these animals showed a significant decrease in all alpha-diversity parameters, including species richness, diversity, and dominance of the major bacterial groups that form the core part of a healthy mouse gut microbiome. Administration of cefazolin (Ab group) further reduced microbial diversity and worsened dysbiosis in treated mice. In contrast, administration of CC-195

Table 1: Numbers of reads generated for different experiments and their accession numbers at the SRA NCBI database

Name*	Number of Reads	Total Number of Bases	Mean Read Length	Phred Type	Quality Score Mean	SRA accession
1. NC	2,956,903	680,458,038	230.12	33	30.39	SRR23874751
2. PC	1,527,931	326,268,274	213.53	33	30.06	SRR23874750
3. CC-195	1,615,574	347,163,991	214.88	33	30.50	SRR23874749
4. Ab	1,100,013	234,676,883	213.34	33	29.92	SRR23874748
5. Ab_CC-195	1,590,379	338,482,136	212.83	33	30.28	SRR23874747

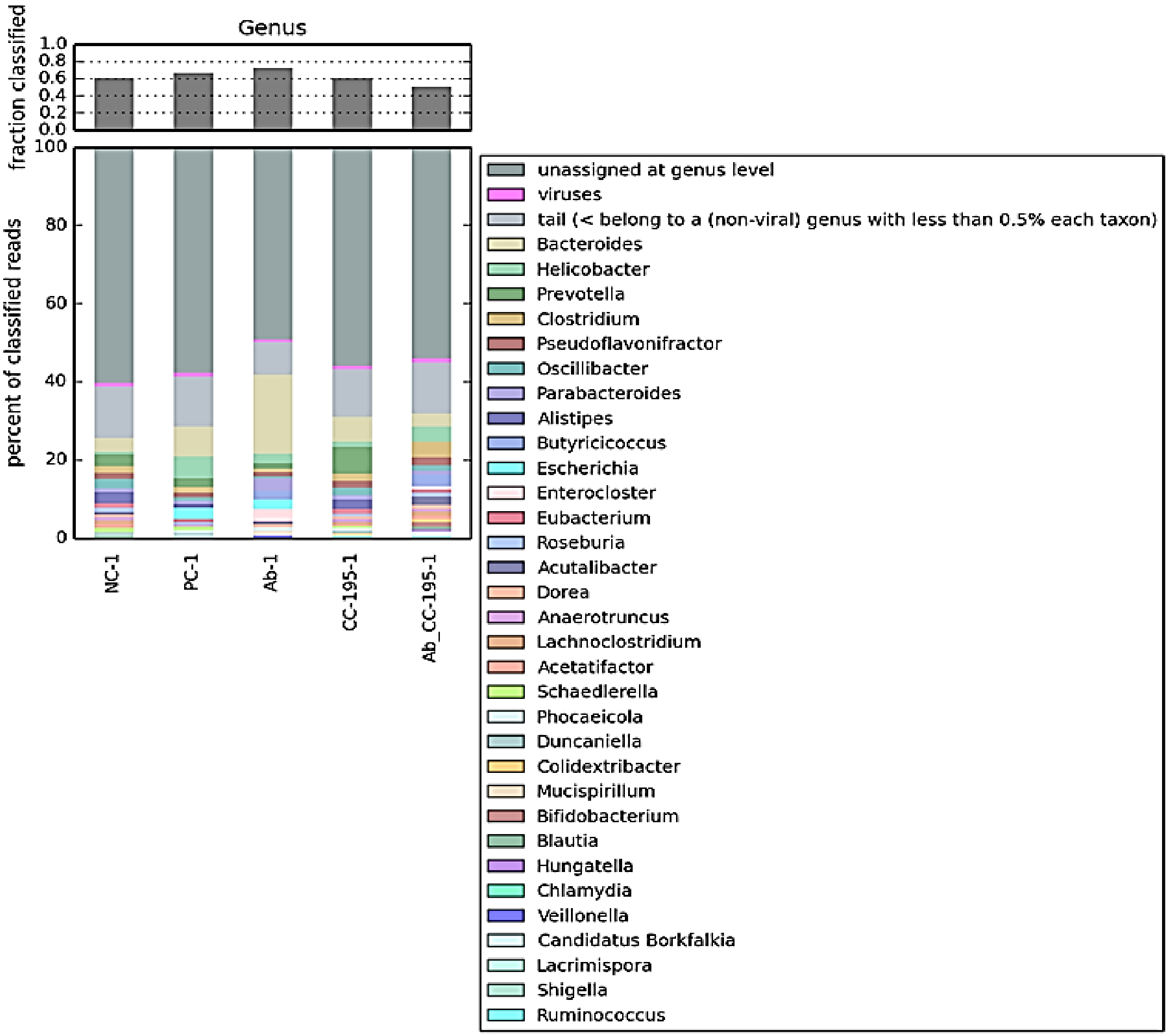

Fig. 1: Comparison of taxonomic composition of the metagenomic samples obtained from the five experimental settings.

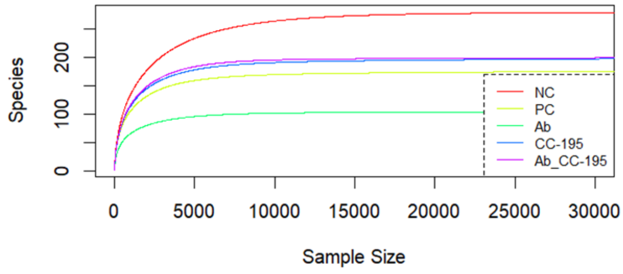

Fig. 2: Rarefaction curves obtained for the five sampled microbiomes.

promoted pronounced restoration of gut microbiota diversity and composition, bringing it close to the parameters observed in the NC group. Combined use of cefazolin and CC-195 (Ab_CC-195 group) also led to restoration of the normal gut microbiome, mitigating the adverse effects of antibiotic treatment alone.

To compare the microbial community composition of the different samples, a Bray-Curtis distance matrix was calculated using R. Raw OTU count values were normalized to relative abundance values by dividing each taxon count by the total number of reads per sample and correcting for differences in sequencing depth. The distance matrix was calculated using the *vegdist* function in the R vegan package. Principal Coordinate Analysis (PCoA) and hierarchical clustering were performed on the normalized data matrix to visualize inter-sample variation (Fig. 4 and Fig. 5).

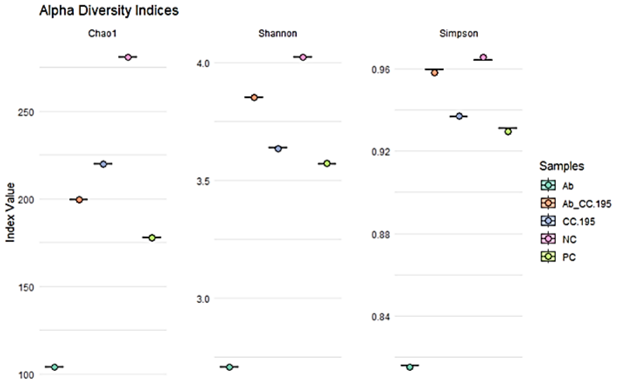

Fig. 3: Alpha diversity of five sampled mouse gut microbiomes.

PCoA demonstrated spatial separation of the microbiota between the studied groups. The PC group showed a shift in the point in the diagram, indicating a change in microbiota structure compared to the control group. The samples of the group treated with cefazolin (Ab) were also separated from the other clusters and showed variability, reflecting the destabilization of the microbial community. The group receiving CC-195 formed a separate cluster closer to the NC group, indicating partial recovery of the microbiota. The samples of the group with combined treatment (Ab + CC-195) were also skewed toward healthy animals.

The Bray-Curtis distance matrix was used to infer a dendrogram of gut microbiome similarity using the Neighbor-Joining algorithm implemented in the R. The resulting dendrogram, shown in Fig. 5, demonstrates a close compositional similarity between the gut microbiome of mice treated with CC-195 and that of the NC group, whereas other microbiomes showed varying levels of deviation from the normal gut microflora, with the Ab group being the most divergent. The heatmap shown in Fig. 6 provides a detailed comparison of alterations in the microbiota at the bacterial genus level. Only genera with relative abundances exceeding 1% were included in this comparison.

A significant increase in abundance of *Bacteroides* (+9.66), *Helicobacter* (+11.68), and *Escherichia* (+7.19) followed infection of animals with the MDR *S. aureus*. These taxa are associated with inflammatory and pathogenic changes in the intestinal microbiota. At the same time, there was a decrease in the abundance of *Alistipes* (-5.58), *Oscillibacter* (-4.40), and *Roseburia* (-1.39) taxa, which are key producers of short-chain fatty acids (SCFAs).

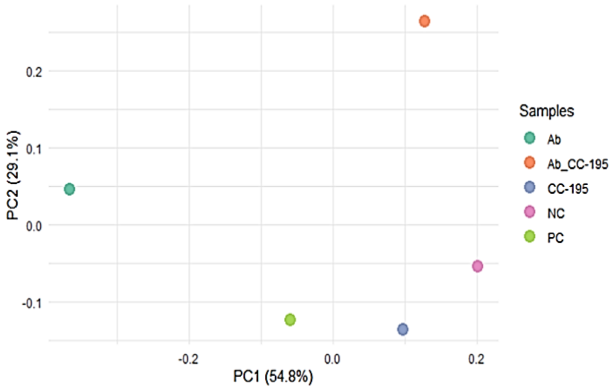

Fig. 4: PCoA analysis of gut microbiota samples.

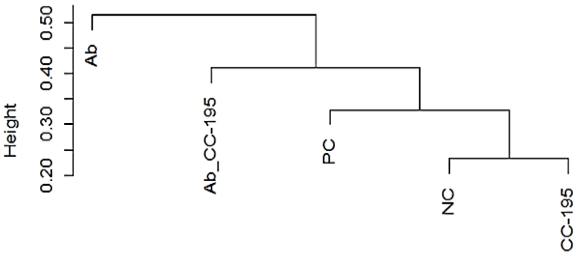

Fig. 5: Dendrogram of clustering of the sampled gut microbiomes.

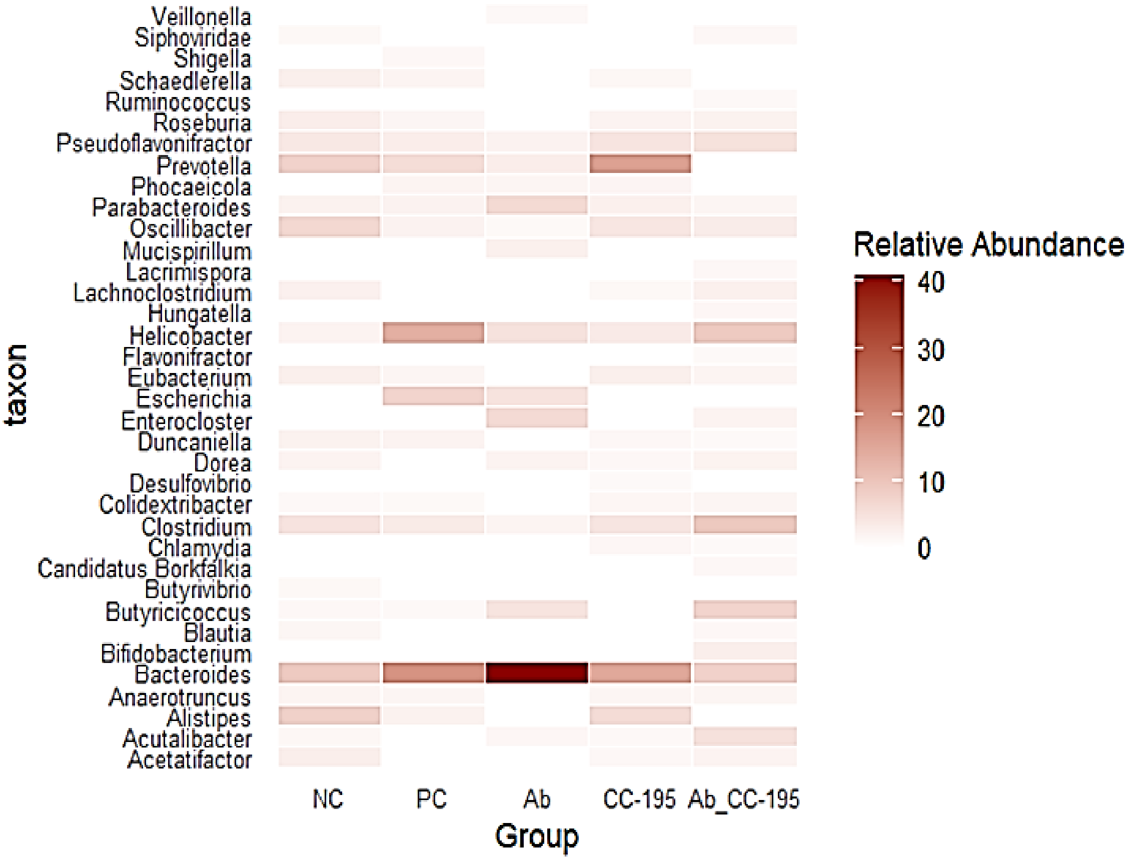

Fig. 6: Heatmap of alterations of dominant gut microbiome taxa in the different groups of the treated and control mice.

DISCUSSION

Metagenomic sequencing of the intestinal samples was carried out to assess the level of disturbance of the gut microbiota of the mice due to an experimental lung infection with MDR *S. aureus*, followed by treatment with the antibiotic cefazolin, iodine-containing complex CC-195, and a combinatorial treatment. Lung infection may significantly affect the gut microbiome owing to the existence of a gut-lung axis involving crosstalk between the respiratory and gut microbiomes (Budden et al. 2017). Cefazolin is considered a preferable antibiotic for the treatment of staphylococcal infections because of its mild side effects on the resident gut microbiota (Loubet et al. 2018). Other reports have suggested that this antibiotic can restore normal gut microbiota after surgery (Luo et al. 2021). The comparison of the metagenomes in Fig. 1 shows that the ability of cefazolin to restore the disturbed gut microbiome was limited. An expectation of this study was that application of the supplementary drug CC-195 would improve healing and restore the resident gut microbiome of infected and antibiotic-treated mice.

Antibiotic administration was accompanied by a further increase in the proportion of the genus *Bacteroides* (+22.04 relative to PC) as well as an increase in *Enterocloster* (+6.24) and *Parabacteroides* (+4.18), indicating their possible resistance to the drugs used. Simultaneously, a significant decrease in *Alistipes*, *Prevotella*, and *Roseburia* was detected, which reflects the sensitivity of these taxa to antibiotics (Zafar and Saier 2021). *Bacteroides spp*. are known to exhibit resistance to β-lactam antibiotics, macrolides, and tetracyclines. *Parabacteroides distasonis* has been reported to be resistant to clindamycin (White et al. 2015), while *Enterocloster clostridioformis* shows resistance to benzyl penicillin and moxifloxacin (Nie et al. 2021).

The use of CC-195 has a positive effect on the restoration of the intestinal microbial balance by increasing the number of beneficial taxa (*Prevotella*, *Clostridium*, and *Oscillibacter*) involved in the production of SCFAs and reducing the content of opportunistic microorganisms, such as *Helicobacter* and *Escherichia* (Chen 2017), reflecting the favorable effect of CC-195 (Fig. 6). An increase in the number of bacteria in the genus *Prevotella* has a favorable effect on intestinal health. These microorganisms are actively involved in the breakdown of dietary fiber and the synthesis of short-chain fatty acids, particularly propionate (Kovatcheva-Datchary et al. 2015). The genera *Clostridium* and *Oscillibacter* are important producers of butyrate, which maintains the integrity of the intestinal epithelium and regulates the immune response (Fusco et al. 2023). The combined use of antibiotics and CC-195 also demonstrated a restorative effect on the disturbed gut microflora of mice by increasing the proportion of beneficial genera *Clostridium* and *Butyricicoccus* and suppressing the growth of opportunistic *Bacteroides spp*., which may indicate the selectivity of therapy against pathogenic forms. These observations are consistent with literature data confirming the significant role of *Clostridium butyricum* in restoring microbial balance and improving intestinal barrier function after antibiotic therapy. *Butyricoccus pullicaecorum*, a producer of butyrate with probiotic potential, reduces inflammatory processes in the intestine and contributes to the normalization of its function (Geirnaert et al. 2014; Zheng et al. 2024).

The positive effect of CC-195 may be associated with the activation of monocytes and granulocytes by this complex, as reported previously by Bukeyeva et al. 2023. The resident microbiota is better adapted to host immune responses than pathogenic bacteria and opportunistic intruders. The use of antibiotics alone often causes an imbalance in the gut microbiota of mice due to differential selective pressure on different bacterial taxa.

Thus, the iodine-containing complex CC-195, both in monotherapy and in combination with an antibiotic, shows a pronounced normalizing effect on the intestinal microbiota and has the potential to be applied in the therapy of dysbacteriosis of various origins.

Conclusion

Although the antimicrobial effect of iodine-containing complexes on several virulent bacteria, including multidrug-resistant pathogens, has been reported previously, this work has demonstrated for the first time that these complexes could mitigate gut dysbacteriosis and stimulate a restoration of the resident intestinal microbiome disturbed during infection and/or antibiotic therapy. This effect was observed when CC-195 was administered alone or in combination with cefazolin. It should be noted that the complex CC-195 was introduced in this study as a promising supplementary xenobiotic to support antibiotic therapy, but not as a replacement for antibiotic therapy. The developed laboratory model and microbiome surveillance system based on metagenomic sequencing will be of practical interest for further studies on the development of new antimicrobials and xenobiotics to overcome the antibiotic resistance problem.

DECLARATIONS

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Conflict of Interest: The authors declare no conflicts of interest.

Data Availability: The obtained sequences with metadata were deposited in the NCBI database as Sequence Read Archive (SRA) records under a BioProject identification number PRJNA945223 (SRA accession numbers are SRR23874747-SRR23874751).

Ethics Statement: The protocol was approved by the Committee of Institutional Animal Care and Use in the JSC Scientific Center for Anti-Infectious Drugs, Almaty (ID: # 21/2 from 03.08.2020).

Author's Contribution: Conceptualization: IK Methodology: SS, TK, LI and AK Software: IK and OR Validation: NZ, OR and TK Formal analysis: SS, NZ and LI Investigation: IK, SS Resources: AK Data curation: TK, and AK Writing-original draft preparation: IK Writing-review and Editing: NZ, SS and OR Visualization: IK Supervision: ST Project administration: ST Funding acquisition: ST All authors have read and agreed to the published version of the manuscript.

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