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The role of resistance plasmids influencing gastrointestinal colonization of multidrug resistant *Klebsiella pneumoniae* in murine model

PhD thesis

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List of abbreviations

AMR:	Antimicrobial resistance
ASVs:	Amplicon sequence variants
ATCC:	American Type Culture Collection
BLIs:	Beta-lactamase inhibitors
CDC:	Centers for Disease Control and Prevention
CFU:	Colony forming unit
CPE:	Carbapenemase-producing *Enterobacteriaceae*
CP-Kp:	Carbapenemase-producing *K. pneumoniae*
CRE:	Carbapenem-resistant *Enterobacteriaceae*
CRKP:	Carbapenem-resistant *K. pneumoniae*
DNA:	Deoxyribonucleic acid
EC:	Abbreviation for the sodium-azide resistant *Escherichia coli* J53 laboratory strain used in our research
EC-CTXM:	Abbreviation for a transconjugant *Escherichia coli* J53 laboratory strain which harbours the *bla*CTX-M-15 gene of the MDR-KP donor strain
EC-OXA:	Abbreviation for a transconjugant *Escherichia coli* J53 laboratory strain which harbours the *bla*OXA-162 gene of the MDR-KP donor strain
ECDC	European Centre for Disease Prevention and Control
EDTA:	Ethylenediaminetetraacetic acid
ESBL:	Extended-spectrum beta-lactamase
ELISA:	Enzyme-linked immunosorbent assay
EUCAST:	European Committee on Antimicrobial Susceptibility Testing
GIT:	Gastrointestinal tract
hAD5 and 6:	human α-defensins 5 and 6
hBD1-4:	human β-defensin 1-4
HGT:	Horizontal Gene Transfer
ICUs:	Intensive care units
IgA:	Immunoglobulin A
Inc groups:	Incompatibility groups
IMP:	Imipenemase

ISE:	Insertion sequence
LB:	Luria-Bertani
KPC:	*Klebsiella pneumoniae* carbapenemase
mAD5:	Murine α-defensin 5
mBD3:	Mouse β-defensin-3
MBL:	Metallo-β-lactamase
MDR:	Multidrug-resistant
MDR-KP:	Abbreviation for a multidrug-resistant *Klebsiella pneumoniae* isolate used in our research
MIC:	Minimal inhibitory concentration
NDM:	New Delhi metallo-β-lactamase
PBS:	Phosphate-buffered saline
PCR:	Polymerase chain reaction
RNA:	Ribonucleic acid
VIM:	Verona integron-encoded metallo-β-lactamase
WHO:	World Health Organization

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1. Introduction

1.1. Importance of the antimicrobial resistance

Antimicrobial resistance (AMR) represents a significant global public health challenge in the effective management of infections caused by bacteria, fungi, parasites, and viruses. AMR occurs when microorganisms develop the ability to resist the effects of drugs, rendering standard treatments ineffective, which leads to prolonged illness, higher medical costs, and increased mortality rates. Resistance occurs through various mechanisms, including genetic mutations or the acquisition of resistance genes from other organisms. Multi-resistance refers to the ability of bacteria to resist multiple antimicrobials, often across different classes, making treatment options even more limited and complex. This phenomenon is particularly concerning in both community-acquired and nosocomial infections, where resistant pathogens are associated with worse clinical outcomes and increased transmission rates (1, 2).

Community-acquired infections caused by resistant bacteria, such as urinary tract infections, skin infections, and pneumonia, are increasingly difficult to treat with first-line antibiotics (3). In parallel, nosocomial infections, which occur in healthcare settings like hospitals, are commonly associated with multidrug-resistant organisms. Pathogens such as *Staphylococcus aureus* (particularly methicillin-resistant *S. aureus*), *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and members of the *Enterobacteriaceae* family (e.g., *Escherichia coli* and *Klebsiella pneumoniae*) are notable for their resistance profiles in hospital settings, contributing significantly to morbidity and mortality (4). In many countries, the prevalence of AMR has steadily increased over recent decades, and this rise is particularly remarkable among Gram-negative bacteria, which are intrinsically harder to treat due to their double membrane structure, limiting drug penetration (5-7).

According to the World Health Organization (WHO), multidrug-resistant (MDR) Gram-negative bacteria, such as carbapenemase producing *Enterobacteriaceae* (CPE), *Acinetobacter spp.*, and *P. aeruginosa*, pose critical or high priority threats globally, particularly in low- and middle-income countries where healthcare resources are limited (8). The European Centre for Disease Prevention and Control (ECDC) reports that more

than 670000 infections in the European Union and European Economic Area were caused by resistant bacteria in 2015 alone, leading to approximately 33000 deaths. In the United States, the Centers for Disease Control and Prevention (CDC) estimate that more than 2.8 million antibiotic-resistant infections occur each year, resulting in over 35000 deaths (9, 10).

The most common resistance mechanisms among bacteria include enzymatic degradation of antibiotics (e.g., β-lactamases that degrade penicillins and cephalosporins), modification of drug targets (e.g., mutations in penicillin-binding proteins), efflux pumps that expel antibiotics from bacterial cells, and alterations in membrane permeability to reduce drug uptake (11). Extended-spectrum β-lactamases (ESBLs) and carbapenemases, such as *K. pneumoniae* carbapenemase (KPC) and New Delhi metallo-β-lactamase (NDM), are particularly concerning in Gram-negative bacteria because they confer resistance to broad-spectrum β-lactams, including carbapenems, which are often used as last-resort treatments. The global spread of these resistance mechanisms has been facilitated by the horizontal transfer of resistance genes through mobile genetic elements like plasmids, transposons, and integrons, which can transfer between different bacterial species(12, 13).

The transmission of resistant bacteria and their resistance genes is further exacerbated by factors such as antibiotic overuse in humans and animals, poor infection control practices, global travel, and inadequate sanitation. In many parts of the world, especially in developing countries, the widespread availability of over-the-counter antibiotics without prescription contributes significantly to the rise in resistance. In high-income countries, hospital-acquired infections caused by resistant pathogens are often associated with invasive procedures, immunocompromised patients, and prolonged hospital stays (14). The dissemination of resistance through nosocomial pathogens, such as CPE and ESBL-producing *Enterobacteriaceae*, is particularly problematic in intensive care units (ICUs), where patients are highly susceptible to infections (15, 16).

Efforts to combat AMR require a multifaceted approach, including the development of new antibiotics, alternative therapeutic strategies such as bacteriophage therapy, and robust infection prevention and control measures. Surveillance systems, such as the WHO's Global Antimicrobial Resistance Surveillance System, have been established to monitor AMR trends and guide public health interventions. However, the development of

new antibiotics has slowed significantly in recent years, with few novel drugs reaching the market. This is compounded by the ability of bacteria to rapidly evolve and adapt, often leading to the emergence of resistance soon after new drugs are introduced (17, 18).

1.2. Horizontal gene transfer

Horizontal gene transfer (HGT) is a fundamental mechanism in bacterial evolution, enabling the rapid dissemination of genetic material across different species, often leading to the acquisition of advantageous traits such as antibiotic resistance and metabolic versatility. Unlike vertical gene transfer, which involves the inheritance of genetic material from parent to offspring, HGT allows bacteria to incorporate foreign DNA (deoxyribonucleic acid) from the environment, other bacteria, or viruses into their genomes. The two key vehicles of HGT in bacteria are plasmids and transposons, both of which play a crucial role in bacterial adaptation and evolution (12, 13, 19).

Plasmids are extrachromosomal DNA molecules that replicate independently of the bacterial chromosome. They often carry genes that confer selective advantages, such as antibiotic resistance, virulence factors, and metabolic capabilities. Plasmids can be classified into various replicon types, which are determined by their origin of replication, or "replicon." The replicon type is essential for controlling the plasmid's replication machinery, determining compatibility with other plasmids, and influencing the plasmid's stability within a host (20). For instance, incompatibility groups (Inc groups) categorize plasmids based on their ability to coexist within the same cell. Plasmids from the same Inc group often cannot stably coexist in a single bacterial cell due to similar replication mechanisms that compete for the host's cellular resources. Therefore, understanding the replicon type of a plasmid is crucial for predicting its behavior in a bacterial population and its potential for horizontal transmission (21).

Plasmids are also classified based on their mobility. Conjugative plasmids carry the necessary genes for self-transfer between bacteria, often through the formation of a pilus that connects donor and recipient cells, allowing the transfer of the plasmid DNA (22). Non-conjugative plasmids lack these transfer genes but can be mobilized if a conjugative plasmid is present in the same cell. This ability to transfer genetic material through conjugation is a powerful driver of bacterial evolution, especially in environments where selective pressures, such as antibiotics, are present. The rapid spread of antibiotic

resistance genes via conjugative plasmids is a significant concern in clinical settings, contributing to the emergence of multidrug-resistant bacterial strains (23).

Transposons, also known as "jumping genes," are another critical element in HGT. These DNA sequences can move from one location in the genome to another, either within the same DNA molecule or between different DNA molecules, such as between the chromosome and plasmids. Transposons often carry genes that provide adaptive advantages, such as resistance to antibiotics or heavy metals, and they can integrate into plasmids, thereby facilitating the spread of these traits across bacterial populations (24).

Transposons are categorized into two main classes based on their mechanism of transposition: Class I transposons, also known as retrotransposons, use an RNA (ribonucleic acid) intermediate during transposition, while Class II transposons, or DNA transposons, move directly from one DNA location to another via a "cut-and-paste" mechanism. In bacteria, most transposons belong to Class II and can be highly active in promoting genetic diversity by inserting into new genomic locations or transferring between plasmids and the bacterial chromosome. This mobility is crucial in environments where bacteria must rapidly adapt to changing conditions, such as the presence of antibiotics or other stressors (24).

The interaction between plasmids and transposons is particularly significant in the context of antibiotic resistance. Resistance genes can be captured by transposons, which then insert into conjugative plasmids. These plasmids can spread resistance genes across bacterial populations through conjugation, leading to the rapid emergence of resistant strains (25). The plasmid replicon type also plays a role in determining how efficiently these resistance genes are propagated. For example, plasmids with broad host ranges can replicate in a wide variety of bacterial species, facilitating the transfer of resistance genes between distantly related bacteria. In contrast, narrow host range plasmids are limited to specific bacterial species or genera, which may constrain the spread of resistance genes but still contribute to genetic diversity within those species (21).

Furthermore, some plasmids carry multiple replicons, known as "multireplicon plasmids." These plasmids can replicate using different replication systems, allowing them to adapt to various host environments and maintain stability in different bacterial species. Multireplicon plasmids often carry a combination of resistance genes, transposons, and other mobile genetic elements, making them particularly efficient

vehicles for HGT. The presence of multiple replicons can also enhance plasmid stability within a bacterial population, as it allows the plasmid to utilize different replication machinery depending on the host's cellular conditions (20).

In addition to plasmids and transposons, integrons can also play a role in the transfer of AMR genes in bacteria. Integrons are frequently found on plasmids, where they can acquire and exchange gene cassettes, further facilitating the spread of resistance genes through HGT (26).

The clinical implications of HGT, particularly involving plasmids and transposons, are profound. The rapid dissemination of antibiotic resistance genes has led to the rise of multidrug-resistant pathogens, posing significant challenges to public health. Understanding the mechanisms of HGT, including the role of plasmid replicon types and transposons, is crucial for developing strategies to combat the spread of resistance. For instance, targeting the conjugation machinery of plasmids or the transposition mechanisms of transposons could be potential therapeutic approaches to limit the spread of resistance genes. Additionally, surveillance of plasmid replicon types in clinical settings can provide insights into the potential for resistance gene transfer and the emergence of resistant strains (19).

1.3. Role of β-lactamases in antimicrobial resistance

B-lactamases are enzymes produced by many bacteria, which confer resistance to β-lactam antibiotics, including penicillins, cephalosporins, monobactams, and carbapenems. They degrade the β-lactam ring, a core structure of these antibiotics, rendering them ineffective. The clinical significance of β-lactamases lies in their contribution to the growing problem of antibiotic resistance, especially among Gram-negative bacteria such as *E. coli* and *K. pneumoniae* (27).

1.3.1 Classifications and general characteristics of β-lactamase enzymes

The classification of β-lactamases is based on two principal systems: the molecular classification proposed by Ambler, and the functional classification by Bush-Jacoby-Medeiros. Ambler’s system categorizes β-lactamases into four classes (A, B, C, and D)

based on their amino acid sequence. Class A, C, and D enzymes use serine in their active site to hydrolyze the β-lactam ring, while class B enzymes, known as metallo-β-lactamase (MBLs), use metal ions, typically zinc, to catalyze the reaction. The functional classification, on the other hand, groups β-lactamases based on their substrate specificity and ability to hydrolyze different classes of β-lactam antibiotics. This system includes 17 functional groups, with enzymes like ESBLs and carbapenemases representing critical threats in clinical settings due to their ability to hydrolyze broad-spectrum antibiotics (27).

ESBLs, primarily found in *Enterobacteriaceae*, such as *E. coli* and *K. pneumoniae*, are a subset of class A β-lactamases that can hydrolyze oxyimino-cephalosporins like cefotaxime and ceftazidime but are inhibited by β-lactamase inhibitors such as clavulanic acid. Carbapenemases, such as the KPC, NDM, and oxacillinases (OXA), represent a more advanced resistance mechanism as they degrade carbapenems, often considered antibiotics of last resort. These carbapenemases can belong to class A (e.g., KPC), class B (e.g., NDM), or class D (e.g., OXA-48) (4).

The prevalence of β-lactamase-producing bacteria is widespread. The global dissemination of β-lactamase-producing bacteria is facilitated by the horizontal transfer of resistance genes, often located on plasmids that can move between bacterial species, thus amplifying the spread of resistance (28).

Various strategies have been proposed to counteract β-lactamase-mediated resistance, including the development of new β-lactamase inhibitors (BLIs) and combination therapies. For example, avibactam and vaborbactam are novel BLIs that have shown efficacy against class A and some class C and D β-lactamases when combined with cephalosporins or carbapenems (29). These inhibitors work by binding to the active site of β-lactamases, preventing them from degrading the antibiotic (30, 31).

1.3.2. General characteristics of CTX-M-type β-lactamases

CTX-M-type β-lactamases are a rapidly growing class of ESBLs that confer resistance to a broad range of β-lactam antibiotics, particularly cephalosporins. First identified in the late 1980s, CTX-M enzymes have now become the most prevalent group of ESBLs globally, largely due to their rapid dissemination among *Enterobacteriaceae*, especially *E. coli* and *K. pneumoniae* (32).

The CTX-M family is divided into five main clusters based on amino acid sequence similarities: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 groups. The CTX-M-1 and CTX-M-9 groups are the most widespread and clinically relevant, with CTX-M-15 (belonging to the CTX-M-1 group) being particularly notorious for its high prevalence and association with multidrug-resistant strains (33, 34). CTX-M-15 has become a dominant variant worldwide and is frequently linked to mobile genetic elements such as plasmids, which facilitate its horizontal transfer across bacterial species and environments. The plasmid-mediated spread of CTX-M-15 has contributed significantly to its global dissemination and is a major public health concern (34).

A key feature of CTX-M enzymes is their potent hydrolytic activity against cefotaxime, which initially gave rise to their name. The “CTX” designation stands for cefotaximase, reflecting this activity (35). However, many CTX-M variants, including CTX-M-15, also exhibit significant activity against ceftazidime, a cephalosporin traditionally used to treat infections caused by resistant Gram-negative bacteria. This broad-spectrum resistance has severely limited the therapeutic options available for treating infections caused by CTX-M-producing pathogens, making carbapenems the last-resort antibiotics in many cases (32, 36).

The widespread distribution of CTX-M-type β-lactamases is attributed not only to their plasmid-mediated transfer but also to their localization on integrons, transposons, and insertion sequences that promote genetic mobility (37). In particular, insertion sequence ISEcp1 (insertion sequences) has been implicated in the mobilization of CTX-M genes from the chromosomes of Kluyvera species, considered the progenitor of CTX-M-type β-lactamases, to plasmids, enhancing their spread among pathogenic bacteria (38). This genetic adaptability has facilitated the rapid evolution and diversification of CTX-M enzymes, contributing to their increasing clinical importance (32, 39).

In addition to their resistance to cephalosporins, many CTX-M-producing strains also harbor resistance genes for other classes of antibiotics, such as fluoroquinolones and aminoglycosides, further complicating treatment. This co-resistance is often due to the presence of multiple resistance determinants on the same plasmids, leading to the selection of multidrug-resistant strains under antibiotic pressure (40). Consequently, infections caused by CTX-M-producing bacteria are associated with increased morbidity, mortality, and healthcare costs (41-43).

Detection of CTX-M-type β-lactamases in clinical isolates relies on phenotypic methods such as the double-disk synergy test, which detects the enhanced hydrolysis of cephalosporins in the presence of clavulanic acid, a β-lactamase inhibitor. However, molecular techniques, including polymerase chain reaction (PCR) and sequencing, are necessary for precise identification of CTX-M variants and monitoring their dissemination (44).

The rise of CTX-M-type β-lactamases has prompted significant changes in clinical practice, particularly regarding the use of carbapenems, which have become the mainstay of treatment for infections caused by CTX-M-producing bacteria. However, the increasing prevalence of carbapenem-resistant *Enterobacteriaceae* (CRE), including those co-producing CTX-M and carbapenemases such as KPC and NDM, represents a growing threat to global public health (45). This has led to the exploration of alternative therapeutic strategies, including the use of newer β-lactam/β-lactamase inhibitor combinations, such as ceftazidime-avibactam and meropenem-vaborbactam, which have shown efficacy against some CTX-M-producing strains (46).

Infection prevention strategies, including antimicrobial stewardship programs and rigorous infection control practices, are essential to mitigate the spread of CTX-M-producing bacteria in healthcare and community settings (47).

1.3.3. General characteristics of carbapenemases

Carbapenemases are a diverse group of β-lactamases degrading carbapenems, leading to severe clinical challenges due to limited treatment options. Carbapenemases are classified into three main classes according to the Ambler classification: class A, class B (MBLs), and class D (oxacillinases), each with distinct biochemical properties and clinical implications (4).

Class A carbapenemases, such as KPC, are particularly significant due to their high prevalence, especially in *Enterobacteriaceae* species. KPC-producing organisms are a leading cause of hospital-associated infections and are found predominantly in intensive care units (48). The gene encoding KPC is often plasmid-mediated, which facilitates HGT and the rapid dissemination of resistance among Gram-negative bacteria. Though class A carbapenemases are inhibited by some β-lactamase inhibitors, such as tazobactam, their clinical utility remains limited in severe infections (49).

Class B carbapenemases, known as MBLs, require zinc ions for their catalytic activity. The most clinically important MBLs include NDM, Verona integron-encoded metallo-β-lactamase (VIM), and imipenemase (IMP) (50). NDM, in particular, has garnered global attention due to its rapid spread across various bacterial species and geographic regions. NDM-1, first reported in *K. pneumoniae* in India in 2008, now poses a global health threat due to its resistance to nearly all β-lactams, except for aztreonam (51). These MBLs are not susceptible to traditional β-lactamase inhibitors but can be inhibited by metal ion chelators, such as EDTA (Ethylenediaminetetraacetic acid). However, this has not led to clinically effective treatments, as chelators are not therapeutically viable (52).

Class D carbapenemases, particularly the OXA-type enzymes, have been associated predominantly with *A. baumannii*, but they also appear in other pathogens like *P. aeruginosa* and *Enterobacteriaceae* (53). OXA-48-like enzymes are a key subgroup within class D carbapenemases. Despite their relatively weak activity against carbapenems, these enzymes are of concern because they also hydrolyze penicillins and some cephalosporins, limiting treatment options (54). OXA-48-producing organisms are often resistant to multiple other antibiotic classes, complicating treatment strategies. The resistance conferred by OXA-type carbapenemases is particularly worrisome in clinical settings, where infection control measures often fail to contain their spread (55).

The rise in carbapenemase prevalence has been exacerbated by the overuse and misuse of antibiotics in both clinical and agricultural settings. HGT, particularly through plasmids, transposons, and integrons, plays a significant role in the rapid dissemination of carbapenemase genes, further complicating infection control measures in healthcare environments (21).

Therapeutic options for infections caused by carbapenemase-producing organisms are limited. Polymyxins, tigecycline, and fosfomycin are among the few remaining treatments, although their efficacy is inconsistent, and they often come with significant toxicity (56). Newer antibiotic combinations, such as ceftazidime-avibactam and meropenem-vaborbactam, have shown promise, particularly against KPC-producing organisms. However, their effectiveness against MBLs and OXA-type carbapenemases remains limited (57).

Diagnostic challenges further complicate the management of carbapenemase-producing infections. Rapid detection methods, such as PCR and whole-genome

sequencing, are crucial for identifying carbapenemase genes and implementing timely infection control measures (58). These methods also help in epidemiological surveillance, allowing health authorities to track the spread of carbapenemase-producing organisms and respond accordingly. Improved diagnostics, coupled with global surveillance programs, are essential for controlling the spread of carbapenemase-producing bacteria (16).

1.3.4. Challenge of global dissemination of β-lactamases

The worldwide dissemination of ESBL-producing *Enterobacteriaceae* has emerged as a critical issue for medical professionals, primarily due to the severe limitations in available therapeutic options that hinder effective treatment strategies for both community-acquired infections and those acquired in hospital settings. ESBL-producing *Enterobacteriaceae* have become closely associated with the concept of multidrug resistance over the last two decades. (37).

Over the past decade, a notable shift has occurred in which CTX-M-type ESBLs have supplanted the previously dominant TEM- and SHV-types among clinical isolates of *Enterobacteriaceae*, indicating a worrying trend in antibiotic resistance patterns (59). The rapid and extensive spread of CTX-M-type β-lactamases throughout various regions of the globe has been aptly termed the "CTX-M pandemic," which is intrinsically linked to the increasing documentation and recognition of these enzymes in diverse geographic locales (60), with variations in prevalence rates observed among different species within the *Enterobacteriaceae* family; however, it is particularly noteworthy that these resistance determinants are most frequently identified in notorious pathogens such as *K. pneumoniae* and *E. coli* (61).

The alarming rise in the incidence of infections caused by MDR Gram-negative bacteria, particularly those that produce ESBLs, has corresponded with a marked increase in the usage of carbapenems as a primary treatment modality for managing these challenging infections (62). This trend has, in turn, contributed to the further emergence and dissemination of CPE, which poses an even greater threat to public health due to their profound resistance mechanisms. While it is important to acknowledge that resistance rates to carbapenems remain relatively low in certain regions of Europe, the recent developments and alarming trends observed in southern and southeastern Europe—areas

historically characterized by the unregulated and unrestrained use of these essential antibiotics—are particularly concerning (63). CPE infections are significantly correlated with high levels of morbidity and mortality, especially among particularly vulnerable groups of patients, which notably include neonates, young children, senior citizens, individuals who are hospitalized, patients with compromised immune systems, as well as those who are critically ill and require intensive medical care. The primary catalyst for the rampant and uncontrolled spread of these strains within healthcare settings is their inherent capacity to endure and proliferate swiftly in such environments; indeed, the production of carbapenemases is frequently associated with successful MDR clones that are commonly linked to nosocomial, or hospital-acquired, infections, as evidenced in numerous studies (64, 65).

Among the various types of carbapenemases, OXA-48-like carbapenemases represent one of the most prevalent forms, with their increasing prevalence particularly noted across Europe, although substantial geographic variations in their distribution are often observed, highlighting the complexities of their epidemiology and public health implications (63, 66). While *K. pneumoniae* stands out as the primary reservoir for the *bla*OXA-48 gene, there has been a noticeable increase in research studies reporting cases involving other *Enterobacteriaceae* species that produce *bla*OXA-48, thereby indicating a concerning global trend (65, 67, 68). Given the high prevalence and widespread nature of *bla*OXA-48-like carbapenemases among both community-associated and nosocomial Gram-negative bacterial infections, effectively curtailing the further dissemination of pathogens that harbor these enzymes poses a formidable challenge for healthcare systems and infection control measures (68, 69). The *bla*OXA-48-like and *bla*OXA-48 carbapenemases are frequently located on plasmids that exhibit a significant tendency to disseminate across various bacterial species through mechanisms of HGT, which complicates the management of these resistant strains (66). It is not an uncommon occurrence to identify different bacterial species containing identical plasmids that carry the *bla*OXA-48 gene, which may be isolated from the same patient, either functioning as colonizers within the microbiome or acting as causative agents of infections, thus further illustrating the complexity and challenges associated with tackling these resistant organisms (70). OXA-48 is linked to various Tn1999 transposon variants and is predominantly identified as the sole antibiotic resistance determinant on the conjugative

IncL (IncL/M) replicon-type plasmids (71, 72). The presence of pOXA-48a-like IncL plasmids has been documented in numerous Gram-negative bacterial species, including *Citrobacter freundii*, *E. coli*, *Enterobacter cloacae*, *K. pneumoniae*, *Klebsiella oxytoca*, and *Raoultella planticola* (73, 74). Certain high-risk clones (e.g., ST11, ST15, ST101, and ST307 for *K. pneumoniae*, as well as ST38 and ST410 for *E. coli*) have been implicated in the worldwide dissemination of various OXA-type carbapenemases (OXA-48, OXA-181, OXA-232, and OXA-204) (70, 75-77). OXA-162, which also belongs to the OXA-48-like carbapenemases, has been identified in diverse gut microbiota, with reports emerging from Turkey, Germany, Greece, and Hungary to date (67, 78-80).

Enterobacteriaceae constitute a significant component of the human gut microbiome, and fecal carriers may serve as a crucial reservoir for the inter-human transmission and propagation of these bacteria. Moreover, colonization of the gut by MDR bacteria has been correlated with an elevated risk of subsequent clinical infections, which are associated with increased mortality rates (64, 81). Consequently, proactive surveillance is essential in curbing the proliferation of such strains. Initiatives aimed at reducing the transmission of carbapenemase-producing *K. pneumoniae* strains emphasize fundamental and advanced infection control protocols, while the significance of the intestinal reservoir of these strains and its modulation by various antibiotic treatments remains largely unexamined (82). The administration of antibiotics is a recognized risk factor contributing to the emergence of resistance; however, its precise role in colonization is yet to be elucidated.

1.4. Gastrointestinal carriage of multiresistant *Klebsiella pneumoniae*

The gastrointestinal tract (GIT) plays a critical role as a reservoir for multiresistant, high-risk clones of *K. pneumoniae*, which can lead to the spread of AMR and severe healthcare-associated infections. Colonization of the GIT by MDR *K. pneumoniae* has been associated with the acquisition of genes conferring resistance to carbapenems, extended-spectrum β-lactams, and other antibiotics. High-risk clones of *K. pneumoniae*, such as ST258, have been identified in numerous studies as key players in the global dissemination of carbapenemase-producing *K. pneumoniae* (CP-Kp), particularly within

healthcare settings, where they often cause life-threatening infections such as pneumonia, urinary tract infections, and sepsis (83, 84).

The ability of *K. pneumoniae* to establish itself within the GIT is linked to several virulence factors, including the production of polysaccharide capsules, siderophores, and adherence mechanisms, which enhance its persistence and ability to evade host immune responses (85). Once colonized, the GIT can act as a reservoir for HGT, allowing *K. pneumoniae* to acquire and disseminate resistance genes to other members of the gut microbiota. This phenomenon is particularly concerning in patients undergoing prolonged hospitalization, especially those receiving broad-spectrum antibiotics, which can disrupt the microbiota and create selective pressures that favor the overgrowth of resistant strains (86).

Studies have demonstrated that the gastrointestinal carriage of MDR *K. pneumoniae* precedes invasive infections, suggesting that colonization is a precursor to subsequent clinical manifestations. In a study by Martin et al. (2017), rectal colonization by *K. pneumoniae* was found to be a strong predictor of infection in immunocompromised patients, particularly those in ICUs. Furthermore, the same study indicated that the duration of colonization can persist for weeks to months, increasing the likelihood of transmission to other individuals or healthcare environments. These findings emphasize the importance of GIT surveillance in identifying colonized individuals and implementing targeted infection control measures to prevent outbreaks of MDR *K. pneumoniae* in healthcare settings (87).

The molecular epidemiology of MDR *K. pneumoniae* strains reveals that high-risk clones, particularly those harboring carbapenemases such as KPC, NDM, and OXA-48, have become increasingly prevalent worldwide. These carbapenemase-producing strains exhibit significant resistance to multiple antibiotic classes, making them exceedingly difficult to treat with conventional therapies (88). The gastrointestinal carriage of these strains is particularly worrisome in immunocompromised populations, including patients with hematological malignancies or solid organ transplants, who are at heightened risk of invasive infection due to their compromised immune systems (89). Routine screening of high-risk patient populations for GIT colonization with MDR *K. pneumoniae* has been suggested as an essential strategy for early detection and prevention of subsequent infections (90).

In recent years, increasing attention has been given to the role of gut microbiota in the dynamics of *K. pneumoniae* colonization and infection. Alterations in the gut microbiome, particularly a loss of microbial diversity due to antibiotic use, have been implicated in the expansion of *K. pneumoniae* and other *Enterobacteriaceae*. Research by Pamer (2016) has shown that restoring the microbiome, for instance through fecal microbiota transplantation, can potentially reduce *K. pneumoniae* colonization and curb the spread of resistant strains. This novel approach highlights the importance of maintaining a balanced microbiome in preventing the establishment and persistence of MDR organisms in the gut (91).

Infections caused by MDR-*Enterobacterales* (particularly *E. coli* and *K. pneumoniae*) are often endogenous, most commonly arising from asymptomatic gut colonization. The connection between the normal gut flora and the production of IgA (immunoglobulin A) as well as defensins has been studied in relation to various gastrointestinal diseases (92-95).

Defensins, a family of cationic antimicrobial peptides, play a crucial role in the innate immune defense of the GIT. These peptides, categorized into α- and β-defensins based on their structural characteristics and disulfide bonding patterns, exhibit broad-spectrum antimicrobial activity and contribute to maintaining intestinal homeostasis by shaping the gut microbiota and providing a barrier against pathogens (96). A-defensins, primarily secreted by Paneth cells in the small intestine, exhibit potent antibacterial activity against a range of Gram-positive and Gram-negative bacteria. These peptides function by disrupting bacterial membranes through electrostatic interactions and hydrophobic insertions, leading to cell lysis. Notably, human α-defensins 5 and 6 (hAD5 and 6) are essential in safeguarding the crypts of Lieberkühn, where they are stored and released in response to microbial or inflammatory stimuli. HD6, in particular, demonstrates a unique ability to form peptide nanonets that entrap bacteria, preventing their translocation and colonization (97). B-defensins, on the other hand, are expressed more ubiquitously across the epithelial cells of the GIT and are inducible by microbial products or pro-inflammatory cytokines. Human β-defensins (hBD1-4) are key contributors to mucosal immunity. While hBD1 is constitutively expressed and provides baseline protection, hBD2, hBD3, and hBD4 are inducible and act in response to bacterial invasion or tissue injury. These defensins not only exhibit direct antimicrobial activity but also modulate

immune responses by recruiting immune cells through chemotactic effects, thereby amplifying the mucosal defense (98).

Secretory IgA plays a pivotal role in immune exclusion by binding to microbial antigens, preventing pathogen adherence, and facilitating their removal through mucus flow. This mechanism is especially significant in limiting the colonization and dissemination of MDR *Enterobacteriaceae*, which pose a significant threat to public health. Secretory IgA interacts with the bacterial surface antigens to neutralize pathogens and prevent their adhesion to the intestinal epithelium. Furthermore, it contributes to microbial homeostasis by selectively promoting the growth of commensal bacteria over pathogenic species (99). However, reduced IgA levels or altered IgA specificity can impair the immune exclusion process, enabling these pathogens to evade mucosal defenses and form reservoirs within the gut. Additionally, IgA-opsonized bacteria are more effectively recognized and eliminated by innate immune cells (100). Dysbiosis, characterized by reduced microbial diversity and overgrowth of pathogenic species, has been linked to impaired IgA production and an increased risk of colonization with resistant strains (101).

Certain *Klebsiella* species are potential pathobionts, which means that they can cause intestinal diseases if certain colonization conditions are met (102). Their pathogenicity is influenced by the colonization state of the intestinal microbiota (92). MDR, high-risk *K. pneumoniae* clones are found globally in healthcare facilities, where they successfully multiply and survive under antibiotic selection pressure due to their favorable genetic constellation. These clones can be present asymptomatically in the GIT, furthermore it is also important to emphasize their significance in terms of the infections they cause and the therapeutic challenges they represent (103, 104). The CTX-M-15-producing *K. pneumoniae* ST15 is a high-risk, globally widespread clone with resistance to multiple antibiotic classes. Due to their high virulence, these strains pose a significant clinical challenge, as they can spread easily, leading to outbreaks (105-107). The clone has the ability to transmit efficiently between hosts, high pathogenicity, and AMR, which has contributed to its widespread prevalence in Europe. The narrow host range IncFII(K) plasmids, which often harbor *bla*CTX-M-15, and the broad host range IncL plasmids, which frequently carry a *bla*OXA-type gene, play a crucial role in the successful dissemination of the bacteria. (21, 59, 108-110).

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2. Objectives

Our aims were

- to investigate the colonization dynamics of a multi-resistant, high-risk clone of *K. pneumoniae* isolated from clinical sample
- to detect and identify bacterial resistance plasmids which are transferred via conjugation and to gain a transconjugant strains
- to detect the alteration of local antibacterial response in the gut to colonization
- to investigate the adaptive humoral immune response by determining the fecal IgA antibody content
- to investigate the non-specific antibacterial response of the gut mucosa by measuring the fecal levels of small antimicrobial peptides, namely murine β-defensin 3 (mBD3) and murine α-defensin 5 (mAD5)
- to detect the alteration of the gut microbiota by 16S metagenomic analysis of fecal samples

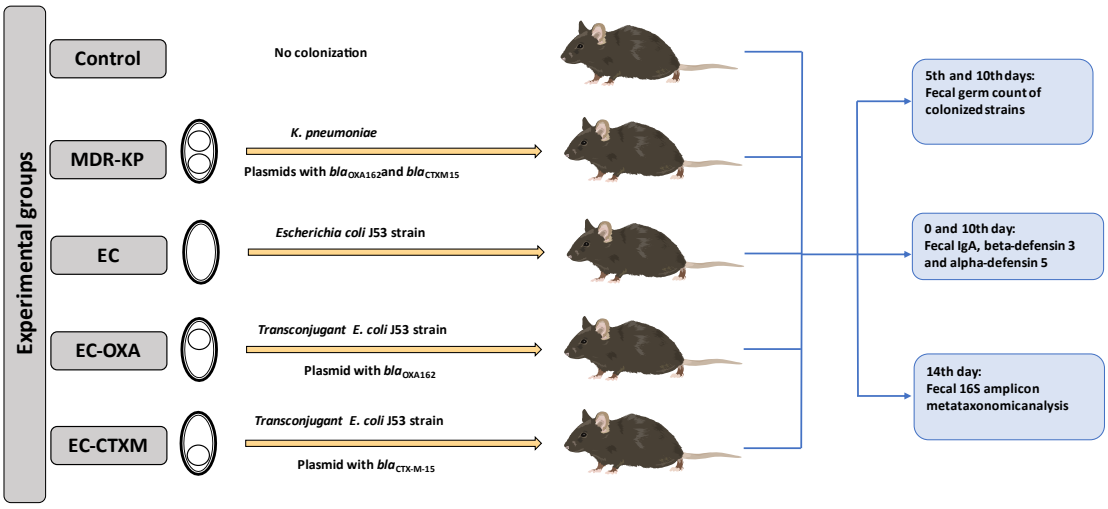

Figure 1. Experimental design (111).

3. Methods

3.1. Bacterial strains

For our research, we acquired a multidrug-resistant, high-risk variant of *K. pneumoniae* ST15 that was isolated from a clinical sample (MDR-KP) at the National Center for Public Health and Pharmacy in Budapest, Hungary. It was also utilized as a donor strain during the conjugation process. An azide-resistant *E. coli* J53 laboratory strain (EC) served as the acceptor strain in the conjugation experiments. This strain is free from plasmids and is sensitive to most antibiotics, including β-lactam antibiotics and fluoroquinolones. Transconjugant *E. coli* J53 strains harboring one of the donor MDR-KP plasmids were generated according to the conjugation assay described below (112).

3.2. Antibacterial susceptibility testing

To investigate the resistance of the original (MDR-KP and EC) strains and transconjugants against the most significant antibiotics related to this study – β-lactams (penicillins, cephalosporins, and carbapenems), ciprofloxacin – antibacterial susceptibility was determined by broth microdilution methodology in accordance with the EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines version 14.0 (www.eucast.org) (113). The incubation occurred at a temperature of 35 °C for a duration of 16–20 hours, and the determination of minimum inhibitory concentrations (MICs) was executed through visual observation. *E. coli* ATCC (American Type Culture Collection) 25922 served as the reference control strain. As a phenotypic verification of the transmission of resistance genes, results were compared between the original strains and the transconjugants (112).

3.3. Conjugation assay

The primary objective of this experiment was to generate transconjugant bacterial strains carrying transmissible resistance genes. To achieve this, conjugation assays were performed using the broth mating technique in Luria-Bertani (LB) broth (Sigma-Aldrich, USA). The clinical isolate of *K. pneumoniae* (MDR-KP) served as the donor strain, while

the sodium-azide resistant *E. coli* J53 strain was employed as the recipient strain. Overnight cultures of both donor and recipient strains were introduced into 8 mL of fresh LB broth. The donor and recipient cultures were mixed at a 1:1 ratio, achieved by adding 300 µL of each culture, and incubated at 37°C for a period of 4 hours to allow for conjugation to occur (112).

Following the incubation, the cultures were centrifuged, and the supernatant was carefully removed to eliminate any residual antibiotics. The bacterial pellet was then re-suspended in fresh LB broth, and aliquots were plated onto LB-agar solid medium containing selective agents: 100 µg/mL sodium azide (Sigma-Aldrich) and 0.1 µg/mL of cefotaxime (Sigma-Aldrich), ertapenem (Sigma-Aldrich), or both, to select for transconjugants that had acquired resistance genes. The selective plates were incubated, and colonies were further cultured on selective agar to confirm their resistance. Colonies demonstrating consistent growth were tested for the presence of ESBLs and carbapenemases. These tests were conducted using CTX-M Multi and Carba 5 immunochromatographic assays (NG Biotech, Guipry, France), which provided rapid detection of ESBL and carbapenemase-producing strains. This confirmed the successful horizontal transfer of resistance genes in the generated transconjugant strains (112).

3.4. Whole genome sequencing

Genomic DNA from *K. pneumoniae* strain KP5825 (MDR-KP), *E. coli* J53 (EC) and transconjugant (EC-CTXM, EC-OXA) strains was extracted using the NucleoSpin Microbial DNA Kit (Macherey-Nagel, Germany), following the manufacturer's recommended protocol. Similarly, plasmid DNA was purified using the NucleoSpin Plasmid DNA Kit (Macherey-Nagel, Germany) under the same standard operating procedures. The purity, integrity, and concentration of the extracted DNA samples were evaluated using a Qubit 4.0 fluorometer (Invitrogen, Waltham, MA, USA) and Tapestation 4150 system (Agilent Technologies, Santa Clara, CA, USA). For next-generation sequencing (NGS) library preparation, the Nextera DNA Flex Library Prep Kit (Illumina, Eindhoven, The Netherlands) was employed, incorporating Nextera DNA CD Indexes for sample multiplexing. Sequencing was carried out on the Illumina MiSeq platform using the MiSeq Reagent Kit v2, producing paired-end reads of 250 base pairs. This process was performed at the Genomics Resource Center at Biomi Ltd. The resulting

FASTQ files were directly imported into the cloud-based computational engine of BioNumerics version 7.6 software (Applied Maths NV, Belgium). For genome assembly, the *de novo* sequence reads were assembled using the SPAdes genome assembler (version 3.7.1) (112). Generated data were further analysed by the SEED and RAST softwares (114).

3.5. Animal studies

For gastrointestinal colonization studies, male C57BL/6 mice aged 6 to 8 weeks, sourced from Jackson Laboratory (Bar Harbor, ME, USA), were used. These mice were housed individually to prevent cross-contamination. They were placed in individually ventilated cages under controlled environmental conditions, which included a 12-hour light–dark cycle and a regulated ambient temperature of 20–22°C. Throughout the study, the mice had unrestricted access to sterile food and water, with sterile bedding provided to maintain a pathogen-free environment. Prior to the initiation of the study, the mice underwent a two-week acclimatization period. To facilitate the colonization of introduced bacterial strains within the GIT, the mice were pre-treated with ampicillin (Sandoz) in their drinking water at a concentration of 0.5 mg/L. This antibiotic regimen was administered for two weeks, which reduced the endogenous bacterial populations and promoted the establishment of exogenously administered bacterial strains. Following this pre-treatment, colonization was achieved through orogastric gavage. Each mouse was colonized with 100 µL solution of phosphate-buffered saline (PBS) containing 10^8 colony-forming units (CFUs) of one bacterial strain: MDR-KP, EC, *E. coli* producing OXA-type β-lactamase (EC-OXA), or *E. coli* harboring CTX-M-type ESBL (EC-CTXM). Mice in the control group were given sterile PBS via the same protocol to serve as a non-colonized baseline comparison. Six mice were assigned to each experimental group. Post-colonization, the mice continued to receive ampicillin in their drinking water at the same concentration (0.5 mg/L) to promote sustained colonization. Fresh fecal samples were collected at specific time points—Days 5, 10, and 14 post-colonization. The fecal samples were promptly weighed and processed for bacterial germ count determination. In addition, aliquots of the fecal material were stored at −80°C for subsequent enzyme-linked immunosorbent assay (ELISA) and DNA extraction. Throughout both the acclimatization period and the experimental phase, mice were

handled with care to minimize stress and maintain overall welfare. All procedures were carried out in strict compliance with ethical standards, as mandated by the institutional animal care and use committee. The study adhered to the guidelines established in the Guide for the Care and Use of Laboratory Animals, with full ethical approval granted by the Animal Care Committee of Semmelweis University (Permission Nos. PE/EA/60-8/2018 and PE/EA/964-5/2018). (111).

3.6. Determination of the fecal germ count of mice

The quantification of fecal shedding of the colonized bacterial strains was performed by determining the bacterial germ count in freshly collected fecal samples. Each fecal sample was first weighed and then suspended in 1 mL of sterile PBS (VWR, Hungary) by mechanically disaggregating the fecal pellet using a sterile inoculation loop. The sample was thoroughly vortexed to create a homogenous suspension. Subsequently, the bacterial suspension was subjected to a tenfold serial dilution. Twenty µL from each dilution were then streaked onto selective chromogenic agar plates and incubated overnight. Next day, bacterial colonies were identified based on their characteristic color and counted manually. The germ count, expressed as CFUs per gram of fecal matter, was calculated. For selective isolation of the MDR-KP strain, Orientation CHROMagar plates supplemented with 0.5 mg/L ciprofloxacin (Fresenius Kabi, Bad Homburg vor der Höhe, Germany) were used to inhibit the growth of competing flora. For the detection of EC, EC-OXA and EC-CTXM, *Enterobacteriaceae* CHROMagar plates containing 100 mg/L sodium azide (Merck, Darmstadt, Germany) were employed. Statistical analysis of the results was performed using a two-tailed Student's t-test to compare germ counts across experimental groups. (111).

3.7. Determination of total IgA and defensin levels in stool by ELISA

The levels of total IgA, murine β-defensin 3, and murine α-defensin 5 were quantified from mouse fecal samples using commercially available ELISA kits (MyBiosource, San Diego, CA, USA; product codes: MBS7725462, MBS7725303, and MBS7725358, respectively). Frozen fecal samples collected during the experiment were thawed and

accurately weighed before being suspended in PBS. The suspensions were then vortexed thoroughly for 1 hour at 4°C to ensure complete homogenization. Following this process, the fecal suspensions were centrifuged at 2500 rpm for 10 minutes, and the resulting supernatants were collected for further analysis. Sandwich ELISA assays were conducted in accordance with the manufacturer's protocols to measure the concentrations of IgA and defensins in the samples. Optical density was measured using a microplate reader at 450 nm, with 690 nm serving as the reference wavelength. Concentration values were determined using calibration curves generated from the kit-provided standards. The results were then expressed as mg/g or pg/g of fecal material to reflect the total IgA and defensin content. Statistical analysis of the results was performed using the Wilcoxon rank-sum test to assess differences between experimental groups, providing non-parametric comparisons where applicable (111).

3.8. Microbiome composition with 16S metagenomic analysis

To assess the impact of different colonizing bacterial strains on the composition of the gastrointestinal microbiota in mice, fecal samples were collected on Day 14 post-colonization. Approximately 80 mg of feces was used for DNA extraction, which was performed using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA, D4300) in accordance with the manufacturer's instructions. The bacterial 16S rRNA gene's V3–V4 region was then amplified using PCR. To facilitate sample identification and sequencing, dual indices (barcodes) and Illumina sequencing adapters were added to the PCR amplicons using the Nextera XT Index Kit (Illumina, Inc., San Diego, CA, USA). This was followed by DNA purification using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA, USA). Individual barcoded DNA samples were quantified using the Qubit dsDNA HS Assay Kit with the Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and further quantified with the DNA 7500 Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The quantified samples were then normalized and pooled. The pooled multiplexed libraries were diluted to a concentration of 7 pM, denatured with NaOH, and prepared for sequencing on the Illumina MiSeq system. Sequencing was performed using the MiSeq reagent kit v3, which provided 600 cycles (2 × 300 bp paired-end reads) (111).

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The resulting sequencing data were uploaded and analyzed using the CosmosID-HUB software version 2.0. Paired-end reads were processed through the DADA2 algorithm. After primer removal, the reads were quality-trimmed with a median Phred score threshold of 20. Forward and reverse reads were trimmed to a uniform length based on read quality, and sequences with at least a 12-base overlap were merged. Chimeric sequences were subsequently removed to ensure accuracy. The processed data were converted into amplicon sequence variants (ASVs), which provided a detailed profile of the microbiota. Taxonomic classification of the ASVs was performed using DADA2's naive Bayesian classifier in conjunction with the Silva version 138 database. The microbial composition of the samples was characterized by the relative abundance of the identified taxa and analyzed using diversity indices such as CHAO1 and Simpson's diversity index. Additionally, the abundance distribution of specific taxa between experimental groups was compared. Statistical analyses were performed using the Wilcoxon rank-sum test to evaluate differences in microbial composition and abundance across the experimental conditions (111).

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4. Results

4.1. Characterisation of the antibiotic susceptibility of the donor, recipient and transconjugant strains used in this study

The data analysis in this study focused on whole-genome sequencing of four distinct bacterial strains: MDR-KP, EC, EC-CTXM, and EC-OXA. For determining the role of mobile genetic elements, we analysed the whole genome sequencing data of a multidrug-resistant clinical isolate of *K. pneumoniae* (MDR-KP). We identified four distinct plasmids in the sequence data, classified under the following incompatibility types: IncF(I)B, IncF(II)K, ColpVC, and IncL. Subsequently, during annotation we located resistance genes on both the chromosome and plasmids. Among the β-lactamases, we identified two ESBL-type enzymes: one *bla*SHV-28 gene located on the chromosome and another *bla*CTX-M-15 gene on a plasmid. Additionally, we discovered two carbapenemase enzyme genes, *bla*OXA-1 and *bla*OXA-162, both residing on plasmids (Table 1.).

Of note among other resistance mechanisms was the detection of fluoroquinolone resistance, given that our selective culturing used ciprofloxacin-containing media. Multiple mechanisms of fluoroquinolone resistance were identified: several point mutations in the *gyr*A and *par*C genes on the chromosome, and the plasmid-located resistance determinant *aac(6')-Ib-cr*. The MDR-KP strain also harbored genes conferring resistance to other antibiotics (*aac(3)-IIa*, *aph(3')-Ia*), making it particularly suitable as a donor in our conjugation experiments. This donor strain's gene repertoire allowed for various transfer experiments with the recipient strain, EC, a laboratory strain devoid of plasmids but capable of plasmid uptake (Table 1.).

Our conjugation experiments confirmed that the recipient strain did not originally contain plasmids. Following conjugation, two transconjugant strains were isolated. The first, EC-CTXM, acquired an IncF(II)K-type plasmid carrying the *bla*CTX-M-15 gene from the donor strain. This gene was embedded within a class 1 mobile genetic element. Additionally, we observed plasmid-mediated transfer of a fluoroquinolone resistance gene. The second transconjugant strain, EC-OXA, acquired an IncL-type plasmid

containing the *bla*OXA-162 carbapenemase resistance gene within a Tn1999.2 transposon (Table 1).

Microdilution confirmed that these genotypes conferred phenotypic resistance, particularly to β-lactam antibiotics such as ampicillin, ceftazidime, and cefuroxime. Carbapenem resistance was assessed against ertapenem, imipenem, and meropenem, while ciprofloxacin was tested as a representative of the fluoroquinolones. The MDR-KP clinical isolate demonstrated resistance to all tested antibiotics, while the recipient strain, EC, showed no elevated MIC values for any antibiotics prior to conjugation. Post-conjugation, the EC-CTXM strain exhibited increased resistance to ampicillin and cephalosporins, and the EC-OXA strain showed a slight elevation in MIC values for ampicillin and carbapenems, although ciprofloxacin MIC values remained unchanged (Table 2).

It is noteworthy that while the transconjugant strains acquired resistance genes, this did not always result in MIC values reaching clinical resistance breakpoints. Nonetheless, the elevated MIC values indicate that these genes moderately enhance the strain's antibiotic resistance capacity. The EC-CTXM strain did not confer a substantial increase in ciprofloxacin MIC, although the fluoroquinolone resistance determinant was successfully transferred.

Table 1. Resistance genes of different bacterial strains used for gastrointestinal colonization (112).

Strains	**MDR-KP**	**EC**	**EC-CTXM**	**EC-OXA**
Plasmid replicon types	IncF(I)B, IncF(II)K, ColpVC, IncL	NA	IncF(II)K	IncL
	B-lactamases			
Chromosomal	*bla*SHV-28	NA	*bla*CTX-M-15	*bla*OXA-162
On mobile genetic elements	*bla*OXA-1, *bla*OXA-162, *bla*CTX-M-15			
Mobile genetic elements	Class I, Tn1999.2	NA	Class I	Tn1999.2
	Quinolon resistance determinants			
Chromosomal	gyrA (S83F, D87A, N645H) parC (S80I, P402A)			
On mobile genetic elements	aac(6')Ib-cr		aac(6')Ib-cr	
Other resistance genes	aac(3)-IIa, aph(3')Ia			

Table 2. Minimal inhibitory concentrations (MIC's) of different bacterial strains used for gastrointestinal colonization (112).

Strains	MDR-KP	EC	EC-CTXM	EC-OXA
Ampicillin	>32	4	>32	>32
Ceftazidime	>32	0.25	>32	0.25
Cefotaxime	>32	0.125	>32	0.25
Ertapenem	>32	<0.0625	<0.0625	0.25
Imipenem	16	0.5	0.5	0.5
Meropenem	>32	<0.0625	<0.0625	0.125
Ciprofloxacin	>32	<0.0625	<0.0625	<0.0625

4.2. Fecal shedding of the colonised strains

For effective and high-density colonization, mice underwent a two-week antibiotic pretreatment, during which ampicillin was administered via their drinking water. To maintain a high gastrointestinal density of the colonizing strains, this treatment was continued throughout the experiment.

In each experimental group, previously characterized MDR-KP, EC, EC-CTXM, and EC-OXA strains were introduced via orogastric gavage, and the bacterial load was quantified in fecal samples collected on the 5th and 10th days of the experiment, measured in CFU per gram of feces. The lowest bacterial load was observed in the EC group, with an average of 3.77x10^7 CFU/g, indicating a lower colonization potential of the laboratory strain lacking resistance plasmids compared to other strains. During the experiment, animals demonstrated higher colonization densities, with the highest CFU counts on the 5th day observed in the MDR-KP group, which contained the most resistance genes, at 8.64x10^9 CFU/g. Notably, substantial bacterial loads were also detected in the EC-CTXM group, with 7.06x10^8 CFU/g, and in the EC-OXA group, with 1.54x10^9 CFU/g, suggesting that the resistance genes present on plasmids enhanced the colonization potential of these strains (Figure 2).

On the 10th day, sustained antibiotic treatment led to uniformly higher CFU counts across all experimental groups. However, the relative abundances of the bacterial strains were consistent with the results from the 5th day. The MDR-KP group continued to show the highest bacterial load at 5.43x10^10 CFU/g, followed by the EC-CTXM group at

3.18x10^9 CFU/g and the EC-OXA group at 2.01x10^10 CFU/g. The lowest CFU counts continued to be observed in the EC group at 8.43x10^8 CFU/g, indicating the comparatively lower colonization ability of this strain (Figure 2).

These findings underscore the role of plasmid-borne resistance genes in enhancing bacterial colonization efficiency in the context of sustained antibiotic exposure. However, it is worth considering that statistical analysis of bacterial counts only revealed a significant difference between the EC-OXA and EC groups. No statistical differences were observed among the other groups, primarily due to the high variance in individual values within each group. Although mean values align with the presence of resistance genes across the bacterial groups, colonization success likely depends on various other factors, which may explain the lack of statistical differences. This supports the notion that additional differences between the groups cannot be solely explained by the bacterial counts achieved during colonization.

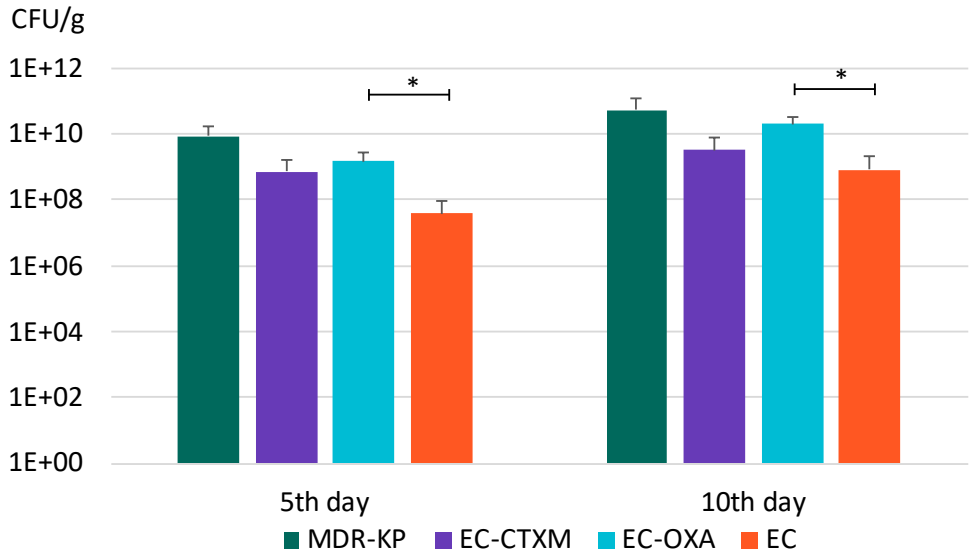

Figure 2. The gastrointestinal colonization rate characterized by germ count (colony-forming units: CFU) in the feces by different bacteria—MDR-KP, EC, EC-OXA, and EC-CTXM—on the fifth and tenth day of colonization. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ (111).

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4.3. Fecal IgA level of the experimental groups

The course of the immune response to colonization was assessed by measuring the total IgA content in feces. For this, baseline fecal samples were collected from each experimental animal on day zero, immediately before the introduction of the various bacterial strains, yielding an average IgA content of 5.89 mg/g. On day 10, total IgA levels in fecal samples were compared to this baseline. In the control group, which did not undergo colonization treatment, the IgA content remained unchanged at 6.26 mg/g. Similarly, no significant difference in IgA levels was observed in the EC group compared to day zero or the control group. These findings suggest that, although the plasmid-free, non-virulent laboratory strain was able to establish itself in high densities within the animals' GITs, it did not elicit an enhanced humoral immune response.

In contrast, bacteria carrying resistance plasmids significantly increased the total fecal IgA content by day 10 following their introduction. Notably, in the group colonized with the MDR-KP strain, which contained the most plasmids, IgA levels were measured at more than twice the baseline value, averaging 13.67 mg/g. In groups colonized with transconjugant strains containing only a single plasmid from the MDR-KP donor strain, an even greater increase—approximately fourfold—was observed. In these groups, total IgA levels in fecal samples reached 22.68 mg/g in the EC-CTXM group and 23.52 mg/g in the EC-OXA group (Figure 3).

It appears that the combined effects of immunomodulatory factors present on multiple plasmids and the chromosome of the clinical *K. pneumoniae* strain (MDR-KP) led to a somewhat lower increase in immunoglobulin production compared to the transconjugant strains (EC-CTXM, EC-OXA). Nevertheless, these findings also indicate that the transfer of plasmids containing resistance genes into strains with a lower innate capacity to stimulate immune responses can significantly alter these strains' immunogenic properties.

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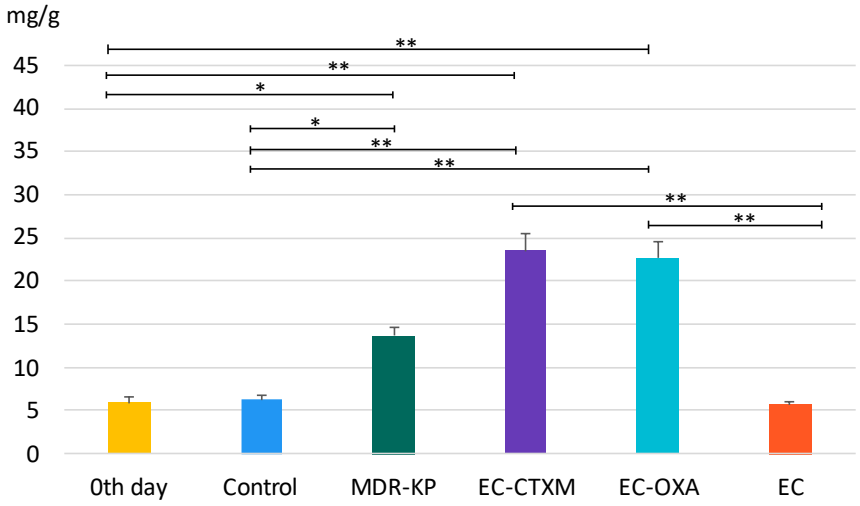

Figure 3. The IgA level in feces of mice colonized by different bacteria MDR-KP, EC, EC-OXA, and EC-CTXM. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ (111).

4.4. Fecal β-defensin 3 level of the experimental groups

Among inducible antimicrobial peptides, mouse β-defensin-3 (mBD3) was successfully detected in the collected fecal samples using ELISA. Similar to the IgA measurements, baseline values were obtained from fecal samples collected on day zero, immediately before colonization, and were compared to samples collected on day 10. At the start of the experiment, prior to colonization, the average mBD3 concentration in the feces was 196.63 pg/g. In the control group, the mBD3 level did not change significantly after 10 days, measuring slightly lower at 154.5 pg/g. These results align with previous observations linking mBD3 level increases to intestinal microbiota instability and low diversity (115). It appears that ampicillin administration, by reducing microbiota diversity, induces significant compositional changes in the intestinal microbiota, which keeps mBD3 levels low, consistent with findings from other studies (Figure 4).

Our experiments aimed to promote colonization of the introduced bacterial strains through oral ampicillin administration while modeling the environment that might occur during antimicrobial therapy. The results across different colonization groups demonstrated that, in the context of a low-diversity, unstable microbiota, resistant strains not only successfully colonize but also significantly enhance the antibacterial responses

of the intestinal mucosa. This was evident both in the IgA production associated with specific immune responses and in defensin production, part of the non-specific immune mechanisms.

Unsurprisingly, the MDR-KP strain, isolated from clinical samples and carrying multiple plasmids, significantly increased fecal mBD3 levels, reaching an average of 1640 pg/g in this group. A similar effect was observed in the EC-CTXM group colonized with a transconjugant strain carrying the CTX-M-15 ESBL gene, where mBD3 levels were even higher, averaging 1825 pg/g. This value was significantly different not only from the baseline and control group but also from the EC-OXA group. These results suggest that the plasmid carrying the *bla*CTX-M-15 gene significantly enhances the bacteria's ability to induce mBD3 production (Figure 4).

In contrast, mBD3 levels in the EC-OXA group, colonized with a transconjugant strain carrying the OXA-162 carbapenemase, were comparable to baseline and control values, averaging 309 pg/g. A statistically significant difference was observed only when compared to the EC-CTXM group, highlighting that plasmids encoding different β-lactamases can exert opposing effects on mBD3 induction (Figure 4).

The plasmid-free E. coli strain, while increasing mBD3 levels compared to baseline, yielded values intermediate between the minimum and maximum levels measured in other groups, with an average of 898 pg/g. However, no statistically significant differences could be demonstrated for this group (Figure 4).

These findings collectively emphasize the influence of specific plasmids on modulating immune responses, particularly the ability to enhance mBD3 production, while underscoring the varied impacts of different β-lactamase-encoding plasmids on host immune mechanisms.

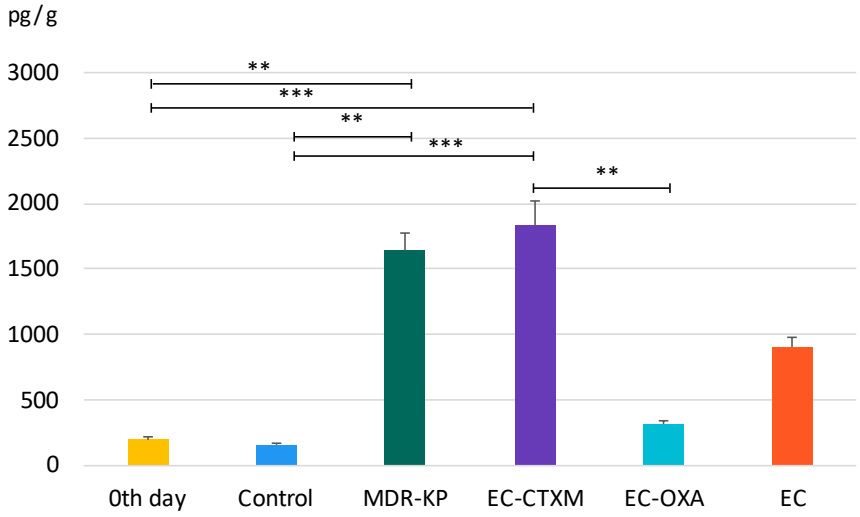

Figure 4. The β-defensin-3 level in feces of mice colonized by different bacteria MDR-KP, EC, EC-OXA, and EC-CTXM. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ (111).

4.5. Fecal α-defensin 5 level of the experimental groups

In the investigation of the antibacterial response to gastrointestinal colonization across different experimental groups, the level of murine α-defensin 5 (mAD5) was measured in addition to total IgA and mBD3 concentrations in fecal samples. As in previous analyses, baseline values from day 0 and the average values from the non-colonized control group were determined as reference points for assessing changes induced by colonization. The baseline mAD5 level on day 0 was 12.6 pg/g on average, and in the control group, it showed no significant change by day 10, with an average of 13.8 pg/g (Figure 5).

In contrast to previous findings, the most significant increase, both in magnitude and statistical significance, was observed in the EC group colonized with the plasmid-free, low-virulence J53 laboratory *E. coli* strain. In this group, the average mAD5 level reached 172 pg/g in fecal samples. A slightly smaller but still statistically significant increase was observed in the MDR-KP group, where the average mAD5 level was 125 pg/g (Figure 5).

In the groups colonized with transconjugant strains containing single plasmids from the donor MDR-KP strain, mAD5 levels increased modestly but did not reach statistical significance. Specifically, the average mAD5 concentration was 104 pg/g in the EC-

CTXM group and only 50 pg/g in the EC-OXA group. These findings suggest that resistance gene-containing plasmids effectively suppress the induction of mAD5 production, an antibacterial response. This suppression was particularly pronounced in the EC-OXA group (Figure 5).

Although the MDR-KP strain contains both plasmids, the results indicate that their effects manifest only in conjunction with other factors not examined in this study. While there is a noticeable reduction in mAD5 induction potential, it is not statistically significant. It is plausible that additional genes within the bacterial genome or other plasmids contribute to enhancing mAD5 production.

Overall, these findings suggest that the IncFIIK and IncL plasmids carrying β-lactamase genes may, to some extent, suppress or inhibit the host antibacterial response. This suppression likely facilitates the adhesion and persistence of resistant strains on the intestinal mucosa, supporting their colonization.

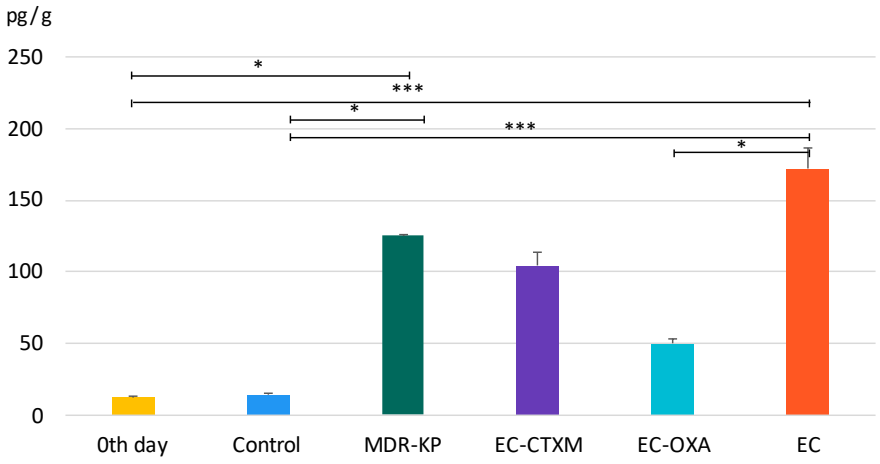

Figure 5. The alfa-defensin-5 in feces of mice colonized by different bacteria MDR-KP, EC, EC-OXA, and EC-CTXM. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$ (111).

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4.6. Alterations in the fecal microbiota composition in the experimental groups

During our experiments, we investigated changes in the microbiota following colonization. Fecal samples were collected on the 14th day after the introduction of various bacterial strains. This interval allowed sufficient time for both the colonizing strains to establish themselves and for the stabilization of changes in the microbiota composition. Based on previous findings, we observed that a consistently high bacterial load was achieved across all colonization groups. Additionally, a control group was established, which received only oral ampicillin treatment (similar to all groups) but was not colonized with any bacterial strain.

To characterize the microbiota in the fecal samples, α diversity indices were calculated from 16S metagenomic analysis data. The results indicated no statistically significant differences in CHAO1 and Simpson diversity indices among the different colonization groups. However, it is noteworthy that the observed values were relatively low, indicating a less diverse, species-poor microbial community across all groups. This is likely attributable to the previously mentioned antibiotic administration, which, while facilitating successful colonization, may have obscured potential differences in diversity (Figure 6 and 7).

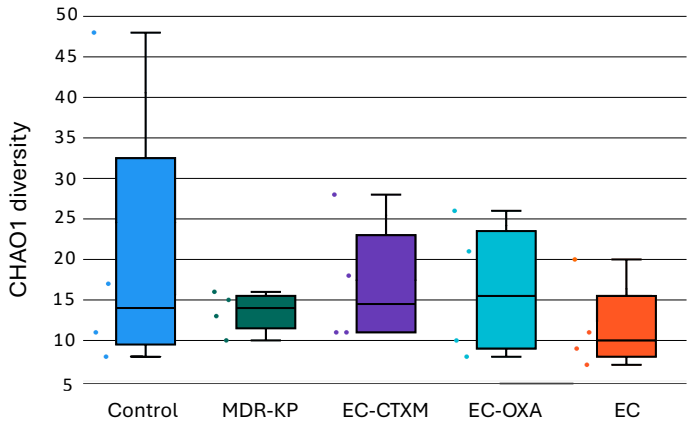

Figure 6. Chao1 α-diversity of fecal samples in the different groups (Control, MDR-KP, EC, EC-CTXM, and EC-OXA). Box plots show the distribution of diversities in each group (111).

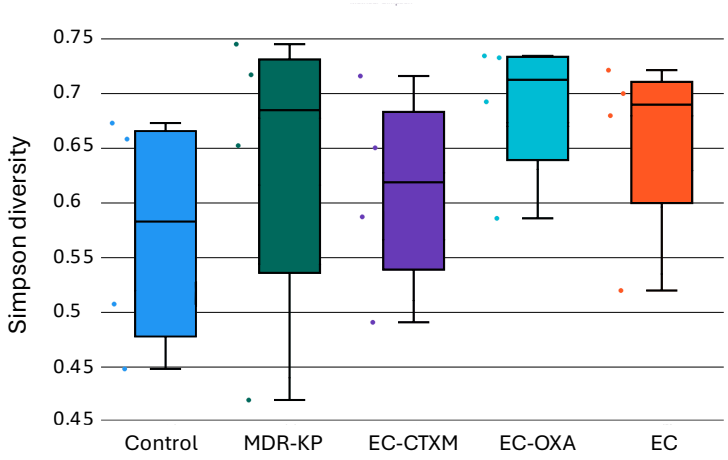

Figure 7. Simpson α-diversity of fecal samples in the different groups (Control, MDR-KP, EC, EC-CTXM, and EC-OXA). Box plots show the distribution of diversities in each group (111).

From another perspective, this suggests that the differences between colonization groups are more likely attributed to the intrinsic properties of the colonizing strains and minor differences in the microbiota composition rather than diversity changes induced by them. Although diversity indices effectively characterize community richness and the distribution of species abundances from various perspectives, they provide no information about the specific composition of the communities. Therefore, to further investigate this aspect, we compared the actual compositions of the groups at different taxonomic levels. Initially, we analyzed the proportions of the most frequently occurring phyla in the samples.

In all experimental groups, the phylum *Proteobacteria* was among the most abundant groups, together with the *Bacteroidota* across the MDR-KP, EC-CTXM, and EC-OXA-162 groups. Whereas the *Firmicutes* phylum was markedly dominant in the EC group (Figure 8). Due to the low diversity, individual phyla were predominantly represented at the family level by only a few taxa. For instance, the *Bacteroidota* phylum was primarily represented by members of the *Muribaculaceae* family in experimental groups colonized by resistant bacterial strains. In contrast, the *Lachnospiraceae* family was prevalent in the EC group and, interestingly, also in the EC-OXA group. At the family level *Muribaculaceae* was significantly enriched ($p < 0.05$) only in the EC-OXA-162 group compared to the EC group, suggesting a positive correlation between the abundance of

Muribaculaceae and the presence of the OXA-162 plasmid. Conversely, the *Lachnospiraceae* family was predominant in the EC group, indicating a potential protective role of *Lachnospiraceae* against colonization by high-risk *Klebsiella* clones and the dissemination of CTX-M15- containing resistance plasmids (Figure 9).

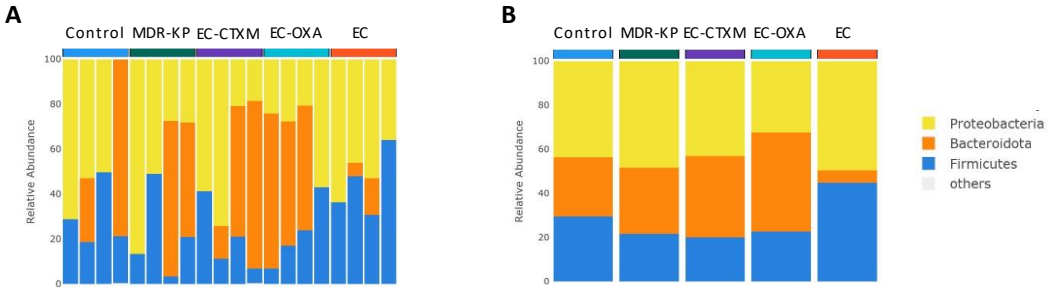

Figure 8. (A) Relative abundances of abundant taxonomic phylum in each mouse. Elements are shown if they have at least 2% relative abundance in at least one of the averaged samples. (B) Average values of relative abundances at the phylum level were calculated for samples from the same treatment groups. Elements are shown if they have at least 2% relative abundance in at least one of the averaged samples (111).

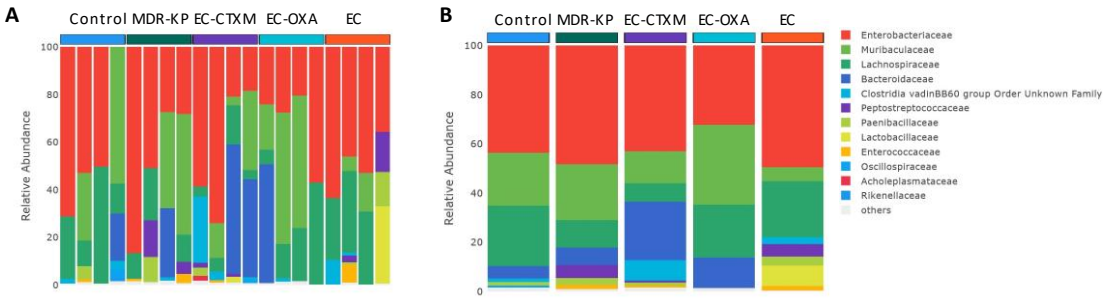

Figure 9. (A) Relative abundances of most abundant taxonomic families in each mouse. Elements are shown if they have at least 2% relative abundance. (B) Average values of relative abundances at the family level were calculated for samples from the same treatment groups. Elements are shown if they have at least 2% relative abundance in at least one of the averaged samples (111).

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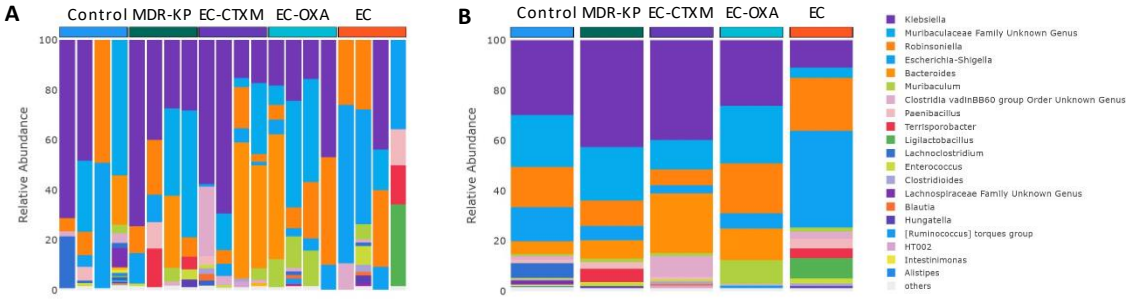

Figure 10. (A) Relative abundances of most abundant taxonomic genera in each mouse. Elements are shown if they have at least 2% relative abundance. (B) Average values of relative abundances at the genus level were calculated for samples from the same treatment groups. Elements are shown if they have at least 2% relative abundance in at least one of the averaged samples (111).

The composition of the microbiota was also analyzed at the genus level, where, consistent with the previously discussed families, only a few representatives were found in higher proportions. These included the *Klebsiella* and *Escherichia* genera from the *Enterobacteriaceae* family, an unidentified genus and the *Muriaculum* genus from the *Muribaculaceae* family, and the *Robinsoniella* genus representing the Lachnospiraceae family (Figure 10). These findings further demonstrate that our experimental model provided a low diversity, dysbiotic microbiota environment suitable for studying the colonization of various strains. The *Muribaculaceae* family exhibited a positive correlation with the high-risk *Klebsiella* clone and resistance plasmids containing CTX-M15 and OXA-162 (Figure 11). In contrast, The abundance of the *Lachnospiraceae* family demonstrated an inverse relationship with gastrointestinal colonization by the MDR-KP strain and the presence of resistance plasmid in the EC-CTXM (Figure 12).

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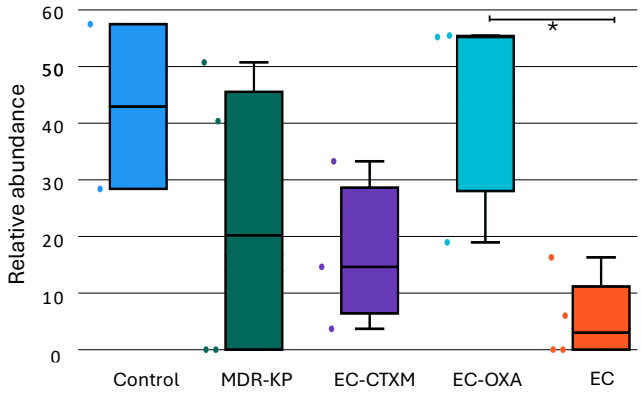

Figure 11. The relative abundance of *Muribaculaceae* family in each group (Control, MDR-KP, EC, EC-CTXM, and EC-OXA). Statistical difference is marked with * $p < 0.05$ (111).

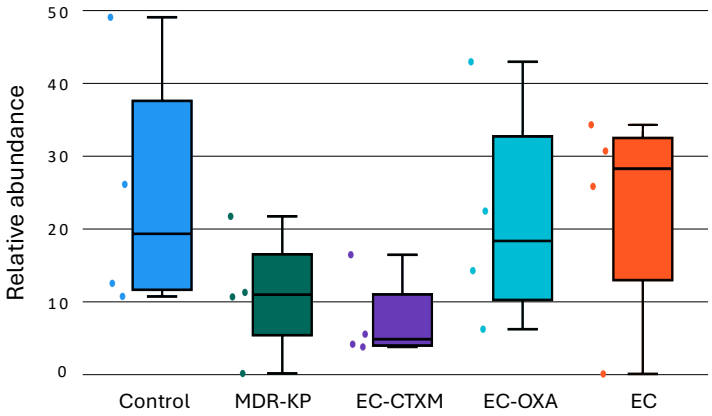

Figure 12. The relative abundance of *Lachnospiraceae* family in each group (Control, MDR-KP, EC, EC-CTXM, and EC-OXA) (111).

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5. Discussion

Gastrointestinal colonization by MDR strains of *Enterobacterales* has garnered significant global attention due to the potential for these strains to disseminate rapidly among healthy individuals and hospitalized patients (64). Among those strains, *K. pneumoniae* represents a significant and alarming etiological agent of hospital-acquired infections, particularly within ICUs (81, 82). The emergence and global dissemination of carbapenem-resistant *K. pneumoniae* (CRKP) has elevated this pathogen to a critical public health concern, prompting its classification by the WHO as a priority 1, critical pathogen due to its association with elevated mortality rates and severely restricted treatment options (116-118). The *K. pneumoniae* donor strain used in our experiments, along with the generated transconjugant strains, serve as suitable models for studying colonization with multidrug-resistant (MDR) bacteria due to their multiple resistance mechanisms, particularly the production of ESBL (CTX-M-15 and SHV-28) and carbapenemase (OXA-1 and OXA-48) enzymes. Successful conjugation also enabled the investigation of the significance of horizontal gene transfer (HGT).

The gut provides a favorable environment for bacterial proliferation, characterized by high microbial density, optimal temperature, and abundant nutrients, which facilitate HGT. This process accelerates the dissemination of resistance genes within the intestinal microbiota, promoting the evolution of MDR strains (119). Numerous studies have identified risk factors associated with colonization by ESBL- and carbapenemase-producing *Enterobacterales*, including prior antibiotic exposure, previous hospitalizations, intensive care unit treatment, and international travel (120-131).

Notably, patients harboring CRKP in their intestinal tract at the time of hospital admission may serve as reservoirs for transmission (132). Furthermore, gastrointestinal colonization with multidrug-resistant *K. pneumoniae* has been linked to an increased risk of subsequent infections and heightened mortality (132, 133). Colonization with carbapenem-resistant strains has been identified as a key risk factor for the development of extraintestinal infections caused by these pathogens. Consequently, identifying patients colonized with CRKP at an early stage is pivotal for implementing targeted infection control measures, thereby reducing the likelihood of progression to active infection and improving patient outcomes (134).

To address this issue, several countries have implemented screening programs for intestinal colonization in healthcare settings, particularly for high-risk groups such as neonates and patients transferred between facilities. Notably, these programs have also been extended to travelers returning from regions with high MDR prevalence (120-125). Efforts to develop decolonization strategies are driven by the recognition that prior gastrointestinal colonization with MDR *Enterobacterales* increases the risk of systemic and community-acquired infections. Additionally, these strains contribute to hospital outbreaks (135). Our animal study has revealed that the colonization of the gut by MDR strains, including *K. pneumoniae* and *E. coli* harboring resistance plasmids such as *bla*CTX-M-15 (IncFII[K]) and *bla*OXA-162 (IncL), results in significant increases in fecal bacterial counts. Interestingly, *E. coli* lacking plasmids exhibited limited colonization capacity, suggesting that the presence of specific plasmids alters colonization dynamics. For example, *bla*CTX-M-15 and *bla*OXA-162 plasmids enhanced the colonization properties of previously sensitive strains. Emerging evidence underscores the interplay between gut microbiota composition and colonization by ESBL-producing *E. coli*. Metagenomic analyses have highlighted shifts in microbiome composition associated with travel-related diarrhea but not with ESBL acquisition (136). Studies examining diversity and relative abundance parameters found no significant differences between individuals colonized or not colonized with ESBL-producing *E. coli* (137, 138). Consistent with these findings, our study observed no changes in α diversity between control groups and those colonized with ESBL- or carbapenemase-producing strains (139-141). However, the dominance of specific bacterial taxa, such as *Bacteroidota*, correlated with MDR features, particularly during colonization by plasmid-carrying *K. pneumoniae* or *E. coli*. Dysbiosis marked by reductions in *Muribaculaceae*, *Rikenellaceae,* and *Lachnospiraceae* was also observed in animal models (139). Our analysis did not reveal a statistically significant difference in the overall abundance of the *Lachnospiraceae* and *Muribaculaceae* families across the studied groups. Nevertheless, notable trends emerged: a significant increase in *Muribaculaceae* abundance was observed in groups colonized EC-OXA strains with plasmid encoded OXA-162 carbapenemase. While a modest difference in Lachnospiraceae levels was detected within the EC and EC-OXA groups. The presence of OXA-162 plasmids, whether in *K.*

pneumoniae or *E. coli* strains, appeared to influence gut microbiota composition, with a pronounced dominance of *Muribaculaceae* strongly associated with plasmid carriage.

The *Muribaculaceae* family is known for its specialization in the fermentation of complex polysaccharides, a metabolic capability that may confer a selective advantage in the gut ecosystem under certain conditions. Genomic analyses further indicate that propionate production, an important short-chain fatty acid, is a widespread metabolic feature within this family. These findings suggest a possible link between the plasmid-driven colonization dynamics and shifts in microbial functional profiles, warranting further investigation into the mechanistic interplay between plasmid presence and microbial community structure. (142, 143).

While considerable attention has been given to the microbiome composition during gastrointestinal colonization by multidrug-resistant (MDR) strains of *K. pneumoniae* and *E. coli*, other mechanisms underlying this process remain poorly understood in both human and animal studies. Notably, the roles of immunoglobulin A (IgA) and defensins in the gastrointestinal tract (GIT) of mice colonized with MDR clones, particularly their influence on long-term colonization dynamics, require further exploration. To elucidate these mechanisms, we quantified IgA and β-defensin levels in mouse fecal samples. IgA, a key mucosal immune component, binds to both commensal bacteria and pathobionts such as *Klebsiella*, thereby limiting their growth and preventing their penetration of the mucus barrier. This protective interaction underscores the importance of IgA in maintaining gut homeostasis and modulating colonization by MDR pathogens. By characterizing the interplay between host immune factors and microbial colonization, this study aims to provide deeper insights into the immune responses that govern the persistence and dissemination of MDR strains within the GIT (143, 144). *Klebsiella* has been shown to actively stimulate IgA production in the gut (92). Similarly, persistent colonization by antibiotic-resistant *E. coli* induces luminal IgA secretion, a response not observed with commensal *E. coli* strains, highlighting the host immune system's selective response to pathogenic or resistant bacteria. (143, 144). Our findings indicate that host immunity selectively recognizes *E. coli* carrying specific resistance plasmids, prompting the development of strain-specific IgA. This IgA response plays a critical role in preventing resistant *E. coli* from accessing the intestinal epithelium. Similarly, *K. pneumoniae* elicits a targeted IgA response, effectively modulating its colonization levels

in the gut. (102). The pathogenicity of *Klebsiella* is closely influenced by the composition of the gut microbiota, with dysbiosis creating conditions that favor *Klebsiella* overgrowth and promote inflammatory responses. (102, 139). Our findings demonstrate that resistance plasmids, specifically the IncFII(K) plasmid carrying *bla*CTX-M-15 and the IncL plasmid with *bla*OXA-162, play a pivotal role in enhancing multidrug-resistant (MDR) colonization within the gastrointestinal tract. Colonization by *K. pneumoniae* was observed to induce the production of human β-defensins, including β-defensin 2 and β-defensin 3, antimicrobial peptides that help regulate gut microbiota composition (145-147). However, the abundance of specific microbiota such as *Klebsiella* did not vary significantly based on β-defensin levels alone, suggesting that while β-defensins are induced by *Klebsiella*, their levels are not solely determinative of *Klebsiella* abundance (146, 147). Additionally, experimental alterations in the gut microbiota composition were shown to influence β-defensin 3 secretion, underscoring the complex interplay between microbiome dynamics, *Klebsiella* colonization, and host antimicrobial peptide production (115). Our data also indicate that *bla*CTX-M-15 is a critical factor influencing the activity of the IncFII(K) plasmid in β-defensin 3 production. *E. coli* plays a similarly significant role in defensin regulation, with distinct strains eliciting variable effects. For instance, the probiotic *E. coli* Nissle 1917 promotes β-defensin 2 production, whereas other strains differentially affect gut microbiota and defensin levels (148). Based on our results, the presence of the *bla*CTX-M-15-containing IncFII(K) plasmid was linked to increased β-defensin 3 production. Interestingly, α-defensin 5 levels were highest in colonization by non-pathogenic *E. coli*, but reduced during colonization with plasmid-carrying strains, suggesting nuanced interactions between defensins and colonizing strains.

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6. Conclusions

Our study utilized a murine model to investigate the multifactorial nature of intestinal colonization by the MDR strains. CTX-M-15- and OXA-162-producing *Klebsiella pneumoniae* ST15 high-risk clone served as good model for this. The findings indicate that colonization within the gastrointestinal tract is not solely attributable to the high-risk MDR clone itself but is also significantly influenced by the presence of resistance plasmids, specifically the IncFII(K) and IncL plasmids. These plasmids play a critical role in modulating colonization efficiency and persistence.

Additionally, several host and microbial factors contribute to the colonization dynamics of this MDR strain. The levels of IgA and antimicrobial peptides, such as mBD3 and mAD5, were found to influence colonization, highlighting the interplay between host immune responses and pathogen persistence. Furthermore, the composition of the intestinal microbiota emerged as another key determinant, underscoring the complex interactions between microbial community structure and the ability of MDR *K. pneumoniae* to establish and maintain colonization. These findings collectively emphasize the intricate and multifaceted mechanisms underlying the colonization of high-risk MDR clones in the GIT.

This study is, to our knowledge, the first to comprehensively evaluate gut microbiome dynamics alongside IgA production and defensin levels during colonization by an MDR *K. pneumoniae* high-risk clone. Our results underscore the critical roles of IgA and β-defensin 3 in mediating colonization and plasmid dissemination. These findings highlight that plasmids carrying resistance genes contribute significantly to the spread of high-risk clones worldwide, with implications extending beyond antimicrobial resistance.

As new scientific findings, our studies demonstrated resistance plasmids, IncFII(K) encoding *bla*CTX-M-15 gene and IncL plasmids encoding *bla*OXA-162 can alter not only the colonization dynamics of host bacterial strain after conjugation, but also cause alteration in the induction of antimicrobial responses. Both plasmids were potent activator of the specific immunity. However, the IncFII(K) plasmid induces mBD3 and in less extent mAD5 production likely to the host MDR-KP strain, the IncL plasmid had a strong inhibitory effect on both, mBD3 and mAD5 production.

Further research is needed to elucidate the direct effects of defensins on resistance plasmids or their indirect effects through microbiota modulation. Future studies should also investigate other MDR high-risk clones of *K. pneumoniae* and *E. coli* harboring diverse resistance plasmids and genes in colonization models to expand our understanding of these mechanisms.

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7. Summary

Antimicrobial resistance (AMR) poses a critical global health challenge. Horizontal gene transfer (HGT) plays a key role in spreading resistance genes, such as those encoding β-lactamases. Multidrug resistant (MDR) bacteria can colonize asymptomatically the gastrointestinal tract, which is a multifactorial process influenced by host factors too. This study investigated the colonization dynamics of MDR *K. pneumoniae*, focusing on plasmid-mediated resistance and the host immune response.

The research utilized MDR *K. pneumoniae* ST15 harboring several plasmids including an IncFII(K) encoding *blaCTX-M-15* and an IncL encoding *blaOXA-162* (MDR-KP), its transconjugant *E. coli* strains harboring only one of the mentioned plasmids (EC-CTXM, EC-OXA) and a plasmid free *E. coli* strain (EC). Their resistance to antibiotics were investigated using broth microdilution. In murine model, following gastrointestinal colonization of mice fecal germ count, total IgA, murine β-defensin 3 (mBD3), murine α-defensin 5 (mAD5) were measured and 16S metagenomic analysis was carried out.

Colonization experiments revealed that resistance plasmid-carrying strains colonized the gastrointestinal tract more effectively than non-resistant strains, triggering stronger immune responses. The MDR group showed the highest bacterial colonization accompanied by elevated fecal IgA and defensin levels.

Carriage of plasmids both colonization efficiency and antimicrobial responses. MDR-KP and EC-CTXM strains strongly induced mBD3 and IgA production, while EC-OXA strain suppressed both mBD3 and mAD5, however IgA induction was strong. The non-resistant EC strain has a limited colonizing capacity and induced only mAD5. Microbiota composition analysis revealed dominance of *Bacteroidota* in MDR-associated groups and *Firmicutes* in non-resistant EC strain, with specific families like *Muribaculaceae* and *Lachnospiraceae* correlating with plasmid presence and colonization outcomes.

These findings highlight the dual role of high-risk MDR clones and resistance plasmids in gut colonization and immune modulation, demonstrating that resistance plasmids not only enhance bacterial survival but also shape immune responses. The results provide novel insights into the interplay between plasmids, host immunity, and microbiota, emphasizing the need for targeted strategies to combat AMR.

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8. Összefoglalás

Az antimikrobiális rezisztencia (AMR) kritikus globális egészségügyi kihívást jelent. A horizontális géntranszfer kulcsszerepet játszik a rezisztencia, például a β-laktamázokat kódoló gének terjedésében. A multirezisztens (MDR) baktériumok tünetmentesen kolonizálhatják a gastrointestinális traktust. A disszertáció a MDR K. pneumoniae kolonizációs dinamikáját vizsgálja, különös tekintettel a plazmid-mediált rezisztenciagének és a gazdaszervezet immunreakciójának szerepére.

A kutatás során MDR K. pneumoniae ST15 törzset (MDR-KP) használtunk, amely több plazmidot hordozott. Többek között egy IncFII(K) típusút, amely blaCTX-M-15 ESBL β-laktamázt kódolt, valamint egy IncL típusút, mely blaOXA-162 karbapenemázt kódolt. Konjugációs akceptorként plazmidmentes E. coli J53 törzset (EC) alkalmaztunk. A létrehozott transzkonjugáns E. coli törzsek közül mindegyik csak az említett plazmidok egyikét hordozta (EC-CTXM, EC-OXA). Egérmodellben a gastrointestinális kolonizációt követően az egerek székletéből csíraszámot, az össz. IgA szintet, a murine β-defenzin 3 (mBD3) és murine α-defenzin 5 (mAD5) mennyiségét határoztuk meg, és 16S metagenomikai elemzést végeztünk.

A kolonizációs kísérletek azt mutatták, hogy a rezisztens törzsek (MDR-KP, EC-CTXM, RC-OXA) esetében a plazmidok jelenléte növelte mind a kolonizáció hatékonyságát, mind az antimikrobiális válaszreakciókat. Az MDR-KP és az EC-CTXM törzsek erőteljesen indukálták az mBD3 és az IgA termelését, míg az EC-OXA törzs elnyomta az mBD3-at és az mAD5-öt, ugyanakkor erős IgA-indukciót váltott ki. A nem rezisztens EC törzs korlátozott kolonizációs képességet mutatott, és csak az mAD5-öt indukálta. A mikrobióta összetételének elemzése kimutatta a Bacteroidota dominanciáját a MDR-hez kapcsolódó csoportokban és a Firmicutes dominanciáját a nem rezisztens EC törzsnél. Továbbá családok szintjén a Muribaculaceae és a Lachnospiraceae aránya korrelált a plazmidok jelenlétével és a kolonizációs eredményekkel.

Eredményeink rávilágítanak a magas kockázatú MDR-klónok és plazmidjaik kettős szerepére a gastronintestinális kolonizációban és az immunmodulációban. Ezzel új betekintést nyújtanak a plazmidok, a gazdaszervezet immunitása és a mikrobióta közötti kölcsönhatásokba, hangsúlyozva a célzott stratégiák szükségességét az AMR elleni küzdelemben.

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10. Bibliography of the candidate's publications

10.1. Publications related to the PhD thesis

1. Stercz B, Domokos J, Dunai ZA, Makra N, Juhasz J, Ostorhazi E, Kocsis B, Szabo D. The roles of a multidrug-resistant *Klebsiella pneumoniae* high-risk clone and its resistance plasmids on the gastrointestinal colonization and host-defense effectors in the gut. Antibiotics (Basel). 2024;13:698.
   Impact factor (expected):4.3
   Scopus – Pharmacology, Toxicology and Pharmaceutics (miscellaneous) SJR indicator: D1
2. Stercz B*, Farkas FB*, Tóth Á, Gajdács M, Domokos J, Horváth V, Ostorházi E, Makra N, Kocsis B, Juhász J, Ligeti B, Pongor S, Szabó D. The influence of antibiotics on transitory resistome during gut colonization with CTX-M-15 and OXA-162 producing *Klebsiella pneumoniae* ST15. Sci Rep. 2021;11:6335.
   Impact factor: 4.997 *Authors contributed equally
   Scopus - Multidisciplinary SJR indicator: D1

10.2. Publications not related to the PhD thesis

1. Kelemen J, Sztermen M, Dakos EK, Budai J, Katona J, Szekeressy Zs, Sipos L, Papp Z, Stercz B, Dunai ZsA, Kocsis B, Juhasz J, Michelisz F, Daku Zs, Domokos J, Szabó D. Complex Infection-Control Measures with Disinfectant Switch Help the Successful Early Control of Carbapenem-Resistant Acinetobacter baumannii Outbreak in Intensive Care Unit. Antibiotics (Basel). 2024;13:869.
   Impact factor (expected): 4.3
2. Monyók Á, Mansour B, Vadnay I, Makra N, Dunai ZsA, Nemes-Nikodém É, Stercz B, Szabó D, Ostorházi E. Change in Tissue Microbiome and Related Human Beta Defensin Levels Induced by Antibiotic Use in Bladder Carcinoma. Int J Mol Sci. 2024;25:4562.
   Impact factor (expected): **4.9**

3. Szabo D, Ostorhazi E, Stercz B, Makra N, Penzes K, Kristof K, Antal I, Rethelyi JM, Zsigmond RI, Birtalan E, Merkely B, Tamas L. Specific nasopharyngeal Corynebacterium strains serve as gatekeepers against SARS-CoV-2 infection. Geroscience. 2023;45:2927-2938.

Impact factor: 5,3

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Impact factor: 4,7

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The influence of antibiotics on transitory resistome during gut colonization with CTX-M-15 and OXA-162 producing *Klebsiella pneumoniae* ST15

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Great efforts have been made to limit the transmission of carbapenemase-producing *Enterobacteriaceae* (CPE), however, the intestinal reservoir of these strains and its modulation by various antibiotics remain largely unexplored. Our aim was to assess the effects of antibiotic administration (ampicillin, ceftazidime, ciprofloxacin) on the establishment and elimination of intestinal colonization with a CTX-M-15 ESBL and OXA-162 carbapenemase producing *Klebsiella pneumoniae* ST15 (KP5825) in a murine (C57BL/6 male mice) model. Whole genome sequencing of KP5825 strain was performed on an Illumina MiSeq platform. Conjugation assays were carried out by broth mating method. In colonization experiments, 5×10^6 CFU of KP5825 was administered to the animals by orogastric gavage, and antibiotics were administered in their drinking water for two weeks and were changed every day. The gut colonization rates with KP5825 were assessed by cultivation and qPCR. In each of the stool samples, the gene copy number of $bla_{OXA-162}$ and $bla_{CTX-M-15}$ were determined by qPCR. Antibiotic concentrations in the stool were determined by high pressure liquid chromatography and a bioanalytical method. The KP5825 contained four different plasmid replicon types, namely IncFII(K), IncL, IncFIB and ColpVC. IncL (containing the $bla_{OXA-162}$ resistance gene within a Tn1991.2 genetic element) and IncFII(K) (containing the $bla_{CTX-M-15}$ resistance gene) plasmids were successfully conjugated. During ampicillin and ceftazidime treatments, colonization rate of KP5825 increased, while, ciprofloxacin treatments in both concentrations (0.1 g/L and 0.5 g/L) led to significantly decreased colonization rates. The gene copy number $bla_{OXA-162}$ correlated with *K. pneumoniae* in vivo, while a major elevation was observed in the copy number of $bla_{CTX-M-15}$ from the first day to the fifteenth day in the 0.5 g/L dose ceftazidime treatment group. Our results demonstrate that commonly used antibiotics may have diverse impacts on the colonization rates of intestinally-carried CPE, in addition to affecting the gene copy number of their resistance genes, thus facilitating their stable persistance and dissemination.

Abbreviations

BSL	Biosafety-level
CFU	Colony-forming unit

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CPE	Carbapenemase-producing *Enterobacteriaceae*
CRKP	Carbapenem-resistant *Klebisella pneumoniae*
ESBL	Extended-spectrum β-lactamase
HGT	Horizontal gene transfer
HPLC	High-performance liquid chromatography
KP5825	*K. pneumoniae* Strain no. 5825
LoD	Limit of detection
LB	Luria -Bertani
MIC	Minimum inhibitory concentration
MDR	Multidrug resistant
NA	Not available
ST	Sequence type

Multidrug-resistant (MDR) Gram-negative bacteria have emerged as a major public health threat. Extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* have disseminated worldwide and have become a serious concern for clinicians, due to limited therapeutic options, both in community-acquired and nosocomial infections[1]. In the last decade, CTX-M-type ESBLs have replaced TEM- and SHV-types among clinical *Enterobacteriaceae* isolates[2]. The explosive dissemination of CTX-M-type β-lactamases around the world has been referred to as the "CTX-M pandemic", associated with their increasing description around the globe[3], and their prevalence rates may vary among different members of the *Enterobacteriaceae* family; nevertheless, they are most common in species, such as *Klebsiella pneumoniae* and *Escherichia coli*[4]. The increasing prevalence of infections caused by MDR Gram-negative bacteria (especially ESBL-producers) was accompanied with the rise in the use of carbapenems for the treatment of these infections[5]. Subsequently, this has further enhanced the emergence and dissemination of carbapenemase-producing *Enterobacteriaceae* (CPE). Although resistance rates to carbapenems remain low in some parts of Europe, the developments in southern and southeastern Europe (which were previously characterized by an unrestricted use of these life-saving drugs) is concerning[6].

CPE infections are associated with high morbidity and mortality, particularly in vulnerable patient populations, including infants, children and the elderly, hospitalized patients, immuncommpromised patients, as well as the critically ill. The major driving force in the uncontrolled dissemination of these strains is their ability to survive and spread rapidly in healthcare environments; in fact, carbapenemase-production is usually linked to successful MDR clones, commonly associated with nosocomial infections[7,8]. The carbapenemase genes in *Enterobacteriaceae* have been shown to be associated with mobile genetic elements including plasmids or transposons, allowing for the transfer among different members of the family. OXA-48-like carbapenemases are one of the most common carbapenemases (with increasing prevalence in Europe, although wide-ranging differences in their geographic distribution may be observed) in *Enterobacteriaceae*, and they are continuously being introduced into regions of non-endemicity, where they may be responsible for nosocomial outbreaks[6,9]. While *K. pneumoniae* is the main reservoir of $bla_{\text{OXA-48}}$, the number of studies reporting cases due to other $bla_{\text{OXA-48}}$ producing *Enterobacteriaceae* species is increasing worldwide[8,10,11].

Due to the high prevalence and pervasiveness of $bla_{\text{OXA-48}}$-like carbapenemases in community-associated and nosocomial Gram-negative bacteria, limiting the additional spread of pathogens producing these enzymes is a difficult task[11,12]. $Bla_{\text{OXA-48-like}}$/$bla_{\text{OXA-48}}$ carbapenemases are found on plasmids that have a high propensity to disseminate among various bacterial species via horizontal gene transfer (HGT)[9]. It is not uncommon to detect different bacteria containing identical plasmids harboring $bla_{\text{OXA-48}}$, obtained from the same patient, both as colonizers or as causative agents of infections[13]. OXA-48 is associated with different *Tn1999* transposon variants and located mainly as the only antibiotic resistance gene on the conjugative IncL (IncL/M) replicon type plasmids[14,15]. The occurrence of pOXA-48a-like IncL plasmids were described in many Gram-negative bacteria, including *Citrobacter freundii, E. coli*, *Enterobacter cloacae*, *K. pneumoniae*, *K. oxytoca* and *Raoultella planticola*[16,17]. Some high-risk clones (e.g., ST11, ST15, ST101 and ST307 for *K. pneumoniae,* and ST38 and ST410 for *E. coli*) have been associated with the global dispersal of many OXA-type carbapenemases (OXA-48, OXA-181, OXA-232 and OXA-204)[13,18–20]. OXA-162—which is also a member of the OXA-48-like carbapenemases—has been observed in different gut bacteria, reported from Turkey, Germany, Greece and Hungary until now[10,21–23].

Enterobacteriaceae are inhabitants of human gut microbiota, and feacal carriers may represent an important reservoir for person-to-person transmission and dissemination of bacteria. Furthermore, gut colonization by MDR bacteria has been associated with a high risk of developing subsequent clinical infection associated with increased mortality[7,24]. Therefore, active surveillance is a key part in preventing the spread of such strains. Efforts to limit the transmission of carbapenemase-producing *K. pneumoniae* strains focus on basic and enhanced infection control measures, while the importance of the intestinal reservoir of these strains and its modulation by various antibiotics remain largely unexplored[25]. Administration of antibiotics is a known risk factor for the development of resistance, however its role in colonization is still unclear. In this study, our aim was to assess the effects of antibiotic administration on the establishment and elimination of intestinal colonization with a CTX-M-15 ESBL and OXA-162 carbapenemase co-producing *K. pneumoniae* in a murine model, followed by administration of ampicillin, ceftazidime or ciprofloxacin (Fig. 1).

Results

Based on the whole genome sequencing the KP5825 strain harboured $bla_{\text{CTX-M-15}}$ ESBL and the $bla_{\text{OXA-162}}$ carbapenemase, as well as other antibiotic resistance-determinants for β-lactam resistance ($bla_{\text{SHV-28}}$ and $bla_{\text{OXA-1}}$). The KP5825 isolate harboured several chromosomal nucleotid mutations resulted in GyrA amino acid alterations in position Ser83Phe, Asp87Ala and Asn645His and in ParC in position Ser80Ile and Pro402Ala, furthermore the

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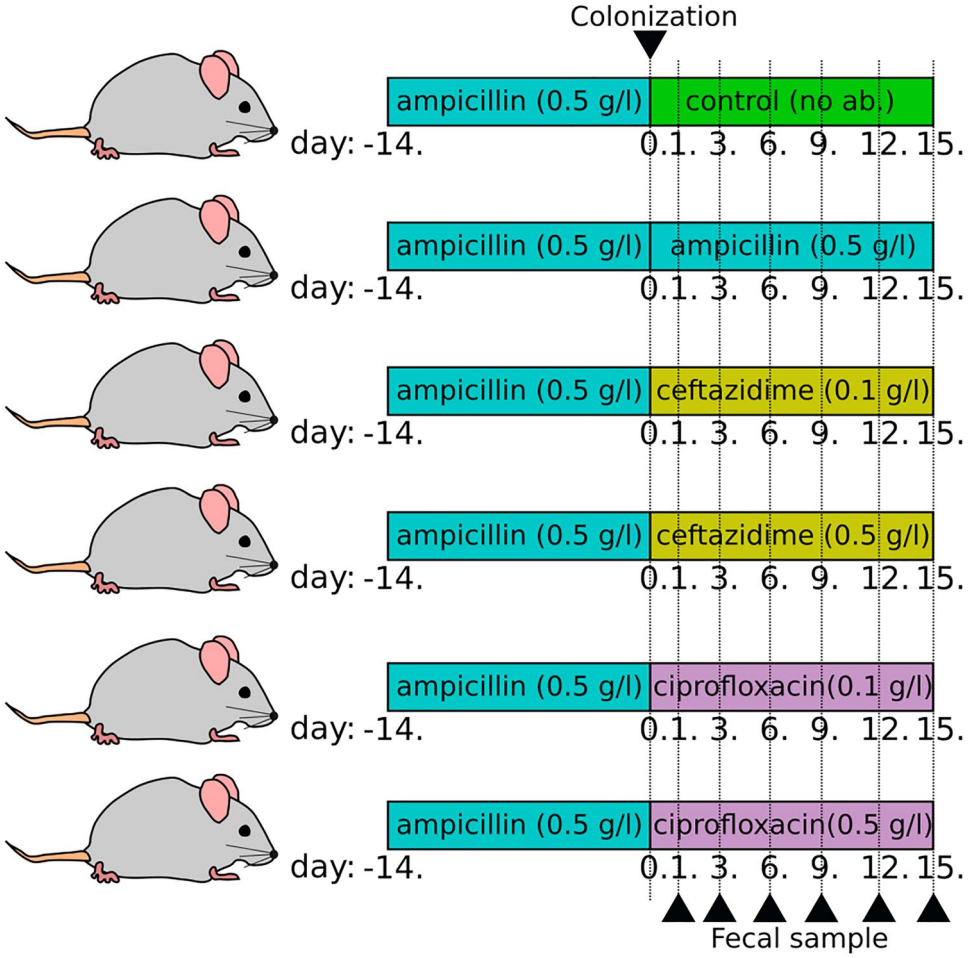

Figure 1. The colonization protocol used in our experiments.

isolate also carried the plasmid-borne *aac(6')-Ib-cr* fluoroquinolone resistance determinant. The isolate carried resistance-determinants for aminoglycoside resistance (*aac(3)-IIa, aph(3')-Ia* and *aac(6')Ib-cr* as well. In addition, four different plasmid replicon types, namely IncFII(K), IncL, IncFIB and ColpVC were detected in the KP5825. The IncL and IncFII plasmids were successfully conjugated, and the IncL plasmid contained only the $bla_{\text{OXA-162}}$ resistance gene within a *Tn1991.2* genetic element, while the IncF(II)K contained the $bla_{\text{CTX-M-15}}$ resistance gene.

The KP5825 showed high level resistance against the beta-lactam and fluoroquinolone antibiotics based on MIC values determined in the broth microdilution assay. The broth mating procedure-based in vitro conjugation assay performed was successful, and the conjugated *E. coli* J53 harbouring the pOXA-162 showed increased resistance in case of ertapenem and meropenem and in case of *E. coli* J53 harbouring pCTX-M-15, resistance to cephalosporins (ceftazidime, cefotaxime) was detected. The characteristics of KP5825 and the conjugated *E. coli* J53 strains are shown in Table 1.

In the colonization studies with 6–8 week-old C57BL/6 male mice the antibiotics were administered in their drinking water for two weeks. The concentration of antibiotics in mouse stool was assessed with high pressure liquid chromatography (HPLC) on the 1st and 15th day after KP5825 colonization. The ampicillin concentration in the stool samples in Amp_0.5 group on the first day was 720.2 ± 247.0 μg/g (average ± SD), while on the fifteenth day 739.3 ± 219.4 μg/g. The average ciprofloxacin concentration in the Cip_0.1 group was 17.2 ± 5.96 μg/g on the first day, and 20.7 ± 4.97 μg/g on the fifteenth day; and in Cip_0.5 group, it was 203.8 ± 46.0 μg/g on the first day and 244.8 ± 61.9 μg/g on fifteenth day. Ceftazidime was undetecable from mice stool samples.

The colonization was performed with the KP5825 strain administered by orogastric gavage and the colonization rate of mice with CTX-M-15 ESBL- and OXA-162 carbapenemase-producing KP5825 was quantified by both a conventional culture analysis method and the qPCR technique, in order to simultaneously determine the absolute and relative colonization rates with the tested isolate. The effect of different treatment regimens on colonization with KP5825 are shown in Fig. 1A,B. The densities of KP5825 detected in feces were assayed on the 3rd, 6th, 9th, 12th and 15th days. If KP5825 organisms were not detected in the stool, the lower limit of detection (~ 2.3 $\log_{10}$ CFU/g) was assigned. In case of all observation periods of ceftazidime treatments, the rate of KP5825 colonies were the highest, while on the other hand, during ciprofloxacin treatments they were the lowest (Fig. 2A). During ampicillin (Amp_0.5) and ceftazidime treatments (Caz_0.1 and Caz_0.5), the absolute

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	Strains			
	KP5825	***E. coli* J53**	***E. coli* J53 Transconjugant-5825/1 pCTX-M-15**	***E. coli* J53 Transconjugant-5825/2 pOXA-162**
Plasmid replicon types	IncF(I)B, IncF(II)K, ColpVC, IncL	NA	IncF(II)K	IncL
Beta-lactamases				
Chromosomal	*bla*SHV-28	NA	*bla*CTX-M-15	*bla*OXA-162
On mobile genetic elements	*bla*OXA-1, *bla*OXA-162, *bla*CTX-M-15			
Mobile genetic elements	*Class I, Tn1999.2,*	NA	*Class I*	*Tn1999.2*
Quinolone resistance determinants				
Chromosomal	*gyrA (S83F, D87A, N645H)*	NA	*aac(6')Ib-cr*	
	parC (S80I, P402A)			
On mobile genetic elements	*aac(6')Ib-cr*			
Other resistance genes	*aac(3)-IIa, aph(3')Ia*	NA		
MIC (mg/L)				
Ampicillin	> 32	4	> 32	> 32
Ceftazidime	> 32	0.25	> 32	0.25
Cefotaxime	> 32	0.125	> 32	0.25
Ertapenem	> 32	< 0.0625	< 0.0625	0.25
Imipenem	16	0.5	0.5	0.5
Meropenem	> 32	< 0.0625	< 0.0625	0.125
Ciprofloxacin	> 32	< 0.0625	< 0.0625	< 0.0625

Table 1. Features of KP5825 and the transconjugated *E. coli* J53 strains. MIC: minimum inhibitory concentrations; NA: not applicable.

colonization rate of the carbapenem-resistant KP5825 slightly increased. Upon treatment with ampicillin, a moderate increase of *K. pneumoniae* cell count was detected. In contrast, during ciprofloxacin treatments in both concentrations (Cip_0.1 and Cip_0.5) and in the control group, the colonization rates have decreased significantly. The most extensive decrease in colonization rate was observed in the group treated with the lower dose of ciprofloxacin (Cip_0.1). These alterations are the most unexpected as the present carbapenemase-producing *K. pneumoniae* shows high level resistance to fluoroquinolones. These results were consequent with qPCR results by observing the $\log_{10}$ fold change of *rpoB1* housekeeping gene designed for KP5825. The relative colonization rate of KP5825 between the first and fifteenth day of colonization showed also differences between antibiotic treatments. In Caz_0.1 group the colonization rate of carbapenem-resistant KP5825 slightly increased, while on the other hand treatment with ampicillin resulted in a moderate increase of KP5825. An extensive decrease in colonization rate was observed in the groups treated ciprofloxacin (Cip_0.1, Cip_0.5) (Fig. 2B).

The effect of antibiotic-treatment regiments on $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-162}}$ genes' copy number in the gut was determined by qPCR from each the stool sample and results were calculated as the fold change of gene normalized to the *rpoB1* reference gene and relative to the control mice. The relative copy number of the ESBL $bla_{\text{CTX-M-15}}$ and the carbapenemase $bla_{\text{OXA-162}}$ were determined and these results were correlated to the *rpoB1* housekeeping gene of KP5825 on the first and on the fifteenth days from the feces of each mouse used in the experiment (Fig. 3). The relative copy number did not change for $bla_{\text{OXA-162}}$ during the observed period in any treatment group. In contrast, a major elevation was observed from the first day to the fifteenth day in the treatment group with the Caz_0.5 treatment yielding 2 and 400-fold absolute gene copy number increase of the $bla_{\text{CTX-M-15}}$ gene. At the same time, the relative copy number of the $bla_{\text{CTX-M-15}}$ gene (which was controlled with the rate of the *rpoB* gene) also increased significantly ($p < 0.05$) from 2- to 5-times relative to the control in the Caz_0.5 treatment group (Fig. 3). Nevertheless, only the original CTX-M-15 and OXA-162-producing *K. pneumoniae* isolate could be reisolated from various feaces samples from mice during the experiment using the selective CHROMagar plates. We could not isolate other ESBL or carbapenenamse-producing bacteria on the appropriate selective culture media, except the original KP5825.

Discussion

Klebsiella pneumoniae is a prevalent and dangerous cause of hospital-associated infections, especially in ICUs[24,25]. Because of their global spread, high mortality and very limited therapeutic options, carbapenem-resistant *K. pneumoniae* (CRKP) was declared a major public health threat, rated as priority 1, critical pathogen by the World Health Organization[26–28]. Patients with intestinal carriage of CRKP upon admission may act as reservoirs[29]; moreover, gastrointestinal colonization with MDR *K. pneumoniae* increases the risk of subsequent infections and mortality[29,30]. Colonization with a carbapenem-resistant *Klebsiella* has been highlighted as a hallmark of a subsequent extraintestinal infection by these pathogens; therefore, the identification of patients whom are positive for CRKP-colonization may be an important step to introduce infection control interventions and to save patients from developing an infection[31].

Our experiments aimed to investigate the effects of various antibiotic treatments on the gastrointestinal colonization, gene dynamics and role in the resistome of the high-risk clone *K. pneumoniae* ST15, producing the

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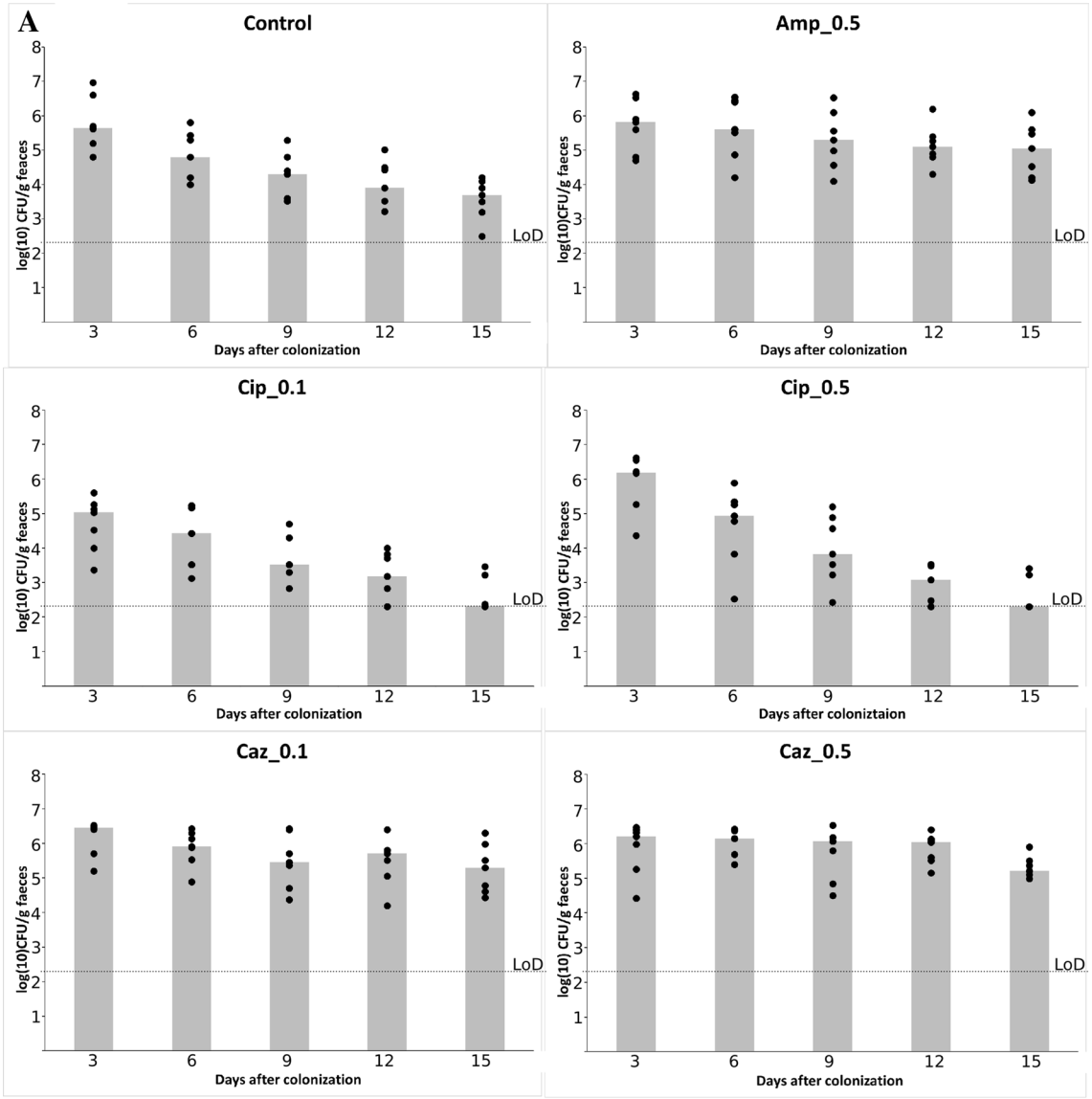

Figure 2. (**A**) Effect of various antibiotic administration on the establishment of intestinal colonization with KP5825 by orogastric gavage on day 0 (n = 7 mice per antibiotic treatment group). Densities of KP5825 are shown on 3rd, 6th, 9th, 12th and 15th days after colonization. The limit of detection (LoD) (~ 2.3 $\log_{10}$ CFU/g) was assigned. Colums represent median values. (**B**) Changes in the relative colonization rate by KP5825 in the antibiotic-treated groups and in the control group. The $\log_{10}$ fold change of *rpoB1* housekeeping gene show the relative colonization rate between the first and fifteenth day of the colonization with the different treatment.

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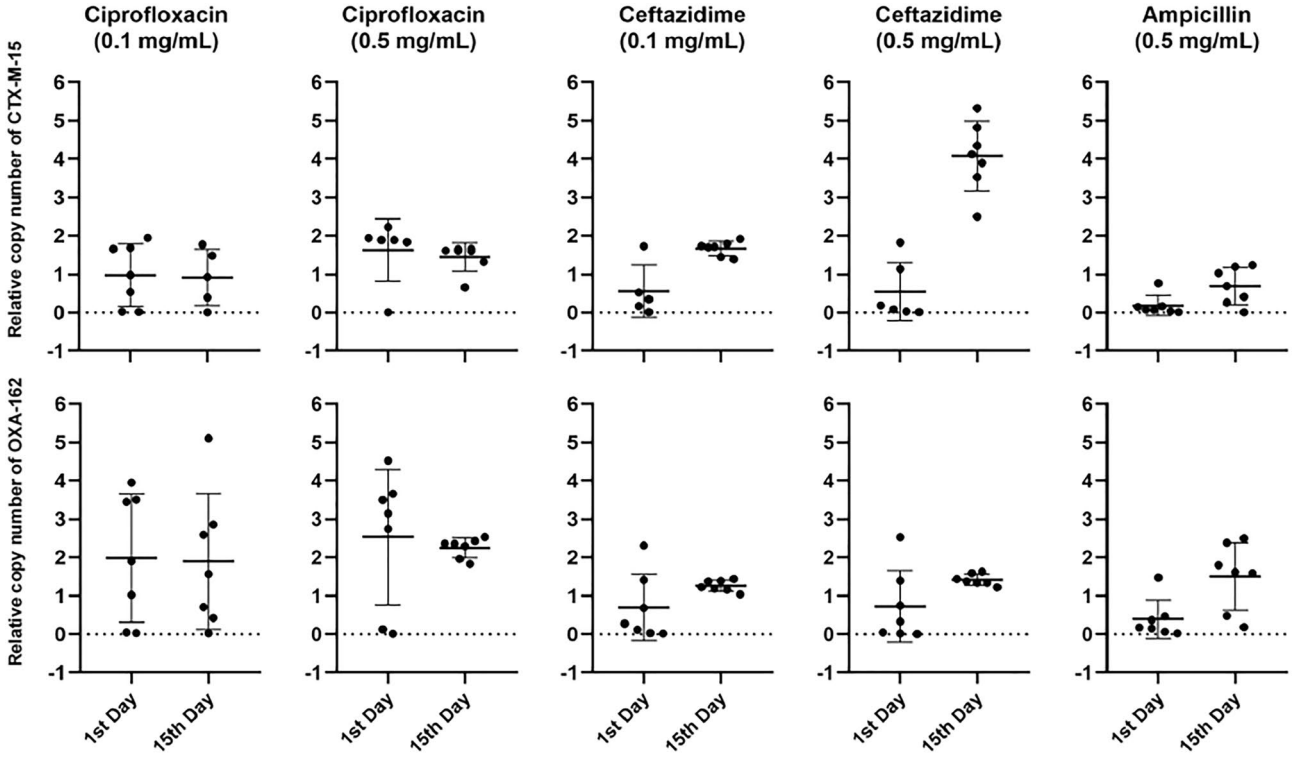

Figure 3. The relative copy number of the $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-162}}$ genes from the feces of individual mice after the different antibiotic-treatment regiments on the first and fifteenth days after colonization with KP5825.

CTX-M-15 and OXA-162 β-lactamases, focusing on the major problem of the emergence and spread of ESBL and carbepenemase genes. In case of OXA-162, the host plasmid IncL and in case of CTX-M-15, the host plasmid IncFII of the high risk clone *K. pneumoniae* play an important role in its international dissemination. In our experiment, both plasmids were shown to be conjugable. OXA-48-like enzymes itselves hydrolyze carbapenems to a lesser extent, as we also observed, however their co-occurrence with other β-lactam resistance mechanisms, such as membrane impermeability, may result in high-level carbapenem-resistance[9–11].

Three antibiotics were included in our study, namely ampicillin, ceftazidime (representatives of β-lactams) and ciprofloxacin. Ampicillin and its derivates (i.e. the aminopenicillins) and ciprofloxacin (a member of the fluoroquinolones) are still one the most widely used drugs in the community, therefore, the assessment of their effect on the gut resistome is of utmost importance[32]. Ceftazidime has been recently sidelined in therapy, due to its availability and the emergence of ESBLs worldwide. Nevertheless, the introduction of ceftazidime and avibactam, a novel cephalosporin/β-lactamase inhibitor combination into the clinical practice—especially for the treatment of OXA-48-type (Class D) carbapenemase producing MDR Gram-negative organisms has provided renewed relevance to this drug[33,34]. Results of our experiments have shown that the studied antibiotic treatment regiments affected the resistome of mice in different ways.

Previous antibiotic therapy is an independent risk factor for colonization with ESBLproducing *Enterobacteriaceae* as demonstrated several studies[35,36]. During our studies, ampicillin pre-treatment was used (for a duration of 14 days) to maintain and promote the colonization of KP5825 in all treatment groups, which was done to model the natural colonization of the host with the microorganism. The rationale behind this was that—based on literature findings—gastro-intestinal colonization with *K. pneumoniae* is difficult to establish in mice via gavage treatment and that antibiotic (ampicillin) pre-treatment has been noted to play a role in disrupting the microbiota of the desired host to allow for the colonization of *K. pneumoniae*[37,38].

There are controversial data regarding the effects of beta-lactam treatment on the gastrointestinal colonization with multi-drug reistant organisms. Several authors have noted that exposure to various β-lactam antibiotics allow for the colonization by ESBLs (an ST131 *E. coli* strain was used in the experiments), regardless of negatively affecting (clindamycin) the members of the Bacteroidales order or not (cefuroxime and dicloxacillin)[39]. Conversely, others reported that the treatment with cephalosporins at the ICU did not increase the acquisition rate of carbapenem-resistant *Enterobacteriaceae*[40]. In our study, as a consequence of the treatment with β-lactam antibiotics, both the colonization rate and—independently from this—the gene copy number of $bla_{\text{CTX-M-15}}$ both increased. Nevertheless, the copy number of $bla_{\text{OXA-162}}$ correlated with the colonization rate of KP5825. In the case of $bla_{\text{CTX-M-15}}$ located on IncF(II)K plasmid, a higher gene copy number was detected in mice stool samples after cephalosporin treatment, thus indicating a shift in resistome. The measurement of the replicon' copy number could have additionally provided valuable information on the underlying reason for the observed increase, however, this experiment was unfortunately not performed. Given that the CTX-15-producing transconjugant could not be isolated from stool samples, highlights that either recipient Enterobacteriales was not detectable

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(or culturable) in feces of mice or the copy number of $bla_{CTX\text{-}M\text{-}15}$ resistance genes was increased only in the host cells. Thus, it may also be assumed that the $bla_{CTX\text{-}M\text{-}15}$ gene may have been transferred to non-culturable bacteria. However, this does not change the fact that $bla_{CTX\text{-}M\text{-}15}$ gene was present in higher levels and the plasmid is capable of conjugation in present of susceptible recipient bacterium.

The fact that ciprofloxacin reduced the colonization rate in our experiments is particularly interesting, especially in light of the fact that the colonizing *K. pneumoniae* strain itself had high-level fluoroquinolone resistance as it had both chromosomal and plasmid-mediated quinolone resistance determinants. Regardless, our carbapenem-resistant *K. pneumoniae* isolate disappeared or its load has significantly decreased in the feces of ciprofloxacin treated mice. These findings support earlier studies where ciprofloxacin did not increase the abundance of antibiotic resistance genes-carrying plasmids and failed to promote colonization with MDR Gram-negative bacteria[37,41]. A potential explanation involves the limited antimicrobial effects of ciprofloxacin on the anaerobic intestinal microbiota[42].

Based on the results of our experiments, it may be assumed that the differences in the colonization effects of the tested antibiotics are mainly rooted in their structre-activity relationships and biological targets, rather then the doses in which they were applied (there were no difference between different doses of the same antibiotic). These results highlights the fact that the that timing of the antimicrobial adiministration relative to CPE exposure is also an important parameter to consider in providing ecological space for the implantation and expansion of the MDR strain.

Conclusions

In summary, our results have shown that in the presence of β-lactam antibiotics, the amount of the high-risk clone of *K. pneumoniae* showed an increase in the absolute and relative colonization rate, as well as gene copy number of $bla_{CTX\text{-}M\text{-}15}$ on the IncF(II) conjugative plasmid. In contrast, gene copy of $bla_{OXA\text{-}162}$—which was also conjugative in vitro on IncL plasmid—correlated with *K. pneumoniae* cell count in vivo. Increases in the degree of colonization in the presence of antibiotics has been described by previous studies, however, a clone-independent change in the copy number of $bla_{CTX\text{-}M\text{-}15}$ resistance genes in vivo has not been previously described. In contrast, a parallel decrease in both the clone and the resistance genes was observed after the treatment of fluoroquinolones. This has already been observed by others, but contrasting observations have also been published. Gastrointestinal colonization of MDR bacteria poses a serious clinical problem, both in community-based and nosocomial settings, and in our study we demonstrated a diverse influence of commonly administered antibiotics (ampicillin, ceftazidime, ciprofloxacin) on intestinally carried multidrug-resistant *K. pneumoniae*.

Methods

Bacterial strains. *K. pneumoniae* ST15 (KP5825) was obtained from National Public Health Centre (Budapest, Hungary)[23]. Azide-resistant *E. coli* J53 was used in the conjugation assays.

Antibacterial susceptibility testing. Antibacterial susceptibility testing was performed by the broth microdilution method according to the EUCAST guidelines v.9.0 (www.eucast.org)[43]. Incubation was performed at 35 °C for 16–20 h and minimum inhibitory concentrations (MICs) were determined visually. *E. coli* ATCC 25922 was used as control strain.

Conjugation assay. Conjugation assays were carried out by broth mating procedure in Lurian-Bertani (LB) broth (Sigma-Aldrich, USA) with the KP5825 isolate as donor and the *E. coli* J53 azide resistant strain as recipient[43]. Overnight cultures of donor and recipient strains grown in LB broth were added to 8 mL fresh LB broth at a donor-recipient ratio of 1:1 (300 µL of cultures each), and incubated for 4 h at 37 °C. The mixed cultures were centrifuged and the supernatant was removed in order to get rid of the antibiotics, to avoid the inhibitory effect against *E. coli* J53. The pellet was re-suspended in fresh culture and plated onto a LB-agar containing 100 µg/mL azide (Sigma-Aldrich) and 0.1 µg/mL of cefotaxime (Sigma-Aldrich) and/or 0.1 µg/mL of ertapenem (Sigma-Aldrich)[44]. Colonies growing on the selective agar plates and again on subculture agar were subjected to confirmatory tests of ESBLs and carbapenemase by CTX-M Multi and Carba 5 immunochromatographic assays (NG Biotech, Guipry, France).

Mouse model of in vivo colonization with KP5825. All experiments were carried out using 6–8 week-old C57BL/6 male mice weighted 24–26 g (Jackson Laboratory, Bar Harbor, Maine, USA) and housed in sterile cages with irradiated food and acidified water. Each group contained seven mice. For experiments involving antibiotic treatment, 0.5 g/L of ampicillin (Sandoz GmbH) was administered to animals in the drinking water for fourteen days and changed every day. For colonization experiments, 5×10^6 CFU of *K. pneumoniae* KP5825 was administered by orogastric gavage in a 200 µl volume on the fourteenth and fifteenth day of ampicillin pretreatment. After the oral colonization with KP5825 the following antibiotics—0.5 g/L ampicillin (Amp_0.5), 0.1 g/L ceftazidime (GlaxoSmithKline) (Caz_0.1), 0.5 g/L ceftazidime (Caz_0.5), 0.1 g/L ciprofloxacin (Bayer AG) (Cip_0.1) and 0.5 g/L ciprofloxacin (Cip_0.5)—were further administered to the animals in the drinking water for two weeks and changed every day (Fig. 1).

Mice were single-housed at the time of colonization experiment. Animals were maintained in a specific pathogen-free facility at Institute of Medical Microbiology, Semmelweis University. All mouse handling, cage changes and feacal pellet collection were performed in a biosafety level 2 (BSL-2) facility, with personnel wearing sterile gowns, masks and gloves.

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Oligonucleotides	Sequences
rpoB1 forward primer	5′ CCC ACT ACG GTC GCG TAT G 3’
rpoB1 reverse primer	5′ CAG ACC GAT GTT CGG ACC TT 3’
rpoB1 probe	5′ VIC-CCG ATC GAA ACG CCT-MGB 3’
oxa-162 forward primer	5′ GGG CGA ACC AAG CAT TTT T 3’
oxa-162 reverse primer	5′ GCG ATC AAG CTA TTG GGA ATT T 3’
oxa-162 probe	5′ FAM-CCC GCA TCT ACC TTT-MGB-NFQ 3’
ctx-m-15 forward primer	5′ CGA CGT TAA ACA CCG CCA TT 3’
ctx-m-15 reverse primer	5′ TGC CCG AGG TGA AGT GGT A 3’
ctx-m-15 probe	5′ FAM-CGG GCG ATC CGC GTG-MGB-NFQ 3’

Table 2. Oligonucleotide probes and primers used in qPCR assays.

Sequencing. Genomic DNA from KP5825 was isolated by NucleoSpin Microbial DNA Kit (Macherey Nagel), and plasmid DNA was isolated by NucleoSpin Plasmid DNA Kit (Macherey Nagel) according to the manufacturer's instructions. The quality and quantity of isolated DNA was assessed by measurements using a Qubit 4.0 fluorometer (Invitrogen, Waltham, USA) and Tapestation 4150 systems (Agilent, Santa Clara, USA). The NGS libraries were prepared using the Nextera DNA Flex Library Prep Kit (Illumina, Eindhoven, The Netherlands) with Nextera DNA CD Indexes[45]. The NGS libraries were sequenced on an Illumina MiSeq instrument using the MiSeq Reagent Kit v2 using paired end 250 bp reads at the Genomics Resource Center at the Biomi Ltd. The fastq files were imported directly from Illumina BaseSpace to the BioNumerics version 7.6 software's (Applied Maths NV, Belgium) cloud-based calculation engine[45]. De novo sequence assemblies were made with the SPAdes *de novo* genome assembler (version 3.7.1).

Accession numbers, data deposition. The genomic assembly of the OXA-162 and CTX-M-15 producing *K. pneumoniae* KP5825 have been deposited at European Nucleotide Archive at study PRJEB38863. The assembly of the plasmid containing the OXA-162 submitted under ERZ1461529 accession number and the plasmid containing the CTX-M-15 submitted under ERZ1462751 accession number to the European Nucleotide Archive.

Determination of the antibiotic concentrations in the fecal samples of mice. The concentrations of antibiotics in the stool samples of each mice were determined by HPLC at two different time points: on the first and fifteenth day after colonization with KP5825. For the determination of ampicillin, mouse fecal pellets were extracted with acetonitrile–water mixture after homogenization and derivatized with formaldehyde. The fluorescent derivative was separated on a Phenomenex Kinetex EVO C18 column and detected at $\lambda_{ex} = 346$ nm and $\lambda_{em} = 422$ nm wavelenghts. Ciprofloxacin was extracted from mouse faeces with 0.1 M phosphoric acid. The sample extract was separated on the same column and detected at $\lambda_{ex} = 310$ nm and $\lambda_{em} = 445$ nm wavelenghts using fluorescent detection. Ceftazidime was extracted with water and separated on an Agilent Polaris 3 C18-Ether column followed by UV detection at 261 nm.

Assessment of the colonization rate with KP5825 by cultivation during different antibiotic treatments. To quantify the burden of KP5825, fresh stool samples were collected on the 3rd, 6th, 9th, 12th and 15th days after the colonization with KP5825. Fresh stool specimens were used for the quantitative culture of KP5825. Serially diluted aliquots were inoculated onto a selective CHROMagar (Mast Diagnostika, Reinfeld, Germany) containing 0.1 µg/mL cefotaxime. Plates were incubated at 37 °C for 48 h and the CFU per gram of stool was calculated. The color and morphological characteristics of the colonies grown were assessed on CHROMagar (Mast Diagnostika) after 24 h and 48 h of incubation in ambient air at 35 °C.

Assessment of the colonization rate with KP5825 and copy number of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}162}$ by qPCR assay during different antibiotic treatments. Genomic DNA of KP5825 was extracted by QiaAmp Power fecal kit (QIAGEN, Venlo, NL) strictly based on manufacturer protocols. Oligonucleotid primers and FAM (fluorescein amidite)- and VIC (2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein)-labelled probes were designed by Primer Express 3.0 software (Table 2). The qPCR was carried out in a Step One Real-Time PCR System (Applied BioSystems, Thermo Fisher Scientific) in default setting. The copy number of resistance gene results were evaluated using the $2^{-\Delta\Delta Ct}$ method[46]. Utilizing the $2^{-\Delta\Delta Ct}$ method, results are presented as the fold change of gene normalized to the *rpoB1* reference gene and relative to the control mice. The number of *rpoB1* housekeeping gene for the determinaton of the *K. pneumoniae* relative amount in the feces, and the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}162}$ genes for determining the relative amount of resistance genes compared to KP5825 were determined on the first and on the fifteenth days.

Statistical analysis. Statistical analysis were performed using SSPS version 17.0 software (SPSS Inc., Chicago, IL, USA) and Microsoft Office Excel 2007 (Microsoft, Redmond, WA, USA). The variables such as the copy number of the *rpoB* housekeeping gene, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}161}$ genes were compared by Wilcoxon rank-sum

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test. A *p*-value of less than 0.05 was considered statistically significant. P-values are represented by arterisks (*, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$).

Ethics approval. Animals were maintained and handled in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals and the experiments were approved by the Animal Care Committee of Semmelweis University (Permission No. PE/EA/60-8/2018, PE/EA/964-5/2018).

Consent to participate. Not applicable.

Data availability

The dataset supporting the conclusions of this article is included within the article.

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Author contributions

B.S., J.D., A.T. performed the in vitro studies: culturing, conjugation assay, molecular studies; F.B.F. an B.K. performed the qPCR; N.M. performed the measurements; V.H. performed HPLC analysis; M.G. drafted the manuscript and designed the figures; S.P. was involved in study design; B.L., and J.J. processed the experimental data; E.O., D.Sz. and S.P. aided in interpreting results, supervised scientific work and developed the manuscript. All authors discussed the results and commented on the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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antibiotics

MDPI

Article

The Roles of a Multidrug-Resistant *Klebsiella pneumoniae* High-Risk Clone and Its Resistance Plasmids on the Gastrointestinal Colonization and Host-Defense Effectors in the Gut

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Abstract: The asymptomatic gastrointestinal colonization of multidrug-resistant (MDR) bacteria can lead to difficult-to-treat infections. We investigated the role of host factors influencing colonization in an orogastrical murine infection model using a CTX-M-15- and OXA-162-producing *Klebsiella pneumoniae* ST15 (MDR-KP) strain, as well as *Escherichia coli* J53 (EC) and *E. coli* transconjugants with an IncFII(K) plasmid carrying CTX-M-15 (EC-CTXM), and with an IncL plasmid carrying OXA-162 (EC-OXA) genes. The fecal bacterial count in colony-forming unit/gram stool (CFU/g) was determined by cultivation, IgA and defensin levels by ELISA, and gut microbiota by 16S rRNA analysis. The CFU was the lowest in EC, followed by EC-OXA and EC-CTXM, and the highest in the MDR-KP group. The IgA level in feces increased in MDR-KP, EC-CTXM, and EC-OXA, and did not change in EC. The beta-defensin 3 level markedly increased in all groups, with the highest values in MDR-KP and EC-CTXM. Alpha-defensin-5 increased in all groups especially in EC. In microbiota, the *Bacteroidota* phylum was dominant in MDR-KP, EC-CTXM, and EC-OXA, whereas *Proteobacteria* was dominant in EC. The *Muribaculaceae* family was significantly more common in the MDR-KP and EC-OXA groups, while the *Lachnospiraceae* family was dominant in the EC group. While fecal IgA levels positively correlated with colonizing bacterial CFU, the alpha-defensin 5 levels inversely correlated with CFUs and IgA levels. The presence of the IncFII(K) plasmid induced beta-defensin 3 production. The amounts of the *Muribaculaceae* family members exhibited a correlation with the IncL plasmid. The detected amounts of the *Lachnospiraceae* family indicated the protective role against the high-risk clone and the resistance plasmids' dissemination. Our results suggest that not only the MDR-KP clone itself but also the resistance plasmids play a primary role in the colonization rate in the gastrointestinal tract. Both the MDR-KP clone as well as the IncFII(K) and IncL resistance plasmids provide survival and colonization benefits in the gut.

Keywords: colonization; gut; multidrug resistance; mouse model; defensins; microbiome; ESBL; CTX-M; OXA-carbapenemase

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1. Introduction

The global spread and increasing prevalence of multidrug-resistant (MDR) *Enterobacterales* pose a significant threat to our healthcare systems. These Gram-negative pathogens often resist commonly used third-generation cephalosporins and carbapenems due to the

production of extended-spectrum β-lactamases (ESBLs), such as CTX-M types, and carbapenemases, including KPC, metallo-beta-lactamase, and OXA-type carbapanemase [1]. Making antibiotic-resistance dissemination more widespread, the genes encoding these ESBLs and carbapenemases are located on mobile genetic elements [2]. Infections caused by ESBL- or carbapenemase-producing *Enterobacterales* are associated with increased morbidity and mortality rates compared to infections caused by less resistant organisms [3]. In response to this growing threat, the World Health Organization in 2018 designated both ESBL-producing and/or carbapenemase-producing *Enterobacterales* as critical priority pathogens for the research and development of new therapeutic strategies and rapid diagnostics [4].

Since *Enterobacterales* are common commensal bacteria of the intestinal microbiota, infections caused by MDR-*Enterobacterales*—particularly, *Escherichia coli* and *Klebsiella pneumoniae*—often originate from prior asymptomatic gut colonization. The relationship between the intestinal microbiota and IgA production, as well as defensin production, has been investigated in connection with several primarily gastrointestinal diseases [5–8]. Defensins help to maintain the balance of microbiota by controlling the growth of pathogens and promoting the survival of beneficial bacteria [9]. Generally, IgA production in the gut plays a crucial role in the immune response. *K. pneumoniae*, as a common gut pathobiont, can induce intestinal inflammation [10]. However, the pathogenicity of *Klebsiella* is sensitive to the colonization status of gut microbiota [5].

High-risk *K. pneumoniae* clones are detected worldwide in hospital settings, and these are capable of acquiring diverse antibiotic-resistance mechanisms that enable them to survive in the hospital environment. Furthermore, high-risk clones can asymptomatically colonize the gut, and these clones are responsible for a high number of difficult-to-treat infections, because these exhibit multidrug resistance; therefore, limited number of effective antibiotics are available for treatment [11,12].

K. pneumoniae ST15 is an internationally disseminated high-risk clone that has been identified globally and appears to be resistant to multiple antibiotics, including cephalosporins, carbapenems, and fluoroquinolones. The high prevalence and virulence of *K. pneumoniae* ST15 strains make them a significant clinical and public health concern, particularly in hospital settings where they can spread rapidly and induce outbreaks [13–15].

CTX-M-15-producing *K. pneumoniae* ST15 is a widely disseminated clone that has been identified globally, particularly in Europe. The clone has been found to be highly transferable and has undergone multiclonal spread, contributing to its widespread presence in different parts of Europe. In the context of hospital settings, the presence of IncFII(K) plasmids carrying CTX-M-15 can contribute to the spread of multidrug-resistant bacteria, strongly limiting treatment options. IncL plasmids are often associated with antimicrobial-resistance genes, such as OXA-type carbapenemase [2,16].

The purpose of the current study was to determine different host factors influencing the gastrointestinal colonization of multidrug-resistant *Enterobacterales* strains. Our goal was to examine separately the role of resistance plasmids during colonization. We aimed to assess the effects of intestinal colonization with a CTX-M-15 ESBL- and OXA-162 carbapenemase-producing *K. pneumoniae* ST15 high-risk clone; and a sensitive, laboratory *E. coli* J53 strain and its transconjugants—either with a CTX-M-15-harboring IncFII(K) plasmid or with an OXA-162-harboring IncL plasmid—in a murine model to quantify the effects of the *K. pneumoniae* ST15 high-risk clone itself and the resistance plasmids on the establishment and elimination of intestinal colonization [17].

One particular aim of our study was to perform gastrointestinal colonization in oral-ampicillin-pretreated mice (C57BL/6) with a clinical CTX-M-15 ESBL- and OXA-162 carbapenemase-producing *K. pneumoniae* ST15 strain (MDR-KP), as well as its *E. coli* J53 transconjugants with an IncFII(K) plasmid containing the $bla_{\text{CTX-M-15}}$ resistance gene (EC-CTXM), *E. coli* J53 transconjugants with an IncL plasmid containing the $bla_{\text{OXA-162}}$ resistance gene within a Tn1991.2 genetic element (EC-OXA), and *E. coli* J53 strain itself (EC) [17]. After the orogastric colonization of the mice with the strains listed above, the

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bacterial count of the feces for the colonizing bacteria, the content of IgA, and the levels of beta-defensin-3 and alpha-defensin-5 were determined at different time points (Figure 1).

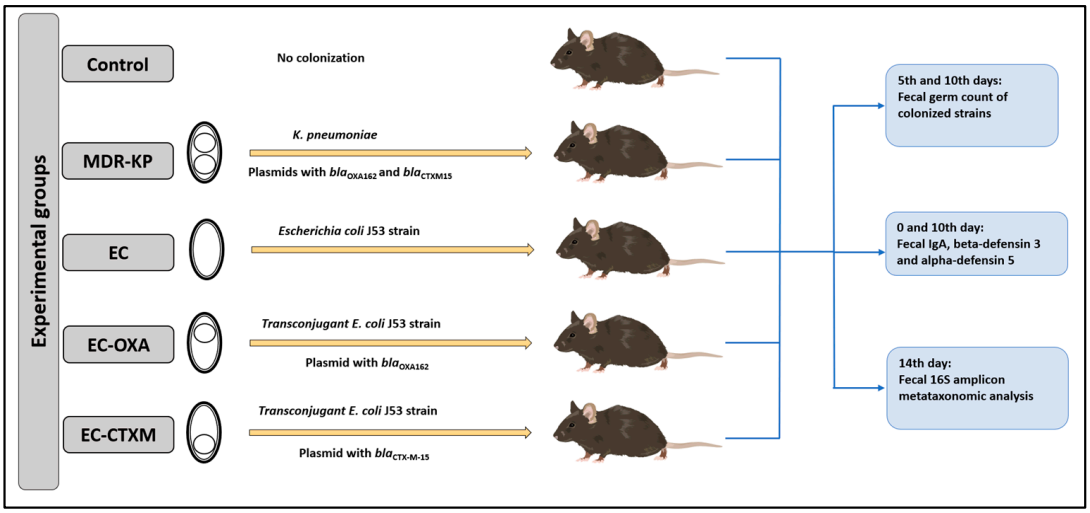

Figure 1. Experimental design.

2. Results

2.1. Bacterial Loads in Feces During Gastrointestinal Colonization

The mice were colonized orogastrically with MDR-KP (CTX-M-15- and OXA-162-producing *K. pneumoniae* ST15 strain), EC (*E. coli* J53), EC-CTXM (*E. coli* J53 transconjugant with an IncFII(K) plasmid containing the $bla_{\text{CTX-M-15}}$ resistance gene), and EC-OXA (*E. coli* J53 transconjugant with an IncL plasmid containing the $bla_{\text{OXA-162}}$ resistance gene within a Tn1991.2 genetic element). Fecal samples were collected on the fifth and tenth day of the colonization in order to determine the colonizing bacteria amount in the feces. On the fifth day of colonization, the CFU was the lowest at 3.77×10^7 CFU/g in the EC group, indicating the low colonization capability of laboratory-sensitive strains. The colonization rate was the highest in the MDR-KP group with a mean value of 8.64×10^9 CFU/g, and was also high in the EC-CTXM group at 7.06×10^8 CFU/g and in the EC-OXA group at 1.54×10^9 CFU/g, indicating the elevated gastrointestinal colonization ability of the transconjugant *E. coli* strains. The determination of germ counts from stool samples taken on the tenth day after colonization exhibited similar trends. On the tenth day, the fecal mean bacterial load was in the MDR-KP group at 5.43×10^{10} CFU/g, in the EC group at 8.43×10^8 CFU/g, in the EC-CTXM group at 3.18×10^9 CFU/g, and in the EC-OXA group at 2.01×10^{10} CFU/g (Figure 2A).

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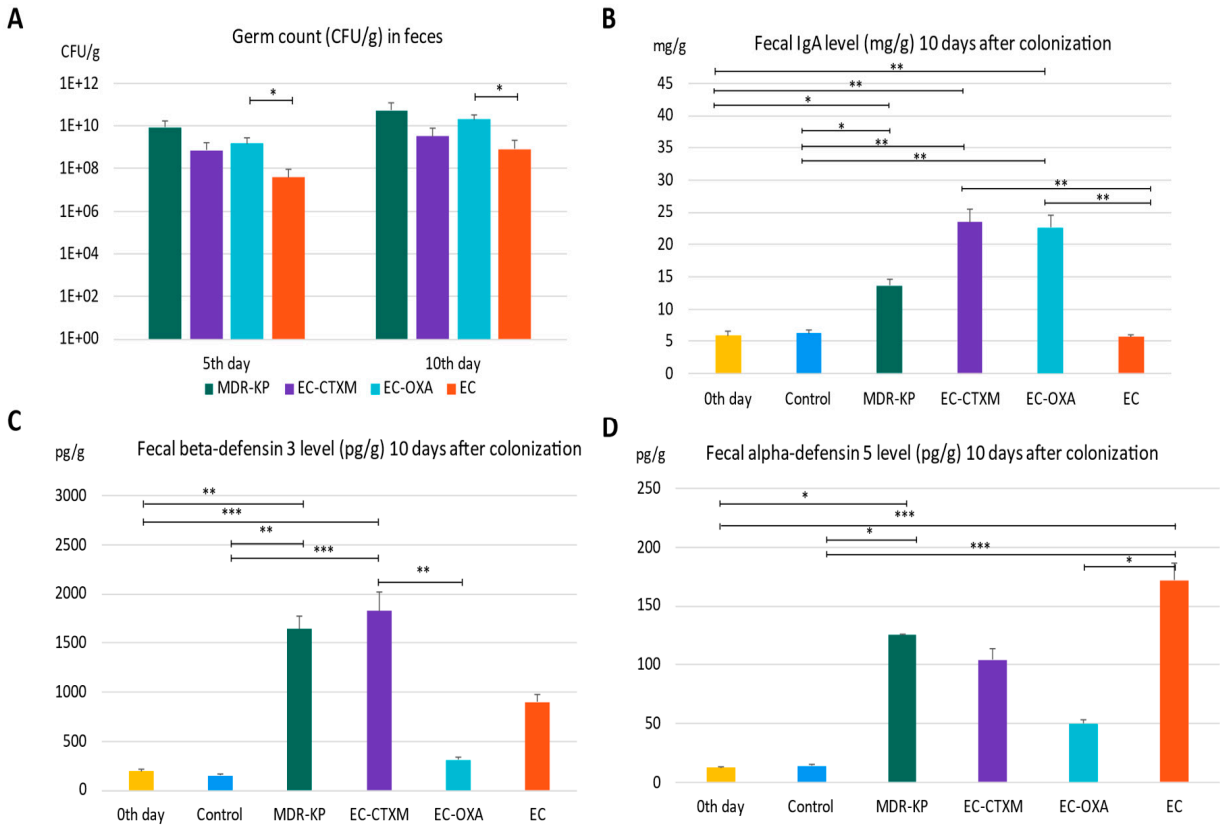

Figure 2. (**A**) The gastrointestinal colonization rate characterized by germ count (colony-forming units: CFU) in the feces by different bacteria—MDR-KP, EC, EC-OXA, and EC-CTX-M—on the fifth and tenth day of colonization. (**B**) The IgA level in feces of mice colonized by different bacteria MDR-KP, EC, EC-OXA, and EC-CTXM. (**C**) The beta-defensin-3 level in feces of mice colonized by different bacteria MDR-KP, EC, EC-OXA, and EC-CTXM. (**D**) The alfa-defensin-5 in feces of mice colonized by different bacteria MDR-KP, EC, EC-OXA, and EC-CTXM. Statistical differences are marked with * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

2.2. Fecal IgA Levels During Gastrointestinal Colonization

The IgA level was measured in feces just before the colonization (mean of 5.89 mg/g) and it was increased in the MDR-KP group (mean of 13.67 mg/g) and did not change in the EC group (mean of 5.66 mg/g). However, it markedly increased in the group EC-CTXM (mean of 22.68 mg/g) and in the group EC-OXA (mean of 23.52 mg/g) by Day 10 (Figure 2B).

2.3. Fecal Beta-Defensin 3 Levels During Gastrointestinal Colonization

In the feces, the baseline mean beta-defensin 3 level was a mean of 196.63 pg/g. The beta-defensin-3 level was increased to a mean of 1640 pg/g in the MDR-KP group, to a mean of 898 pg/g in the EC group, to a mean of 309 pg/g in the EC-OXA group, and to a mean of 1825 pg/g in the EC-CTXM group, indicating the dominant effect of the $bla_{\text{CTX-M-15}}$-containing IncFII(K) plasmid on beta-defensin 3 production (Figure 2C).

2.4. Fecal Alpha-Defensin Levels During Gastrointestinal Colonization

The level of alpha-defensin 5 increased to a mean of 125 pg/g in the MDR-KP group, to a mean of 172 pg/g in the EC group, to a mean of 104 pg/g in the EC-CTXM group, and to a mean of 50 pg/g in the EC-OXA group, and it was increased in the EC groups. Based on these results, the fecal alpha-defensin 5 levels were inversely correlated with CFUs and IgA levels (Figure 2D).

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2.5. Fecal Microbiota Composition During Gastrointestinal Colonization

A 16S rRNA taxonomic analysis was performed on the feces samples on Day 14. There were no significant differences in the alpha-diversity by the Chao1 and Simpson tests and in the beta-diversity among the groups. The *Bacteroidota* phylum was the most dominant phyla in the MDR-KP, EC-CTXM, and EC-OXA-162 groups, whereas, in the EC group, the *Proteobacteria* phylum was dominant (Figure 3). At the family level, the *Muribaculaceae* family was significantly ($p < 0.05$) more common in the EC-OXA-162 groups than in the EC group, showing a correlation with the presence of the OXA-162 plasmid. The *Lachnospiraceae* family was dominant in the EC group, indicating the protective effect of the *Lachnospiraceae* family against the high-risk *Klebsiella* clone and the CTX-M15- and OXA-162-containing resistance plasmid dissemination (Figure 4).

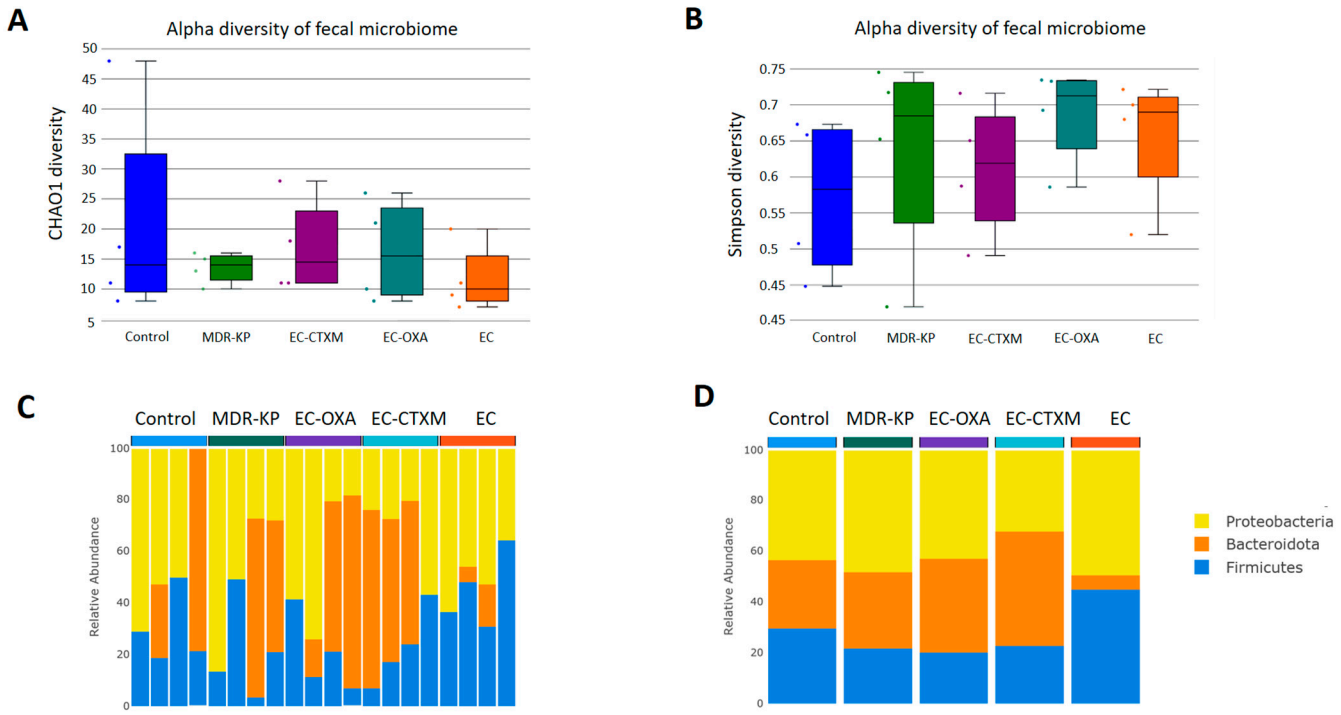

Figure 3. (**A**) Relative abundances of abundant taxonomic phylum in each mouse. Elements are shown if they have at least 2% relative abundance in at least one of the averaged samples. (**B**) Average values of relative abundances at the phylum level were calculated for samples from the same treatment groups. Elements are shown if they have at least 2% relative abundance in at least one of the averaged samples. (**C**) Chao1 alpha-diversity of fecal samples in the different groups (Control, MDR-KP, EC, EC-CTXM, and EC-OXA). Box plots show the distribution of diversities in each group. (**D**) Simpson alpha-diversity of fecal samples in the different groups (Control, MDR-KP, EC, EC-CTXM, and EC-OXA). Box plots show the distribution of diversities in each group.

On one hand, the abundance of the *Lachnospiraceae* family showed an inverse relationship with the gastrointestinal carriage of the MDR-KP strain and harboring of the resistance plasmid in the EC-CTXM and EC-OXA-162 groups. On the other, the *Enterobacteriaceae* family showed a correlational relationship with the high-risk *Klebsiella* clone and the CTX-M15- and OXA-162-containing resistance plasmids (Figure 5).

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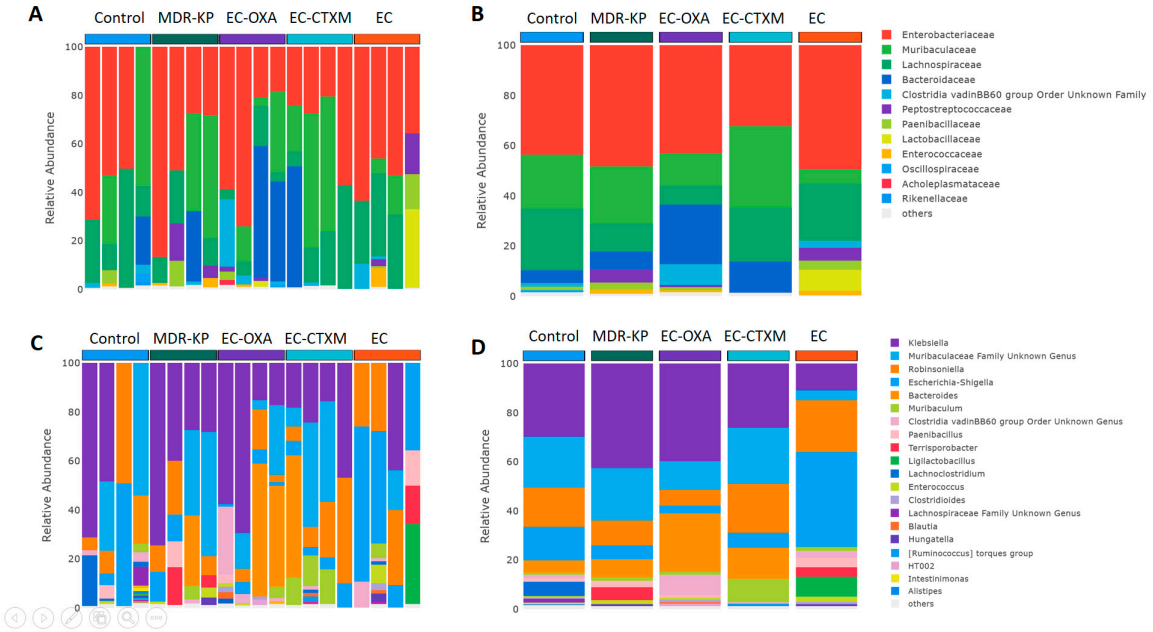
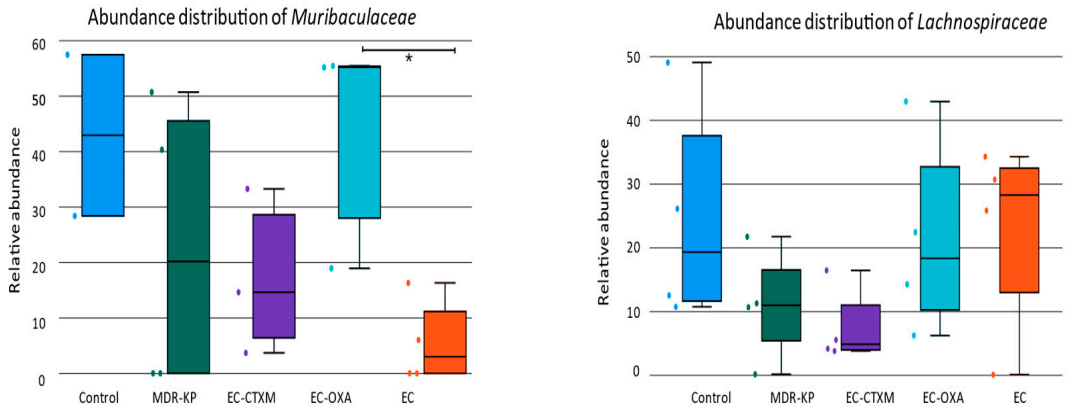


Figure 4. (**A**) Relative abundances of most abundant taxonomic families in each mouse. Elements are shown if they have at least 2% relative abundance. (**B**) Average values of relative abundances at the family level were calculated for samples from the same treatment groups. Elements are shown if they have at least 2% relative abundance in at least one of the averaged samples. (**C**) Relative abundances of most abundant taxonomic genera in each mouse. Elements are shown if they have at least 2% relative abundance. (**D**) Average values of relative abundances at the genus level were calculated for samples from the same treatment groups. Elements are shown if they have at least 2% relative abundance in at least one of the averaged samples.



(**A**) (**B**)

Figure 5. (**A**) The relative abundance of *Muribaculaceae* family in each group (Control, MDR-KP, EC, EC-CTXM, and EC-OXA). (**B**) The relative abundance of *Lachnospiraceae* family in each group (Control, MDR-KP, EC, EC-CTXM, and EC-OXA). Statistical difference is marked with * $p < 0.05$.

3. Discussion

Gastrointestinal colonization by multidrug-resistant strains of Enterobacterales has been the focus of attention worldwide because these multidrug-resistant strains (e.g., ESBL- and/or carbapenemase-producing *K. pneumoniae*, and *E. coli*) can spread easily from person to person between healthy individuals as well as among hospitalized patients. The gut environment provides optimal conditions (e.g., a high bacterial density, optimal

temperature, and a source of nutrients for bacteria) for horizontal gene transfer, that enhances the further dissemination of resistance genes among intestinal bacteria, and multidrug-resistant strains can evolve [18,19].

Several studies have investigated the risk factors of colonization with multidrug-resistant ESBL- and carbapenemase-producing Enterobacterales, such as earlier antibiotic treatment, previous hospitalization, intensive care unit treatment, travel abroad, etc. [20–31]. The screening for intestinal colonization with ESBL- and carbapenemase-producing Enterobacterales strains has been implemented in healthcare settings in several countries. Its importance is well-described among patients (e.g., newborns, and patients who are transferred between hospitals); however, healthy people after travel can be also screened [20–25].

Different decolonization strategies have been investigated in order to diminish the intestinal colonization of ESBL- and carbapenemase-producing Enterobacterales strains, because the prior gastrointestinal colonization can induce systemic infections and can initiate community-acquired infections. Furthermore, intestinally carried MDR-Enterobacterales strains can induce several outbreaks in hospitals as well [32].

In our animal study, after the colonization assay, the amount of MDR-*Klebsiella* and the *E. coli* strains harboring the CTX-M or the OXA-162 plasmids markedly increased in feces. Interestingly, the *E. coli* strain without the plasmid was not able to colonize the gastrointestinal tract; however, *K. pneumoniae* and *E. coli* harboring different resistance plasmids were able to successfully colonize.

It seems that the presence of the IncFII(K) plasmid with $bla_{\text{CTX-M-15}}$ and the IncL plasmid with $bla_{\text{OXA-162}}$ in *E. coli* changed the colonization properties of the original all-sensitive *E. coli* strain. Several underlying mechanisms can explain the reason, that the carriage of plasmids was accompanied by an increase in the bacterial cell count in stool samples.

In recent years, the relationship between the intestinal microbiome composition and ESBL-producing *E. coli* has been investigated. Davies et al. conducted a point-prevalence metagenomics study on fecal samples from international travelers before and after travel, observed changes in the microbiome composition during travel, and found that these changes were primarily associated with the development of travelers' diarrhea rather than the acquisition of ESBL-producing *E. coli* [33]. In another study, no differences were found in the diversity parameters or relative abundance of bacterial species in the gut microbiome between healthy individuals, who were colonized or not colonized with ESBL-producing *E. coli* [34]. Recently, Ducarmon et al. analyzed the potential role of the gut microbiome in controlling the colonization of ESBL-producing *E. coli*, and no differences in the diversity parameters or in the relative abundance were observed between ESBL-producing *E. coli* and the negative groups [35]. Our obtained results are in good correlation with the previous literature data that describe human results, and we also found no difference in the alpha-diversity between the control group that does not carry ESBL or carbapenemase genes, and the group of ESBL- and carbapenemase-carrying strains [36–38]. Based on our findings, the abundance of *Bacteroidota* phylum was correlated with multidrug-resistance features. It was clearly dominant in the colonization with *Klebsiella* or with *E. coli* containing either the ESBL or OXA-162 plasmids. The gut microbiota exhibits remarkable alteration after colonization with the carpanemase-producing *K. pneumoniae* in animal studies with a specific dysbiosis characterized by a consistently marked decrease in *Muribaculaceae*, *Rikenellaceae*, and Lachnospiraceae_NK4A136_group [36]. However, we could not detect a significant difference in the abundance of the *Lachnospiraceae* and *Muribaculaceae* groups. Having said that, a significant difference could be observed in favor of ES and EC-OXA-162 in terms of *Muribaculaceae*, and a small difference could be detected in the EC group in terms of *Lachnospiraceae* in our study. The presence of the OXA-162 plasmids either in the *K. pneuomoniae* or *E. coli* strain led to changes in the gut microbiota composition, with a *Muribaculaceae* dominance, showing a correlation with the presence of the plasmid. The *Muribaculaceae* family specializes in the fermentation of complex polysaccharides. A genomic analysis has also suggested that the capacity for propionate production is widespread in the family [39,40].

Apart from the composition of the microbiome behind the gastrointestinal colonization with multidrug-resistant *Klebsiella* or *E. coli*, we are unaware of other mechanisms investigated in human or animal studies. The role of IgA and defensins in the gastrointestinal tract of mice colonized with a multidrug-resistant clone and its long-term colonization remains to be studied in detail. In order to identify the mechanisms involved in the gastrointestinal colonization of multidrug-resistant strains, we quantified the IgA and beta-defensin levels in mouse feces.

IgA binds to commensal bacteria and pathobionts like *Klebsiella*, which, in turn, can inhibit their growth and penetration of the mucus layer [40,41]. Interestingly, *Klebsiella* itself can induce IgA production in the gut [5]. Persistent colonization by resistant *E. coli* induces the secretion of luminal IgA, while commensal *E. coli* strain does not [40,41]. However, our results demonstrate that host immunity selectively recognizes pathobiont *E. coli* with the specific resistance plasmids, and develop specific IgA. The induced IgA specific to resistant *E. coli*, in turn, contributes to preventing the resistant strains from accessing the epithelium. *K. pneumoniae* induces a targeted IgA response in the gut, which helps to control its own colonization levels [10]. However, *Klebsiella* pathogenicity depends on the overall composition of the gut microbiota, with a dysbiotic state favoring *Klebsiella* overgrowth and inflammation [10,36]. Our results indicate that the presence of resistance plasmids—the IncFII(K) plasmid with CTXM-15 and the IncL plasmid with OXA-162—play a primary role in the MDR colonization rate in the gastrointestinal tract.

K. pneumoniae colonization in the gut can induce the production of human beta-defensins, which are antimicrobial peptides that help to regulate the gut microbiome [42,43]. Specifically, *Klebsiella* infection leads to increased levels of human beta-defensin 2 and human beta-defensin 3 in the intestine [44]. However, the total number of specific intestinal microbiota like *Klebsiella* does not differ significantly based on the different beta-defensin levels, suggesting that, while *Klebsiella* induces beta-defensin production, the defensin levels alone do not determine the abundance of *Klebsiella* in the gut [43,44]. Experimentally altering the gut microbiome composition can lead to changes in the beta-defensin-3 secretion, indicating a complex interplay between the microbiome, *Klebsiella* colonization, and host antimicrobial peptide production in the intestine [45]. Our results indicate that $bla_{\text{CTX-M-15}}$ plays a dominant effect in containing the IncFII(K) plasmid in beta-defensin 3 production.

E. coli is known to play a significant role in the production and regulation of defensins in the gut. *E. coli* can induce the production of defensins in response to various stimuli, including the presence of pathogens. Different *E. coli* strains can have distinct effects on defensin production and gut health. For example, the probiotic *E. coli* Nissle 1917 can induce human beta-defensin 2 production, while other strains may have different effects on the gut microbiota [46]. Based on our results, the $bla_{\text{CTX-M-15}}$-containing IncFII(K) plasmid presence plays an important role in human beta-defensin 3 production. Surprisingly, the alpha-defensin 5 level was the highest in the case of colonization with the apathogen *E. coli* and lower during the colonization with the plasmid carrying strains in this study. These findings highlight the importance of *E. coli* in the production and regulation of defensins in the gut, a process that plays a crucial role in maintaining gut health and controlling the growth of pathogens.

To our knowledge, this is the first paper that studied not only the gut microbiome dynamics, but also the role of IgA production and defensin levels during colonization by a multidrug-resistant *K. pneumoniae* high-risk clone. We documented that IgA levels and human beta-defensin 3 production have a crucial role in colonization and plasmid dissemination. All these findings confirm and emphasize that plasmids carrying resistance genes play a significant role in the spread of high-risk clones worldwide, whose role goes beyond the spread of resistance. The further identification of plasmid-mediated factors involved in colonization requires additional studies.

The limitations of the study are as follows: Further studies have to identify the direct roles of defensins on the pathogenic-resistance plasmid or indirect effects through the

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microbiota modification. Other MDR high-risk clones of *K. pneumoniae* and *E. coli* that carry different resistant plasmids and resistance genes should be tested in a colonization model.

4. Materials and Methods

4.1. Bacterial Strain and Conjugation Assay

For colonizing the experimental groups of mice, different bacterial strains were used that were generated previously and described in detail [17]. We provide here a summary of the relevant information related to these experiments. A multiresistant *K. pneumoniae* isolate ST15 (5825) (MDR-KP) was used as a conjugation donor. It harbors different resistance plasmids, among them an IncF(II)K-replicon-type plasmid with the gene $bla_{\text{CTX-M-15}}$ and an IncL-replicon-type plasmid with the gene $bla_{\text{OXA-162}}$. MDR-KP were also resistant to ciprofloxacin, that was used as selection agent during cultivation for germ count determination. *E. coli* J53 (EC) strain was used as an acceptor in the conjugation assay. It is resistant to sodium-azide, used as selection agent for acceptor and transconjugant strains. Two transconjugant strain were isolated, one isolate harboring IncL plasmid with bla_{OXA162} (EC-OXA) and one isolate harboring IncF(II)K plasmid with $bla_{\text{CTX-M-15}}$ (EC-CTXM). These four different bacterial strains were propagated on Luria–Bertani (LB) agar (Biolab, Budapest, Hungary) (EC) and LB agar with 8 mg/L ampicillin (Sandoz, Schaftenau, Austria) (MDR-KP, EC-OXA, and EC-CTXM). A suspension in phosphate buffer saline (PBS, VWR, Debrecen, Hungary) was made containing 10^9 CFU/mL for gastrointestinal colonization of mice.

4.2. Animal Study

For gastrointestinal colonization, C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME, USA), aged 6–8 weeks, were used. The mice were individually housed in standard ventilated cages (IVC) under a 12 h light–dark cycle, with controlled temperature (20–22 °C). They had ad libitum access to sterile food and water, along with sterile bedding material. To prevent potential interference from natural gut bacteria, each mouse was housed individually throughout the experiment. Prior to the commencement of the experiments, the mice underwent a two-week acclimation period to minimize stress and ensure adaptation to the new environment.

Before colonization, the mice were treated with ampicillin (Sandoz) in their drinking water at a concentration of 0.5 mg/L for two weeks to facilitate the establishment of bacterial strains in the gut environment.

Colonization was then achieved via orogastric gavage. Each mouse received a dose of 10^8 colony-forming units (CFUs) in 100 microliters of PBS for each bacterial strain. The bacterial strains used for colonization were MDR-KP, EC, EC-OXA, and EC-CTXM. The control group was treated with sterile PBS following the same protocol. Each experimental groups contained six mice. After orogastric colonization, ampicillin treatment through the drinking water (0.5 mg/L) was maintained until the end of the experiment. Fresh fecal samples were collected and weighed for further investigation on Days 5, 10, and 14 after colonization. For the determination of the germ count, samples were used immediately after collection. For ELISA and DNA extraction, separated samples were stored at -80°C until they were investigated.

During the acclimation period and throughout the experiment, mice were handled gently to maintain their welfare. Experimental procedures were conducted in compliance with ethical guidelines and approved by the institutional animal care and use committee. Animals were maintained and handled in accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals and the experiments were approved by the Animal Care Committee of Semmelweis University (Permission No. PE/EA/60-8/2018, PE/EA/964-5/2018).

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4.3. Determination of the Fecal Germ Count of Mice

Fecal shedding of the colonized bacterial strains was quantified by determination of germ count in feces. Freshly collected fecal samples were weighed and immediately suspended by mechanical dissection of fecal pellet with sterile inoculation loop in 1 mL sterile PBS (VWR, Hungary) followed by thorough vortexing to gain a homogenous suspension. The suspension was serially diluted tenfold, and twenty microliters from each dilution were streaked onto selective chromogenic agar plates and incubated overnight. The following day, colonies were identified by appropriate color and counted. The germ count was calculated based on the colony numbers and dilution factors, expressed as CFUs per gram of fecal mass. For the multiresistant *K. pneumoniae* (MDR-KP), Orientation CHROMagar plates containing 0.5 mg/L ciprofloxacin (Fresenius Kabi, Bad Homburg vor der Höhe, Germany) were used to selectively support their growth. For *E. coli* J53 (EC) and transconjugant *E. coli* strains (EC-OXA and EC-CTXM), *Enterobacteriaceae* CHROMagar plates containing 100 mg/L sodium-azide (Merck, Darmstadt, Germany) were used. Results were statistically compared with two-tailed Student's *t*-test.

4.4. Determination of Total IgA and Defensin Levels in Stool by ELISA

Total IgA, murine beta-defensin 3, and murine alpha-defensin 5 were determined from mouse feces by commercial ELISA kits (MyBiosource, San Diego, CA, USA, MBS7725462, MBS7725303, and MBS7725358). Collected frozen fecal samples were thawed and weighed before they were suspended in PBS and vortexed thoroughly for 1 h at 4 °C. Suspensions were centrifuged at 2500 rpm for 10 min and the supernatants were used in further studies. Sandwich ELISA measurements were made according to manufacturer's instructions. After stopping the reaction, optical density was measured at 450 nm and 690 nm as reference wavelengths. Results were calculated with a calibration curve gained from included standards. Lastly, total IgA and defensin content were calculated (mg/g or pg/g feces). Results were statistically compared using Wilcoxon rank-sum test.

4.5. Microbiome Composition with 16S Metagenomic Analysis

To investigate the effect of different colonizing bacterial strains on the composition of gastrointestinal microbiota of mice, stool samples were collected on day 14 after colonization. DNA was extracted from ~80 mg of feces using the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA, D4300) according to manufacturer's instructions. The V3–V4 region of the bacterial 16S rRNA genes were amplified by PCR. Dual indices (barcodes) and Illumina sequencing adapters were added to the amplicons using the Nextera XT Index kit (Illumina, Inc., San Diego, CA, USA), followed by DNA purification (Agencourt AMPure XP, Beckman Coulter, Brea, CA, USA). Individual barcoded DNA samples were then quantified with Qubit dsDNA HS Assay kit with Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA), quantified with DNA 7500 kit with Agilent 2100 Bioanalyzer (Santa Clara, CA, USA), normalized, and pooled. Multiplexed libraries were diluted to 7 pM and denatured with NaOH prior to sequencing on the MiSeq system (Illumina) using the MiSeq reagent kit v3 600 cycles (2 × 300 bp; Illumina). Results of the sequencing were uploaded and analyzed with the CosmosID-HUB software v2.0 [47]. Paired-end reads of samples were analyzed by DADA2 algorithm, after primer removal data were quality-trimmed with a threshold of median Phred score 20 over the length of reads. Forward and reversed reads were trimmed to a uniform length based on quality of reads and merged if they have at least 12 base long overlap followed by the removing of chimeric sequences. Data were then processed to amplicon sequence variants (ASVs). The taxonomical annotation of clusters was made using DADA2's naive Bayesian classifier and the Silva version 138 database. Microbial composition of samples was characterized by relative abundance of identified taxa, diversity indices (CHAO1, Simpson), and comparison of abundance distribution of specified taxa between experimental groups. Statistical comparison was made using Wilcoxon rank-sum test.

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5. Conclusions

In our study, a mouse model demonstrated that intestinal colonization with the MDR CTX-M-15- and OXA-162-producing *K. pneumoniae* ST15 high-risk clone is multifactorial. Not only the MDR clone itself but also the resistance plasmids, namely, IncFII(K) and IncL, play a primary role in the colonization rate in the gastrointestinal tract. The levels of IgA, beta-defensin-3, and alpha-defensin-5, as well as the intestinal microbiota composition influence the colonization of the MDR *K. pneumoniae* high-risk clone.

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Data Availability Statement: The datasets supporting the conclusions of this article are included within the article. The sequencing data generated during the current study are available through following the figshare identifier: https://doi.org/10.6084/m9.figshare.26124370.

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