LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

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RECOMBINANT BACTERIOCINS: NOVEL NARROW SPECTRUM ANTIMICROBIAL AGENTS

Doctoral Dissertation
Natural Sciences,
Biology (N 010)

Kaunas, 2025

The dissertation has been prepared at the Laboratory of Clinical and Molecular Gastroenterology of the Institute for Digestive Research of the Medical Faculty of the Lithuanian University of Health Sciences during the period of 2020–2025.

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The dissertation will be defended at the open session of the Biology Research Council of the Lithuanian University of Health Sciences at 12 p.m. on June 26th, 2025, at the Museum of the History of Lithuanian Medicine and Pharmacy.
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REKOMBINANTINIAI BAKTERIOCINAI: NAUJI SIAURO VEIKIMO SPEKTRO ANTIMIKROBINIAI PREPARATAI

Daktaro disertacija
Gamtos mokslai,
biologija (N 010)

Kaunas, 2025

Disertacija rengta 2020–2025 metais Lietuvos sveikatos mokslų universiteto Medicinos fakulteto Virškinimo sistemos tyrimų instituto Klinikinės ir molekulinės gastroenterologijos laboratorijoje.

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Disertacija bus ginama viešame Biologijos mokslo krypties tarybos posėdyje 2025 m. birželio 26 d. 12 val. Lietuvos medicinos ir farmacijos istorijos muziejuje.
Disertacijos gynimo vietos adresas: Rotušės a. 28, LT-01100 Kaunas, Lietuva.

CONTENT

ABBREVATIONS

ATP	–	adenosine triphosphate
AMR	–	antimicrobial resistance
BSI	–	bloodstream infection
CAUTI	–	catheter-associated urinary tract infection
CLAMBSI	–	central line-associated bloodstream infection
CPS	–	polysaccharide capsule
DNA	–	deoxyribonucleic acid
DSS	–	dextran sodium sulfate
EBV	–	Epstein-Barr virus
ECDC	–	European Centre for Disease Prevention and Control
ESBL	–	extended-spectrum β-lactamase
GDP	–	gross domestic product
GIT	–	gastrointestinal tract
GLASS	–	Global Antimicrobial Resistance and Use Surveillance System
GRAM	–	Global Research on Antimicrobial Resistance
GRAS	–	generally regarded as safe
HAI	–	hospital-acquired infection
HGT	–	horizontal gene transfer
HIV	–	human immunodeficiency virus
ICU	–	intensive care unit
kDa	–	kilodalton
LPS	–	lipopolysaccharides
MDR	–	multi drug-resistance
MRSA	–	methicillin-resistant *Staphylococcus aureus*
NI	–	nosocomial infection
OMV	–	outer membrane vesicle
RNA	–	ribonucleic acid
SPF	–	specific-pathogen-free
SSI	–	surgical site infections
TNBS	–	trinitrobenzene sulfonic acid
VAP	–	ventilator-associated pneumonia
WHO	–	World Health Organization

INTRODUCTION

Bacterial infections in hospital settings, particularly within the gastrointestinal tract (GIT), present a significant challenge to healthcare systems worldwide. Hospital-acquired infections (HAIs), also known as nosocomial infections (NIs), are often linked to invasive procedures, antibiotic use, and compromised immune systems of patients. In the GIT, various bacterial pathogens such as *Escherichia coli*, *Clostridium difficile*, and especially *Klebsiella* species can cause infections ranging from mild gastroenteritis to severe conditions such as colitis and life-threatening sepsis [1]. Notably, *Klebsiella* species, was currently named in the top of World Health Organisation (WHO) superbugs list, because of its widespread resistance to third-generation antibiotics in the European Region [2]. With approximately 10% of hospitalized patients worldwide affected by NIs, understanding the mechanisms of bacterial transmission, implementing rigorous infection control strategies, and monitoring antibiotic resistance patterns are paramount in mitigating the impact of these infections on patient outcomes and healthcare facilities.

Klebsiella pneumoniae and *Klebsiella quasipneumoniae* are Gram-negative, rod-shaped, facultative anaerobic bacteria commonly found in hospital settings, these bacteria can be opportunistic pathogens, often colonizing the human GIT and nasopharynx [3]. However, these bacteria are also regularly associated with secondary infections, including pneumonia and urinary tract infections. Their presence in hospitals is concerning, especially in patients with compromised immune systems or those undergoing invasive procedures, as they can lead to severe infections with high morbidity and mortality rates. Additionally, their growing resistance to antibiotics further complicates treatment options, making these infections even harder to manage.

Despite antibiotic treatment being one of the main methods in modern medicine to combat bacterial infections, the abundant and inappropriate use of antimicrobial agents leads to the emergence of multi-drug resistant bacteria (MDR)[4]. Globally, resistance of *Klebsiella* bacteria to antimicrobial drugs currently reaches up to 70%, with mortality rates ranging from 40% to 70%. Due to this reason, the multidrug resistance of bacteria, including *Klebsiella*, has become one of the main public health concerns. Recurrent infections, increasing mortality rates, and rising treatment costs encourage the search for new antimicrobial agents.

Bacteriocins are antibacterial peptides or small proteins synthesized by bacterial ribosomes, capable of acting against the same or closely related species of bacteria. These antimicrobial agents are currently being globally

investigated as a promising option for novel antibiotic-resistant bacterial infections, offering unique advantages over traditional antibiotics. Bacteriocins have a narrow spectrum of bacterial inhibition and are less prone to develop resistance compared to antibiotics. Therefore, recombinant bacteriocins – KvarIa and KvarM – were chosen as the investigational therapeutic agent for treating drug resistant infections.

Understanding the potential of recombinant bacteriocins in combating these infections could provide valuable insights into the development of novel and effective therapeutic approaches, especially in the context of rising antibiotic resistance.

Aim and objectives

The aim of the study was to assess the efficacy of recombinant bacteriocins in the treatment of infections caused by *Klebsiella* strain bacteria using animal experimental models, and to determine their impact on gut microbiota.

Objectives

1. To create lower gastrointestinal tract bacterial colonization model in mice and assess *Klebsiella* bacterial counts.
2. To determine the physicochemical properties of recombinant bacteriocin coating to enable efficient release in murine gastrointestinal models.
3. To evaluate the efficacy of recombinant bacteriocins in treating gastrointestinal *Klebsiella* infections in murine models.
4. To assess the impact of recombinant bacteriocins on the gut microbiota composition in murine models.

The scientific novelty and relevance of the study

To address the growing need for microbiome-sparing antimicrobial strategies, this study investigated the therapeutic potential of bacteriocin-based interventions against *Klebsiella* species, a group of clinically significant pathogens exhibiting increasing multidrug resistance [1, 3]. Gastrointestinal infection models were employed to assess both pathogen virulence and treatment efficacy under conditions that reflect clinically relevant challenges. The main novel scientific contributions of the study, including the development of a novel infection model, characterization of pH-dependent drug release, identification of species-specific treatment needs, and evaluation of targeted bacteriocins with minimal impact on the gut microbiota: 1. This study tested multiple *Klebsiella* species in gastrointestinal colonization models and identified the most virulent strain capable of inducing infection.

This led to the development of a novel model for therapeutic testing that does not require prior antibiotic-induced dysbiosis. 2. The pH levels were measured in gastrointestinal tissues and fecal samples from murine models. The findings identified the drug coatings optimal for targeting both the small and large intestines while avoiding degradation in the acidic stomach environment. 3. *K. quasipneumoniae* is often misidentified as *K. pneumoniae*, leading to inappropriate treatment and therapeutic failure due to differing drug susceptibilities. To address this gap, the study investigated the efficacy of the recombinant bacteriocin klebicin KvarIa as a targeted intervention for *K. quasipneumoniae*-associated gastrointestinal infections. 4. *K. pneumoniae* is a high-priority MDR pathogen and broad-spectrum antibiotics used against it disrupt the GI microbiota. Recombinant bacteriocins KvarM and ColA-Ia reduced gastrointestinal *K. pneumoniae* colonization by 99%, supporting their potential as effective, microbiota-sparing therapies for MDR infections. 5. Although recombinant bacteriocins offer a more targeted approach, their impact on the GI microbiota remained unknown. In this study, the impact of KvarM and ColA-Ia on gut microbial composition was assessed, and results showed no significant alterations in bacterial abundance or diversity. This suggests that these bacteriocins can be used alongside other therapies without compromising the integrity of the gut microbiome, making them suitable candidates for combination treatments, especially in vulnerable patient populations.

THE LAYOUT OF THE DISSERTATION

This thesis describes the results of three different studies: the Pilot Study which investigates the establishment of lower gastrointestinal tract colonization using *K. quasipneumoniae* subs. *similipneumoniae* (DSM® 28212™) and evaluates the efficacy of the recombinant bacteriocin KvarIa in inhibiting and reducing bacterial colonization; the Colonization Study further investigates the establishment of lower gastrointestinal tract colonization using *K. pneumoniae* subs. *pneumoniae* ATCC® 12657™ and *K. pneumoniae* subs. *pneumoniae* ATCC® 43816™; the Therapy study evaluated the efficacy of the recombinant bacteriocins, klebicin KvarM and chimericin ColA-Ia, in reducing bacterial colonization of the gastrointestinal tract and assessed their impact on the gut microbiome. The Pilot study provided a broad overview of the colonization model and identified the most effective therapeutic intervention methods. The subsequent Colonization study established a stable model of lower gastrointestinal tract colonization using opportunistic pathogens, effectively simulating a bacterial infection. Finally, the Therapy study investigated the efficacy of recombinant bacteriocins as narrow-spectrum antimicrobials for combating bacterial colonization, demonstrating their potential to target pathogens without causing off-target damage to the gut microbiome. The results of Pilot study, part of Colonization study, and Therapy study are published in open-acces peer-riewed journals *Gut Pathogens* and *Frontiers in Cellular and Infection Microbiology*, respectively. The layout of the thesis is represented in Fig. 1.

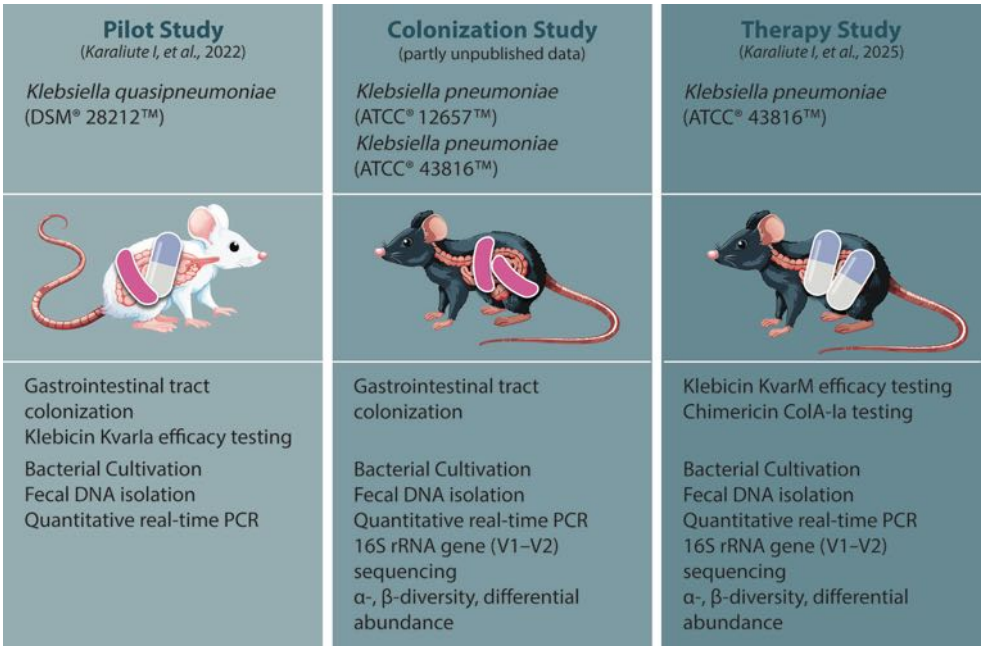

Fig. 1. *The layout of the dissertation*

Table 1. *The summary of the study designs accordingly to the layout of the dissertation*

Group No	Group name	Substances
Pilot Study		
PS1	Ctrl group	–
PS2	Kqpn group	50 µL/10^9 CFU bacterial suspension
PS3	Pen:Strep group	2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU bacterial suspension; 500 mg/L ampicillin
PS4	Pen:Strep:Met group	2000 U/mL penicillin; 2 mg/mL streptomycin; 1 g/L metronidazole; 50 µL/10^9 CFU bacterial suspension; 500 mg/L ampicillin
PS5	VOC group	50 µL/10^9 CFU bacterial suspension; 100 µg uncoated KvarIa
PS6	AbI:Ia group	2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU bacterial suspension; 100 µg Eudragit® S100 coated KvarIa
PS7	AbII:Ia group	2000 U/mL penicillin; 2 mg/mL streptomycin; 1 g/L metronidazole; 50 µL/10^9 CFU bacterial suspension; 100 µg Eudragit® S100 coated KvarIa
PS8	Hi:Ia group	2000 U/mL penicillin; 2 mg/mL streptomycin; 1 g/L metronidazole; 50 µL/10^9 CFU bacterial suspension; 1000 µg Eudragit® S100 coated KvarIa

Table 1. *Continued*

Group No	Group name	Substances
GIT model of *K. pneumoniae* subs. *pneumoniae* (ATCC® 12657™) colonization		
C1	KpnI group	50 µL/10^9 CFU bacterial suspension
C2	Ab:KpnI group	2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU bacterial suspension; 500 mg/L ampicillin
C3	Kpn:Bis group	2 mM bismuth subsalicylate; 50 µL/10^9 CFU bacterial suspension
C4	Bis:Bis group	2 mM bismuth subsalicylate; 50 µL/10^9 CFU bacterial suspension
GIT model of *K. pneumoniae* subs. *pneumoniae* (ATCC® 43816™) colonization		
C5	KpnII group	50 µL/10^9 CFU bacterial suspension
C6	Ab:KpnII group	2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU bacterial suspension; 500 mg/L ampicillin
C7	AbI:KpnII group	2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU bacterial suspension
C8	AbI:Amp group	2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU bacterial suspension; 500 mg/L ampicillin
Group No	**Group name**	**Substances**
KvarM therapy for GIT infection of *K. pneumoniae* subs. *pneumoniae* (ATCC® 43816™)		
M1	VC group	0.125 µg/µL Eudragit® S100; 0.125 µg/µL Eudragit® L100; 50 µL/10^7 CFU bacterial suspension
M2	KvarM:EudS group	12.5 µg/100 µL Eudragit® S100 coated KvarM; 50 µL/10^7 CFU bacterial suspension
M3	KvarM:EudL group	12.5 µg/100 µL Eudragit® L100 coated KvarM; 50 µL/10^7 CFU bacterial suspension
M4	KvarM:EudSL group	12.5 µg/100 µL Eudragit® S100 and Eudragit® L100 coated KvarM; 50 µL/10^7 CFU bacterial suspension
M5	ColA-Ia:EudSL group	12.5 µg/100 µL Colicin-A and KvarIa hybrid coated Eudragit® S100 and Eudragit® L100; 50 µL/10^7 CFU bacterial suspension
M6	Kpn:AB group	2 mg/200 µL ciprofloxacin; 50 µL/10^7 CFU bacterial suspension
Vehicle Control study		
VC1	Cont group	–
VC2	Eud group	12.5 µg/100 µL Eudragit® S100 and Eudragit® L100
VC3	KvarM:Eud group	500 µg/100 µL KvarM coated with Eudragit® S100 and Eudragit® L100
VC4	AB group	2 mg/200 µL ciprofloxacin

PHD CANDIDATE'S CONTRIBUTION

The author, Indrė Karaliūtė, contributed to all phases of the thesis in respect to each of the studies as presented in the layout of the dissertation. This included study conception and design, as well as participation in all aspects of the investigations: animal experiments, bacterial cultivation, DNA extraction from fecal and bacterial samples, calibration curve setup, qRT-PCR, and pH measurements of fecal and tissue samples. Performed data analysis, generated figures and wrote the manuscripts for the publications.

1. LITERATURE REVIEW

1.1. The urgency of action in bacterial infection treatment

The rapid emergence and spread of antibiotic resistant bacterial species pose a significant challenge to modern medicine, threatening the efficacy of antibiotics and complicating the treatment of common infections. Of particular concern are HAIs caused by multidrug-resistant Gram-negative pathogens, which present a major burden to both patients and healthcare systems [5, 6].

In this context, there is an urgent need for alternative therapeutic strategies to combat antibiotic-resistant infections. Hence, bacteriocins, are promising candidates to potentially replace or complement traditional antibiotics [7], as they exhibit potent activity against certain bacteria, including multidrug-resistant strains, while the producer strains remain insusceptible to their bactericidal effects.

This thesis explores the potential of recombinant bacteriocins as narrow-spectrum antimicrobial agents, with a focus on their application in addressing the challenges posed by antibiotic-resistant pathogens. By examining the properties, mechanisms of action, and potential therapeutic applications of bacteriocins, we aim to contribute to the ongoing efforts to develop novel strategies for combating antimicrobial resistance (AMR) and improving patient outcomes in the face of this global health crisis [8].

1.2. Hospital-acquired infections

HAIs – also referred to as NIs – are infections that patients acquire during their stay in healthcare facilities, typically manifesting 48 hours or more after admission. These infections are a significant global health concern due to their prevalence, impact on patient outcomes, and associated economic burden. According to the WHO, HAIs affect approximately 7% of hospitalized patients in high-income countries and 15% in low-income countries, translating to millions of cases annually. The prevalence is particularly high in intensive care units (ICUs), where invasive procedures and prolonged stays increase susceptibility [9].

Common types of HAIs include central line-associated bloodstream infections (CLABSI), catheter-associated urinary tract infections (CAUTI), ventilator-associated pneumonia (VAP), surgical site infections (SSI), and gastrointestinal infections such as *C. difficile* colitis. These infections are caused by a variety of pathogens, including multidrug-resistant bacteria like *Staphylococcus aureus* (including MRSA), *E. coli*, *K. pneumoniae*, and

Pseudomonas aeruginosa. The emergence of multidrug-resistant organisms further complicates treatment and control efforts [2].

The health and economic impacts of HAIs are profound. Patients with HAIs experience longer hospital stays, higher morbidity and mortality rates, and increased medical costs. For instance, the European Centre for Disease Prevention and Control (ECDC) estimated that HAIs result at least 37000 deaths annually and have an economic impact of approximately 7 billion euros each year [2]. The financial burden extends beyond healthcare systems, affecting patients' families and communities. Moreover, HAIs contribute to the spread of AMR, exacerbating the global crisis. Addressing HAIs requires robust infection prevention measures, such as improved hygiene practices, antimicrobial stewardship, and adherence to evidence-based protocols.

1.2.1. Prevalent bacteria in hospital-acquired infections

The WHO's Global Antimicrobial Resistance and Use Surveillance System (GLASS) has identified eight bacterial strains isolated from patients' biological material, who exhibited clinical signs of HAIs. These pathogens pose significant challenges in healthcare settings due to their increasing AMR. Among these, *Acinetobacter* spp., particularly *Acinetobacter baumannii*, are notorious for their ability to survive on various surfaces for extended periods, leading to NIs such as pneumonia and bloodstream infections (BSIs). *E. coli*, a common cause of urinary tract and gastrointestinal infections, has been among the most prevalent pathogens in AMR cases for several decades. *S. aureus*, especially methicillin-resistant MRSA, is a leading cause of skin and soft tissue infections, pneumonia, and surgical site infections. Other significant pathogens include *Streptococcus pneumoniae*, known for causing respiratory infections; *Shigella* spp., responsible for gastrointestinal infections in regions with poor sanitation; *Neisseria gonorrhoeae*, the causative agent of gonorrhea with increasing resistance to multiple antibiotics; and *K. pneumoniae*, a leading cause of pneumonia, BSIs, urinary and GIT infections in healthcare settings. Understanding the prevalence and characteristics of these bacteria is crucial for implementing effective infection control measures and developing targeted treatment strategies to combat the growing threat of antimicrobial resistance in hospital environments [10, 11].

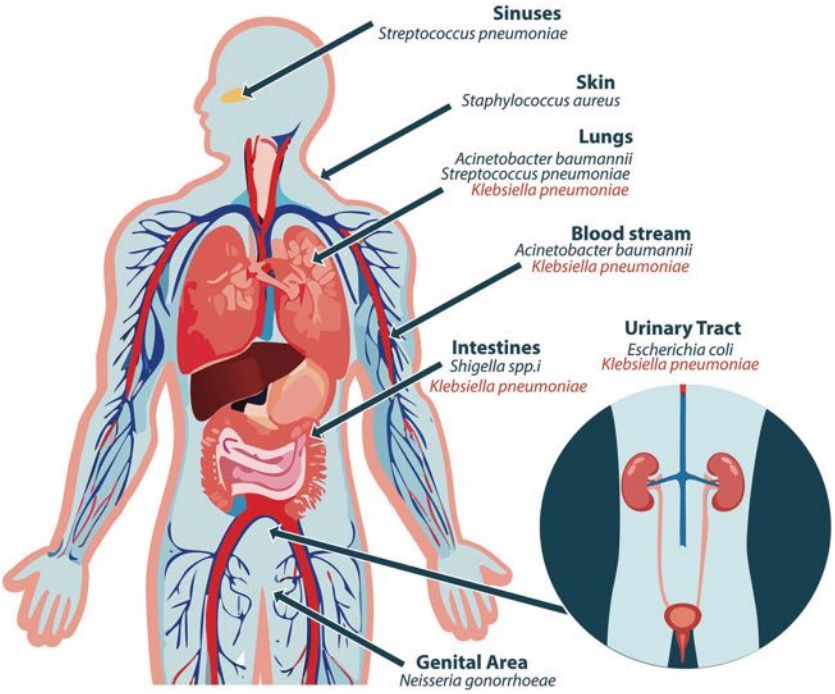

Fig. 1.2.1.1. *The most prevalent bacteria and their infection sites in clinical settings*

K. pneumoniae stands out as a particularly formidable pathogen among HAIs due to its remarkable adaptability and resilience. Unlike many other bacteria, *K. pneumoniae* possesses a thick polysaccharide capsule that acts as a protective shield, enhancing its survival in harsh hospital environments and conferring resistance to phagocytosis by immune cells. This capsule, combined with its ability to form biofilms, allows *K. pneumoniae* to persist on various surfaces, including medical devices, for extended periods. Furthermore, *K. pneumoniae* exhibits an exceptional capacity for acquiring and disseminating antimicrobial resistance genes, often through horizontal gene transfer (HGT) (*see Section 1.3.3*), leading to the rapid emergence of multidrug-resistant strains. The bacterium's genomic plasticity enables it to quickly adapt to antibiotic pressures, with some strains developing resistance to last-resort antibiotics like carbapenems. Perhaps most alarmingly, *K. pneumoniae* has given rise to hypervirulent strains that can cause severe, invasive infections even in healthy individuals, a trait uncommon among typical opportunistic pathogens. This combination of antibiotic resistance, environmental persistence, and enhanced virulence makes *K. pneumoniae* a uniquely challenging threat in healthcare settings, demanding urgent attention and innovative strategies for control and treatment [9, 12, 13].

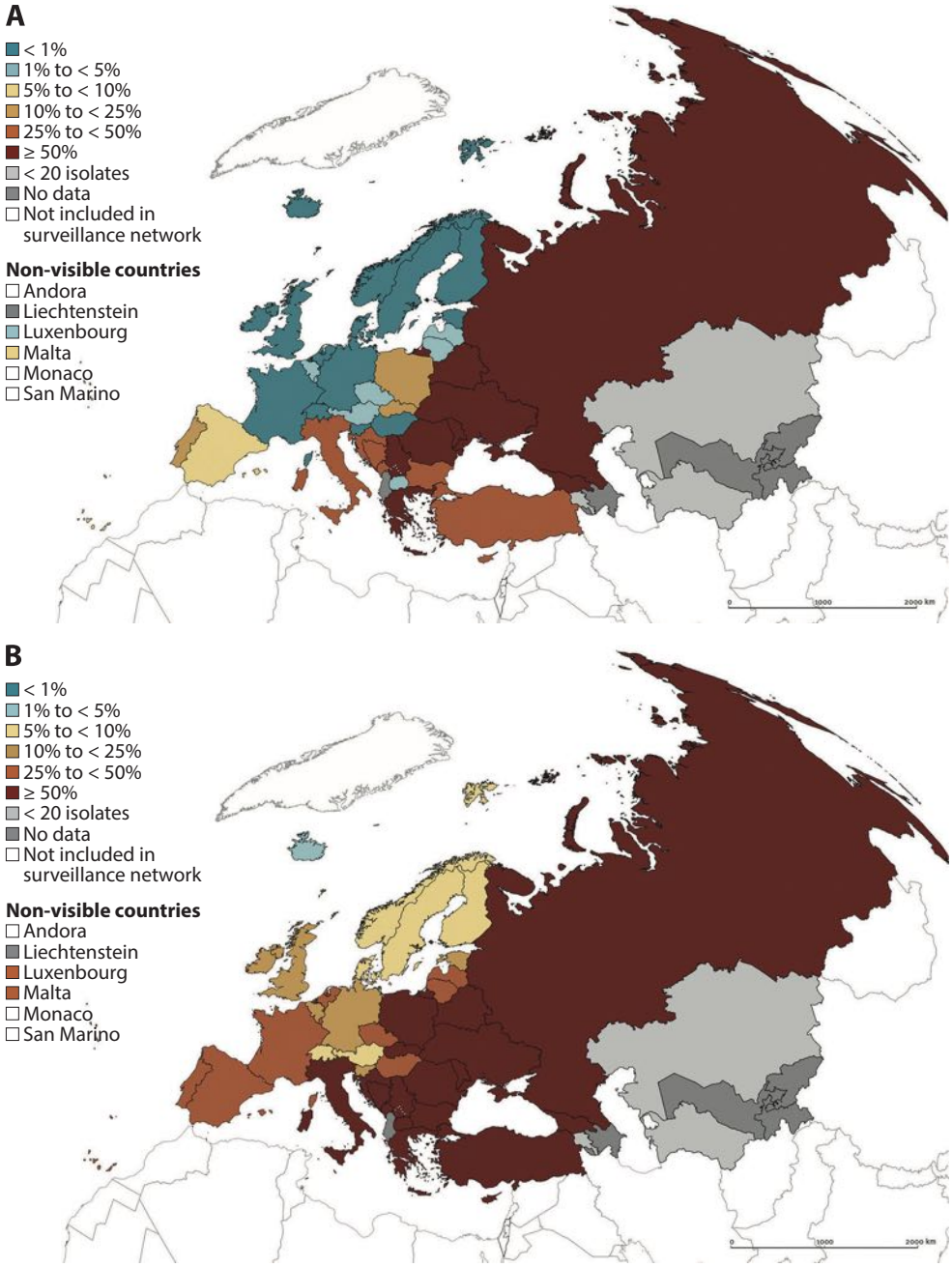

Fig. 1.2.1.2. (A) *Percentage of Klebsiella pneumoniae invasive isolates resistant to third-generation cephalosporins (cefotaxime/ceftriaxone/ceftazidime), by country, WHO European Region, 2021;* ***(B)*** *Percentage of Klebsiella pneumoniae invasive isolates resistant to carbapenems (imipenem/meropenem), by country, WHO European Region, 2021* [2]

1.3. *Klebsiella* spp.

Klebsiella is a notable member of the Enterobacteriaceae family and a significant pathogen in healthcare settings. This bacterium is primarily known for its role in causing a variety of infections, particularly in immunocompromised patients. Its ability to thrive in diverse environments, including the human GIT, soil, and water, contributes to its widespread presence and potential for opportunistic infections. The increasing prevalence of multidrug-resistant strains of *Klebsiella* has raised alarms within the medical community, highlighting the urgent need for effective strategies to combat this resilient pathogen. Therefore, *K. pneumoniae* carbapenemase (KPC) producing organisms can be classified as ESKAPE organisms, which is a group consisting of the six most important microorganisms resistant to antimicrobials worldwide (*Enterococcus faecium*, *S. aureus*, KPC*, Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* spp.) [14].

1.3.1. Phylogenetic groups of *Klebsiella*

The classical method of *Klebsiella* classification is into several phylogenetic groups based on genetic similarities and differences. The four main groups are KpI, KpII, KpIII, and KpIV. KpI predominantly consists of *K. pneumoniae* strains associated with clinical infections in humans, making it the most clinically relevant group. KpII primarily includes *K. variicola*, which is less frequently linked to human infections, but more commonly found in environmental samples. KpIII comprises *K. quasipneumoniae* and *K. quasivariicola*, which have been implicated in both human and animal infections but are less common in clinical settings. Finally, KpIV includes *K. michiganensis*, a newer group that has been associated with plant infections. On the other hand, *K. pneumoniae* complex that is multidrug-resistant, hypervirulent, and opportunistic groups based on the accessory genome, has been classically and taxonomically divided into the just KpI, KpII, and KpIII phylogroups. Recently, it has been suggested that these phylogroups, which are collectively known as the KPC, can be classified as separate species as follows: *K. pneumoniae* (KpI), *K. quasipneumoniae* (KpII), and *K. variicola* (KpIII) [15]. Understanding these phylogenetic distinctions is crucial for scientific studies and infection control measures aimed at managing the spread of this pathogen, as no approved potential therapy currently exists for hypervirulent strains of this bacteria.

1.3.2. Characteristiscs and pathogenicity of KpI

K. pneumoniae (KpI) is a bacterium known for its encapsulated, non-motile nature and ability to ferment lactose. This facultative anaerobe possesses a genome of 167989 bp with a GC content of 39.6% and 295 predicted genes [16]. Nevertheless, *K. pneumoniae* has high chances of transitioning from opportunistic bacterium to a pathogenic one through a combination of virulence factors and environmental conditions. Firstly, KpI pathogenicity is attributed to a few virulence factors. Primary virulence factor is characterized as a thick polysaccharide capsule (CPS) of bacteria, which protects against host immune responses, particularly protecting the bacterium from phagocytosis and complement proteins [17]. This factor also contributes to the ability of forming biofilms on surfaces or attachment to host tissues, using type 1 and type 3 fimbriae, allowing abundant colonization [18]. Contribution of supporting the growth, virulence, and antibiotic resistance is also highly due to siderophores, which helps bacteria to feed from its environment, and lipopolysaccharides (LPS) in the outer membrane, which helps immune evasion. Furthermore, environmental factors are just as important for the pathogenicity of *K. pneumoniae*, as it can easily colonize mucosal surfaces, particularly in the GIT and the spread to other tissues in individuals with weakened immune systems or those with underlying health conditions [19]. Obviously, *K. pneumoniae* is quite capable of genetic adaptations, like additional virulence or antibiotic resistance genes, which allows the bacteria to persist despite the treatments [20]. This combination of traits enables *K. pneumoniae* to cause severe infections, including pneumonia, urinary tract infections, and BSIs, particularly in individuals with underlying health conditions or those undergoing invasive procedures.

1.3.3. Characteristiscs and pathogenicity of KpII

K. quasipneumoniae (KpII) is quite recently defined Gram-negative, rod-shaped bacterium within the *Klebsiella* genus, often misidentified as *K. pneumoniae* due to similar phenotypic traits [21]. As per latest reviews, *K. quasipneumoniae* has been classified as KpII subs. *quasipneumoniae* and KpIV subs. *similipneumoniae*. Originally linked to opportunistic environmental pathogens, both subspecies are now increasingly reported as pathogens associated with HAIs [22]. KpII possesses virulent factors quite like KpI, which leads to urinary tract infections, BSIs, gastrointestinal infections, and pneumonia. This similarity often leads to the misidentification of the primary pathogen responsible for the disease [23]. Although *K. quasipneumoniae* generally exhibits higher antimicrobial susceptibility than *K. pneumoniae*, it can easily acquire carbapenemase-encoding genes and harbors various resistance

genes (like fosfomycin resistance gene, *ompK36* gene, *ompK37* gene etc.) highly contributing to MDR [23]. Alarmingly, *K. quasipneumoniae* uses horizontal gene transfer quite effectively for acquiring new antimicrobial mechanisms (outer membrane vesicles (OMVs) [24], plasmid acquisition, mobile genetic elements, diverse gene profiles, environmental adaptation [25]). Thus, the increasing recognition of *K. quasipneumoniae* as a distinct pathogen highlights its clinical significance, particularly in HAIs, necessitating improved measures to address its virulence and tendency to multidrug – resistance.

1.3.4. Clinical significance and associated infections of KpI and KpII

K. pneumoniae and *K. quasipneumoniae* are clinically significant pathogens responsible for a wide range of HAIs. VAP occurs at least 48 hours after endotracheal intubation and is caused by the microaspiration of bacteria colonizing the oropharynx and upper airways [26]. CAUTIs are mostly caused by bacterial colonization of urinary catheters, with one exceptional trait being the ability to form biofilms [27]. SSIs occur when bacteria, in immunocompromised, colonize surgical wounds, leading to complications such as abscesses or wound dehiscence [28]. BSIs usually results from primary infections like pneumonia or intra-abdominal infections after surgical or intravenous interventions [29]. GIT infections often result from translocation of bacteria from the gut microbiota to sterile sites, it can lead to intra-abdominal abscesses or secondary BSIs [21]. Wound infections occur when bacteria enter through breaks in the skin caused by surgery or trauma, leading to cellulitis, necrotizing fasciitis, or other soft tissue infections [20]. Undoubtedly, infections caused by MDR *Klebsiella* species are associated with higher morbidity and mortality rates, with hypervirulent and carbapenem-resistant bacterial infections showing mortality rates as high as 50% [20, 26]. These challenges show the need for ongoing surveillance, strict infection control protocols, and research into novel therapeutic approaches, like bacteriocins.

1.4. Antimicrobial resistance

AMR has emerged as one of the most significant global health challenges of the 21st century, threatening the efficacy of modern medicine. According to the WHO, bacterial AMR was directly responsible for 1.27 million deaths worldwide in 2019 and contributed to an additional 4.95 million deaths, underscoring its devastating impact on public health [9]. Projections from the Global Research on Antimicrobial Resistance (GRAM) Project suggest that AMR could directly cause over 39 million deaths between 2025 and 2050,

with annual deaths attributable to AMR rising from 1.14 million in 2021 to an estimated 1.91 million by 2050 [30]. This crisis is driven primarily by the misuse and overuse of antimicrobials in humans, animals, and agriculture, which accelerates the development of drug-resistant pathogens with progressively advanced AMR mechanisms [31].

MDR bacteria have developed more than a few mechanisms to bypass the effects of antimicrobial agents. These mechanisms can be categorized into four primary types. I. Limiting drug uptake reduces the permeability of the bacterial cell membrane or modifies porin channels to restrict antibiotic entry. For example, most *K. pneumoniae* and *K. quasipneumoniae* can alter the expression of OmpK35 and OmpK36 porins, limiting the influx of β-lactams and carbapenems [32]. II. Target modification alter the structure or expression of the antibiotic's target site, rendering the drug ineffective. III. Drug inactivation mechanism produces the enzymes that modify or degrade antibiotic molecules. For example, the production of β-lactamases by *Klebsiella* species, which hydrolyze the β-lactam ring of penicillins, cephalosporins, and carbapenems [32]. IV. Active drug efflux actively expel antibiotics' molecules from the bacterial cell, reducing their overall intracellular concentration. In *K. pneumoniae*, systems like AcrAB-TolC and OqxAB play crucial roles in conferring resistance to multiple antibiotics [33].

1.4.1. Antimicrobial resistance mechanism in *K. quasipneumoniae* and *K. pneumoniae*

K. quasipneumoniae and *K. pneumoniae* share several resistance mechanisms that contribute to MDR profiles. Both *Klebsiella* species produce β-lactamases, including extended-spectrum β-lactamases (ESBLs) such as CTX-M, SHV, and TEM types, AmpC β-lactamases like DHA-1, and carbapenemases such as NDM, IMP, KPC, and OXA-48 variants, which hydrolyze β-lactam antibiotics and makes them ineffective [1]. Along with employment of target modification strategies where mutations or structural alterations in antibiotic target sites reduce drug efficacy. Antibiotic entry into the bacterial cell can also be limited employing decreased permeability through porin alterations (like OmpK35 and OmpK36 mutations). Both bacteria use active efflux pump systems (AcrAB-TolC and OqxAB). Furthermore, one of the more distinct characteristics is biofilm formation, which enhances AMR by shielding bacterial cells from antibiotics and facilitating HGT of resistance genes via plasmids [34].

Despite the similarities, *K. quasipneumoniae* exhibits some distinct resistance mechanisms in comparison to *K. pneumoniae*. *K. quasipneumoniae* harbors species-specific chromosomally encoded class A β-lactamases, which

have exceptional resistance to ampicillin but are less effective against other β-lactams compared to the blaSHV variants typically found in *K. pneumoniae* [23]. Additionally, ESBL production is usually less prevalent in *K. quasipneumoniae*, and it generally shows higher susceptibility to certain antibiotics such as ampicillin/sulbactam and trimethoprim/sulfamethoxazole. Moreover, plasmid-mediated resistance varies between the two species. *K. quasipneumoniae* isolates have been found to harbor unique plasmid profiles containing genes such as blaCTX-M-2, blaKPC, and fosA [35].

1.4.2. Economic and socioeconomic challenges posed by MDR strains

Globally MDR strains pose significant economic and socioeconomic challenges. The World Bank estimates that AMR could result in at least US$ 1 trillion additional healthcare costs by 2050 and US$ 1–3.4 trillion in annual GDP losses by 2030 [31]. Currently, AMR increases healthcare costs by US$ 66 billion annually, projected to rise to US$ 159 billion by 2050 if resistance rates follow historical trends [36]. The burden falls disproportionately on low- and lower-middle-income countries, with the median cost of treating a resistant infection ranging from US$ 100–30000 per hospital admission, depending on the country's income level [36]. Socioeconomic factors also play a role, with studies showing a small but consistent negative association between socioeconomic status and overall antimicrobial resistance rates [37]. The economic impact extends beyond healthcare, affecting productivity, agriculture, and tourism. Without effective interventions, AMR could lead to 22.2 million additional deaths by 2050, reducing the global workforce by 8 million [36]. These challenges underscore the urgent need for strategies on controlling AMR, including improved access to high-quality treatments, development of new antibiotics, and addressing socioeconomic inequality.

1.5. Bacteriocins

Bacteriocins have emerged as a promising class of novel antimicrobial agents with potential to address the growing challenges in AMR field. Unlike traditional antibiotics, bacteriocins often target specific bacterial strains, reducing the risk of any following complications including undisturbed microbiota. Their structural diversity, stability under extreme conditions, and ability to combat multidrug-resistant pathogens make them attractive candidates for therapeutic applications. Additionally, advancements in bioengineering enables the further discovery and optimization of bacteriocins'

formulations, paving the way for their integration into clinical and industrial settings as alternatives to conventional antibiotics [38].

1.5.1. Definition and general characteristics of bacteriocins

Bacteriocins are defined as proteinaceous toxins, ribosomally synthesized antimicrobial peptides or proteins, produced by bacteria to inhibit the growth of similar or closely related bacterial strains [39]. These peptides have different structures depending on bacteria strains, but typically are small, heat-stable and prevalent in environments with high microbial competition, like microbiota or soil [40]. Their production enables bacteria to establish a competitive advantage using several key characteristics distinguishing them from traditional antibiotics. I. Pathway of synthesis differs from antibiotics usual multi-enzyme complexes synthesis as bacteriocins are ribosomally synthesized and can undergo post-translational modifications. II. Many bacteriocins have a narrow spectrum activity, affecting closely related bacterial species, though chimericins can be used for broader spectrum. III. Diverse structures ranging from linear to cyclic in small peptides or larger proteins. IV. Mechanisms of action include pores formation in the cell membrane of targeted bacteria leading to cell death, and some bacteriocins (microcin B17; J25; C; H47) may have intracellular targets (RNA polymerase, aspartyl-tRNA synthetase). V. Producer immunity helps the producing bacteria to protect themselves from their own antimicrobial peptides, using immunity proteins (Nisl protein, PedB), efflux systems (as-48EFGH), membrane modifications (lipid profile anchoring Nisl). VI. Stability is demonstrated by many bacteriocins under various environmental conditions, including high temperatures and a wide pH range. VII. Low toxicity to eukaryotic cells was observed, making bacteriocins potentially safer alternatives than traditional antibiotics [41].

1.5.2. Classification and types of bacteriocins

The classification of bacteriocins have changed a few times over the dacades as new types have been discovered and characterized. Currently, most widely accepted categorization is based on bacteriocin producing organisms (Gram-positive or Gram-negative bacteria), structure, mechanisms of action and some genetic characteristics. Gram-positive bacteriocins classification include Class I: Lantibiotics, such as nisin, lacticin or mersacidin, which are small peptides (< 5 kDa) containing unique amino acids (lanthione and β-methyllanthionine) and have three subclasses (Ia; Ib; Ic) [42]. Class II: Non-lantibiotic Bacteriocins, like Pediocin PA-1, Enteroci A and else, are small (< 10 kDa) heat-stable peptides without lanthionine and have five

subclasses (IIa, IIb, IIc, IId, IIe) [43]. Class III: Large bacteriocins, such as lysostaphin and helveticin J, are heat-labile proteins (> 30 kDa). They are divided into two subclasses: Class IIIa (bacteriolysins), which cause cell wall lysis, and Class IIIb (non-lytic bacteriocins), which kill cells by disrupting metabolic processes without lysis. Class IV: Complex bacteriocins, such as sublancin and glycocin F, contain lipid or carbohydrate moieties that contribute to their antimicrobial activity. These bacteriocins exhibit unique structural features that enhance their functionality. Gram-negative bacteriocins classification include colicins, such as A, B, E2, and E3, are large proteins (30–80 kDa) produced by *E. coli*. They are protease-sensitive and heat-sensitive bactericidal proteins that target closely related species. Colicins typically enter bacterial cells by hijacking specific receptors like porins or iron transport systems and disrupt essential cellular processes such as membrane integrity or DNA replication. Colicin-like bacteriocins: S-piocins and klebicins, share structural similarities with colicins but are produced by other Gram-negative bacteria like *Pseudomonas* and *Klebsiella*. These bacteriocins also rely on receptor hijacking for entry into target cells and often exhibit species-specific activity. Microcins: microcin B17, C7, and E492, are small peptides (< 10 kDa) produced by members of the *Enterobacteriaceae* family. Microcins are highly stable under extreme conditions (like proteases or pH variations) and act as "*Trojan horse*" compounds by mimicking vital molecules to penetrate sensitive bacteria. They typically inhibit intracellular targets like DNA gyrase or RNA polymerase. Phage-tail-like bacteriocins: R- and F-piocins produced by *P. aeruginosa*, resemble the tail structures of bacteriophages. These high-molecular-weight bacteriocins (20–100 kDa) function by puncturing the membranes of target cells, leading to rapid cell death[38, 44–47].

1.5.3. Recombinant bacteriocins

Recombinant bacteriocins represent a significant advancement in the field of antimicrobial research, providing a highly customizable approach to producing these naturally occurring antimicrobial peptides. By using genetic engineering and biotechnological tools (CRISPR-Cas9; Vector Systems; Gene Fusion; etc.), recombinant production allows for broader yields, improved stability, and the ability to modify bacteriocins for specific applications. This approach is particularly promising in addressing the global challenge of MDR pathogens, while minimizing side and/or off-target effects. Recombinant production of bacteriocins offers increased yield and scalability using recombinant systems, such as *E. coli* or *Corynebacterium glutamicum* [44]. As well as genetic engineering allows to enhance bacteriocins' antimicrobial

activity, stability or broaden spectrum of action [48]. Recombinant platforms, overall, allow the expression of diverse bacteriocins and uses Generally Regarded As Safe (GRAS) organisms, including synthesis of bacteriocins which are difficult to produce naturally due to complex post-translational modifications [44]. Recombinant Bacteriocins have been explored in many fields because of their broad applications. Firstly, and most importantly, one of the newer applications is as novel agents for MDR pathogens, being explored for therapeutic application of infections caused by Gram-positive and Gram-negative bacteria. Secondly, recombinant bacteriocins have been used in the food industry as natural preservatives to inhibit foodborne pathogens without altering food properties. Lastly, bacteriocins can serve as biotechnological platforms as they enable the simultaneous production of multiple bacteriocins from a single operon to delay resistance development in target species [5].

1.5.4. Mechanisms of action of bacteriocins

Bacteriocins employ various mechanisms to exert their antimicrobial effects, targeting various cellular components and processes in susceptible bacteria. These mechanisms can be broadly categorized into two classes – membrane-targeted and intracellular-targeted. Membrane-Targeted actions include pore formation in the cytoplasmic membrane of targeted bacteria, which leads to the dissipation of the proton motive force, causing rapid cell death, e.g., lacticin Q forms huge toroidal pores (4.6–6.6 nm) causing leakage of large intracellular components (proteins, ATP). Additionally, certain lantibiotics (e.g. nisin), can exhibit dual mechanism by binding to lipid II, which is crucial in cell wall biosynthesis. Lastly, some bacteriocins (e.g., bacillus subtilis GAS101) can directly disrupt the bacterial cell membrane. Intracellular-targeted actions employ a bit more mechanisms starting with inhibition of cell wall synthesis, which can include binding to lipid II or blocking the incorporation of glucose and D-alanine in peptidoglycan precursors. As well as, inhibition of nucleic acid and protein synthesis, targeting RNA synthesis without affecting DNA synthesis or intracellular ATP levels. Microcin-like bacteriocins can also inhibit essential enzymes like DNA gyrase and topisomerase IV, converting them into toxic molecules that fragment bacterial DNA (similar to quinolone antibiotics). Finaly, microcins (e.g., microcin C) can employ "*Trojan Horse*" strategy: microcin C is processed inside the target cell to release a toxic entity that blocks aspartyl-tRNA synthetase, thereby inhibiting the transcription [49]. The diversity in mechanisms of action contributes significantly to the effectiveness of bacteriocins against a wide range of bacterial species.

1.5.5. Klebicins and other *klebsiella*-specific bacteriocins

Klebicins are a diverse class of bacteriocins produced by *Klebsiella* species. These proteinaceous toxins are classified based on their mechanisms of action, including pore-forming klebicins (e.g., Klebicin B and Klebicin E), nuclease klebicins (e.g., Klebicin A), and peptidoglycan-degrading klebicins (e.g., ColM-like klebicins such as KvarM and KpneM). Klebicins exert their antimicrobial effects through various mechanisms, such as forming pores in bacterial membranes, disrupting nucleic acid synthesis using DNase or RNase activity, or degrading peptidoglycan to compromise cell wall integrity. They often rely on receptor-mediated entry using outer membrane proteins like OmpC and the Ton system. The antimicrobial spectrum of klebicins varies, with some, like Microcin E492, exhibiting broad activity against *Klebsiella* spp., *E. coli*, *Pseudomonas* spp., and *Acinetobacter* spp., while others, like KvarM, target a majority of tested *Klebsiella* strains. KvarM is a ColM-like bacteriocin produced by *K. variicola* that exhibits a broad spectrum of antimicrobial activity against various *Klebsiella* species, including *K. pneumoniae*, *K. quasipneumoniae*, *K. oxytoca*, *K. variicola*, and *K. aerogenes*. Notably, KvarM demonstrates significant efficacy against 85% of antibiotic-resistant clinical isolates of *K. pneumoniae*. Its mechanism of action involves disrupting peptidoglycan biosynthesis, which compromises bacterial cell wall integrity and leads to cell death. In vitro studies have shown that KvarM can reduce bacterial colony-forming units (CFU) by three to four orders of magnitude in liquid cultures and by more than two logs in biofilm assays, making it effective against both planktonic bacteria and biofilms. In vivo experiments using murine models have further highlighted its potential as a therapeutic agent. Intravenous administration of KvarM in a sepsis model resulted in a dose-dependent reduction in bacterial burden, while oral administration using an Eudragit® coating effectively reduced gastrointestinal colonization by *K. pneumoniae* without disrupting the gut microbiota. These findings underscore KvarM's promise as both a treatment for systemic infections and a preventive measure against gut colonization by multidrug-resistant *Klebsiella* strains, offering a targeted and microbiota-sparing alternative to conventional antibiotics [50,51].

Another promising klebicin – KvarIa is a colicin-like bacteriocin produced by *K. variicola*, demonstrating potent antimicrobial activity against several *Klebsiella* species, including *K. quasipneumoniae*, *K. oxytoca*, and *K. aerogenes*. This bacteriocin employs a pore-forming mechanism, disrupting bacterial membrane integrity, leading to leakage of cytoplasmic contents and cell death. KvarIa has shown remarkable efficacy in reducing bacterial viability, achieving reductions of four to nine orders of magnitude in liquid

culture assays and two to six logs in biofilm assays. Despite its narrow spectrum of activity, which excludes most *K. pneumoniae* strains, KvarIa is highly effective against its close relative, *K. quasipneumoniae*. In vivo studies using the *Galleria mellonella* larvae model demonstrated KvarIa's ability to rescue larvae from lethal infections at doses ranging from ~0.07–0.9 μmol/kg of body weight. Furthermore, encapsulated KvarIa delivered orally in a murine gastrointestinal infection model significantly reduced colonization by *K. quasipneumoniae*, highlighting its potential for treating multidrug-resistant infections of the gut [50, 51].

Overall, klebicins show promise in therapeutic applications, including treatment of multidrug-resistant infections, biofilm disruption, and targeted therapy that minimizes microbiota disruption [50]. Klebicins could help for the development of narrow-spectrum antimicrobials, which represents a novel approach to combat bacterial infections while minimizing collateral damage to the host microbiome. Targeted therapies that can effectively eliminate specific pathogens without disrupting beneficial bacterial communities are becoming more recognized in their value. By targeting specific pathogens, narrow-spectrum agents can preserve the diversity and function of the host microbiome. However, there are still some challenges in optimizing production, delivery methods, and understanding resistance mechanisms of klebicins.

1.6. Narrow-spectrum activity of recombinant bacteriocins

The development of narrow-spectrum antimicrobials, represents a novel approach to combat bacterial infections while minimizing collateral damage to the host microbiome. Targeted therapies that can effectively eliminate specific pathogens without disrupting beneficial bacterial communities are becoming more recognised in their value. By targeting specific pathogens, narrow-spectrum agents can preserve the diversity and function of the host microbiome. As well as, minimizing the risk of antibiotic-associated complications, like *C. difficile* infections, which often result from disruption of the gut microbiota. Most importantly, using narrow-spectrum antimicrobials help to limit the development of resistance in non-target bacteria, potentially slowing the spread of AMR [52].

1.7. Experimental animal models

Animal models are indispensable tools in infectious disease research, offering critical insights into pathogenesis, immune responses, and therapeutic interventions. Among these, murine (mouse) models are the most widely

used due to their genetic similarity to humans, cost-effectiveness, ease of handling, and the availability of genetically modified strains. Researchers can effectivelly mimic various conditions because approximately 95% of protein-coding genes in mice are homologous to humans. Additionally, the ability to knock out or modify specific genes allows to study precise immune mechanisms and host-pathogen interactions, as well as, inbred mouse strains provide genetically identical individuals, reducing variability in experiments. Murines are relatively small animals making them easy to house, and reproduce rapidly, enabling efficient studies. Despite being widely used for research animal models have some limitations. While mice share many similarities with humans, some aspects of their immune responses differ, which can limit direct translation to human biology. Nevertheless, using different experimental methods (e.g., infection routes or anesthesia) can affect reproducibility and comparability across studies. Most importantly, ethical concerns and strictly adhering to the 3R principles (Replacement, Reduction, Refinement) is critical to minimize animal use while maximizing scientific output [53].

1.7.1. Murine models of infectious gastrointestinal tract diseases

Murine models are invaluable tools for studying infectious diseases of the GIT, providing critical insights into disease mechanisms and potential therapies. These models aim to replicate human gastrointestinal disease conditions, offering researchers a platform to investigate host-pathogen interactions, immune responses, and therapeutic interventions.

Bacterial infection models: often specific bacteria are used to induce intestinal inflammation in mice, allowing the researchers to evaluate disease progression and immune responses. For example, *Citrobacter rodentium* infection in mice serves as a model for human enteropathogenic and enterohemorrhagic *E. coli* infections. This model has been instrumental in elucidating the roles of innate and adaptive immunity in controlling enteric bacterial infections [54].

Microbiota studies: gut microbiota plays a crucial role in protecting against GIT infections. Studies using germ-free mice, mice with defined microbial communities or specific-pathogen-free (SPF) mice have revealed the importance of the microbiome in modulating host susceptibility to pathogens [55].

Chemically induced models: using various chemical substances, like dextran sodium sulfate (DSS)-induced colitis or trinitrobenzene sulfonic acid (TNBS)-induced colitis, can be used to study how pre-existing inflammation affects susceptibility to enteric pathogens. DSS is directly toxic to colonic

epithelial cells, mimicking acute or chronic inflammatory conditions observed in diseases like Crohn's disease. These models are particularly useful for investigating the interplay between inflammation and infection in the GIT [56].

Humanized models: these models, which usually consist of immunodeficient mice engrafted with human immune cells or tissues, allow for the study of human-specific pathogens that do not naturally infect mice. Humanized mice through decades have been used not only in GIT diseases as *H. pylori* infection, but also for human-specific pathogens like Epstein-Barr virus (EBV), or HIV [55].

Genetically modified: genetically engineered mouse models allow researchers to dissect the roles of specific genes in GI infections. One of the most popular models is mice deficient in Nod2 – a gene linked to Crohn's disease – exhibit increased susceptibility to bacterial infections. Additionally, transgenic mice expressing human gastrin genes infected with *Helicobacter* have been used to study gastric cancer development [57]. Overall, murine models provide a versatile platform for studying infectious GIT diseases by combining bacterial infection approaches with microbiota studies, chemically induced inflammation models, humanized systems, and genetic modifications. These models have significantly advanced understanding of disease mechanisms and therapeutic strategies while highlighting the complex interplay between pathogens, host immunity, and the gut microbiome.

2. MATERIALS AND METHODS

2.1. Ethics statement

The studies were approved by the Lithuanian Ethics Committee of Biomedical Research on three different occasions: for pilot study Protocol no. G2-119 and G2-184 (Supplements 1 and 2), and for the therapeutics testing study Protocol no. G2-242 (Supplement 3). All regulated procedures on living animals were conducted in accordance with Directive 2010/63/EU of the European Parliament and the Council of European Union.

2.2. Study designs

2.2.1. Pilot study: lower GIT model *of K. quasipneumoniae* subs. *similipneumoniae* (DSM® 28212™) colonization and KvarIa therapy

Pilot study of lower GIT colonization was conducted using commercial in *Klebsiella quasipneumoniae* clinical isolate DSM® 28212™ in BALB/c mice. Study design was composed of four groups (total n = 12 mice (male n = 2; female n = 1 per group); 8–12 week-old): (PS1) Ctrl group with a natural microbiome, without any additional procedures during the experimental period; (PS2) Kqpn group without prior antibiotic disruption of the host microbiota and bacterial colonization of *K. quasipneumoniae* (50 µL suspension 10^9 CFU once per day, for three days, orally); (PS3) Pen:Strep group with antibiotic pre-treatment (n = 3 days) of penicillin (2000 U/mL) and streptomycin (2 mg/mL) (mechanism of action against Gram-negative and Gram-positive bacteria) and bacterial colonization of *K. quasipneumoniae*; (PS4) Pen:Strep:Met group with with antibiotic pre-treatment (n = 3 days) of metronidazole (1 g/L), penicillin (2000 U/mL) and streptomycin (2 mg/mL) (used to treat gastrointestinal and anaerobic infections) and bacterial colonization of *K. quasipneumoniae*. For the graphical study design, see Fig. 2.2.1.1.

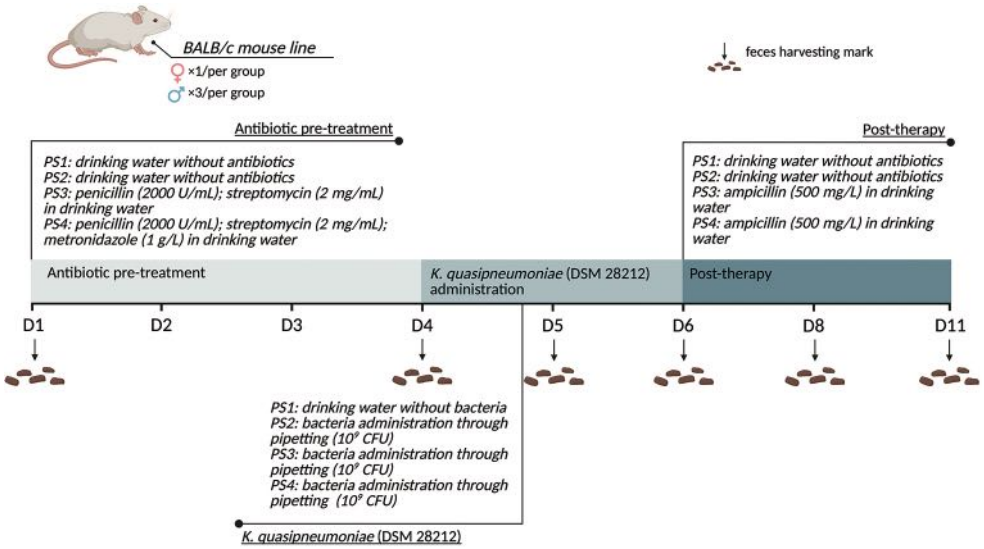

Fig. 2.2.1.1. *The scheme of Klebsiella quasipneumoniae (DSM® 28212™) Pilot Study colonization model design*

For KvarIa therapy testing, the same antibiotic pre-treatment and similar bacterial setup as previously described (*Section 2.2.1*) was conducted using commercial *Klebsiella quasipneumoniae* clinical isolate DSM® 28212 ™ in BALB/c mice. Study design was composed of four groups (total n = 12 mice (male n = 2; female n = 1 per group); 8–12 week-old): (PS5) Vehicle-only Control or VOC group received 100 µg of uncoated KvarIa after *K. quasipneumoniae* colonization (50 µL suspension orally; 10^9 CFU); (PS6) AbI:Ia group received 100 µg of Eudragit® S100-coated KvarIa after *K. quasipneumoniae* colonization (50 µL suspension orally; 10^9 CFU) with antibiotic pre-treatment (penicillin (2000 U/mL), streptomycin (2 mg/mL)) before; (PS7) AbII:Ia group received 100 µg of Eudragit® S100-coated KvarIa after *K. quasipneumoniae* colonization (50 µL suspension orally; 10^9 CFU) with antibiotic pre-treatment (penicillin (2000 U/mL), streptomycin (2 mg/mL), metronidazole (1 g/L)) before bacterial colonization; (PS8) Hi:Ia group received high dose (1000 µg) of Eudragit® S100-coated KvarIa after *K. quasipneumoniae* colonization (50 µL suspension orally; 10^9 CFU) with antibiotic pre-treatment (penicillin (2000 U/mL), streptomycin (2 mg/mL), metronidazole (1 g/L)) before bacterial colonization. Groups PS6; PS7 and PS8 were also given low-dose ampicillin therapy (500 mg/L) during bacterial inoculation to minimize the viability of non-*K. quasipneumoniae* bacteria. Fecal samples were collected at various time points for each mouse, as detailed in Fig. 2.2.1.2.

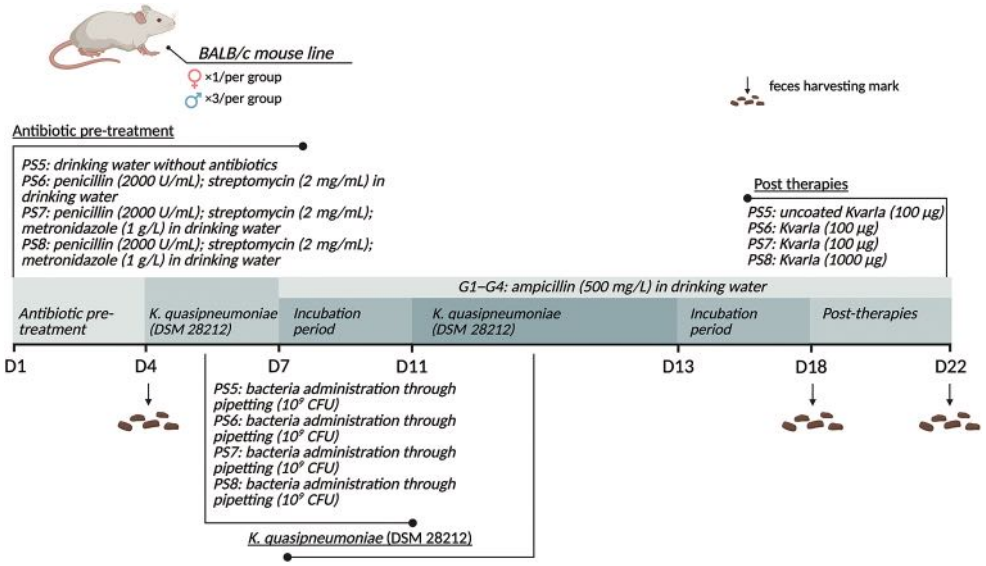

Fig. 2.2.1.2. *The scheme of Pilot study klebicin KvarIa therapy testing design*

2.2.2. GIT model of *K. pneumoniae* subs. *pneumoniae* (ATCC® 12657™) colonization

A new murine model of *K. pneumoniae* colonization, was conducted using commercial ATCC® 12657™ isolate in C57BL/6J strain mice. Study design was composed of four groups (n = 8 (m = 3; f = 1 per group); 8–12 week-old): (C1) KpnI group without antibiotic pre-treatment was given 50 µL suspension of *K. pneumoniae* orally (10^9 CFU); (C2) Ab:KpnI group with antibiotic pre-treatment (penicillin (2000 U/mL), streptomycin (2 mg/mL)) before *K. pneumoniae* colonization (50 µL suspension orally; 10^9 CFU) and low dose ampicillin therapy (500 mg/L) during and after bacterial inoculation; as an additional model based on the literature [58] two groups with bismuth subsalicylate were designed (C3) Kpn:Bis group with additional bismuth subsalicylate three days before *K. pneumoniae* inoculation; (C4) Bis:Bis group with bismuth subsalicylate prior, during and after *K. pneumoniae* inoculation. Fecal samples were collected at various time points for each mouse, as detailed in Fig. 2.2.2.1.

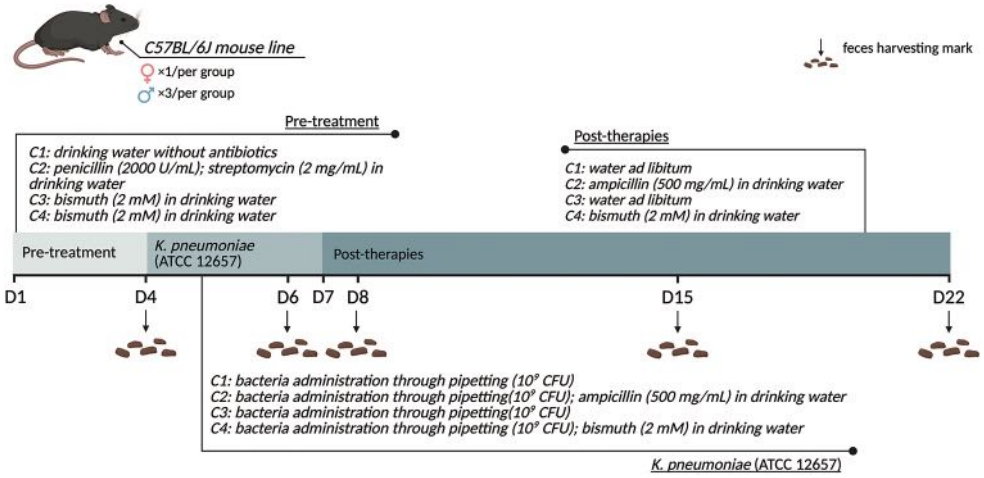

Fig. 2.2.2.1. *The scheme of Klebsiella pneumoniae (ATCC® 12657™) Colonization study design*

2.2.3. GIT model of *K. pneumoniae* subs. *pneumoniae* (ATCC® 43816™) colonization

Subsequently, after evaluation of study described in Section 2.2.2 an additional clinical isolate of *K. pneumoniae* ATCC® 43816™ was selected for testing in similar experimental design settings using the same C57BL/6J strain mice. The study design was comprised of four groups (n = 6 (m = 2; f = 1 per group); 8–10 week-old): (C5) KpnII group was inoculated with 50 µL suspension of *K. pneumoniae* (10^9 CFU) without using any additional therapies; (C6) Pen:Strep group with antibiotic pre-treatment (penicillin (2000 U/mL) and streptomycin (2 mg/mL)) in drinking water (ad libitum) before bacterial colonization for three days, followed by low-dose ampicillin therapy (125 µg/mL) during and after the colonization period (*K. pneumoniae* 50 µL suspension 10^9 CFU once per day, for three days, orally); (C7) AbI:Kpn group pre-treated with singular dose of streptomycin and penicillin (2000 U) before *K. pneumoniae* (10^9 CFU) 50 µL suspension inoculation for two consecutive days without any further treatments; (C8) AbI:Amp group pre-treated with high, singular dose of streptomycin and penicillin (2000 U) ad libitum, before *K. pneumoniae* (10^9 CFU) 50 µL suspension inoculation for two consecutive days and low dose ampicillin therapy (125 µg/mL). During this study, fecal samples from the mice were collected on the days indicated in Fig. 2.2.3.1.

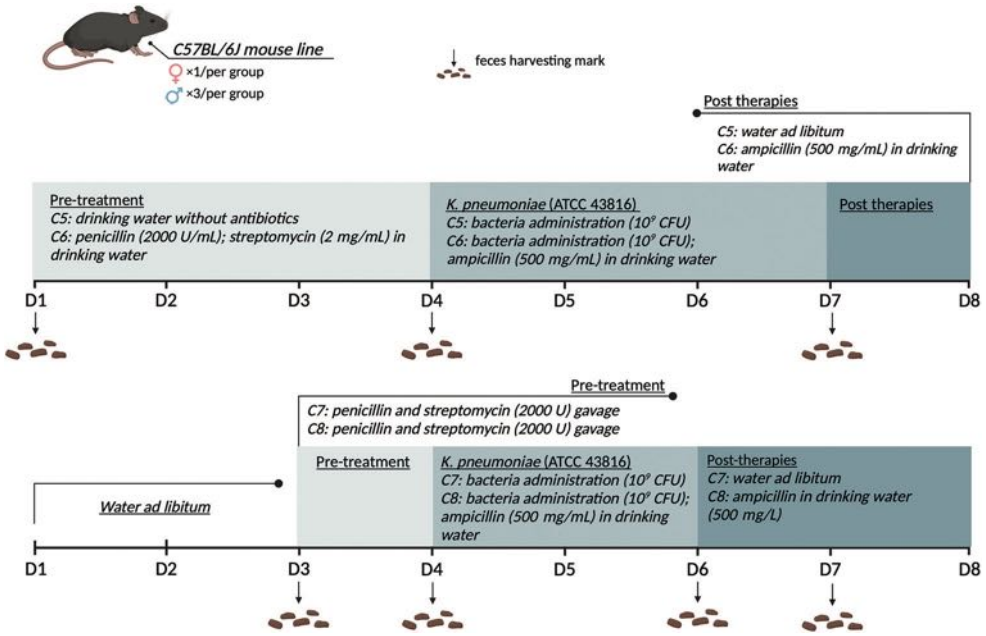

Fig. 2.2.3.1. *The scheme of Klebsiella pneumoniae (ATCC® 43816™) Colonization study design*

2.2.4. KvarM therapy for GIT infection of *K. pneumoniae* subs. *pneumoniae* (ATCC® 43816™)

Experimental animal study was design to evaluate the efficacy and assess the impact on microbiome of Eudragit-coated klebicin KvarM in the experimental murine model of *K. pneumoniae* colonization using C57BL/6J strain and composed of six groups (n = 25 (m = 3; f = 2 per group); 8–10 week-old): (M1) Vehicle Control or VC group was given Eudragit® S100 (0.125 μg/μL) and Eudragit® L100 (0.125 μg/μL) mix after *K. pneumoniae* inoculation of 50 μL suspension (10^7 CFU) for two consecutive days; (M2) KvarM:EudS group with Eudragit® S100 coated KvarM therapy (12.5 μg/100 μL) after *K. pneumoniae* inoculation of 50 μL suspension (10^7 CFU) for two consecutive days; (M3) KvarM:EudL group was given Eudragit® L100 coated KvarM therapy (12.5 μg/100 μL) after *K. pneumoniae* inoculation of 50 μL suspension (10^7 CFU) for two consecutive days; (M4) KvarM:EudSL group had Eudragit® S100 coated KvarM and of Eudragit® L100 coated KvarM therapies mix (2.5 μg/100 μL) after *K. pneumoniae* inoculation of 50 μL suspension (10^7 CFU) for two consecutive days; (M5) ColA-Ia:EudSL group, received a Colicin-A and KvarIa hybrid coated with Eudragit® S100 and Eudragit® L100 (12.5 μg/100 μL) after *K. pneumoniae* inoculation of 50 μL suspension (10^7 CFU) for two consecutive days, it was tested to evaluate

whether the hybrid has the same effect against *K. pneumoniae* colonization as KvarM, a bacteriocin specifically targeting KvarM; (M6) Kpn:AB group with antibiotic (ciprofloxacin 2 mg/200 µL) therapy after *K. pneumoniae* inoculation of 50 µL suspension (10^7 CFU) for two consecutive days. Fecal samples were collected at various time points based on the therapy administered. For more details, refer to Fig. 2.2.4.1.

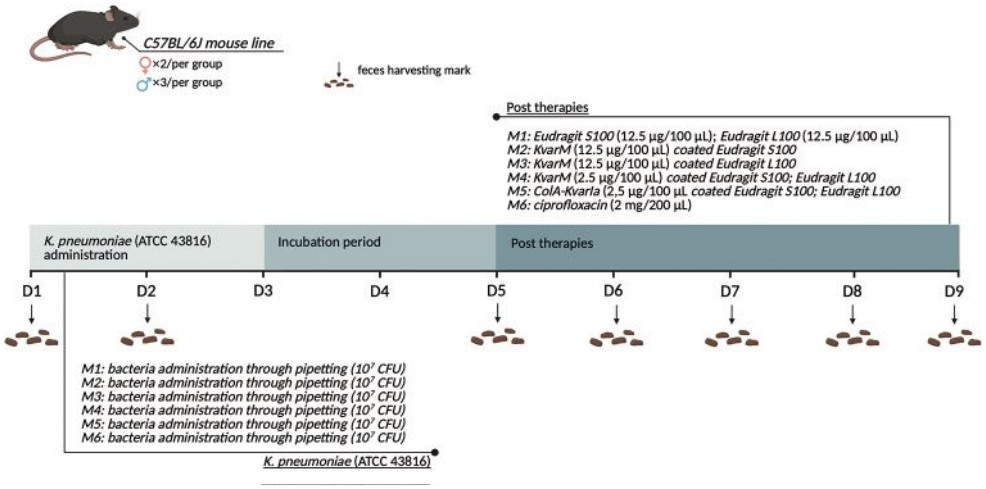

Fig. 2.2.4.1. *Schematic representation of the Therapeutic study design involving klebicin KvarM and chimericin ColA-Ia*

2.2.5. Vehicle Control study

The experimental study was designed to examine microbiome changes in vehicle-only control groups, meaning that the substances used in previous experiments were tested independently. The murine model was conducted using C57BL/6J strain mice without any bacterial colonization and composed of four groups (n = 20 (m = 3; f = 2 per group); 8–12 week-old): (VC1) Control or Cont group with a natural microbiome, without undergoing any procedures or therapies; (VC2) Eud group received Eudragit® S100 and Eudragit® L100 (12.5 µg/100 µL); (VC3) KvarM:Eud group was given KvarM coated with Eudragit® S100 and Eudragit® L100 (500 µg/100 µL); (VC4) AB group received ciprofloxacin (2 mg/200 µL) to assess the antibiotic's impact on commensal microbiome. The detailed experimental design and fecal sampling days are shown in Fig 2.2.5.1.

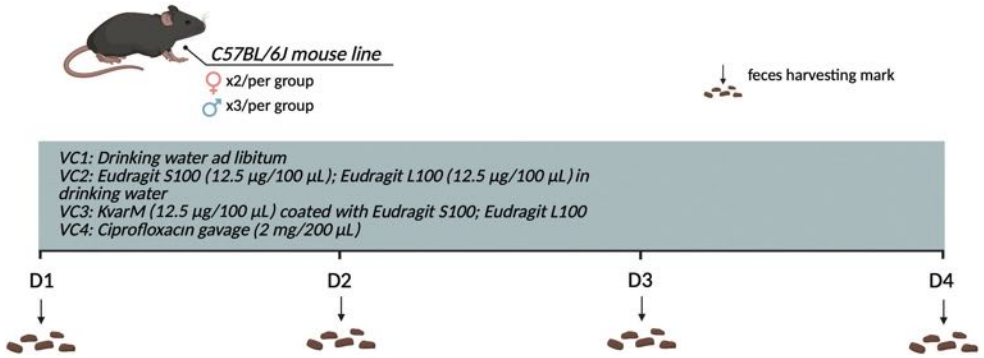

Fig. 2.2.5.1. *Study design scheme for testing the effects of therapies on the microbiome*

2.3. Klebicin production in plants and purification

The extraction and purification of bacteriocins were carried out by our partners Nomads, UAB. Briefly, for their production, the klebicin-coding gene with plant-optimized codons was cloned into a magnICON® tobacco mosaic virus-based vector. The resulting binary expression vector was introduced into *Agrobacterium tumefaciens*. The transformed agrobacteria were then used for transient expression of bacteriocin in plants by infiltrating the bacterial strain into the leaves of young *Nicotiana benthamiana* plants under vacuum. The bacteriocin was subsequently purified to homogeneity from the crude plant extracts using a two-step protein chromatography process. For further details, please refer to the published study by E. Denkovskiene et al. from our partners, Nomads, UAB [50].

2.4. Determination of pH of the gastrointestinal tract

Fecal samples were collected at multiple time points during the pilot study described in Section 2.2.1 to determine GIT pH and optimize the formulation for klebicins. The acquired samples were homogenized with deionized water at a 1:10 ratio until a uniform mass was achieved. The pH was measured using a Mettler Toledo pH meter (Ohio, US) equipped with an InLab Ultra-Micro electrode. Additionally, pH measurements were taken from intestinal samples collected from mice during laparotomy. The GIT was divided into three sections: the stomach, duodenum, and rectum for sampling (Fig. 2.4.1). Altogether, these measurements provided essential data for developing an effective bacteriocin formulation with Eudragit®, designed to withstand the pH conditions within the GIT.

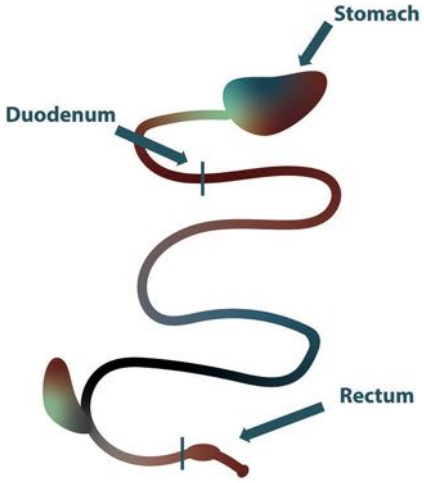

Fig. 2.4.1. *GIT location where the tissue samples were collected*

2.5. Coating of the klebicins

2.5.1. Eudragit® S100 coating

Initially, Eudragit® S100 was used in the pilot study as a formulation for klebicin to prevent its premature release in the digestive tract. A 5% Eudragit® S100 solution was prepared by dissolving 0.5 g of Eudragit® S100 (Evonik Industries, Germany) in 10 mL of Milli-Q water, followed by 30 minutes of sonication in an ultrasonic bath at 25 °C. 250 µg of klebicin was then mixed with 200 µg of the 5% Eudragit® S100 solution and the resulting mixture was freeze-dried at – 51 °C for 24 hours.

2.5.2. Eudragit® S100 and Eudragit® L100 coating

Following the pilot studies of pH measurements, it was decided to use two types of bacteriocin formulations, Eudragit® S100 and Eudragit® L100. This approach was chosen to prevent the dissolution of the Eudragit coating in the alkaline environment of the mouse oral cavity and to avoid the premature release of antimicrobial agents before they reach the upper and lower digestive tract, where *K. pneumoniae* bacteria typically colonize. To prepare a 5% Eudragit® L100 solution, 100 mg of Eudragit® L100 powder (Evonik Industries, Germany) was dissolved in 2 mL of 50 mM phosphate buffer, with 2M NaOH added until the pH reached 6–7 to ensure full dissolution. Similarly, a 5% Eudragit® S100 solution was created by dissolving 100 mg of Eudragit® S100 powder (Evonik Industries, Germany) in 2 mL of 50 mM phosphate buffer, with 2M NaOH added until the pH reached 8 for

complete dissolution. Lyophilized bacteriocin samples (0.25 mg each) were dissolved in 250 µL of distilled water, then mixed with 250 µL of the 5% Eudragit® L100 or Eudragit® S100 solution. The bacteriocin-Eudragit mixtures were then carefully acidified with 2M HCl until the pH reached 4–5, inducing Eudragit polymerization, as indicated by the change from a clear solution to a white suspension. The resulting samples were lyophilized and dissolved in distilled water prior to use.

2.6. Bacteria cultivation

2.6.1. *K. quasipneumoniae* cultivation

K. quasipneumoniae DSM® 28212™ was obtained from the Leibniz Institute (DSMZ) and used in the pilot studies. The bacterial culture was grown in LB nutrient medium (Gibco by Life Technologies, USA) supplemented with 100 µg/mL ampicillin to maintain selective growing. Cultures were incubated for approximately 72 hours at 37 °C with shaking at 180 rpm to promote optimal bacterial growth. All cultivation steps followed the supplier's guidelines to ensure the viability and integrity of the bacterial strain.

2.6.2. *K. pneumoniae* cultivation

K. pneumoniae subsp. *pneumoniae* ATCC® 12657™ and *K. pneumoniae* subsp. *pneumoniae* ATCC® 43816 ™ strains were obtained from the Global Bioresource Center (ATCC®). *K. pneumoniae* ATCC® 12657™ was used in the GIT model of *K. pneumoniae* colonization study (*see Section 2.2.2*), similarly *K. pneumoniae* ATCC® 43816™ was used in the GIT model of *K. pneumoniae* colonization (*see Section 2.2.3*) and KvarM therapy for GIT infection (*see Section 2.2.4*) studies. Both bacterial cultures were grown and cultivated using the same methodology as *K. quasipneumoniae*. Bacterial cultures were grown according to the supplier's recommendations in LB medium (Gibco by Life Technologies, USA), with *K. pneumoniae* ATCC® 12657™ cultured using 50 µg/mL ampicillin, and *K. pneumoniae* ATCC® 43816™ cultured with 25 µg/mL ampicillin. Cultures were incubated for approximately 72 hours at 37 °C with shaking at 180 rpm to promote optimal bacterial growth.

2.7. Nucleic acid extraction and data analysis

2.7.1. Nucleic acid extraction and synthesis of cDNA

Bacterial deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted from rectal excrement samples using the AllPrep PowerFecal DNA/RNA Kit (Qiagen, Germany). Extraction procedures utilized up to 200 mg of fecal material. The concentration and purity of the extracted nucleic acids were assessed using either the NanoDrop 2000 (Nanodrop Technologies, Wilmington, DE, USA) or the Qubit 4 (Invitrogen, Carlsbad, CA, USA), depending on the intended application, such as sequencing or real-time quantitative PCR (qRT-PCR). All steps followed the manufacturer's protocol. Complementary DNA (cDNA) was subsequently synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Lithuania). All procedures adhered to the manufacturer's guidelines.

2.7.2. Quantative assesments of *Klebsiella* spp.

The *khe* gene, which encodes for haemolysin, was selected as a marker for identifying the *Klebsiella* genus. A standard curve was established using DNA samples from *K. quasipneumoniae* DSM® 28212™ (Fig. 2.7.2.1), *K. pneumoniae* ATCC® 12657™ (Fig. 2.7.2.2), and *K. pneumoniae* strain ATCC® 43816™ to enable quantitative assessment (Fig. 2.7.2.3).

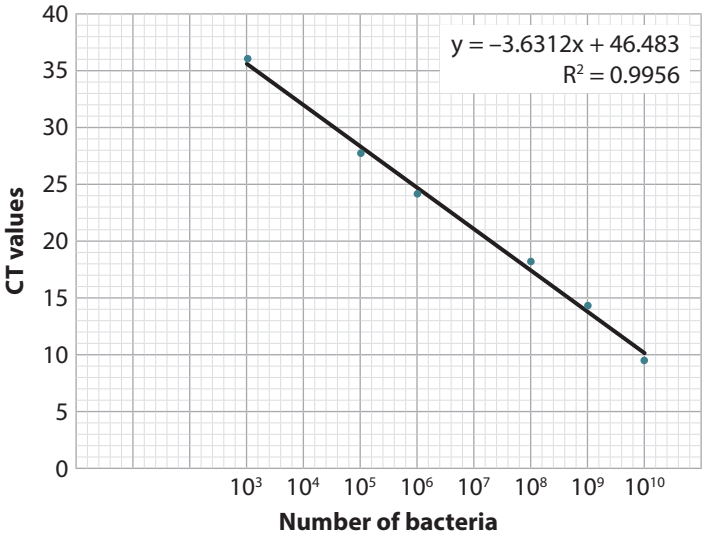

Fig. 2.7.2.1. *Standard curve of Klebsiella quasipneumoniae DSM® 28212™*

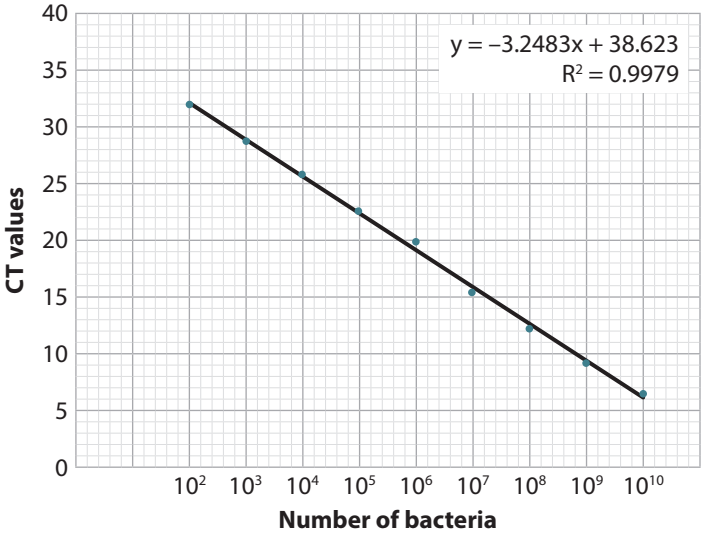

Fig. 2.7.2.2. *Standard curve of Klebsiella pneumoniae ATCC® 12657™*

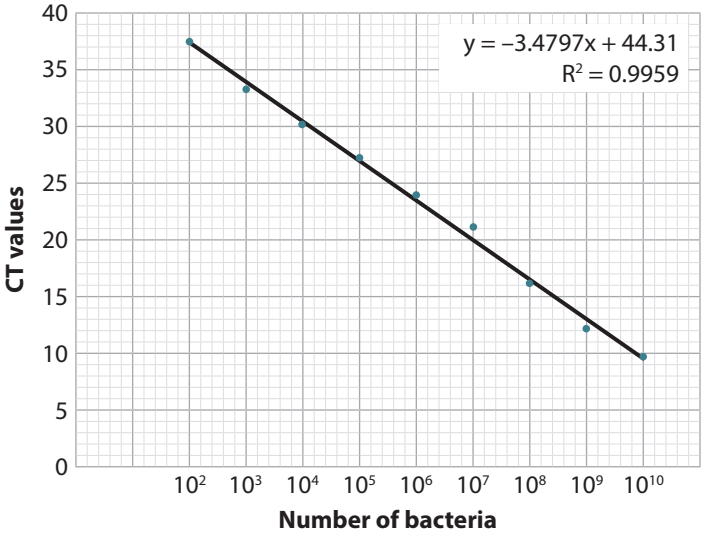

Fig. 2.7.2.3. *Standard curve of Klebsiella pneumoniae ATCC® 43816™*

qRT-PCR was performed using TaqMan Universal Master Mix II with UNG, a TaqMan probe (5'-6-FAM-CGCGAACTGGAAGGGCCCG-TAMRA-3'), and primers (Forward: 5'-GATGAAACGACCTGATTGCATTC-3' and Reverse: 5'-CCGGGCTGTCGGGATAAG-3') from Applied Biosystems, USA, following the manufacturer's protocol. Amplification of the *khe* gene was conducted on an ABI Fast 7500 System (Life Technologies, Carlsbad, CA, USA) using the standard protocol.

For controls, DNA isolated from *K. pneumoniae* served as the positive control, while DNA from *E. coli* was used as the negative control. For each qRT-PCR reaction, we aimed to normalize all samples to approximately 60 ng of DNA per reaction, with only minimal variation, depending on the specific procedures performed on the mice during the study. The DNA-based standard curve was generated using *K. quasipneumoniae* concentrations ranging from 10^3 to 10^{10} CFU in 200 µL, with DNA extracted from bacteria culture using the QIAamp Fast DNA Stool Mini Kit, following the protocol for liquid samples.

2.8. 16S rRNA gene sequencing of V1–V2 hypervariable regions

To analyse the microbiome, 16S rRNA gene sequencing was conducted on an Illumina MiSeq platform using the MiSeq Reagent kit V3 (2 × 300 cycle reagent kit) (Illumina, Inc., San Diego, USA). Amplicons targeting the V1–V2 hypervariable region were produced following a custom library preparation protocol, as detailed in our previous publication [59]. The library was prepared using the Platinum SuperFi PCR Master Mix kit (Invitrogen, USA) along with barcoded forward (27F – 5′-AGAGTTTGATCCTGG CTCAG-3′) and reverse (338R – 5′-TGCTGCCTCCCGTAGGAGT-3′) primers with dual indexes on both pairs of pimers which are incorporated during the PCR process. Each sample was processed in two technical replicates. The cycling conditions were as follows: initial denaturation at 98 °C for 30 seconds, followed by 34 cycles of 98 °C for 9 seconds, 50 °C for 1 minute, and 72 °C for 20 seconds, with a final extension at 72 °C for 10 minutes and a hold at 10 °C. PCR products were purified and normalized using the SequalPrep Normalization Plate Kit (Invitrogen, Thermo Fisher Scientific). All procedures followed the manufacturer's protocols and recommendations.

2.9. Bioinformatical and statistical analysis

Statistical and bioinformatic analyses were conducted using various software platforms. Statistical evaluations were performed using the RStudio software (2019–2022 RStudio, PBS), *Friedman's test* and *Kruskal-Wallis test* were utilized to compare qRT-PCR data tendencies between the studied groups, afterwards using *Mann-Whitney U* test for non-parametric data points. Results were considered statistically significant when $p \leq 0.05$.

Bioinformatic processing of microbiome data was also carried out in the R environment, as previously described in our study [60]. Paired-end fastq files, after removal of barcodes and adapters, underwent quality control, denoising,

and preparation for further analysis using the dada2 package [61]. The amplicon sequence variant (ASV) table was annotated with bacterial sequences using the Silva database (version 138.1). Data normalization and α-diversity, β-diversity analyses across different taxonomic levels were performed using the DESeq2 package, with statistical significance determined by an adjusted p-value (p.adj) < 0.05.

Differences in bacterial abundance between study groups were evaluated by PERMANOVA statistical tests, using 9999 permutations. Bacterial alpha diversity, representing richness and evenness of microbial species within a specific sample group, was examined based on bacterial abundance (Simpson, Shannon, Richness diversity indexes). Statistical differences were identified using either the Mann-Whitney, Kruskal-Wallis, or DESeg2 algorithm tests, followed by Benjamini-Hochberg correction for multiple comparisons. The analysis of bacterial beta diversity representing the variation in microbial composition and structure between the groups was performed only on the bacteria which were present in more than 10% or 25% of the samples in at least one of the comparison groups. Kaplan-Meier curves were used for the correlation of the survival data. Log-Rank of Mantel-Cox with 95% confidence interval followed the Kaplan-Meier analysis to determine statistical differences. For all tests, differences were considered statistically significant when the corrected p-value (q-value of p_{adj}) was 0.05. Principal Coordinate Analysis (PcoA) was performed using weighted and unweighted Unifrac distance matrices to visualize the distribution of complex data.

3. RESULTS

3.1. Pilot study: lower GIT model of *K. quasipneumoniae* (DSM® 28212™) colonization and KvarIa therapy

3.1.1. pH measurements along the GIT

To evaluate the effective coating and delivery of klebicins in the GIT, the pH levels in feces and along various regions of the GIT tissues (post-mortem) were measured. The pH in feces was tracked through different time points across groups seen in Fig. 3.1.1.1. In the Ctrl group, pH levels remain relatively stable, ranging between 6.5 and 8.1 with an average of 7.3 ± 0.52. The Kqpn group showed similar pH values compared to the Ctrl group with the average of 7.5 ± 0.74 and the peak on day 11 reaching pH of 9.94. In the Pen:Strep group, values fluctuated within a narrow range between 7.2 and 8.2 with the average of 7.7 ± 0.29. Meanwhile, the Pen:Strep:Met group showed more fluctuation with a drop in pH on Day 4 (av. 6.57 ± 0.26) but followed by a recovery by Day 11 with an average of 8.11 ± 0.27.

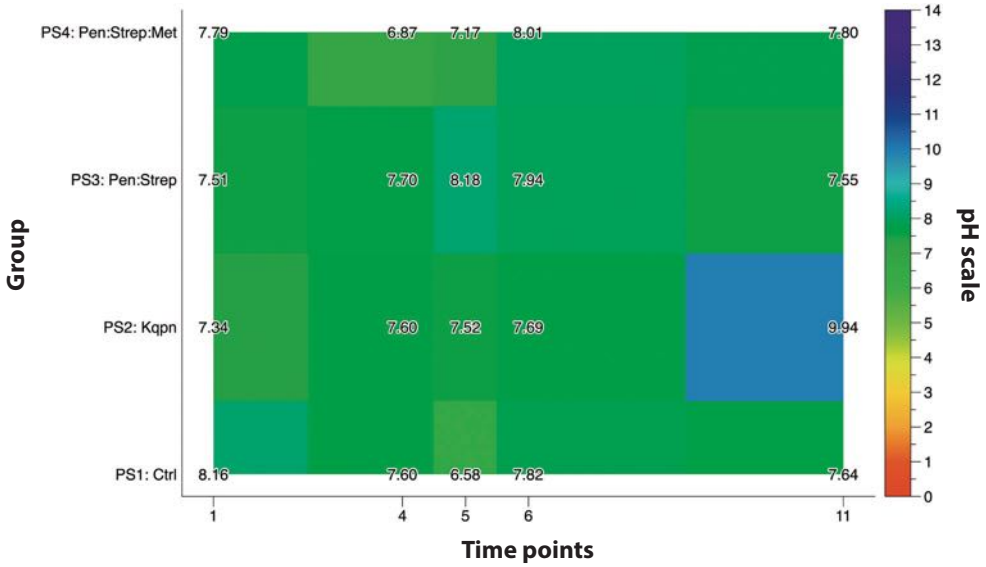

Fig. 3.1.1.1. *The fecal pH measurements through different time points in groups*

Time points correspond to sample collection days. PS1 Ctrl group: Control group with a natural microbiome. PS2 Kqpn group: 50 µL/10^9 CFU *Klebsiella quasipneumoniae* colonization. PS3 Pen:Strep group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 500 mg/L ampicillin therapy. PS4 Pen:Strep:Met group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 1 g/L metronidazole; 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 500 mg/L ampicillin therapy.

The pH analysis across different sections of the GIT was performed using tissues taken post-mortem (Fig. 3.1.1.2). The fecal samples showed the same trends as in feces pH analysis. Within the small intestine (duodenum) the Pen:Strep group displayed the lowest pH (av. 6.07 ± 0.08), suggesting increased acidity potentially induced by antibiotic treatment. The large intestine (rectum) showed relatively stable pH values, ranging from 6.5 to 7.7 across all groups, with the average of 7.48 ± 0.42. The stomach exhibited the most acidic environment, with pH values between 2.34 and 4.73, with the average of 7.69 ± 0.39, clearly seen in the Pen:Strep:Met and Kqpn groups. The significant differences in pH measures were found between the Ctrl group and Pen:Strep group ($p = 0.004$) in small intestine, as well as, between Ctrl group and Pen:Strep:Met group ($p = 0.04$) in large intestine.

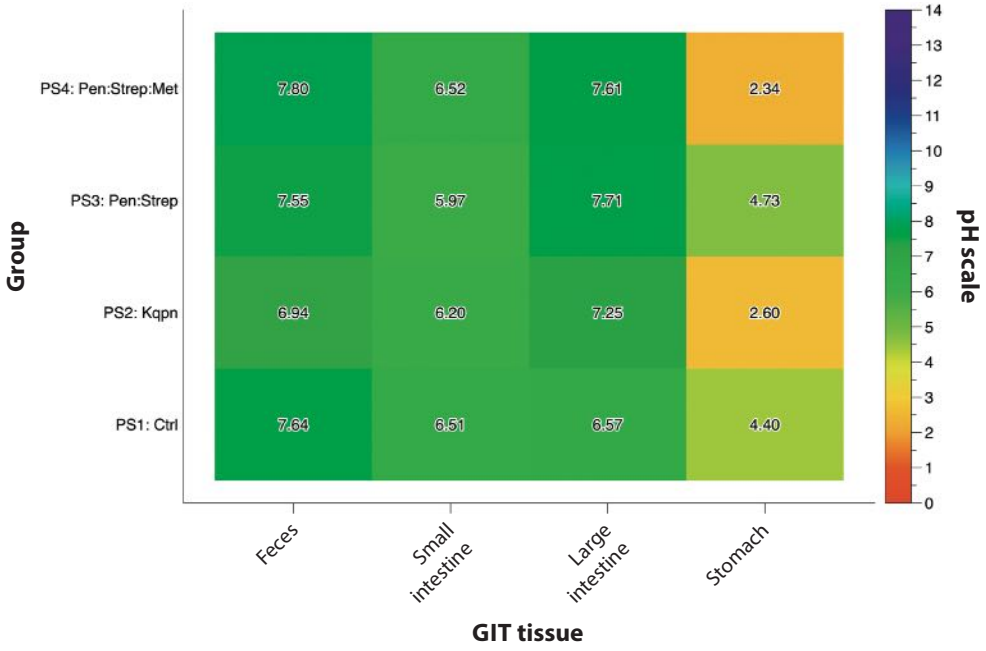

Fig. 3.1.1.2. *The pH measurements of GIT tissues post-mortem*

GIT tissue scale shows the locations of biopsies within the gastrointestinal tract. PS1 Ctrl group: Control group with a natural microbiome. PS2 Kqpn group: 50 µL/10^9 CFU *Klebsiella quasipneumoniae* colonization. PS3 Pen:Strep group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 500 mg/L ampicillin therapy. PS4 Pen:Strep:Met group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 1 g/L metronidazole; 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 500 mg/L ampicillin therapy.

3.1.2. Colonization model of GIT with antibiotic pre-treatment

The GI tract colonization model was established in mice using *K. quasipneumoniae*. It was, primarily, designed to reflect bacterial colonization in the host after the disruption of natural microflora with antibiotics therapy and to test the colonization model with and without antibiotic pre-treatment. CT values of *khe* gene remained constant (undetectable) throughout the time points in the Ctrl group and Kqpn group, indicating no detectable bacterial load or amplification. The use of antibiotic pre-treatment (Fig. 3.1.2.1), in order to disrupt the host gut microbiota, showed sustained bacterial colonization from day 4 to day 8 in Pen:Strep (av. 5.26×10^8 CFU; $p = 2.73 \times 10^{-7}$) and Pen:Strep:Met (av. 5.16×10^8 CFU; $p = 10^{-6}$) groups. Nonetheless, the bacterial counts remained relatively stable on day 11 showing the average of 3.12×10^8 CFU in Pen:Strep group and 4.04×10^8 CFU in Pen:Strep:Met group, with seeing no significant difference in both groups from day 8 indicating stabilization. The data strongly suggest that colonization of mice gut by *K. quasipneumoniae* can be more effectively established after the disruption of natural gut microbiota.

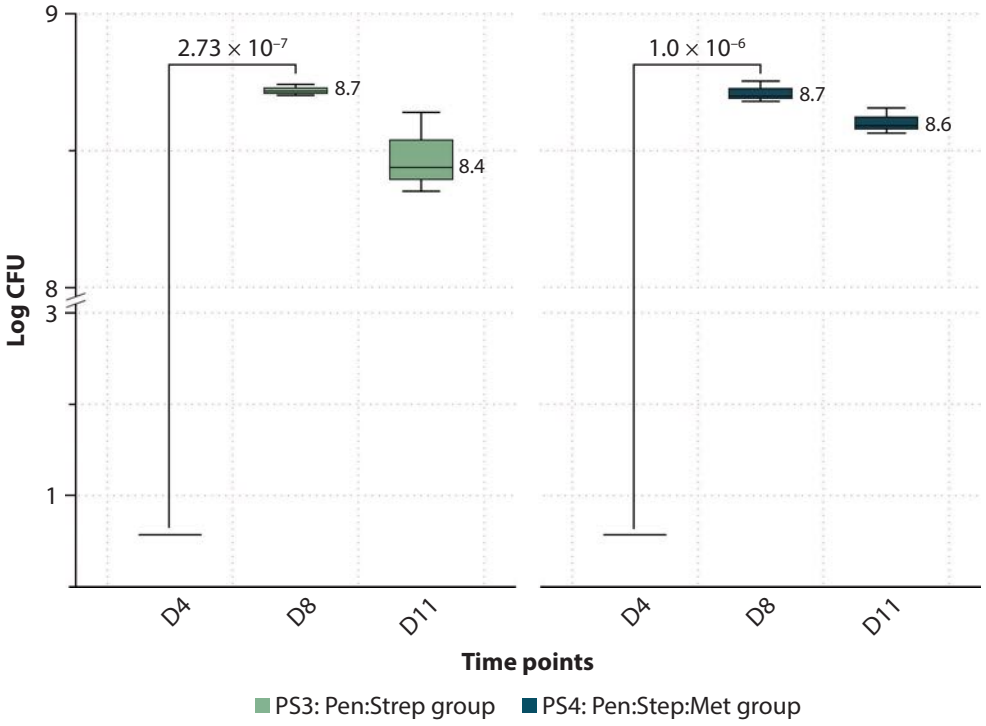

Fig. 3.1.2.1. *The colonization of Klebsiella quasipneumoniae in groups with antibiotic pre-treatment*

p-values represented on the top line; whiskers represent minimum and maximum values, middle line – median. Time points correspond to sample collection days (D4, D8, D11). Log CFU shows *Klebsiella quasipneumoniae* bacterial counts. PS3 Pen:Strep group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 500 mg/L ampicillin therapy. PS4 Pen:Strep:Met group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 1 g/L metronidazole; 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 500 mg/L ampicillin therapy.

3.1.3. Reducing *K. quasipneumoniae* colonization of GIT using KvarIa

In this study, four groups of mice (Fig. 2.2.1.2) were used to evaluate the effects of different antibiotic pre-treatments and post-therapies on bacterial colonization. To reduce the bacterial colonization of GIT using bacteriocin, three different combinations of recombinant klebicin KvarIa were used. VOC group served as a control with uncoated Kvarla (100 µg) post-therapy, while groups AbI:Ia group and AbII:Ia group received coated Kvarla (100 µg) post-therapy after varying pre-treatments (Fig. 3.1.3.1), and Hi:Ia group received higher dose of KvarIa (1000 µg). Fecal samples were collected on days 4, 18, and 22 to measure bacterial load using the amplification of the *khe* gene as a marker. The bacterial load of *K. quasipneumoniae* decreased significantly in the AbI:Ia and AbII:Ia groups. In the AbII:Ia group, the bacterial load dropped from 6.3 x 10^7 CFU on day 18 to 3.9×10^5 CFU on Day 22 ($p = 1.0 \times 10^{-2}$). Similarly, in the Hi:Ia group, the bacterial load decreased from 4.0×10^7 CFU on day 18 to 1.6×10^5 CFU on day 22 ($p = 9.0 \times 10^{-3}$). No significant changes in bacterial loads were observed in the VOC group after administering uncoated KvarIa and in the AbI:Ia group after administering post-treatments (Fig. 3.1.3.1).

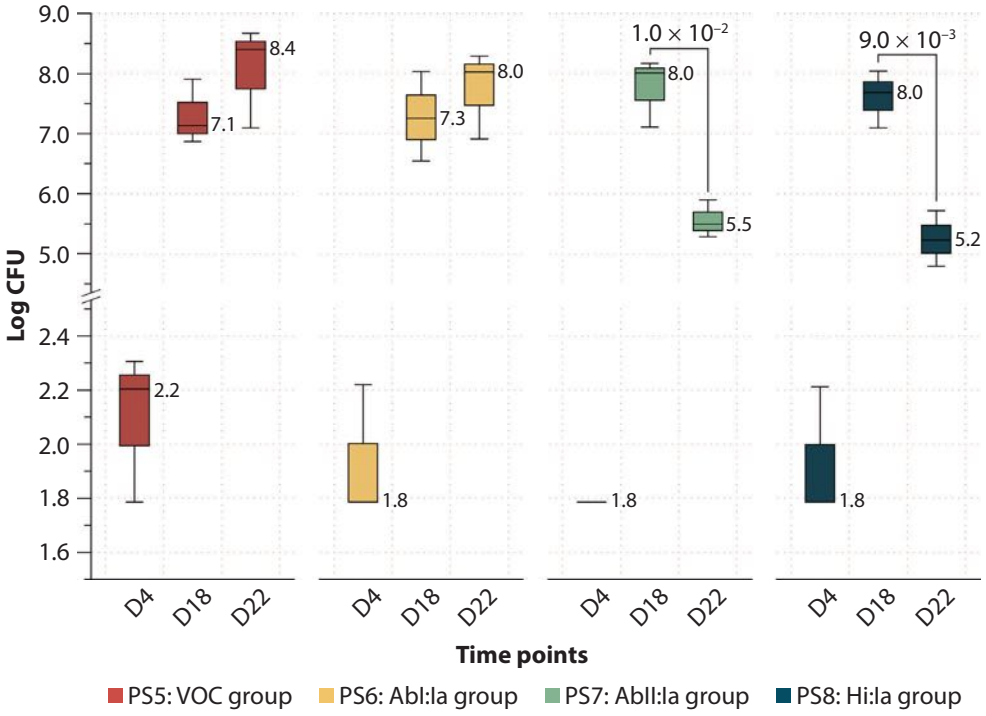

Fig. 3.1.3.1. *Klebsiella quasipneumoniae colonization in the gut after administration of klebicin KvarIa*

D – days; p-values represented on the top line; whiskers represent minimum and maximum values; middle line – median. Time points correspond to sample collection days (D4, D18, D22). Log CFU shows *Klebsiella quasipneumoniae* bacterial counts. PS5 Pen:Strep group: 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 100 µg uncoated KvarIa. PS6 AbI:Ia group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 100 µg Eudragit® S100-coated KvarIa. PS7 AbII:Ia group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 1 g/L metronidazole; 50 µL/10^9 CFU *Klebsiella quasipneumoniae* suspension; 100 µg Eudragit® S100-coated KvarIa. PS8 Hi:Ia group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 1 g/L metronidazole; 50 µL/10^9 CFU bacterial suspension; 1000 µg Eudragit® S100-coated KvarIa.

3.2. GIT model of *K. pneumoniae* (ATCC® 12657™) colonization

3.2.1. *K. pneumoniae* (ATCC® 12657™) colonization

Further, we worked on the design of the model for GIT *K. pneumoniae* colonization. The groups received pre-treatment with or without antibiotics, or bismuth. Fecal samples were collected at different time points (days 4, 6, 8, 15, and 22) to measure the bacterial load. The data showed that the Ab:KpnI group consistently exhibited the highest bacterial loads across all time points with the average of 8.64×10^7 CFU. The KpnI group experienced

a significant decline in bacterial colonization with the average of 1.21×10^4 CFU on day 8 and 6.10×10^1 CFU on day 22 ($p = 1.06 \times 10^{-3}$), suggesting the potential of microbiome self-clearance. The Kpn:Bis (av. 2.69×10^5 CFU) and Bis:Bis (av. 3.33×10^5 CFU) groups showed stable and notably lower bacterial loads, with no significant differences to them. Overall, Ab:KpnI group showed statistically significant in almost all time points compared to other groups, repeating previous results as antibiotic pre-treatment greatly increases bacterial colonization.

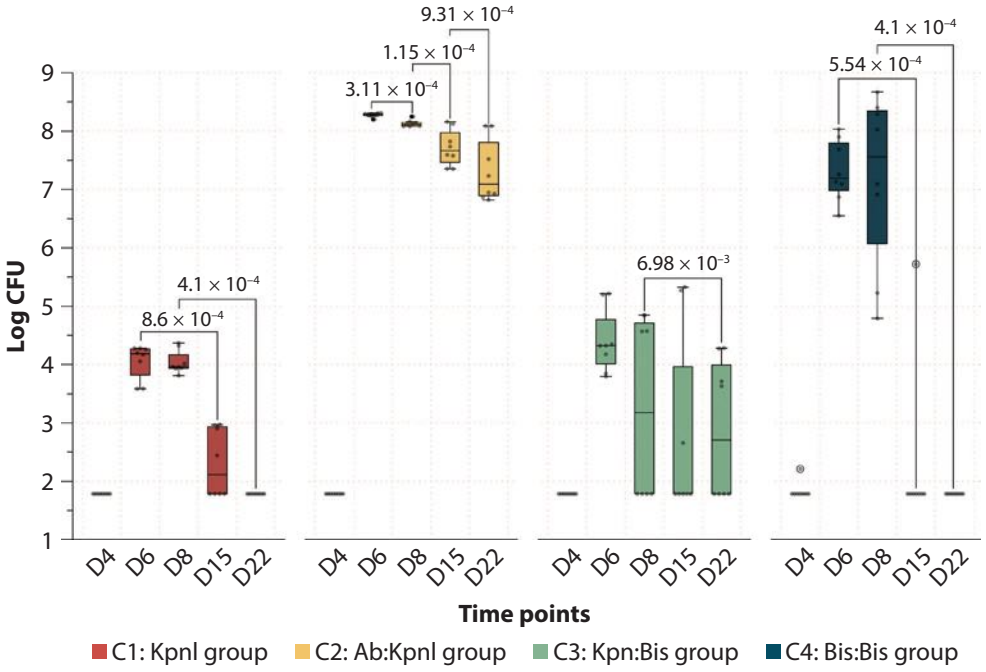

Fig. 3.2.1.1. *Klebsiella pneumoniae colonization model using antibiotic pre-treatment and bismuth*

D – days; p-values represented on the top line; whiskers represent minimum and maximum values; middle line – median. Time points correspond to sample collection days (D4, D6, D8, D22). Log CFU shows *Klebsiella pneumoniae* bacterial counts. C1 KpnI group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C2 Ab:KpnI group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C3: Kpn:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C4: Bis:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension.

3.2.2. pH measurements along the GIT of *K. pneumoniae* (ATCC® 12657TM) colonization

To support the results of the pilot studies further, the pH measurements were taken throughout this study period (Fig. 2.2.2.1) using fecal samples. The samples showed relatively stable trends across the three study groups with no significant shifts between days. Ab:KpnI group showed the pH values ranging between 5.93 and 8.28, with the avarage of 7.00 ± 0.53. Kpn:Bis group measures were ranging from 6.37 to 8.33, with the average of 7.03 ± 0.39. Bis:Bis group pH values were between 6.58 and 8.60, had the average of 7.14 ± 0.43. However, KpnI group showed statistically significant decrease in pH on day 15 compared to day 1, with changes from 6.25–7.00 to 7.02–7.66 ($p = 3.43 \times 10^{-3}$).

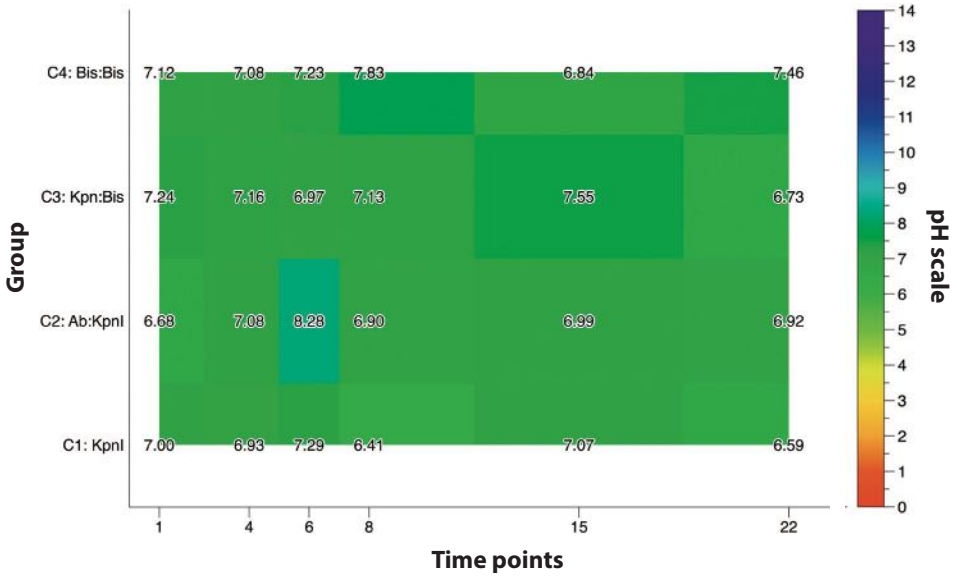

Fig. 3.2.2.1. *The fecal pH measurements through different time points in groups*

Time points correspond to sample collection days (1, 4, 6, 8, 15, 22). C1 KpnI group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C2 Ab:KpnI group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C3 Kpn:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU Klebsiella pneumoniae suspension. C4 Bis:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension.

3.2.3. Changes in mice microbiome during *K. pneumoniae* (ATCC® 12657™) colonization

To characterize the microbiome profiles of the KpnI, Ab:KpnI, Kpn:Bis and Bis:Bis groups in the *K. pneumoniae* (ATCC® 12657™) colonization study, a compositional analysis of fecal samples was performed using relative abundances of microbial taxa at the phylum, family, and genus levels. Samples collected on days 4, 6, and 8 revealed that the fecal microbiome across all groups was comprised of seven bacterial phyla. Among these, three phyla consistently dominated across all groups and time points: *Bacteroidota* (55.8%), *Proteobacteria* (21.82%), *Firmicutes* (21.1%), with the latter being more abundant on day 4 but showing a slight decrease afterwards (Fig. 3.2.3.1).

Further, metrics of diversity (α-diversity, β-diversity) were analyzed in all groups across the different time points. Bacterial diversity (α-diversity), represented by the Shannon diversity index, show significant differences only within the Ab:KpnI group when comparing antibiotic pre-treatment and bacterial colonization on day 6 to the subsequent low-dose ampicillin post-treatment on day 8 ($p = 5.26 \times 10^{-4}$). No significant differences in bacterial diversity were observed in any of the other groups (Fig. 3.2.3.2).

Additionally, microbial community clusters (β-diversity) based on Bray-Curtis, UniFrac weighted and UniFrac unweighted dissimilarity indexes showed similar trends indicating significant sample clustering between Ab:KnpI group and KpnI, Kpn:Bis, Bis:Bis groups ($p < 0.05$), whereas group Ab:KpnI formed a separate cluster (Fig. 3.2.3.3).

Thereafter, analysis of differential abundance was performed to further investigate the differences in microbiome composition between this experimental study group. Abundance of bacterial families such as *Enterobacteriaceae*, *Erysipelatoclotridiaceae*, *Desulfovibrionaceae* and *Atopobiaceae* were enriched, while *Lactobacillaceae*, *Clostridiaceae*, *Lactobacilacea*, *Erysipelotrichaceae*, *Butyricicoccaceae* were depleted following experimental days throughout study groups KpnI, Kpn:Bis and Bis:Bis. The antibiotic-treated group Ab:KpnI showed distinct differences from other groups, characterized by enrichment of *Bacteroidaceae* and *Muribaculaceae* at the family level, as well as *Bacteroides* and *Muribaculum* at the genus level. Thus, depletion was observed in *Clostridiaceae* and *Enterobacteriaceae* families, alongside a reduction of *Klebsiella* at the genus level.

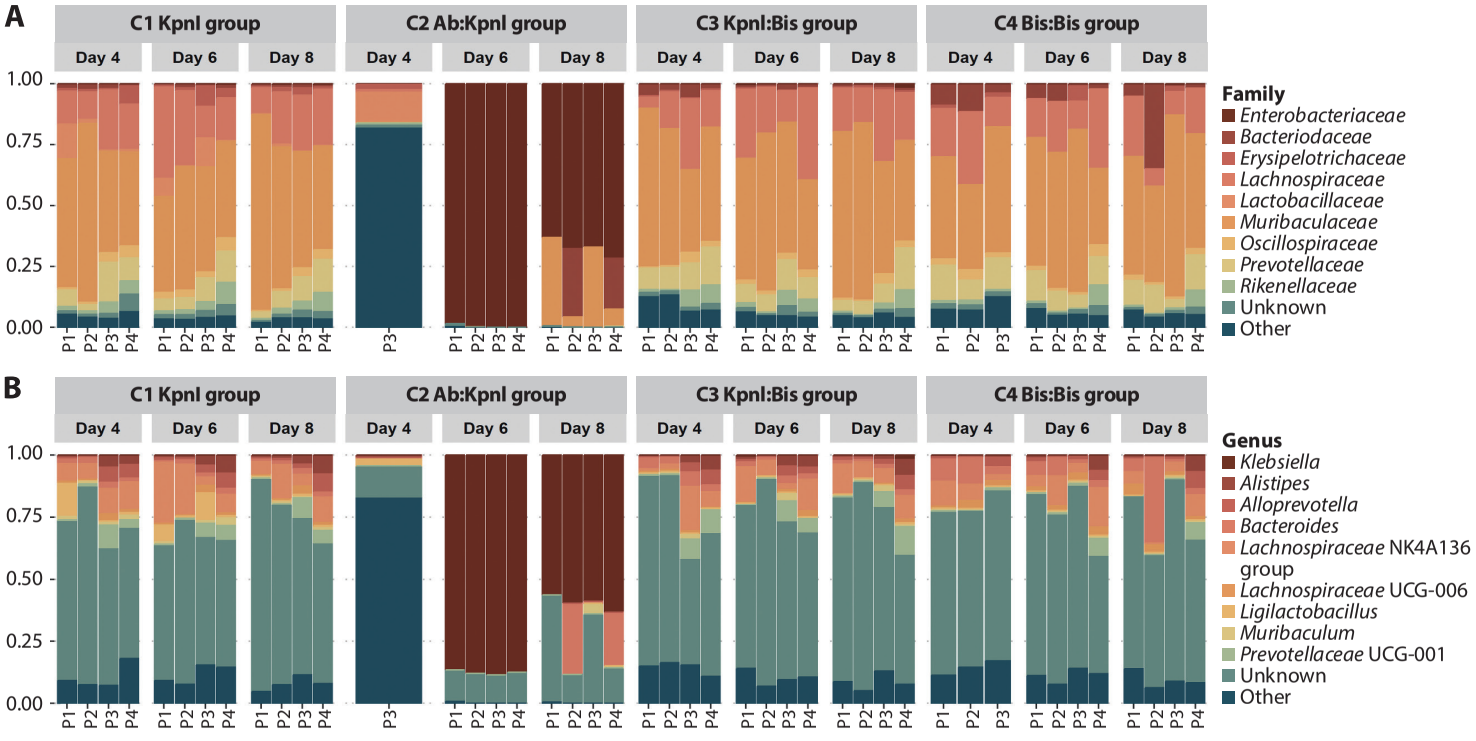

Fig. 3.2.3.1 (A–B). *The composition of mice fecal microbiome during Klebsiella pneumoniae (ATCC® 12657™) colonization*

(A) Represents bacterial composition of fecal microbiome on Family level; **(B)** represents bacterial composition of fecal microbiome on Genus level. Time points correspond to sample collection days (Day 4, Day 6, Day 8); the number of the experimental animal marked P1–4. C1 KpnI group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C2 Ab:KpnI group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C3 Kpn:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C4 Bis:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension.

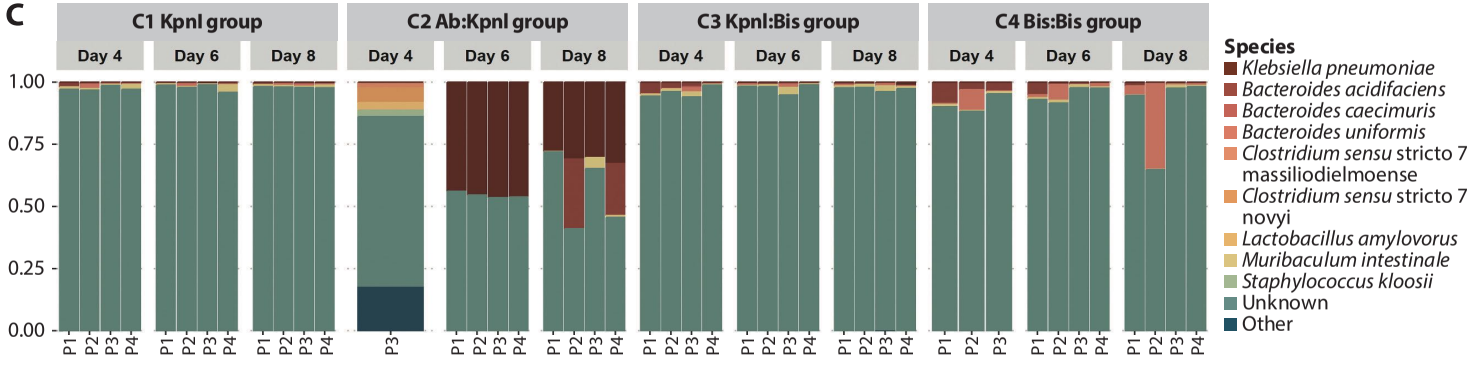

Fig. 3.2.3.1 (C). *The composition of mice fecal microbiome during Klebsiella pneumoniae (ATCC® 12657™) colonization*

(C) represents bacterial composition of fecal microbiome on Species level. Time points correspond to sample collection days (Day 4, Day 6, Day 8); the number of the experimental animal marked P1–4. C1 KpnI group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C2 Ab:KpnI group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C3 Kpn:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C4 Bis:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension.

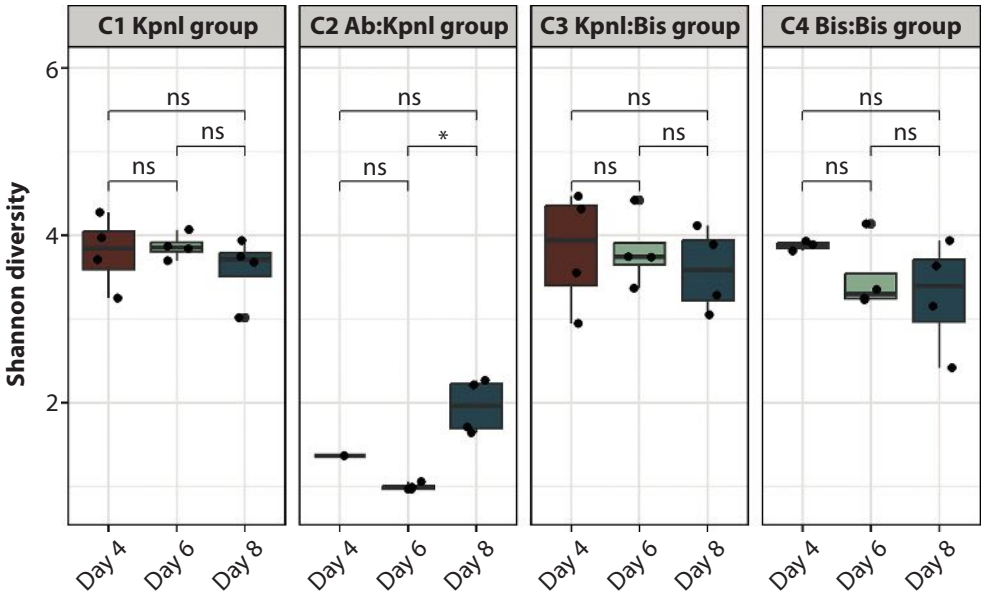

Fig. 3.2.3.2. *Shannon diversity index of mice fecal microbiome during Klebsiella pneumoniae (ATCC® 12657™) colonization*

α-diversity represented by the Shannon diversity index. Time points correspond to sample collection days (Day 4, Day 6, Day 8); whiskers represent minimum and maximum values; middle line – median; ns – no significant difference; *p = 5.26×10^{-4} on Day 8. C1 KpnI group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C2 Ab:KpnI group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C3 Kpn:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C4 Bis:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension.

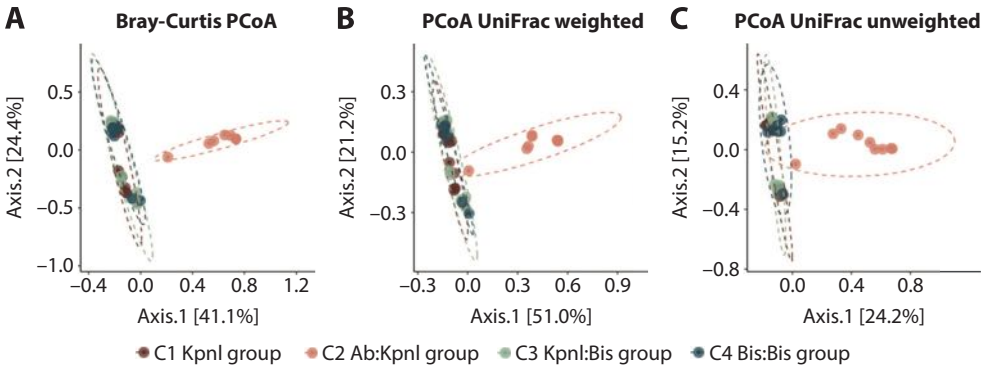

Fig. 3.2.3.3. *β-diversity analysis of mice fecal microbiome during Klebsiella pneumoniae (ATCC® 12657™) colonization.*

β-diversity based on Bray-Curtis, UniFrac weighted and UniFrac unweighted principal coordinates analysis (PcoA). C1 KpnI group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C2 Ab:KpnI group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C3 Kpn:Bis group: 2mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C4 Bis:Bis group: 2 mM bismuth subsalicylate; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension.

3.3. GIT model of *K. pneumoniae* (ATCC® 43816™) colonization

3.3.1. *K. pneumoniae* (ATCC® 43816™) colonization

Following up previous GIT colonization model, we developed a new model using different strain of *K. pneumoniae* (ATCC® 43816™). Four experimental groups were pre-treated with or without antibiotics before bacterial administration (Fig. 2.2.3.1). Fecal samples were collected at multiple time points (days 1, 2, 4 and 8) to measure bacterial load throughout the study period. The data showed that the AbI:Amp group consistently had the highest bacterial loads across all time points, with an average of 2.94×10^9 on day 4 and 1.34×10^9 on day 8, though the reduction showed a consistent trend, it did not reach statistical significance. The KpnII group showed relatively high colonization with 9.24×10^6 CFU on day 8, but significantly lower bacterial colonization compared to Ab:KpnII group ($p = 6.3 \times 10^{-3}$), AbI:KpnII group ($p = 4.9 \times 10^{-3}$), and AbI:Amp group ($p < 10^{-6}$). Furthermore, the Ab:KpnII group demonstrated intermediate bacterial loads, with the average of 3.05×10^8 CFU on day 8, showing statistically significant increased colonization from day 4 ($p < 0.01$). The AbI:KpnII group showed rapid increase of bacterial colonization on day 4 with the average of 3.58×10^7 CFU, but the colonisation significantly decreased on day 8 with the average of 1.39×10^6 CFU ($p = 4.2 \times 10^{-2}$). Overall, statistical analysis

confirmed that antibiotic pre-treatment significantly increased bacterial colonization at key time points (day 4 and 8), highlighting the impact of antibiotics on gut microbiome dynamics during *K. pneumoniae* colonization, but showing that low dose ampicillin post-treatment had no impact on keeping bacterial load stable.

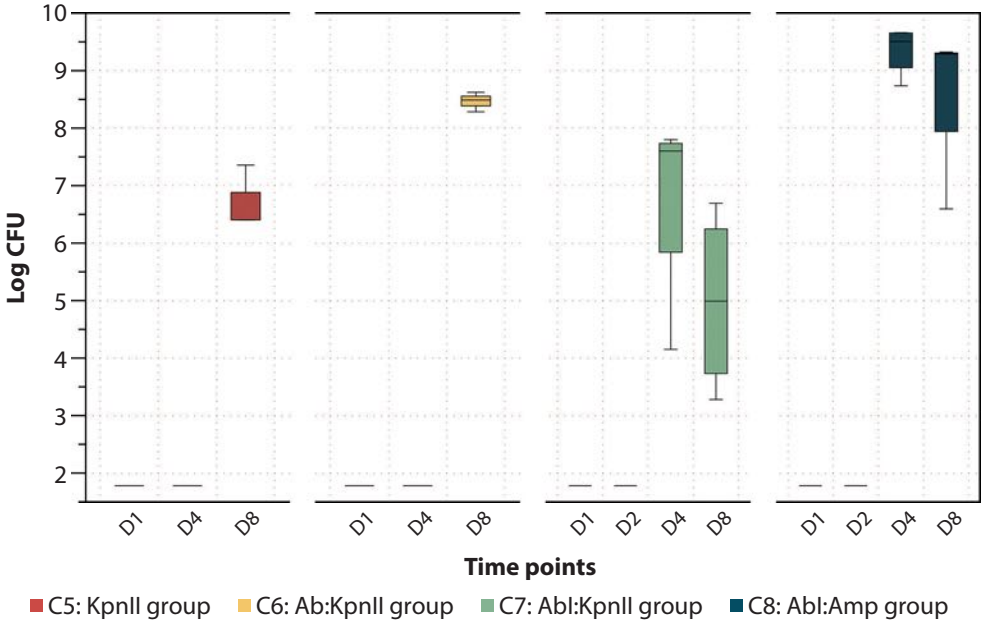

Fig. 3.3.1.1. *K. pneumoniae (ATCC® 43816™) colonization of mice GIT during different time points*

D – days; p-values represented on the top line; whiskers represent minimum and maximum values; middle line – median; log CFU shows *Klebsiella pneumoniae* bacterial counts; time points correspond to sample collection days (D1, D2, D4, D8). C5 KpnII group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C6 Ab:KpnII group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C7 Ab:KpnII group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C8 AbI:Amp group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin.

3.3.2. Changes in mice microbiome during *K. pneumoniae* (ATCC® 43816™) colonization

To determine the microbiome profiles of the KpnII, Ab:KpnII, Ab:KpnII, and Ab:Amp groups in the *K. pneumoniae* (ATCC® 43816™) GIT colonization study, a compositional analysis of fecal samples, collected on days 1, 4, and 8, was performed using relative abundances of microbial taxa at the phylum, family, and genus levels. Across all groups, the microbiome was composed of six bacterial phyla, with three phyla consistently dominating at the beginning of the study: *Bacteroidota* (43.5%), *Proteobacteria* (27.4%), *Firmicutes* (26.71%). However, in the Ab:KpnII and Ab:Amp groups, a clear redistribution in microbial composition was observed following antibiotic pre-treatment and bacterial inoculation, with *Bacteroidota* decreasing to 3.4%, and *Proteobacteria* increasing to 70.5%, while *Firmicutes* remained relatively stable at 20.8%.

Additionally, diversity metrics (α-diversity, β-diversity) were analyzed across all groups at the previously mentioned three time points. Bacterial diversity (α-diversity), represented by the Richness index, showed significant differences only within the AbI:KpnII group comparing all time points (p_{adj} = 0.043). No significant differences in bacterial diversity were observed in other groups (Fig. 3.3.2.2).

To conclude, KpnII, Ab:KpnII, AbI:KpnII, AbI:Amp were enriched with *Enterobacteriaceae* family, as well as, *Klebsiella* at genus level comparing time points before bacterial inoculation (day 1 and 4) versus after the bacterial inoculation (day 8). To further check potential differences between colonization models of KpnII, Ab:KpnII, AbI:KpnII and AbI:Amp groups in this study, changes in the abundance of the *Klebsiella* genus were analyzed at the last time point (day 8); however, no significant differences in bacterial counts were observed. This indicates that any type/model of antibiotic pre-treatment has no impact on colonization compared to the bacterial inoculation alone.

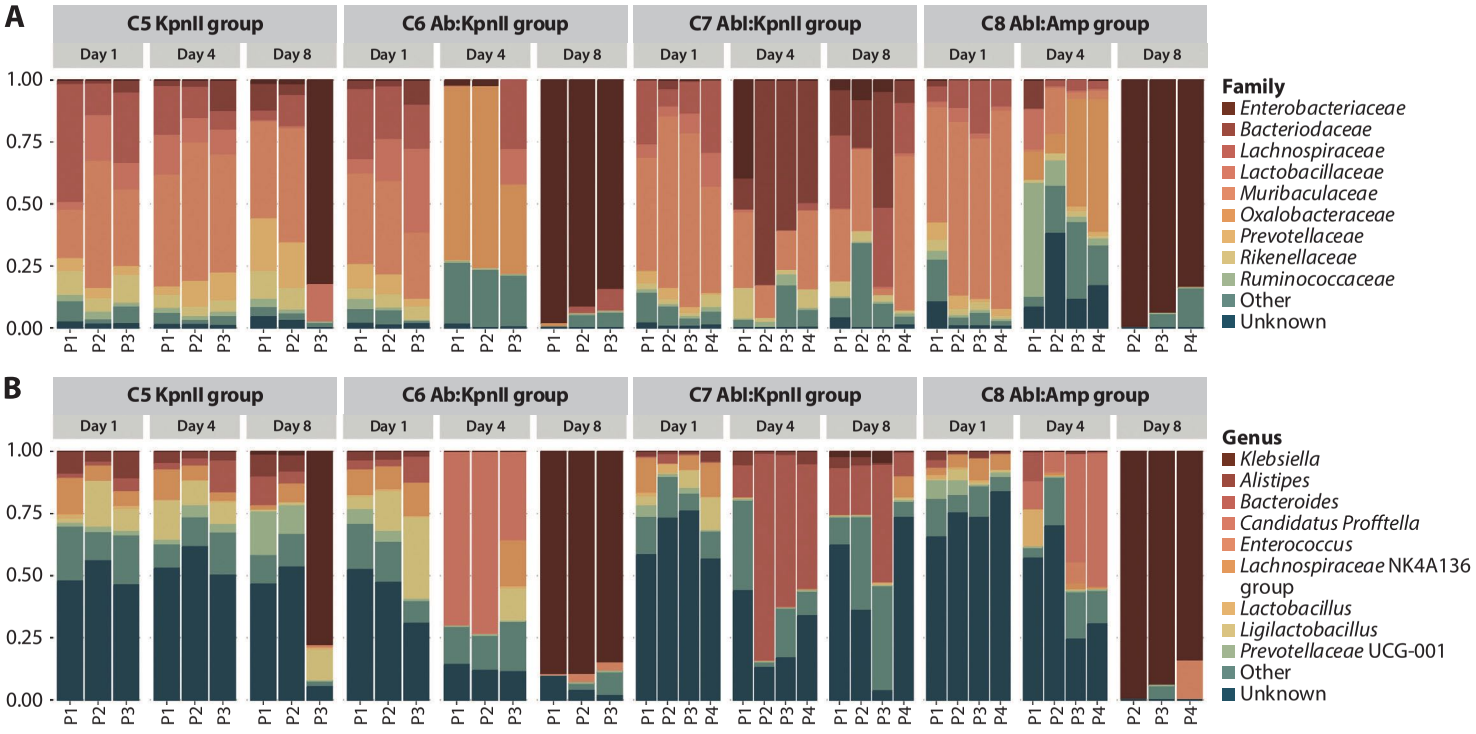

Fig. 3.3.2.1 (A–B). *The composition of mice fecal microbiome during Klebsiella pneumoniae (ATCC® 43816™) colonization*

(A) Represents bacterial composition of fecal microbiome on Family level; **(B)** represents bacterial composition of fecal microbiome on Genus level. Time points correspond to sample collection days (Day 1, Day 4, Day 8); the number of the experimental animal marked P1–4. C5 KpnII group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C6 Ab:KpnII group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C7 Ab:KpnII group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C8 AbI:Amp group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin.

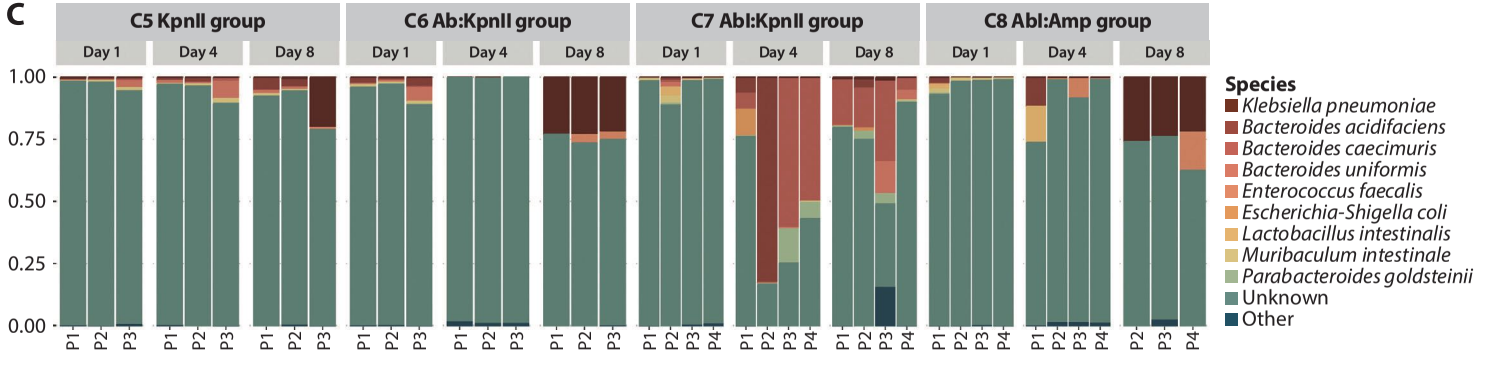

Fig. 3.3.2.1 (C). *The composition of mice fecal microbiome during Klebsiella pneumoniae (ATCC® 43816™) colonization*

(C) represents bacterial composition of fecal microbiome on Species level. Time points correspond to sample collection days (Day 1, Day 4, Day 8); the number of the experimental animal marked P1–4. C5 KpnII group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C6 Ab:KpnII group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C7 Ab:KpnII group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C8 AbI:Amp group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin.

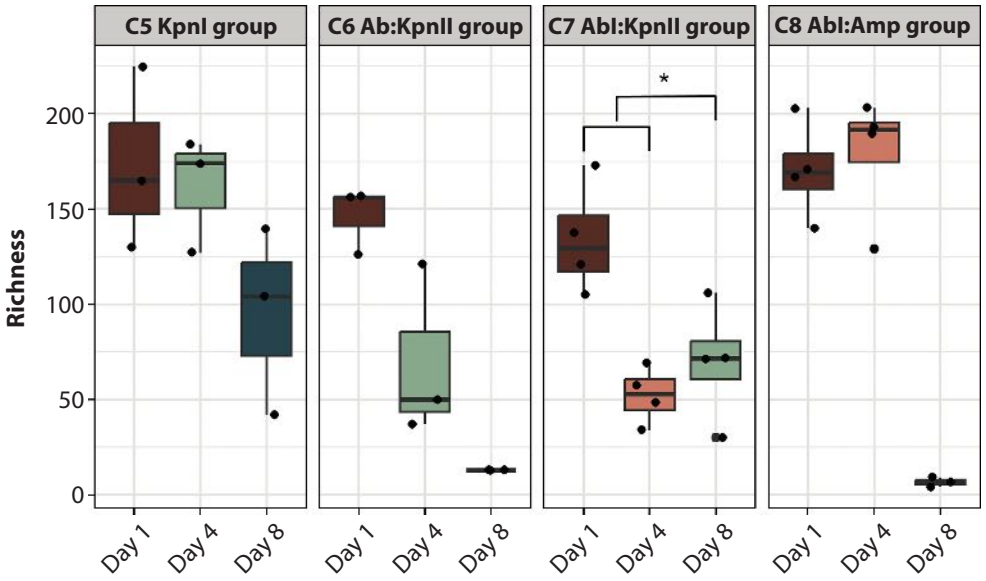

Fig. 3.3.2.2. *Richness index of mice fecal microbiome during Klebsiella pneumoniae (ATCC® 43816™) colonization*

α-diversity represented by the Richness index. Time points correspond to sample collection days (Day 4, Day 6, Day 8); whiskers represent minimum and maximum values; middle line – median; * p_{adj} = 0.043 during all time points within C7 group. C5 KpnII group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C6 Ab:KpnII group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C7 Ab:KpnII group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C8 AbI:Amp group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin.

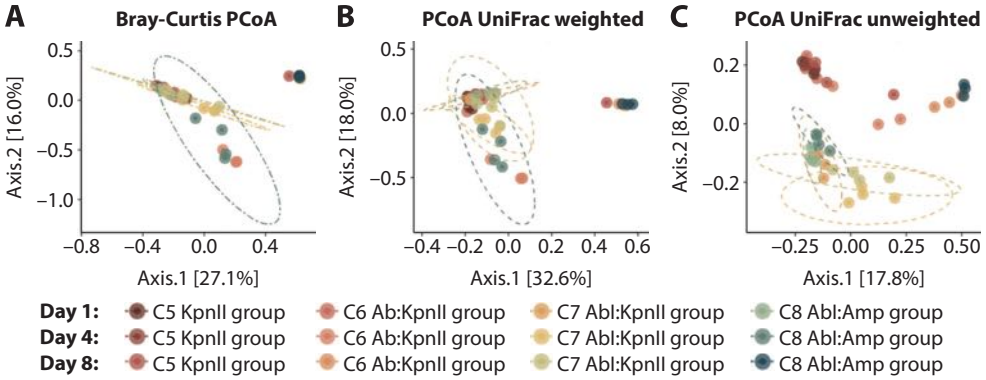

Fig. 3.3.2.3. *β-diversity analysis of mice fecal microbiome during Klebsiella pneumoniae (ATCC® 43816™) colonization*

β-diversity based on **(A)** Bray-Curtis; **(B)** UniFrac weighted; **(C)** UniFrac unweighted principal coordinates analysis (PCoA). Colors correspond to sample collection days (Day 1, Day 4, Day 8) and different groups. C5 KpnII group: 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C6 Ab:KpnII group: 2000 U/mL penicillin; 2 mg/mL streptomycin; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin. C7 Ab:KpnII group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension. C8 AbI:Amp group: 2000 U streptomycin and penicillin mix; 50 µL/10^9 CFU *Klebsiella pneumoniae* suspension; 500 mg/L ampicillin.

3.4. KvarM therapy for GIT infection of *K. pneumoniae* (ATCC® 43816™)

3.4.1. Klebicin KvarM therapy for *K. pneumoniae* (ATCC® 43816™) infection

Based on the above-described results of gut colonization models, the decision has been made to use *K. pneumoniae* ATCC® 43816™ strain for evaluation of therapeutic agents, as it demonstrated a higher colonization level without any additional interventions (9.24×10^6 CFU) compared to *K. pneumoniae* ATCC® 12657™ (1.21×10^4 CFU). Six experimental groups were treated with various formulations of klebicin KvarM or chimericin ColA-Ia, including ciprofloxacin, following bacterial administration (Fig. 3.2.4.1). Fecal samples were collected at multiple time points (days 1, 2, 5, 6, 7, 8 and 9) to monitor bacterial load dynamics during the study period. The data showed distinct trends among the groups. The VC group showed stable bacterial loads (av. 1.85×10^5 CFU) with no significant changes over time. The KvarM:EudS group exhibited the bacterial colonization on day 5 (av. 1.61×10^6 CFU), followed by a drop on day 6 (av. 1.48×10^4 CFU) after KvarM coated Eudragit® S100 therapy, with similar bacterial values through

the rest of the study period (av. 6.45×10^5 CFU). The KvarM:EudL group showed peak bacterial colonization on day 5 (av. 1.3×10^8 CFU) with the drop on day 6 (av. 2.8×10^5 CFU) after KvarM coated Eudragit® L100 therapy repeating the KvarM:EudS group trends across the 7, 8 and 9 days of the study (av. 6.99×10^3 CFU). The KvarM:EudSL group showed substantial bacterial colonization on day 5 (av. 2.18×10^5 CFU), followed by a clear drop on day 6 (av. 1.27×10^3 CFU) after KvarM coated Eudragit® S100 and Eudragit® L100 therapy. The bacterial load remained consistently low through the rest of the study period (av. 4.24×10^5 CFU), indicating effective and sustained reduction in colonization post-treatment. The ColA-Ia:EudSL group showed the effective bacterial colonization on day 5 (av. 1.73×10^5 CFU), following the same manner as KvarM:EudSL group reducing bacterial colonization on day 6 (av. 1.12×10^3 CFU) after ColA-Ia coated Eudragit® S100 and Eudragit® L100 therapy. The bacterial load remained consistently low and stable through the remainder of the study period (av. 1.67×10^7 CFU), indicating that chimericin suppress the bacterial colonization as effective as klebicin. The Kpn:AB group followed similar trends in bacterial colonization on day 5 (av. 1.70×10^7 CFU) and demonstrated reduction in bacterial load following ciprofloxacin treatment on day 6 (3.94×10^7 CFU). The reduction was maintained throughout the remainder of the study period, with bacterial loads remaining consistently low (av. 1.9×10^5 CFU). Even though, any of the therapies did not reach statistical significance, it showed strong trends with the effectiveness being (day 5 compared to day 7–9) 98.43% in KvarM:EudSL group, 99.9% in ColA-Ia:EudSL group, and 99.3% in Kpn:Ab group.

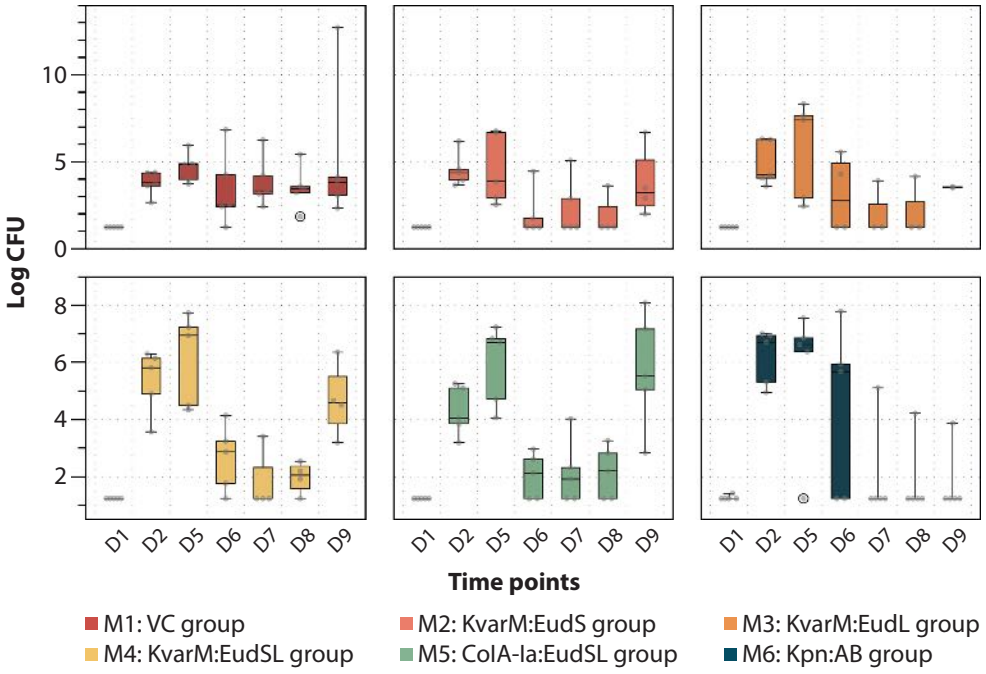

Fig. 3.4.1.1. *Bacteriocin therapy on reducing K. pneumoniae (ATCC® 43816™) infection*

D – days; p-values represented on the top line; whiskers represent minimum and maximum values; middle line – median; dots – samples; log CFU shows *Klebsiella pneumoniae* bacterial counts; time points correspond to sample collection days (D1, D2, D5, D6, D7, D8, D9). M1 VC group: vehichle control group 0.125 µg/µL Eudragit® S100; 0.125 µg/µL Eudragit® L100; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M2 KvarM:EudS group: 12.5 µg/100 µL Eudragit® S100 coated KvarM; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M3 KvarM:EudL group: 12.5 µg/100 µL Eudragit® L100 coated KvarM; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M4 KvarM:EudSL: 12.5 µg/100 µL Eudragit® S100 and Eudragit® L100 coated KvarM; 50 µL/107 CFU *Klebsiella pneumoniae* suspension. M5: ColA-Ia:EudSL group: 12.5 µg/100 µL Colicin-A and KvarIa hybrid coated Eudragit® S100 and Eudragit® L100; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M6 Kpn:AB group: 2 mg/200 µL ciprofloxacin; 50 µL/107 CFU *Klebsiella pneumoniae* suspension.

3.4.2. Changes in mice microbiome during *K. pneumoniae* (ATCC® 43816TM) reduction with KvarM

To determine the impact of bacteriocin KvarM and chimericin ColA-Ia on the gut microbiome following bacterial colonization, compositional analysis was conducted of fecal samples collected on days 1, 5, and 9. Relative abundances of microbial taxa at the phylum, family, and genus levels were compared across the VC, KvarM:EudSL, ColA-Ia:EudSL, and Kpn:Ab groups. Across all groups, the microbiome was composed of six bacterial phy la, with two phyla being the most pronounced throughout the whole study period: *Firmicutes* (42.6%) and *Proteobacteria* (1.14%).

Additionally, diversity metrics (α-diversity, β-diversity) were analyzed across all groups at the previously mentioned time points. Bacterial diversity (α-diversity), represented by the bacterial Richness index, Shannon diversity and Simpson diversity, showed significant differences between the Kpn:Ab and VC, KvarM:EudSL, and ColA-Ia:EudSL groups at the last time point of the study ($p_{adj} = 0.032$) in bacterial richness analysis (Fig. 3.4.2.2). No significant differences in bacterial diversity were observed on the day 1 and 5.

Further, the microbial community clusters (β-diversity) based on Bray-Curtis, UniFrac weighted, and UniFrac unweighted dissimilarity indexes showed similar trends indicating significant sample clustering between Kpn:Ab group and VC, KvarM:EudSL, ColA-Ia:EudSL groups on the day 9 after treatment interventions ($p < 0.05$). All groups clustered together at the other time points (Fig. 3.4.2.3).

Thereafter, differential abundance analysis was performed to evaluate the presence and amount of bacteria families differences within each VC, KvarM:EudSL, ColA-Ia:EudSL, and Kpn:Ab group before (day 5) and after (day 9) treatment interventions. Groups VC, KvarM:EudSL, and ColA-Ia:EudSL showed no differences in bacterial abundance between time points. However, abundance of bacterial families, in Kpn:Ab group, such as *Enterobacteriaceae*, *Prevotellaceae*, *Rikinelaceae* and others were depleted. To further check potential differences between impact of the treatments on gut microbiome VC, KvarM:EudSL, ColA-Ia:EudSL, and Kpn:Ab groups were compared on day 9. The differential abundance analysis showed clear tendencys when comparing Kpn:Ab group to other groups, indicating that neither KvarM nor ColA-Ia impacted the profile of microbiome.

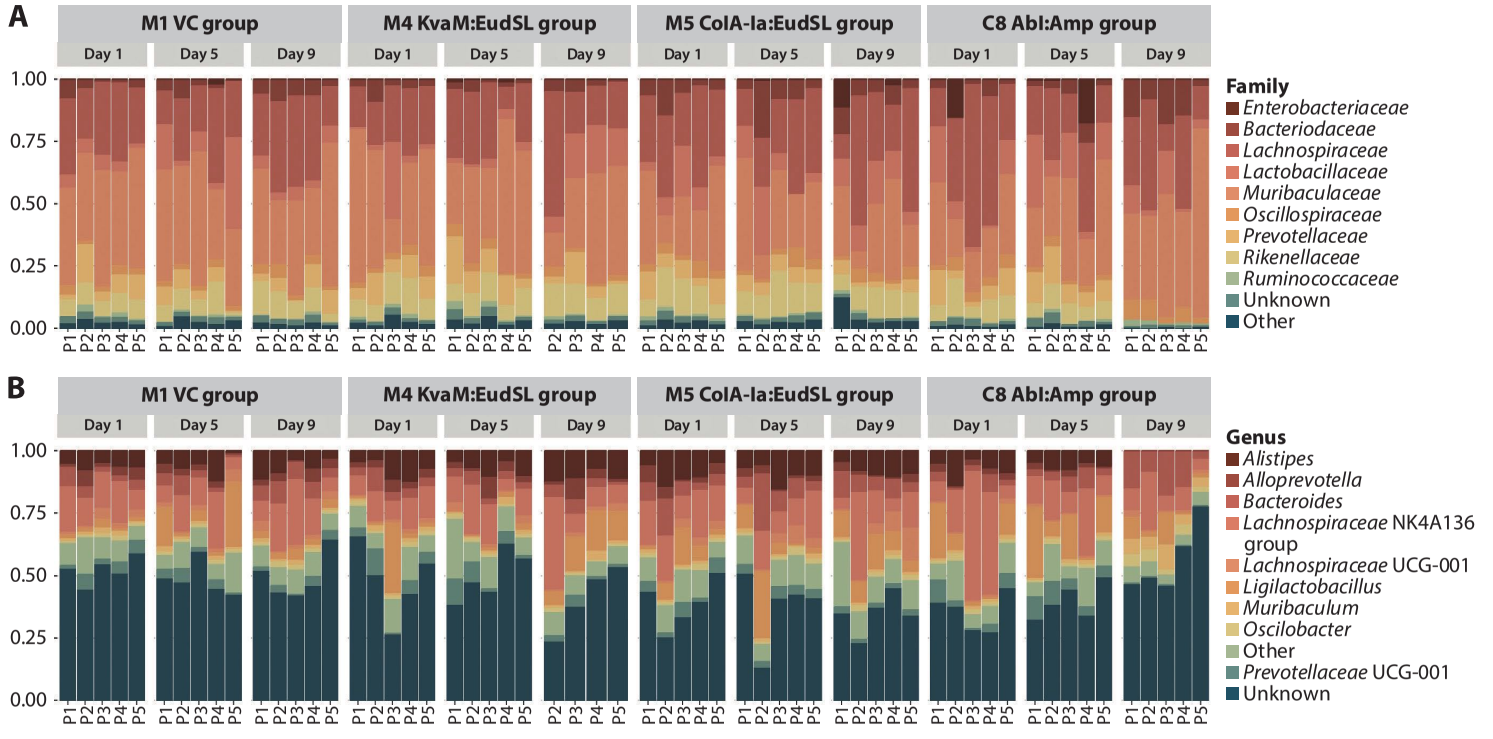

Fig. 3.4.2.1 (A–B). *The composition of mice fecal microbiome during recombinant bacteriocins therapy*

(A) Represents bacterial composition of fecal microbiome on Family level; **(B)** represents bacterial composition of fecal microbiome on Genus level. Time points correspond to sample collection days (Day 1, Day 4, Day 8); the number of the experimental animal marked P1–5. M1 VC group: vehichle control group 0.125 μg/μL Eudragit® S100; 0.125 μg/μL Eudragit® L100; 50 μL/10^7 CFU *Klebsiella pneumoniae* suspension. M4 KvarM:EudSL: 12.5 μg/100 μL Eudragit® S100 and Eudragit® L100 coated KvarM; 50 μL/10^7 CFU *Klebsiella pneumoniae* suspension. M5: ColA-Ia:EudSL group: 12.5 μg/100 μL Colicin-A and KvarIa hybrid coated Eudragit® S100 and Eudragit® L100; 50 μL/10^7 CFU *Klebsiella pneumoniae* suspension. M6 Kpn:AB: 2 mg/200 μL ciprofloxacin; 50 μL/10^7 CFU *Klebsiella pneumoniae* suspension.

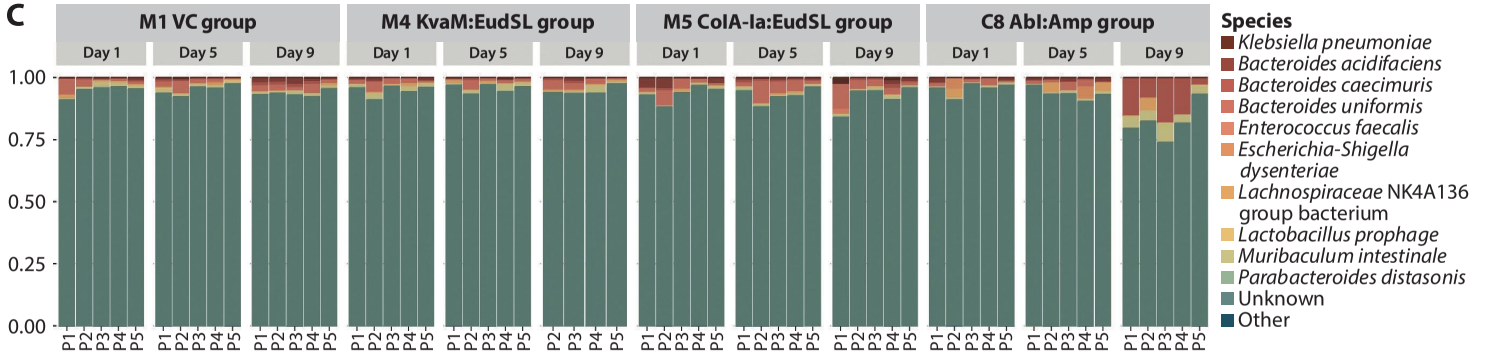

Fig. 3.4.2.1 (C). *The composition of mice fecal microbiome during recombinant bacteriocins therapy*

(C) Represents bacterial composition of fecal microbiome on Species level. Time points correspond to sample collection days (Day 1, Day 4, Day 8); the number of the experimental animal marked P1–5. M1 VC group: vehicle control group 0.125 µg/µL Eudragit® S100; 0.125 µg/µL Eudragit® L100; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M4 KvarM:EudSL: 12.5 µg/100 µL Eudragit® S100 and Eudragit® L100 coated KvarM; 50 µL/ 10^7 CFU *Klebsiella pneumoniae* suspension. M5: ColA-Ia:EudSL group: 12.5 µg/100 µL Colicin-A and KvarIa hybrid coated Eudragit® S100 and Eudragit® L100; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M6 Kpn:AB: 2 mg/200 µL ciprofloxacin; 50 µL/ 10^7 CFU *Klebsiella pneumoniae* suspension.

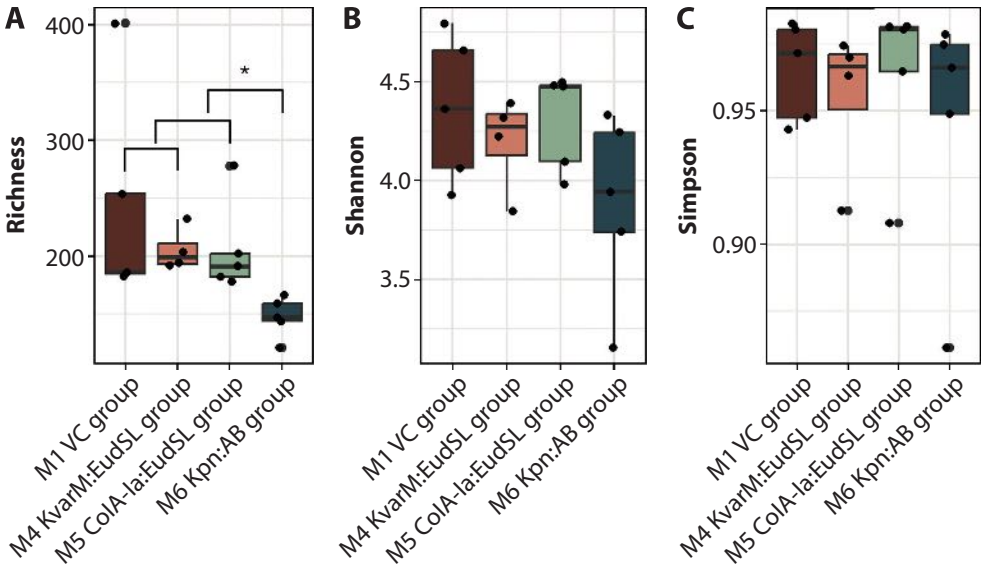

Fig. 3.4.2.2. *α-diversity analysis of mice fecal microbiome during recombinant bacteriocins therapy*

α-diversity represented by the **(A)** bacterial richness; **(B)** Shannon diversity; **(C)** Simpson diversity; *p_{adj} = 0.032 between M6 group and M1, M4, M5 groups on the day 9. M1 VC group: vehichle control group 0.125 µg/µL Eudragit® S100; 0.125 µg/µL Eudragit® L100; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M4 KvarM:EudSL: 12.5 µg/100 µL Eudragit® S100 and Eudragit® L100 coated KvarM; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M5: ColA-Ia:EudSL group: 12.5 µg/100 µL Colicin-A and KvarIa hybrid coated Eudragit® S100 and Eudragit® L100; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension. M6 Kpn:AB: 2 mg/200 µL ciprofloxacin; 50 µL/10^7 CFU *Klebsiella pneumoniae* suspension.

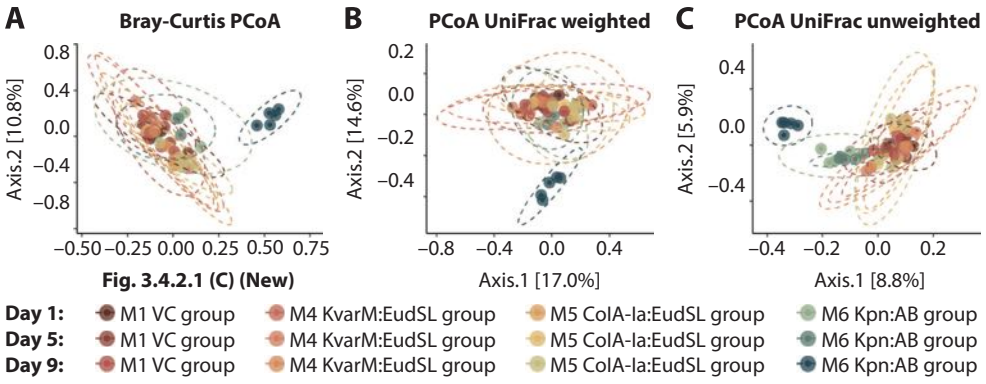

Fig. 3.4.2.3. *β-diversity analysis of mice fecal microbiome during recombinant bacteriocins therapy*

β-diversity based on **(A)** Bray-Curtis; **(B)** UniFrac weighted; **(C)** UniFrac unweighted principal coordinates analysis (PCoA). Colors correspond to sample collection days (Day 1, Day 5, Day 9) and different groups. M1 VC group: vehichle control group 0.125 μg/μL Eudragit® S100; 0.125 μg/μL Eudragit® L100; 50 μL/10^7 CFU *Klebsiella pneumoniae* suspension. M4 KvarM:EudSL: 12.5 μg/100 μL Eudragit® S100 and Eudragit® L100 coated KvarM; 50 μL/10^7 CFU *Klebsiella pneumoniae* suspension. M5: ColA-Ia:EudSL group: 12.5 μg/100 μL Colicin-A and KvarIa hybrid coated Eudragit® S100 and Eudragit® L100; 50 μL/10^7 CFU *Klebsiella pneumoniae* suspension. M6 Kpn:AB: 2 mg/200 μL ciprofloxacin; 50 μL/10^7 CFU *Klebsiella pneumoniae* suspension.

3.5. Changes of the commensal mice gut microbiome under different treatments

To assess the impact of recombinant bacteriocins, Eudragit® S100 and Eudragit® L100, and antibiotics on the commensal microbiome profiles compositional analysis of fecal samples collected on the first study day (before interventions) and last study day (after interventions) was conducted. Relative abundances of microbial taxa at the phylum, family, and genus levels were compared across the Cont, Eud, KvarM:Eud and AB groups. Across all groups, the microbiome was composed of six bacterial phyla, with *Firmicutes* (41.43%) being the most abundant.

Moreover, diversity metrics (α-diversity, β-diversity) were analyzed across all groups at the previously mentioned time points. Bacterial diversity (α-diversity), represented by the bacterial Richness index, Shannon diversity and Simpson diversity, showed significant differences between the AB and Eud, KvarM:Eud groups (p_{adj} = 0.048) in bacterial richness analysis (Fig. 3.5.2).

In addition, the microbial community clusters (β-diversity) based on Bray-Curtis, UniFrac weighted, and UniFrac unweighted dissimilarity indexes showed similar trends indicating significant sample clustering between AB group and VC, KvarM:EudSL, ColA-Ia:EudSL groups on the day 4 after treatment interventions ($p < 0.05$), while all the groups were clustered together before antibiotic treatment (Fig. 3.5.3).

To further analyse the trend of only antibiotic treatment disrupting the gut microbiome analysis of differential abundance was performed. Cont, Eud, KvarM:Eud had no changes in bacterial abundance on family and genus levels, while AB group showed high bacterial abundance changes on the last day of the experiment. Abundance of bacterial families such as *Anaerostipes, Muribaculum, Incartea Sedis, Candidatus Profftella, Bacteroides* and others were enriched, while *Alistipes, Prevotellacea, Desulfovibrio*, *Mucispirillum* and others were depleted, supporting the findings that neither recombinant bacteriocins nor Eudragit polymers have impact on commensal microbiome.

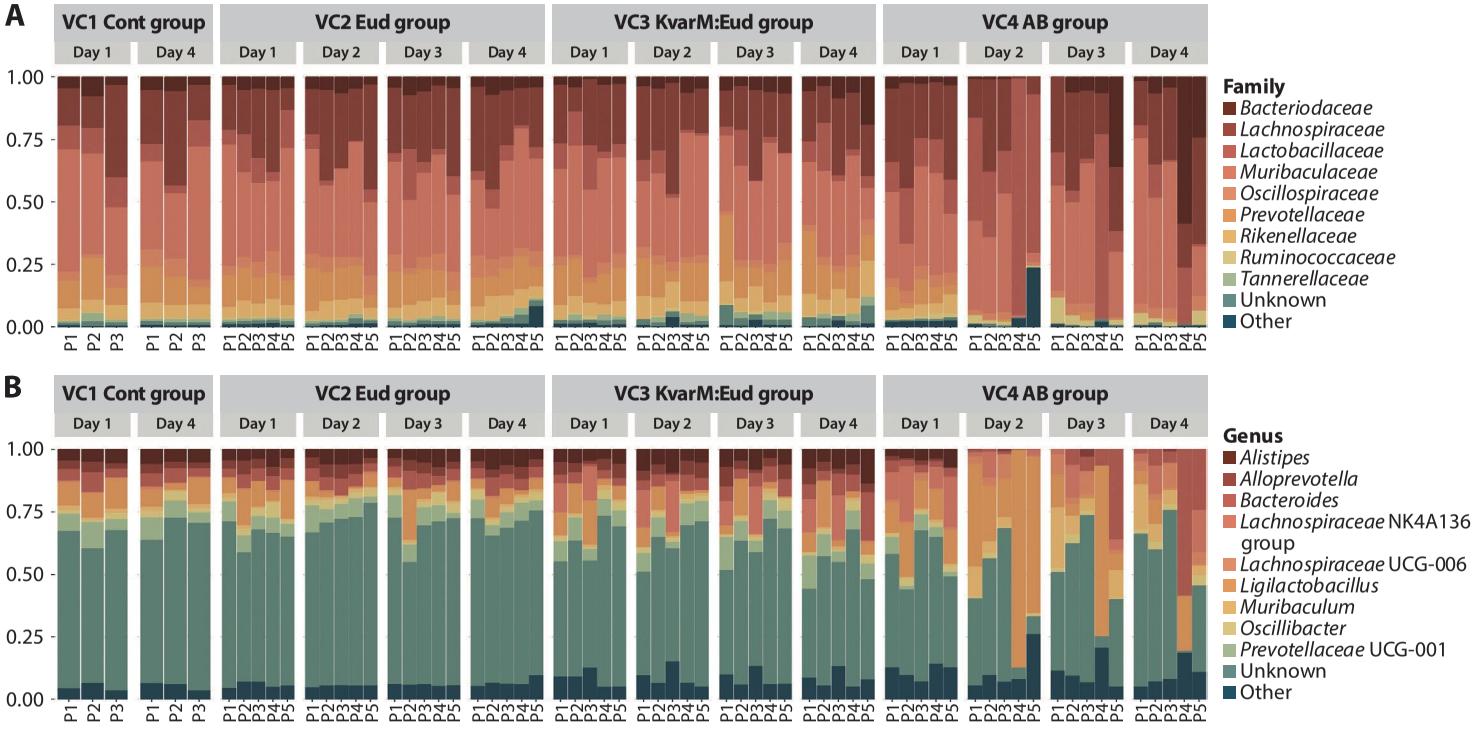

Fig. 3.5.1 (A–B). *The composition of mice fecal microbiome under different treatments*

(A) Represents bacterial composition of fecal microbiome on Family level; **(B)** represents bacterial composition of fecal microbiome on Genus level. Time points correspond to sample collection days (Day 1, Day 2, Day 3, Day 4); the number of the experimental animal marked P1–5. VC1 Cont group: Control group with natural microbiome. VC2 Eud group: 12.5 µg/100 µL Eudragit® S100 and Eudragit® L100 mix. VC3 KvarM:Eud group: 500 µg/100 µL KvarM coated with Eudragit® S100 and Eudragit® L100. VC4 AB group: 2 mg/200 µL ciprofloxacin.

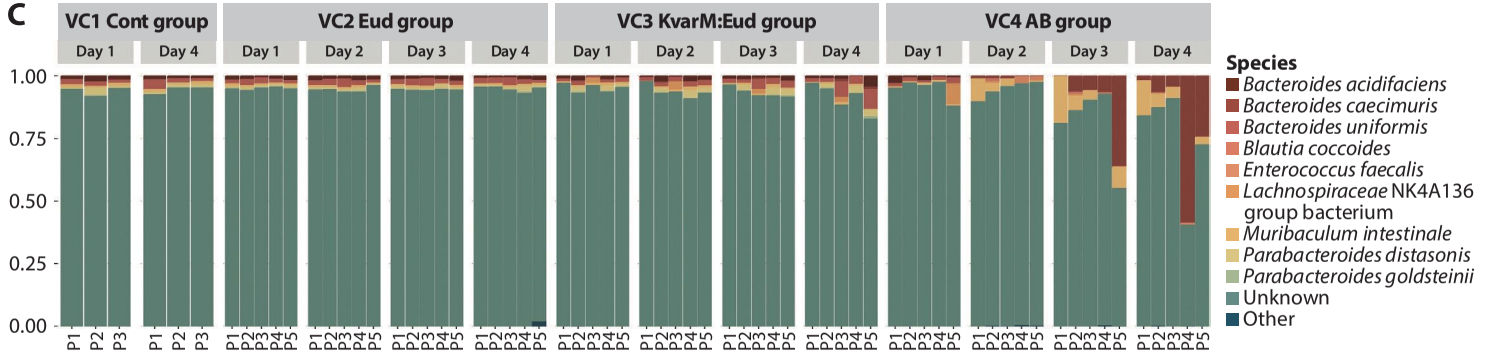

Fig. 3.5.1 (C). *The composition of mice fecal microbiome under different treatments*

(C) Represents bacterial composition of fecal microbiome on Species level. Time points correspond to sample collection days (Day 1, Day 2, Day 3, Day 4); the number of the experimental animal marked P1–5. VC1 Cont group: Control group with natural microbiome. VC2 Eud group: 12.5 μg/100 μL Eudragit® S100 and Eudragit® L100 mix. VC3 KvarM:Eud group: 500 μg/100 μL KvarM coated with Eudragit® S100 and Eudragit® L100. VC4 AB group: 2 mg/200 μL ciprofloxacin.

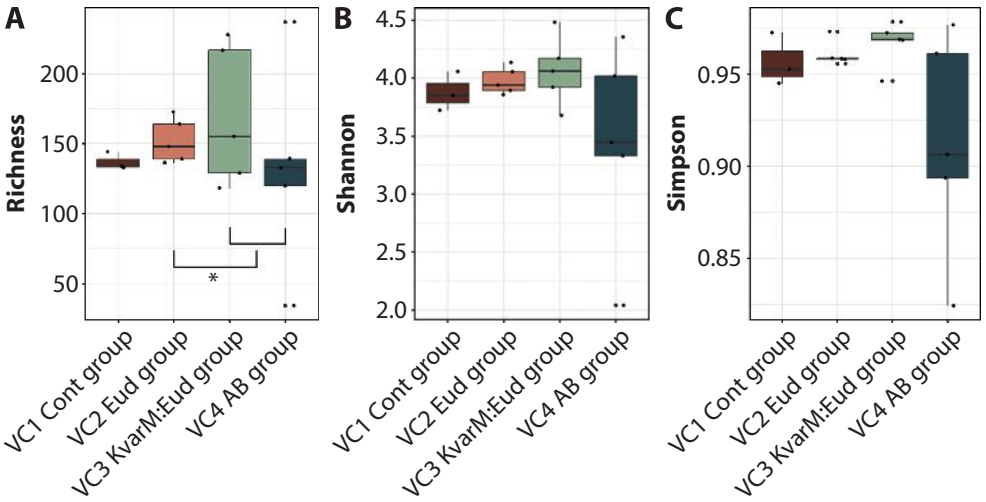

Fig. 3.5.2. *α-diversity analysis of mice fecal microbiome under different treatments (day 4)*

α-diversity represented by the **(A)** bacterial richness; **(B)** Shannon diversity; **(C)** Simpson diversity; *p_{adj} = 0.048 between VC4 group and VC3, VC2 groups on the day 4. VC1 Cont group: control group with natural microbiome. Shannon diversity index, showed significant differences between the AB and Eud, KvarM:Eud groups (p_{adj} = 0.048). VC2 Eud group: 12.5 µg/100 µL Eudragit® S100 and Eudragit® L100 mix. VC3 KvarM:Eud group: 500 µg/100 µL KvarM coated with Eudragit® S100 and Eudragit® L100. VC4 AB group: 2 mg/200 µL ciprofloxacin.

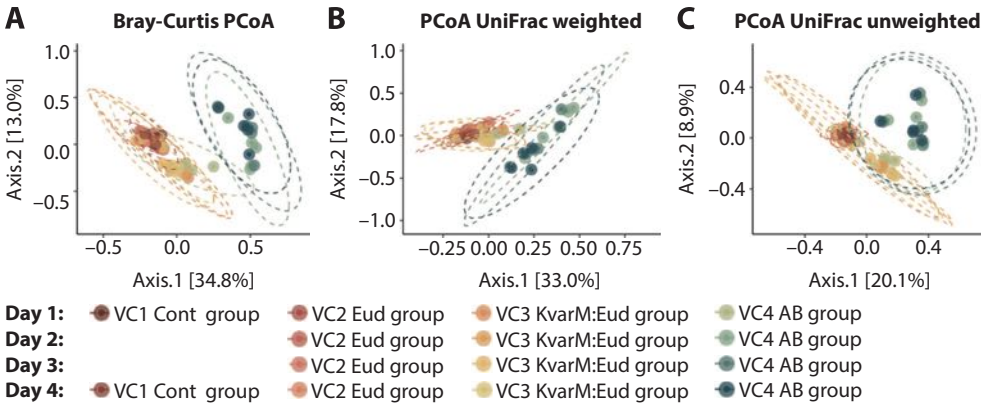

Fig. 3.5.3. *β-diversity analysis of mice fecal microbiome under different treatments*

β-diversity based on **(A)** Bray-Curtis; **(B)** UniFrac weighted; **(C)** UniFrac unweighted principal coordinates analysis (PCoA). Colors correspond to sample collection days (Day 1, Day 5, Day 9) and different groups. VC1 Cont group: control group with natural microbiome. VC2 Eud group: 12.5 μg/100 μL Eudragit® S100 and Eudragit® L100 mix. VC3 KvarM:Eud group: 500 μg/100 μL KvarM coated with Eudragit® S100 and Eudragit® L100. VC4 AB group: 2 mg/200 μL ciprofloxacin.

4. DISCUSSION

4.1. Pilot study

In this Pilot study, we successfully established a lower gastrointestinal tract (GIT) colonization model for *K. quasipneumoniae* (DSM® 28212™) in mice and evaluated the efficacy of KvarIa therapy in reducing bacterial colonization. Notably, we selected *K. quasipneumoniae* as primary agent due to recent studies highlighting its potential virulence and increasing drug resistance, despite being considered an asymptomatic carriage isolate.

The present colonization study aligns with prior research findings [62, 63], indicating that antibiotic pre-treatment effectively disrupts the natural gut microbiota, facilitating stable colonization by *K. quasipneumoniae*. In our study, sustained bacterial colonization was observed in antibiotic-treated groups (Pen:Strep and Pen:Strep:Met), with bacterial counts stabilizing by day 8, indicating successful establishment of *K. quasipneumoniae* within the altered gut environment. No bacterial colonization was detected in the control group without antibiotic pre-treatment, as determined by *khe* gene amplification. Similar results have been reported in other *Klebsiella* murine models, where gut microbiota disruption by amoxicillin was not only required, but also associated with enhanced of the virulence of *K. variicola* [64].

The pilot study was designed to identify the most effective coating formulation for delivering different concentrations of recombinant bacteriocin KvarIa to the large intestine. Given the critical role of GIT pH in drug solubility, stability, and mucosal absorption, pH measurements were necessary to the formulation strategy. GIT pH is usually influenced by factors such as diet, feeding state (fed or fasted), medications, microbiome composition, stress, and daily fluid intake. Accordingly, our study included analysis of GIT pH (feces and tissues) to find an optimal coating for KvarIa delivery. The results revealed elevated rectal pH levels in *K. quasipneumoniae*-colonized mice treated with antibiotics. Untreated mice group pH levels did not fluctuate as much by comparison to other groups. These findings are consistent with prior research by Shimizu et al., which reported significantly increased fecal and colonic pH in antibiotic-treated mice housed under specific pathogen-free conditions [65]. In this study, antibiotic pre-treatment groups (Pen:Strep and Pen:Strep:Met) exhibited notable pH fluctuations in the small intestine, with the Pen:Strep group showing the lowest average duodenal pH (6.07 ± 0.08). Taking these variations into consideration we selected a coating designed to release the bacteriocin at pH levels higher than 5.5, ensuring targeted delivery to the lower gastrointestinal tract.

Our coleages [50] demonstrated the antibacterial efficacy of *Klebsiella*-derived bacteriocins, known as klebicins, in vitro using clinical *Klebsiella* isolates. Among them, the recombinant pore-forming bacteriocin KvarIa was identified as one of the most potent klebicins, exhibiting the highest activity againts *K. quasipneumoniae.* Its efficacy was further validated in vivo using the non-mammal animal model *Galleria mellonella* larvae model, where it showed a significant antibacterial effect. During this study the GIT model was established using *K. quasipneumoniae* after disrupting the natural gut microbiota with antibiotic pre-treatment, which facilitated stable bacterial colonization. Antibiotic-treated groups (Pen:Strep and Pen:Strep:Met) demonstrated sustained colonization, with bacterial counts stabilizing by day 11 at averages of 3.12×10^8 CFU and 4.04×10^8 CFU, respectively. Furthermore, results of this pilot study demonstrated a significant reduction in bacterial load following treatment with recombinant bacteriocin. In the AbII:Ia group, bacterial load dropped from 6.3×10^7 CFU on day 18 to 3.9×10^5 CFU on day 22. Similarly, in the Hi:Ia group, a higher dose of coated KvarIa (1000 µg) reduced bacterial load from 4.0×10^7 CFU on day 18 to 1.6×10^5 CFU on day 22. In contrast, uncoated KvarIa (VOC group) and AbI:Ia formulations showed no significant reductions in bacterial loads, emphasizing the importance of coating and dosage optimization for therapeutic efficacy. Altogether, these findings show the potential of using a pH-responsive oral delivery system to achieve targeted release of recombinant bacteriocin KvarIa in the lower gastrointestinal tract, resulting in a significant reduction of *K. quasipneumoniae* colonization in the murine models.

4.2. Colonization study

This study aimed to investigate the colonization dynamics of *K. pneumoniae* strains (ATCC® 12657™ and ATCC® 43816™) in murine GIT under varying conditions, including antibiotic pre-treatment and bacterial inoculation. The pH measurements across experimental groups in *K. pneumoniae* ATCC® 12657™ colonization model showed relatively stable trends, with no significant shifts observed between days. It suggests that *K. pneumoniae* colonization does not extensively alter GIT pH over extended periods. When observed pH levels remained stable between different measuring points.

The *khe* gene analysis showed that antibiotic pre-treatment significantly enhanced *K. pneumoniae* colonization at key time points during both *K. pneumoniae* strains experimental models. The ATCC® 43816™ strain achieved consistently higher bacterial loads during GIT colonization, especially in the antibiotic-treated groups. In the AbI:Amp group, bacterial loads

peaked at 2.94×10^9 CFU on day 4 and remained relatively stable at 1.34×10^9 CFU on day 8. In contrast, the ATCC® 12657™ strain showed lower colonization levels, with no comparable peak in bacterial load. Antibiotics compromise gut barrier function and promote pathogen overgrowth by disrupting the native microbiota. In particular, *K. pneumoniae* colonization combined with antibiotic treatment can alter the host microbiota and lead to the emergence of a transient super-shedder phenotype. This phenotype is characterized by enhanced bacterial transmission within the GIT [66, 67]. During the current study no significant differences in *Klebsiella* genus differential abundance were observed between colonization models. Indicating that bacterial inoculation alone can achieve successful GIT colonization regardless of pre- and post-treatment conditions.

Microbiome profiling demonstrated distinct changes in bacterial composition across groups and time points. In the Ab:KpnI group, antibiotic pre-treatment led to significant enrichment of *Bacteroidaceae* and *Muribaculaceae* families, alongside depletion of *Clostridiaceae* and *Enterobacteriaceae* families (*K. pneumoniae* ATCC® 12657™). In the AbI:Amp group of the second colonization model (*K. pneumoniae* ATCC® 43816™), *Proteobacteria* increased significantly following antibiotic pre-treatment (70.5%), while *Bacteroidota* decreased (3.4%). These findings highlight the disruptive effect of antibiotics on commensal microbiome, helping pathogens as *Klebsiella* persist. While the study by Sequeira et al. provides significant insights into the role of the microbiome in protecting against *K. pneumoniae* colonization [66]. Researchers demonstrated that commensal *Bacteroidota* in the intestines protect against *K. pneumoniae* colonization through IL-36 signalling, a pathway that also involves macrophages. Importantly, host-to-host transmission of *K. pneumoniae* primarily occurs from its intestinal reservoir, and CCF-producing *Bacteroidota* were shown to be sufficient to block this spread. Particular bacterial families' disruption can lead to higher GIT colonozition. Findings by Sequeira et al. may explain why *K. pneumoniae* ATCC® 43816™ exhibited higher colonization in our study.

4.3. Therapy study

This study evaluated the efficacy of bacteriocin klebicin KvarM and chimericin ColA-Ia, narrow spectrum antimicrobial proteins produced by bacteria, as a potential alternative to traditional antibiotics. Additionally, the impact of these bacteriocin therapies on the gut microbiome was assessed in comparison to ciprofloxacin treatment. The results showed promising trends in bacteriocin-based therapies as effective alternatives to antibiotics for

controlling *K. pneumoniae* infections while maintaining the commensal gut microbiome.

The results demonstrated that both KvarM:EudSL and ColA-Ia:EudSL therapies effectively reduced bacterial loads post-treatment, with reductions of 98.43% and 99.9%, respectively, from peak colonization levels on day 5 to sustained low levels on days 7–9. These reductions were comparable to ciprofloxacin (Kpn:Ab group), which achieved a 99.3% reduction. Specifically, KvarM shows a sustained and prolonged reduction in bacterial colonization over time, suggesting a potential for longer-lasting therapeutic effects over bacterial colonization. Complementing these findings, a study by N. Carpena et al. explored the targeted delivery of narrow-spectrum protein antibiotics to the lower gastrointestinal tract using a murine model of *E. coli* colonization. Their findings emphasize the importance of site-specific drug delivery in enhancing therapeutic efficacy while minimizing off-target effects. By employing encapsulation strategies, a localized and sustained release of protein antibiotics was achieved, leading to a significant reduction in *E. coli* colonization [68]. Together these studies show that tailored antimicrobial therapies, such as bacteriocins and encapsulated protein antibiotics, can be used in achieving improved efficacy and reduced side effects compared to conventional treatments.

The delivery method using Eudragit® S100 and L100 coatings appears critical for the success of these therapies. The dual-coating approach likely facilitated targeted release in the GIT, enhancing the efficacy of the bacteriocins. Notably, the KvarM:EudSL and ColA-Ia:EudSL groups were more effective in colonization reduction than KvarM:EudS and KvarM:EudL targeting just small or large intestine retrospectively, supporting the hypothesis that both bacteriocins are equally effective when delivered using this method. A. Nikam et al. showed that this can be attributed to the distinct solubility thresholds of Eudragit® L100 and S100, which dissolve at pH values above 6.0 and 7.0, respectively, enabling precise drug release in specific regions of the GIT [69].

The main advantage of bacteriocin therapies is their minimal impact on the commensal gut microbiome compared to antibiotics. In this study, α-diversity and β-diversity analyses revealed significant disruptions in microbiome composition in the ciprofloxacin-treated group (Kpn:Ab) by day 9, including notable depletion of bacterial families such as *Enterobacteriaceae*, *Prevotellaceae*, and *Rikinelaceae*. In contrast, no significant changes in microbial diversity or abundance were observed in the KvarM:EudSL or ColA-Ia:EudSL groups. The preservation of microbiome integrity is a critical factor in maintaining host health and reducing the risk of secondary infections or dysbiosis-related conditions.

Supporting this, a study by A. Palleja et al. demonstrated that antibiotic-induced changes to the microbiome can persist for up to six months before α-diversity returns to baseline [70]. Moreover, Theriot et al. showed that such microbiome disruptions increase susceptibility to life-threatening *C. difficile* infections by altering the gut metabolic landscape to favor pathogen germination and growth [71]. These shifts include a decrease in protective secondary bile acids and an increase in nutrients that promote *C. difficile* outgrowth. Demonstrating antibiotic therapy often creates dysbiosis leading to opportunistic infections. This highlights the major benefit of bacteriocin-based therapies, which achieved pathogen suppression without disturbing the gut microbiome.

4.4. Wrap-up

Overall, the study successfully established GIT colonization models for *K. quasipneumoniae* and *K. pneumoniae* in mice, demonstrating the critical role of antibiotic pre-treatment in facilitating stable colonization, though during the microbiome analysis showing that virulent *K. pneumoniae* species are able colonize without any additional interventions. Targeted delivery of recombinant bacteriocin KvarIa using pH-sensitive coatings effectively reduced bacterial colonization in GIT, emphasizing the importance of coating and dosage optimization. As mentioned, GIT models for *K. pneumoniae* strains (ATCC® 12657™ and ATCC® 43816™) revealed strain-specific colonization dynamics, with antibiotic pre-treatment significantly enhancing bacterial colonization and altering gut microbiota composition. Furthermore, the study evaluated recombinant bacteriocins, KvarM and ColA-Ia, as narrow-spectrum antimicrobials, demonstrating their efficacy in reducing GIT infection comparable to ciprofloxacin while preserving gut microbiome diversity. Unlike ciprofloxacin, bacteriocin therapies had minimal effect on gut microbial diversity, addressing concerns about dysbiosis and resistance development. Altogether, these studies show strong potential of bacteriocins as safer alternatives to traditional antibiotics, offering targeted efficacy with reduced side effects and microbiome disruption. However, the limitations being small group sizes, further studies with larger sample sizes should be appropriate to confirm statistical significance and explore long-term outcomes.

CONCLUSIONS

1. The lower gastrointestinal tract models were established using three different *Klebsiella* strains. *Klebsiella quasipneumoniae* (DSM® 28212™) colonization model showed sustained colonization in antibiotic pre-treatreted groups. *Klebsiella pneumoniae* (ATCC® 12657™) colonization model showed sustained colonization in antibiotic pre-treatreted groups. *Klebsiella pneumoniae* (ATCC® 43816™) colonization model showed sustained colonization in all groups with or without antibiotic pre-treatment.
2. The average pH levels of mice gastrointestinal tract were determined: 3.3 ± 0.92 in the stomach, pH 6.5 ± 0.34 in the small intestine, and pH 7.4 ± 0.42 in the large intestine. These values led to the bacteriocin coating being formulated to release at pH levels above 5.5, ensuring targeted delivery beyond the stomach.
3. In a pilot study, tested concentrations of 100 µg and 1000 µg of klebicin KvarIa significantly reduced *Klebsiella quasipneumoniae* (DSM® 28212™) bacterial loads. Additionally, 2.5 µg/100 µL of klebicin KvarM and 12.5 µg/100 µL of chimericin ColA-Ia showed the trends toward *Klebsiella pneumoniae* (ATCC® 43816™) bacterial load reduction.
4. Recombinant bacteriocins KvarM and chimericin ColA-Ia did not significantly disrupt the composition or diversity of the gut microbiota. The dominance of phyla Firmicutes was maintained, with no significant changes in relative abundance before and after treatment with klebicin KvarM and chimericin ColA-Ia.

SANTRAUKA

Įvadas

Hospitalinės bakterinės virškinamosios sistemos (VS) infekcijos yra sunki ir opi sveikatos problema visame pasaulyje, lemianti komplikacijų riziką bei pacientų mirštamumo didėjimą gydymo įstaigose. Šiai sveikatos problemai išspręsti reikalingi milžiniški ekonominiai bei žmogiškieji ištekliai. Hospitalinės infekcijos (HI), dar vadinamos nozokominėmis, siejamos su atliekamomis invazinėmis, diagnostikos, gydymo procedūromis, neracionaliu antimikrobinių vaistų vartojimu arba susilpnėjusiu imuniniu pacientų atsaku. VS infekcijas lemia įvairūs patogenai: pirmuonys, virusai ir bakterijos, pvz., *Escherichia coli*, *Clostridioides difficile* ar *Klebsiella* genties atstovai. Į organizmą patekę patogenai sukelia skirtingo sunkumo pažeidimus – nuo lengvo gastroenterito iki sunkių, gyvybei grėsmingų būklių (pvz., sepsio) [1, 2, 4].

Klebsiella genties bakterijos neseniai buvo įtrauktos į Pasaulio sveikatos organizacijos (PSO) sąrašą kaip vienos pavojingiausių bakterijų, turinčių genetinius pokyčius, lemenčius jų atsparumą ampicilinui ir stiprėjantį atsparumą trečiosios kartos cefalosporinams Europoje. Net apie 10 proc. visų hospitalizuotų pacientų pasaulyje susiduria HI, todėl itin svarbu išsiaiškinti hospitalinių bakterijų kamienų plitimo mechanizmus, kelius, taikyti griežtas infekcijų kontrolės priemones, diegti antimikrobinio valdymo programas bei nuolat stebėti atsparumo antibiotikams tendencijas tiriant patogenų genetines mutacijas [2, 9, 12].

Klebsiella pneumoniae ir *Klebsiella quasipneumoniae* – tai fakultatyvinės, anaerobinės, gramneigiamosios lazdelės, dažnai nustatomos ligoninėse kaip oportunistinės infekcijos, kolonizuojančios žmogaus VS ir nosiaryklę. Neretai šie patogenai sukelia pneumoniją arba šlapimo takų infekcijas. Bakterijos ypač pavojingos nusilpusį imunitetą turintiems pacientams ar asmenims, kuriems atliktos sunkios invazinės procedūros, nes sukelia infekcijas, lemiančias komplikacijas arba padidėjusią mirtingumo tikimybę [14, 21, 64, 72].

Antibiotikai – veiksmingiausia bakterinių (tarp jų, ir *Klebsiella* genties) infekcijų gydymo priemonė. Tačiau netinkamas jų vartojimas lemia bakterijų, kurioms būdingas dauginis atsparumas (DA), atsiradimą bei plitimą.

Klebsiella atsparumas antibiotikams kai kuriose pasaulio šalyse siekia net iki 70 proc., o su juo susijęs mirštamumas – 40–70 proc. Ši situacija skatina ieškoti alternatyvių antimikrobinių priemonių. Viena alternatyva – bakteriocinai – ribosomų sintetinami peptidai ar maži baltymai, veikiantys

artimas bakterijų rūšis. Bakteriocinai turi siaurą veikimo spektą ri mažesnę atsparumo išsivystymo riziką [12, 13, 32].

Dėl šios priežasties tyrime pasirinkti rekombinantiniai bakteriocinai – KvarIa ir KvarM – kaip medžiagos, atsparių *Klebsiella* bakterijų sukeltoms infekcijoms gydyti. Šis darbas gali prisidėti prie naujų, veiksmingų gydymo strategijų kūrimo didėjant atsparumui antibiotikams.

Tyrimo tikslas ir uždaviniai

Tyrimo tikslas – taikant eksperimentinius gyvūnų modelius, įvertinti rekombinantinių bakteriocinų veiksmingumą, gydant *Klebsiella* genties bakterijų sukeltas infekcijas, ir nustatyti jų poveikį žarnyno mikrobiotai.

Uždaviniai:

1. Sukurti *Klebisiella* genties padermių apatinės virškinimo sistemos bakterinės kolonizacijos modelius ir įvertinti jų tinkamumą bakteriocinų veiksmingumui nustatyti.
2. Nustatyti tinkamas rekombinantinių bakteriocinų apvalkalų savybes, kurios būtų efektyviai taikomos modeliuose.
3. Įvertinti rekombinantinių bakteriocinų veiksmingumą gydant *Klebsiella* bakterijų sukeltas virškinimo sistemos infekcijas.
4. Nustatyti rekombinantinių bakteriocinų poveikį pelių žarnyno mikrobiotai.

Darbo naujumas ir aktualumas

Atsižvelgiant į didėjančią būtinybę kurti naujas antimikrobines terapines strategijas, išsaugojančias žarnyno mikrobiotą, šiame darbe analizuotas bakteriocinų poveikis *Klebsiella* genties bakterijoms – klinikinei praktikai svarbius patogenus, pasižyminčius vis didėjančiu atsparumu antibiotikams. Tyrime taikyti skirtingi virškinimo sistemos infekcijos modeliai, siekiant įvertinti bakteriocinų veiksmingumą esant skirtingoms eksperimentinėms sąlygoms: 1. Įvertintos *Klebsiella* bakterijų kamienų kolonizacijos virškinimo sistemoje galimybės. Remiantis gautais rezultatais identifikuotas tinkamiausiais *Klebsiella* kamienas, lemiantis bakterijų kolonizaciją be antibiotikų vartojimo. 2. Nustatyta, kad pelių virškinimo sistemos audiniuose ir išmatose pH didesnis kaip 5,5. Todėl pasirinkti vaistų apvalkalai, kurie bakteriocinus atpalaiduoja plonojoje ir (arba) storojoje žarnoje, o ne rūgštinėje skrandžio terpėje. 3. *Klebsiella quasipneumoniae* dažnai klaidingai identifikuojama kaip *K. pneumoniae* dėl panašių klinikinių požymių, tai lemia netinkamai parenkamą gydymą ir mažą terapinį veiksmingumą. Siekiant spręsti šią prob-

lemą, tyrime buvo vertinamas rekombinantinio bakteriocino KvarIa veiksmingumas. Rezultatai parodė reikšmingą žarnyno kolonizacijos sumažėjimą, patvirtinantį šio bakteriocino poveikį gydant *K. quasipneumoniae* infekcijas. 4. Tirtas KvarIa poveikis *K. quasipneumoniae* infekcijai gydyti. Patvirtinta, kad apvalkalu padengtas bakteriocinas KvarIa (100 μg ir 1000 μg) veiksmingai mažina *K. quasipneumoniae* kolonizaciją žarnyne. 4. *K. pneumoniae* yra prioritetinis DA patogenas dėl vis didėjančio atsparumo trečiosios kartos cefalosporinams. Sveikatos priežiūros įstaigose patogenas dažiausiai sukelia antrines infekcijas, kurios gydomos plataus veikimo spektro antibiotikais. Darbų metu analizavome antimikrobinį bakteriocinų poveikį, mažinantį *K. pneumoniae* bakterijų kiekį žarnyne. Nustatėme, kad KvarM, ColA-Ia ir chimerinas lemia iki 99 proc. *K. pneumoniae* bakterijų kolonizacijos sumažėjimą pelių VS, tai parodo šių medžiagų, kaip naujų gydymo priemonių, potencialą. 5. Skirtingai nuo įprastų antibiotikų, kurie suardo žarnyno mikrobiotos pusiausvyrą ir didina oportunistinių infekcijų riziką, rekombinantiniai bakteriocinai veikia tik tam tikras gimininges bakterijas. Šiame tyrime parodėme, kad KvarM ir ColA-Ia neturi reikšmingo poveikio pelių žarnyno mikrobiotai, todėl gali būti saugiai vartojami terapiniais tikslais gydant *Klebsiella* infekcijas.

Medžiagos ir metodai

Tyrimai atlikti vadovaujantis Europos Parlamento ir Tarybos direktyva 2010/63/ES bei patvirtinti Lietuvos bioetikos komiteto (protokolai Nr. G2-119, G2-184 ir G2-242). Eksperimentuose naudoti BALB/c ir C57BL/6J pelių modeliai siekiant ištirti *K. quasipneumoniae* ir *K. pneumoniae* kolonizacijos mechanizmus bei rekombinantinių bakteriocinų (KvarIa, KvarM ir ColA-Ia) terapinį poveikį virškinimo sistemos infekcijoms.

Sukurtos kelios eksperimentinės gyvūnų grupės, kurioms taikytos skirtingos antimikrobinės profilaktikos ir terapijų tyrimų planai, vertinant mažų ir didelių dozių bakteriocinų veiksmingumą. *K. quasipneumoniae* ir *K. pneumoniae* kolonizacija žarnyne ir mikrobiotos pokyčiai įvertinti atlikus atvirkštinės transkripcijos polimerazinę grandininę reakciją (RT–PGR) ir sekoskaitos tyrimus. Tyrimams naudoti pelių išmatų mėginiai. Siekiant užtikrinti tikslų vaisto išsiskyrimą, bakteriocinai buvo dengiami pH jautriais polimerais Eudragit® S100 (tirpus, jei pH 7 ir daugiau) ir L100 (tirpus, jei pH 5,5 ir daugiau), remiantis pelių virškinimo sistemos pH matavimais.

Bakteriocinai sintetinami augaluose, naudojant *Nicotiana benthamiana* raiškos sistemą, ir toliau gryninti chromatografijos metodu. Bakterijų kultū-

ros augintos standartinėmis sąlygomis lizogeninio sultinio (LB) terpėje, pritaikant ampiciliną.

Klebsiella bakterijų kiekiui nustatyti taikytas RT–PGR metodas naudojant *khe* geną kaip biologinį žymenį. *khe* genas pasirinktas vadovaujantis kitų mokslininkų darbais, kurie įrodė šio geno tinkamumą *Klebsiella* bakterijoms identifikuoti. Mikrobiotos pokyčiai žarnyne įvertinti atlikus naujos kartos sekoskaitą , amplifikuojant 16S rRNR genų V1–V2 sritis. Gauti duomenys analizuoti „*R Project for Statistical Computing"* programinės įrangos aplinkoje naudojant *dada2* ir *DESeq2* paketus. Nesant normalaus duomenų pasiskirstymo, statistinė analizė atlikta taikant neparametrinius metodus (*Kruskal–Wallis, Mann–Whitney*), PERMANOVA analizę bei *Kaplan–Meier* išgyvenamumo modelį. Rezultatai laikyti statistiškai reikšmingais, jei $p < 0,05$ arba $p_{adj} < 0,05$.

Rezultatai

Bandomuoju tyrimu nustatyta, kad *K. quasipneumoniae* kolonizuoja pelių žarnyną tik po antibiotikų terapijos (penicilino 2000 U/ml, streptomicino 2 mg/ml, metronidazolo 1 mg/l), kuri sutrikdo natūralią mikrobiotą. Kolonizacijos lygis antibiotikais paveiktose pelių grupėse išliko tolygus 4–8 eksperimento dienomis (*Pen:Strep* grupėje vidutiniškai siekė $5,26 \times 10^8$ KSV, o *Pen:Strep:Met* grupėje – $5,16 \times 10^8$ KSV ($p < 0,001$)), nors 11-ąją dieną šiek tiek sumažėjo (atitinkamai $3,12 \times 10^8$ ir $4,04 \times 10^8$ KSV). Pelių virškinimo sistemos pH matmenys padėjo sėkmingai parinkti Eudragit® apvalkalus ir juos panaudoti bakteriocinams (KvarIa ir KvarM) padengti – taip užtikrintas vaistų išsiskyrimas plonojoje ir storojoje žarnose bei išvengta jų skilimo rūgštinėje skrandžio terpėje.

Rekombinantinis bakteriocinas KvarIa statistiškai reikšmingai sumažino *K. quasipneumoniae* (DSM® 28212™) kiekį pelių žarnyne, kai buvo naudojamos Eudragit® padengtos šio bakteriocino formos. AbII:Ia pelių grupėje bakterijų kiekis sumažėjo nuo $6,3 \times 10^7$ KSV (18-ąją dieną) iki $3,9 \times 10^5$ KSV (22-ąją dieną) ($p = 0,01$), o *Hi:Ia* grupėje – nuo $4,0 \times 10^7$ iki $1,6 \times 10^5$ KSV ($p = 0,009$), zondo pagalba suleidus 100 μg ir 1000 μg terapines dozes. Tačiau VOC grupėje, kuriai buvo skiriamas nepadengtas KvarIa, reikšmingo bakterijų kiekio sumažėjimo nepastebėta. Gauti rezultatai patvirtina padengto KvarIa veiksmingumą mažinant *K. quasipneumoniae* bakterijų kolonizaciją žarnyne.

Kurdami *K. pneumoniae* kolonizacijos modelius, nustatėme, kad tradicinės antimikrobinės priemonės (antibiotikai) turi skirtingą poveikį *K. pneumoniae* kamienų kolonizacijai. Tyrimo metu pastebėjome, kad *K. pneumoniae*

kamieno (ATCC® 12657™) kolonizacija pelių žarnyne, nenaudojant antibiotikų greitai mažėjo – 8-ąją dieną siekė $1{,}21 \times 10^4$ KSV, o 22-ąją dieną – tik $6{,}10 \times 10^1$ KSV ($p = 1{,}06 \times 10^{-3}$)). Tačiau antibiotikais veiktose grupėse (Ab:KpnI) bakterijų kiekis išliko didelis – vidutiniškai $8{,}64 \times 10^7$ KSV viso tyrimo metu (22-ų dienų laikotarpiu). Kitos *K. pneumoniae* padermės (ATCC® 43816™) tyrimai atskleidė priešingus rezultatus: bakterijos tolygiai kolonizavo žarnyną visose pelių grupėse, neatsižvelgiant į tai, ar gyvūnams buvo skiriami antibiotikai ar ne (vidutinis kiekis $7{,}72 \times 10^8$ KSV). Pastebėjus stiprias kolonizacijos ypatybes, šis kamienas pasirinktas bakteriocino KvarM antimikrobiniam veiksmingumui ištirti. Klebicino KvarM terapija (2,5 µg/100 µl) sumažino bakterijų kiekį nuo $2{,}18 \times 10^5$ iki $1{,}27 \times 10^3$ KSV, o ColA-Ia (12,5 µg/100 µl) – nuo $1{,}73 \times 10^5$ iki $1{,}12 \times 10^3$ KSV. Gauti rezultatai taip pat patvirtino stiprų klebicino KvarM antibakterinį poveikį, ypač jeigu jis buvo padengtas Eudragit® S100 ir L100 polimerais.

Kitais etapais atlikta pelių žarnyno mikrobiotos analizė. Nustatyta, kad klebicinas KvarM, chimerinas ColA-Ia ar naudojami polimerai (Eudragit® S100 ir L100) neturi reikšmingo poveikio sveikų pelių mikrobiotai – bakterijų įvairovė ir gausa išlika stabili ($p > 0{,}05$). Tačiau ciprofloksacino terapija (Kpn:Ab grupė) sukelia ryškius žarnyno mikrobiotos pokyčius: sumažėja *Clostridiaceae*, *Enterobacteriaceae*, *Lactobacillaceae* ir *Butyricicoccaceae* šeimų gausa ($p_{adj} < 0{,}05$), padidėjo *Bacteroidaceae*, *Muribaculaceae*, *Prevotellaceae* ir *Rikenellaceae*. Alfa įvairovės analizė (Shannon indeksas) parodė reikšmingą įvairovės sumažėjimą tik antibiotikais paveiktų pelių grupėje ($p_{adj} = 0{,}032$), o įvetinę beta įvairovę (UniFrac indeksai) pastebėjome, kad Kpn:Ab pelių grupė formuoja atskirą telkinį, skirtingą nuo VC, KvarM: EudSL ir ColA-Ia:EudSL grupių ($p < 0{,}05$). Tai įrodo, kad rekombinantiniai bakteriocinai neveikia mikrobiotos struktūros, priešingai nei plataus veikimo spektro antibiotikai, pvz., ciprofloksacinas.

Apibendrinant, tyrimo rezultatai patvirtina, kad rekombinantiniai bakteriocinai KvarIa, KvarM ir ColA-Ia veiksmingai mažina antibiotikams atsparių *Klebsiella* kamienų kolonizaciją virškinimo sistemoje. Gydymo metu bakterijų kiekis sumažėja daugiau nei 99 proc. ir šis poveikis ypač ryškus, kai bakteriocinai yra padengti pH jautriais polimerais, kurie neleidžia jiems atsipalaiduoti skrandyje. Skirtingai nei plataus veikimo spektro antibiotikai (ciprofloksacinas), bakteriocinai netrikdo natūraliosios žarnyno mikrobiotos (nei bendros taksonominės sudėties, nei įvairovės rodiklių). Gauti duomenys padės praplėsti jau turimas žinias apie bakteriocinus, prisidės prie naujų terapinių priemonių kūrimo, įgalinančių efektyviai kovoti su patogenais ir kartu išsaugoti žarnyno mikrobiotos pusiausvyrą.

Išvados

1. Sukurti trys apatinės virškinimo sistemos kolonizacijos modeliai naudojant skirtingus *Klebsiella* kamienus. *Klebsiella quasipneumoniae* (DSM® 28212™) kolonizacija buvo stabili antibiotikais paveiktose grupėse. *Klebsiella pneumoniae* (ATCC® 12657™) kolonizacija išliko stabili tik antibiotikais paveiktoms pelėms, o *Klebsiella pneumoniae* (ATCC® 43816™) nustatyta nekintama kolonizacija visose grupėse – tiek su, tiek be antibiotikų.
2. Nustatyti pelės virškinimo sistemos vidutiniai pH rodikliai: 3,3 ± 0,92 skrandyje, 6,5 ± 0,34 plonojoje žarnoje ir 7,4 ± 0,42 storojoje žarnoje. Remiantis šiais duomenimis, bakteriocinams padengti buvo parinktas apvalkalas, užtikrinantis veikliųjų medžiagų išsiskyrimą esant pH > 5,5 – išvengiama irimo skrandžio terpėje ir pasiekiamos tikslinės žarnyno vietos.
3. Klebcino KvarIa 100 µg ir 1000 µg koncentracijos sumažino *Klebsiella quasipneumoniae* (DSM® 28212™) kiekį. Infekcijos modelyje taip pat nustatyta, kad klebicinas KvarM 2,5 µg/100 µl ir chimericinas ColA-Ia 12,5 µg/100 µl tendencingai mažina *Klebsiella pneumoniae* (ATCC® 43816™) bakterijų kiekį.
4. Rekombinantiniai bakteriocinai, klebicinas KvarM ir chimericinas ColA-Ia, nepaveikė žarnyno mikrobiotos sudėties ar įvairovės. Reikšmingų bakterijų santykinio gausumo pokyčių prieš ir po gydymo nenustatyta, labiausiai vyraujančios išliko *Firmicutes* tipo bakterijos.

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2. Karaliūtė, I., Tilindė, D., Ramonaitė, R., Kupčinskas, J., Misiūnas, A., Denkovskienė, E., Gleba, Y., Ražanskienė, A., Skiecevičienė, J.. (2023, kovo 1). Klebicin as a novel therapy for Klebsiella infection management. *Journal of Crohn's and Colitis – JCC : Abstracts of the 18th Congress of ECCO (European Crohn's and Colitis Organisation) : Copenhagen, Denmark, March 1–4, 2023 European Crohn's and Colitis Organisation; Editor-in-Chief Laurence J. Egan. Oxford : Oxford University Press., 2023, Vol. 17, Suppl. 1, February*. https://doi.org/10.1093/ecco-jcc/jjac190.0189 [T1a1] [Field of science: M001] [Citav. rodiklis: 8.3, bendr. cit. rod.: 4.971, kvartilis: Q1 (2023. InCites JCR SCIE)]
3. Karaliūtė, I., Tilindė, D., Ramonaitė, R., Kupčinskas, J., Misiūnas, A., Denskovienė, E., Nikitina, D., Gleba, Y., Ražanskienė, A., Skiecevičienė, J. (2023). KvarM lowers Klebsiella pneumoniae colonization in gastrointestinal tract without disruption of gut microbiota. *Microbiota in Health and Disease : EHMSG – 36th International Workshop on Helicobacter and amp; Microbiota in Inflammation and amp; Cancer : September 7–9, 2023, Antwerp, Belgium : Accepted Abstracts European Helicobacter and Microbiota Study Group (EHMSG).*, *5*, 7–7. https://doi.org/10.26355/mhd_20239_857 [T1c] [Field of science: M001]
4. Karaliūtė, I., Ramonaitė, R., Kupčinskas, J., Misiūnas, A., Denkovskienė, E., Gleba, Y. Y., Ražanskienė, A., Skiecevičienė, J. (2022, vasario 16). Treatment of the lower gastrointestinal tract Klebsiella infections by recombinant bacteriocin KvarIa. *Journal of Crohn's and Colitis : Abstracts of the 17th Congress of ECCO : February 16–19, 2022, Vienna, Austra European Crohn's and Colitis Organisation. Oxford : Oxford University Press., 2022, Vol. 16, Suppl. 1*. https://doi.org/10.1093/ecco-jcc/jjab232.233 [T1a1] [Field of science: M001] [Citav. rodiklis: 8, bendr. cit. rod.: 6.2, kvartilis: Q1 (2022. InCites JCR SCIE)]

5. Karaliūtė, I., Ramonaitė, R., Kupčinskas, J., Misiūnas, A., Denkovskienė, E., Skiecevičienė, J., Gleba, Y., Ražanskienė, A. (2021). The Establishment of Klebsiella Quasipneumoniae Gastrointestinal Mice Model and Eradication of Pathogen by Oral Delivery of Encapsulated Klebicin Kvaria. *United European Gastroenterology Journal : 29th United European Gastroenterology Week Virtual : 2–6 October 2021, Vienna, Austria : Abstract Issue Organizer: United European Gastroenterology. London : Sage Publications, 2021, Vol. 9, Iss. Suppl. 8, October.*, 591–592. https://doi.org/10.1002/ueg2.12144 [T1a1] [Field of science: M001] [Citav. rodiklis: 6.866, bendr. cit. rod.: 7.182, kvartilis: Q1 (2021. InCites JCR SCIE)]
6. Karaliūtė, I., Ramonaitė, R., Kupčinskas, J., Misiūnas, A., Denkovskienė, E., Skiecevičienė, J., Gleba, Y., Ražanskienė, A. (2021). Rekombinantinių bakteriocinų naudojimas Klebsiella quasipneumoniae sukeltai žarnyno infekcijai gydyti. *14-oji Lietuvos jaunųjų Mokslininkų Konferencija BIOATEITIS: Gamtos ir gyvybės mokslų perspektyvos: pranešimų santraukos : 2021 m. lapkričio 25 d., Kaunas. Lietuvos mokslų akademijos Biologijos, Medicinos ir Geomokslų skyrius. Lietuvos sveikatos mokslų universitetas ; [leidinį sudarė Jadvyga Olechnovičienė ir Vaidutis Kučinskas]. Vilnius : Lietuvos mokslų akademija, 2021. ISBN 9789986080855.*, 41–41. https://hdl.handle.net/20.500.12512/112296 [T2] [Field of science: M001]

SUPPLEMENTS

Supplement 1

VALSTYBINĖ MAISTO IR VETERINARIJOS TARNYBA

LEIDIMAS
ATLIKTI BANDYMO SU GYVŪNAIS PROJEKTĄ

2019-06-12 Nr. G2-119
Vilnius

Vadovaujantis Lietuvos Respublikos gyvūnų gerovės ir apsaugos įstatymo 16 straipsnio 4 dalimi, Mokslo ir mokymo tikslais naudojamų gyvūnų laikymo, priežiūros ir naudojimo reikalavimais, patvirtintais Valstybinės maisto ir veterinarijos tarnybos direktoriaus 2012 m. spalio 31 d. įsakymu Nr. B1-866 „Dėl Mokslo ir mokymo tikslais naudojamų gyvūnų laikymo, priežiūros ir naudojimo reikalavimų patvirtinimo“, Europos konvencija dėl eksperimentiniais ir kitais mokslo tikslais naudojamų stuburinių gyvūnų apsaugos (OL *2004 m. specialusis leidimas*, 15 skyrius, 4 tomas, p. 325) ir remiantis Lietuvos bandomųjų gyvūnų naudojimo etikos komisijos prie Valstybinės maisto ir veterinarijos tarnybos 2019-06-04 išvada Nr. 13 „Dėl leidimo atlikti bandymus su gyvūnais“,

l e i d ž i a m a šiam ūkio subjektui atlikti bandymo su gyvūnais projektą:

duomenys apie ūkio subjektą:

pavadinimas Lietuvos sveikatos mokslų universiteto Biologinių tyrimų centras,

adresas Tilžės g. 18, Kaunas,

kodas Juridinių asmenų registre 302536989;

duomenys apie bandymo su gyvūnais projektą:

pavadinimas „Augalinėje sistemoje pagaminto baltymo antimikrobinio efektyvumo įvertinimas pelių modelyje“,

vadovas Vilma Petrikaitė,

naudojami gyvūnai 156 pelės;

duomenys apie bandymo su gyvūnais projekto atlikimo vietą:

pavadinimas Lietuvos sveikatos mokslų universiteto Biologinių tyrimų centras,

adresas Tilžės g. 18, Kaunas.

Duomenys apie veterinarinius vaistus, vaistinius preparatus ar kitas medžiagas (toliau – vaistai), kurie bus naudojami vykdant bandymo su gyvūnais projektą*.

Eil. Nr.	Vaisto pavadinimas	Gamintojas	Vaistinė forma	Kiekis, reikalingas vykdant bandymo su gyvūnais projektą
1.	PENICILINAS IR STREPTOMICINAS	Sigma Aldrich, USA	Injekcinis tirpalas	1000 ml
2.	AMPICILINAS	Sigma Aldrich, USA	Injekcinis tirpalas	100 ml

Leidimas atlikti bandymo su gyvūnais projektą (toliau – leidimas) galioja iki 2022 m. gruodžio 31 d.

Direktoriaus pavaduotojas Vidmantas Paulauskas

* – Nurodomi naudojamo (-ų) vaisto (-ų) pavadinimas (-ai), gamintojas (-ai), vaistinė (-ės) forma (-os), kiekis, reikalingas vykdant bandymo su gyvūnais projektą. Jei vykdant bandymo su gyvūnais projektą vaistai gyvūnams nenaudojami, leidime įrašomas žodis „Nenaudojama“

Supplement 2

VALSTYBINĖ MAISTO IR VETERINARIJOS TARNYBA

LEIDIMAS
ATLIKTI BANDYMO SU GYVŪNAIS PROJEKTĄ

2021-05-31 Nr. G2-184
Leidimo pakeitimo data: 2021-07-

Vilnius

Vadovaujantis Lietuvos Respublikos gyvūnų gerovės ir apsaugos įstatymo 16 straipsnio 4 dalimi, Mokslo ir mokymo tikslais naudojamų gyvūnų laikymo, priežiūros ir naudojimo reikalavimais, patvirtintais Valstybinės maisto ir veterinarijos tarnybos direktoriaus 2012 m. spalio 31 d. įsakymu Nr. B1-866 „Dėl Mokslo ir mokymo tikslais naudojamų gyvūnų laikymo, priežiūros ir naudojimo reikalavimų patvirtinimo", Europos konvencija dėl eksperimentiniais ir kitais mokslo tikslais naudojamų stuburinių gyvūnų apsaugos (OL *2004 m. specialusis leidimas,* 15 skyrius, 4 tomas, p. 325) ir remiantis Lietuvos bandomųjų gyvūnų naudojimo etikos komisijos prie Valstybinės maisto ir veterinarijos tarnybos 2021-05-18 išvada Nr. 6 „Dėl leidimo atlikti bandymus su gyvūnais",

l e i d ž i a m a šiam (šiems) ūkio subjektui (-ams) atlikti bandymo su gyvūnais projektą:

duomenys apie ūkio subjektą (-us):

pavadinimas Lietuvos sveikatos mokslų universiteto Biologinių tyrimų centras,

adresas: Tilžės g. 18, Kaunas,

kodas Juridinių asmenų registre 302536989;

duomenys apie bandymo su gyvūnais projektą:

pavadinimas „Rekombinantinių baltymų aktinomikrobinio efektyvumo ir poveikio navikams įvertinimas",

vadovas dr. prof. Vilma Petrikaitė,

naudojami gyvūnai 170 pelių;

duomenys apie bandymo su gyvūnais projekto atlikimo vietą:

pavadinimas Lietuvos sveikatos mokslų universiteto Biologinių tyrimų centras,

adresas: Tilžės g. 18, Kaunas.

Duomenys apie veterinarinius vaistus, vaistinius preparatus ar kitas medžiagas (toliau – vaistai), kurie bus naudojami vykdant bandymo su gyvūnais projektą*:

Eil. Nr.	Vaisto pavadinimas	Gamintojas	Vaistinė forma	Kiekis, reikalingas vykdant bandymo su gyvūnais projektą
1.	PENICILINAS IR STREPTOMICINAS	Sigma Aldrich, Jungtinės Amerikos Valstijos	Tirpalas	10 fasuočių po 100 ml
2.	AMPICILINAS	Sigma Aldrich, Jungtinės Amerikos Valstijos	Tirpalas	10 fasuočių po 10 ml
3.	SPECIALAUS FORMULAVIMO BAKTERIOCINAI	Pagamintas atliekant tiriamąsias veiklas	Tirpalas (su ištirpintu baltymu)	10 mg/100 µl – terapinė dozė
4.	ŽMOGAUS VIRŠKINIMO SISTEMOS VĖŽINĖS LĄSTELIŲ LINIJOS	ATCC	Ląstelės	10^6 ląstelių linijos

Leidimas atlikti bandymo su gyvūnais projektą (toliau – leidimas) galioja nuo 2021-06-01 iki 2024-06-01.

Direktoriaus pavaduotojas,
pavaduojantis direktorių Vidmantas Paulauskas

*– Nurodomi naudojamo (-ų) vaisto (-ų) pavadinimas (-ai), gamintojas (-ai), vaistinė (-ės) forma (-os), kiekis, reikalingas vykdant bandymo su gyvūnais projektą. Jei vykdant bandymo su gyvūnais projektą vaistai gyvūnams nenaudojami, leidime įrašomas žodis „Nenaudojama"

Supplement 3

VALSTYBINĖ MAISTO IR VETERINARIJOS TARNYBA

LEIDIMAS
ATLIKTI BANDYMO SU GYVŪNAIS PROJEKTĄ

2023-03-21 Nr. G2-242
Vilnius

Vadovaujantis Lietuvos Respublikos gyvūnų gerovės ir apsaugos įstatymo 16 straipsnio 4 dalimi, Mokslo ir mokymo tikslais naudojamų gyvūnų laikymo, priežiūros ir naudojimo reikalavimais, patvirtintais Valstybinės maisto ir veterinarijos tarnybos direktoriaus 2012 m. spalio 31 d. įsakymų Nr. B1-866 „Dėl Mokslo ir mokymo tikslais naudojamų gyvūnų laikymo, priežiūros ir naudojimo reikalavimų patvirtinimo“, Europos konvencija dėl eksperimentiniais ir kitais mokslo tikslais naudojamų stuburinių gyvūnų apsaugos (OL *2004 m. specialusis leidimas*, 15 skyrius, 4 tomas, p. 325) ir remiantis Lietuvos bandomųjų gyvūnų naudojimo etikos komisijos prie Valstybinės maisto ir veterinarijos tarnybos 2023-03-16 išvada „Dėl LSMU Biologinių tyrimų centro 2023-02 paraiškos dėl bandymo su gyvūnais procedūrų projekto „Bakteriocinų antimikrobinio efektyvumo ir poveikio navikams įvertinimas“:

l e i d ž i a m a šiam ūkio subjektui (-ams) atlikti bandymo su gyvūnais projektą:
duomenys apie ūkio subjektą (-us):
pavadinimas Lietuvos sveikatos mokslų universiteto Biologinių tyrimų centras,
adresas: Tilžės g. 18, Kaunas,
kodas Juridinių asmenų registre 302536989;
duomenys apie bandymo su gyvūnais projektą:
pavadinimas „Bakteriocinų antimikrobinio efektyvumo ir poveikio navikams įvertinimas“,
vadovas Indrė Karaliūtė,
naudojami gyvūnai: 256 pelės (genetiškai pakeisti gyvūnai),
duomenys apie bandymo su gyvūnais projekto atlikimo vietą:
pavadinimas Lietuvos sveikatos mokslų universiteto Biologinių tyrimų centras,
adresas: Tilžės g. 18, Kaunas.

Duomenys apie veterinarinius vaistus, vaistinius preparatus ar kitas medžiagas (toliau – vaistai), kurie bus naudojami vykdant bandymo su gyvūnais projektą*:

Eil. Nr.	**Vaisto pavadinimas**	**Gamintojas**	**Vaistinė forma**	**Kiekis, reikalingas vykdant bandymo su gyvūnais projektą**
1.	PENICILINAS IR STREPTOMICINAS	Sigma Aldrich, USA	Tirpalas	12 pak. po 100 ml
2.	AMPICILINAS	Sigma Aldrich, USA	Tirpalas	10 pak. po 10 ml
3.	SPECIALAUS FORMULAVIMO BAKTERIOCINAI	Pagamintas atliekant tiriamąsias veiklas	Tirpalas (su ištirpintu baltymu)	10 mg/100 µl – terapinė dozė, bendras kiekis priklausys nuo tyrimo rezultatų
4.	CEFUROKSIMAS	Sigma Aldrich, USA	Milteliai	3 pak.
5.	MEROPENEMAS	Sigma Aldrich, USA	Milteliai	3 pak.
6.	CEFTRIAKSONAS	Sigma Aldrich, USA	Milteliai	3 pak.
7.	CEFTAZIDIMAS	Sigma Aldrich, USA	Milteliai	3 pak.
8.	CIPROFLOKSACINAS	Sigma Aldrich, USA	Milteliai	4 pak.

Leidimas atlikti bandymo su gyvūnais projektą (toliau – leidimas) galioja nuo 2023-04-06 iki 2026-04-03.

Direktoriaus pavaduotoja,
atliekanti direktoriaus funkcijas Audronė Mikalauskienė

LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS

LIETUVOS SVEIKATOS MOKSLŲ UNIVERSITETAS
PODIPLOMINIŲ STUDIJŲ CENTRAS
LITHUANIAN UNIVERSITY OF HEALTH SCIENCES
CENTRE FOR POSTGRADUATE STUDIES

PAŽYMĖJIMAS
CERTIFICATE

Indrė Karaliūtė

Šiuo pažymėjimu patvirtinama, kad nurodytas asmuo išklausė intensyvaus mokymo kurso programą **MOKSLO IR MOKYMO TIKSLAIS NAUDOJAMŲ GYVŪNŲ LAIKYMO, PRIEŽIŪROS IR NAUDOJIMO REIKALAVIMAI**, 2021-01-07/2021-03-04, Lietuvos sveikatos mokslų universiteto Veterinarijos akademijoje (LSMU VA), Tilžės g. 18, Kaunas, Lietuva.

This certificate is to verify that the person named above, has successfully fulfilled the intensive course ***KEEPING, CARE AND USAGE REQUIREMENTS FOR ANIMALS USED FOR SCIENTIFIC AND EDUCATIONAL PURPOSES*** *held from January 7th – March 4th, 2021 in Lithuanian University of Health Sciences, Veterinary Academy, Tilžės 18, Kaunas, Lithuania.*

Mokymo programos trukmė 80 val. (3 kreditai). Mokymų būdas: paskaitos, praktiniai užsiėmimai grupėse, individualios užduoties atlikimas, projekto parengimas ir egzaminas raštu.

The training program consisted of 80 hours (3 ECTS credits) of lectures, hands-on exercises, work in groups, presentation of an individual task and project and final passed the written examination.

Kurso mokymo programa suderinta su Valstybine maisto ir veterinarijos tarnyba 2013-10-16 raštu Nr. B6-(1.9)-2625, atnaujinta 2017-03-28, raštas Nr. B6-(1.9)-852. Programa atitinka 2010 m. rugsėjo 22 d. Europos Parlamento ir Tarybos direktyvą 2010/63 ES. Mokymų kursų dalyvis turi reikiamų žinių, yra atitinkamai apmokytas atlikti bet kurią iš funkcijų, nurodytų Direktyvos 23 straipsnyje (a, b, c, d).

The course program was approved by The State Food and Veterinary Service by official letter No. B6-(1.9)-2625 of 16 October 2013 and updated 28 th of March, 2017, official letter No B6-(1.9)-852. The curriculum fulfills requirements of the directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010. Participant of this training course is adequately educated and trained to perform any of the functions mentioned in Article 23 (a, b, c, d) of the Directive 2010/63 EU.

Prof. dr. Mindaugas Malakauskas
LSMU VA kancleris
Chancellor of Veterinary Academy
Lithuanian University of Health Sciences

Prof. dr. Rasa Želvytė
LSMU VA Anatomijos ir fiziologijos katedros vedėja
Head of Anatomy and Physiology Department
Lithuanian University of Health Sciences

Pažymėjimo reg. Nr. PK022896
Certificate registration N. PK022896

Data: 2021.03.04
Date of issuing: 04/03/2021

UAB
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expedit pel Rector
de la Universitat Autònoma de Barcelona a

CERTIFICADO

expedido por el Rector
de la Universitat Autònoma de Barcelona a

Indre Karaliute

per la seva participació,
amb avaluació positiva, al curs

Training Course In Laboratory Animal Science For Scientists Responsible For The Design Or Conduct Of Animal Experiments (January)

de 5 crèdits ECTS *(European Credit Transfer System)*, organitzat, en el curs 2021-2022, i acreditat per FELASA.
Reference: FELASA accredited course F002/03 Functions: A+B+C+D

Bellaterra (Cerdanyola del Vallès), 15 de març de 2022

por su participación,
con evaluación positiva, en el curso

Training Course In Laboratory Animal Science For Scientists Responsible For The Design Or Conduct Of Animal Experiments (January)

de 5 créditos ECTS *(European Credit Transfer System)*, organizado, en el curso 2021-2022, y acreditado por FELASA.
Reference: FELASA accredited course F002/03 Functions: A+B+C+D

Bellaterra (Cerdanyola del Vallès), 11 de abril de 2022

La persona interessada,

Indre Karaliute

El Rector,

Francisco Javier Lafuente Sancho

La Vicerectora d' Estudis i d' Innovació Docent,

Maria Valdés Gázquez

Reg. nº 2022-00162

I. UNIVERSAL, S.L.

CURRICULUM VITAE

Name Surname: **Indrė Karaliūtė**
E-mail: indre.karaliute@lsmu.lt; karaliute.in@gmail.com
Phone: +370 651 14404

Education

2020–present **PhD candidate in Natural Sciences, Biology, Lithuanian University of Health Sciences, Kaunas, Lithuania**
Thesis: „Recombinant bacteriocins as narrow spectrum antimicrobial agents“. *Supervisor*: prof. J. Skiecevičienė

2018–2020 **MSc by Research, Integrative Neuroscience, The University of Edinburgh, Edinburgh, United Kingdom**
Thesis: „The Relationship between Multiple Sclerosis and Lymphopenia, Drugs and Treatment“. *Supervisor*: dr. Peter Connick

2014–2018 **BSc, Medical and Veterinary Genetics, Lithuanian University of Health Sciences, Kaunas, Lithuania**
Thesis: „*CYP2D6, CYP2C9, CYP2C19, VKORC1* polymorphisms in cases of drugs intolerance“. *Supervisor*: prof. V. Ašmonienė

Professional experience

2020–present **Institute for Digestive Research, Laboratory of Clinical and Molecular Gastroenterology, Lithuanian University of Health Sciences, Kaunas, Lithuania**
Position: junior researcher

2022–2024 Council of Doctoral Students, Lithuanian University of Health Sciences, Kaunas, Lithuania
Position: president, senator

2017–2017 **Institute for Veterinary Genetics, University of Aleksandras Stulginskis, Kaunas, Lithuania**
Position: laboratory technician

Research projects

2022–present Junior researcher, *Project:* Development of mice model for microscopic colitis: implementation of microbiota transplantation (McMouse, no. S-MIP-23-100), funded by Research Council of Lithuania

2022–present Junior researcher, *Project:* Lyophilized fecal microbiome transfer for primary Clostridioides difficile infection (DONATE study): a multicenter randomized controlled trial (no. JPIAMR2022-020), funded by EU Structural Funds (HORIZON) and Research Council of Lithuania

2022–2023	Junior researcher, *Project:* Gut-blood-liver axis: Circulating microbiome as non-invasive biomarker for Inflammatory bowel disease (IBD) and Primary sclerosing cholangitis (PSC) (no. S-BMT-21-11), funded by EEA grants and Research Council of Lithuania
2022–2023	Junior researcher, *Project:* The intestinal organoid model: a system for drug and personalized therapy testing of inflammatory bowel diseases (grant no. 01.2.2-LMT-K718-04-003), funded by EU Structural Funds and Research Council of Lithuania

Scientific internships

2023	Oslo University Hospital, Research Institute for Internal Medicine, Oslo, Norway
2017	Clinica Neurologica e Malttie Neurometaboliche, Universiteta di Siena U.O.C., Siena, Italy
2017	Laboratory of Neurooncology and Genetics, LSMU Neuroscience Institute, Kaunas, Lithuania
2017	Neurology Department, Vilnius University Hospital, Vilnius, Lithuania
2016	Laboratory of Opthalmology, LSMU Neuroscience Institute, Kaunas, Lithuania
2015–2016	Genetics and Molecular Medicine Clinic, Hospital of Lithuanian University of Health Sciences Kauno klinikos, Kaunas, Lithuania

Additional courses and certificates

2022	Training course in laboratory animal science for scientists responsible for the design or conduct of animal experiments (functions a, b, c, d according to Directive EU63/2010), Barcelona, Spain (No. 202200162)
2021	Keeping, care and usage requirements for animals used for scientific and educational purposes, Kaunas, Lithuania (No. PK022896)
2020	„Modern treatment of migrene and other actualities of general medicine 2020“, Vilnius, Lithuania (No. M-1242-526)
2019	Childhood Autism and Comorbidities, Vilnius, Lithuania (No. 1500000-K-98)
2015	Biomarkers of Chronic Diseases, Kaunas, Lithuania (No. PS-10-101-21639)

ACKNOWLEDGMENTS

I would like to express my heartfelt gratitude to everyone who supported me and generously shared their knowledge and experience throughout the process of preparing this dissertation. Each of you inspired me to pursue better results and grow as a researcher. Thank you for your patience, understanding, invaluable advice, and constant support during this challenging stage of my life.

First and foremost, I extend my sincere and deepest appreciation to Prof. Dr. Jurgita Skiecevičienė, my dissertation supervisor. Her scientific insight, critical thinking, and tireless support were fundamental throughout every stage of this work. She consistently encouraged me to think independently and to approach challenges with confidence and curiosity. Her high standards and thoughtful feedback shaped not only the quality of this dissertation but also my development as a researcher. I am especially grateful for her patience, her trust in my abilities, and her willingness to provide guidance even in the most demanding circumstances. It has been a privilege to work under her supervision.

I would also like to extend my sincere thanks to the entire NOMADS UAB team, with whom I had the privilege to collaborate during the course of this dissertation. Their expertise, technical assistance, and active involvement in the experimental work, data analysis, and preparation of publications were truly valuable. I am especially grateful for the productive and professional partnership, which enhanced both the scientific depth and the translational significance of this research.

I am sincerely grateful to the entire team at the Institute for Digestive Research, whose collaborative spirit and friendly atmosphere were an important part of my scientific work. Your support extended beyond professional matters, creating a sense of community that helped me navigate both professional and personal challenges. I am especially thankful to PhD student Deimantė Tilindė, Dr. Rokas Lukoševičius, Dr. Darja Nikitina, Dr. Rima Ramonaitė, whose work and input greatly contributed to the execution of the experiments and the interpretation of the results. I also wish to thank Dr. Violeta Šaltenienė, who welcomed me into the institute and helped me find my footing, and to Dr. Rūta Inčiūraitė, whose knowledge, generosity in sharing it, and constant readiness to help made her an invaluable mentor and colleague throughout this journey.

My sincere thanks go to Prof. Habil. Dr. Limas Kupčinskas and Prof. Dr. Juozas Kupčinskas, whose leadership and belief in my potential played a crucial role in my academic development. Their commitment to advancing

translational research and fostering collaboration between clinical and biological sciences created a rich environment in which I was able to grow both intellectually and professionally. I am especially grateful for the opportunities they provided to engage in multidisciplinary projects, to participate in scientific discussions that bridged laboratory and clinical perspectives, and to gain firsthand experience in the broader context of gastroenterological research. Their guidance helped shape not only this dissertation, but also my long-term vision as a scientist.

I am also deeply thankful to Prof. Habil. Dr. Vaiva Lesauskaitė and Prof. Dr. Ingrida Ulozienė for their consistent support throughout my doctoral studies and especially during my term as President of the Doctoral Student Council. I am truly grateful for their organizational guidance, administrative backing, and scientific insight, all of which contributed significantly to my growth – not only as a researcher, but also as a leader. Their trust and encouragement empowered me to take on new responsibilities with confidence and to develop the skills needed to navigate both academic and institutional challenges.

I extend my sincere gratitude to the Research Centre of the Lithuanian University of Health Sciences team for their continuous and sincere assistance throughout my doctoral studies. Whether it involved administrative, technical, or procedural matters, the Centre was always ready to help – efficiently, kindly, and without hesitation. Your dependable support ensured that many behind-the-scenes aspects of this journey ran smoothly, allowing me to focus fully on the scientific work.

To all my friends and colleagues who took an interest in my work, shared their thoughts, and offered a positive outlook – thank you for your encouragement, your conversations, and your presence along the way.

Finally, I wish to express my heartfelt appreciation to my family. Thank you for always standing by my side, for trusting my decisions, and for encouraging me in moments of doubt. You are my greatest source of motivation and the reason I strive to grow both as a person and as a scientist. I am truly grateful for the care, reassurance, and love you offer me at every step of the way.