Multimodal treatment of intestinal carriage of multi-drug resistant bacteria with probiotics and prebiotics

Murad Ishnaiwer

DOCTORAT BIOLOGIE BRETAGNE SANTE LOIRE

Nantes Université

THESE DE DOCTORAT DE

NANTES UNIVERSITE

ECOLE DOCTORALE N° 605
Biologie Santé
Spécialité : *Microbiologie, virologie, parasitologie (BS)*

Par
Murad Ishnaiwer

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Thèse présentée et soutenue à Nantes, le 21/09/2022
Cibles et médicaments des infections et de l'immunité, IICiMed, UR 1155

Rapporteurs avant soutenance :

Geneviève Hery-Arnaud, PU-PH du CHU de Brest, Université de Bretagne occidentale, Inserm UMR 1078 GGB
Etienne Ruppé Professeur de médecine des Universités de Paris, MCU-PH à l'hôpital Bichat

Composition du Jury :

Président :	Geneviève Hery-Arnaud	PU-PH du CHU de Brest, Université de Bretagne occidentale, Inserm UMR 1078 GGB
Examinateurs :	Patricia Lepage	DR INRAE, Jouy en Josas
Dir. de thèse :	Michel Dion	PU, Nantes université
Co-dir. de thèse :	Eric Batard	PU-PH CHU Nantes, Nantes Université
Invité	Hervé Blottière	DR INRAE, Nantes Université Equipe Phan

DEDICATION

I would like to dedicate my thesis and express *my* deepest gratitude and utmost appreciation to my beloved parents, who gave me supportive words of encouragement and push for tenacity. “My success is their blessing”.

To my beloved brothers and sisters,

Not forget to give special thanks to my friends, particularly those who joined me during the daily life in Nantes,

To my beloved country Palestine,

To my second-home, Nantes

Acknowledgment

In the long run, I would like to wholeheartedly thank my great advisors, Professor Michel Dion and Professor Eric Batard, for guiding me throughout my study with their inspirational leadership, plentiful experience, and a fruitful discussion and notes. "THANK YOU" for being friendly with me, for answering my questions, for your reliable encouragement, and for steering me to be autonomous. Indeed, you were like a candle who have lit up my study path, until achieving this final dissertation. I look forward to collaborate with you in the future.

Prof. Michel Dion, I was lucky, and I owe a deep debt of gratitude to your day-to-day supervision, which has enabled me to earned a lot of research experience and knowledge. You have taught me how to be more precise and thinker in doing my experiments, and to be a real researcher.

Prof. Eric Batard, I was lucky, and I owe a deep debt of gratitude to you, for accepting me to join the MIHAR lab, and for enlighten me with part of your experience in statistical analysis, and for your smart way of analysing the data and solving experiment struggles.

I would like to thank Professor Geneviève Hery-Arnaud for giving me the honor to be a rapporteur of my dissertation, and for spending the time to read and judge it.

I would like to thank Professor Etienne Ruppé for giving me the honor to be a rapporteur of my dissertation, and for spending the time to read and judge it.

I would like to thank Professor Patricia Lepage for giving me the honor to be an Examiners of my dissertation, and for spending the time to read and judge it.

I would like to thank Professor Hervé Blottière for giving me the honor to be a member of my dissertation committee, and for spending the time to read and judge it.

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List of abbreviations

(ADC) *Acinetobacter*-derived cephalosporinase

(CRE) Carbapenem-resistant *Enterobacterales*

(CTX-M) Cefotaximase

(CMY) Cephamycins

(CFU) Colony-forming units

(comEc) Commensal *E. coli*

(CSF) Cerebrospinal fluid

(DP) Degree of polymerization

(DHFR) Dihydrofolate reductase

(EDTA) Ethylenediaminetetraacetic acid

(EUCIC) European committee on infection control

(EuSCAPE) European survey of carbapenemase-producing *Enterobacterales*

(ESCMID) European society of clinical Microbiology and infectious diseases

(ESBL Ec) Extended-spectrum β-lactamase producing *E. coli*

(ESBL-EB) Extended-spectrum β-lactamase producing *Enterobacterales*

(FMT) Fecal microbiota transplantation

(1GCs) First-generation cephalosporins

(2GCs) Second-generation cephalosporins

(3GCs) Third- generation cephalosporins

(4GCs) Fourth-generation cephalosporins

(5GCs) Fifth-generation cephalosporins

(FAO) Food and agriculture organization of the United Nations

(FOS) Fructo-oligosaccharide

(GOS) Galacto-oligosaccharides

(IMP) Imipenemase

(IBD) Inflammatory bowel disease

(KPC) *K. pneumoniae* carbapenemase

(LAB) Lactic acid bacteria

(MBLs) Metallo-β-lactamases

(MRSA) Methicillin-resistant *Staphylococcus aureus*

(MDR) Multi-drug resistant bacteria

(MDR-EB) Multi-drug resistant *Enterobacterales*

(MDRO) Multidrug resistant organisms

(MDR-GNB) Multidrug-resistant gram-negative bacteria

(NDM) New delhi metallo-β-lactamase

(OM) Outer membrane

(OXA) Oxacillinase enzymes

(PABA) para-aminobenzoic acid precursors

(PBPs) Penicillin binding proteins

(PPI) Proton pump inhibitor

(SCFAs) Short chain fatty acids

(SHV) Sulfydryl variant

(TEM), Temoneira β-lactamase

(Toho) Toho university, Tokyo

(TMP) Trimethoprim

(VRE) Vancomycin-resistant *Enterococci*

(VIM) Verona imipenemase

(WHO) World health organization

1 Introduction

1.1 Multi-drug resistant bacteria (MDR)

1.1.1 Prevalence and temporal trends in multi-drug resistance

Since the beginning of 20th century, antibiotic therapy has been extensively administered as a powerful treatment for bacterial infections, but several bacterial strains displayed new antibiotic resistance that made them difficult to treat. The prevalence of these multi-drug resistant bacteria (MDR) particularly methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Enterobacterales* (CRE), Extended-spectrum β-lactamase (ESBL)–producing *Enterobacterales*, vancomycin-resistant Enterococci (VRE) has gradually increased (1-4).

For instance, among *Klebsiella pneumoniae* European isolates, resistance to 3rd generation cephalosporins (3GCs) increased and reached 79% in Bulgaria and 65 % in Greece in 2019, while fluoroquinolone resistance reached 74% in Greece and 67% in Bulgaria (5). In France, the resistance of *K. pneumoniae* for 3GCs, fluoroquinolone, aminoglycosides isolates have respectively increased from 4 %, 7%, and 5% in 2005 to 28%, 28%, and 19% in 2019 (Figure1) (5).

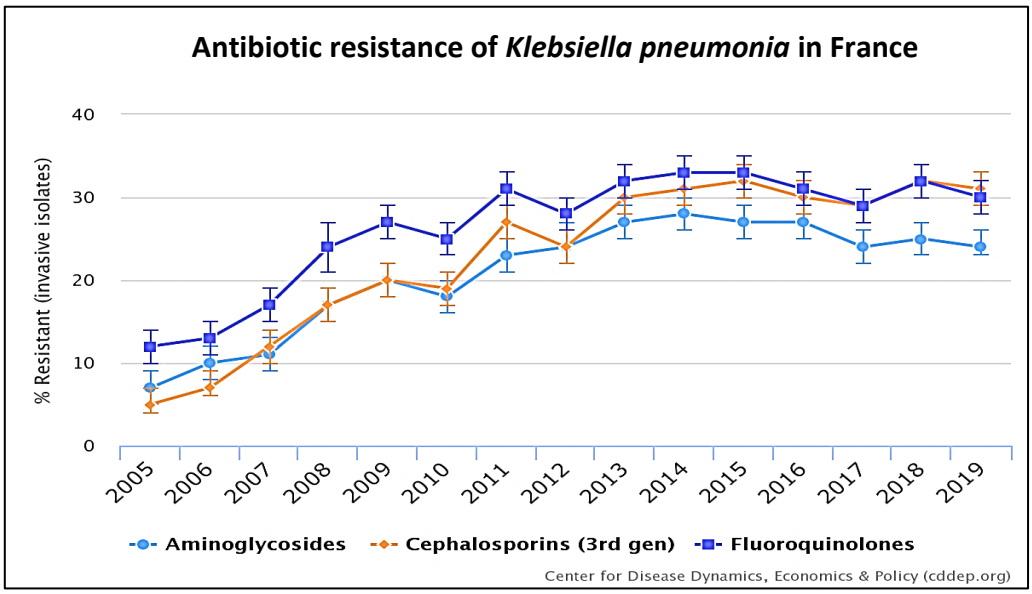

Figure 1: Evolution of the rate of *Klebsiella pneumoniae* resistance to 3GCs, aminoglycosides, and fluoroquinolones in France, (CSF and blood invasive isolates), from 2005 to 2019 (5).

However, although (MDR) issue has emerged globally in developing and developed countries, developed countries have endorsed different strategies to reduce the incidence of MDR. For instance, the European region have constituted the “European strategic action plan

on antibiotic resistance. Similarly, in 2014 US government released the 'National strategy for combating antibiotic-resistant bacteria". By contrast, developing countries still have a weak antimicrobial stewardship governed by the Healthcare System. Additionally, people could self-utilized antibiotics from drug sellers without medical prescriptions, and with many clinical misuse of antimicrobials, and limited diagnostic facilities. This has contributed to the overburdened of antibiotic resistance in these countries and increased the global resistance rate (Figure2, 3) (5).

For instance, high percentage of carbapenem resistance *Escherichia coli* (2019) have been reported in countries of Bangladesh, Egypt, India, and Pakistan, with 26 %, 37%, 41%, and 21 % respectively. Also, percentage of 3GCs resistant *E. coli* were extremely high in these countries, and reached up to 89 % in Pakistan (2019) (Figure 2) (5).

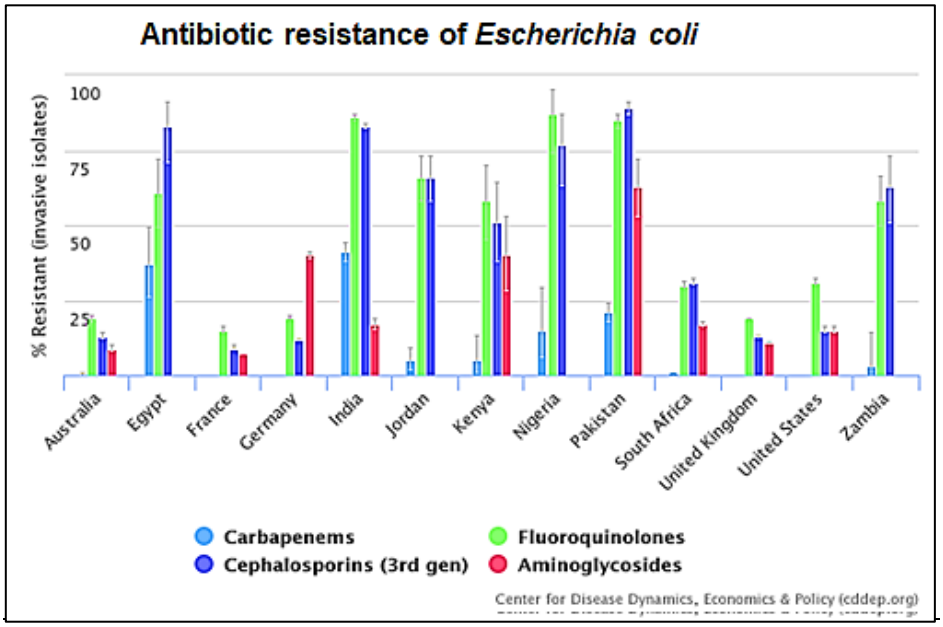

Figure 2: The global resistance rate of *E. coli* resistance to 3GCs, carbapenems, fluroquinolones, and aminoglycosides, (CSF and blood invasive isolates), 2019 (5).

Similarly, percentage of 3GCs and carbapenem resistance in these countries was also higher for *of K. pneumoniae* isolates, with nearly 75% of carbapenem resistance *K. pneumoniae* in Egypt, and with 73% to 96% of 3GCs resistant *K. pneumoniae* in south Africa and Egypt respectively (Figure 3) (5).

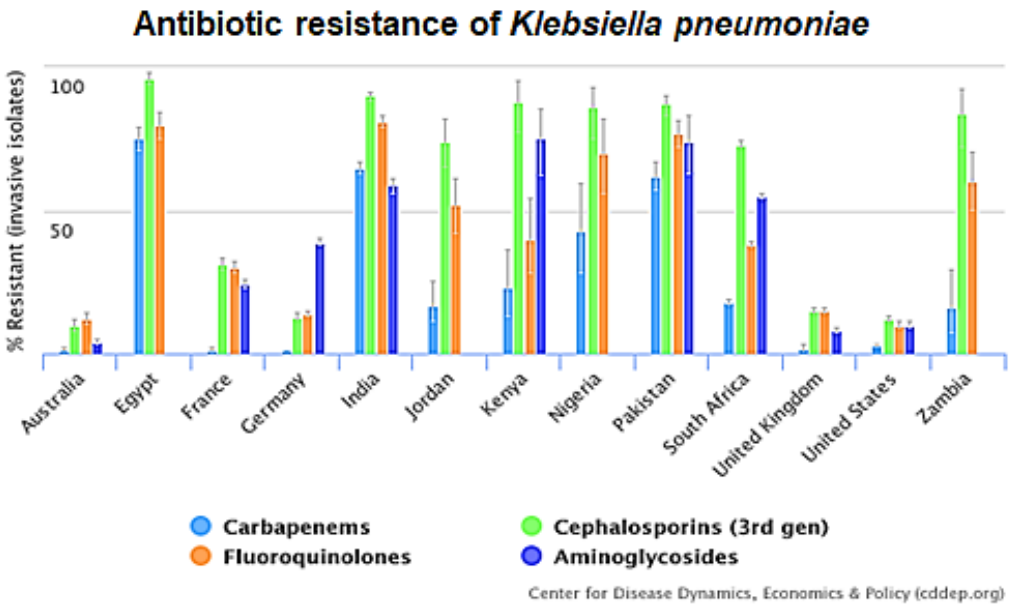

Figure 3 : The global resistance rate of *K. pneumoniae* resistance to 3GCs, carbapenems, fluoroquinolones, and aminoglycosides, (CSF and blood invasive isolates), 2019 (5).

1.1.2 Morbidity and mortality attributable to MDR

In Europe

In Europe and European economic area, the median number of attributable deaths related to infections by 3GCs resistant *E. coli* increased by 4.12-fold, from 2139 in 2007 to 8750 in 2015, and from 891 to 3508 for 3GCs resistant *K. pneumoniae* (6). Furthermore, the highest numbers of deaths attributable to MDRO were associated to 3GCs resistant *E. coli* and *K. pneumoniae*, aminoglycoside-resistant and fluoroquinolone-resistant *Acinetobacter* spp, *Pseudomonas aeruginosa* resistant to at least three antimicrobial groups, and finally to carbapenem resistant or colistin resistant *E. coli*, *K. pneumoniae*, *Acinetobacter* spp, and *P. aeruginosa* (6).

In addition, a recent study (2020-2022) related more than 670,000 infections with MDR yearly at the European Union/European Economic Area (EU/EEA), with 33 ,000 attributable death (7).

In the world

In 2019, 1.27 million deaths were globally attributed to MDR (8). The most causative bacteria are *E. coli*, followed by *S. aureus*, *K. pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *P. aeruginosa* (8). Accordingly, in case of continuation of the same antibiotics delivery policy especially at developing countries, more than 300 million deaths are globally

predicted in 2050, and the number of annual deaths attributable to resistance would reach about 10 million per year in 2050 (9). Therefore, researchers sought to find new alternatives to antibiotics, knowing that they are non-selective agents affecting not only the target bacteria, but also the pattern of normal microbiota leading to more health perturbation.

1.2 Action mechanisms of antibiotics

Multi-drug resistance bacteria are defined as acquiring resistance to at least one agent in three or more of antibiotic classes (e.g., a macrolide, a β-lactams, an aminoglycoside). Antimicrobial agents can be grouped by their mechanism of action into: agents that inhibit synthesis of cell wall, inhibit protein synthesis, inhibit nucleic acids synthesis, inhibit metabolic pathways, and disrupt the bacterial membrane structure (Figure 4) (10).

Inhibition of bacterial cell wall synthesis is considered as the main strategy since it targets bacteria peptidoglycan that is lacking at human cells, thus avoiding side effect on human cells. Some antibiotics can inhibit cell wall synthesis by the inactivation of enzymes involved in peptidoglycan construction, especially the transpeptidases. β-lactams and glycopeptides are the major antibiotics classes that target cell wall synthesis, especially those of gram-positive bacteria whose peptidoglycan is more accessible. β-lactams are antibiotic molecules that contains a β-lactam nucleus in their molecular structures that is required for their antimicrobial activity (10, 11,12). Common β-lactams includes penicillins derivatives, cephalosporins, carbapenems, and monobactams. Besides, glycopeptides are composed of a glycosylated cyclic, whose the two major representants are vancomycin and teicoplanin. They are commonly possessing effectivity against MRSA and *Enterococci* which are often less sensible to β-lactams (11,12).

Antibiotics could also stop or slow down the growth of bacteria through the inhibition of bacterial protein synthesis. The majority of antibiotics can inhibit protein synthesis through preventing the progression of bacterial mRNA translation, by interacting and blocking the activity of 30S or 50S ribosome subunits. Macrolides, aminoglycosides, and tetracyclines are the commonest antibiotics that inhibit bacteria protein synthesis through binding to the 30S ribosomal subunit. It precisely acts through blocking the attachment of aminoacyl-tRNA to the A site of ribosome, thereby driving to mRNA mistranslations. Meanwhile, chloramphenicol's are another common protein synthesis inhibitors, able to interfere with peptidyl-transfer center of the 50S ribosomal subunit and impeding the elongation of proteins (11, 12).

Antibiotics can also inhibit bacterial nucleic acids synthetic pathway. Such antibiotics suppress bacterial RNA transcription by interfering with DNA-dependent RNA polymerase, as with rifampin antibiotics. Instead, other family like quinolones, can block DNA replication

through acting on DNA topoisomerases like gyrase enzymes; This kind of enzyme are crucial in the DNA replication process since it allows the DNA to be relaxed (11,12).

Moreover, some antibiotics act through suppressing some bacterial metabolic pathways leading to a bacteriostatic effect. The most used antibiotics of this class are sulphonamides and trimethoprim (TMP): they mainly act through inhibiting the pathway of folic acid synthesis, a critical coenzyme for nucleic acids and amino acids synthesis. Normally, bacterial cells produce their own folic acid from the para-aminobenzoic acid precursors (PABA). Combined treatment of these two antibiotics can act synergistically against a wide variety of bacteria like *E. coli, Shigella, S. aureus*, *S. pneumoniae*. Sulphonamides inhibit certain enzymes required for PABA to-folic acid conversion, while TMP's acts on the dihydrofolate reductase (DHF) (11, 12).

Bacterial cell membrane disruption is another strategy used by some antibiotics. For example, polymyxins can bind to the negatively charged bacterial cell membrane thanks to their positive charge, thereby altering the membrane structure, inducing extensive permeability which leads to the death of gram-negative bacteria (11, 12).

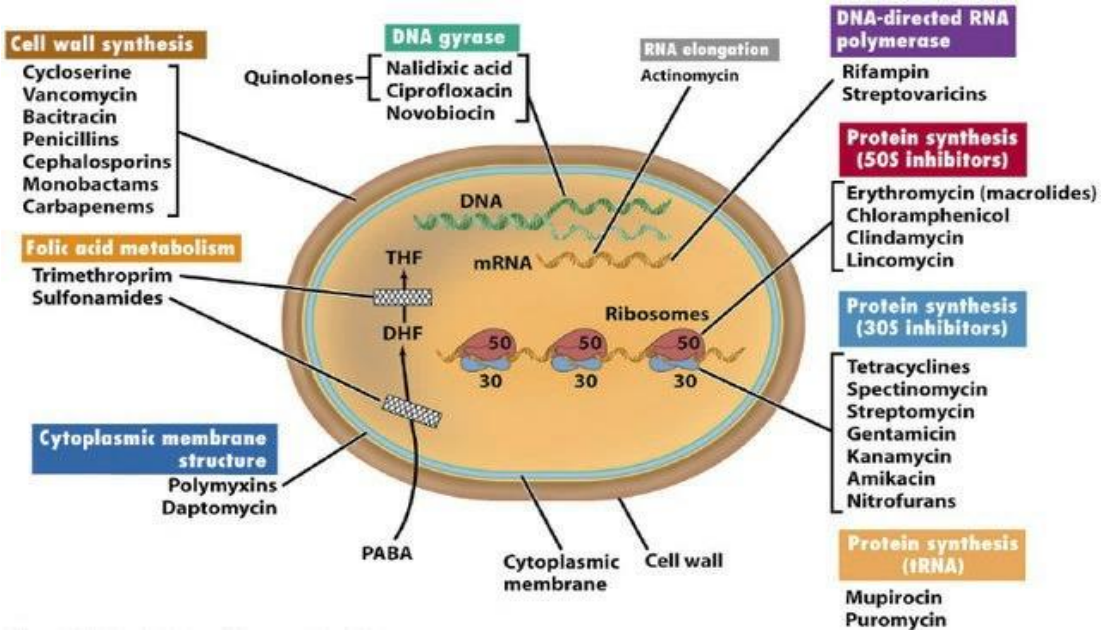

Figure 4 : Classes of antibacterial agents with their modes of action against bacteria (10).

1.2.1 β-lactam antibiotics

β-lactam antibiotics are a widely used antimicrobial agents that are typically used to treat a broad range of gram-positive and gram-negative bacteria infections; it was estimated that more than 65% of injectable antibiotics prescriptions in United States between 2004-2014 contained β-lactams treatment (12-14). β-lactam antibiotics include carbapenems, Penicillins,

cephalosporins, and monobactams. Their activities are based on inhibition of bacterial cell wall synthesis, through a covalently binding of their β-lactam ring to transpeptidases enzymes belonging to penicillin binding proteins (PBPs), which are responsible for building the bacterial cell wall. Thus, it leads to leaky peptidoglycan that is degraded through cellular lysis enzymes, resulting in cell lysis. Generally, β-lactam antibiotics are more effective against gram-positive bacteria, as it can easily pass through their accessible cell wall and target the PBPs (12-14). By contrast, gram-negative bacteria are less susceptible to β-lactams, as these molecules must cross the outer-membrane which sometimes block the β-lactams passage. however, some β-lactams antibiotics have been chemically modified to be effective against gram-negative bacteria: for instance, amoxicillin can penetrate into the periplasm of gram-negative bacteria by diffusing through their outer membrane channels called porins, and then target the PBPs (12-14).

At present, five main classes of β-lactams antibiotics structures have been clinically identified, including penams, cephems, carbapenems, penem, and monobactams (Figure5) (14-16). This classification depends on the chemical structure of the ring and radicals fused to the active β-lactam nucleus, forming a functional bicyclic scaffold (14-16).

Penams are a Penicillins large class of β-lactams, which contains a bicyclic structure of 6-aminopenicillanic acid constructed from a dipeptide bonding of L-cysteine and L-valine, forming a five-membered thiazolidine ring fused to a β-lactam ring that is essential for Penicillins antibacterial activity (14-16).

Cephems are another class of β-lactams antibiotics that include cephalosporin, cephamycins, carbacephems antibiotics. In cephems, the β-lactam ring is fused to the 6-membered dihydrothiazine ring to form the cephem scaffold. The β-lactam-dihydrothiazine moiety allows the cephems antibiotics to be more resistant to β-lactamase activity of bacteria. Cephalosporins have been grouped into five generations that are basically different in their antimicrobial activity each new generation presenting broader activity spectra than the preceding ones, especially on gram-negative bacteria. 1GCs are very effective against gram-positive bacteria including cocci, and to a little extent against gram-negative bacteria like *E. coli* (14,15).

Meanwhile, 2GCs are effective against both gram-positive and gram-negative bacteria, but have more activity against gram-negative rods like *E. coli* and *Klebsiella, Enterobacter* than against gram-positive bacteria. 3GCs have extended spectrum of activity against gram-negative bacteria, and strains that showed resistance to CG1s and CG2s, including *Enterobacter, E. coli, P. aeruginosa, Meningococci*, *Staphylococci*, and others genera. 3GCs are often used to treat sepsis caused by unknow gram-negative bacteria, and to treat

meningitis thanks to their ability to cross the central nervous system barriers. 4GCs exhibited effectivity against resistant bacteria to previous generations. They have more activity coverage to gram-negative bacteria, *Streptococci* and methicillin-susceptible *Staphylococci.* 5GCs including ceftaroline, possesses extended activity against resistant bacteria including MRSA, penicillin-resistant *Streptococci*, and beta-lactamase–producing *Enterococcus faecalis* (14,15). The list of different cephalosporins according to their antimicrobial activity and generation are shown at (Table1) (13).

First-generation	Second-generation	Third-generation	Fourth-generation	Fifth-generation
Cephalothin	Cefamandole	Cefotaxime	Cefepime	Ceftobiprole
Cephapirin	Cefuroxime	Ceftizoxime	Cefpirome	Ceftaroline
Cefazolin	Cefonicid	Ceftriaxone		
Cephalexin[a]	Ceforanid	Ceftazidime		
Cephradine[a]	Cefoxitin[b]	Cefoperazone		
Cefadroxil[a]	Cefmetazole[b]	Cefixime[a]		
	Cefminox[b]	Ceftibuten[a]		
	Cefotetan[b]	Cefdinir[a]		

Table 1 : Major cephalosporins based on antimicrobial activity (13).

Carbapenem is another class of β-lactam antibiotics. Carbapenems are a critical β-lactam antibiotics that have a broad-spectrum activity against gram-negative bacteria, and to little extent against gram-positive bacteria. It is considered among the last-line agents for the treatment of multidrug-resistant gram-negative bacteria. Carbapenems have similar penicillin five-membered ring structure, but with a sulfur-carbon replacement at C1, and a double bond at C2-C3. Carbapenems including imipenem, meropenem, doripenem, ertapenem are commonly used to treat bacterial infections related to gram-negative *Enterobacterales* species like *E. coli, K. pneumoniae*, and *P. aeruginosa*. They can penetrate into gram-negative bacteria through porin channels and target PBPs, thus inhibit cell wall synthesis. These antibiotics are particularly useful for cephalosporin-resistant bacteria which produce ESBL. Carbapenem also exhibits strong activity against gram-positive bacteria like methicillin-sensitive *Staphylococcus* and *Streptococcus.* However, because of the broad potency of carbapenems activity against gram-positive and gram bacteria, several recent studies underlined the increased rates of carbapenem-resistant bacteria, particularly CRE (15,16).

Monobactams antibiotics contain a non-fused monocyclic β-lactam ring, in contrast to other β-lactams classes structures. Monobactams including aztreonam , a common clinical monobactam, exhibited a narrow spectrum of activity against only gram-negative bacteria and aerobic bacteria like *Neisseria*, *Pseudomonas,* while it is inactive against gram-positive and anaerobic bacteria (17).

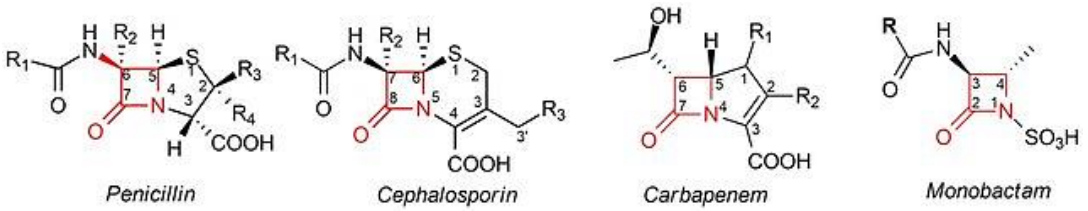

Figure 5: β -lactams core ring structures (14).

On other hand, β-lactamase inhibitors including clavams is another strategy that could combat hydrolytic activity against β-lactamase-producing bacteria. The clavams acid have similar structure to penams, but with an oxygen - sulfur replacement in the β-lactam ring. Clavulanic acid is a critically important clavams that is naturally synthesized from *Streptomyces clavuligerus* , and capable of inhibiting β-lactamases activity. Oftenly, clavulanic acid is widely prescribed in combination with amoxicillin to form a broad-spectrum therapeutic activity, as the case in the highly commercial product augmentin (18).

1.2.2 Antibiotic resistance mechanisms

Bacterial resistance (intrinsic/Acquired)

Bacterial resistance to antibiotics is either natural (intrinsic) or acquired.

- **Intrinsic resistance**

Intrinsic resistance is a natural resistance of bacteria against particular antimicrobial agents. Such resistance is normally controlled by bacterial chromosomes, and conferred to offspring through vertical gene transfer. It may commonly be due to the impassable drug entrance, or to unique structures of some bacteria, for example specific cell wall of Mycobacteria (19-22). See Table 2 for bacterial intrinsic resistance examples of some bacteria (21).

Organisms	Natural resistance against	Mechanism
Anaerobic bacteria	Aminoglycosides	Lack of oxidative metabolism to drive uptake of aminoglycosides
Aerobic bacteria	Metronidazole	Inability to anaerobically reduce drug to its active form
Gram-positive bacteria	Aztreonam (a beta-lactam)	Lack of penicillin binding proteins (PBPs) that bind and are inhibited by this beta lactam antibiotic
Gram-negative bacteria	Vancomycin	Lack of uptake resulting from inability of vancomycin to penetrate outer membrane
Lactobacilli and *Leuconostoc*	Vancomycin	Lack of appropriate cell wall precursor target to allow Vancomycin to bind and inhibit cell wall synthesis
P. aeruginosa	*Sulfonamides,trimethoprim, tetracycline, or chloramphenicol*	Lack of uptake resulting from inability of antibiotics to achieve effective, intracellular concentrations
Enterococci	*Aminoglycosides*	Lack of sufficient oxidative metabolism to drive uptake of aminoglycosides
	All cephalosporins	Lack of PBPs that effectively bind and are inhibited by these beta lactam antibiotics

Table 2 : Common bacteria with intrinsic resistance mechanisms (21).

- ## Acquired resistance

Bacteria could acquire resistance to antibiotics. Indeed, acquired resistance could be raised in some bacterial strains through the horizontal gene transfer between bacteria, or through specific mutations of chromosomal genes, which constitutes a vertical gene transfer; meanwhile, horizontal transfer comprises the acquisition of exogenous movable genetic materials, such as plasmids or transposons via conjugation, transduction and transformation. Up to 80 % of human clinical samples are usually associated with resistance of exogenous acquisition of bacterial resistance plasmids (23). Indeed, bacteria could resist to several antibiotics by the acquisition of several resistance plasmids, or by a single plasmid with several antibiotic resistance genes. The more relevant plasmids among gram-negative pathogenic bacteria includes incompatibility plasmids of IncF and IncI groups (24).

IncI is a plasmid that found in *E. coli* and *Salmonella enterica. E. coli* strain ESBL2057 contains plasmid IncI1 that include aadA5, sul2, $bla_{CTX\text{-}M\text{-}1}$, and dfrA7 resistance genes, which encoding antibiotic resistance to aminoglycosides, sulphonamides, β-lactams (CTX-M variant), and trimethoprim respectively (25). By contrast, *S. enterica* comprises billion of clinical cases of enteric diseases annually. Infections with *S. enterica* could give particularly severe human diseases like typhoid, enterocolitis, and bacteremia. This bacterium can be multidrug resistant thanks to its plasmids IncA/C that contain resistant genes against

aminoglycosides, β-lactams, chloramphenicol, sulfisoxazole, tetracyclines, and trimethoprim (26,27).

Moreover, *E. coli* strain ESBL3277, contains plasmid IncFIB/FIC displaying resistance genes *dfrA14, tetAR, bla*$_{CTX\text{-}M\text{-}55}$*, aac3-Iia, aadA1(2x), sul3, floR*, involved in the resistance against trimethoprim, tetracycline, β-lactams, aminoglycoside, sulphonamides, and fluoroquinolone respectively (24). Moreover, multi-antibiotic-resistant *A. baumanni*, one of the leading causes of the clinical nosocomial infections, harbors the GR6 plasmid containing ESBL *bla*$_{OXA\text{-}58}$ gene and aminoglycoside-resistant *aphA6* gene (28).

Generally, several resistance mechanisms have evolved in bacteria. and could be mainly maintained into four main ways: inactivation of antibiotics; modification of antibiotic targets; reduction of antibiotic uptake, active antibiotic efflux (Figure 6) (29-32,12). These resistance mechanisms could coexist in one bacterial cell, providing multiple antibiotic resistance strategies, or could be restricted to only one specific mechanism (29-32,12).

Inactivation of antibiotics

Enzymatic inactivation of antibiotics is the major mechanism of antibiotic resistance. Resistant bacteria have developed several enzyme variants that could hydrolyze main antibiotics classes like β-lactams, macrolides, aminoglycosides, and phenicols. For instance, *Enterobacterales β*-lactamases enzymes could inactivate *β*-lactam antibiotics by degrading the amide bond of the *β*-lactam ring. Some species of *Enterococcus, Staphylococcus, and Streptococcus*, reduced affinity of aminoglycoside molecules for 30S subunit binding through aminoglycoside enzymatic modifications, involving phosphotransferases, acetyltransferases or adenyltransferases (29-32,12).

Alteration/change in the target site of the antibiotic

Bacteria could also acquire resistance through modifying antibiotic target sites. Hence, changes in the bacterial target sites that interact with the antibiotic could reduce their binding affinity and lead to antibiotic resistance. Target sites alterations often resulted from spontaneous mutations in specific chromosomal genes. Common alterations include changes at ribosomal 16S or 23S subunits, which drop the affinity of macrolides, chloramphenicol, or tetracycline. Alterations could also occur at the RNA polymerases and topoisomerases (DNA gyrase), leading to resistance to rifamycins and quinolones respectively. Resistance could also concern alterations in penicillin-binding proteins (PBPs), resulting in resistance to β-lactam antibiotics. Moreover, resistance involving change of D-alanyl-alanine to D-alanyl-

lactate in the wall tetrapeptide is the most frequently observed with glycopeptides like vancomycin and teicoplanin (12, 29-32,).

Reduction of antibiotic uptake

Some bacteria like *P. aeruginosa* could harbors specific mutations that decrease outer membrane (OM) permeability to antibiotics. Such mutations often concern the porin channels, which are used by some antibiotics including β-lactams and quinolones to cross the outer membrane. Thereby it leads to bacterial resistance through reduction of intracellular accumulation of these antimicrobial agents (12,29-32,).

Efflux pumps

Bacteria could also resist antibiotics through their efflux pumps that can export antibiotics outside the cell. This efflux pumps could be either antibiotic specific (e.g Tet (A) that reject tetracycline) or multi-drug transporters, such as MexAB-OprM efflux pump that confer *P. aeruginosa* multi resistance to quinolones, macrolides, novobiocin, chloramphenicol, tetracyclines, lincomycin, and β-lactam antibiotics (12,29-34) . Also, MDR *Acinetobacter* contains AbeM efflux pump that confer resistance against aminoglycoside and fluoroquinolone antibiotics.

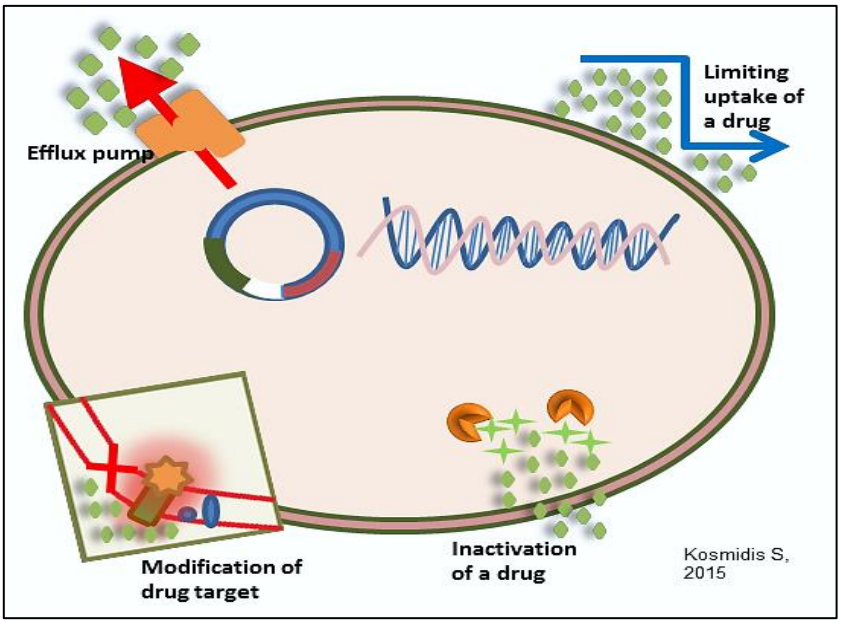

Figure 6: Main antimicrobial resistance mechanisms (32).

- **Mechanism of Resistance to β-lactam antibiotics through β-lactamases (acquired resistance)**

The bacterial resistance to β -lactam antibiotics occurs predominantly through the production of β-lactamases. These β-lactamases enzymes confer resistance to β-lactam antibiotics like Penicillins, cephalosporins, cephamycin's, monobactams and carbapenems (ertapenem). β-lactamases catalyse β-lactam nucleus hydrolysis, causing the inactivation of the molecule (35,36). β-Lactamases are clinically most relevant in gram-negative bacteria, especially in *P. aeruginosa*, and *Enterobacterales* species like *E. coli* and *K. pneumoniae* (35,36).

- **Classification schemes for bacterial β-lactamases**

Due to the high diversity and emergent of β-lactamases, different classifications have been proposed. One classification is based on β-lactamase genes (*bla*) location, but this classification was used shortly, since the corresponding *bla* genes can be mobilized from chromosomes into plasmids or transposons and vice versa. Hence, two classification systems for β-lactamases are currently in use. The molecular classification of Ambler system, and the functional classification of Bush-Jacoby-Medeiros. Indeed, the Ambler system, the most widely used, separates β-lactamases into four classes A, B, C, D along their differences in amino acids motif and variations in hydrolytic mechanism (Figure 7) (37). Classes A, C, and D (serine β-lactamases; SBLs) comprises the most clinically relevant β-lactamases It contains a serine active-site motif (Ser–X–X–Lys motif) that interacts with the carbonyl group of β-lactam ring and opens it leading to the antibiotic hydrolysis (Figure 8A) 38). It precisely occurs through an acylation-deacylation processes. During acylation, the activated nucleophilic serine covalently binds to the carbonyl carbon of the β- lactam amide bond. Then, the deacylation process, mediates the activation of deacylating water for hydrolysis step (Figure 8B) (35). The significant enzyme families within this class includes: temoneira β-lactamase (TEM), sulfydryl variant (SHV), cefotaximase (CTX-M) and *K. pneumoniae* carbapenemase (KPC) for class *A. cephamycins* (CMY) and *Acinetobacter*-derived cephalosporinase (ADC) for class C. and oxacillinase (OXA) enzymes for Class D. (35,37).

By contrast, class B β-lactamases (metallo-β-lactamases, or MBLs), link their active-site metal ion (zinc) to carbonyl group of lactam ring to drive the hydrolytic reaction (Figure 8A) 38). The MBLs contains verona imipenemase (VIM) and new delhi metallo-β-lactamase (NDM).

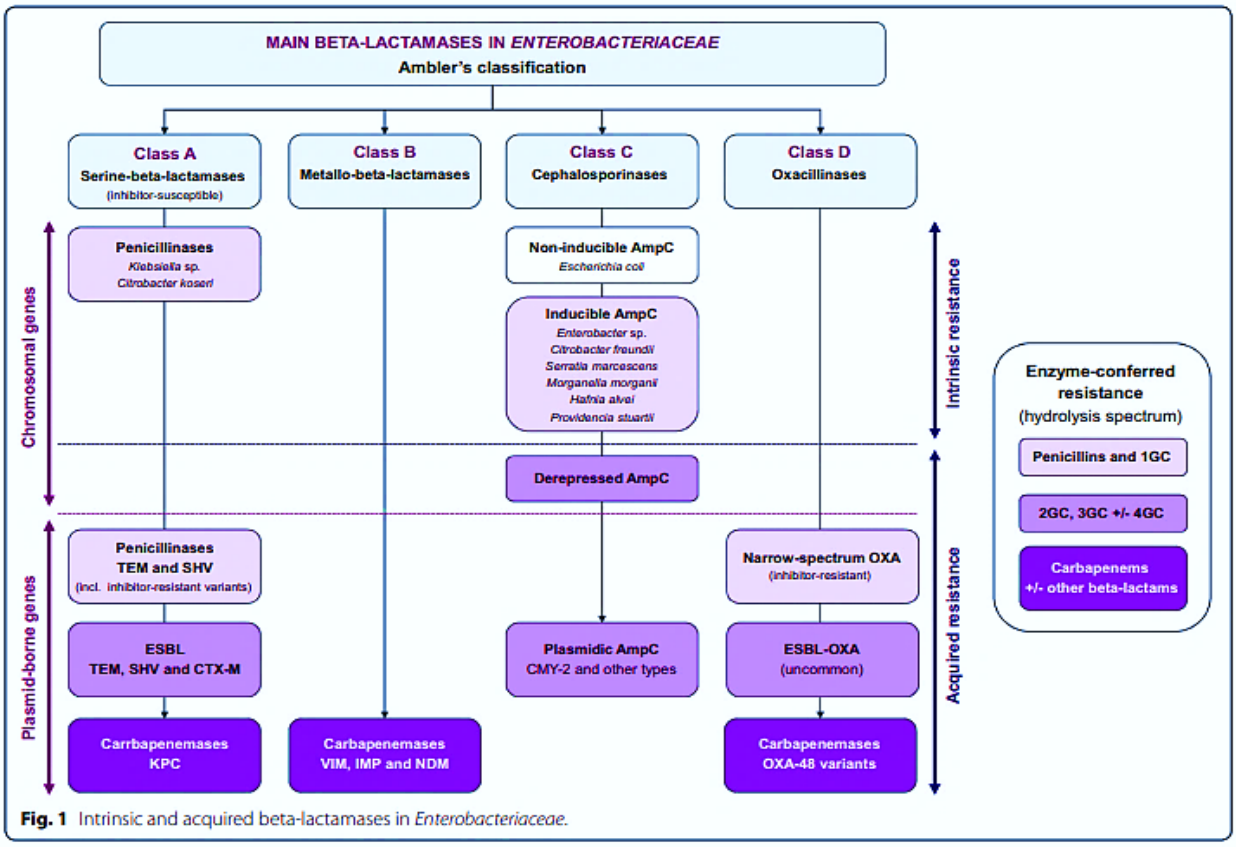

Figure 7: Major ß-lactamase classes in *Enterobacterales* in basis of Ambler's classification (37).

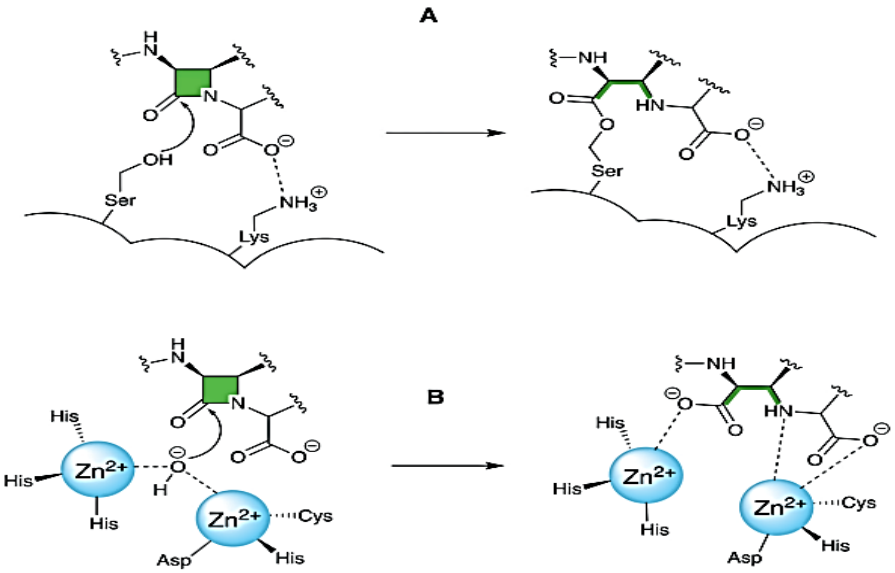

Figure 8A: β-lactam inactivation by serine-based β-lactamases (A), or by metallo-β-lactamases (B) (38).

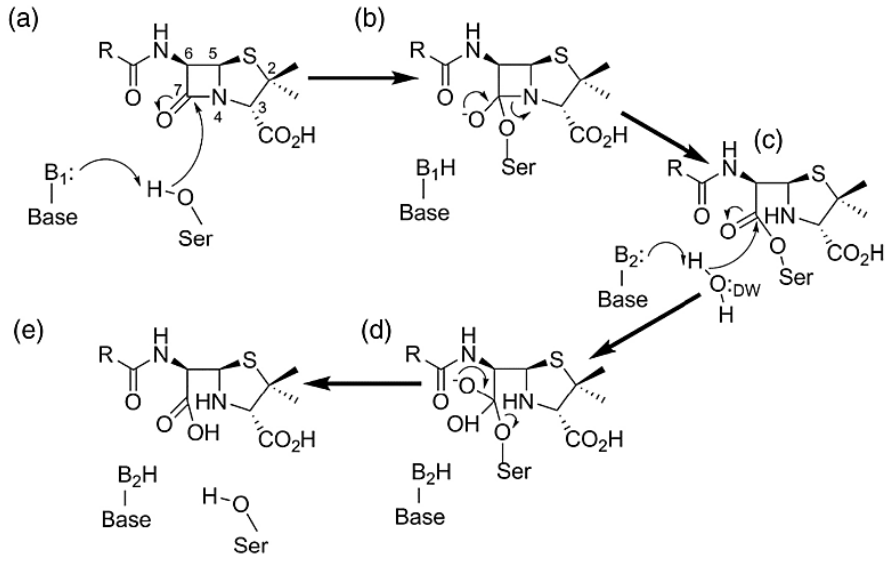

Figure 8B: Mechanistic overview of β-lactam inactivation by serine-based β-lactamases (35).

However, among all of these, few enzymes have been remarkedly emerged, which are TEM, SHV, CTX-M and KPC in class A as well oxacillinase (OXA) from class D. The majority of these enzymes have been widely disseminated on plasmids and mobile elements across gram-negative pathogens, particularly among *Enterobacterales* species such as *E. coli*, *K. pneumoniae, A. baumannii* and also *P. aeruginosa i*n addition to the ability of some enzymes to hydrolyze oxyiminocephalosporins such as cefotaxime and ceftazidime generating the ESBL phenotype. Hence, the clinically important enzyme families will be discussed in more details below (35,37).

In contrast, the Bush-Jacoby-Medeiros took place initially in 1989, and was updated in 1995, for the classification of β-lactamases according to their functional role. Although, this classification is less common than Ambler, it can admittedly help to observe the clinical hydrolytic activity of β-lactamases, and their degree of inhibition by β-lactamase standard inhibitors such as clavulanic acid, tazobactam, and EDTA. Therefore, it divided β -lactamases into 3 main groups 1-3; Group1 includes class C of “Ambler” enzymes (cephalosporinases), group 2 includes (classes A and D), and group 3 metallo-β-lactamases (class B). Additionally, several new subgroups are also included (35,37).

TEM β-lactamase

TEM β-lactamase are plasmid-mediated lactamases that are commonly found in gram-negative bacteria particularly *E. coli*. They were named in 1963 from a Greece patient Temoneira, whom it was found at fecal *E coli* strain. *TEM* genes are typically born by plasmids

which facilitates their dissemination among bacterial strains including *P. aeruginosa*, *Neisseria gonorrhoeae, Haemophilus influenzae*, and *K. pneumoniae* (39,40).

Up to 90 % of ampicillin resistant *E. coli* is related to the TEM-1 secretion. TEM-2 is the first variant of TEM-1 that has been characterized in 1984 in France. It results from a single amino acids substitution of TEM-1, and has the same hydrolytic activity but differs in the isoelectric point (39,40). However, single amino acid substitutions within the TEM enzyme residues including glutamate to lysine at position 104, arginine to either serine or histidine at position 164, glycine to serine at position 238, and glutamate to lysine at position 240, are typically responsible for the (ESBL) phenotype. TEM-3, was the first TEM variant that displayed the ESBL phenotype in 1989. Moreover, point mutations at Met- 69, Arg-244, Arg-275, and Asn-276 residues has further resulted in the inhibitor-resistant TEM phenotype that showing resistance to clavulanic acid and sulbactam. Multiple amino acids substitutions extended the activity of TEM-type ESBLs to degrade oxyimino-cephalosporins such as ceftazidime and cefotaxime (39,40). More than 200 TEM variants have emerged to date, most of them being ESBLs, but not types TEM-1, TEM-2 and TEM-13 because they only show activity against penicillins (35,41-43). Figure 9 shows the TEM ESBL variants (39).

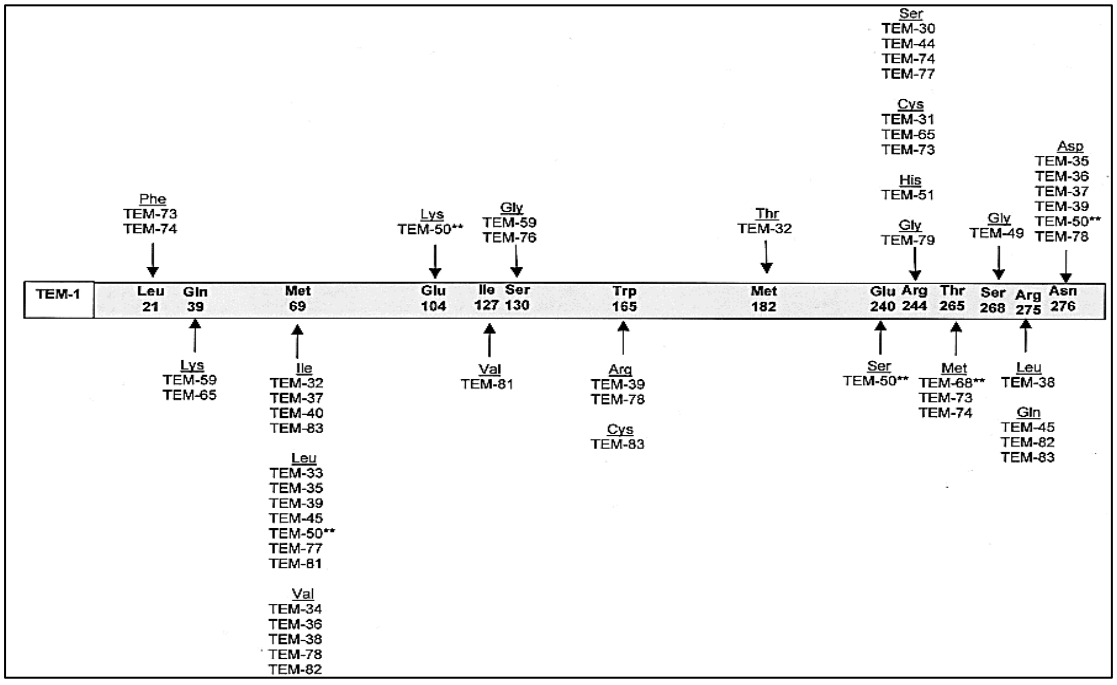

Figure 9 : Main amino acid substitutions within TEM enzymes (39).

SHV β-lactamases

β-lactamase class A comprises the plasmid-encoded enzyme SHV (sulfhydryl variant) ; it is grouped as 2 β-lactamases according to the Bush-Jacoby classification. It's frequently expressed by *Enterobacterales* and correlates with the ampicillin resistance to for apparently

20% of cases. SHV has a plasmid support in *E. coli* strains, but is coded by the chromosome for the majority of *K. pneumoniae* strains. SHV-1 was detected in *K. pneumoniae* of neonates feces and it provides resistance to Penicillins and 1GCs. It shared up to 68% of sequence identity and hydrolytic activity with TEM-1. The enduring amino acid substitution over the years, have evolved several SHV variants with a subtle change around active site, that elicit a broad spectrum of activity toward monobactams and carbapenems hydrolysis (35,39,41,44). The majority of SHV variants are encompassed (ESBL) phenotype, with estimation of 189 SHV β-lactamase variants. SHV-2 is the first variant designated by a serine for glycine substitution at position 238 of SHV-1. It was described in *K. ozanae* and showed a high resistance to cefotaxime and a mild one to ceftazidime. Basically, SHV β-lactamases are allocated into three main classes according to their molecular and functional antimicrobial activities: (i) 2b class with nearly 37 variants, where it exhibited activity against penicillins and early cephalosporins but inhibited by clavulanic acid and tazobactam. (ii) 2br class included 7 variants, and possess resistance to clavulanic acid. (iii) 2be class includes 46 variants, and is able to hydrolyze oxyimino β-lactams. Common amino acid substitutions in SHV ESBL derivatives are shown at Figure 10 (35,39,41,44).

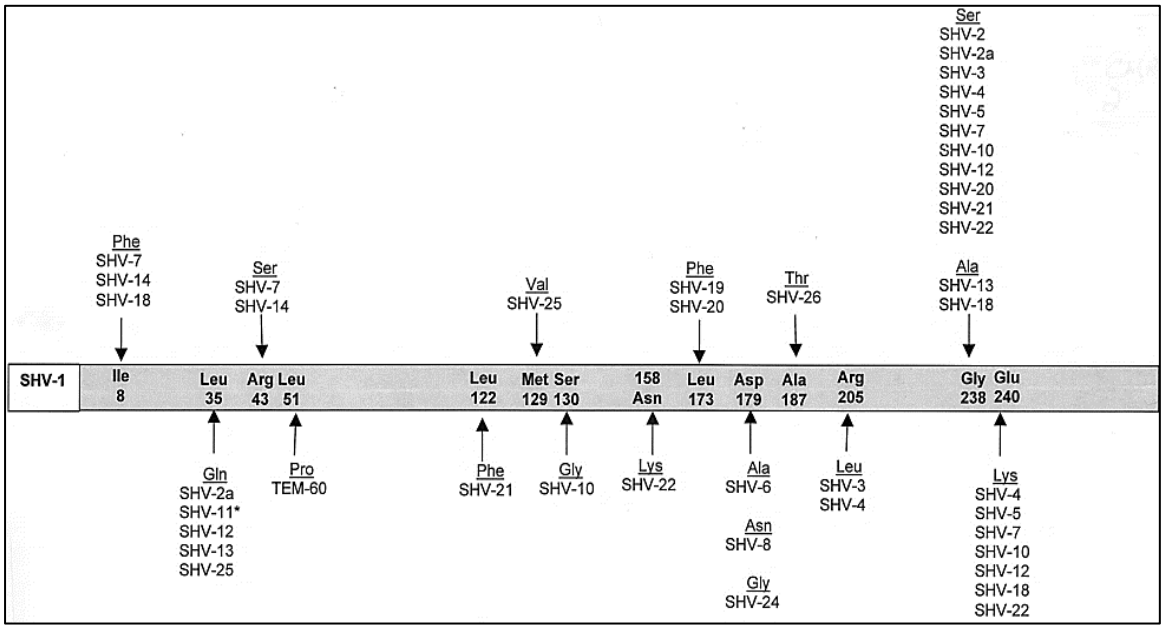

Figure 10 : Main amino acid substitutions within SHV ESBL derivatives (39).

CTX-M β-lactamases

CTX-M β-lactamases are a group of class A enzymes that are clinically detected in gram-negative bacteria *Enterobacterales* spp. They are considered as the highest prevalence of ESBLs enzymes, and characterized by their hydrolytic activity against oxyiminocephalosporin

cefotaxime. Although CTX-M enzymes are mostly reported in *E. coli* and *K. pneumoniae* strains they have been also reported in other bacterial species like *Acinetobacter spp, Serratia marcescens, V. cholerae,* and *P. aeruginosa,* which highlight the possibility of gene transmission of $bla_{CTX\text{-}M}$ genes to non-*Enterobacterales* species (19,42,45-47).

However, the CTX-M enzymes are well different from other β-lactamases, as they show only 40% of identity with common β-lactamases such as SHV and TEM. Amino acid comparisons of CTX-M variants revealed six main clusters including CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25, and KLUC. Clusters are different from each other by ≥10% amino acid residues (Figure 11) (47), and each cluster comprises different allelic variants that are distinct from each other by ≤5% amino acid residues. More than 70 CTX-M ESBLs have been described. The first CTX-M was described in Japan under the name FEC-1 in a cephem resistant *E. coli* strain (Matsumoto et al. AAC 1988 (45). Moreover, the first extended-spectrum β-lactamase (ESBL) of the CTX - M type(CTX-M-1) has emerged in Munich (1990) in a cefotaxime-resistant *E. coli* isolate. This strain was susceptible to ceftazidime, and produced neither-TEM, nor SHV, therefore it was labelled as CTX-M**,** (referred to cefotaxime-Munich), and became the predominant ESBL in *E. coli*. New CTX-M variants have also evolved during the 1990-1995 period, the Toho-1 and Toho-2 ESBL produced by cefotaxime-resistance *E. coli* isolate from Japan in 1993, and 1995 respectively, (Toho refers to Toho University, Tokyo), where they later renamed as CTX-M-44 (Toho-1) and CTX-M-45 (Toho-2) since their high sequence identity with CTX-M-2**.** Moreover, recent clinical studies have described new CTX-M variants that resulted from CTX-M enzymes amino acid substitutions particularly D240G and P167S, which makes them opposed ceftazidime. These substitutions have been described in CTX-M-1, -2, -9, and -25 variants (42,45-47).

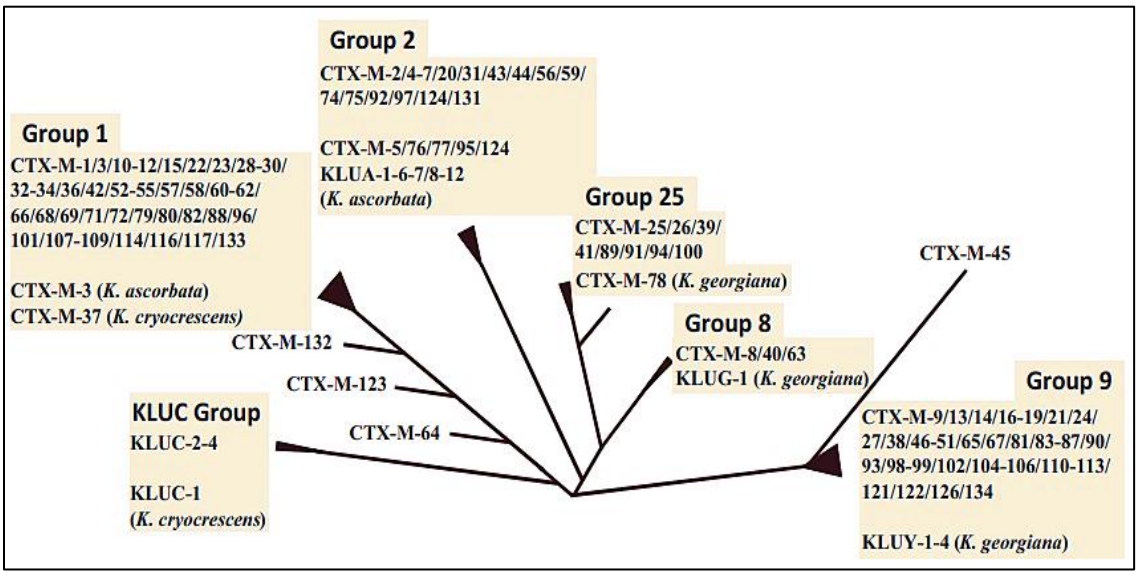

Figure 11 : Phylogenetic tree showing the similarity between the CTX-M-type β-lactamases (47).

Genetically, The *CTX-M* encoding genes were originated from *Kluyvera* species including *K. scorbate, K. gorgiana* and *K. cryocrescens*, that are found in normal human microbiota. Chromosomal *bla*, and *klu* genes that exist in *Kluyvera* species present the main ancestors for the *CTX-M* clusters. In contrast, the *CTX-M* chromosomal genes are generally acquired on conjugative plasmid that mediate their rapid spreading among clinical isolates mainly *Enterobacterales* (Figure 12) (45). This plasmid acquisition could be probably mediated through transposable insertion sequences that are mostly located upstream the $bla_{CTX\text{-}M}$ genes, and are capable of mobilizing flanking DNA fragments from the *Kluyvera* spp chromosome to *Enterobacterales* plasmids (42,45-47).

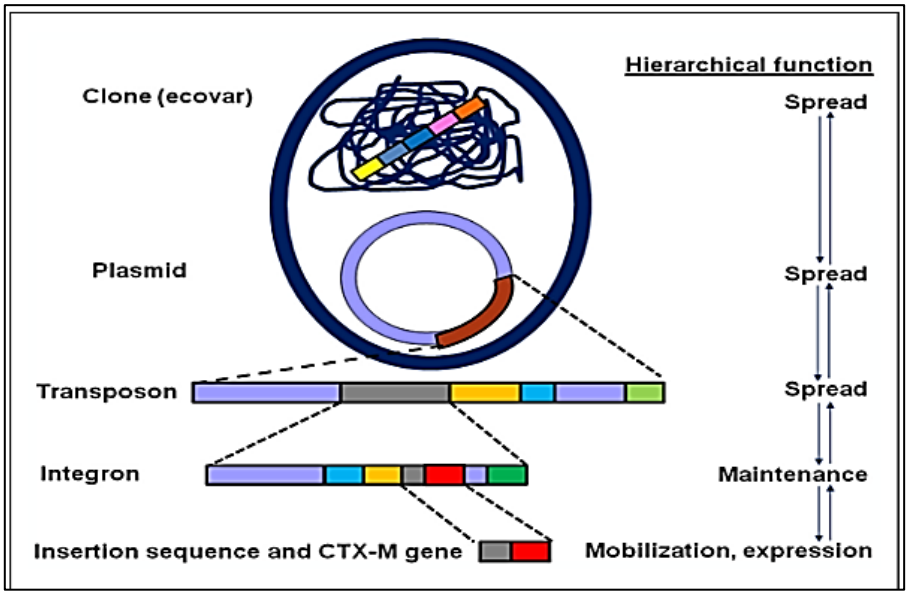

Figure 12 : Overview of $bla_{CTX\text{-}M}$ genes dissemination (45).

OXA type ß-lactamases

The OXA enzymes are a class D ß-lactamases that encompasses high hydrolytic activity against oxacillin, cloxacillin and penicillin. Most OXA- ß-lactamases are non-ESBL as they susceptible to 3GCs, but however, new OXA- ß-lactamases variants has recently emerged a hydrolytic activity against carbapenem, and cephalosporins 2GCs, 3GCs, 4GCs generations. The first OXA-ESBLs was early reported in Turkey 1991 in a clinical *P. aeruginosa* isolate. Variants of OXA-11, OXA-10, OXA-13, OXA-14, OXA-16, OXA-17, OXA-19, and OXA-28 have also been characterized in *P. aeruginosa*. Generally, recent identification reported five OXA- ß-lactamases variants that confer carbapenem resistance. Four of which (OXA-23, OXA-24/40, OXA-51 and OXA-58) were described at *A. baumannii*, and are a plasmid encoded, except OXA-51 that are exhibited a chromosomal encoding. While the OXA-48

enzymes respective genes are found on plasmids that assessed their transmission between *Enterobacterales* species (48,49).

The OXA-48 enzyme is generally able to degrade penicillins, carbapenems and early cephalosporins; but possesses weak broad-spectrum activity on cephalosporins. The first OXA-48 carbapenemase was first reported in Turkey 2001 in a *K. pneumoniae* isolate then in *E coli*. It has been proposed that the waterborne *Shewanella* spp are the main progenitor for the bla_{OXA-48} gene. In addition, bla_{OXA-48} and other variants such as $bla_{OXA-199}$ and $bla_{OXA-204}$ have been also proposed at *S. xiamenensis.* Since then, several OXA-48 variants differing by one to five amino acids substitutions or deletion with more than 97% homology, have been described in Europe countries. These variants are not necessarily having the same hydrolytic activity like OXA-48. Common clinical variants including OXA-162 (single substitution at Thr213Ala), OXA-232 (single substitution at Arg214Ser), and OXA-245 (single substitution at Glu125Tyr) that were early identified from *K. pneumoniae* isolates in Turkey, France, and Spain respectively. At that time there was multiple outbreaks in Turkey and French hospitals as well as other countries including Belgium, UK, Morocco, Lebanon, Tunisia, and others (Table3) (48,49).

Indeed, the emergence of the OXA enzymes is associated by the rapid dissemination of the conjugative plasmid that harboring the bla_{OXA} genes through mobile genetic elements. ISAba1, Saba3, and IS1999 are the most common insertion sequences that are associated with gene expression and mobilization for bla_{OXA} genes. ISAba1was originated from *A. baumannii*, were it promoted bla_{ADC} gene expression that revealed cephalosporins resistance, it also mediated bla_{OXA} genes expression to confer carbapenem resistant. It is associated with genes encoding for OXA-type beta-lactamases, such as the bla_{OXA-23}-like genes, bla_{OXA-51}-like genes, bla_{OXA-58}-like genes, and $bla_{OXA-235}$-like genes. In contrast, the insertion sequence IS*Aba3 was identified in A. baumannii* isolate*, and commonly associated with bla*$_{\text{OXA-58}}$-like genes (48,49).

IS1999 is the insertion sequence that have been inserted upstream $bla_{OXA\text{-}48}$ gene in the conjugative plasmid of some *Enterobacterales* species, especially *E. coli*. This transposon element is associated with bla_{OXA-48} gene expression and mobilization to other bacterial species. Tn1999 is another transposon sequence that upstreamly flanked bla_{OXA-48} and the carbapenemase genes and mediated their expression and mobilization as was described in *K. pneumoniae* isolates. The $bla_{\text{OXA-48}}$ gene is usually flanked by two main insertion sequences IS*1999 and* Tn*1999,* located upstream and downstream respective gene. These transposons elements mediate the $bla_{\text{OXA-48}}$ gene expression and mobilization at the conjugative plasmids. Further Tn1999 variants have been further identified (Tn1999.2-

Tn1999.5). The Tn1999.2 and Tn1999.3 are the most common variants, and were early identified in *K. pneumoniae* and *E. coli* isolates respectively. The Tn1999.2 contains IS*1R* element inserted into the upstream IS*1999* element located upstream of *bla*$_{OXA-48}$ gene. While the Tn1999.3 variant contains IS*1R* located downstream the *bla*$_{OXA-48}$ gene (48,49).

Enzyme group	Enzyme(s)	No. of enzymes in group	Location(s)	Host species
OXA-23-like	OXA-23, OXA-27, OXA-49, OXA-73, OXA-102, OXA-103, OXA-105, OXA-133, OXA-134, OXA-146, OXA-165–OXA-171, OXA-225, OXA-239	19	C and P	*A. baumannii, A. junii, A. radioresistens, A. pittii, Proteus mirabilis, Acinetobacter* phenon 5, *Acinetobacter* phenon 6/ct 13TU, *A. nosocomialis, Acinetobacter* genomic species 10/11, *A. lwoffii, Klebsiella pneumoniae, A. baylyi*
OXA-40-like	OXA-40, OXA-25, OXA-26, OXA-72, OXA-139, OXA-160, OXA-207	7	C and P	*A. baumannii, A. haemolyticus, A. pittii, A. baylyi, Pseudomonas aeruginosa, A. calcoaceticus, K. pneumoniae*
OXA-51-like	OXA-51, OXA-64–OXA-71, OXA-75–OXA-80, OXA-82–OXA-84, OXA-86–OXA-95, OXA-98–OXA-100, OXA-104, OXA-106–OXA-113, OXA-115–OXA-117, OXA-120–OXA-128, OXA-130–OXA-132, OXA-138, OXA-144, OXA-148–OXA-150, OXA-172–OXA-180, OXA-194–OXA-197, OXA-200–OXA-203, OXA-206, OXA-208, OXA-216, OXA-217, OXA-219, OXA-223, OXA-241, OXA-242, OXA-248–OXA-250, OXA-254	95	C and P	*A. baumannii, A. nosocomialis, Enterobacter cloacae, Escherichia coli, K. pneumoniae*
OXA-58-like	OXA-58, OXA-96, OXA-97, OXA-164	4	C and P	*A. baumannii, A. pittii, A. nosocomialis, Acinetobacter* phenon 6/ct 13TU, *A. junii, Acinetobacter* genomic species 9, *A. bereziniae, A. calcoaceticus, A. radioresistens, E. cloacae, Comamonas testosteroni, E. coli, K. pneumoniae, Delftia acidovorans*
OXA-134a-like	OXA-134a, OXA-186–OXA-191	7	C	*A. lwoffii*
OXA-143-like	OXA-143, OXA-182, OXA-231, OXA-253, OXA-255	5	P	*A. baumannii, A. pittii*
OXA-213	OXA-213	17	C	*A. calcoaceticus*
OXA-214-like	OXA-214, OXA-215	5	C	*A. haemolyticus*
OXA-211-like	OXA-211, OXA-212, OXA-309	6	C	*A. johnsonii*
OXA-229-like	OXA-228–OXA-230, OXA-257	8	C	*A. bereziniae*
OXA-235-like	OXA-235–OXA-237, OXA-278	7	C	*A. schindleri*
OXA-48-like	OXA-48, OXA-48b, OXA-162, OXA-163, OXA-181, OXA-199, OXA-204, OXA-232, OXA-244, OXA-245, OXA-247	11	C and P	*E. cloacae, K. pneumoniae, E. coli, Shewanella xiamenensis, Citrobacter freundii, Serratia marcescens, Providencia rettgeri, Klebsiella oxytoca, Enterobacter sakazakii, A. baumannii*

Table 3 : OXA-type Carbapenemases (48).

K. pneumoniae carbapenemase

K. pneumoniae carbapenemases (KPC) belongs to ambler class A β-lactamases; KPC enzymes confer high carbapenem resistance, they are encoded by *bla*$_{KPC}$ gene flanked by Tn4401 genetic transposon. This transposon element facilitated the insertion of *bla*$_{KPC}$ gene into plasmids different gram-negative bacteria. This plasmid acquisition is associated with the rapid dissemination for up to 23 KPCs variants carrying bacterial species worldwide. KPC enzymes was initially identified in *K. pneumoniae* isolate at united states 1996, then it has emerged globally. Greece and Italy were exceedingly suffered from the KPC national outbreaks. The *K. pneumoniae* carbapenem resistant rate was 60.5% in Greece 2012, and 28.8% in Italy 2009. High prevalence was also detected in Brazil, China, Colombia and others (50).

According to the analysis of European survey of carbapenemase-producing *Enterobacterales* conducted between 2013-2014. KPC represent the dominant carbapenemases with (45.5%)

among 944 genomes of *K. pneumoniae* resistant to carbapenems. This was followed by high rate with OXA-48 of (36.3%), then NDM (11.5%), VIM (8.2%), and IMP (0.4%). The most frequent carbapenemases detection was occurred in Italy, were KPC represent the majority. Then Turkey and Spain were OXA-48 displayed the major carbapenemases (Figure 13) (50,51).

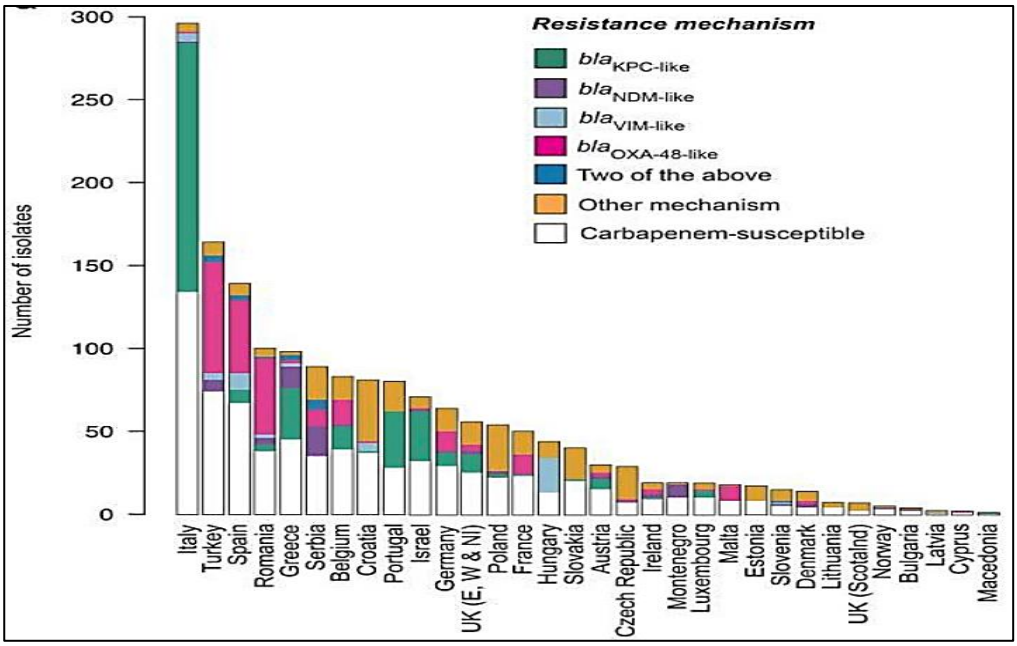

Figure 13 : Distribution of carbapenem resistance mechanisms among clinical isolates included at the EuSCAPE survey (2013-2014) (51).

1.3 Digestive colonization by ESBL-EB and CPE

- **Extended-spectrum beta-lactamase-producing *Enterobacterales* (ESBL-EB)**

ESBLs are rapidly evolving β-lactamases. They were initially identified in *K. pneumoniae* in 1983. Then a subsequent transmission has driven an early ESBL case in France 1986, and then in 1990 where it was estimated that up to 35 % of *K. pneumoniae* nosocomial infections had an ESBL phenotypes. Recently, during the early 2000s, studies reported high prevalence of ESBLs in northern Europe countries, Latin America and Asia-Pacific regions (52,53).

ESBLs are primarily originated from an excessive mutation in *TEM-1*, and *SHV-1* genes, that change amino acid arrangement around β-lactamases active site. Normally, ESBLs are distinguished by their hydrolytic activity against penicillins, early and third generation cephalosporins and aztreonam, but inhibited by clavulanic acid. ESBLs activities are most often detected among class A SHV, TEM, and CTX-M types. Moreover, being a plasmid encoded, ESBLs is rapidly disseminated among bacterial isolates. *Enterobacterales*

members particularly: *Klebsiella* spp and *E. coli* present the main ESBLs hospitals acquired infections. Indeed, based on the evolving reclassifications of the bacteria taxa in the literature, we considered the term *Enterobacterales* to replace *Enterobacteriaceae*, except at qPCR and metagenomic data where the names were defined according to a conserved databases and analysis.

Additionally, ESBLs could also be transmitted to other bacterial species like *Enterobacter spp.*, *Proteus spp*, *Citrobacter spp*, *Salmonella spp* and others. The ESBL plasmids contains other resistance genes that confer bacterial resistance to main antibiotics including aminoglycosides, fluoroquinolones, and sulphonamides. Notably, new ESBL-producing isolates exhibited carbapenem resistance phenotypes have recently emerged (53).

On other hand, the clinical detection for ESBLs is primarily carried out by either genetic or phenotypic approaches. The genetic method is basically relied on the molecular PCR detection and then sequencing for the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes. The detected Isolates were considered ESBL positive when the amplicon sequence matched significantly with previously identified ESBL (53-55).

By contrast, phenotypic detection of ESBL phenotype is typically based on the ability of ESBL isolates to hydrolyze 3GCs , as well as their inhibition by clavulanate. Such guiding's have been well described though *Enterobacterales* genera/species of *E. coli, Klebsiella*, and *Proteus*. But notably, detection within other ESBL- producing species such as *Enterobacter* and *Citrobacter* could be impractical. Although these species hydrolyze 3GCs, they show resistance to clavulanic acid and β-lactamases inhibitors due to the activity of AmpC enzymes (53-55).

Moreover, new ESBL carriage detection approaches have been arisen recently, like chromogenic selective agar medium containing 3GCs (ESBL-Bx; BioMérieux, Marcy l'Etoile, France), through which ESBL producing colonies were colorized with either pink-burgundy, blue-green, orange to brown along their genus. Although this approach could do rapid ESBL identification, but however genotypic characterization of isolates remains more effective (53-55).

- **Carbapenemase-producing *Enterobacterales* (CPE)**

Carbapenemase are group of β-lactamases that confer hydrolytic activity against carbapenems as well as penicillins, cephalosporins, and monobactams. The majority of these enzymes are produced by *K. pneumoniae* and *E. coli*, and to little extent by *P. aeruginosa* and *A. baumanni.* Carbapenemases are carried by mobile plasmids, which facilitates their global aggressive dissemination (56).

Carbapenemase are members of Ambler classes A, B, and D β-lactamases. Most prevalent carbapenemase are including *K. pneumoniae* carbapenemase (KPC), a plasmid encoded enzyme of Ambler class A β-lactamases. OXA-type β-lactamases of class D, detected in *Enterobacterales* and *A. baumannii,* the metallo-β-lactamases of class B β-lactamases including imipenemase (IMP), the new delhi metallo-β-lactamase (NDM), and the verona integron-encoded metallo-β-lactamase (VIM). OXA, and KPC type β-lactamases are of particular concern (50,56).

- **Prevalence of ESBL-EB/CRE intestinal colonization**

The gut is a reservoir of multidrug resistant organisms (MDRO), particularly in patients who have had extensive antibacterial therapy. Asymptomatic intestinal colonization by MDRO may evolve to various infections, mainly urinary, digestive and blood leading to death (57). Moreover, it can also cause contamination of the environment and thereby increase of MDRO prevalence (57).

A recent systematic review (57) recruited the data of 66 studies of 28909 healthy asymptomatic individuals, from 1978 to 2015 in WHO–defined regions. This review aimed to assess the prevalence of ESBL-EB class A fecal colonization. Results revealed that 14% of individuals were colonized with ESBL-EB. The highest burden was in west Pacific (46%), then in southeast Asia and Africa (22%), followed by 15% in the eastern Mediterranean, 4% in Europe (3% in central to 4% in northern and 6% in southern Europe). The lowest ESBL-EB prevalence was in Americas with 2% (Figure 14) (57). Moreover, CTX-M enzymes represented the dominant type of ESBL enzymes with 69% among isolates, then 21% for TEM, and 10% for SHV enzymes.

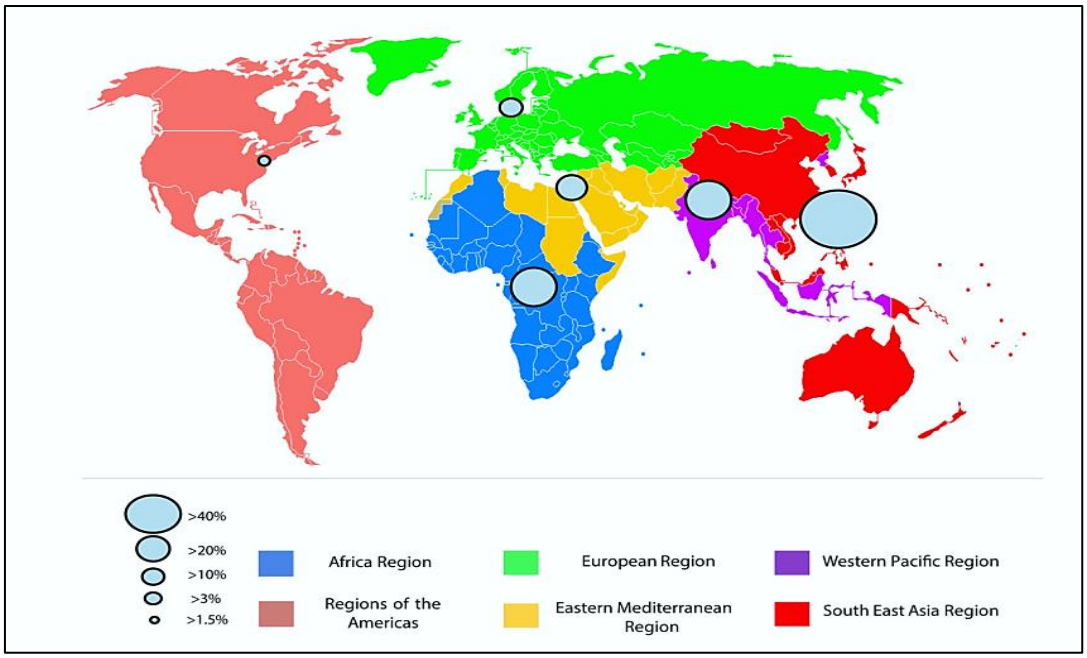

Figure 14 : Prevalence and fecal colonization rates of ESBL-EB among WHO region (57).

Moreover, Bezabih et al. reported in his recent systemic review the global abundance of the human gut colonization by ESBL-Ec, including 62 studies for the period 2000-2020 (58). The pooled global proportion of ESBL-Ec intestinal carriage was 16.5 % in healthy individuals. South-East Asia had a substantially higher ESBL Ec carriage of 27%, than Europe (6 %). The prevalence of ESBL Ec intestinal carriage increased from 2.6% in 2003–05 to 21.1% in 2015–18. Furthermore, the same author, performed a meta-analysis (accepted in JAC-Antibio Resistance, 2022) of 133 articles to compare prevalences of ESBL-Ec among healthy individuals and inpatients. Intestinal ESBL Ec was more prevalent among inpatients (21.1%) than among healthy individuals (17.6%). Moreover, the incidence carriage rate increased 3-fold among inpatients from 7% in 2001-2006 to 25.7% during the 2016-2020 period, but it increases ten-fold among healthy individuals from 2.6% to 26.4% for the same periods. The highest prevalences of carriage were found in South-East Asia for healthy individuals (35.1%) and in Eastern Mediterranean region (45.6 %).

1.4 Strategies for MDR decolonization

The latest clinical guidelines of the European society of clinical Microbiology and infectious diseases, could not recommend any treatment for colonization by multidrug-resistant gram-negative bacteria (MDR-GNB), especially MDR-EB. Accordingly, the ESCMID-EUCIC panel has basically suggested the urgent need to develop innovative non-antibiotic therapies like FMT, prebiotics, probiotics, and bacteriophage therapy, and to conduct clinical trials of decolonization with oral colistin and neomycin. Thus far, to date, all non-antibiotic trials of fecal microbiota

transplantation (FMT) (59-64), bacteriophages (65-67), and probiotics (68,69) that have been explored to treat or prevent MDR-EB intestinal colonization in human and mice, had no efficacy. They only may modestly reduce the average MDR-EB fecal titer or significantly decline it in subgroup of mice. Moreover, there is difficulties to allocate a rational effective dose, and to assess the long-term safety issues.

1.4.1 Antibiotics to treat MDRO colonization

Colistin is a polymyxin type E antibiotic produced by *Bacillus polymyxa*. It is clinically available in two forms of either cationic colistin sulfate, applied orally or subcutaneously, or anionic colistimethate that is normally given through intramuscular and intravenous route (70,71). Neomycin is an aminoglycoside produced by *Streptomyces fradiae*, acting on 30S ribosomal subunit (72). Their use as selective digestive decontamination have been studied, but their efficacy has not been convincingly demonstrated so far (73). The ESCMID-EUCIC panel has suggested conducting clinical trials of decolonization with:

- Oral colistin sulphate and neomycin sulphate to temporarily suppress 3GCs carriage in patients with severe neutropenia.
- Oral colistin sulphate with or without gentamicin sulphate to temporarily suppress CRE carriage in high-risk patients.

Using these antibiotics would expose to the risk of selecting colistin and/or aminoglycoside resistance in the intestinal microbiota. Such trials should be performed with careful monitoring of neomycin or colistin resistance (73).

1.4.2 Fecal microbiota transplantation (FMT)

FMT is a possible strategy to treat the MDR-EB intestinal colonization It is mainly applied through transferring fecal bacteria of one healthy donor into another recipient gut.

Hence, most successful FMT trials to treat MDR-EB in humans have been restricted to individual-clinical cases. Examples of some FMT efficacy are summarized at Table 4 (59-62).

Studies of the effect of FMT on MDR-EB has been also explored in mouse model but to little extents. Mahieu et al. revealed that FMT was not effective to eradicate intestinal carriage of *E. coli* producing a new delhi metallo-β-lactamase-1 (NDM-1) in Swiss mice after 26 days follow-up (63,64). Hence, in spite of some evident effectiveness of FMT, it still has several limitations. There is a weakliness in the process of screening and identifying FMT microorganisms, thereby there is a risk of transferring MDR strains to recipient, as recently being reported by DeFilipp et al. for the transmission of ESBL-Ec from donated feces (74). Besides, recipient patients might

also have post-abdominal disorders like bloating, nausea, rectal discomfort (75), in addition to the insufficient allocation of effective dosage as well the monitoring of future uncertain impacts. Additionally, FMT cannot routinely envisaged, without availability of capsules of microbiota.

References	Experimental conditions	Study design	(MDR)	Participants	FMT efficacy
(59)	Antibiotics administrations over 24 h, followed by FMT	Case report	KPC OXA-48	82-year-old female	complete eradication of KPC OXA-48
(60)	PPI intervention prior and during FMT, then single FMT	Uncontrolled cohort	MDR-GNB Multidrug-mainly ESBL-EB and CPE	Adult patients	A) 60 % (15/25) Decolonization after 1 month follow-up B) 93% (13/14) Decolonization after 6 months follow-up
(61)	Single FMT, without prior antibiotics.	Case report	ESBL-Ec	60-year-old male	-negative ESBL-Ec rectal culture at week two
(62)	5-day antibiotics administration, then single FMT	Randomized clinical trials	ESBL-E and CPE	≥18 years patients Colonized by ESBL-E (*n* = 36) and/or CPE (*n* = 11)	(41%) of patients negative for ESBL-E/CPE, but also (29%) of control were ESBL-E/CPE negative; this difference was not significative

Table 4 : Examples of some FMT clinical trials to treat MDR-EB in humans (59-62).

1.4.3 Bacteriophages

The use of bacteriophages is another strategy that have been gradually assessed against MDR colonization. Bacteriophages have been initially described by Félix d'Hérelle in 1917, and consist in bacterial viruses that can infect and kill specifically bacteria without deleterious effect for mammalian cells (76,77). Several studies have described the potential efficacy of bacteriophages against intestinal pathogens in mammals. Although they described a reduction of different pathogens, the overall efficacy was limited, and only concerned some subgroups of

individuals. For instance, in mice, bacteriophages were able to reduce the fecal titers of different associated pathogens including *Clostridioides difficile, V. cholerae*, and *P. aeruginosa (*78). Meanwhile, Sheng et al. reported significant elimination of fecal *E. coli* O157:H7 using oral treatment with SH1 and KH1 bacteriophages (65). Conversely, Javaudin et al. have shown no efficacy of novel lytic phages to reduce ESBL-Ec digestive carriage in mice (66). Similarly, Porter et al. found no significant effect for a bacteriophages cocktail to reduce mice intestinal carriage of MDR *E. coli* (ST131)-*H*30R (67).

Bacteriophage therapy was sometimes successful in humans: phages were able to prevent or treat intestinal bacteria dysentery associated with of *Shigella, Salmonella, Proteus, and E. coli* bacterial infections, in addition to their efficacy at different body site (77,78). However, bacteriophages still present different drawback, like the difficulty to purify them and thereby to insure of the mixture safety. Moreover, bacteriophages isolates are very specific of their target which reduce their activity spectra. More human clinical studies are required to conclude about their utility in preventing or treating intestinal colonization by MDRO (77).

1.4.4 Innovative antimicrobial strategies

New innovative antimicrobial technologies were further developed to prevent or treat the MDR carriage. The Phage-delivered CRISPR-Cas9 system was combined with phages to target specific MDR strains. In this approach, the CRISPR-Cas9 system is delivered to the bacterial cells through engineered phage-based vectors, targeting precisely antibiotic resistance genes and resulting in cell death (with antibiotic treatment). Such approach has succeeded in vivo to cleave $bla_{SHV\text{-}18}$ or $bla_{NDM\text{-}1}$ resistance genes in *E. coli*, and the kanamycin and *mecA* genes in *S. aureus*. However, this technique remains recent and limited to a narrow host range, adding that safety issues remain to be validated. (79, 80). Besides, DAV132 colon-targeted adsorbent is another innovative medical product designed by Da Volterra company to tackle MDR infections. The inactivating carbon agent is surrounded by a pectin beads coat, hydrolyzed by colonic bacterial enzymes for activation, which drastically reduces the colonic concentration of residual antibiotics without affecting the plasma concentration, and prevents intestinal microbiota dysbiosis. (81). Such activity of DAV132 preserved human gut microbiota from moxifloxacin dysbiosis in a clinical trial (82), It also protected hospitalized patients from *Clostridioides difficile* infections following fluoroquinolones administration (83). However, the DAV132 activity has been only tested on limited number of antibiotics, and more time needed to validate its safety, and broad activity on different antibiotics. Furthermore, oral administration of beta-lactamases could also save gut microbiota from the beta-lactamines disruption, and

reduce the emergence of MDR. For instance, Ribaxamase has reduced the ceftriaxone microbiota damages (84), which has provided colonization resistance against *C. difficile* infections in dogs and pigs. (85,86). Meanwhile, beta-lactamases activity was tested mostly for a limited number of antibiotics. Their long-term effect on indigenous microflora following administration is also still not clear.

1.4.5 Probiotics

The use of probiotics is maybe a more promising strategy to treat or prevent MDR-EB colonization. Probiotics are non-pathogenic live microorganisms, that could confer a health benefit on the host, when administered in adequate amount (FAO/WHO) (87).

Competitive exclusion is a crucial mechanism employed by some probiotics to exclude specific pathogens (Figure 13) (88). First, probiotics could competitively exploit essential nutrients in detriment of other bacteria and pathogens in a specific niche by secretion of extracellular nutrients-degrading enzymes (88,89). Second, exclusion could occur through direct competition with specific pathogen for receptor sites on the host, as has been reported for some *Lactobacilli* and *Bifidobacteria* species which were able to exclude some pathogens, like enterohemorrhagic *E. coli*, *Salmonella* and *Helicobacter pylori* (88,89). Third, probiotics could also exert competition against pathogens through the secretion of antimicrobial compounds, which could be bacteriocins (88,89). These bacteriocins have either a narrow or wide spectrum of activity, depending on their type. They present antibacterial activity against pathogens through inhibition of cell wall synthesis, or membrane pore formation (88,89). For instance, some *Bifidobacteria* strains produce Bifidocin B that targets gram-positive bacteria, while bacteriocin of some *Lactobacilli* strains can inhibit some gram-negative bacteria like *H. pylori* (88,89). *L. reuteri* bacteria colonizes the gastro-intestinal tract of humans and animals, and secretes Reuterin and Reutericyclin that inhibit a wide range of pathogenic gram-negative bacteria. It also produces histamine that reduces intestinal inflammation (89).

Moreover, some probiotics are able to produce short chain fatty acids (SCFAs) like butyrate, propionate, succinate, acetate, through fermenting non-digestible carbohydrates fibers and carbohydrates. These SCFAs provides different health benefits for the host gut. *Bifidobacterium* spp., *Lactobacillus* spp, *Eubacterium* spp, *Faecalibacterium* spp, *Bacteroides* spp, and *Ruminococcus* spp are the main SCFA-producers in humans (90,91). SCFAs production is basically accompanied by lowering the colonic pH from 6.5 to 5.5, which selectively promote the growth of health associated bacteria, and inhibits acids-sensitive entero-pathogens like *Shigella* spp., *E. coli*, and *Salmonella* spp. They could also increase

microbiota adhesion, by stimulating epithelial cell secretion of mucin and host antimicrobial peptides (91,92). Besides serving as an energy source, SCFAs are associated with a number of health benefits for the host. Studies have reported their ability to reduce and prevent different intestinal diseases, including ulcerative colitis, inflammatory bowel disease (IBD), and diarrhea (90,91). Moreover, SCFAs are also substrates for some other bacteria through cross feeding mechanisms. Bacterial cross-feeding plays an important role in the gut and influences the microbiota composition (92). Furthermore, probiotics could also act by colonizing the intestinal mucosa and improve epithelial barrier, thus preventing pathogens attachment (Figure 15) (88,90). For instance, *E. coli* Nissle 1917 can strengthen the intestinal epithelial, by modulating expression of junction proteins that restores intestinal barriers against enteropathogenic *E. coli* (90). Finally, probiotics could further act to improve the immune response toward pathogenic bacteria, through direct stimulation of the immune and epithelium cells (Figure 15) (88,90).

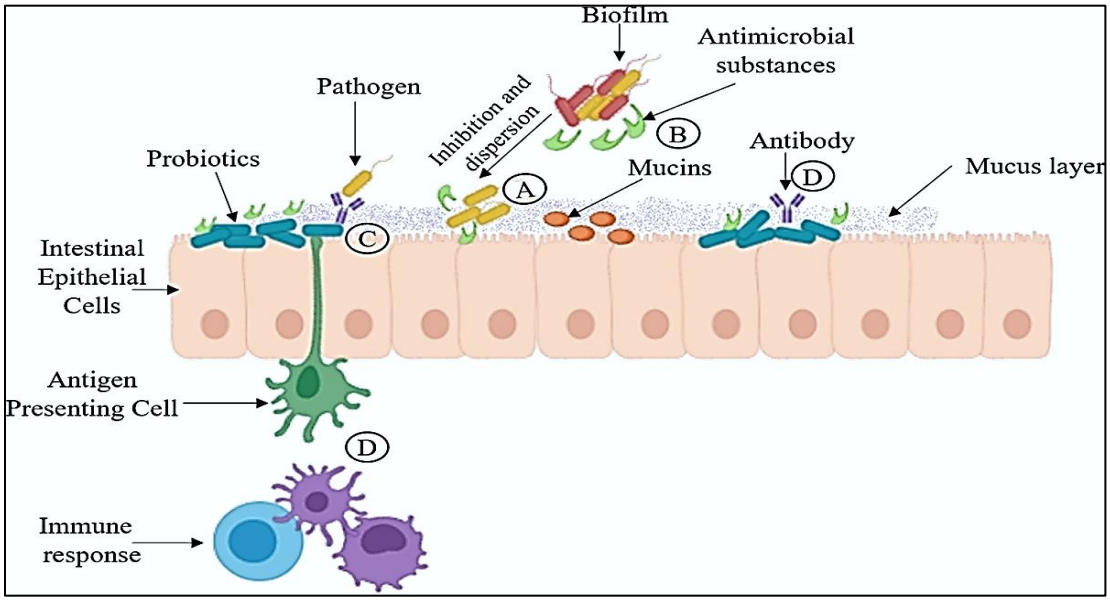

Figure 15 : Major probiotic mechanisms of action (A) Competitive exclusion of pathogens, (B) Production of antimicrobial compounds. (C) Adhesion to the intestinal mucosa and amelioration of epithelial barrier. (D) stimulation of the immune system (88).

1.4.5.1 Lactic acid bacteria

To date, lactic acid bacteria (LAB) species (*Lactococcus, Lactobacillus, Streptococcus* and *Enterococcus*) and *Bifidobacterium* are the most widely studied probiotics. (Table 5) (88). The first LAB was described in 1873 by Joseph Lister, and it was called *Bacterium lactis* (current name: *Lactococcus lactis*). LAB have been widely used in dietary food as it could promote health benefits if consuming in adequate amounts (93).

Lactobacilli	***Bifidobacterium***	**Other bacteria**
Lactobacillus acidophilus	*Bifidobacterium bifidum*	*Saccharomyces bourlardii*
Lactobacillus amylovorus	*Bifidobacterium infantis*	*Propionibacterium freudenreichii*
Lacticaseibacillus casei	*Bifidobacterium lactis*	*Enterococcus faecalis*
Lactobacillus crispatus	*Bifidobacterium adolescentis*	*Enterococcus faecium*
Lactiplantibacillus plantarum	*Bifidobacterium animalis subsp. lactis*	*Lactococcus lactis*
Lacticaseibacillus casei Shirota	*Bifidobacterium longum R0175*	*Leuconstoc mesenteroides*
Lacticaseibacillus paracasei	*Bifidobacterium breve*	*Pediococcus acidilacticiei*
Lacticaseibacillus rhamnosus		*Sporolactobacillus inulinus*
Limosilactobacillus reuteri		*Streptococcus thermophilus*
Lactobacillus johnsonii		*Escherichia coli 1917*
Lactobacillus helveticus R0052		*Saccharomyces cerevisiae var. boulardi*
Limosilactobacillus fermentum		*Bacillus coagulans*

Table 5 : The most common probiotics (88).

Using live bacteria to counter intestinal colonization by MDRO after antimicrobial therapy is becoming increasingly plausible with the improving knowledge of the structure and functions of the intestinal microbiota. However, to date, probiotics treatments including LAB have shown modest activity against MDR-EB infections. For instance, no inhibitory effect was obtained *with Lacticaseibacillus rhamnosus*GG (LGG) on ESBL-Ec colonization in adults (69). Another study reported only 12.5 % ESBL-EB decolonization from chronic carriers, following administration of a consortium of 8 live probiotic strains (Vivomixx), *Lactiplantibacillus plantarum, Lacticaseibacillus paracasei, Lactobacillus acidophilus, Lactobacillus delbrueckii ssp.*

bulgaricus, Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve and Streptococcus thermophilus (94). Indeed, the failure of LAB probiotics to compete effectivity with the MDR-EB could be explained by the following reasons: Generally, it is proposed that probiotics effects are both strain specific and diseases specific (95). This means that likely effective LAB strains should be identified against each type of MDR-EB. It is also might refer that a LAB cocktail will not show the same degree of efficacy in different patients, due to their different intestinal microbiota compositions. Moreover, from a methodological point of you, most current LAB clinical trials are not yet standardized for selection of effective dose or mixture. Also, the LAB titers are done most of the time in the feces, but the exact titers at the gut remain poorly described. Indeed, it is important to check that the potential probiotic reached a significant titer in the gut to confer colonization resistance.

Besides, concerns have been raised regarding adverse effects of current LAB probiotics. For instance, a daily intervention of probiotics mixture has impaired post antibiotic restoration of normal indigenous microbiota in humans and murine model (96). Likewise, probiotics opposed post-antibiotics reduction of antimicrobial resistance genes (97). Furthermore, Wieërs et al. found that a probiotic mixture of *lactobacilli, Bifidobacteria* and *Saccharomyces* gave rise to an elevated ESBL colonization after antibiotic treatment in elderly patients (98).

1.4.5.2 ***Bacillus spp***

Bacillus spp are a rod-shaped gram-positive, strictly aerobic and endospore-forming bacteria belonging to the Firmicutes phylum. They are commonly ingested with vegetables, leading to titers of *Bacillus* spores in human feces around 10^5 colony-forming units (CFU) per gram. *B. subtilis*, *B. polyfermenticus*, *B. clausii*, *B. coagulans*, *B. pumilus*, and *B. licheniformis* are the most common non-pathogenic *Bacillus* species (99).

Notably, bacterial sporulation is adapted as a response to nutritional deprivation at stationary phase. The endospore is metabolically inactive, but can later germinate to a vegetative cell when favorable conditions like nutrients abundance and moderate temperature are available (100).

Bacillus probiotic spores may confer an advantage to *Bacillus* spp over other potential probiotics. They are widely used as feed additives for animals. This is due to their ability to resist extreme conditions of feed manufacturing like high temperature, pressure, and long-term storage. Spores are also able to survive to harsh intestinal conditions upon ingestion (100-102). Notably, endospores resist to the stomach acidity, low oxygen level, bile salts. However, the vegetative cells are more sensible to the intestine conditions, in particularly to the lack of oxygen since *Bacillus* is a strict aerobic bacterium (100-102). *Bacillus* spores can germinate in different parts of gut. Germination of a low fraction of spores can be induced in the stomach, which could kill acid

sensitive vegetative cells. However, nutrient-rich small intestine is the best site of germination, where they secrete different hydrolyses enzymes and antimicrobial compounds. Finally spores late germination can occur in the large intestine but it is rather the site where at least one part of vegetative cells sporulate again. Indeed, there is a strong competition with other bacteria and oxygen availability is the lowest in this gut compartment (101,102).

Besides, the vegetative *Bacillus* cells may confer their activity through the production of antimicrobial compounds, as described for *B. subtilis and B. coagulans* species. Hence, *B. subtilis* is capable to produce bacteriocins subtilin and subtilosin that display activity against some strains of gram-positive bacteria (101). Besides, *B. coagulans* produces coagulin bacteriocin that exhibits antimicrobial activity against human entero-microbes, able to attenuate the severity of susceptible diseases including abdominal pain, intestinal inflammation of irritable bowel syndrome and colitis (102). In addition to the production of antimicrobial compounds, some *Bacillus spp* like *B. coagulans* can produce different metabolites like SCFA, vitamins that may enhance intestinal barrier functions in human intestine (102). However, despite the protective effect that have been demonstrated of some *Bacillus* strains on the human gut health, no studies have reported *Bacillus* ability to reduce asymptomatic intestinal colonization of multi-resistant *Escherichia coli*. Accordingly, *Bacillus* spp. were the first probiotics that we tested for this aim.

1.4.5.3 *Akkermansia muciniphila*

A. muciniphila is a gram-negative, strictly anaerobic bacterium belonging to the phylum Verrucomicrobia. It constitutes up to 4% of the human gut microbial community, and has been reported to be a contributor to the maintenance of the gut balance and function (95,96) *A. muciniphila* was initially isolated in 2004 by Derrien et al from human feces by cultivating it on pig mucin as its main nitrogen and carbon source (103). It can also be grown on rich media like brain heart infusion (BHI) but with a lower extent than on mucin (103-105). Mucins are mucus glycoproteins secreted by intestinal mucosal epithelium and cover the intestinal track. They are composed of a peptide backbone core rich in serine and threonine residues completed by glycan oligosaccharides containing sugars like N-acetylglucosamine, N-acetylgalactosamine, galactose and fucose (Figure 16A) (106,107).

The adherence of *A. muciniphila* to the gut mucus layer helps to provide thick steady mucosa that sustain intestinal barrier function and protect epithelial cells from pathogen attacks (Figure 16B) (108). However*, A. muciniphila* is also able to hydrolyze intestinal mucins leading to different sugars release which can be then fermented in SCFAs. These SCFAs can be utilized by other intestinal bacteria including *Anaerostipes caccae, Eubacterium*

hallii and *Faecalibacterium prausnitzii* through cross-feeding mechanism (108). The fermentation of these mucin-degraded monosaccharides by these bacteria could eventually result in the production of essential metabolites like vitamin B_{12}, butyrate, and propionate, as well as immune regulatory proteins, which could be respectively used as sources of energy, immunoregulators, and modulators of human metabolism and health (108). Hence, several studies addressed the potential *A. muciniphila* effect to combat human intestinal diseases including inflammatory and metabolic disorders; they described an inverse correlation between *A. muciniphila* abundance and appendicitis, inflammatory bowel disease (IBD), endotoxemia and obesity diseases (109,110).

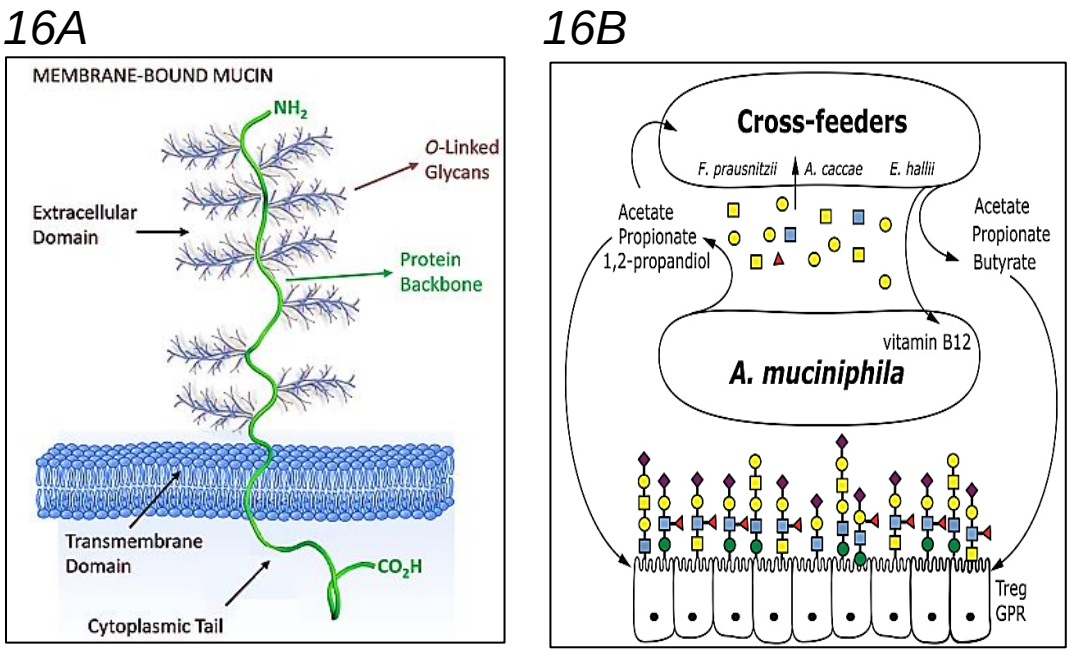

Figure 16 : A, basic structure of intestinal mucin (99)**. B**, cross-feeding network of *A. muciniphila* at the *mucus* layer (108).

Consequently, *A. muciniphila* seems to be a promising probiotic. No work presented its effect on MDRO intestinal carriage: M Grégoire et al. only demonstrated that mice with low level fecal colonization by ESBL-producing *K. pneumoniae* were associated with high fecal abundance of *A. muciniphila* (111), That's why we wanted to test its potential preventive activity against ESBL-Ec.

1.4.5.4 *Escherichia coli*

E. coli is a gram-negative facultative anaerobic bacterium; it is a commensal inhabitant of the mammalian large intestine, and accounts for nearly 0.1% of human intestinal microbiota. Commensal *E. coli* (comEc) exert different beneficial effect for the host as the colonization

resistance against pathogens through direct competition for similar nutrients and niche (112,113). ComEc can also resist through the direct secretion of antimicrobial compounds including bacteriocins (112,113), or by stimulating host defense, and improving intestinal barrier.

Several therapeutic activities have been reported for *E. coli*. In human, several *E. coli* strains isolated from the human gut, have been commercialized as probiotic products for the treatment of different gastro-intestinal diseases. The most effective products are Mutaflor that includes *E. coli* strain Nissle 1917 (114), and Symbioflor-2 which contains six *E. coli* strains (115). Mutaflor attenuated intestinal inflammation, chronic colitis, reduced the duration of infants diarrhea, treated sever constipation and irritable bowel syndrome (113,114). Besides, Symbioflor was able to reduce IBD of adults (115).

Some interesting results were also obtained in mice: streptomycin-treated mice pre-colonized by the combination of comEc strain HS and the probiotic *E. coli* Nissle 1917 *EcN*, protected from an *E. coli* EHEC strain gut colonization (116). Also, comEc strain COMEC15 reduced severity of chronic colitis in mice (117).

However, to date, *E. coli* probiotics have been only studied as a curative treatment of MDR-EB intestinal colonization. However, these experiments conducted piglets were not successful (118). Furthermore, the choice of the probiotic strain - especially the host from whom originates - may be crucial. Hence, a murine *E. coli* isolate would be a better candidate as a treatment of MDR-EB colonization in mice than a strain of human origin, and this treatment may be more active to prevent colonization than to cure it. Hence, we looked for murine E. *coli* strains and tested their preventive activity against intestinal colonization with MDR-Ec.

1.4.6 Prebiotics

Prebiotics are non-digestible substrate that may modulate the host microbiota through eliciting the growth of beneficial bacteria (119).

This concept was initially described by Roberfroid in 1995 by showing that some bacteria, mainly *Bifidobacteria* and *Lactobacillus,* were remarkably linked the health of individuals and that they were stimulated by some prebiotics, particularly fructo-oligosaccharide (FOS) (120,121). Prebiotics exists naturally in different plant derivatives such as asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke. There are three main criteria that feature the prebiotics: 1) the resistance to gastric enzymatic degradation, acidity, and absorption. 2) they should also be susceptible to microbiota fermentation in the large intestine. 3) they selectively promote the growth and activity of intestinal health promoting bacteria. Hence, prebiotics

fermentation leads to different essential products including SCFAs, amino acids and vitamins, that may improve the intestinal health (120,121).

The most common prebiotics types are inulin, (FOS), and galacto-oligosaccharides (GOS), (120,121). Inulin and FOS displaying a linear poly-fructose chain with β-(1,2) linkage, differing in their degree of polymerization (DP) that correspond to the number of monosaccharide units, DP <10 for FOS, and up to 60 DP for inulin (120). Bacterial utilization of prebiotics depends of the length of the chain: for example, only few species like *Bifidobacterium, Lactobacillus, Sutterella*, and *Akkermansia* can ferment long chains of inulin, whereas a large number of bacteria can degrade FOS (120-122). GOS, another prebiotic type, are obtained by transglycosylation from lactose submitted to β-galactosidase activity, and consists in β-linked galactose moieties with glucose or galactose at the reducing end. It was shown that GOS could enhance the growth of some intestinal bacteria such as *Bifidobacteria*, *Lactobacilli*, and *Enterobacteria, Bacteroidetes, Firmicutes* to lesser extent (120,121).

Potential therapeutic effect of prebiotics was described to restore the unbalanced-microbial composition implicated in some intestinal diseases. For instance, they can alleviate severity of colonic inflammation, inflammatory bowel diseases and irritable bowel syndrome in humans (123-126). They also further decreased necrotizing enterocolitis in neonatal rats (127). However, to date, the efficacy of prebiotics to treat intestinal MDR-EB colonization has not been reported.

1.4.6.1 Inulin

Inulin is a widely used prebiotic naturally found in plant roots like onion, chicory and Jerusalem artichoke (128). It was initially discovered in 1804 by Valentin Rose from roots of *Inula helenium* plant, and was approved on 2018 by the FDA as a dietary fiber (Figure 17) (122,129). It is a non-digestible fiber that passes through the GIT and induce fermentation by intestinal microbiota that generates diverse SCFAs in the colon (128). These SCFAs triggered gut barrier rectification and bacterial metabolism by cross feeding (128). It also induces secretion of antimicrobial compounds that confer enteric protection against various pathogens (130). The potential therapeutic effect of inulin has been well studied in human and mice. In humans, it can alleviate severity of colonic inflammation, inflammatory bowel diseases and irritable bowel syndrome (130-132). Moreover, a daily intake of 12 g of native chicory inulin improved stool frequency in patients with chronic constipation (133). In mice, inulin administration was shown to attenuate glucose and lipid metabolism disorder in a leptin mutant obese (ob/ob) mouse (134). It also reduced mice endotoxemia and colitis inflammation, and inhibited enteric *Candida albicans*, and systematic

Listeria monocytogenes and *Salmonella typhimurium* infections (135,136). Inulin elevated SCFAs producing bacteria including *Bifidobacterium*, *Anaerostipes*, *Faecalibacterium*, and *Lactobacillus* in humans (137-139). It also induces the growth of *Sutterella* and *Akkermansia* that were reduced in mice with MDR-EB colonization (96,111,135, 140). However, the efficacy of inulin to treat MDR-EB colonization has not been reported, Consequently Inulin-type prebiotic seems promising to slow down the MDR-Ec intestinal colonization.

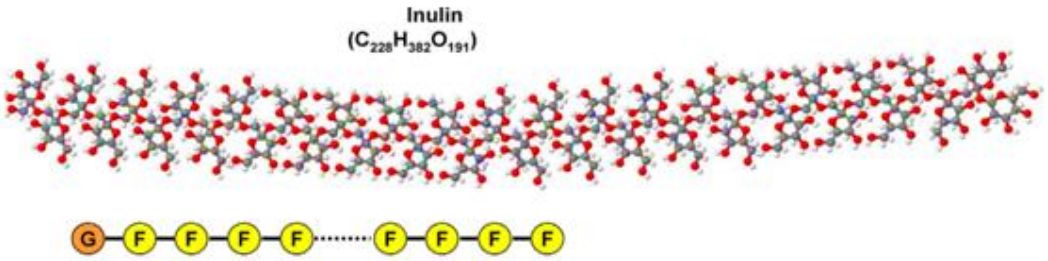

Figure 17 : Main structure of inulin**,** long chain of β-(2,1) fructosyl-glycosidic polymer**,** linked to terminal glucosyl residue by an α-(1,2) bond. G: Glucose; F: Fructose (F: up to 60 for native inulin from chicory root) (122).

1.5 Objectives of the PhD

Main Objectives

Our main objectives were to assess the efficacy of treatments with *Bacillus subtilis, Escherichia coli, Akkermansia muciniphila* and inulin, to decrease fecal titers of ESBL-Ec in a murine model amoxicillin-induced dysbiosis. The efficacy of treatments were assessed by comparing the proportion of mice with low ESBL-Ec fecal titers, as defined by the area under the curve of fecal titers between 1- and 8-days post-inoculation. We also aimed to identify taxa displaying higher abundances in effectively treated mice in comparison with untreated and ineffectively treated mice, and to assess their activity on ESBL-Ec colonization.

2. Studies

2.1 Article n°1: In vitro and in vivo activity of new strains of *Bacillus* subtilis against ESBL-producing *Escherichia coli*: an experimental study

Received: 7 June 2021 | Revised: 5 October 2021 | Accepted: 7 October 2021

DOI: 10.1111/jam.15329

Journal of Applied Microbiology sfam

ORIGINAL ARTICLE

In vitro and in vivo activity of new strains of *Bacillus subtilis* against ESBL-producing *Escherichia coli*: an experimental study

Murad Ishnaiwer[1] | **YihieneIw Bezabih**[2] | **François Javaudin**[1,3] | **Mohamed Sassi**[4] | **Pascale Bemer**[1,5] | **Eric Batard**[1,3] | **Michel Dion**[1]

[1]MiHAR Lab, EE1701, University of Nantes, Nantes, France

[2]WellStar Atlanta Medical Center, Atlanta, Georgia, USA

[3]Emergency Department, CHU Nantes, Nantes, France

[4]Inserm, BRM [Bacterial Regulatory RNAs and Medicine] - UMR_S 1230, Université de Rennes 1, Rennes, France

[5]Department of Bacteriology, University Hospital, CHU Nantes, Nantes, France

Correspondence
Michel Dion, MiHAR, 22 Boulevard Benoni-Goullin, F-44200 Nantes, France.
Email: michel.dion@univ-nantes.fr

Funding information
Société d'Accélération du Transfert de Technologies, Grant/Award Number: DV3924; Université de Nantes

Abstract

Aims: The gastro-intestinal tract is a major reservoir of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli*. *Bacillus* spores may be used as probiotics to decrease digestive colonization by ESBL-*E. coli*. Our aim was to assess the in vitro and in vivo activity of new *Bacillus* strains against ESBL-*E. coli*.

Methods and Results: We screened the in vitro activity of 50 *Bacillus* strains against clinical isolates of ESBL-*E. coli* and selected *B. subtilis* strains CH311 and S3B. Both strains decreased ESBL-*E. coli* titers by 4 $\log_{10}$ CFU L^{-1} in an in vitro model of gut content, whereas the *B. subtilis* CU1 strain did not. In a murine model of intestinal colonization by ESBL-*E. coli*, CH311 and S3B did not decrease fecal titers of ESBL-*E. coli*. Ten sequences of putative antimicrobial peptides were identified in the genomes of CH311 and S3B, but not in CU1.

Conclusions: Two new *B. subtilis* strains showed strong in vitro activity against ESBL-*E. coli*.

Significance and Impact of Study: Despite strong in vitro activities of new *B. subtilis* strains against ESBL-*E. coli*, intestinal colonisation was not altered by curative *Bacillus* treatment even if their spores proved to germinate in the gut. Thus, this work underlines the importance of in vivo experiments to identify efficient probiotics. The use of potential antimicrobial compounds identified by genome sequencing remains an attractive alternative to explore.

KEYWORDS

antimicrobial compound, antimicrobial resistance, *Bacillus subtilis*, Enterobacteriaceae, ESBL, *Escherichia coli*, extended-spectrum beta-lactamase, gut colonisation, probiotic

INTRODUCTION

Third-generation cephalosporins and carbapenem-resistant Enterobacteriaceae are critical pathogens according to the World Health Organisation priority list of antibiotic resistant bacteria. The intestinal microbiota is a major reservoir of multidrug-resistant Enterobacteriaceae (MDR-EB), and intestinal colonization by MDR-EB is promoted by intestinal dysbiosis (Sorbara et al., 2019). Intestinal colonization by MDR-EB may evolve from an asymptomatic carriage to various infections including urinary, gastrointestinal and bloodstream infections. Furthermore, the intestinal carriage of MDR-EB can lead to environmental contamination and transmission to healthy or diseased subjects. Hence, treatments to decrease the intestinal carriage of MDR-EB are crucial to limit the global spread of antimicrobial resistance. Various

strategies have been assessed but none has proved to be effective so far (Tacconelli & Pezzani, 2019).

Probiotics may be considered to restore dysbiotic intestinal microbiota, to treat symptomatic intestinal infections and/or to decrease asymptomatic intestinal colonization by MDR-EB. Probiotic preparations, usually consisting of *Bifidobacteria*, *Lactobacilli*, *Enterococci* and/or *Streptococci* alter intestinal microbiota (Chae et al., 2016) and showed variable efficacy in preventing experimental intestinal infections due to *E. coli* in animals (Fukuda et al., 2011; Guerra-Ordaz et al., 2014). Besides, their efficacy in humans on improving microbiome reconstitution after an antibacterial treatment has not been demonstrated, and a commercial probiotic preparation even delayed and decreased post-antibiotic restoration of the gut microbiota and prevented post-antibiotics reduction of antimicrobial resistance genes (Montassier et al., 2021; Suez et al., 2018). Furthermore, few randomized controlled trials have been conducted to assess if probiotics may decrease intestinal colonization by MDR-EB in humans (Newman & Arshad, 2020). Hua et al. found no effect of a commercial probiotic on rectal colonization by ESBL-producing bacteria in preterm newborns, although subgroup analysis suggested an effect in non-breastfed patients (Hua et al., 2014). *Saccharomyces boulardii* and other commercial probiotic preparations consisting of *Lactobacillus*, *Bifidobacterium* and *Streptococcus* (Vivomixx®) or *S. boulardii*, *L. acidophilus*, *B. animalis subsp. lactis and Lacticaseibacillus paracasei* (Bactiol Duo®) failed to significantly reduce intestinal colonization by Extended Spectrum Beta-Lactamase producing Enterobacteriaceae (ESBL-EB) in adult patients (Ljungquist et al., 2020; Wieërs et al., 2020). Hence, there is a need for probiotics that would reduce intestinal colonization by MDR-EB. *Bacillus* spp. consists of different species of spore-forming soil bacteria that are commonly ingested with vegetables and reach a concentration of about 10^{4-5} colony-forming units (CFU) per gram of animal and human faeces (Elshaghabee et al., 2017). *Bacillus* spores are resistant to the acidity of gastric contents and are stable for long periods in various environments (Cutting, 2011). After germination, vegetative cells of *Bacillus* produce antimicrobial compounds (AMCs) that confer an antimicrobial activity against various pathogens (Caulier et al., 2019). Most of these compounds have bactericidal activity against gram positive bacteria but a few, such as Gramicidin S and Polymyxins, are active against gram negative bacteria (Mogi & Kita, 2009). Unfortunately, the latter antimicrobial peptides (AMPs) have toxicity and can induce colistin resistance in other enterobacteria (Halaby et al., 2013).

Therefore, alternative *Bacillus* strains that could serve as a source for safer AMCs have been sought (Caulier et al., 2019; Guo et al., 2017; Latorre et al., 2016). Treatment with *Bacillus* spores has been shown to prevent various gastrointestinal infections, including infections due to *E. coli* in piglet, chicken, rabbit and mouse (D'Arienzo et al., 2006; Guo et al., 2017; La Ragione et al., 2001; La Ragione & Woodward, 2003; Tsukahara et al., 2013). *Bacillus* also abrogated asymptomatic intestinal colonization of methicillin resistant *S. aureus* in mice (Piewngam et al., 2018). However, whether *Bacillus* treatment decrease the intestinal colonization by MDR*Ec* remains unknown.

Consequently, we searched for new strains of *Bacillus* that would inhibit ESBL-producing *E. coli* (ESBL-*E. coli*) under in vitro conditions that mimic the gut environment. We also characterised their genome and tested their efficacy as a curative treatment of intestinal colonization by an ESBL-*E. coli* in a murine model.

MATERIALS AND METHODS

Bacterial strains and media

Clinical isolates of ESBL-*E. coli* were collected at the University Hospital of Nantes. One of them was also resistant to carbapenems through an OXA-48 carbapenemase and was used in the in vitro culture test and in the murine model of intestinal colonization. The reference strain for *Bacillus subtilis* was the commercially available CU1 strain (Lefevre et al., 2015). For bacterial numeration, we used CHROMID^{TM} ESBL agar plates (Biomerieux) for ESBL-*E. coli*, and LB agar NaCl plates (75 g L^{-1} NaCl) for *Bacillus and Staphylococcus* spp. strains.

Isolation of *Bacillus* strains from the environment and identification

Samples were collected from different environmental sources such as soil, mice food, lake and river mud as well as faeces from a variety of organisms (human, mouse, dog and snail). Each sample was mixed with water. One ml of each mix was heated at 80°C for 20 min to kill vegetative cells, leaving *Bacillus* spores. Then they were spread on LB NaCl agar plates. For identification, 16S RNA genes were amplified with universal primers D88 (5′-AGAGTTTGATCCTGGCTCAG-3′) and F17 (5′-CCGTCAATTCCTTTGAGTTT), and *gyrA* genes were amplified with primers gyrA-f (5′-CAGTCAGGAAATGCGTACGTCCTT) and gyrA-r (5-′CAAGGTAATGCTCCAGGCATTGCT). Sequences were blasted in NCBI.

Spores production

To produce large amounts of spores, an overnight culture of *Bacillus* in 10 ml LB medium was inoculated into a flask containing 400 ml of Difco Sporulation Medium (Monteiro et al., 2005). After 3 days of shaking at 37°C, most of the *Bacillus* bacteria were in a sporulated form. The culture was then centrifuged, washed and resuspended in 3 ml of sterile water. Titration of spores was carried out after heating this suspension for 20 min at 80°C and then stored (about 10^{10}–10^{11} spores ml^{-1}) at −80° C.

Colony diffusion assay

The in vitro inhibitory activity of *Bacillus* against ESBL-*E. coli* was screened as follows. Two μL of fresh *Bacillus* suspension (10^6 CFU ml^{-1}) were inoculated on the surface of a dried LB or minimal medium with 0, 2% inulin agar plates where an ESBL-*E. coli* isolate had been spread. Fifteen clinical isolates of ESBL-*E. coli* were used. After incubation for 24 hr at 37°C, the areas of inhibition around *Bacillus* colonies were determined. The in vitro activity of strains was classified as strong, medium and weak for inhibition diameter higher than 20 mm, between 10 and 20 mm, and lower than 10 mm, respectively.

Co-culture of *Bacillus* and ESBL-*E. coli* isolates in murine faeces

In order to mimic the culture conditions in colonic content (including low oxygen pression), 50 mg of faeces from an antibiotic naive mouse were suspended in 1 ml of sterile water and shaken at 20 Hz (Mixer Mill MM 400, RETSCH's) for 5 min to make a homogenous suspension. Then, 100 μl of this suspension was mixed with suspensions of vegetative cells of *Bacillus* and ESBL-*E. coli* strains (in a 1:1 ratio) to obtain 10^6 CFU ml^{-1} of each bacterium (final volume, 1 ml), supplemented or not with 0,2% inulin (Bulk powders). Inulin was tested since it is a prebiotic, which could be used by *Bacillus* as a carbon source. The 2-ml tubes (containing a 1ml suspension) were shaken horizontally for 24 hr at 37°C (orbital shaker-incubator ES-20, Grant bio). Titres of *E. coli* and *Bacillus* were assessed after incubation at 37°C for 24 hr and 48 hr, respectively. All experiments were done in triplicate.

Murine model of intestinal colonization by ESBL-*E. coli*

All experiments were approved by the Animal Experiment Committee of Pays de la Loire (France, authorization number APAFIS#18120) and ARRIVE Guidelines were followed. Six-week-old male Swiss mice (Janvier Labs, Saint-Berthevin, France) were kept in individual cages with free access to food and water. First, intestinal dysbiosis was induced with amoxicillin (0, 5 g L^{-1}) in drinking water for 3 days. Pantoprazole (0, 1 g L^{-1}) and inulin 0.2% (Bulk powders) were added to drinking water of all mice, from the experiment first day and the day of amoxicillin discontinuation, respectively. Pantoprazole was added because it is known to suppress the gastric acid production and to promote intestinal colonization by certain bacteria (Stiefel et al., 2006). ESBL-*E. coli* (10^6 CFU) was instilled intragastrically the day of amoxicillin discontinuation. At 1 day post *E. coli* inoculation (dpi), 10^9 spores of *Bacillus* CH311 were daily administered by intragastric instillation, for 4 days. The experimental protocol was slightly modified to assess activity of *Bacillus* S3B: amoxicillin was administered for 5 days, and ESBL-*E. coli* was inoculated 2 days after amoxicillin was stopped. Faecal samples were collected daily. Faecal titres of *Bacillus*, *Staphylococci* and ESBL-*E. coli* were assessed by plating serially diluted faeces. There were 4–7 mice per group. No mouse was excluded from analysis. Researchers were aware of the group allocation during the experiment.

To enumerate the total number of *Bacillus* (spores and vegetative cells) per gram of faeces, faeces were homogenized in sterile water before plating serial dilutions in LB NaCl agar and incubating at 37C° for 24 h. To enumerate the total number of spores, suspensions were heated for 20 min at 80°C and then spread on the same medium. The difference in the number of CFU between the non-heat-treated and heat-treated suspensions was considered as the number of spores that germinated. The spores of S3B and CH311 in frozen stocks were used as control to check the efficiency of this procedure.

Genome sequencing and assembly

Whole-genome sequencing was performed using the Illumina MiSeq platform, with a paired-end library (2 x 150 bp paired-end read mode). Illumina reads were trimmed using Trimmomatic (Bolger et al., 2014), quality filtered using the Fastx-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and then assembled using the SPAdes software (Bankevich et al., 2012; Nurk et al., 2013). SIS and GapFiller version 1.10 (Boetzer & Pirovano, 2012; Nadalin et al., 2012) were used to improve the initial set of contigs, and the closest complete genome was used as the reference to order and orient the contigs. The draft genome sequences of CH311 and S3B were deposited in NCBI under accession numbers JAAUXF000000000 and JAAUXE000000000, respectively.

Genome annotation, comparative genomics and phylogeny

The nucleotide sequences of CH311 and S3B strains were also submitted to ResFinder 3.2 servers (https://cge.cbs.dtu.dk) for identification of acquired resistance genes. All annotated proteins with unknown function were submitted to Deep-AmPEP30 (Yan et al., 2020) to predict peptides with antimicrobial activities. The genomes of 423 *B. subtilis* strains were downloaded from Genbank (http://www.ncbi.nlm.nih.gov/genome), redundant sequenced strains and very poor genome quality was removed resulting in a total number of 405 genomes. The 405 *B. subtilis* genomes were submitted to CSIphylogeny (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). The core genome polymorphic sites were retrieved and phylogenetic tree constructed using Fasttree (Price et al., 2010). To normalize genes predictions, all genomes were annotated using Prokka (Seemann, 2014). The pangenomic analysis was performed using Roary software (Page et al., 2015). The gene homologies between the strains were assessed with >98% nucleotide identity and >80% sequence coverage cutoffs.

Statistical analyses

Means were reported with standard deviations. Difference in mean titres were tested with t test or ANOVA according of the number of compared groups. The efficacy of treatments was assessed using linear mixed effect models, where faecal titre was the dependent variable, treatment and time being the fixed independent variable, and mouse the random effect. Statistical analyses were performed with R 3.6.3, R Foundation for Statistical Computing, Vienna, Austria.

Results

Identification of *Bacillus* strains from the environment and screening for inhibitory activity against ESBL-*E. coli*

Fifty different *Bacillus* strains were isolated from environmental samples. Based on their 16S RNA and *gyr*A genes sequences, they were identified as *B. subtilis*, *B. licheniformis* or *B. pumilus*. We screened their inhibitory activity against 15 clinical isolates of ESBL-*E. coli* isolates using the colony diffusion assay. Nine *Bacillus* isolates inhibited growth of ESBL-*E. coli* isolates (Table 1). Among these, two *Bacillus subtilis* strains CH311 and S3B, isolated from dog and snail faeces respectively, presented the highest inhibition against 12 or 13 different strains of clinical isolates of ESBL-*E. coli*. For several strains, inhibition diameters were higher in minimal medium with inulin than in LB (data not shown).

In vitro activity against ESBL-*Escherichia coli*

To assess the activity of these strains in conditions mimicking the gut environment, co-cultures of an ESBL-*E. coli* and *Bacillus* strains were performed in faeces suspension with or without inulin, and ESBL-*E. coli* titres were

TABLE 1 *Bacillus* strains: Identification and screening for activity against ESBL-*Escherichia coli*

Strain	Origin	Identification method	Identification	Level of activity against ESBL *E. coli* (number of *E. coli* strains)[a]				
				Strong	Medium	Weak	Any activity	Total
CU1	Commercial	-	*Bacillus subtilis*	0	0	0	0	15
A532	Bogoria Lake	16S RNA	*B. licheniformis*	0	0	0	0	15
S2	Snail faeces	gyrA	*B. subtilis*	2	4	6	12	15
S3B	Snail faeces	gyrA	*B. subtilis*	6	3	3	12	15
S28	Snail faeces	16S RNA	*B. pumilus*	4	4	3	11	15
CH311	Dog faeces	gyrA	*B. subtilis*	10	1	2	13	15
Bac2	Mice aliment	gyrA	*B. subtilis*	4	0	5	9	15
Bac4	Mice aliment	gyrA	*B. subtilis*	2	2	6	10	14
MD1	Human faeces	gyrA	*B. subtilis*	2	0	4	6	15
Mi1	Mice faeces	gyrA	*B. subtilis*	3	0	5	8	15
Mi2	Mice faeces	gyrA	*B. subtilis*	2	5	4	11	15

[a]Activity was assessed using a colony diffusion assay on LB agar against 15 clinical isolates of ESBL-*E. coli*. A532 strain was kept to provide a negative control.

determined after 24 h. *Bacillus* titres increased after 24-h culture in the medium without inulin and remained steady in the medium with inulin (Figure 1a). However, after a 24-h culture, titres of *Bacillus* isolates in the medium without inulin showed a high inter-isolate variability, whereas they did not when cultivated with inulin.

After a 24-h culture without *Bacillus*, mean ESBL-*E. coli* titres increased in the medium with or without inulin. ESBL-*E. coli* titres after a 24-h co-culture with *Bacillus* without inulin were significantly different from the control for one *Bacillus* isolate (isolate Bac2, 7.4 ± 0.3 vs 8.1 ± 0.3 $\log_{10}$ CFU ml^{-1}, t test P-value, 0.04, Figure 1b). The combination of *Bacillus* and inulin was more effective, as 5 among 11 *Bacillus* strains significantly decreased ESBL-*E. coli* titres, the latter ranging from 3.7 ± 0.5 to 4.8 ± 1.0 $\log_{10}$ CFU ml^{-1}. Regardless of the culture conditions, the commercial *B. subtilis* strain (CU1) did not show inhibition (Figure 1a, B). For subsequent *in vivo* experiments, we selected the 2 most active strains on ESBL-*E. coli* (*B. subtilis* CH311 and S3B). Of note, these strains also had strong in vitro activity against *S. aureus* (data not shown).

In vivo activity in a murine model of intestinal colonization by ESBL-*Escherichia coli*

Then, we assessed the activity of curative treatment with *Bacillus* spores on ESBL-*E. coli* faecal titres. First, we tested the *B. subtilis* S3B strain. Between the 2nd and the 5th day after starting treatment with *Bacillus* S3B (i.e. between 3 and 6 days post *E. coli* inoculation), fecal titers of *Bacillus* in treated mice exceeded those of control mice by 3.0 ± 0.2 $\log_{10}$ CFU g^{-1} (P-value <0.00001, Figure 2a). Although *Bacillus* titers decreased when *Bacillus* administration was discontinued, they were still higher than controls at 6 dpi (4.5 ± 0.2 $\log_{10}$ CFU g^{-1}). Germination

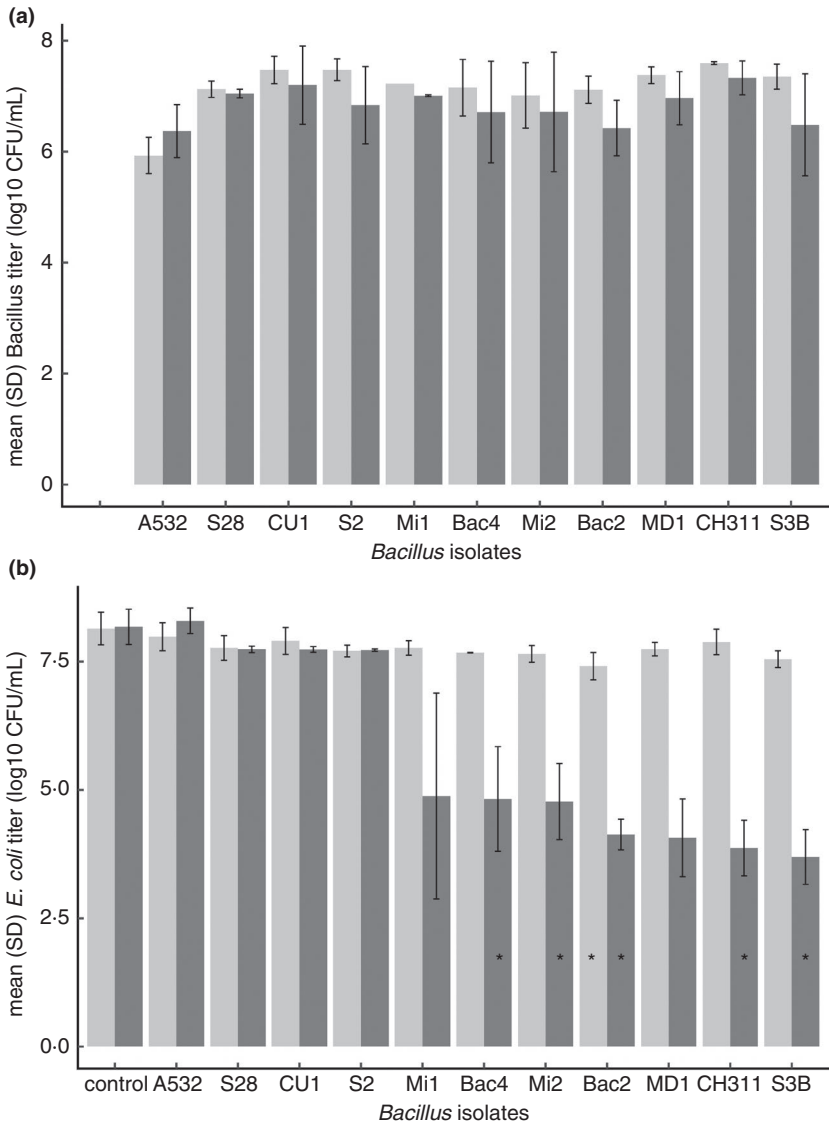

FIGURE 1 In vitro activity of *Bacillus* against ESBL-*Escherichia coli*. *Bacillus* (a) and ESBL-*E. coli* (b) mean titres after 24 hr cultivation with or without 0.2% inulin in a liquid medium containing murine native faeces (5 mg ml-1). Dark, with inulin; light, without inulin. Error bars, standard deviation. Stars show statistically significant differences with control

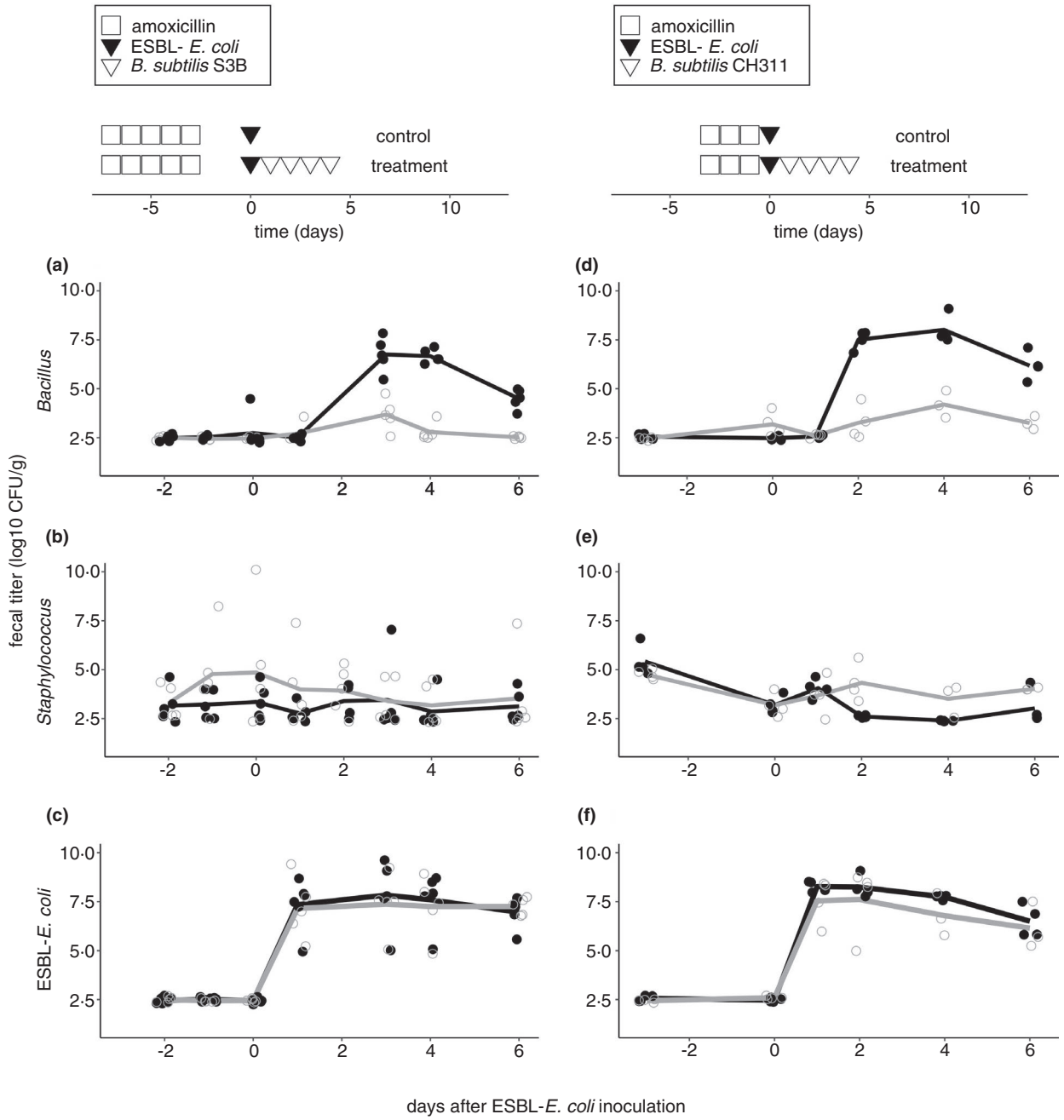

FIGURE 2 Effect of treatment with *Bacillus* on faecal titres of *Bacillus*, commensal *Staphylococci* and ESBL-*Escherichia coli*. Faecal titres of *Bacillus* (a and d), *Staphylococcus* (b and e) and ESBL-*E. coli* (c and f). Mice were treated with spores of *B. subtilis* S3B (left) or *B. subtilis* CH311 (right). The experimental design is shown at the top of the figure: mice were treated with amoxicillin before being inoculated with ESBL-*E. coli*. Spores of *B. subtilis* S3B and CH311 were inoculated from 1 to 3 dpi, and from 1 to 4 dpi, respectively. Grey lines and circles, control; black lines and circles, *Bacillus* treatment

rates of a *Bacillus* spores in faeces are reported in Table 2. During the same period, neither ESBL-*E. coli* or commensal *Staphylococcus* titers were significantly altered (differences with control, -0.2 ± 0.7 $\log_{10}$ CFU g^{-1}, P-value, 0.82; and -0.3 ± 0.7 $\log_{10}$ CFU g^{-1}, P-value 0.64, respectively, Figure 2b and 2c).

Second, we tested the activity of *B. subtilis* CH311. Fecal titers obtained during and after treatment with *Bacillus* CH311 were slightly higher than those with *Bacillus* S3B. Indeed, between the 1st and the 5th day after starting treatment with *Bacillus* CH311, *Bacillus* fecal titers were higher by 3.7 ± 0.4 $\log_{10}$ CFU g^{-1} in comparison with control mice (P-value <0.00001, Figure 2d). Meanwhile, *Staphylococcus* faecal titers were lower by 1.3 ± 0.3 $\log_{10}$ CFU g^{-1} (*p*-value <0.00001; Figure 2e). However, ESBL-*E. coli* titers were not significantly altered by *Bacillus* treatment (difference with control, -0.5 ± 0.6 $\log_{10}$ CFU g^{-1}, P-value, 0.42, Figure 2f).

Genome sequencing of *S3B* and *CH311* strains

Genomes of S3B and CH311 strains were sequenced in order to (a) determine whether they are novel strains and (b) try to identify the genes responsible for the strong *in vitro* inhibitory activity against *E. coli*. Comparative genomics statistics are summarized in Table 3. To specify the relationship between the isolates, we performed a pangenomic analysis of the 405 *B. subtilis* genomes. We predicted a total of 894 orthologous genes representing the core genome (genes shared by all strains) and 46,942 orthologous genes and singletons representing the accessory genome (genes encoded in one or more isolates, but not in all). Strain CH311 shared 86% of gene content with strain GXA-28 and 85% with strain H19. The strain S3B shared 97% of gene content with strains MSP1 and MSP5. The strains CH311 and S3B shared 83% of their gene content. We further analysed SNPs in the core genome and detected 38,016 polymorphic sites between all *B. subtilis* genomes. For strain CH311 we detected 1,521 SNPs with the closest strain H19. For strain S3B we detected 140 SNPs with the closest strains MSP1 and MSP5. We also detected 2,198 SNPs between strains CH311 and S3B. Altogether, phylogenetic and comparative genomic analyses demonstrated a polyclonal population, suggesting that CH311 and S3B are phylogenetically distinct from other strains (Figure S1 available as supplementary data).

We then searched 50 AMCs encoding genes (Caulier et al., 2019; Sumi et al., 2015) within the annotated genomes of the two newly identified CH311 and S3B *B. subtilis* strains. Nine different AMCs were detected in both strains, including four genes reported to have a mild inhibitory activity against gram-negative bacteria (Table 4). Of note, these four genes have also been reported in CU1 genome. Then, we pursued this analysis, and all peptides with unknown function were submitted to the software Deep-AmPEP30 (Yan et al., 2020) to predict potential new peptides with anti-microbial activities. As presented by

TABLE 2 Percentage of spores and vegetative cells of *Bacillus* in faeces

Isolate	CH311	S3B
Total cells, CFU/g	$2.4{\cdot}10^6 \pm 1.8{\cdot}10^6$	$1.2{\cdot}10^5 \pm 8.2{\cdot}10^4$
Spores, CFU/g (%)	$1.4{\cdot}10^6 \pm 1.4{\cdot}10^6$ (51% ±12%)	$5.6{\cdot}10^3 \pm 3{\cdot}10^3$ (5% ±2%)
Vegetative cells, CFU/g (%)	$1{\cdot}10^6 \pm 5{\cdot}10^5$ (49% ±12%)	$1.2{\cdot}10^5 \pm 7.9{\cdot}10^4$ (95% ±2%)

Faeces were sampled in mice treated with *B. subtilis* S3B and CH311 3 and 2 days after inoculation of ESBL-*Escherichia coli*. *Bacillus* cells were enumerated in faeces before (total cells) and after 20 min heating at 80°C (spores). Vegetative cells were estimated as the difference between total cells and spores.

TABLE 3 Genomic and comparative genomic statistics

	CH311	S3B
Genome properties		
Genome size (bp)	4,190,428	4,038,907
Number of contigs	5	6
Total genes	4,377	4,138
Protein-coding genes	4,209	3,995
Comparative genomics		
Number of unique genes	48	8
Closest genomes (number of SNPs; % of shared orthologous genes)	H19 strain (1,521 SNPs; 85.8%)	MSP1 and MSP5 strains (140 SNPs; 97, 4%)
Genome annotation		
Number of genes with unknown function	712	552
Number of predicted antimicrobial peptides with Deep-AmPEP30, product probability >0.7	5	9
Predicted antibiotic resistance genes (Phenotype)	*mph*(K) gene (spiramycin, telithromycin) *aadK* gene (streptomycin) *tet*(L) gene (doxycycline, tetracycline)	*mph*(K) gene (spiramycin, telithromycin) *aadK* gene (streptomycin)

TABLE 4 Genes associated with antimicrobial compounds in three strains of *Bacillus subtilis*

	Inhibition spectrum			Gene detected in strain		
Antimicrobial compound	**Gram-positive bacteria**	**Gram-negative bacteria**	**Fungi**	**CU1**	**CH311**	**S3B**
Subtilin	+		+			+
Subtilosin-A	+	+		+	+	+
Surfactin	+		+	+	+	+
Bacilysin	+	+	+	+	+	+
Bacillaene	+	+		+	+	+
Plipastatin			+	+	+	+
Bacillibactin			+	+	+	+
Kanosamine			+		+	+
Bacilysocin		+	+	+	+	+

Note: Ribosomal peptide is indicated in bold character. +, target of the antimicrobial compound (AMC) or gene coding for peptide or AMC-synthesising enzyme.

the Table 3, we found, respectively, 5 and 9 peptides in the genomes of CH311 and S3B with a probability above 0.7. All of them are different but 4 predicted peptides from CH311 genome are also present in the CU1 genome. Thus sequences of putative AMPs only found in CH311 and S3B are shown in the Figure S2 (available as supplementary data).

DISCUSSION

From our environmental sample screening, we isolated new *Bacillus* strains. About 10% of them displayed a strong *in vitro* activity against ESBL-*E. coli* not only in the colony diffusion assays but also in conditions that mimic faecal microbiota. Thus, most of the collected strains of *Bacillus* did not show such bactericidal activity, as did the reference CU1 *Bacillus* strain and in agreement with previous reports (Caulier et al., 2019).

The in vitro activities of the newly isolated *Bacillus* in gut mimicking conditions were strongly related to the presence of inulin, albeit *Bacillus* grew well in faeces without this oligosaccharide. However, without addition of these *Bacillus*, inulin 0.2% alone was not able to decrease ESBL-*E. coli* titres in a suspension of antibiotic naïve mouse faeces. The in vitro efficacy of *Bacillus* with inulin on ESBL-*E. coli* may be mediated by the fermentation of inulin by *Bacillus*, production of short-chain fatty acids (SCFAs) and colonic acidification. Indeed, *Bacillus* produces SCFAs, mainly acetate, and the combination of pH under 5, 75 and SCFAs proved to be deleterious for *E. coli* survival (Nakano et al., 1997; Sorbara et al., 2019). However, this mode of action was not predominant in our experiments, as pH after a 24-h co-culture ranged between 6 and 7 (data not shown). Alternatively, inulin may induce the synthesis of AMCs by *Bacillus* as it was shown for the antifungal compound bacillomycin (Qian et al., 2015). It is, thus, likely that the in vitro bactericidal activities of *Bacillus* were due to the production of AMCs.

Despite using inulin and the strains S3B and CH311 that exhibited the most potent in vitro activity, we could not decrease ESBL-*E. coli* faecal titres in a mouse model of intestinal colonization. Several causes could explain this result. First, inulin concentration in the gut was not assessed and we cannot affirm that it was high enough to induce the activity of *Bacillus*. Second, the intestinal transit time of the mouse is as short as a few hours, which quickly eliminates this genus of the intestinal flora, as shown by the rapid decrease of *Bacillus* titres in our mice faeces. Third, although the *Bacillus* strains proved to germinate at quite high level, they are subjected to harsh environmental conditions including low oxygen tension and high faeces concentration. Inhibitors may reduce growth and/or gene expression of *B. subtilis* in comparison with what we observed in our in vitro experiments. As the bactericidal activity of *Bacillus* on *Staphylococci* was previously described in mice gut (Piewngam et al., 2018), we tested it as a control of the metabolic activity of our strains in these conditions. Activity against endogenous *Staphylococci* was found for the CH311 strain, but not for S3B, suggesting that AMCs are differentially expressed in the gut. This result suggests that the lack of activity of *Bacillus* in our murine model is likely to be caused by insufficient expression/activity of the AMCs.

Genome sequencing of CH311 and S3B showed that these strains potentially expressed at least nine AMCs. Four of these AMCs are reported to have inhibitory activity against gram-negative bacteria, but these effects are often described as mild, and all of them are also potentially produced by the CU1 strain that showed no in vitro

activity against *E. coli*. Only two compounds, subtilin and kanosamine, were found in CH311 and/or S3B and not in the CU1 strain but they have no activity against gram-negative bacteria (Caulier et al., 2019; Sumi et al., 2015). Therefore, it was not possible to attribute the inhibitory activity against ESBL-*E. coli* to any of these compounds, although it cannot be excluded that it could be due to different levels of gene expression of the gram-negative-active AMCs. In addition, some *Bacillus* AMCs likely remain unidentified and those that have been detected using DeepAmPEP30 must be investigated. Thus, further biochemical analyses are required to identify the origin of the bactericidal activity of these new strains of *Bacillus* against *E. coli*.

In conclusion, we described two new strains of *Bacillus* exhibiting an in vitro activity against ESBL-*E. coli*. Further studies are needed to elucidate their mechanisms of action and to assess if they could be used to treat infections or decrease colonization due to MDR *E. coli*.

ACKNOWLEDGEMENT

This work was supported in part by the SATT Ouest valorisation (France).

We thank Maxime Naour, Ibtissem Ben Brahim, Melissa Riffaut and Sarah Luca for their excellent technical help.

CONFLICT OF INTEREST

No conflict of interest declared.

AUTHOR CONTRIBUTION

Substantial contribution to conception and design or the acquisition and analysis of data: Murad Ishnaiwer, Yihienew Bezabih, François Javaudin, Mohamed Sassi, Eric Batard and Michel Dion. Drafting or critically revising the manuscript: Murad Ishnaiwer, Yihienew Bezabih, Eric Batard, Pascale Bemer, François Javaudin, Mohamed Sassi and Michel Dion.

ORCID

Mohamed Sassi https://orcid.org/0000-0002-0527-1171
Michel Dion https://orcid.org/0000-0002-5443-1362

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Ishnaiwer, M., Bezabih, Y., Javaudin, F., Sassi, M., Bemer, P., Batard, E. & et al. (2021) In vitro and in vivo activity of new strains of *Bacillus subtilis* against ESBL-producing *Escherichia coli*: an experimental study. *Journal of Applied Microbiology*, 00, 1–10. https://doi.org/10.1111/jam.15329

Supporting information

Figure S1: (A) Phylogenetic tree of 405 strains of *B. subtilis*. The 405 *B. subtilis* genomes were submitted to CSIphylogeny (https://cge.cbs.dtu.dk/services/CSIPhylogeny/). The core genome polymorphic sites were retrieved and phylogenetic tree constructed using Fasttree. (B) Phylogenetic tree of 10 closest *B. subtilis* genomes to S3B and CH311. According to core genome SNPs analysis, 10 closest genomes were selected for phylogenetic analysis Maximum likehood (ML) phylogeny was constructed using iqtree2 with 100 non-parametric bootstrap iterations (Nguyen *et al.* 2015)

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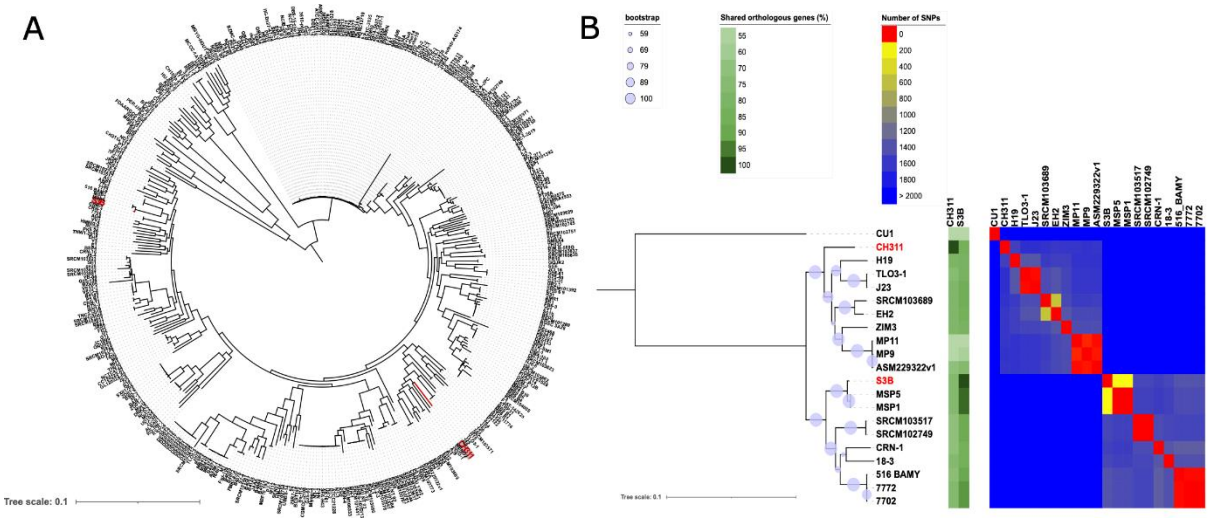


Figure S2: Sequences of putative antimicrobial peptides of strains CH311 and S3B that were not present in the strain CU1, as determined by the software Deep-AmPEP30

CH311

MTIYEQIKDALKNKINELVSPQEVKKTLQEKYGTNPDSIILSDYCYNRYNKGISFNKHLFEYMN
RSSYKYLGENSLYTGLIFRKSKGEDKEVIVGEWVNGVKSLREASVTNNQINDQAEIISKEQLV
NLYNEYNQILR

S3B

1. MIQYASESINLPGEIAFKDVREIFFYQIAKISCFYFLLFCAIFAAVNFINGWPRIVYGSDA
LNLFMSMLIIVMSVLFTLLLLLLLYVKFSRAYKKNERMKSKRTYTLNQEGIRICSKKYDLIFNW
NEITAVFEYKNIFRINTSSSQYIAIPKHFFHSEVEMNRFKEIILKNTETKKLKFKKDQH
2. MKILGVTGVILICLLAISVLMDMLQGFSLTKAVYNNMSSFKMTTFAEWVVILFFVLVLV
REMYVIYKSKKKNP
3. MTEKKQQNKPNENPEHNDLTDPIPNEELKENMNDEKHKRQQRDNSQSERDYDTK
4. MFRIFKMSFAVIIIILALIAFNYTEHTSVIQSVMLVFLGAVMFMQGLEERKKENDGSGA
FNIYTAVFVWSVSLIGFTLHII
5. MVREAAMLHIKEGLEQEFEDAFRQAAPIISGMKGYITHSLSKCMEETHKYLLLVEWE
TLEDHTEGFRGSSEYQEWKALLHRFYTPFPTVEHFQDV
6. MLQTPIGRLRTMGFIEGMSLLILLFIAMPLKYWAGLPLAVTIVGSVHGGLFILYLLVLAY
ATFSVKWPLKWSAAGFVAAFVPFGNFLYDRGLRKYK
7. MNYKGITLLCVMMLLFSAIASFPVSAQAKDQDAGILIIYSTLDGKESSQVKMLDLLAG
HFTSHVTVKKDLDVEASDFKGKDHVIYYGQTKRKLSKKLLSLISGVKNPVVAIGYNAGQINQF
SGLSLARKENVFQVHSRSEKADVSLESGLNVLSISGLKGTALYTFKADEGTTHSFIWKTKKG
NVYIGLTNLLNDNLIVAKQLREAFGEKAGTTLLYLRLEDISPMSDEKLLLQAGTYLHKRHIPFIL
AVIPVYLNPETGDKVYLSDKPKMVKVLKKLQSMGGSIIVHGYTHAYRYSETGEGFEFWDAK
ADQPITSGNAEDPPSILEKEQDFPNEQAYHSYLEPFREKEETYTKQKLTRAIEDLTSSGLYPL
AFEAPHYTMSEYGYQIASQYFTSIFGQVQLSSTTWKTSGAPPFVTAPSMLHGMTLYPETIGF
VDTSKQNPLGEMEERISQMIDFEGGVAGGFYHPYLGMKYLPELVDQMERIPDSEWLDLKKT
KQTVKTDKVEIHTSGDGTIQVKNGVSPIDEFFDHHRQTPLEKALWILSAVVLLFVIMFVSYTFY
LRATLKKRIFKERRSLG
8. MLSFLVSLVVAIVIGLIGSAIVGNRLPGGIFGSMIAGLIGAWIGHGLLGTWGPSLAGFAI
FPAIIGAAIFVFLLGLIFRGLRKEAMPWSMKDYPASLKNLEKPVRKKAIDIANAMIDEGYEEGR
AIPIATSKAKEWAENASTDEIDDFLTHDDETERDADPSSGSGPELMNKAEHVIKHKKGWAVK
AEGAKRVSEIKDTKKEAIERAKEIAAHKGIEVIVHLADGSVQRKIKTGS

Manuscript n°1: Microbiota composition influences the effect of the combination of inulin and pantoprazole on intestinal colonization by multidrug resistant *E. coli* in mice with amoxicillin-induced dysbiosis

Murad Ishnaiwer, Quentin Le Bastard, Emmanuel Montassier, Eric Dailly, Maxime Naour, Ivan Rychlik, Eric Batard, and Michel Dion

IICiMed UR1155
Institute for Health Research 2 (IRS2)
Nantes Université

ABSTRACT

Background: By altering intestinal microbiota, inulin may limit the level and/or prevalence of intestinal colonization by multidrug-resistant *Enterobacterales*. Our objective was to assess the efficacy of inulin and pantoprazole to prevent the intestinal colonization by ESBL producing *E. coli* (ESBL-Ec) in mice with amoxicillin-induced intestinal dysbiosis.

Methods: After being exposed to amoxicillin, mice were inoculated with ESBL-Ec. Before ESBL-Ec inoculation mice were treated with inulin (administered either early or lately) and pantoprazole, pantoprazole alone or inulin alone. The effect of treatment was assessed on ESBL-Ec fecal titers. Microbiome was assessed by quantitative PCR and shotgun metagenomic sequencing.

Results: The early inulin-pantoprazole (EIP) combination reduced ESBL-Ec fecal titers by 0.7±0.3 $\log_{10}$ CFU/g and the effect increased from 1 to 8 days post-inoculation (dpi) by 0.3±0.1 $\log_{10}$ CFU/g/day. Late inulin and pantoprazole (LIP) reduced ESBL-Ec fecal titers by 1.2±0.3 $\log_{10}$ CFU/g more than EIP. Pantoprazole and inulin alone had no or litle effect on ESBL-Ec. QPCR and shotgun sequencing identified 5 and 26 taxa that predicted the efficacy of the EIP combination, respectively, including *Adlercreutzia caecimuris and A. muris*. Preventive treatments with *A. caecimuris* or *A. muris* reduced ESBL-Ec fecal titers. Mice colonized at high level were enriched in tetracycline antibiotic resistance genes (ARGs). Conversely, mice with low colonization were enriched in beta-lactam, kasugamycin, aminoglycoside, fosmidomycin and vancomycin ARGs. Relative abundancy of beta-lactam ARGs was significantly correlated with fecal beta-lactamase activity (Spearman r, 0.55, P-value, 0.05) and with AUC of ESBL-EC fecal titers (Spearman r, -0.73; P-value, 0.005).

Conclusion: The combination of pantoprazole and inulin decreased fecal colonization by ESBL-Ec. Among other taxa, *Adlercreutzia caecimuris* and *A. muris* were predictive of treatment efficacy, decreased fecal colonization and should be combined as potential probiotics with inulin and pantaprazole. Increased beta-lactam ARG was associated with subsequent lower colonization by ESBL-Ec.

1 INTRODUCTION

The gut is the main reservoir of multidrug resistant *Enterobacterales* (MDR-EB). Intestinal colonization by MDR-EB promotes various infections including urinary, gastrointestinal and bloodstream infections, and contaminates the environment and/or healthy or diseased subjects (Feehan & Garcia-Diaz, 2020). Different non-antibiotic approaches such as probiotics and fecal microbiota transplantation have been assessed to treat or prevent MDR-EB intestinal colonization in mice and humans, but treatments that are both effective and easy to administer remain to be identified (Ishnaiwer et al., 2022; Ljungquist et al., 2020; Saha et al., Dall et al., 2019; 2019; Gopalsamy et al., 2018; Singh et al., 2018; Tannock et al., 2011). Hence, there is an urgent need to validate new treatments of intestinal colonization by MDR-EB.

Prebiotics are nondigestible carbohydrates. They modulate the composition of gut microbiota and induce colonization resistance against enteropathogenic bacteria (Ishnaiwer et al., 2022; Rastall, 2010; Rastall & Gibson, 2015; Roberfroid, 2007). Inulin, one of the most commercialized prebiotics, originates from plant roots such as onion, chicory and Jerusalem artichoke (Mensink et al., 2015). Its structure consists of 10 to 60 units of β-D-fructosyl subgroups linked together by (2→1) glycosidic bonds which is not digested by gut enzymes (Mensink et al., 2015). Inulin is selectively fermented by certain intestinal bacteria in healthy mice, and induces the production of short chain fatty acids (SCFAs) in the colon, including butyrate, acetate, succinate and propionate (Bäckhed et al., 2015; Baxter et al., 2019; Igarashi et al., 2020; Parada Venegas et al., 2019). The most common SCFA producing bacteria of the intestinal microbiota are *Bacteroides* (Chijiiwa et al., 2020), *Muribaculaceae* (Ormerod et al., 2016), *Eubacterium rectale*, (Wang et al., 2019)*, Lactobacillus* (Ai et al., 2021; Li et al., 2020; Pan et al., 2009) , *Bifidobacterium* (Li et al., 2020; Parada Venegas et al., 2019), *Akkermansia* (Parada Venegas et al., 2019; Zhu et al., 2017), *Allobacullum*, *Dubosiella* (Ai et al., 2021; Li et al., 2020), and most of them are stimulated by inulin. SCFAs trigger colonic acidification, boost the gut barrier function, modulate bacterial metabolism and stimulate the host inflammatory and immune responses (Parada Venegas et al., 2019; Sorbara et al., 2019). SCFAs also induce secretion of antimicrobial compounds against various pathogens (Bosscher et al., 2006), and reduce endotoxemia and colitis inflammation (Li et al., 2020; Wang et al., 2019). Higher colonic content in SCFAs has been associated with protection with intestinal colonization by MDR-EB (Sorbara et al., 2019). Inulin also inhibits enteric infections by *Candida albicans*, *Listeria monocytogenes, Salmonella typhimurium* and enterotoxigenic *Escherichia coli* (Buddington et al., 2002; Fuhren et al., 2021). Inulin promotes the growth of genera reported to be lower in mice that are susceptible to colonization by MDR-EB, including *Sutterella* (Grégoire et al., 2021; Li et al., 2020) and *Akkermansia* (Juhász et al., 2021; Wang et al., 2019; Zhu et al., 2017). Inulin also decreases intestinal titers of *Streptococcus*, that is reported to be more abundant in mice colonized by MDR-EB (Juhász et al., 2021; Li et al., 2020; Sasaki et al., 2019). Hence, by altering intestinal microbiota, inulin may limit the level and/or prevalence of intestinal colonization by MDR-EB.

In this study we tested the efficacy of inulin to prevent the intestinal colonization by an ESBL-Ec in mice with amoxicillin-induced intestinal dysbiosis. In our model, pantoprazole was used to decrease gastric acidity and promote the colonization by ESBL-Ec (Ishnaiwer et al., 2022; Stiefel et al., 2006). We found that inulin decreased the level of ESBL-Ec colonization in mice also treated with pantoprazole, but inulin had no activity in mice that did not receive pantoprazole. We also identified taxa predictive of inulin efficacy.

2 MATERIALS AND METHODS

2.1 Bacterial strains and media

Inulin sourced from chicory roots was purchased from Bulk Powders; 0,2 % (w/w) solution was prepared by dissolving inulin powder in distilled water before autoclaving. A clinical isolate of ESBL-Ec was collected from University Hospital of Nantes (Ishnaiwer et al., 2022. ESBL-Ec enumeration was realized on ChromID^{TM} ESBL agar plates (Biomerieux). *Adlercreutzia* strains were grown under anaerobic conditions of N_2-CO_2 (80:20) on supplemented BHI medium (Oxoid) with glucose 0.4% (Braun), cysteine 0.05% (Sigma-Aldrich), and yeast extract 0.04% (Biokar) (Danylec et al., 2020).

2.2 Murine model of inulin effect against ESBL-Ec intestinal colonization

All experiments were approved by the Animal Experiment Committee of Pays de la Loire (France, authorization number APAFIS#18120) and ARRIVE Guidelines were followed. Six weeks old male Swiss mice (Janvier Labs, Saint-Berthevin, France) were housed individually to avoid inter-individual contamination, in pathogen free conditions with free access to food and water. Intestinal microbiota was first altered by amoxicillin administration in drinking water (0.5 g·L^{-1}) for 3 days to make mice susceptible to ESBL-Ec colonization. Inulin (0.2%) was administered in drinking water, either 5 or 2 days before inoculation (respectively, early and late treatment with inulin). In some mice, pantoprazole was added to drinking water (0.1 g·L^{-1}) on the first day of amoxicillin, because it is known to suppress the gastric acid production and to promote intestinal colonization by MDR bacteria ((Stiefel et al., 2006). Two days after amoxicillin discontinuation, ESBL-Ec was intragastrically inoculated (10^6 CFU per mouse). Mice were monitored for 8 days and feces were serially collected.

Mice were randomly divided into 5 groups (Figure 1A): control (n=19); early inulin (n=10); pantoprazole (n=11); early inulin and pantoprazole (EIP) (n=12); late inulin and pantoprazole (LIP) (n=8).

Fecal samples were collected daily and then frozen at -80˚C until further use. ESBL-Ec enumeration from stool was performed with ~ 50 mg of daily collected mouse feces in 1 ml of sterile water which were shaken at 20 Hz (Mixer Mill MM 400, RETSCH's) for 5 min to make a homogenous suspension. ESBL-Ec fecal titers were assessed by daily culture from 0 to 8 days post-inoculation (dpi) and incubation at 37°C for 24 hr in aerobic conditions. Fecal samples were then frozen at -80˚C until further use.

2.3 β-Lactamase detection assay

Fecal pellets from mice were collected on the first day after amoxicillin discontinuation (i.e., 2 days before ESBL-Ec inoculation) and resuspended in 1ml of phosphate-buffered saline (PBS). Samples were left for 5 min to allow particulate matter to sediment. Next, 180 µl of the suspension were incubated 30 min at 37°C with 20 µl of Nitrocefin 0.5 mg/ml (Merck). Then the β-Lactamase activity was determined in the supernatant by absorption at 492 nm.

2.4 Amoxicillin fecal concentrations

Fecal pellets from mice were collected on the first day after amoxicillin discontinuation (i.e., 2 days before ESBL-Ec inoculation), weighed, resuspended and homogenized in water (10 mg/100 µL) by sonication. The material obtained was centrifuged (5 min, 13000 g, + 4 ° C). The supernatant was mixed with acetonitrile solution containing the internal standard ($^{13}C_6$ amoxicillin). After centrifugation of the mixture (5 min, 13000 g, +4°C), the supernatant was diluted in formic acid 0.1% v/v (1/2 v/v) and injected into the chromatographic system. The system consisted of a Kinetex® 2.6 µm C18 50 mm x 2.1 mm column (Phenomenex, Le Pecq, France) in a thermostatically controlled oven at 40°C, mobile phases with a binary gradient [(acetonitrile/formic acid 0.1% v/v) and (ultrapure water/formic acid 0.1% v/v)] at a flow rate of 0.5 mL/min and a tandem mass spectrometry monitoring (3200 QTRAP® Sciex, Villebon-sur-Yvette, France).

2.5 Microbiome analysis using qPCR

DNA from fecal samples (30 mg) were extracted with Nucleospin DNA stool kit (Macherey Nagel) and eluted in 30 µl of buffer. The following taxa were sought in 1 µl of these samples by qPCR using previously published primers: *Akkermansia muciniphila* (Collado et al., 2007), Bacteroidetes (Guo et al., 2008), *Muribaculaceae* (Remi Le Guern), *Bacteroides* (Manz et al., n.d.), *B. thetaiotaomicron, B. uniformis* and *B. ovatus* (Kollarcikova et al., 2020), Firmicutes (Guo et al., 2008), *E. coli* (Tillman et al., 2015), *Enterobacteriaceae* (Bartosch et al., 2004), *Enterococcus spp*. (Blake et al., 2019). For the amplification of Equol Producing Bacteria (EPB) of the *Coriobacteriaceae* family, we used the specific primers of *tdr* gene (Vázquez et al., 2017). Primers for the specific amplification of *Bacteroides acidifaciens* 5'-CGATGAAGACGGAAGAAGTGG (Baci3) and 5'-TTCAAGTTCATAAAGCTCATCATTC (Baci4) were designed as follows: *B. acidifaciens* genomes were acquired from NCBI public database. Coding sequences (CDSs) from these genomes were clustered together by CD-HIT v4.8.1 (Fu et al., 2012) at a 95% nucleotide identity threshold in global alignment. Genes that were present in all genomes were taken for further analysis. CDSs of other species (off-targets) were downloaded from the NCBI. Conserved genes from *B. acidifaciens* were clustered with the genes from most similar species at the 80% nucleotide identity threshold. Sequences that clustered together with off-targets were discarded. The target gene was selected and searched for dissimilarity by BLAST in NCBI. Primers were designed on the NCBI web server using Primer-BLAST (Ye et al., 2012) and checked for homo- and heteroduplexes using the OligoAnalyzer® tool from Integrated DNA Technologies, Inc. Those for *Duncaniella muris* 5'-TCACCATCCGTGAGATGCCTCC (Dunca-f), 5'- ATAGAGGAAAGCCGCCCAGCAG (Dunca-r) and *Adlercreutzia caecimuris* 5'-AGTCACGCACCCCCGTATTCTC (ACA-f), 5'-CGCGCCATTCGATGATGCTTCC (ACA-r) were designed by comparing the genome of *D. muris and A. caecimuris* with the genome of 25 bacteria specially selected for the identification of unique sequences. We selected bacteria affiliated or not with the same taxonomic genus in order to achieve the widest possible analysis. All bacterial genomes were

acquired from NCBI public database. These genomes were used to develop a BLAST database and then to compare genomes using the shell command Bash « blastn » (https://github.com/ncbi/blast_plus_docs). The unique sequences were retrieved and quality control was performed using the NCBI BLASTn and Uniprot BLAST tools. The primers have been designed by the Eurofins Genomics webserver «PCR Primer Design Tool» (https://eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/). The selection criteria for the primers were as follows: a melting temperature close to 60°C, a percentage of GC greater than 50% and an amplicon length between 300 and 500 bps. Their specificity were controlled by PCR amplification from fecal DNA and sequencing of the amplicon. Briefly, the PCR reactions were performed in a total volume of 20µl. All taxa were detected by using the PowrUp Sybr Green Master Mix (Life Technologies), with 10 pmol of each of the forward and reverse primers for each reaction. Most of the PCR reaction conditions were 94°C for 3 min, then 40 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec. For the PCR of Bacteroides species, EPB, D. *muris* and *A. caecimuris* the conditions were the following: 94°C for 3 min, then 40 cycles of 94°C for 30 sec, 59°C for 1min. The calibrations were done with each PCR fragment amplified from the respective strains and whose concentration was adjusted to 5.10^6 molecules/µl. The specificity of each primer pair was verified by controlling that only one band of the expected size was obtained on a 2% agarose gel after amplification from the fecal DNA and that its sequence was as expected. The titers of each bacteria was inferred from the shift of the threshold cycle (CT), obtained by amplifying target from the fecal DNA in comparison to that of reference DNA. Corrections were made when the target was the 16S RNA gene since it is differently repeated in the bacterial genomes.

2.6 Metagenomic analysis

Fresh stools samples were immediately frozen and stored at −80 °C until DNA extraction. The DNA was then extracted using the PowerSoil Pro HTP (Qiagen Inc., Venlo, The Netherlands) following the manufacturer's protocol. Sequencing was then performed using NovaSeq S1 150PE platform at the University of Minnesota Genomic Center. Raw sequences were filtered and trimmed using FastQC (v. 0.11.9) and Trimmomatic (v. 0.36) (Bolger et al., 2014). Metagenomic taxonomic classification was performed using Kraken2 (v. 2.1.2) and Bracken (v. 2.5.0) against the mouse specific MGBC database (release 2.0) (Beresford-Jones et al., 2022; Lu et al., 2017; Wood et al., 2019)

2.7 Resistome analysis

Analysis of antibiotic resistance gene content. For ARG quantification, ARG-OAP v.2.0 pipeline was used. (Yin et al., 2018). Subsampled FASTQ files were also processed with ARG-OAP v.2.0 to obtain the annotation of ARG profiles. ARG-OAP v.2.0 provides model-based identification of assembled sequences using SARGfam, a high-quality profile Hidden Markov Model containing profiles of ARG subtypes and including cell number quantification by using the average coverage of essential single-copy marker genes. We used ARG-OAP with default settings. ARG abundances were normalized by cell number. Each reference sequence was tagged with its functional gene annotation (ARG subtype) and membership within a class of antibiotics targeted by the gene (ARG type). The ARG-OAP pipeline includes an ARG database with curated and complete ARG sequences, improving the coverage of ARG detection.

2.8 Statistical analyzes

Median are reported with 1st and 3rd quartiles, and means with SD. Treatment efficacy at different time points was assessed through comparison of ESBL-Ec fecal titers using linear mixed effect models. Area under the curve (AUC) of ESBL-Ec fecal titers was calculated by the trapezoidal method between 1 and 8 dpi. Continuous variables were described by median (1st and 3rd quartile) and compared using the Mann-Whitney test. Dunnett test was used to compare qPCR results of effectively treated mice with untreated and ineffectively treated mice.
Alpha diversity analyses were performed using vegan R package (version 2.5-7) (Oksanen et al., 2020). The Shannon and observed-species biodiversity indices were computed and comparisons were performed using Kruskal-Wallis Wallis (KW) with Dunn's tests (Ogle et al., 2022). Beta diversity analyses were performed using Bray-Curtis distances in the vegan R package and were compared between groups with ANOSIM using vegan R package (version 2.5-7) (Oksanen et al., 2020). Relative abundances of species were compared using Kruskal-Wallis with Dunn's tests and p-values were adjusted using false discovery rate (FDR) and with generalized fold change from SIAMCAT (Wirbel et al., 2021). Taxa which were not present in at least 20% of the samples were removed. FDR-corrected p-value under 0.10 was considered significant. All statistical analyses were performed with R 3.6.3, R Foundation for Statistical Computing, Vienna, Austria.

3 RESULTS

3.1 In vivo activity of inulin and/or pantoprazole in a murine model of intestinal colonization by ESBL *E. coli*

A clinical isolate of ESBL-Ec was collected from University Hospital of Nantes (Ishnaiwer et al., 2022). Whole-genome sequencing was performed on this ESBL-Ec strain (Illumina technology). It displayed resistance to 3GCs through blaCTX-M-27, and to carbapenem through an OXA-181 carbapenemase. This strain belongs to the phylogroup B2 and is of serotype O25. It also contains the following virulence factors and resistance genes. (Supplementary material, Tables S1 and S2 respectively).
However, the efficacy of different treatments was assessed by comparing mean ESBL-Ec fecal titers in treated and control mice using a multivariate model between 1 and 8 dpi (**Supplementary material, Table S3**). Titers in the control group slightly decreased with time, by 0.2 ± 0.1 $\log_{10}$ CFU/g/day. Early inulin (i.e., administered from -5 dpi) initially increased titers (estimate, +0.9± 0.3 $\log_{10}$ CFU/g), but this effect was abolished by a significantly negative interaction between early inulin and time (-0.3± 0.1 $\log_{10}$ CFU/g/day), producing observed titers at 8 dpi in the control and early inulin group of 7.7±1.6 and 6.1±1.9 $\log_{10}$ CFU/g, respectively (**Figure 1**). Pantoprazole alone had no significant activity. The combination of early inulin and pantoprazole (EIP) showed a better efficacy than single treatments. Indeed, it significantly decreased ESBL-Ec titers by 0.7 ± 0.3 $\log_{10}$ CFU/g and the effect gradually increased over time by 0.3 ± 0.1 $\log_{10}$ CFU/g each day (**Figure 1c, Table S4**). Hence, mean observed titers in control and pantoprazole with early inulin at 1 dpi were 8.6±0.5 and 7.5±1.9 $\log_{10}$ CFU/g, respectively. At 8 dpi, they were 7.7±1.6 and 4.5±1.8 $\log_{10}$ CFU/g, respectively. The combination of late inulin and pantoprazole (LIP) significantly decreased ESBL-Ec titers by 1.2 ± 0.3 $\log_{10}$ CFU/g, with no significant interaction with time.

The same model was then applied to mice treated with either early inulin or the combination of early inulin and pantoprazole. In comparison with early inulin, the combination decreased ESBL-Ec titers by 1.6 ± 0.4 log_{10} CFU/g (P-value, < 0.001), without significant interaction between time and treatment.
Inspection of ESBL-Ec titers of each mice suggested that some treatments might be highly effective in some mice, and not effective in other mice. To support this hypothesis, we computed for each mouse the AUC of the time variation of ESBL-Ec titers between 1 and 8 dpi. AUCs ranged between 22 and 64 log_{10} CFU·day·g^{-1} (Supplementary Figure S1). AUCs were grouped in 3 classes (low, medium and high, defined as ≤ 39, between 39 and 50 or ≥50 log_{10} CFU·day·g^{-1}, respectively. Among 19 control mice, none had low AUC. Conversely, among 20 mice treated with pantoprazole with early or late inulin, 11 (55%) had low titers (comparison with control, P-value, < 0.001).

3.2 Quantitative PCR of fecal taxa

To assess the effect of amoxicillin on fecal microbiota, we analyzed using qPCR feces of control mice sampled just before starting amoxicllin (-5 dpi) and just before ESBL-Ec inoculation (0 dpi). Amoxicillin decreased significantly DNA fecal concentration and titers of Bacteroidetes, *Muribaculaceae*, *A. caecimuris*, *B. acidifaciens*, *B. ovatus*, *D. muris* and EPBs, and increased *Enterococcus* (**Table 1 and Supplementary figures S2 and S3).**
Then, we assessed the impact of each treatment on microbiota by comparing feces sampled just before ESBL-Ec inoculation (0 dpi) in control and treated mice. Notably, *Bacteroidetes, Bacteroides, B. acidifaciens, A. caecimuris* and EPBs were significantly increased both in EIP and LIP groups (**Supplemental material, Figure S3)**. *B. ovatus, B. thetaiotaomicron* and *E. coli* were also increased in EIP-treated mice, and *Akkermansia* was increased in the LIP group. Moreover, pantoprazole increased the abundance of *Bacteroidetes, Muribaculaceae*, *Duncaniella muris* and EPBs. Surprisingly, inulin alone induced no significant changes among tested taxa, including *Lactobacillus spp* and *Bifidobacterium spp* that have been previously reported to be increased by inulin (data not shown). In comparison with control, pantoprazole and EIP increased significantly the amount of DNA in feces (**data not shown**).
In order to identify taxa that may be associated with the efficacy of treatments on ESBL-Ec decolonization, we compared effectively treated mice with both ineffectively treated and untreated mice (**Table 2)**. Effective and ineffective treatments were defined by low and high AUCs of ESBL-Ec titers, respectively. In comparison with mice that were untreated or ineffectively treated with EIP, mice effectively treated with EIP had significantly higher DNA fecal concentration and higher titers of *Enterobacteriaceae, Muribaculaceae, Duncaniella muris, E. coli, A. caecimuris* and EPBs, and lower titers of *Enterococcus* (**Supplemental material, Figures S2, S4 and S5**). By contrast, mice effectively treated with LIP had only significant gain in *A. caecimuris* and EPBs when compared to control and ineffectively treated mice (**Supplemental material, Figure S5**). Only DNA titers and EPBs were significantly increased in mice that were effectively treated with pantoprazole, when compared to untreated and ineffectively treated mice (**Supplemental material, Figure S6**). This analysis could not be applied to mice treated with inulin alone, as we identified none of these mice as being effectively treated.
To complete this analysis, we estimated the total concentration of fecal bacteria by adding titers of Bacteroidetes, Firmicutes, *Enterobacteriaceae* and *Akkermansia*. Regardless of the treatment, the median total concentration of bacteria at 0 dpi was 10.0 (9.7 - 10.4) log_{10} CFU/g, and was significantly correlated to the AUC of the ESBL-Ec titers between 1 and 8 dpi (Spearman test P-value, 0.009).

Finally, to decipher the effect of inulin in pantoprazole treated mice, we compared mice treated with EIP and LIP with those treated with pantoprazole alone, regardless of their level of ESBL-Ec colonization. We found that EIP increased significantly *B. ovatus* and *B. thetaiotaomicron*, while LIP increased *Akkermansia*.

3.3 β-lactamase activity and amoxicillin concentration in the feces samples

Since β-lactamase producing bacteria have been reported to decrease amoxicillin fecal concentration (González-Bello et al., 2020) and prevent the effect of amoxicillin on the microbiota, we assessed β-lactamase activity and amoxicillin concentration in the feces sampled just after amoxicillin discontinuation (-2 dpi). Pantoprazole and EIP increased fecal β-lactamase activity (Dunnett test P-values, 0.05 and 0.004, respectively) and tended to decrease amoxicillin concentration (Dunnett test P-values, 0.08 and 0.07, respectively; **Supplemental material, figure S7A, S7B)**. These 2 parameters were significantly associated, independently of the treatment (linear regression estimate, -10±4; P-value, 0.02; **Supplemental material, figure S7C**). Furthermore, there was a significant association between the AUC of ESBL-Ec titers and either β-lactamase activity at -2 dpi (linear regression estimate, -4±1; P-value, 0.008) or amoxicillin fecal concentration (linear regression estimate, 0.2±0.1; P-value, 0.01), independently of the treatment (**Supplemental material, Figure S7D and S7E**). We found a significant correlation between β-lactamase activity at -2 dpi and DNA fecal concentrations 2 days later (**Supplemental material, Figure S7F**; Pearson ρ, 0.75; P-value, <0.001). There were also significant correlations between β-lactamase activity at -2 dpi and titers estimated by qPCR at 0 dpi for Bacteroidetes (Pearson ρ, 0.59), *Enterobacteriaceae* (Pearson ρ, 0.86), *Muribaculaceae* (Pearson ρ, 0.65), *Enterococcus* (Pearson ρ, -0.50), *Duncaniella muris* (Pearson ρ, 0.86), *E. coli* (Pearson ρ, 0.76), *A. caecimuris* (Pearson ρ, 0.55) and EPBs (Pearson ρ, 0.86, **Supplemental material, Figure S8**). Furthermore, 5 *Enterobacteriaceae* isolates randomly picked from feces sampled from 5 mice with low level of colonization by ESBL-Ec were resistant to amoxicillin.

3.4 Metagenomic analysis

Fecal microbiome composition was assessed using shotgun metagenomic sequencing in control mice (n=7, including 6 high and 1 medium level ESBL-Ec colonization) and mice treated with early inulin and pantoprazole (n=11, including 4, 1 and 6 high, medium and low level ESBL-Ec colonization, respectively).

Spectrum of fecal microbiome disruption

To understand how the fecal microbiome changed according to colonization status (i.e., high vs medium vs low colonization, n = 18 mice), we first used alpha diversity measures. The significance of change in alpha diversity was tested on a per species level basis using a Kruskal-Wallis test. Based on Shannon index and Observed Species alpha diversity indices, we observed a significant change between high medium and low colonized mice (KW test, p-value = 0.003 for Shannon index and p-value = 0.011 for Observed Species, **Supplementary material, Figure 9A & 9B**). Using Dunn's test, we found that the difference was only significant between low vs high colonized mice (Dunn's test, FDR p-value = 0.003 for Shannon index and FDR p-value = 0.009 for Observed Species).

Differences in fecal microbiome composition among samples can be visualized by means of the Principal Coordinates Analysis (PCoA), such that each point represents a single fecal sample. Similar samples are located relatively close to each other, and clusters of distinct microbiome compositions can be appreciated. Here, we found that samples from high, medium or low colonized mice had a significant different overall architecture (Bray-Curtis distance, PERMANOVA, p-value = 0.001, r^2 = 0.496; Kruskal-Wallis test, Principal Coordinate 1, p-value = 0.001; Principal Coordinate 2, p-value = 0.019, **Figure 2**). Along Principal Coordinate 1, we found that the difference was significant between high and low colonized mice (Dunn's test, FDR p-value = 0.0009), whereas, along Principal Coordinate 2, the difference was significant between medium and low colonized mice (FDR p-value = 0.028). Control and treated mice with high level of colonization were not different for alpha diversity measures (Shannon index, p-value = 0.5224; Observed Species, p-value = 0.2008). Moreover, their microbiome architecture was not different (PERMANOVA, r^2 = 0.08572, p-value = 0.534).

Predictive microbiome signature

We then tested differences between high, medium and low level of ESBL-colonization in control or EIP-treated mice at the species level, using a Kruskal-Wallis test. At a FDR level of 0.10, we identified a panel of 74 species that were differentially abundant between samples from mice who had high, medium or low colonization (**Supplemental material, table S4 and Figure S10**). We found that mice colonized at high level were enriched in relative abundance of 2 species including *Enterococcus faecalis.* Conversely, mice colonized at low level were enriched in 72 species, and more specifically in 28 species of the *Muribaculaceae* family (including *Duncaniella muris* and 3 other *Duncaniella spp*, 4 *Muribaculum spp* and 2 *Paramuribaculum spp*), 23 species of the *Lachnospiraceae* family (including 2 *Dorea spp*), 4 species of the *Enterobacteriaceae* family (including *E. coli, Klebsiella pneumoniae* and *Klebsiella variicola),* 4 species of the *Rikenellaceae* family (*Alistipes spp*) and 2 species of the Coriobacteriia class (*Adlercreutzia caecimuris* and Adlercreutzia_sp004793465).

Microbiome alteration in mice treated with early inulin and pantoprazole

When we subset the microbiome analysis to samples from mice treated with early inulin and pantoprazole (high level colonization mice, n=4; low level colonization mice, n=6), we confirmed the microbiome disruption between high and low colonized mice. Based on Shannon index and Observed Species alpha diversity indices, we observed a significant change between high and low colonized mice (KW test, p-value = 0.011 for Shannon index and p-value = 0.02 for Observed Species, **Supplementary material, Figure S11**). We also found that samples from high colonized mice had a significant different overall architecture compared to those with low colonization (Bray-Curtis distance, PERMANOVA, p-value = 0.008, r^2 = 0.473; Kruskal-Wallis test, Principal Coordinate 1, p-value = 0.011; Principal Coordinate 2, p-value = 0.831, **Supplementary material, Figure S12**). At a level of FDR of 0.10, we identified 26 species that were differentially abundant between samples from mice who had high and low level of colonization (**Supplementary material, Table S5**). We found that high colonized mice were enriched in relative abundance of species including *Enterococcus faecalis*, whereas low colonized mice were enriched in species among family *Enterobacteriaceae* (including *Klebsiella pneumoniae*, *Klebsiella variicola*, *Escherichia coli*, *Enterobacter himalayensis), Prevotella massilia*, 2 species among genus *Alistipes*, and 11 species among family *Muribaculaceae* (including *Duncaniella muris)*.

3.5 *Adlercreutzia* activity againt ESBL-Ec colonization

Quantitative PCR and metagenomic analyses showed that higher titers of EPBs of the Coriobacteriia class, as well as *Adlercreutzia caecimuris* and Adlercreutzia_sp004793465 were predictive of the efficacy of the combination of inulin and pantoprazole. *Adlercreutzia* was the only genus of the Coriobacteriia class identified by metagenomic analyses as discriminating mice with low, medium and high ESBL-Ec titers. Hence, we assessed whether treating mice with *Adlercreutzia caecimuris* or any EPB-producing *Adlercreutzia* would decrease the level of ESBL-Ec colonization. To isolate a murine isolate of EPB-producing *Adlercreutzia*, we selected a mouse with a high titer of EPBs at 0 dpi and subsequently colonized at low level by ESBL-Ec. Fecal samples were cultivated on a medium designed for isolate *Adlercreutzia* and added with colimycin 20µg/ml. Several colonies were tested by PCR with the EPB primers and the positive colony 1552 was further characterized. Its 16S RNA gene sequence gave 99% identity on 900bp with that of *Adlercreutzia muris* strain SP-7 (DSM 29508). A *tdr* gene fragment (100bp) sharing a 97% homology with that of *A. equolifaciens celatus* was also detected in this strain, which has not been previously reported for *A. muris*. We also purchased the *A. caecimuris* DSM 21839. In a new set of experiments, mice were treated with 7 $\log_{10}$ CFU of either *A. caecimuris* DSM 21839 or *A. muris* 1552. Shotgun metagenomic sequencing showed that *A. caecimuris* relative abundancy tended to be higher than 10^{-5} in mice treated with A. *caecimuris* whereas it was lower than 10^{-5} in control mice (**Supplemental material, Figure S13**). Furthermore, while Adlercreutzia_sp004793465 was undetectable in control mice, its relative abundancy was higher than 10^{-5} in mice treated with *Adlercreutzia muris*. In comparison with control mice, preventive treatments with *A. caecimuris* and *A. muris* decreased significantly ESBL-Ec titers by 2.0 (0.5) and 1.6 (0.5) $\log_{10}$ CFU/g, respectively (**Figure 3, TableS6**).

3.6 Resistome analysis

Fecal resistome was assessed in 7 control mice (including 6 and 1 high and medium level colonization) and 11 EIP treated mice (4, 1 and 6 high, medium and low level colonization, respectively). Fecal resistome alpha diversity was significantly higher in EIP treated mice than in control mice (ARG subtypes, KW test, p-value = 0.019 for Shannon index and p-value = 0.005 for Observed Species). Furthermore, resistome alpha-diversity was also higher in effectively treated mice (i.e., low level colonization) than in control mice (ARG subtypes, Dunn's test, FDR p-value = 0.046 for Shannon index, FDR p-value = 0.01 for Observed Species), and then in ineffectively treated mice (ARG subtypes, Dunn's test, FDR p-value = 0.028 for Shannon index, FDR p-value = 0.013 for Observed Species, **Supplementary file, Figure 14A and 14B**). We also found a significant difference in resistome architecture when comparing effectively treated mice with control and ineffectively treated mice (Bray-Curtis distance, PERMANOVA, p-value = 0.003, $r^2 = 0.51$; Kruskal-Wallis test, Principal Coordinate 1, p-value = 0.02; Principal Coordinate 2, p-value = 0.30). Along Principal Coordinate 1, we found that the difference was significant between high and low colonized mice (Dunn's test, FDR p-value = 0.02, **Supplementary file, Figure 14C**). We then tested differences between high, medium and low colonized mice at species level using a Kruskal-Wallis test. At a level of FDR of 0.10, we identified a panel of 6 ARG types and 73 ARG subtypes that were differentially abundant between samples from mice who had high, medium or low colonization (**Supplementary file,**

Figure 15 and Table S7). Mice colonized at high level were enriched in tetracycline ARGs. Conversely, mice with low colonization were enriched in beta-lactam, kasugamycin, aminoglycoside, fosmidomycin and vancomycin ARGs. The relative abundancy of 7 dominant families (defined as a mean relative abundancy higher 10^{-5}) was correlated significantly with beta-lactam resistance genes abundancy with a Spearman r higher than 0.7 or lower than -0.7: *Muricabaculaceae* (r, 0.85; *Desulfovibrionaceae,* (r, 0.75); *Enterobacteriaceae*, (r, 0.74); *Rikenellaceae*, (r, 0.71); *Oscillospiraceae*, (r, 0.70); *Lachnospiraceae*, (r, 0.70); *Enterococcaceae*, (r, -0.92, **Supplementary file, Figure 16**). Furthermore, relative abundance of beta-lactam ARG was correlated with fecal beta-lactamase activity (Spearman r, 0.55, P-value, 0.05) and with AUC of ESBL-EC fecal titers (Spearman r, -0.73; P-value, 0.005).

4 DISCUSSION

Here we showed that a preventive treatment with a combination of inulin and pantoprazole decreased ESBL-Ec fecal titers in a murine model of MDRO intestinal colonization with amoxicillin-induced dysbiosis, unlike inulin or pantoprazole alone. The treatment efficacy was highly variable among mice and could be predicted by the structure of microbiota just before ESBL-Ec inoculation.

In our model, amoxicillin induced a profound dysbiosis, as it decreased or tended to decrease DNA fecal concentration and titers of most of the taxa assessed by qPCR. As previously reported, amoxicillin also increased *Enterococcus* fecal titers (Ubeda et al., 2010). Unlike inulin alone, combinations of inulin and pantoprazole, and pantoprazole alone, partially restored the amoxicillin-altered fecal microbiota by increasing DNA fecal concentration and titers of various taxa that we subsequently found to be associated with activity on ESBL-Ec colonization. We initially used pantoprazole in our murine model to decrease gastric acidity and promote high level colonization in control mice, as previously reported (Stiefel et al., 2006). As the combination of early inulin and pantoprazole proved effective to decrease ESBL-Ec fecal titers, we tested the efficacy of each treatment alone. Inulin alone was not active, and pantoprazole had no or limited effect, indicating a synergy between pantoprazole and inulin.

By preventing amoxicillin-induced dysbiosis, pantoprazole favored microbiota resistance to the digestive colonization by the ESBL-Ec. Pantoprazole may decrease the susceptibility of microbiota to amoxicillin by stimulating efflux pumps, as previously described for tigecycline (Ni et al., 2014). Our results suggest another mechanism of action, as we showed that pantoprazole also increased fecal beta-lactamase activity and tended to decrease amoxicillin concentrations. Furthermore, higher beta-lactamase activity, higher relative abundance of beta-lactam resistance genes and lower fecal concentration of amoxicillin were predictive of lower subsequent level of colonization by ESBL-Ec. We therefore hypothesize that pantoprazole maintains the richness and diversity of the microbiota exposed to amoxicillin, including beta-lactamase-producing bacteria, which would decrease the concentration of amoxicillin in the gut lumen and attenuate the effect of amoxicillin treatment on the overall microbiota, thus contributing to the resistance of the intestinal microbiota to ESBL-Ec colonization. The effect of pantoprazole on intestinal microbiota remains insufficiently understood. For example, some studies found that PPI decreased alpha-diversity while others did not (Le Bastard et al., 2021). In agreement with our results,

a small-size study previously found that PPI use was associated with higher alpha-diversity in older inpatients treated with more than 2 antibiotics, suggesting that PPIs may prevent antibiotic-induced dysbiosis (O'Donogue 2014). These results should be confirmed.

Treatment with inulin had no effect on DNA fecal concentration and titers of more than 15 taxa tested by qPCR including *Lactobacillus* and *Bifidobacterium.* This result is consistent with the poor efficacy of early inulin on ESBL-Ec colonization, and highlights the fact that the effect of prebiotics on the microbiota of healthy mice should not be extrapolated to mice exposed to antibacterial treatments. Adding inulin to pantoprazole did not frankly alter microbiota in comparison to pantoprazole alone, within the limits of the taxa assessed by qPCR. EIP increased titers of *B. ovatus* and *B. thetaiotaomicron, although* only

B. ovatus being known to metabolize inulin. However, titers of these taxa remained relatively low (4 to 6 $\log_{10}$ CFU/g), and were not predictive of EIP efficacy. Other taxa able to use inulin (e.g., *Muribaculaceae*, *B. acidifaciens*, *Akkermansia*) were not increased in EIP treated mice in comparison with pantoprazole alone. More interestingly, 3 taxa (*Enterobacteriaceae*, *E. coli*, *A. caecimuris*) were predictive of efficacy in EIP without being increased in pantoprazole-treated mice, thus being potentially involved in inulin efficacy when combined with pantoprazole. Furthermore, *Enterobacteriaceae* sampled from feces of mice that subsequently displayed a low level of ESBL-Ec colonization were mainly resistant to amoxicillin. These results suggest that the combination of inulin and pantoprazole favored the growth of amoxicillin-resistant *Enterobacterales* that consequently decreased the amoxicillin concentration in feces, thus preventing the amoxicillin-induced dysbiosis and limiting the subsequent level of colonization by ESBL-Ec.

Preservation of the microbiota mediated by decreased concentration of amoxicillin in the digestive lumen is not a sufficient mechanism to explain the activity of EIP on ESBL-Ec colonization, as pantoprazole had no effect on ESBL-Ec titers whereas it altered fecal beta-lactamase activity and amoxicillin concentration. Indeed, we found 72 species whose high titer predicted the efficacy of EIP, including *Adlercreutzia caecimuris* and another *Adlercreutzia spp*. We confirmed the involvement of *Adlercreutzia caecimuris* and *Adlercreutzia muris* by showing that treatment with each of these species decreased level of ESBL-Ec colonization, in a stringent experiment where mice had no preventive treatment of amoxicillin-induced dysbiosis with pantoprazole and inulin. However, the highly variable efficacy of these treatments on ESBL-Ec colonization suggest the involvement of other taxa.

qPCR experiments also showed that higher titers of equol producing Coriobacteriia were predictive of treatment efficacy for EIP, LIP and pantoprazole alone. Equol is an isoflavonoid produced from dietary daidzein by several taxa, mainly of the *Coriobacteriaceae* family, including *Adlercreutzia equolifaciens* (Mayo et al., 2019). Equol inhibits the *in vitro* biofilm formation by carbapenem resistant *E. coli* (Kim & Eom, 2021). The primer that we used in this study was designed for the tdr gene involved in equol production in the Coriobacteriia class. We found this gene in the genome of the *A. muris* strain that we isolated from murine feces, but not in *A. caecimuris* DSM21839. Of note, among the 72 species that we found to be predictive of treatment activity, *Alistipes* and *Eubacterium* may be involved in equol production (Liang et al., 2020; Yu et al., 2008). These results support the involvement of equol in colonization by ESBL-Ec in our murine model. Of note, we can not exclude that assessment of microbiota composition at other times (either after of earlier before ESBL-Ec inoculation) could have identified other taxa associated with treatment efficacy.

Another mechanism of action of inulin, in combination with pantoprazole, may be the production of SCFA from inulin fermentation. Indeed, microbiota-produced SCFAs protect from digestive colonization by MDR-EB ((Sorbara et al., 2019). From taxa that were predictive of efficacy of EIP in our study, *Muribaculaceae* (Ormerod et al., 2016) including *Duncaniella muris* and *Alistipes* (Parker et al., 2020; Vacca et al., 2020) known to produce SCFA. which is not the case of *Adlercreutzia* (Maruo et al., 2008). We also found that *Akkermansia*, that produces SFCA, had higher titers in mice treated with LIP, but increased titers were not predictive of treatment efficacy.
On average, the combination of early inulin and pantoprazole had a moderate efficacy, as it reduced ESBL-Ec mean titers by ~ 3 $\log_{10}$ CFU/g at the end of study. More specifically, the treatment was highly effective in half of mice, in which it decreased ESBL-Ec titers to low level, while other treated mice remained as heavily colonized as control mice. This approach, in which we assume that a given treatment is not uniformly active on all mice, allowed us to identify taxa that predict treatment efficacy, hence being potentially used as probiotics in combination with inulin and pantoprazole. We found that *Adlercreutzia* is a good candidate to be associated with inulin and pantoprazole, but consortia with other taxa, including *Muribaculaceae* and equol producing bacteria, should be considered. Conversely, although higher titers of *Enterobacteriaceae* including *E. coli* and *Klebsiella* were predictive of EIP efficacy, using these taxa as adjunct probiotics to treat MDR-EB colonization should be considered cautiously considering the of transferable resistance from the MDR-EB to the probiotics.

Conclusion

The combination of pantoprazole and inulin decreased fecal colonization by ESBL-Ec in a murine model of amoxicillin-induced dysbiosis. Various taxa, including *A. caecimuris*, *A. muris, Muribaculaceae* and equol producing bacteria were found to be predictive of the treatment efficacy and should be considered as potential probiotic in combination with inulin and pantoprazole. These results open new insight for the future use of prebiotics and probiotics for the prevention of ESBL-Ec colonization. Further studies are needed to determine the ultimate applicability of this treatments to decrease the intestinal carriage of ESBL-Ec in humans.

5 Table titles and notes

5.1 Table 1. Fecal taxa in control and treated mice, assessed by quantitative PCR

	control	naive		early inulin with pantoprazole		late inulin with pantoprazole		pantoprazole		early inulin	
	median titer (log10cfu/g)	median titer (log10cfu/g)	P-value	median titer (log10cfu/g)	P-value	median titer (log10cfu/g)	P-value	median titer (log10cfu/g)	P-value	median titer (log10cfu/g)	P-value
Bacteroidetes	6.1 (5.8-7)	10.2 (10.1-10.4)	0.003	9.9 (9.5-10.3)	< 0.001	10.2 (9.8-10.3)	< 0.001	10.3 (9-10.4)	0.002	8 (6.1-8.6)	0.97
Firmicutes	9.4 (8.9-9.6)	10.3 (10.1-10.5)	0.069	9.3 (9.1-9.7)	1.00	9.7 (9.3-10)	0.63	9.3 (9.2-9.4)	1.00	9.8 (9.3-9.8)	1.00
Enterobacteriaceae	3.7 (3.5-4.8)	5.3 (5.3-5.5)	0.76	5.9 (4.1-8.5)	0.100	6.7 (4.4-6.8)	0.40	6.6 (6.5-7.1)	0.050	2.5 (2.5-2.5)	0.54
Muribaculaceae	5.5 (4.8-6.9)	10.3 (10.1-10.4)	0.005	9.4 (5.4-10.3)	0.099	8.8 (7.5-10)	0.069	9.2 (7.6-10)	0.024	4.2 (3.9-5.2)	0.80
A. caecimuris	2.5 (2.5-2.5)	7.9 (7.5-8)	< 0.0001	4.5 (2.5-6.5)	0.048	4.2 (2.5-7.6)	0.007	4.4 (2.5-4.6)	0.23	2.5 (2.5-3.1)	0.99
Akkermansia	3.5 (3.5-3.6)	4.6 (3.8-6.1)	0.20	3.4 (2.6-4.1)	1.00	6.8 (4.7-8.2)	< 0.001	3.1 (2.5-4.3)	1.00	3 (2.6-3)	0.96
Bacteroides	5.7 (3.9-6)	8.3 (8.1-8.7)	0.093	9.1 (8.4-9.6)	0.004	9.9 (8.4-10.3)	0.006	7.5 (5.8-10.1)	0.11	6.2 (4.5-7.9)	0.91
B. acidifaciens	4.3 (2.5-5.7)	8.3 (8-8.9)	0.024	8.2 (7.7-8.7)	0.019	9.6 (8.1-10.6)	0.002	6.8 (5.2-9.2)	0.087	3.3 (2.5-4.2)	0.98
B. ovatus	2.5 (2.5-2.7)	6.6 (6.6-6.8)	0.011	6 (4.6-7.9)	0.002	5.1 (3.7-6.4)	0.21	3.8 (3.2-4.4)	0.96	2.5 (2.5-2.5)	0.95
B. thetaiotaomicron	2.5 (2.5-2.5)	4.3 (3.6-4.8)	0.42	5 (3.6-6.4)	0.002	3.9 (2.5-5.1)	0.083	3.4 (2.5-3.6)	0.93	2.5 (2.5-3.1)	1.00
B. uniformis	4.6 (4-5.4)	6.8 (6.5-7.2)	0.31	4.6 (4.1-6)	1.00	6.5 (4.9-9.8)	0.10	5.5 (4-8.4)	0.80	4 (2.9-5.7)	1.00
Duncaniella muris	4.1 (3.7-6.3)	9 (8.6-9.2)	0.002	7.5 (4.5-8.3)	0.16	7.1 (5.6-7.9)	0.12	7.9 (7-8.4)	0.014	4.5 (4.4-5)	1.00
E. coli	2.5 (2.5-2.5)	4.4 (4.3-4.9)	0.056	4 (2.6-7.4)	0.002	3.8 (2.5-4.6)	0.20	2.5 (2.5-4.4)	0.60	2.5 (2.5-2.5)	1.00
Enterococcus	9.4 (8.9-9.6)	5.6 (5.3-5.8)	< 0.001	9.2 (8.1-9.7)	0.95	7.9 (6.1-9.4)	0.031	9 (8.6-9.2)	0.68	9.4 (9.1-9.6)	0.94
EPB	2.5 (2.5-3.4)	7.5 (6.9-7.7)	< 0.001	4.5 (2.6-7)	0.049	5.8 (4.3-7.2)	0.001	4.6 (4.1-6.5)	0.020	4.2 (4-4.6)	0.54

Note. Median fecal titers (1st and 3rd quartile) just before ESBL-Ec inoculation (0 dpi), except for feces of naive mice that were sampled just before starting amoxicillin (-3 dpi). P-values, comparison with control group (Dunnett test). The primer used for *Enterobacteriaceae* is an approximation for the former *Enterobacteriaceae* family as it was used to assess the combined titers of *E. coli*, *Shigella* spp and *Salmonella* spp. *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

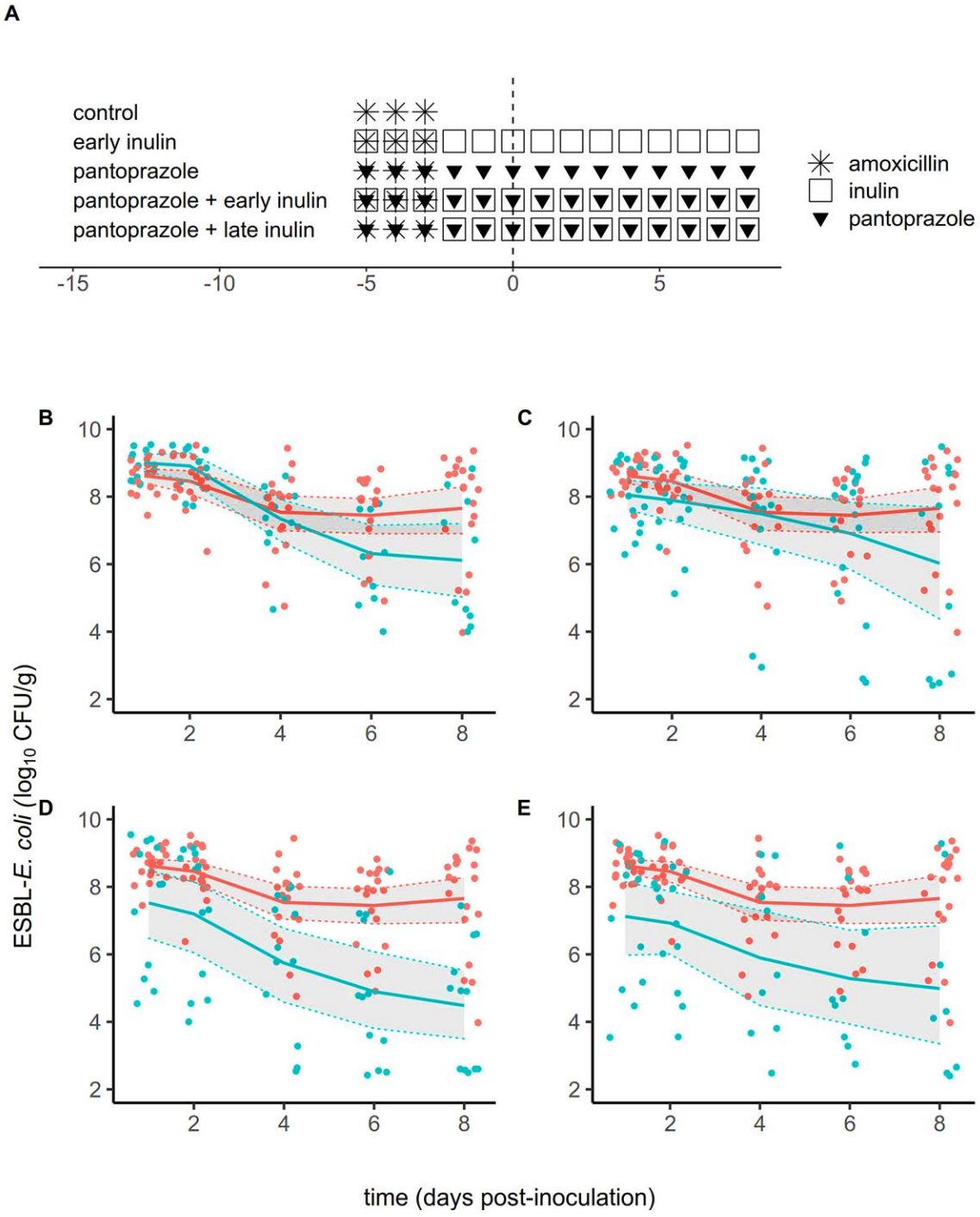
5.2 Table 2. Fecal taxa in control and treated mice, assessed by quantitative PCR: comparison of control mice with treated mice with high level or low-level colonization by ESBL-Ec

	early inulin + pantoprazole			late inulin + pantoprazole			pantoprazole		
taxa	**treated, low median titer** (log10cfu/g)	**treated, low vs control**	**treated, low vs treated, high**	**treated, low median titer** (log10cfu/g)	**treated, low vs control**	**treated, low vs treated, high**	**treated, low median titer** (log10cfu/g)	**treated, low vs control**	**treated, low vs treated, high**
Bacteroidetes	10.3 (10-10.4)	-3.5 (-5.1 to -1.9); P, < 0.0001	-1.8 (-3.9 to 0.3); P, 0.097	10.3 (10.2-10.3)	-3.4 (-5.5 to -1.4); P, 0.002	-0.9 (-3.6 to 1.9); P, 0.65	10.5 (10.4-10.6)	-3.8 (-6.8 to -0.7); P, 0.016	-1.9 (-5.2 to 1.4); P, 0.29
Firmicutes	9.1 (8.3-9.3)	0.4 (-0.1 to 1); P, 0.12	0.9 (0.2 to 1.6); P, 0.011	9.4 (9.1-9.9)	-0.3 (-0.9 to 0.4); P, 0.47	0.2 (-0.6 to 1.1); P, 0.69	9.4 (9.4-9.4)	-0.1 (-0.8 to 0.6); P, 0.87	-0.2 (-1 to 0.6); P, 0.70
Enterobacteriaceae	8.1 (7.1-9.2)	-3.4 (-5.1 to -1.7); P, < 0.001	-4.2 (-6.4 to -2); P, < 0.001	6.7 (5.6-6.8)	-1.3 (-3.5 to 0.8); P, 0.25	-0.5 (-3.4 to 2.4); P, 0.87	6.8 (6.7-6.9)	-2.4 (-4.8 to -0.1); P, 0.041	-0.3 (-2.9 to 2.3); P, 0.92
Muribaculaceae	10.3 (10-10.3)	-4.2 (-6.3 to -2.1); P, < 0.001	-4.8 (-7.4 to -2.1); P, < 0.001	10.4 (9.6-10.4)	-3.9 (-6.8 to -1.1); P, 0.008	-2.7 (-6.3 to 0.9); P, 0.15	10.8 (10.8-10.9)	-4.9 (-8.3 to -1.4); P, 0.007	-3.2 (-7 to 0.6); P, 0.10
A. caecimuris	6.5 (4.1-6.6)	-2.7 (-4.1 to -1.3); P, < 0.001	-1.8 (-3.5 to 0); P, 0.049	7.5 (7-8)	-4.8 (-5.7 to -3.9); P, < 0.0001	-4.9 (-6.2 to -3.7); P, < 0.0001	2.5 (2.5-2.5)	0.2 (-1.5 to 1.8); P, 0.96	1.6 (-0.2 to 3.5); P, 0.088
Akkermansia	4 (3.7-4.8)	-0.5 (-1.2 to 0.2); P, 0.14	-1.4 (-2.3 to -0.6); P, 0.001	6.8 (5.3-8.2)	-3.2 (-5.4 to -0.9); P, 0.006	-1.3 (-4.2 to 1.6); P, 0.46	3.4 (3-3.9)	0.2 (-1 to 1.4); P, 0.87	0 (-1.3 to 1.2); P, 0.99
Alistipes finegoldii	2.5 (2.5-4.8)	-1 (-2.6 to 0.6); P, 0.26	-0.2 (-2.2 to 1.9); P, 0.96	3.3 (2.5-4.7)	-1.2 (-2.6 to 0.2); P, 0.10	-0.6 (-2.6 to 1.5); P, 0.71	3.5 (3-4)	-0.9 (-2.1 to 0.4); P, 0.18	-0.4 (-1.8 to 1); P, 0.72
Bacteroides	9.5 (8.5-10.2)	-3.4 (-5.6 to -1.2); P, 0.003	-0.7 (-3.5 to 2.1); P, 0.79	9.9 (9.6-10.2)	-4.1 (-7.1 to -1.2); P, 0.007	-1.4 (-5.3 to 2.5); P, 0.60	9.4 (9.1-9.8)	-3.8 (-7.4 to -0.1); P, 0.042	-2.8 (-6.8 to 1.2); P, 0.18
B. acidifaciens	8 (7.7-8.6)	-3.3 (-6 to -0.6); P, 0.016	-0.8 (-4.2 to 2.6); P, 0.81	9.6 (9.2-10.2)	-5.1 (-8.7 to -1.5); P, 0.006	-1.4 (-6.1 to 3.2); P, 0.67	6.6 (5.3-7.9)	-2 (-6.4 to 2.5); P, 0.43	0 (-4.9 to 4.8); P, 1.00
B. ovatus	6 (5-7.7)	-3.3 (-6.2 to -0.3); P, 0.031	-0.5 (-4.2 to 3.3); P, 0.93	6 (5-6.9)	-2.6 (-5.4 to 0.2); P, 0.064	-2.6 (-6.2 to 1.1); P, 0.18	5 (4.4-5.6)	-1.7 (-5.1 to 1.7); P, 0.35	-1.7 (-5.4 to 2); P, 0.41
B. thetaiotaomicron	4.3 (3.6-5.1)	-1.7 (-3.5 to 0.1); P, 0.073	2.1 (-0.2 to 4.4); P, 0.071	3.9 (3.5-5.1)	-2 (-3.9 to -0.1); P, 0.040	-1.5 (-4 to 1); P, 0.27	3 (2.8-3.3)	-0.3 (-1.6 to 0.9); P, 0.67	0 (-1.4 to 1.3); P, 0.99
Duncaniella muris	8.3 (8.2-8.9)	-3.5 (-5.1 to -1.9); P, < 0.001	-4.1 (-6.2 to -2); P, < 0.001	7.7 (7.4-8.3)	-3 (-5.2 to -0.8); P, 0.008	-2.7 (-5.6 to 0.3); P, 0.077	9.1 (8.7-9.4)	-4.1 (-7.2 to -1.1); P, 0.009	-2.4 (-5.8 to 0.9); P, 0.16
E. coli	6.4 (4-8.5)	-3.6 (-5.4 to -1.8); P, < 0.001	-3.1 (-5.4 to -0.7); P, 0.010	3.5 (2.5-5)	-1.5 (-2.7 to -0.2); P, 0.021	0.1 (-1.5 to 1.8); P, 0.97	3.5 (3-4)	-1 (-2.1 to 0.1); P, 0.084	-0.6 (-1.8 to 0.6); P, 0.39
Enterococcus	8.4 (7.3-9.1)	1 (0.2 to 1.8); P, 0.010	1.5 (0.5 to 2.5); P, 0.004	7.9 (6.3-8.9)	2 (0.2 to 3.7); P, 0.026	1.5 (-0.8 to 3.8); P, 0.21	8.9 (8.9-9)	0.4 (-1.7 to 2.4); P, 0.85	-0.8 (-3 to 1.5); P, 0.58
EPB	7 (5.2-7.1)	-3.3 (-4.4 to -2.2); P, < 0.0001	-3.7 (-5.1 to -2.2); P, < 0.0001	7 (6.5-7.5)	-4 (-5.1 to -2.9); P, < 0.0001	-3.7 (-5.3 to -2.2); P, < 0.0001	7 (6.7-7.3)	-4 (-6.1 to -1.9); P, < 0.001	-2.8 (-5.1 to -0.5); P, 0.019

Note. Treated mice with low level and high level colonization by ESBL-Ec were considered as effectively and ineffectively treated mice, respectively. Median titers in effectively treated mice are reported as medians (1st and 3rd quartiles; treated, low). Differences between titers in effectively treated mice and (i) ineffectively treated mice (treated, high) and (ii) control mice were tested using Dunnett test. No mouse treated with early inulin had low titers of ESBL-Ec. The primer used for *Enterobacteriaceae* is an approximation for the former *Enterobacteriaceae* family as it was used to assess the combined titers of *E. coli*, *Shigella* spp and *Salmonella* spp. *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

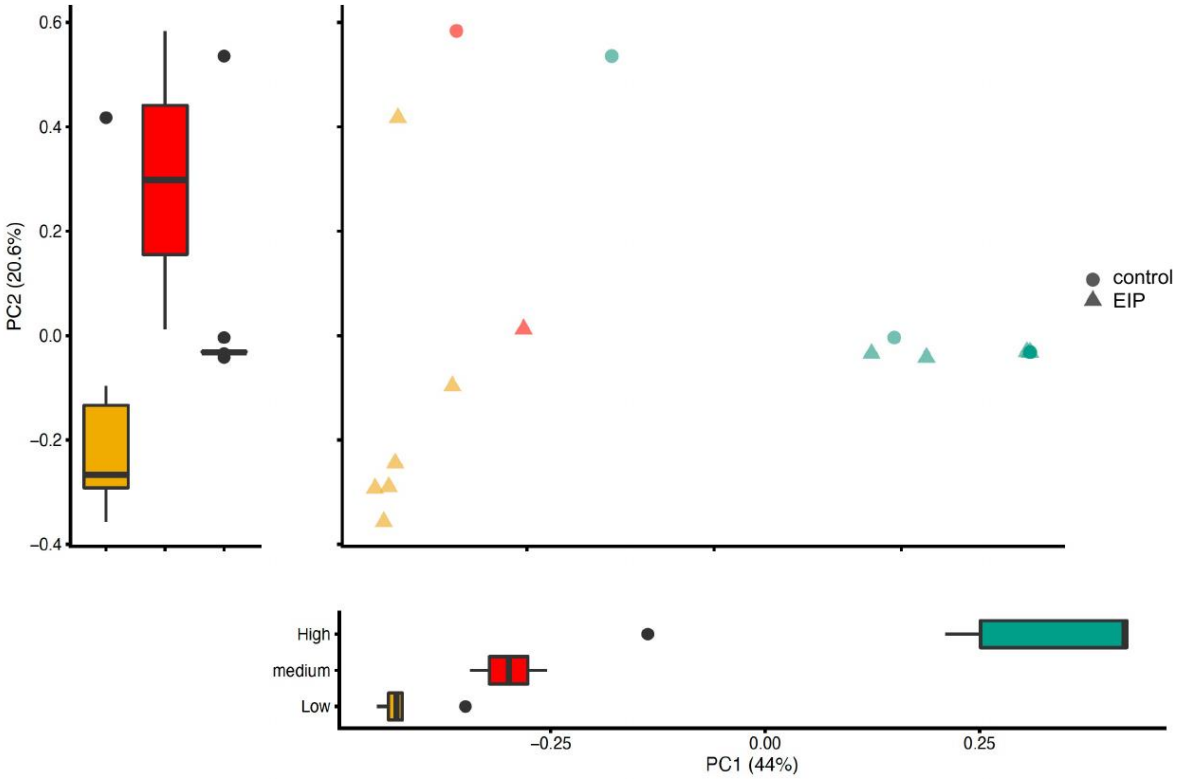
6 Figure titles and notes

6.1 Figure 1. Effect of inulin, pantoprazole and combination on ESBL-Ec fecal titers



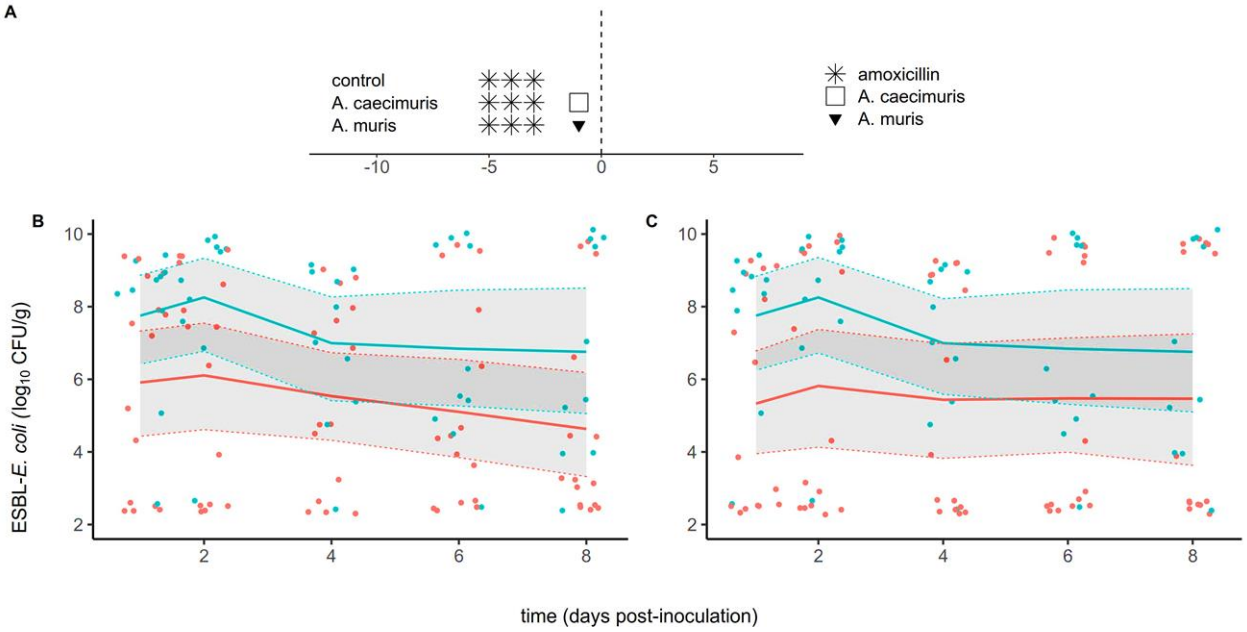
Note. (A) Experimental design; (B) Control versus early inulin; (C) Control versus pantoprazole; (D) Control versus pantoprazole and early inulin; (E) Control versus pantoprazole and late inulin. Full line, mean titer. Dotted line, 95% confidence interval. Red, control; blue, treatments

6.2 Figure 2. Beta-diversity of fecal microbiota just before ESBL-Ec inoculation (0 dpi) in control mice and in mice treated with early inulin and pantoprazole (EIP).



Note. Mice were classified as low, medium or high level of ESBL-Ec colonization, according to the AUC of ESBL titers (≤ 39, >39 and <50, and ≥ 50 $\log_{10}$ CFU·day·g^{-1}, respectively). Green, high; red, medium; yellow, low.

6.3 Figure 3. Preventive treatment with *Adlercreutzia caecimuris* and *Adlercreutzia muris* on ESBL-Ec fecal colonization



Note. (A) Experimental design; (B) Control versus treatment with *Adlercreutzia caecimuris*; (C) Control versus treatment with *Adlercreutzia muris*. Full line, mean titer. Dotted line, 95% confidence interval. Red, treatments; green, control.

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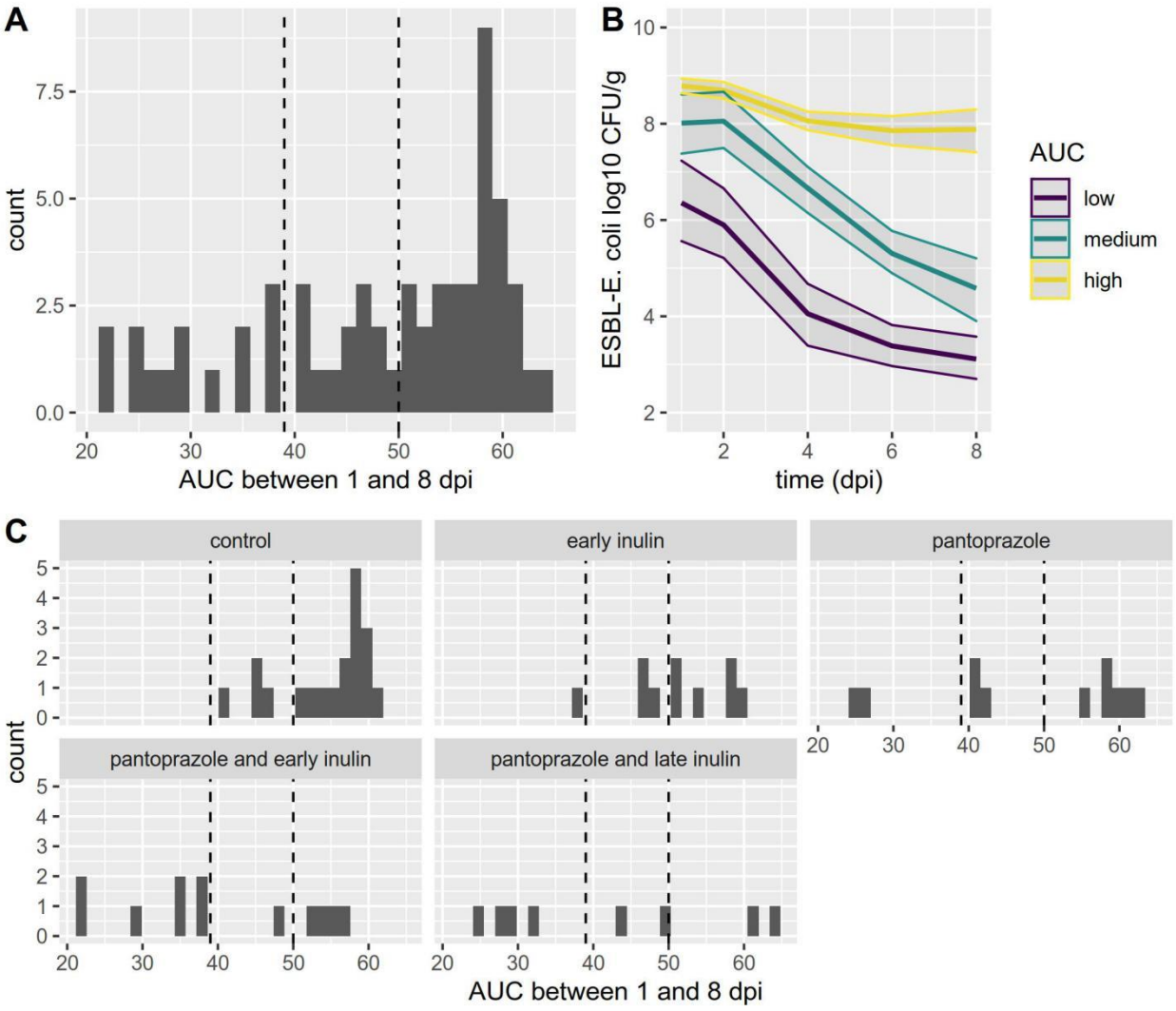
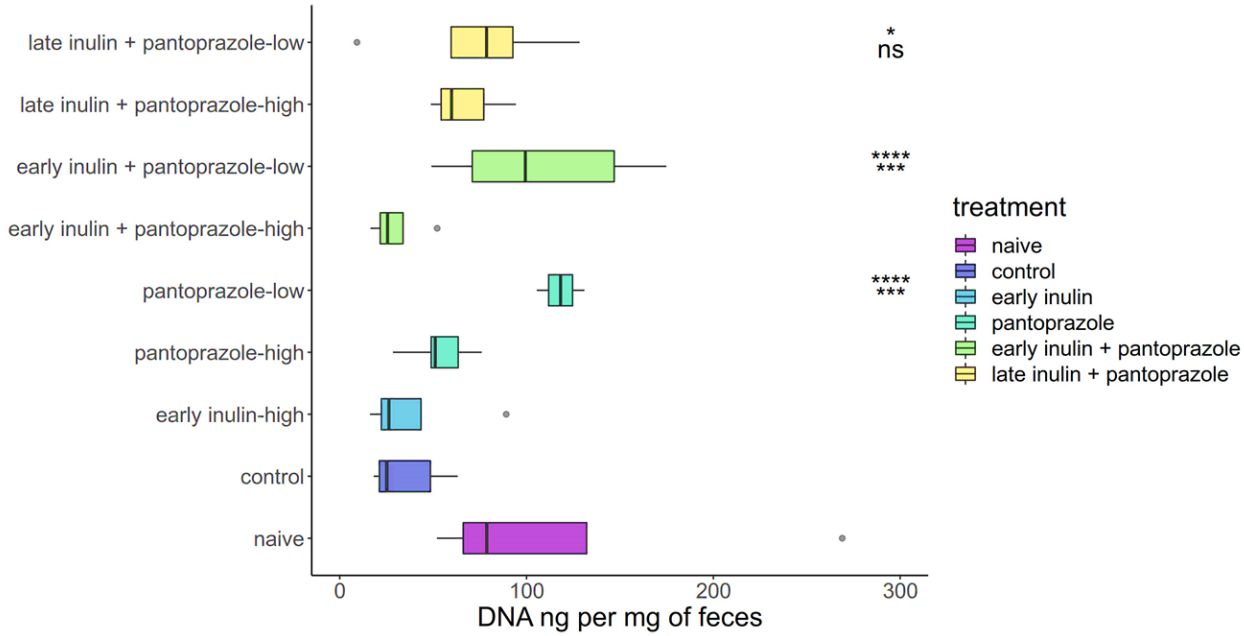
Supplementary Material

Figure S1. Classification of mice according to their ESBL-Ec titers



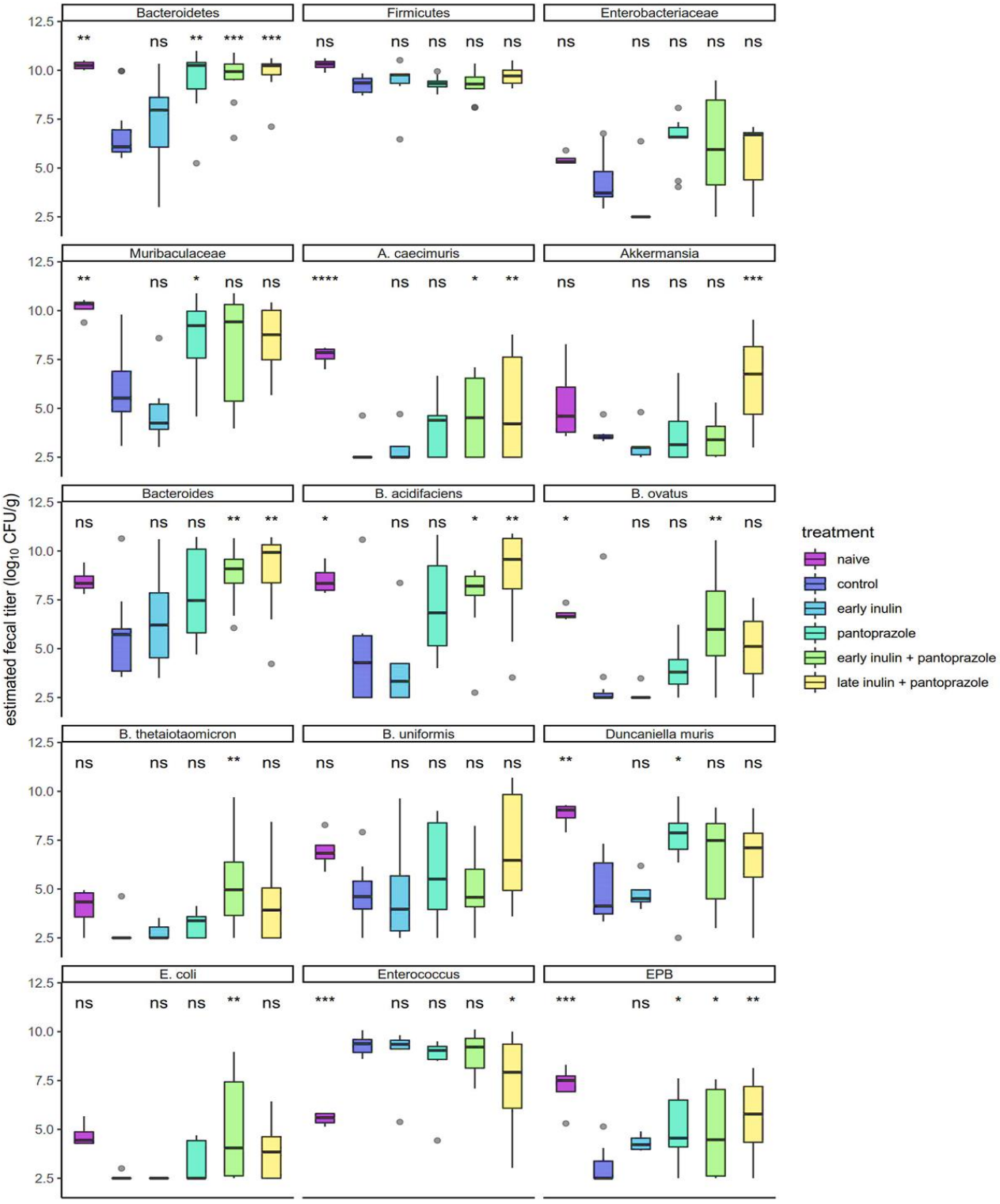
Note. For each mouse the area under the curve of the time variation of ESBL-Ec titers was computed between 1 and 8 dpi. (A) Distribution of AUCs for all mice. AUCs were grouped in 3 classes (low, medium and high). (B) Mean (95% confidence interval) fecal titers of ESBL-Ec according to the AUC group. (C) Distribution of AUCs according to the treatment.

Figure S2. Fecal DNA concentrations according to treatment efficacy



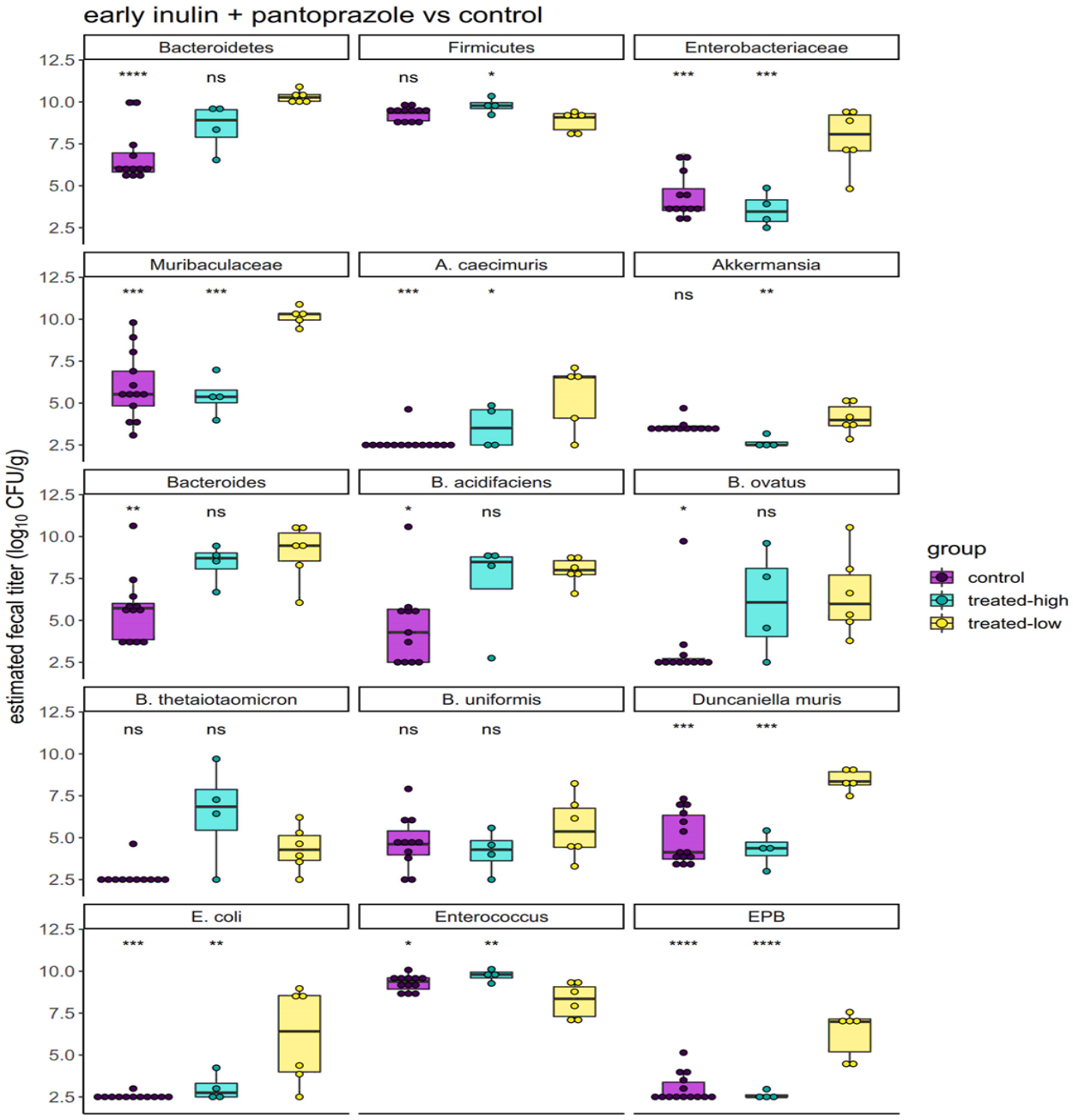
Note. Treated mice were classified as low (effective treatment) or high (ineffective treatment) level of ESBL-Ec colonization, according to the $AUC_{1\text{-}8}$ of ESBL titers (≤ 39 and ≥ 50 $\log_{10}$ CFU·day·g^{-1}). For each treatment, low level treated mice were compared with control mice (upper line) and high level treated mice (lower line). DNA concentrations were significantly higher in naive feces than in control (P-value, 0.005).

Figure S3. Comparison of fecal titers of selected taxa, using quantitative PCR, in control and treated mice.



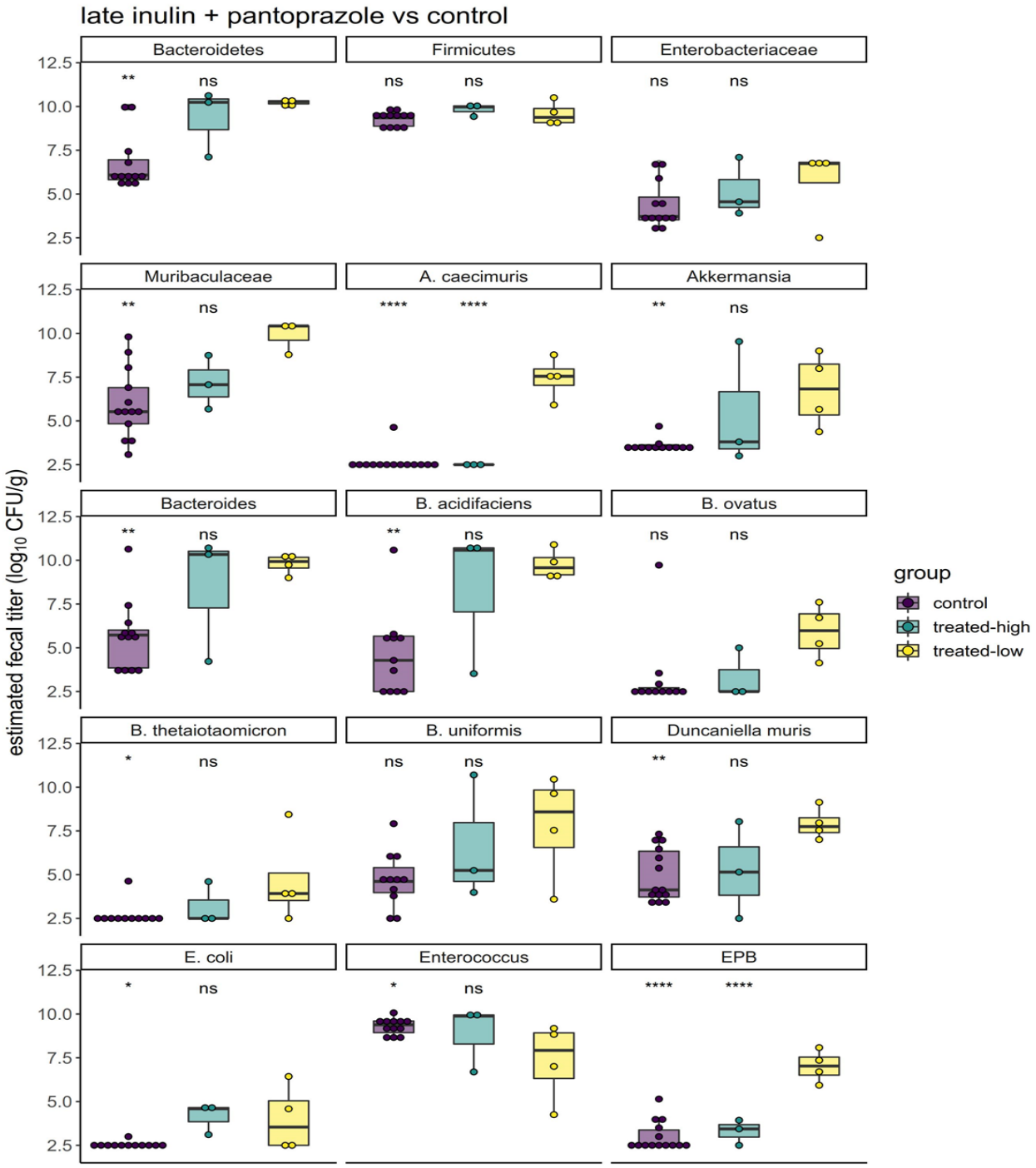
Note. Fecal titers just before ESBL-Ec inoculation (0 dpi), except for feces of naive mice that were sampled just before starting amoxicillin (-3 dpi). P-values, comparison with control group (Dunnett test). ns, not statistically significant. EPB, equol producing bacteria. The primer used for *Enterobacteriaceae* is an approximation for the former *Enterobacteriaceae* family as it was used to assess the combined titers of *E. coli*, *Shigella* spp and *Salmonella* spp. *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

Figure S4. Comparison of fecal titers of selected taxa, using quantitative PCR, in control mice and mice treated effectively and ineffectively with early inulin and pantoprazole (EIP).



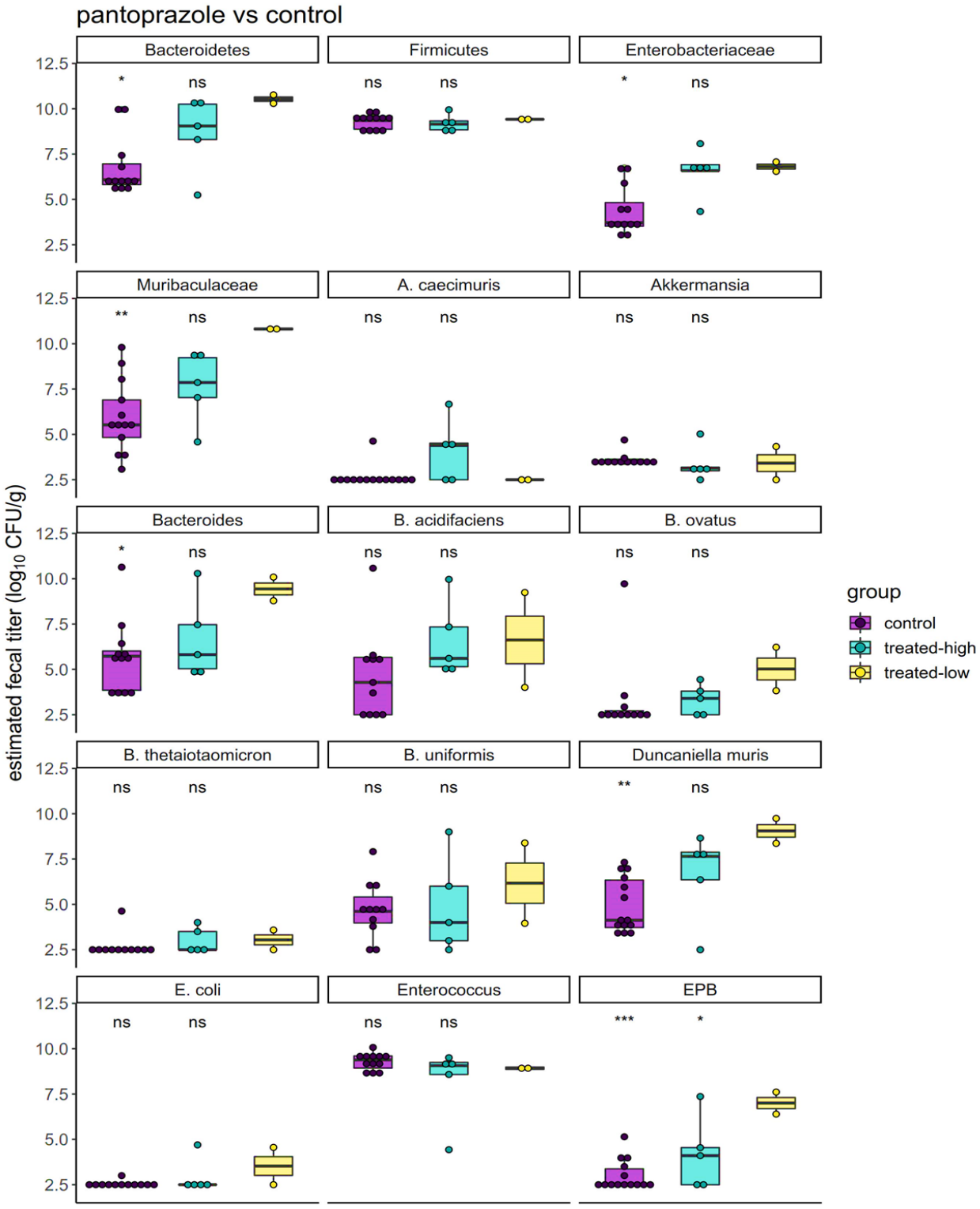
Note. Treated mice were classified as low (effective treatment) or high (ineffective treatment) level of ESBL-Ec colonization, according to the $AUC_{1\text{-}8}$ of ESBL titers (≤ 39 and ≥ 50 $\log_{10}$ CFU·day·g^{-1}). Low level treated mice were compared with control and high level treated mice. EPB, equol producing bacteria. The primer used for *Enterobacteriaceae* is an approximation for the former *Enterobacteriaceae* family as it was used to assess the combined titers of *E. coli*, *Shigella* spp and *Salmonella* spp. *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

Figure S5. Comparison of fecal titers of selected taxa, using quantitative PCR, in control mice and mice treated effectively and ineffectively with late inulin and pantoprazole (LIP)



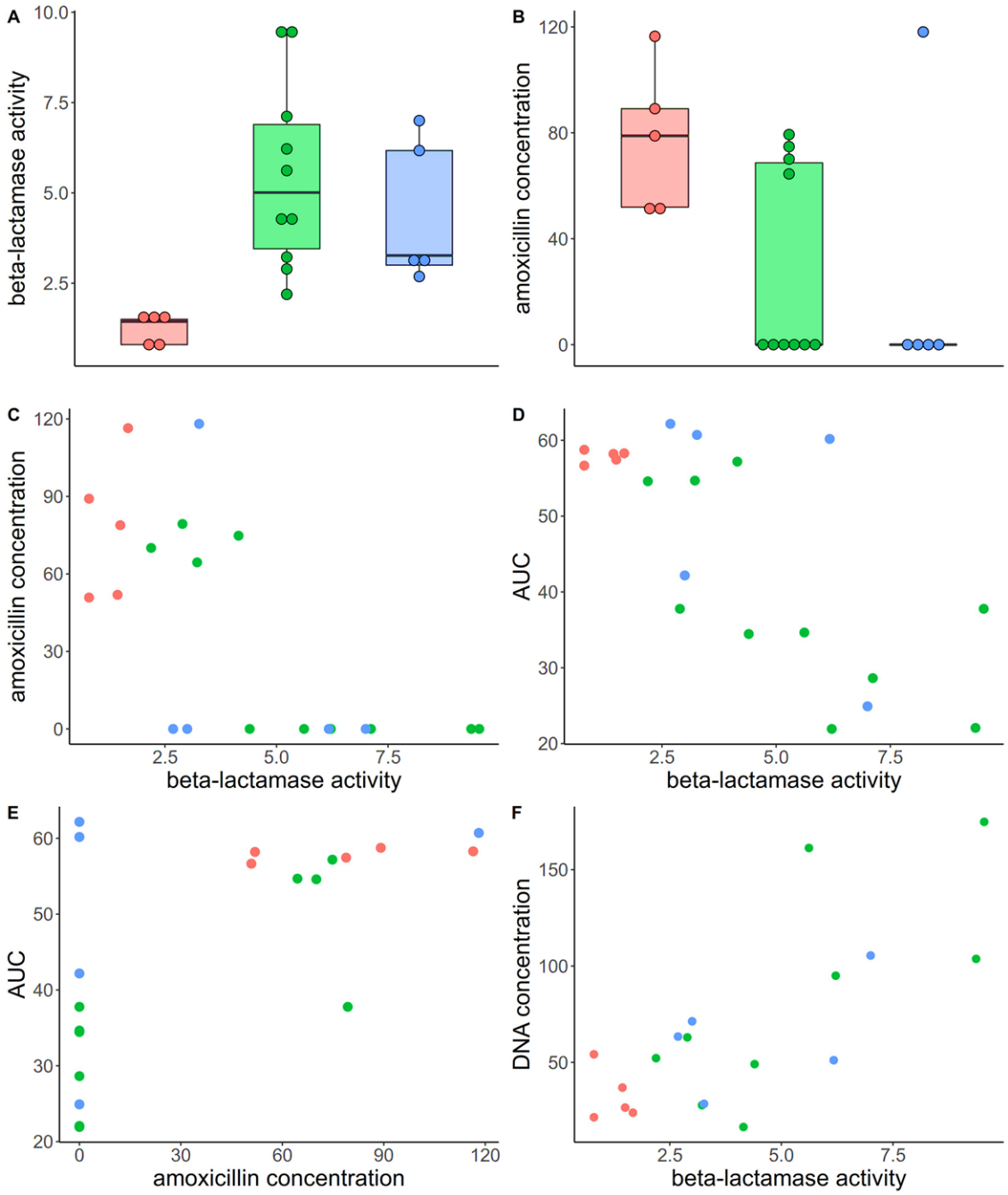
Note. Treated mice were classified as low (effective treatment) or high (ineffective treatment) level of ESBL-Ec colonization, according to the $AUC_{1\text{-}8}$ of ESBL titers (≤ 39 and ≥ 50 $\log_{10}$ CFU·day·g^{-1}). Low level treated mice were compared with control and high level treated mice. EPB, equol producing bacteria. The primer used for *Enterobacteriaceae* is an approximation for the former *Enterobacteriaceae* family as it was used to assess the combined titers of *E. coli*, *Shigella* spp and *Salmonella* spp. *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

Figure S6. Comparison of fecal titers of selected taxa, using quantitative PCR, in control mice and mice treated effectively and ineffectively with pantoprazole.



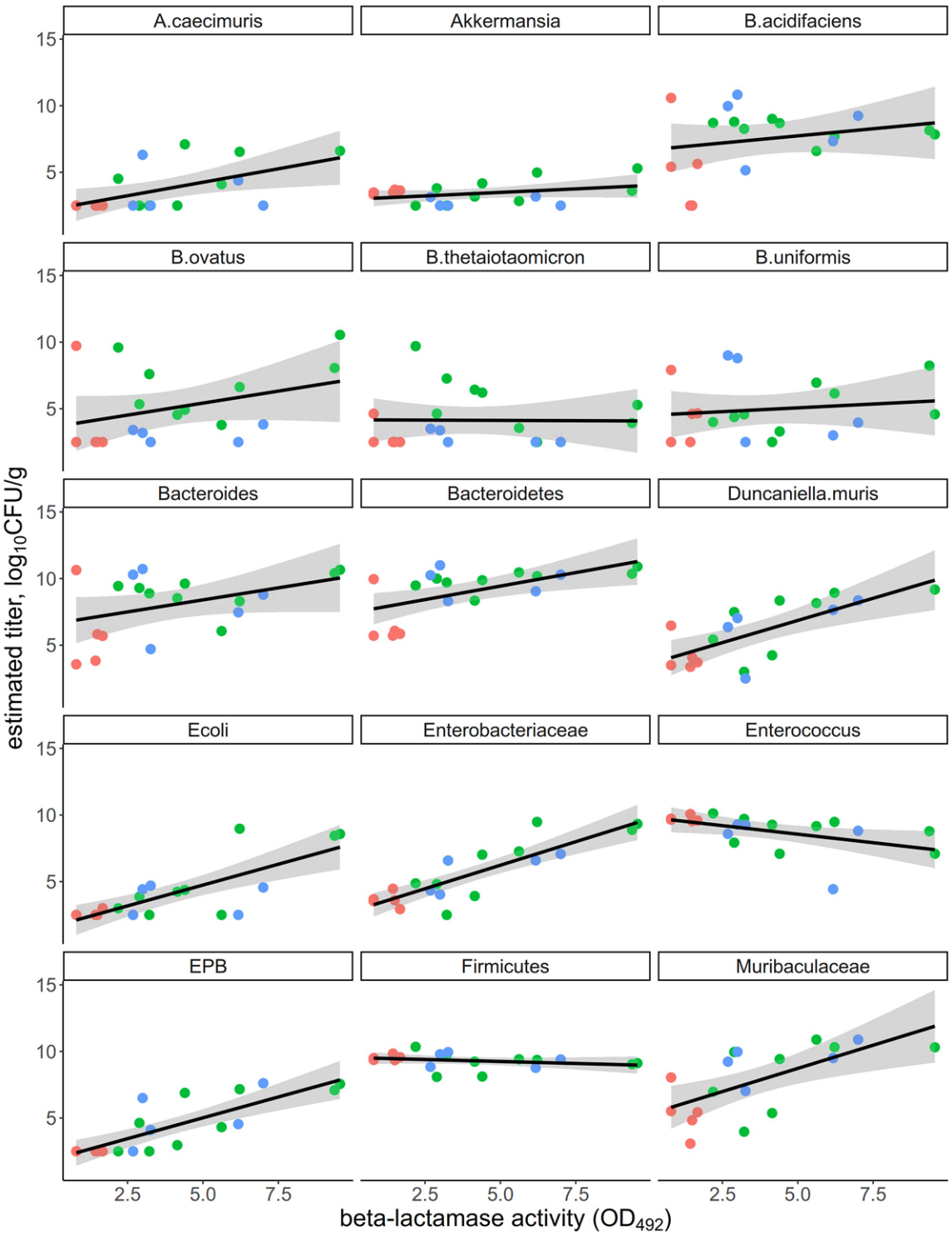
Note. Treated mice were classified as low (effective treatment) or high (ineffective treatment) level of ESBL-Ec colonization, according to the $AUC_{1\text{-}8}$ of ESBL titers (≤ 39 and ≥ 50 $\log_{10}$ CFU·day·g^{-1}). Low level treated mice were compared with control and high level treated mice. EPB, equol producing bacteria. The primer used for *Enterobacteriaceae* is an approximation for the former *Enterobacteriaceae* family as it was used to assess the combined titers of *E. coli*, *Shigella* spp and *Salmonella* spp. *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

Figure S7. Relationships between fecal β-activity, level of ESBL-Ec colonization, and amoxicillin and DNA fecal concentrations



Note. Fecal β-lactamase activity (OD at 492 nm) and amoxicillin concentrations (µg/g) were assessed 2 days before ESBL-Ec inoculation (-2 dpi). DNA fecal concentration (ng/mg) was assessed the day of inoculation (0 dpi). AUC of fecal titers of ESBL-Ec was computed between 1 and 8 dpi. Red, control mice; blue, pantoprazole; green, early inulin and pantoprazole.

Figure S8. Relationship between fecal β-activity and titers of selected taxa.



Note. Fecal β-lactamase activity and titers were assessed at -2 dpi and 0 dpi, respectively. Red, control; blue, pantoprazole; green, early inulin and pantoprazole. Correlations between β-lactamase activity and titers were not significant for *Firmicutes*, Akkermansia, *Bacteroides*, *B. acidifaciens, B. ovatus, B. theraiotaomicron* and *B. uniformis. Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data

Figure S9. Alpha-diversity of fecal microbiota just before ESBL-Ec inoculation (0 dpi) in control mice and in mice treated with early inulin and pantoprazole, according to the subsequent level of ESBL-Ec inoculation

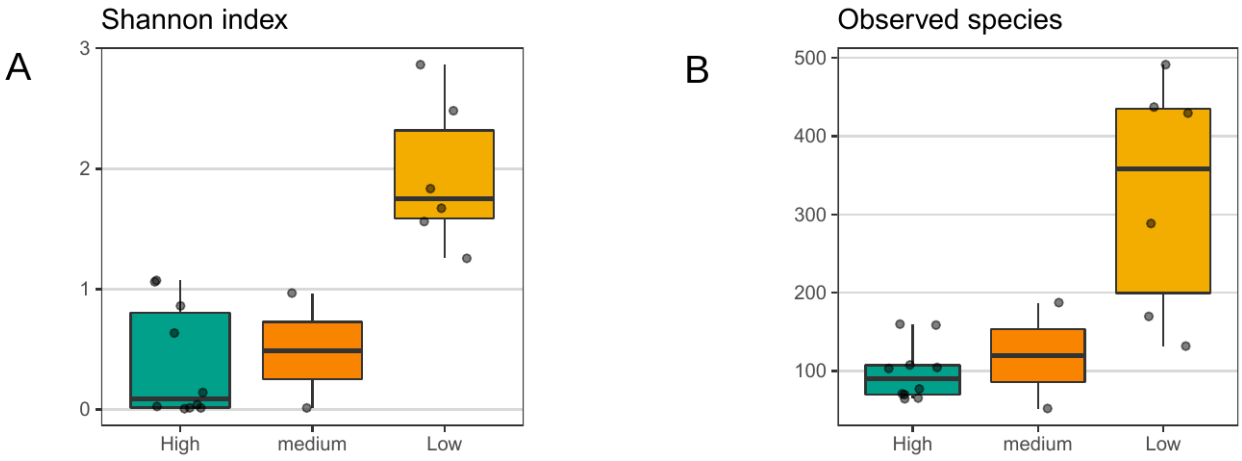
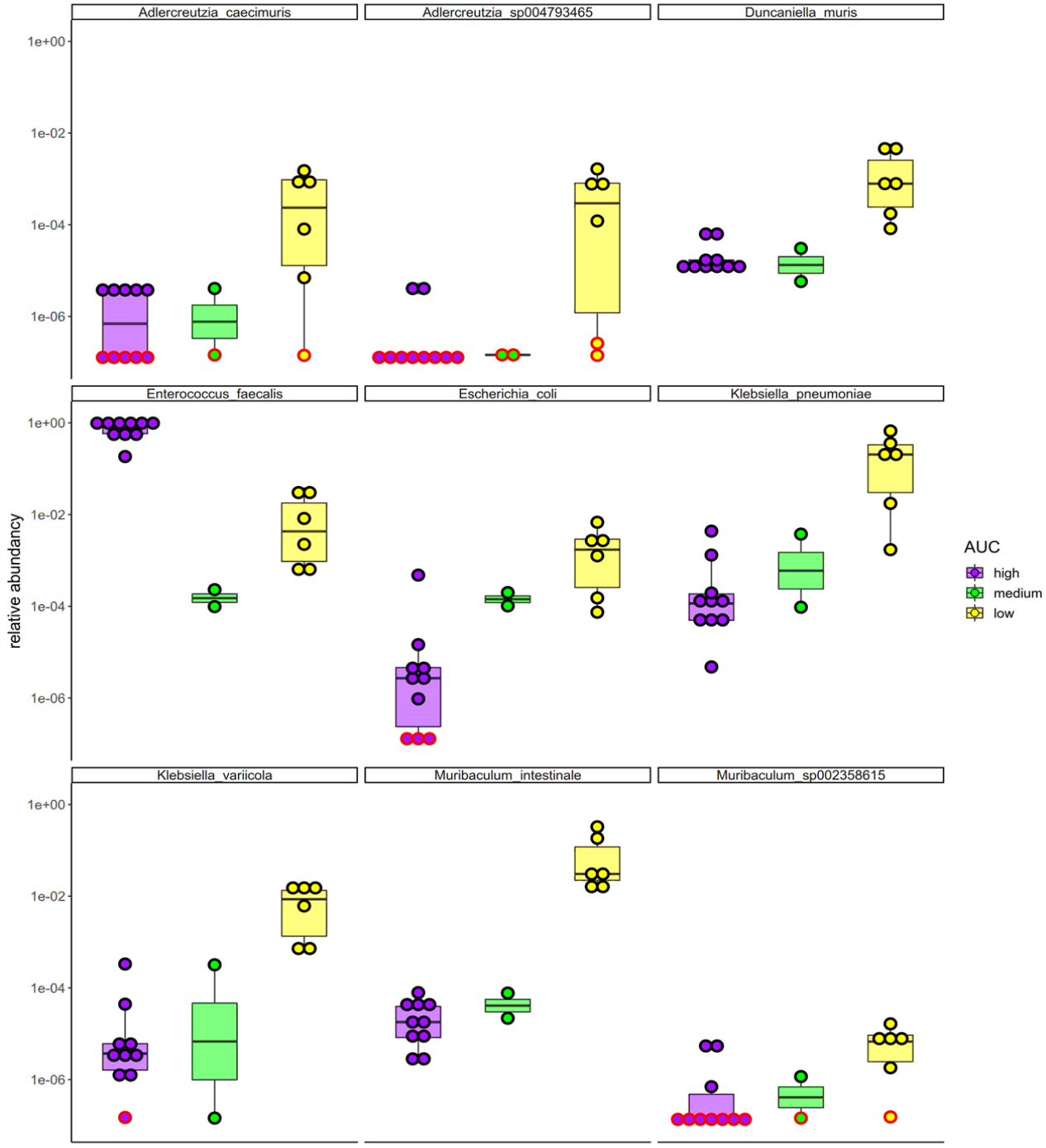


Figure S10. Differences in microbiota composition, according to the level of ESBL-Ec colonization in control mice and mice treated with early inulin and pantoprazole



Note. Shotgun metagenomic sequencing identified 74 species that had significantly different relative abundances between high, medium and low level ESBL-Ec colonization. This figure shows 9 selected species. Complete results are reported in Supplementary table S3. Red strokes indicate undetected taxon (lower limit of detection).

Figure S11. Alpha-diversity of fecal microbiota just before ESBL-Ec inoculation (0 dpi) in mice treated with early inulin and pantoprazole, according to the subsequent level of ESBL-Ec inoculation

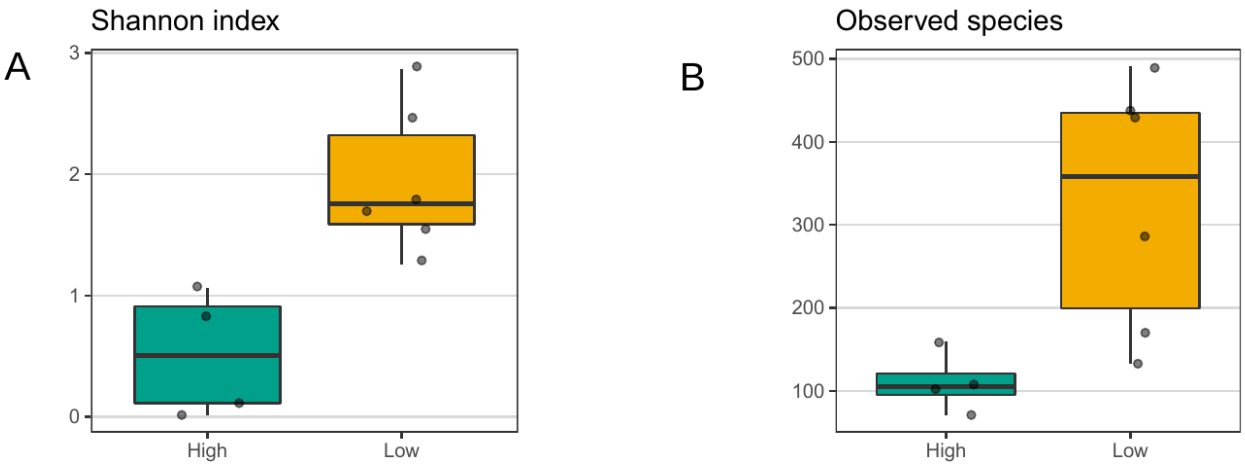
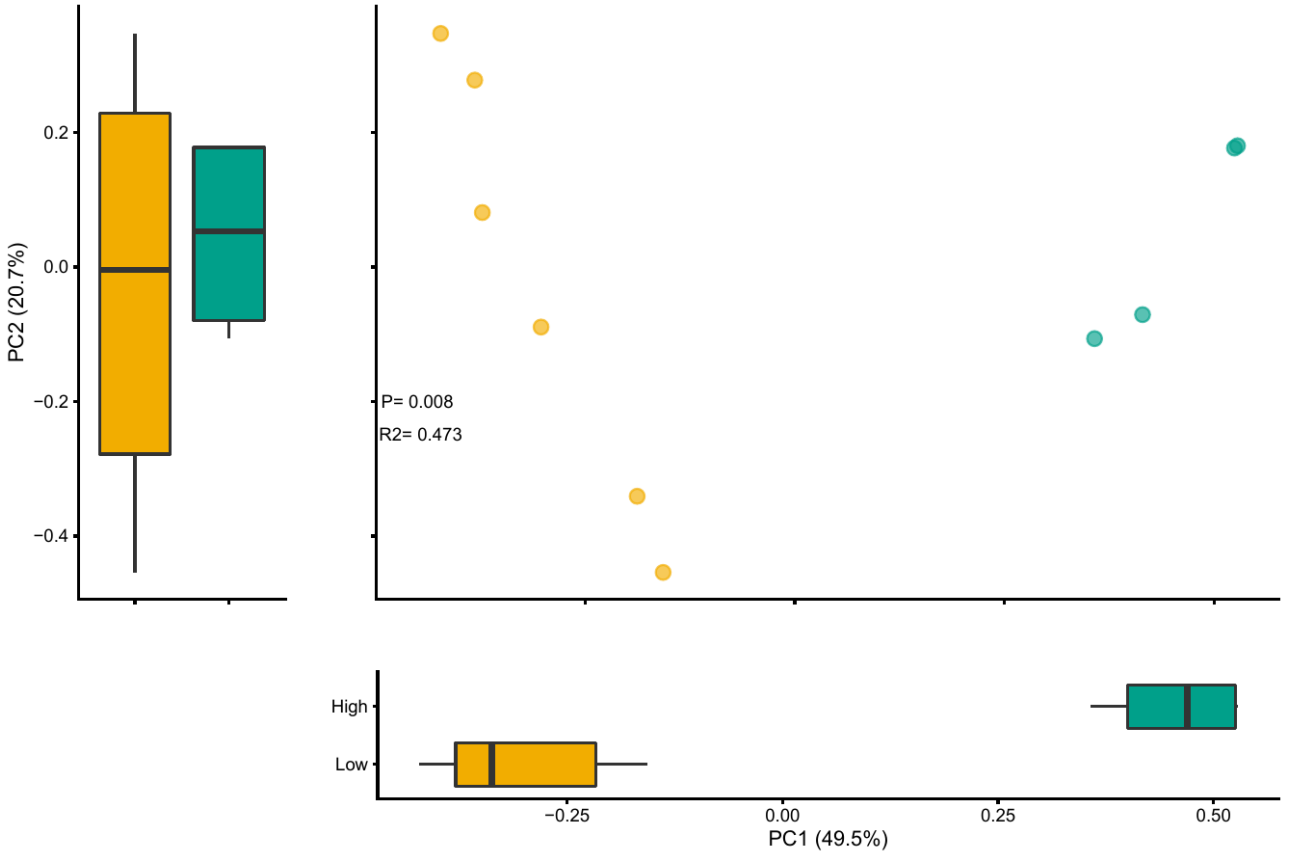
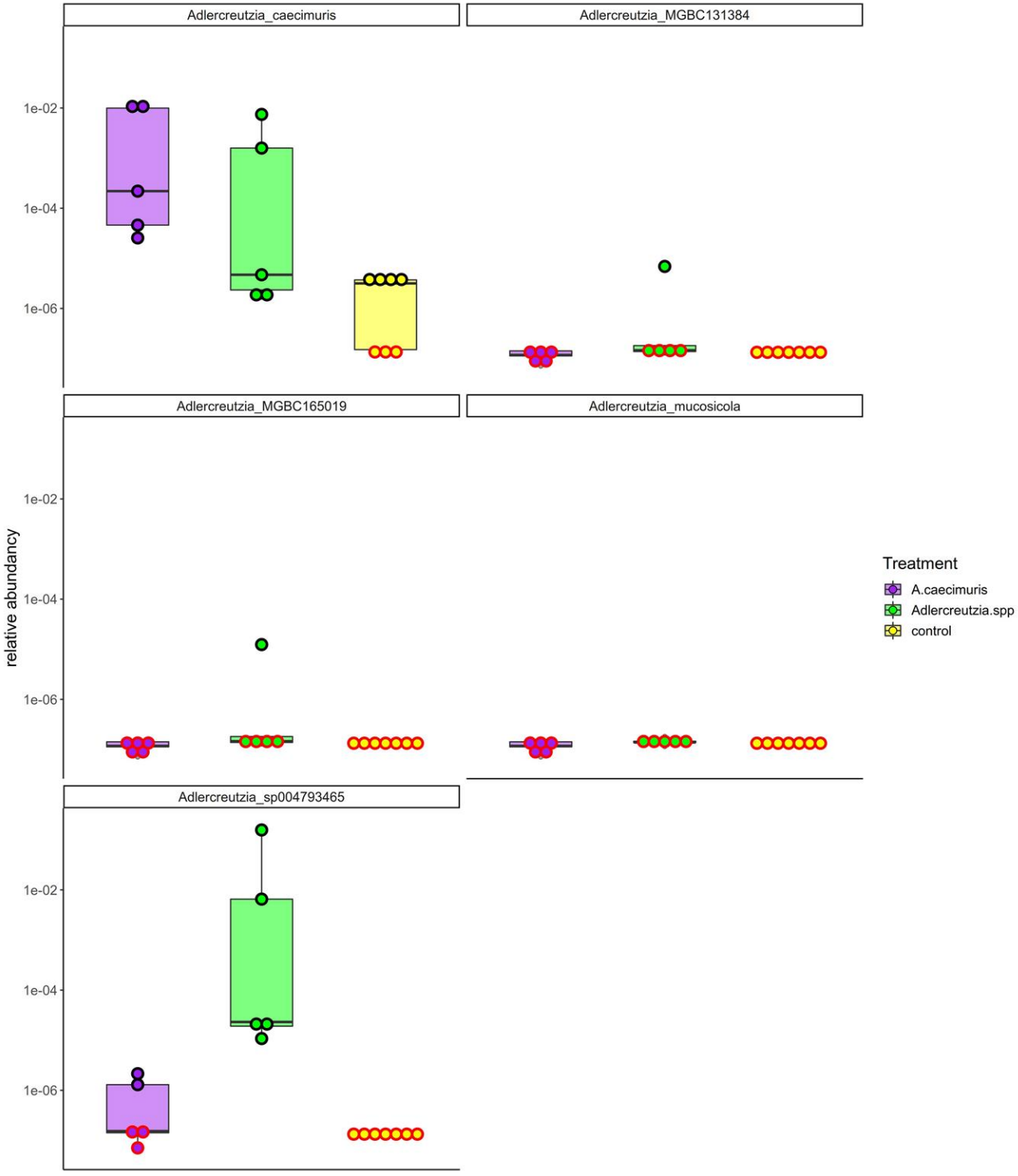


Figure S12. Beta-diversity of fecal microbiota just before ESBL-Ec inoculation (0 dpi) in mice effectively (low subsequent titers) and ineffectively (high subsequent titers) treated with early inulin and pantoprazole.



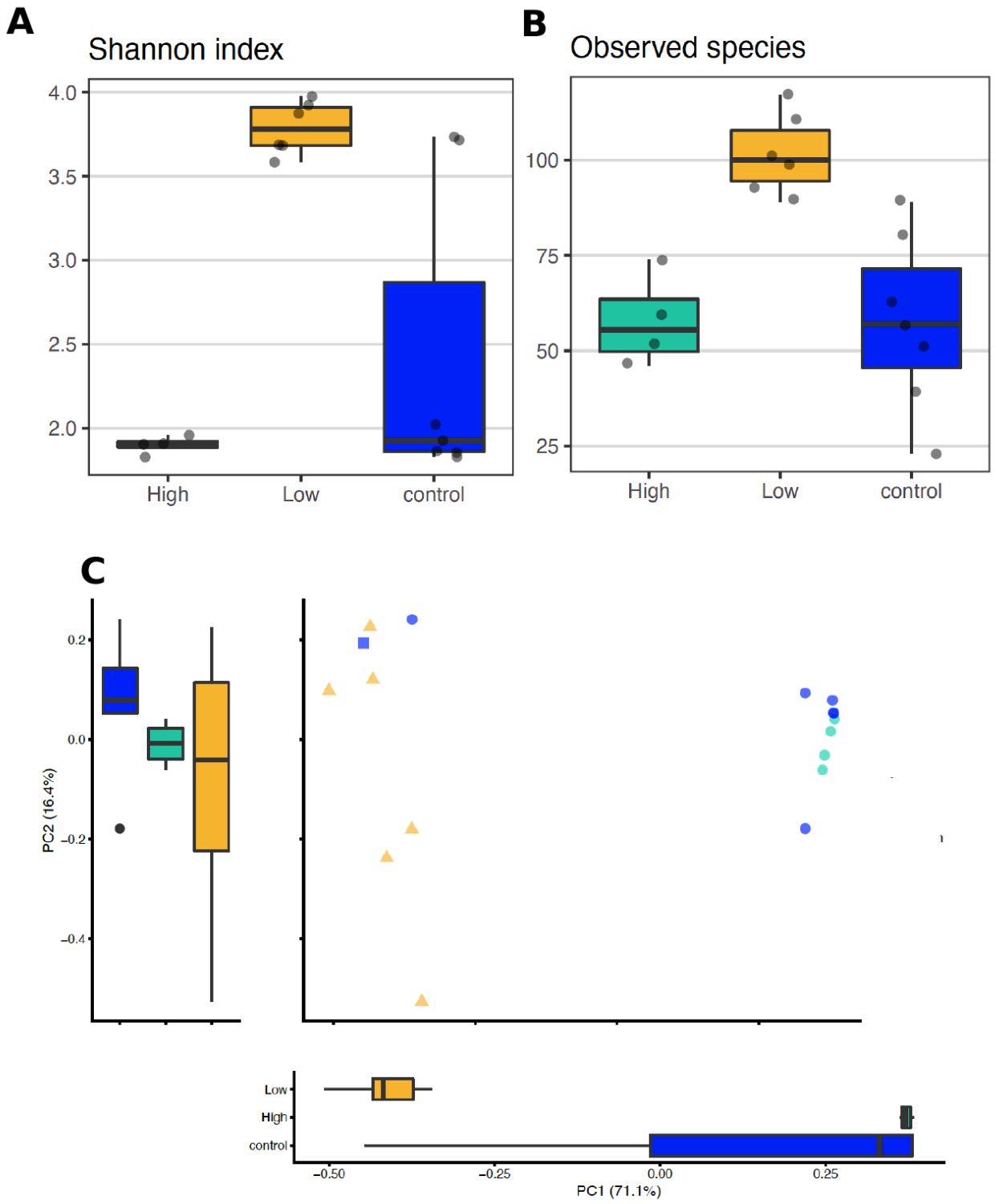
Green, high; yellow, Low.

Figure S13. Relative abundancies of 5 species of the *Adlercreutzia* genus in control mice and mice treated with *A. caecimuris* or *A. muris*



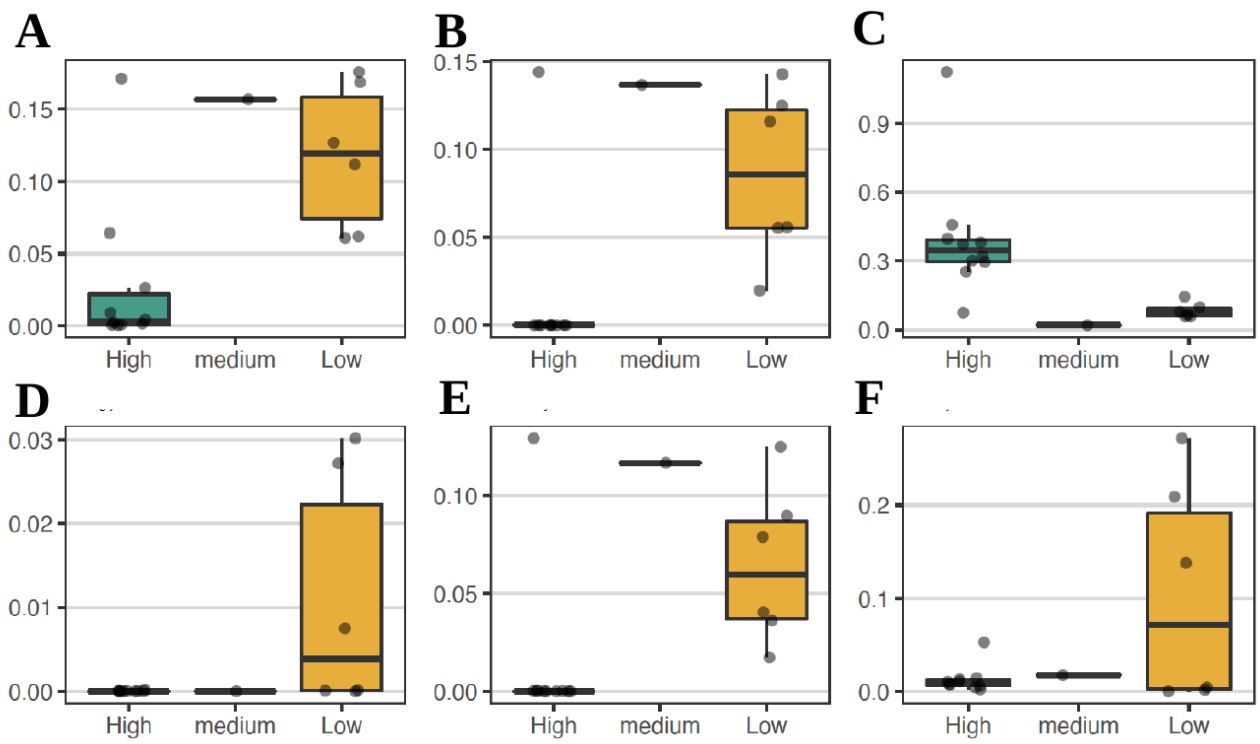
Note. Relative abundancies were assessed using shotgun metagenomic sequencing in feces sampled 1 day after treatment with *A. caecimuris* or *A. muris*. Red stroke, below the detection threshold (i.e., undetected); black stroke, above the detection threshold.

Figure S14. Fecal resistome of control, effectively and ineffectively treated mice.



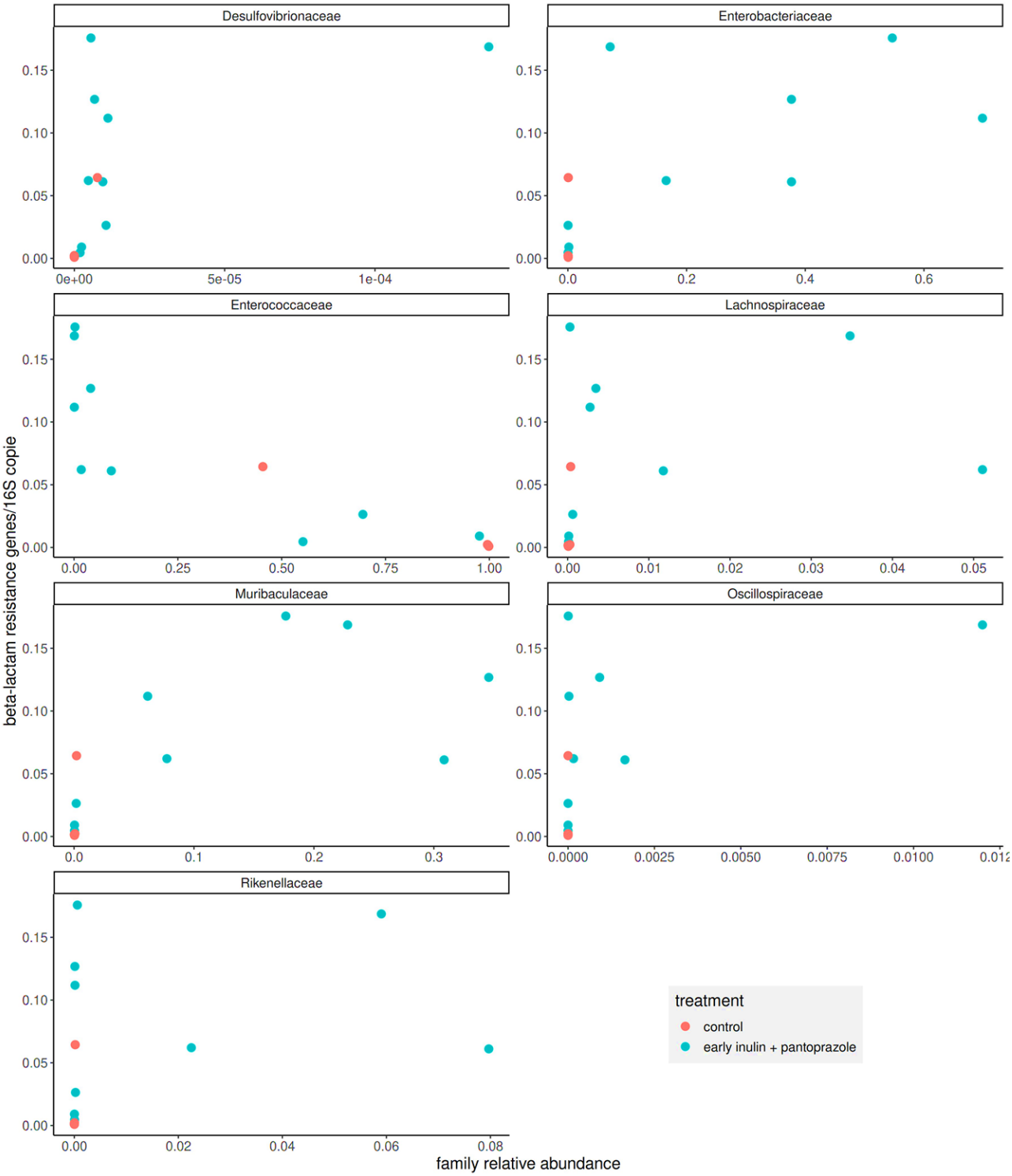
Note. Alpha diversity (A and B) and resistome architecture (C). Blue, control mice; orange, mice effectively treated with early inulin and pantoprazole (low level AUC of ESBL-Ec titers between 1- and 8-day post inoculation); green, mice ineffectively treated with early inulin and pantoprazole (high level AUC of ESBL-Ec titers).

Figure S15. Antibiotic Resistance Genes types in control and early inulin pantoprazole treated mice, according to their level of ESBL-Ec colonization



Note. A, β-lactams; B, kasugamycin; C, tetracycline; D, aminoglycoside; E, fosfidomycin ; F, vancomycin.

Figure S16. Association between relative abundancy of main families of the fecal microbiota and beta-lactam resistance genes



Note. Metagenomic and resistome data was available for 4 control mice and 9 mice treated with early inulin and pantoprazole. The *Enterobacteriaceae* family was defined according the MGBC database used for metagenomic analyses.

Table S1: list of virulence factors for ESBL-Ec of our murine model

Virulence factor	Protein function
cea	Colicin E1
cea	Colicin E1
chuA	Outer membrane hemin receptor
fyuA	Siderophore receptor
gad	Glutamate decarboxylase
gad	Glutamate decarboxylase
iha	Adherence protein
irp2	High molecular weight protein 2 non-ribosomal peptide synthetase
iss	Increased serum survival
iucC	Aerobactin synthetase
iutA	Ferric aerobactin receptor
kpsE	Capsule polysaccharide export inner-membrane protein
kpsMII_K5	Polysialic acid transport protein; Group 2 capsule
ompT	Outer membrane protease (protein protease 7)
papA_F43	Major pilin subunit F43
sat	Secreted autotransporter toxin
senB	Plasmid-encoded enterotoxin
sitA	Iron transport protein
terC	Tellurium ion resistance protein
usp	Tellurium ion resistance protein
yfcV	Uropathogenic specific protein

Table S2: list of Resistance genes for ESBL-Ec of our murine model

Resistance genes	Phenotype
aph(6)-Id	Aminoglycoside resistance
aadA5	Aminoglycoside resistance
aph(3'')-Ib	Aminoglycoside resistance
sitABCD	Disinfectant resistance
qacE	Disinfectant resistance
mph(A)	Macrolide resistance
dfrA17	Trimethoprim resistance
tet(A)	Tetracycline resistance
sul2	Sulphonamide resistance
sul1	Sulphonamide resistance
qnrS1	Quinolone resistance
blaOXA-181	Carbapenem resistance
blaCTX-M-27	Third generation cephalosporin resistance

Table S3. Activity of a preventive treatment with inulin and/or pantoprazole on digestive colonization by ESBL-Ec

parameter	estimate	P-value of the estimate	estimate of the interaction with time	interaction P-value	proportion of mice with low level titers
intercept	8.6 (0.2)	< 0.0001			
time (dpi)	-0.2 (0.1)	0.042			
treatment: pantoprazole	-0.3 (0.3)	0.27	-0.1 (0.1)	0.65	2/11 (18%)
treatment: early inulin	0.9 (0.3)	0.008	-0.3 (0.1)	0.021	1/10 (10%)
treatment: early inulin + pantoprazole	-0.7 (0.3)	0.027	-0.3 (0.1)	0.015	7/12 (58%)
treatment: late inulin + pantoprazole	-1.2 (0.3)	< 0.001	-0.2 (0.1)	0.24	4/8 (50%)

Note. ESBL-Ec titers were compared between 1 and 8 dpi using a linear mixed effects model. The intercept represents the theoretical ESBL-Ec titer of the control group at 0 dpi. The time estimate represents the decrease of ESBL-Ec titers with time (0.2 $\log_{10}$ CFU/g/day). For example, the mean predicted ESBL-Ec titer in control mice is 8.4 $\log_{10}$ CFU/g (8.6- 0.2●1) at 1 dpi, and 7.0 $\log_{10}$ CFU/g (8.6- 0.2●8) at 8 dpi. If there is no significant interaction between time and treatment, treatment estimates represent the mean effect of treatment during the study period: for example, the combination of late inulin and pantoprazole decreased ESBL-Ec titers by 1.2 $\log_{10}$ CFU/g in comparison with control. When the time-treatment interaction is significant, its estimate should be added to the treatment effect. For example, the combination of early inulin and pantoprazole decreased ESBL-Ec titers by 1.4 $\log_{10}$ CFU/g (1.2 + 0.2●1) at 1 dpi, and by 2.8 $\log_{10}$ CFU/g (1.2 + 0.2●8) at 8 dpi. Among 19 control mice, none had low ESBL-Ec titers.

Table S4. Differences in microbiota composition, according to the level of ESBL-Ec colonization in control mice and mice treated with early inulin and pantoprazole.

Feature	level of ESBL-Ec colonization			FDR corrected P-value
	high	medium	low	
f__Enterococcaceae.g__Enterococcus.s__Enterococcus_faecalis	8e-01	2e-04	1e-02	0.003
f__Metamycoplasmataceae.g__UBA710.s__UBA710_sp002298905	0e+00	0e+00	8e-06	0.003
f__Muribaculaceae.g__CAG.873.s__CAG.873_MGBC112867	7e-05	1e-05	2e-02	0.003
f__Muribaculaceae.g__Muribaculum.s__Muribaculum_arabinoxylanisolvens	2e-07	0e+00	5e-06	0.015
f__Muribaculaceae.g__CAG.485.s__CAG.485_MGBC118072	0e+00	0e+00	9e-06	0.029
f__Desulfovibrionaceae.g__Mailhella.s__Mailhella_MGBC110551	0e+00	0e+00	4e-06	0.029
f__Lachnospiraceae.g__COE1.s__COE1_sp003513705	8e-07	4e-06	5e-05	0.029
f__Muribaculaceae.g__CAG.485.s__CAG.485_MGBC152290	0e+00	0e+00	1e-06	0.029
f__Lachnospiraceae.g__UBA3282.s__UBA3282_MGBC107550	0e+00	0e+00	9e-06	0.029
f__Muribaculaceae.g__CAG.873.s__CAG.873_MGBC104416	0e+00	0e+00	7e-07	0.029
f__Muribaculaceae.g__UBA7173.s__UBA7173_sp004102805	1e-08	0e+00	2e-06	0.029
f__Muribaculaceae.g__UBA7173.s__UBA7173_MGBC123817	0e+00	0e+00	8e-07	0.029
f__Enterobacteriaceae.g__Klebsiella.s__Klebsiella_variicola	4e-05	2e-04	9e-03	0.029
f__Muribaculaceae.g__Muribaculum.s__Muribaculum_MGBC161558	5e-08	0e+00	1e-06	0.029
f__Rikenellaceae.g__Alistipes.s__Alistipes_MGBC104726	1e-07	0e+00	2e-05	0.031
f__Muribaculaceae.g__CAG.873.s__CAG.873_MGBC163040	0e+00	0e+00	4e-06	0.031
f__Lachnospiraceae.g__Lachnospiraceae_NOV.s__Lachnospiraceae_NOV_MGBC105353	0e+00	7e-07	5e-06	0.031
f__Lachnospiraceae.g__COE1.s__COE1_sp002358575	2e-05	1e-05	1e-04	0.031
f__Muribaculaceae.g__CAG.485.s__CAG.485_MGBC108169	0e+00	0e+00	4e-06	0.036
f__Rikenellaceae.g__Alistipes.s__Alistipes_MGBC143807	2e-07	0e+00	6e-06	0.044

f__Lachnospiraceae.g__UBA3282.s__UBA3282_sp003611805	1e-06	0e+00	2e-05	0.050
f__Lachnospiraceae.g__Acetatifactor.s__Acetatifactor_muris	0e+00	0e+00	6e-06	0.051
f__Lachnospiraceae.g__Eubacterium_J.s__Eubacterium_J_MGBC139490	4e-07	0e+00	6e-06	0.051
f__Lachnospiraceae.g__UBA9502.s__UBA9502_MGBC000489	6e-07	0e+00	1e-05	0.051
f__Enterobacteriaceae.g__Escherichia.s__Escherichia_sp000208585	2e-06	1e-05	3e-05	0.051
f__Anaerotignaceae.g__ASF356.s__ASF356_MGBC163564	0e+00	1e-06	3e-06	0.051
f__Acutalibacteraceae.g__Eubacterium_R.s__Eubacterium_R_MGBC121343	3e-07	0e+00	6e-06	0.051
f__Enterobacteriaceae.g__Klebsiella.s__Klebsiella_pneumoniae	6e-04	2e-03	2e-01	0.051
f__Muribaculaceae.g__UBA7173.s__UBA7173_sp001689485	1e-05	1e-05	6e-05	0.054
f__Muribaculaceae.g__Paramuribaculum.s__Paramuribaculum_sp900553585	2e-06	0e+00	3e-05	0.059
f__Muribaculaceae.g__UBA3263.s__UBA3263_sp001689615	3e-06	1e-06	2e-05	0.062
f__Lachnospiraceae.g__Lachnospiraceae_NOV.s__Lachnospiraceae_NOV_MGBC163085	0e+00	0e+00	4e-06	0.062
f__Muribaculaceae.g__CAG.485.s__CAG.485_sp002362485	4e-05	2e-05	8e-03	0.062
f__Lachnospiraceae.g__UBA7160.s__UBA7160_sp003612585	1e-06	2e-06	3e-03	0.062
f__Lachnospiraceae.g__UBA9502.s__UBA9502_MGBC129914	0e+00	0e+00	4e-06	0.062
f__Lachnospiraceae.g__CAG.95.s__CAG.95_MGBC161056	0e+00	0e+00	9e-06	0.062
f__Muribaculaceae.g__CAG.485.s__CAG.485_MGBC105045	5e-07	0e+00	3e-06	0.062
f__UBA1381.g__CAG.41.s__CAG.41_MGBC132292	3e-06	0e+00	0e+00	0.062
f__Muribaculaceae.g__CAG.873.s__CAG.873_sp001689415	0e+00	0e+00	9e-07	0.062
f__Lachnospiraceae.g__Lachnospiraceae_NOV.s__Lachnospiraceae_NOV_MGBC165132	0e+00	0e+00	3e-06	0.062
f__Enterobacteriaceae.g__Escherichia.s__Escherichia_coli	5e-05	2e-04	2e-03	0.064
f__Eggerthellaceae.g__Adlercreutzia.s__Adlercreutzia_caecimuris	2e-06	2e-06	6e-04	0.068
f__Muribaculaceae.g__Muribaculum.s__Muribaculum_sp002358615	1e-06	6e-07	7e-06	0.069
f__Muribaculaceae.g__Muribaculaceae_NOV.s__Muribaculaceae_NOV_MGBC128991	1e-05	1e-04	1e-02	0.069
f__Lachnospiraceae.g__Lachnospiraceae_NOV.s__Lachnospiraceae_NOV_MGBC131161	0e+00	0e+00	7e-06	0.073

f__Eggerthellaceae.g__Adlercreutzia.s__Adlercreutzia_sp004793465	8e-07	0e+00	6e-04	0.074
f__Lachnospiraceae.g__Dorea.s__Dorea_MGBC115029	0e+00	0e+00	5e-06	0.074
f__Lachnospiraceae.g__UBA9502.s__UBA9502_MGBC105122	0e+00	0e+00	2e-04	0.074
f__Erysipelatoclostridiaceae.g__Longibaculum.s__Longibaculum_muris	0e+00	3e-07	6e-06	0.076
f__Oscillospiraceae.g__Marseille.P3106.s__Marseille.P3106_MGBC161418	0e+00	0e+00	6e-04	0.076
f__Erysipelatoclostridiaceae.g__Erysipelatoclostridiaceae_NOV.s__Erysipelatoclostridiaceae_NOV_MGBC113645	2e-07	2e-05	2e-05	0.076
f__Lachnospiraceae.g__Dorea.s__Dorea_MGBC109699	0e+00	4e-06	7e-06	0.076
f__Lachnospiraceae.g__Schaedlerella.s__Schaedlerella_MGBC166500	0e+00	1e-06	3e-06	0.076
f__Muribaculaceae.g__Duncaniella.s__Duncaniella_MGBC102213	0e+00	0e+00	2e-06	0.076
f__Lachnospiraceae.g__Hungatella_A.s__Hungatella_A_MGBC000080	0e+00	0e+00	2e-05	0.076
f__Muribaculaceae.g__CAG.485.s__CAG.485_MGBC165708	2e-07	0e+00	5e-06	0.076
f__Muribaculaceae.g__Paramuribaculum.s__Paramuribaculum_sp001689565	9e-07	2e-06	1e-05	0.076
f__CAG.239.g__CAG.495.s__CAG.495_MGBC104355	0e+00	0e+00	4e-06	0.076
f__Muribaculaceae.g__CAG.873.s__CAG.873_MGBC111877	0e+00	8e-06	6e-04	0.078
f__Lachnospiraceae.g__CAG.95.s__CAG.95_MGBC114603	0e+00	0e+00	2e-05	0.079
f__Muribaculaceae.g__Duncaniella.s__Duncaniella_sp001689425	1e-04	2e-05	2e-03	0.079
f__Muribaculaceae.g__Duncaniella.s__Duncaniella_sp002492665	5e-05	4e-06	4e-04	0.079
f__Muribaculaceae.g__Duncaniella.s__Duncaniella_muris	2e-05	2e-05	2e-03	0.079
f__Muribaculaceae.g__Muribaculum.s__Muribaculum_intestinale	3e-05	5e-05	1e-01	0.082
f__Lachnospiraceae.g__Eubacterium_F.s__Eubacterium_F_MGBC164771	2e-06	6e-06	1e-05	0.083
f__Anaerovoracaceae.g__Emergencia.s__Emergencia_MGBC000541	1e-06	0e+00	1e-05	0.083
f__Tannerellaceae.g__Parabacteroides.s__Parabacteroides_merdae	2e-07	0e+00	9e-07	0.089
f__Helicobacteraceae.g__Helicobacter_C.s__Helicobacter_C_typhlonius	4e-06	4e-06	3e-05	0.089
f__Muribaculaceae.g__UBA7173.s__UBA7173_sp002491305	3e-05	4e-06	5e-03	0.089

f__Rikenellaceae.g__Alistipes.s__Alistipes_sp002362235	1e-05	8e-06	3e-02	0.089
f__Rikenellaceae.g__Alistipes.s__Alistipes_sp003979135	4e-06	1e-06	1e-05	0.089
f__Muribaculaceae.g__CAG.485.s__CAG.485_sp002491945	8e-08	0e+00	4e-05	0.089
f__Lachnospiraceae.g__14.2.s__14.2_MGBC116940	2e-07	1e-06	4e-06	0.097
f__Lachnospiraceae.g__Kineothrix.s__Kineothrix_MGBC162921	0e+00	0e+00	1e-05	0.098

Table S5. Differences in microbiota composition, according to the level of ESBL-Ec colonization in mice treated with early inulin and pantoprazole (high vs low level of ESBL-Ec colonization)

	ESBL-Ec mean titers			
species	**high**	**low**	**generalized fold change**	**FDR corrected P-value**
Alistipes_MGBC111863	5e-07	1e-03	-1,3	0.051
UBA7173_sp002491305	6e-06	5e-03	-2,6	0.051
Duncaniella_muris	2e-05	2e-03	-1,6	0.051
CAG.485_sp003979075	5e-06	9e-04	-1,3	0.051
CAG.485_sp002493045	1e-04	2e-02	-1,5	0.051
Muribaculaceae_NOV_MGBC128991	2e-05	1e-02	-2,4	0.051
CAG.873_MGBC111877	0e+00	6e-04	-2,2	0.051
CAG.873_MGBC112867	4e-05	2e-02	-2,8	0.051
Muribaculum_intestinale	5e-05	1e-01	-2,9	0.051
Escherichia_flexneri	1e-04	3e-02	-1,8	0.051
Escherichia_coli	4e-06	2e-03	-2,8	0.051
Klebsiella_variicola	1e-05	9e-03	-2,8	0.051
Klebsiella_pneumoniae	4e-04	2e-01	-2,7	0.051
Enterococcus_faecalis	8e-01	1e-02	2,3	0.051
UMGS1370_MGBC104963	5e-06	8e-03	-1,9	0.051
UBA7160_sp003612585	3e-06	3e-03	-2,6	0.051
Alistipes_sp002362235	1e-05	3e-02	-2,2	0.080
Enterobacter_himalayensis	3e-05	9e-02	-0,8	0.080
Marseille.P3106_MGBC161418	0e+00	6e-04	-2	0.083
UBA9502_MGBC105355	0e+00	2e-03	-1,5	0.083
Parvibacter_MGBC000220	0e+00	3e-04	-1,5	0.083
Prevotellamassilia_sp002933955	7e-05	1e-02	-1,5	0.094
RC9_sp002298075	5e-05	9e-02	-2,2	0.094
Duncaniella_sp001689425	5e-05	2e-03	-1,5	0.094
CAG.485_sp002362485	6e-05	8e-03	-1,9	0.094
CAG.873_MGBC142695	1e-05	2e-03	-1,3	0.094

Table S6. Activity of a preventive treatment with *Adlercreutzia muris* or *Adlercreutzia caecimuris* on digestive colonisation by ESBL-Ec

parameter	estimate	P-value of the estimate
intercept	7.8 (0.4)	< 0.0001
time (dpi)	-0.2 (0.1)	0.67
treatment: *A. caecimuris*	-2.0 (0.5)	< 0.0001
treatment: *A. muris*	-1.6 (0.5)	< 0.001

Note. ESBL-Ec titers were compared between 1 and 8 dpi using a linear mixed effects model. The intercept represents the theoretical ESBL-Ec titer of the control group at 0 dpi. The time estimate represents the decrease of ESBL-Ec titers with time, and was not significantly different from 0. Treatment estimates represent the mean effect of treatment during the study period: for example, preventive treatment with *A. caecimuris* decreased ESBL-Ec titers by 2.0 $\log_{10}$ CFU/g in comparison with control.

Table S7. Mean relative abundance of antibiotic resistance genes in control and early inulin pantoprazole treated mice, according to their level of ESBL-Ec colonization

	Mean relative abundance				
Antibiotic resistance gene	**high level**	**medium level**	**low_level**	**P-value**	**q-value**
multidrug__multidrug_ABC_transporter	6e-03	1e-02	1e-03	< 0.0001	0.002
multidrug__EmrB-QacA family major facilitator transporter	6e-01	3e-02	5e-02	< 0.0001	0.005
multidrug__mdtD	2e-03	2e-02	3e-02	< 0.001	0.007
multidrug__omp36	3e-03	3e-02	5e-02	< 0.001	0.007
macrolide-lincosamide-streptogramin__lsa	2e-01	2e-04	3e-03	< 0.001	0.013
multidrug__mdtM	6e-05	0e+00	4e-02	< 0.001	0.024
multidrug__ompF	1e-02	1e-01	1e-01	< 0.001	0.026
multidrug__acrF	2e-03	1e-02	2e-02	0.001	0.027
multidrug__emrB	8e-03	7e-02	7e-02	0.001	0.027
beta-lactam__SHV-112	0e+00	0e+00	2e-03	0.001	0.027
beta-lactam__SHV-152	0e+00	0e+00	9e-04	0.002	0.027
multidrug__mexB	2e-04	2e-03	1e-03	0.002	0.027
unclassified__truncated putative response regulator ArlR	1e-01	0e+00	2e-02	0.002	0.027
multidrug__bpeF	4e-05	5e-04	4e-04	0.002	0.027
unclassified__bacterial regulatory protein LuxR	4e-05	0e+00	5e-02	0.002	0.027
beta-lactam__SHV-1	1e-05	0e+00	4e-03	0.002	0.027
tetracycline__tetA	2e-05	0e+00	2e-02	0.002	0.027
beta-lactam__SHV-6	5e-06	0e+00	4e-03	0.003	0.027
multidrug__mexX	1e-02	9e-02	8e-02	0.003	0.027
beta-lactam__SHV-53	0e+00	0e+00	9e-03	0.003	0.028
beta-lactam__penA	2e-05	1e-04	1e-02	0.003	0.028
tetracycline__tetO	4e-03	0e+00	6e-06	0.003	0.028
beta-lactam__SHV-39	1e-05	0e+00	1e-02	0.003	0.028
beta-lactam__SHV-167	0e+00	0e+00	1e-03	0.004	0.031
multidrug__major_facilitator_superfamily_transporter	1e-01	1e-01	4e-02	0.004	0.033
multidrug__smeE	0e+00	5e-05	2e-05	0.005	0.034
beta-lactam__SHV-4	0e+00	0e+00	3e-03	0.005	0.034
tetracycline__tetM	4e-01	0e+00	6e-03	0.006	0.040
beta-lactam__PBP-1B	8e-05	8e-04	5e-04	0.006	0.041
multidrug__mdtN	4e-05	0e+00	5e-03	0.007	0.045
beta-lactam__SHV-51	0e+00	0e+00	6e-04	0.007	0.045
beta-lactam__SHV	0e+00	0e+00	8e-04	0.007	0.045
beta-lactam__SHV-12	0e+00	0e+00	3e-03	0.008	0.046
multidrug__oprC	7e-05	8e-04	5e-04	0.009	0.051

unclassified__cAMP-regulatory protein	1e-02	1e-01	9e-02	0.009	0.051
multidrug__acrA	2e-02	2e-01	1e-01	0.010	0.051
beta-lactam__OXA-9	3e-04	3e-03	2e-03	0.010	0.051
kasugamycin__kasugamycin resistance protein ksgA	1e-02	1e-01	9e-02	0.010	0.053
fosfomycin__fosX	0e+00	0e+00	1e-03	0.011	0.053
unclassified__cob(I)alamin adenolsyltransferase	7e-03	7e-02	4e-02	0.012	0.055
beta-lactam__SHV-70	0e+00	0e+00	5e-05	0.012	0.055
multidrug__emrA	1e-02	1e-01	7e-02	0.013	0.057
beta-lactam__SHV-172	0e+00	0e+00	7e-05	0.013	0.057
vancomycin__vanT	0e+00	0e+00	3e-03	0.013	0.057
macrolide-lincosamide-streptogramin__macA	9e-03	8e-02	5e-02	0.013	0.057
multidrug__emrD	1e-02	1e-01	8e-02	0.014	0.057
multidrug__bicyclomycin-multidrug_efflux_protein_bcr	1e-02	8e-02	6e-02	0.014	0.057
multidrug__TolC	2e-02	2e-01	1e-01	0.015	0.059
beta-lactam__SHV-140	0e+00	0e+00	7e-05	0.015	0.059
multidrug__mdtL	1e-02	1e-01	6e-02	0.016	0.060
vancomycin__vanY	2e-04	3e-04	3e-02	0.016	0.060
vancomycin__vanC	4e-06	0e+00	9e-03	0.017	0.063
tetracycline__tetS	5e-04	0e+00	1e-04	0.021	0.076
macrolide-lincosamide-streptogramin__macB	1e-02	9e-02	5e-02	0.022	0.079
fosmidomycin__rosA	5e-03	5e-02	3e-02	0.023	0.079
beta-lactam__SHV-28	0e+00	0e+00	1e-04	0.023	0.080
multidrug__mdtF	3e-04	2e-03	6e-03	0.025	0.085
multidrug__mdtA	1e-02	8e-02	5e-02	0.026	0.086
aminoglycoside__aph(3'')-III	5e-06	0e+00	5e-03	0.027	0.086
aminoglycoside__aadE	0e+00	0e+00	6e-03	0.028	0.086
multidrug__ompR	2e-02	1e-01	8e-02	0.028	0.086
beta-lactam__SHV-5	0e+00	0e+00	5e-05	0.028	0.086
multidrug__mdtC	1e-02	9e-02	5e-02	0.029	0.089
vancomycin__vanR	4e-03	2e-02	3e-02	0.030	0.089
multidrug__mdtB	1e-02	1e-01	6e-02	0.030	0.089
unclassified__DNA-binding_protein_H-NS	4e-03	4e-02	2e-02	0.032	0.091
multidrug__acrB	2e-02	2e-01	1e-01	0.032	0.091
unclassified__transcriptional regulatory protein CpxR cpxR	2e-02	1e-01	8e-02	0.032	0.091
fosmidomycin__rosB	8e-03	7e-02	4e-02	0.033	0.091
beta-lactam__SHV-154	0e+00	0e+00	1e-04	0.035	0.095
multidrug__mdtH	1e-02	1e-01	7e-02	0.035	0.096
multidrug__mdtG	1e-02	1e-01	6e-02	0.036	0.096
tetracycline__tetracycline_resistance_protein	1e-02	0e+00	7e-04	0.038	0.100

Note. Only ARG that were differentially abundant between samples from mice who had high, medium or low colonization at a level of FDR of 0.10 are shown.

2.3 *Adlercreutzia spp.:* supplementary informations

Adlercreutzia (the original name is *Enterorhabdus*) is a gram-positive non-motile strictly anaerobic genus belonging to Actinobacteria phylum, Coriobacteriia class and *Eggerthellaceae* family (141,142); (Figure18, MIHAR lab). *Adlercreutzia* was named by H. Adlercreutz (Professor at university of Helsinki, Finland) (142), He described it as a greyish white, circular, smooth colony with 1–2 mm diameter on blood liver agar plates. *Adlercreutzia* is not a SCFA producing bacterium, and it contains specific enzymes of glutamate decarboxylase and arginine dihydrolase that enable it to utilize arginine, but no enzymes able to hydrolyze polysaccharides. Besides, the rhamnose is its only usable sugar (143).

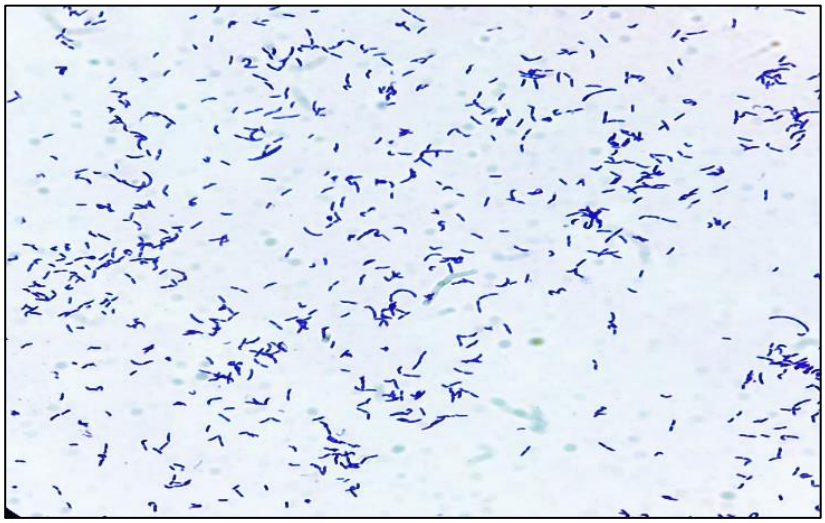


Figure 18: *Adlercreutzia* gram staining, MIHAR lab, Nantes university, 2022.

There are several species of *Adlercreutzia* including *A. equolifaciens, A. caecimuris, A. muris* , *A. caecicola* , *A. hattorii* , *A .mucosicola,* and *A. rubneri*. Some of them like *A. equolifaciens* produce Equol from soybean extract (141,142,144)). Equol is a metabolite of the isoflavone daidzein, found in various plants especially soybeans (142). Scarce studies referred that Equol metabolite displays anti-bacterial and anti-virulence activities (145-147). It has been also shown that it was able to inhibit *in vitro* the carbapenemase expression of *E. coli* (CR-Ec) OXA-48 strain (145).

Other species like *A. caecimuris (*previously *Enterorhabdus caecimuris*) does not produce Equol, and is resistant to cefotaxime. *Adlercreutzia muris* (previously *Enterorhabdus muris)*, seems to be not able to produce equol since synthesis genes are not found in its genome (143). In fact, most studies on *Adlercreutzia* concerned the taxonomic analyses of this genus, and little is known about its activity. Consequently, up to now we are not able to explain their activity to partially prevent the ESBL-Ec colonization of mice.

Manuscript n°2: Effect of *Escherichia coli* and *Akkermansia muciniphila* with or without pantoprazole on the intestinal colonization by ESBL – producing *E. coli* in a murine model

Murad Ishnaiwer, Michel Dion and Eric Batard
UR1155
Institute for Health Research 2 (IRS2)
Nantes University

General Introduction

Third generation cephalosporins and carbapenems are critically important antimicrobials. Resistance to these antibiotics is often associated with multidrug resistance in *Enterobacterales*. The main reservoir of multidrug resistant *Enterobacterales* (MDR-EB) is the gut, and asymptomatic intestinal colonization by MDR-EB may evolve to infections and death, and to contamination of the environment and healthy and diseased subjects (Feehan & Garcia-Diaz, 2020; Sorbara et al., 2019). Therefore, there is an urgent need to find new treatments of MDR-EB intestinal colonization. Hence, several non-antibiotic approaches such as fecal microbiota transplantation (FMT) (Huttner et al., 2019; Saha et al., 2019; Davido et al., 2017; Dinh et al., 2018; Singh et al., 2018; Mahieu et al., 2017) or bacteriophages (Javaudin et al., 2021) have been explored so far, but effective treatments remain to be identified. Currently, there is a growing interest in using probiotics to eradicate MDR-EB intestinal colonization (Ishnaiwer et al., 2022; Silva et al., 2021; Wieërs et al., 2021; Ljungquist et al., 2020; Romario-Silva et al., 2020). Probiotics as live bacteria may contribute to restore perturbations in the composition of intestinal microbial and to the maintenance of healthy gut. However, to date, currently used probiotics have shown little or no activity in treating MDR-EB intestinal carriage (Kumar et al., 2021;Dall et al., 2019; Tannock et al., 2011). Therefore, new probiotics against MDR-EB colonization should be assessed.

2.4.1 Effect of *E. coli* with or without pantoprazole on ESBL-producing *E. coli* intestinal colonization

ABSTRACT

Aims: *Escherichia coli* naturally colonizes the mammalian gastrointestinal tract and may contribute to the maintenance of gut health. Our objective was to assess the efficacy of commensal *E. coli* (comEc) on digestive colonization by extended spectrum β-lactamase producing *E. coli* (ESBL-Ec) in a murine model.

Methods: Intestinal dysbiosis was induced in male Swiss mice by amoxicillin (0.5 g/L) in drinking water for 3 days. comEc was intragastrically administered 1 day before the intragastric inoculation of the ESBL-Ec. ESBL-Ec fecal titers were assessed by daily culture from 0 to 8 days post-inoculation (dpi). Microbiome was assessed by shotgun metagenomic sequencing.

Results: In comparison with control mice, the preventive treatment with a mix of 3 strains of comEc had higher activity than single strains. It decreased significantly ESBL-Ec titers by 2.6 (0.3) log10 CFU/g (P-value, <0.0001), but the effect gradually decreased between 1 and 8 dpi. Metagenomic analysis found no taxa associated with treatment efficacy.

Conclusions: Our findings showed a temporary preventive activity of comEc against intestinal colonization by ESBL-Ec. This study may open new avenues for treatment of intestinal colonization by multidrug resistant *Enterobacterales*.

Keywords: Extended spectrum β-lactamase producing E. coli (ESBL-Ec), commensal Escherichia coli (comEc), multidrug resistant *Enterobacterales* (MDRE).

Introduction

Commensal *E. coli* naturally colonize the mammalian gastrointestinal tract and may contribute to the maintenance of gut health (Maltby et al., 2013). It is a facultative anaerobic bacterium, which could provide colonization resistance against pathogens through direct competition for similar nutrients and niche, or by secreting antimicrobial compounds. (Conway & Cohen, 2015; Khan et al., 2019). The combination of *E. coli* strain HS and *E. coli* Nissle 1917 provided prophylactic activity against enterohemorrhagic *E. coli* (EHEC) strain EDL933 (O157:H7) infections in streptomycin-dysbiosed mice (Maltby et al., 2013). Likewise, *E. coli* strain CEC15 reduced severity of chronic colitis in mouse (Escribano-Vazquez et al., 2019). Moreover, our research group have found during metagenomic sequencing of the DNA of mice feces spontaneously decolonized that *E. coli* was over-represented in mice effectively treated by the combination of inulin and pantoprazole (see manuscript 1 treating of ESBL-Ec colonization with inulin and pantoprazole).

This study addressed the efficacy of murine commensal *E. coli* (comEc) as a preventive treatment of intestinal colonization by ESBL-Ec in a murine model. The combination of comEc with pantoprazole was also tested, as pantoprazole has been shown to reduce gastric acidity and to facilitate the bacterial colonization in mice (Stiefel et al., 2006). We also tried to predict the efficacy of the treatment with comEc from microbiota architecture assessed by metagenomic analysis of feces.

MATERIALS AND METHODS

Bacterial strains and media

The clinical ESBL-Ec isolate was collected from University Hospital of Nantes (Ishnaiwer et al., 2022), It is resistant to amoxicillin, and to cephalosporines and carbapenems through an ESBL and an OXA-48 carbapenemase respectively. ComEc strains (Z, Q and 3) were isolated from feces of mice treated with amoxicillin. All are resistant to amoxicillin. ComEc isolates were identified through PCR amplification of *gadE* gene (Tillman et al., 2015). We checked by ERIC PCR that they were genetically different (Adamus-Bialek et al., 2009) (data not shown)**.** Total *E. coli* titers (Commensal + ESBL-Ec) were determined by culture on LB agar with chromogenic substrate X-GlcA of glucuronidase (0.006%) and 0.1% desoxycholate, while ChromID^{TM} ESBL agar plates (Biomerieux) were used to enumerate ESBL-Ec*.*

Intestinal colonization of ESBL-Ec in a murine model

All *in vivo* experiments were handled by applying the animal ethics rule, and with consent of the Animal experiment committee of Pays de la Loire (France, authorization number APAFIS#18120). Six weeks old male Swiss mice (Janvier Labs, Saint-Berthevin, France) were housed separately to avert cross contamination, in sterile pathogen-free conditions with free access to food and water. Drinking water was initially supplemented with amoxicillin (0, 5 g L-1) for 3 days to disrupt intestinal microbiota and facilitate mice ESBL-Ec colonization. Pantoprazole was also added to drinking water for some mice at the first day of amoxicillin (Stiefel et al., 2006). ESBL-Ec (10^6 CFU per mouse) was intragastrically inoculated 2 days after amoxicillin cessation (0 dpi). ComEc was intragastrically administered for each mouse (10^8 CFU,) the day before the ESBL-Ec inoculation (-1 dpi). Mice were randomly allocated to: control (n=19), pantoprazole (n=11), comEc strain 3 (n=23), comEc strain 3 combined with pantoprazole (n=4), comEc strain Z (n=7), comEc

strain Q (n=7), and the combination of comEc strains Z, Q and 3 (comEc mix, n=12). Experimental design is shown in Figure 2A.

Fecal samples were collected daily and frozen at -80˚C. Bacterial isolation and enumeration were performed from homogeneous suspension prepared by shaking ~ 50 mg of daily collected mouse feces in 1 ml of sterile water at 20 Hz (Mixer Mill MM 400, RETSCH's) for 5 min. ESBL-Ec, and comEc fecal titers were determined by daily culture from 0 to 8 days post-inoculation (dpi) and incubation at 37°C for 24 h in aerobic conditions. ComEc titers was determined by subtracting ESBL-Ec titers from total *E. coli* titers.

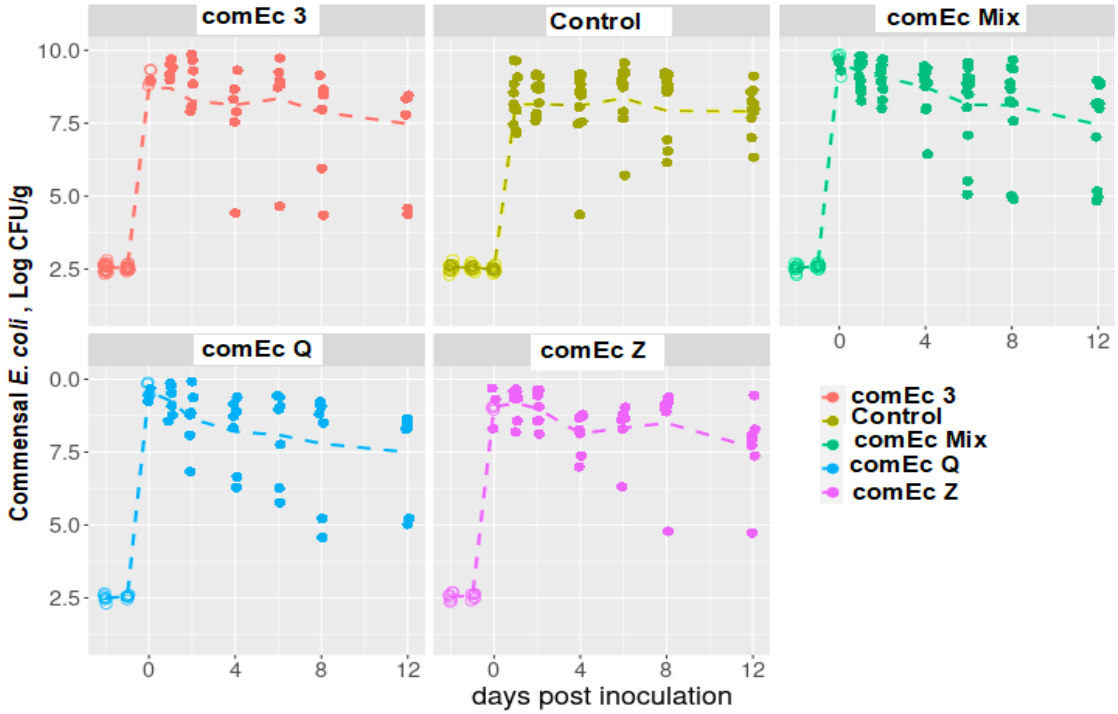
Statistical and metagenomic analyzes were performed as described in the manuscript on inulin.

RESULTS

In vivo activity of commensal *E. coli* in a murine model of ESBL-Ec colonization

We first assessed the fecal titers of comEc before and after inoculation. Hence, after 1 day of inoculation, all comEc treated mice had high titers, with average ranging from 9.4 ±0.4 $\log_{10}$ CFU/g at 0 dpi (i.e. one day after comEc inoculation) and 9.4 ±0.16 $\log_{10}$ CFU/g at 1 dpi (i.e. 2 days after comEc inoculation). By contrast, endogenous comEc fecal titers of control group was not detected at 0 dpi (titers of 2.5±0.1 $\log_{10}$ CFU/g), and 8.1±0.8 $\log_{10}$ CFU/g at 1 dpi (**Figure 1**).

Figure 1: Effect of treatment on fecal titers (log_{10} cfu / g) of commensal *E. coli*



Note. Commensal *E. coli* titers were assessed by subtracting ESBL-Ec titers from total *E. coli* titers. They are the sum of endogenous *E. coli* and comEc administered as an experimental treatment.

We then assessed the influence of different preventive treatments on ESBL-Ec fecal titers. To this end, ESBL-Ec fecal titers were compared between treated and control mice using multivariate model assessment for the period between 1 and 8 dpi (**Table1**).

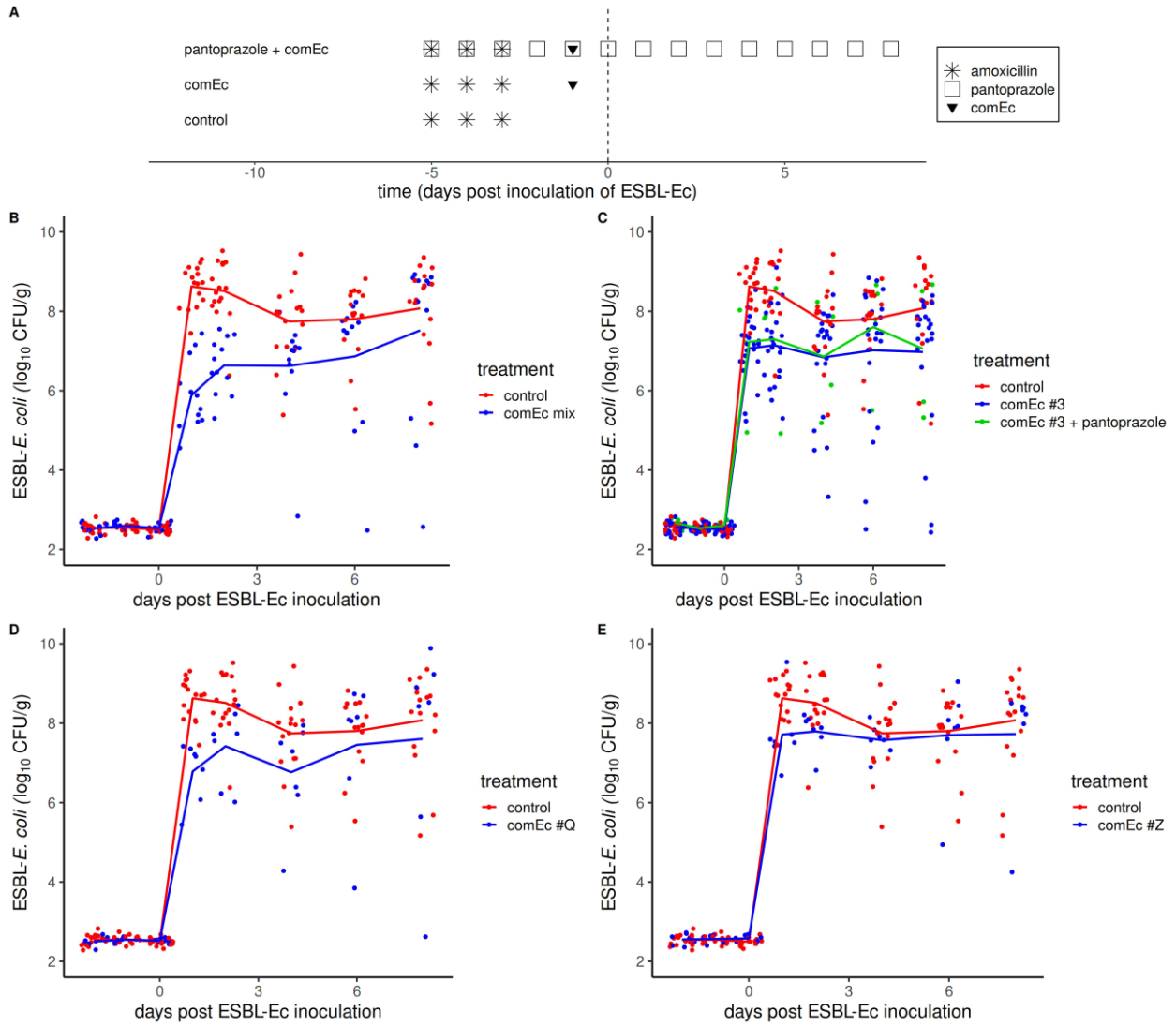
TABLE1: Activity of a preventive treatment of commensal *E. coli* strains with/without pantoprazole on digestive colonization by ESBL-Ec.

parameter	estimate	P-value of the estimate	Estimate of the interaction with time	p-value of the interaction	Proportion of mice with low level titers
Intercept	8.6(0.2)	<0.0001	NA	NA	NA
Time(dpi0)	-0.2(0.1)	0.042	NA	NA	NA
Treatment: pantoprazole	-0.3(0.3)	0.27	-0.1(0.1)	0.65	2/11 (18%)
Treatment: comEc mix	-2.6(0.3)	<0.0001	0.3(0.1)	0.025	2/12 (17%)
Treatment: comEc#3	-1.5(0.3)	<0.0001	0.1(0.1)	0.47	4/23(17%)
Treatment: comEc#3+pantoprazole	-1.3(0.4)	0.003	0.1(0.2)	0.61	1/4 (25%)
Treatment: comEc#Z	-0.8(0.4)	0.020	0.1(0.2)	0.54	0/7(0%)
Treatment: comEc#Q	-1.7(0.4)	<0.0001	0.2(0.2)	0.22	1/7(14%)

Note. dpi, days post-inoculation. NA, not applicable. A linear mixed effects model was used to estimate ESBL-Ec fecal titers between 1 and 8 dpi. The model fixed effects were treatment effect, time effect and the interaction between time and treatment. Control was the reference group for treatment. The proportion of control mice with low level titers was 0 among 19 (0%).

After a strong increase, ESBL-Ec fecal titers slightly decreased with time in the control group, by 0.2 ± 0.1 log10 CFU/g/day. Hence, in comparison with control group, the preventive treatment with comEc mix decreased significantly ESBL-Ec titers by 2.6 (0.3) log10 CFU/g (P-value, <0.0001), but the effect gradually decreased between 1 and 8 dpi (**Figure 2B**). ComEc strain3 with and without pantoprazole decreased ESBL-Ec titers by 1.4 (0.2) log10 CFU/g (P-value, <0.0001) and 1.3 (0.4) log10 CFU/g (P-value, 0.002), respectively, with a constant effect over time (**Figure 2C**). The comEc strain Z without pantoprazole decreased ESBL-Ec titers by 0.8 (0.4) log10 CFU/g (P-value, <0.023) with a constant effect over time. The comEc strain Q without pantoprazole decreased ESBL-Ec titers by 1.7 (0.4) log10 CFU/g (P-value, <0.0001), but the effect gradually decreased by 0.2 (0.2) log10 CFU/g (P-value, <0.22) each day (**Figure 2D**). No significant efficacy was achieved with pantoprazole alone.

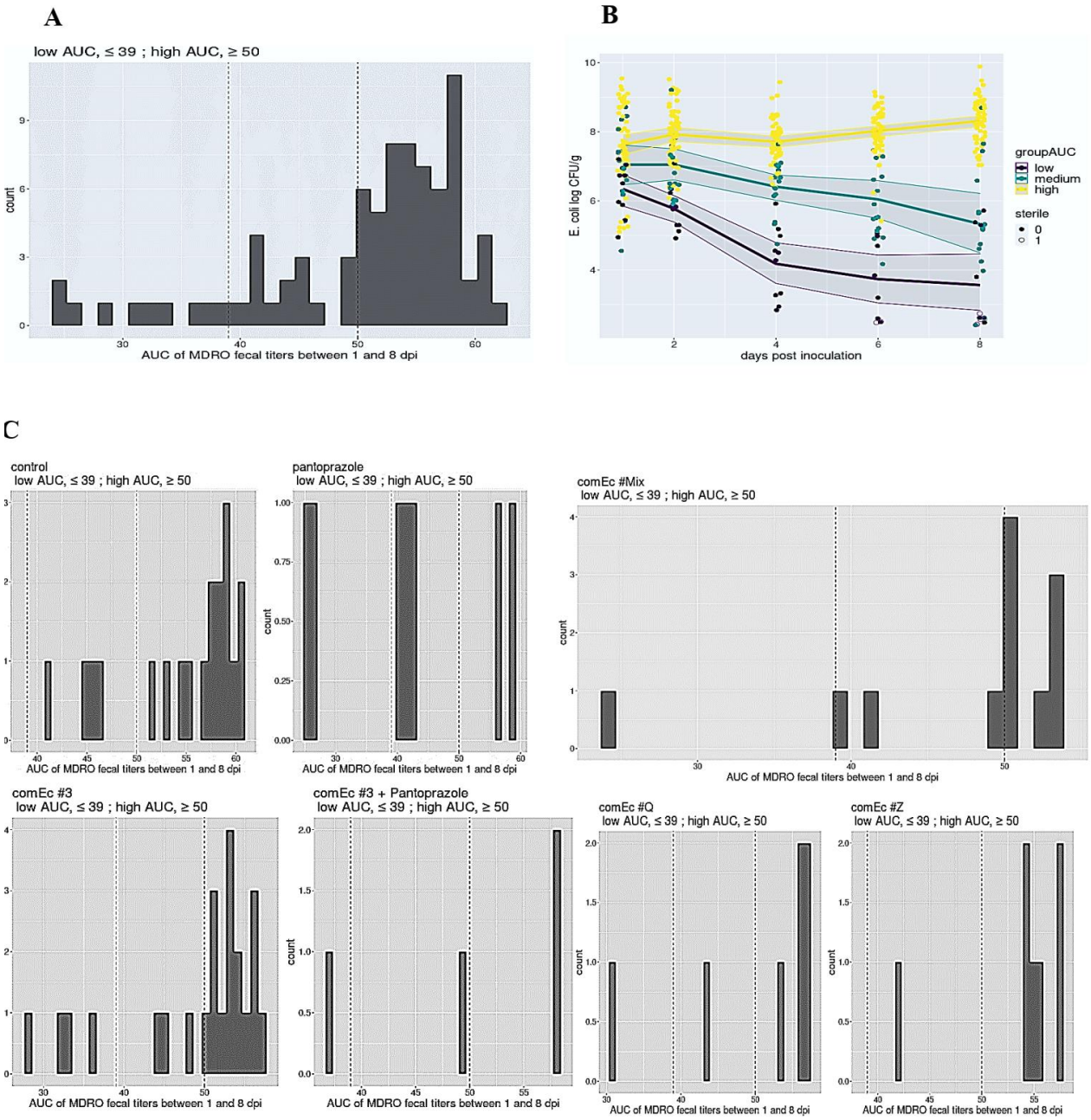
FIGURE 2: Effect of treatment with commensal isolates of *E. coli* and/or pantoprazole on fecal colonization by ESBL producing *E. coli*



Note. (A) murine model experimental design; Control versus (B) comEc mix, (C) comEc3 ± pantoprazole (D) comEc Q (E) comEcZ.

The treatment activity on ESBL-Ec titers was summarized for each mouse by computing the area under curve (AUC) of ESBL-Ec titers between 1 and 8 dpi. Overall AUCs ranged between 24 and 62 $\log_{10}$ CFU·day·g^{-1} (**Figure 3**). According to their AUC, mice were grouped in 3 levels of ESBL-Ec colonization (low, medium and high, defined as ≤ 39, between 39 and 50 or ≥50 log10 CFU·day·g^{-1}, respectively). No mice had low AUC among the 19 controls; while 8 among 53 (15%) commensal *E. coli* treated mice had low titers (Chi-square test, P-value, 0.17). By contrast, among 11 pantoprazole treated mice, 2 (18%) mice had low AUC.

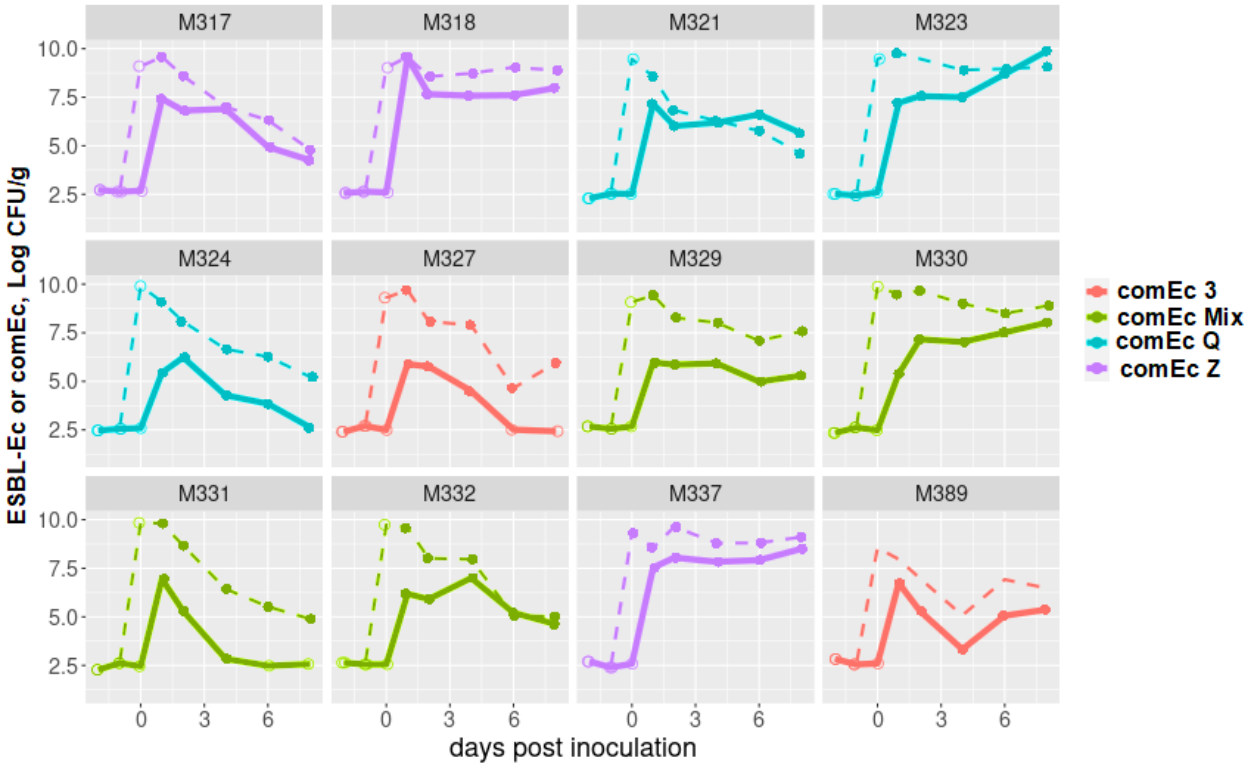
Figure 3*:* Classification of mice according to their ESBL-Ec titers with different treatments: comEc, control, and pantoprazole



Note. Area under curve of the time variation of ESBL-Ec titers was computed for each mouse between 1 and 8 dpi. (A) Distribution of AUCs for all mice. AUCs were divided in 3 groups (low, medium and high). (B) Mean (95% confidence interval) fecal titers of ESBL-Ec according to the AUC group. (C) Distribution of AUCs according to the treatment.

Moreover, analysis of individual mice titers revealed that comEc titers evolved similarly to ESBL-Ec titers: they decreased in mice with low level colonization by ESBL-Ec and remained high in mice in whom *E. coli* probiotic was not effective (**Figure 4**).

Figure 4: Parallel association between ESBL-Ec and comEc titers in mice subgroups.



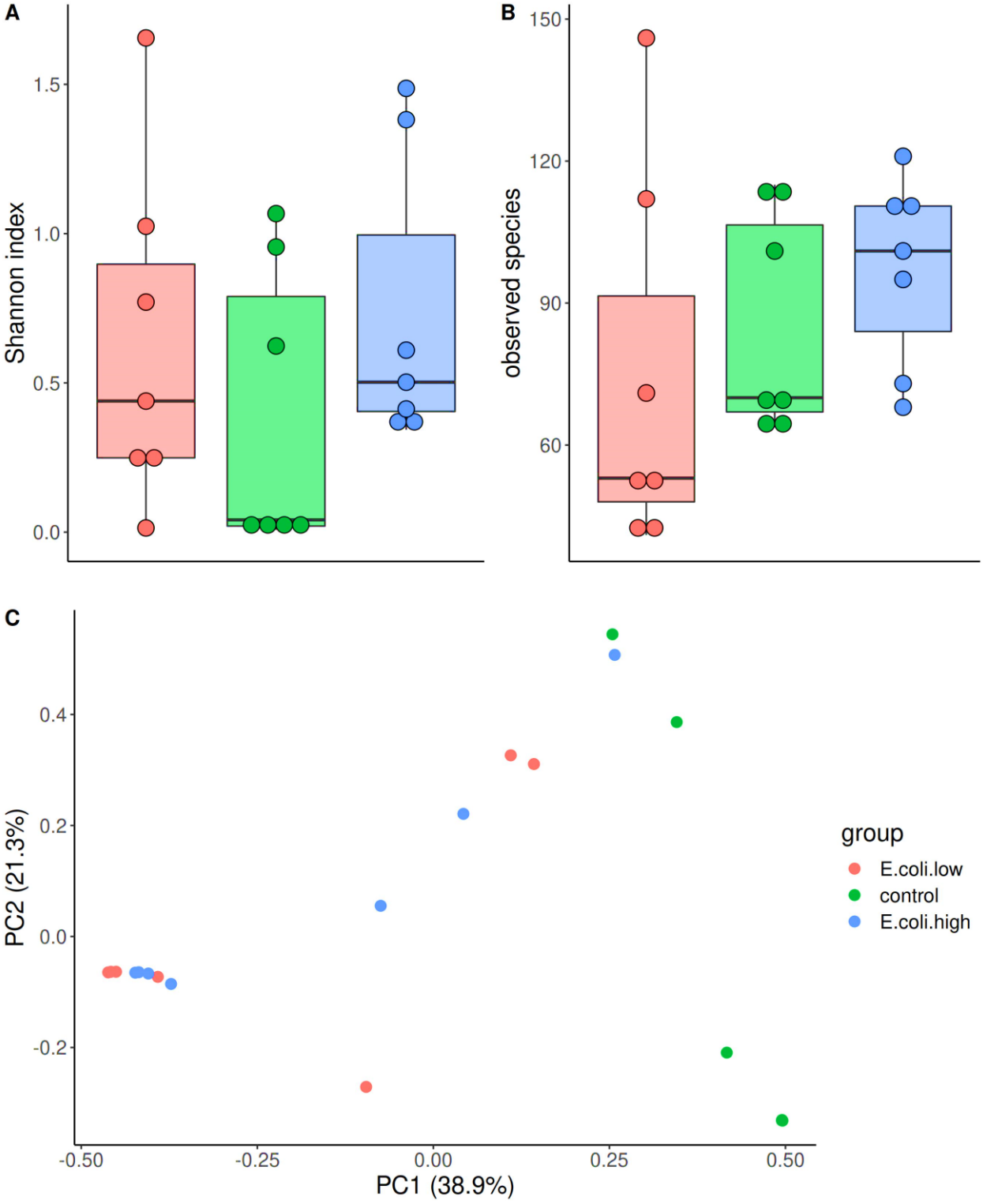
Note: Full line: ESBL-Ec; dashed line: comEc

Metagenomic analysis

We assessed fecal microbiome composition by using shotgun metagenomic sequencing of fecal DNA in 7 control mice (including 6 high and 1 medium level ESBL-Ec colonization), 7 effectively treated (low level ESBL-Ec colonization) and 7 ineffectively treated (high level ESBL-Ec colonization) mice. Among 923 species, 184 were detected in at least 4 samples and were compared between these 3 groups.

We found that alpha-diversity was not significantly different among 3 groups of mice: Kruskall-Wallis test P-value for Shannon index and number of observed species were 0.43 and 0.27 respectively (Figure 5A and 5B). Microbiota architecture, assessed using Principal Coordinates Analysis (PcoA), was not frankly different in the 3 groups of mice (Figure 5C).

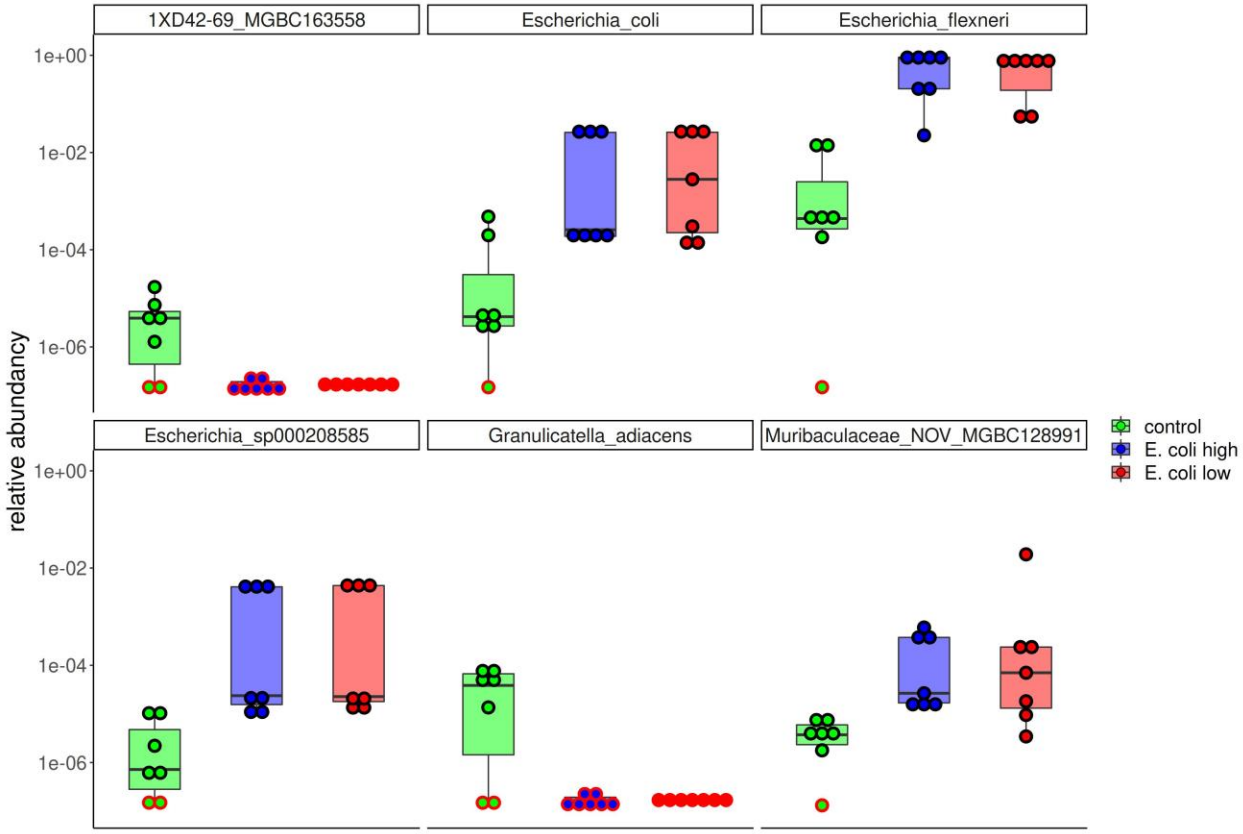
Figure 5. Comparison of metagenomes of control mice and mice treated effectively of ineffectively with comEc



Note. Alpha diversity (A and B) and microbiome architecture (C). Green, control mice; red, mice effectively treated with comEc (low level AUC of ESBL-Ec titers between 1- and 8-day post inoculation); blue, mice ineffectively treated with comEc (high level AUC of ESBL-Ec titers).

Among 182 taxa, none was identified as predictive for treatment efficacy by FDR corrected Kruskall-Wallis test at the prespecified 0.10 threshold. However, the FDR corrected Kruskall-Wallis was slightly above 0.10, i.e., between 0.102 and 0.121, for 5 species. These taxa were *Escherichia*_sp000208585, the so-called *Escherichia_flexneri, Granulicatella_adiacens,* 1XD42-69_MGBC163558 and *Muribaculaceae*_NOV_MGBC128991. However, Dunns test showed that none of these taxa had different relative abundances between effectively and ineffectively treated mice (Figure 6).

Figure 6. Unsuccessful search for taxa predictive of the efficacy of treatment with commensal *E. coli*



Note. Shotgun metagenomic sequencing identified no species that had significantly different relative abundances at 0 dpi (i.e. just before ESBL-Ec inoculation) between effectively treated mice (*E coli* low), ineffectively treated mice (*E. coli* high) and control at the FDR corrected P-value threshold of 0.10 (Kruskall-Wallis test). Five species had differences near to statistical significance, and are represented here in addition with *E. coli*. Red strokes indicate undetected taxon (lower limit of detection).

DISCUSSION

We found a significant activity of comEc strains combined or not with pantoprazole, to decrease ESBL-Ec fecal titers. However, this activity was temporary and low ESBL-Ec titers were only obtained in 15 % of comEc treated mice. Therefore, it was essential to study the microbiota composition between effectively, ineffectively treated and control mice by metagenomic analysis. These analyses allowed first to check the increase of *E. coli* after their administration.

Hence, we found an early activity of comEc single and multi-strains mixture to decrease ESBL-Ec. Such activity could be related to the known ability of comEc to compete with other *E. coli* strains for nutrients /niche, or by producing antimicrobial compounds (Khan et al., 2019; Conway & Cohen, 2015;). Moreover, we found better activity with comEc multi-strains mixture than with single strains, suggesting a synergy between these strains, since all comEc treatments were given with the same total dose of comEc (10^8 CFU per mouse). However, the parallel decrease of comEc with ESBL-Ec in strongly decolonized mice indicates that another parameter(s) contribute to decolonization, like other taxa which remain to be identified. We therefore performed metagenomic analysis of mice microbiota DNA submitted to commensal *E. coli* treatments, to identify these potential microbiota taxa. However, these analyses are to be refined.

Conclusions

Treatment with comEc are only shortly efficient for ESBL-Ec decolonization, and are not modulated by pantoprazole. Other partner taxa were not yet identified in the microbiota to strengthen the early effect. Thus, this treatment alone seems not very appropriated to prevent the ESBL-Ec colonization.

2.4.2. Effect of *Akkermansia muciniphila* with or without pantoprazole on intestinal colonization by ESBL-producing *Escherichia coli*

Murad Ishnaiwer, Michel Dion and Eric Batard

Microbiota, Hosts, Antibiotics and Bacterial Resistance (MiHAR)

Institute for Health Research 2 (IRS2)

Nantes University

ABSTRACT

Aims: *Akkermansia muciniphila* naturally colonize the mammalian gastrointestinal tract and may contribute to the maintenance of gut health. Our objective was to assess the efficacy of these potential probiotics on digestive colonization by extended spectrum β-lactamase producing *E. coli* (ESBL-Ec) in a murine model.

Methods: Intestinal dysbiosis was induced in male Swiss mice by amoxicillin (0.5 g/L) in drinking water for 3 days. Pantoprazole was also appended in drinking water for some mice at the first day of amoxicillin. *A. muciniphila* was intragastrically administered 1 day before the intragastric inoculation of the ESBL-Ec. ESBL-Ec fecal titers were assessed by daily culture from 0 to 8 days post-inoculation (dpi). Microbiome was assessed by quantitative PCR for *A. muciniphila* and pantoprazole combination.

Results: In comparison with control mice, the combination of *A. muciniphila* and pantoprazole decreased significantly ESBL-Ec titers by 1.8 (0.3) log10 CFU/g (P-value, <0.0001), with a consistent effect over time. No significant efficacy was obtained for either pantoprazole or *A. muciniphila* alone. Moreover, qPCR analysis of 0 dpi feces, have not detected the taxa that could be associated with the efficacy of *A. muciniphila* and pantoprazole combination. By contrast, EPBs taxa were predictive for the efficacy of pantoprazole, in comparison with untreated and ineffectively treated mice.

Conclusions: Our findings showed an interesting effect of the combination of *A. muciniphila* and pantoprazole against intestinal colonization by ESBL-Ec. The effect of pantoprazole on the gut microbiota could explain its activity in the presence of *A. muciniphila*. This study may open new avenues for treatment of intestinal colonization by multidrug resistant *Enterobacterales*.

Keywords: Extended spectrum β-lactamase producing E. coli (ESBL-Ec), *Akkermansia muciniphila*, multidrug resistant *Enterobacterales* (MDRE).

INTRODUCTION

Akkermansia muciniphila naturally colonize the mammalian gastrointestinal tract and may contribute to the maintenance of gut health (Derrien et al.,2017). *A. muciniphila* is a strictly anaerobic bacterium belonging to the phylum *Verrucomicrobia* (Collado et al., 2007; Derrien et al., 2004) that is capable of using intestinal mucin as a sole source of carbon and nitrogen. It is one of the major SCFA-producing bacteria that may improve gut barrier integrity through triggering colonic acidification (Baxter et al., 2019; Louis & Flint, 2017; Parada Venegas et al., 2019). Administration of *A. muciniphila* proved to ameliorate ulcerative colitis and endotoxemia in mice (Derrien et al., 2017; Reunanen et al., 2015). It has been demonstrated in our lab that higher fecal titers of *A. muciniphila* were associated with lower level of colonization by ESBL-producing *Klebsiella pneumoniae* in antibiotic-dysbiosed mice (Grégoire et al., 2021).

This study addressed the efficacy of *A. muciniphila* as a preventive treatment of intestinal colonization by ESBL-Ec in a murine model. We also combined *A. muciniphila* treatment with pantoprazole since it has been shown to reduce gastric acidity, to facilitate the bacterial colonization in mice and to act in synergy with inulin to decrease fecal colonization by multidrug resistant *E. coli* (Stiefel et al., 2006). We also evaluated fecal microbiota composition through qPCR.

MATERIALS AND METHODS

Bacterial strains and media

The clinical ESBL-Ec isolate was collected from University Hospital of Nantes (Ishnaiwer et al., 2022), It is resistant to amoxicillin, and to cephalosporines and to carbapenem through an ESBL and an OXA-48 carbapenemase, respectively.

A. muciniphila CIP 107961T was used for the *in vivo* experiments. This strain was grown on BHI (Oxoid) supplemented with L-threonine 2% (Alfa Aesar), yeast extract 0.2% (Biokar), peptone 0.2% (Biokar), inulin 0.2% (Bulk Powders), Sodium bicarbonate 0.4% (Corning), Hemin 0.05% (Acros Organics), N-Acetylglucosamine 0.56% (Alfa Aesar), Cystein 0.05% (Sigma-Aldrich), Agar 1,5%, and adjusted at pH 6.5 (adapted from (BELZER & Vos, 2016; Derrien et al., 2004). *A. muciniphila* inoculum was determined by culture on this medium and fecal titers were assessed by quantitative PCR as previously described in the manuscript on the effect of inulin and pantoprazole.

Intestinal colonization of ESBL-Ec in a murine model

The same protocol as that used for assessing the efficacy of commensal *E. coli* was followed, except the following changes: *A. muciniphila* (10^7 CFU) was intragastrically administered for each mouse the day before the ESBL-Ec inoculation (-1 dpi). Mice were randomly allocated to: control (n=19), pantoprazole (n=11), *Akkermansia* (n=10) and *Akkermansia* combined with pantoprazole (n=11). The experimental design is shown in **Figure 8A**.

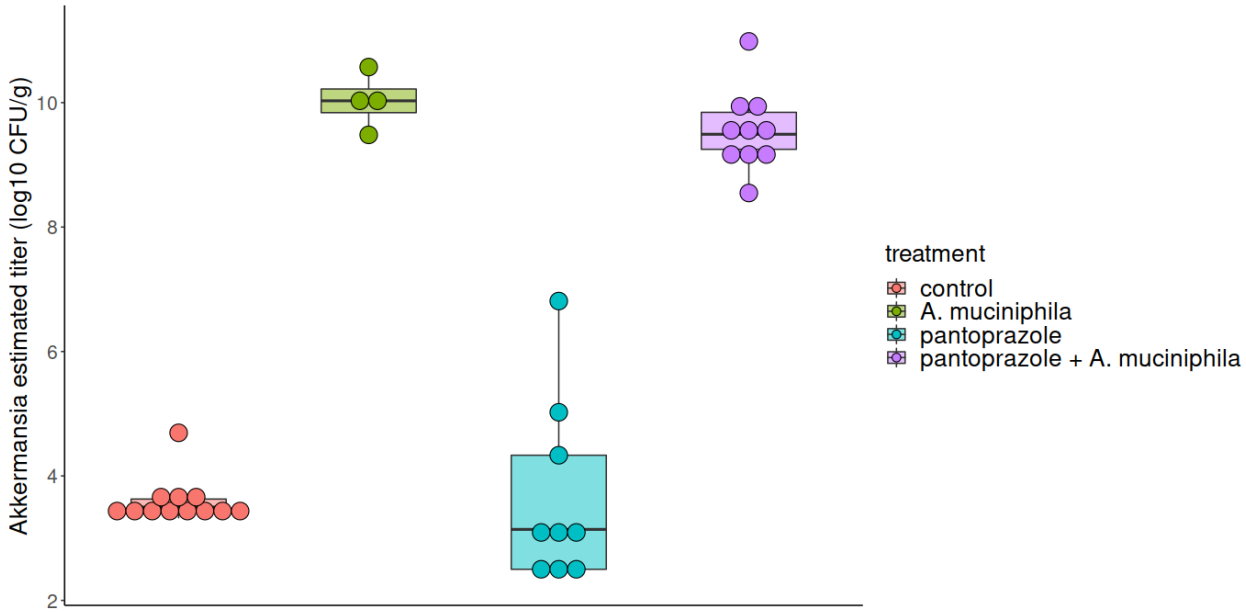
Statistical analyzes and qPCR were performed as described in the manuscript on the efficacy of inulin.

Results

In vivo activity of potential probiotics in a murine model of intestinal colonization by ESBL-Ec

We first assessed the fecal titers of *A. muciniphila* by qPCR one day after *Akkermansia* inoculation (i.e. at 0 dpi, just prior ESBL-Ec administration). *A. muciniphila* titers with and without pantoprazole were 9.3 [9.2-9.4] log10 CFU/g and 10 [9.8-10] respectively, in comparison to control 3.5 [3.5-3.6] log10 CFU/g or Pantoprazole 3.1[2.5-4.3] (**Figure7**).

Figure 7: Fecal titers of *A. muciniphila* one day after *Akkermansia* inoculation.



We then assessed the influence of different preventive treatments on ESBL-Ec fecal titers. To this end, ESBL-Ec fecal titers were compared between treated and control mice using multivariate model assessment for the period between 1 and 8 dpi (**Table2**).

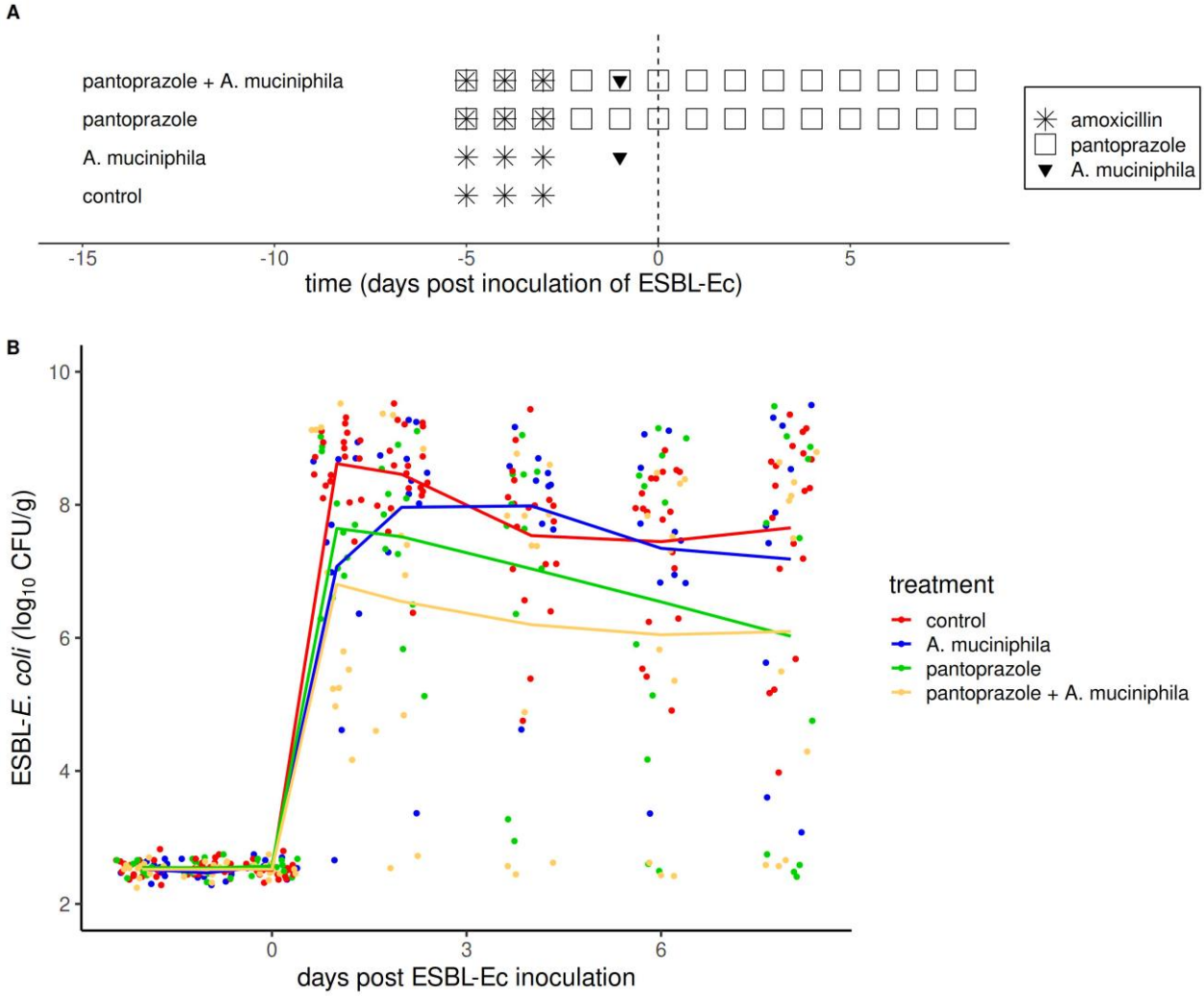
TABLE2: Activity of a preventive treatment of *A. muciniphila* with or without pantoprazole on digestive colonization by ESBL-Ec.

parameter	Estimate (SD)	P-value of the estimate	Estimate (SD) of interaction with time	P-value of the interaction estimate	Proportion of mice with low level titers
Intercept	8.7 (0.4)	<0.0001	-	-	NA
Time	-0.2 (0.1)	0.005	-	-	NA
Treatment					
pantoprazole	-0.9 (0.6)	0.15	0.0 (0.1)	0.90	2/11 (18%)
Akkermansia	-1.2 (0.6)	0.045	0.2 (0.1)	0.083	1/10 (10%)
Akkermansia +pantoprazole	-1.8 (0.6)	0.005	0.2 (0.1	0.13	4/11(36%)

Note. dpi, days post-inoculation. NA, not applicable. A linear mixed effects model was used to estimate ESBL-Ec fecal titers between 1 and 8 dpi. The model fixed effects were treatment effect, time effect and the interaction between time and treatment. Control was the reference group for treatment. The proportion of control mice with low level titers was 0 among 19 (0%).

After a strong increase just after ESBL-Ec inoculation, ESBL-Ec fecal titers slightly decreased with time in the control group, by 0.2 ± 0.1 log10 CFU/g/day. In comparison with control group, combined treatment with *A. muciniphila* and pantoprazole reduced ESBL-Ec titers by 1.8 (0.6) $\log_{10}$ CFU/g (P-value, <0.0001), with a consistent effect over time. Treatment with *A. muciniphila* alone reached initial significant activity, but lost its activity over the experiment days as shown by the nearly significant interaction term (Table 2, **Figure 8B**).

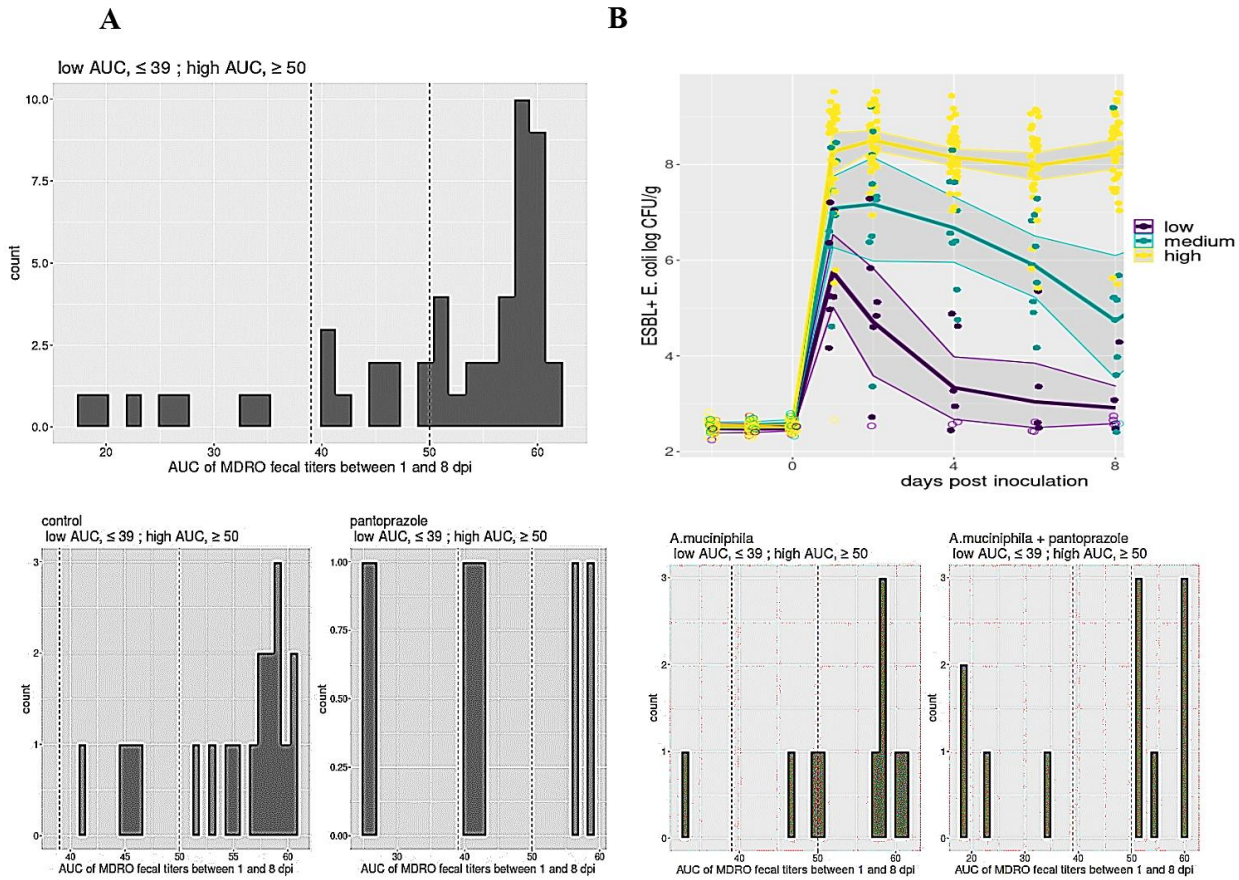
FIGURE 8: Effect of *A. muciniphila* with or without pantoprazole on ESBL-Ec titers



Note. (A) murine model experimental design; (B) Control versus *A. muciniphila* ± pantoprazole, *A. muciniphila,* and pantoprazole.

AUCs of ESBL-Ec titers were computed between 1 and 8 dpi, and allocated in three levels (low, medium and high defined as ≤ 39, between 39 and 50 or ≥50 $\log_{10}$ CFU·day·g^{-1}, respectively). Overall AUCs ranged between 18 and 61 $\log_{10}$ CFU·day·g^{-1} (**Figure 9**). No mice had low AUC among the 19 controls, while 4 among 11 (36%) had low titers in mice treated with pantoprazole and *A. muciniphila* (Chi^2 test P-value, 0.02).

Figure 9: Classification of mice according to their ESBL-Ec titers of A. *muciniphila* treatments ± pantoprazole, control, and pantoprazole

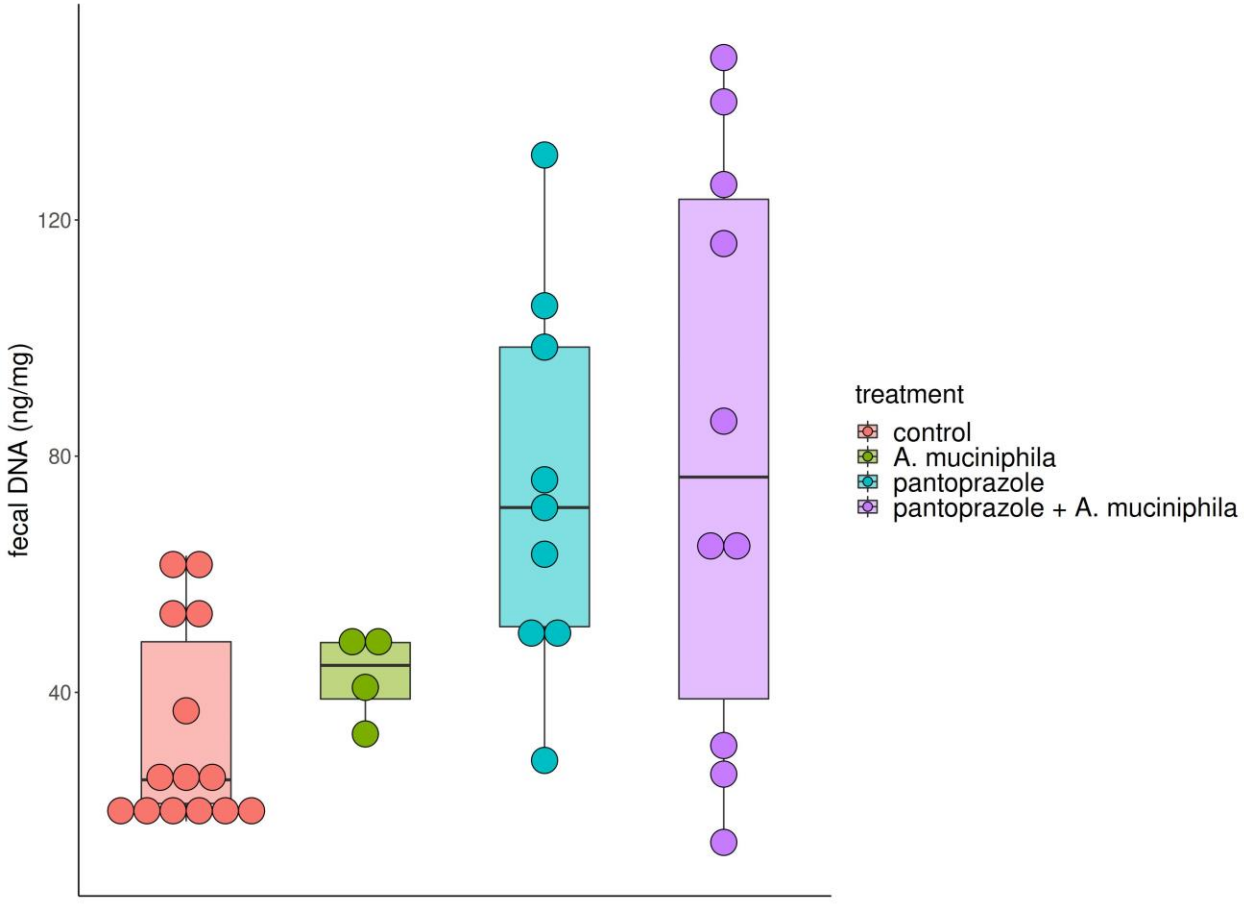


Note. Area under curve of the time variation of ESBL-Ec titers was computed for each mouse between 1 and 8 dpi. (A) Distribution of AUCs for all mice. AUCs were divided in 3 groups (low, medium and high). (B) Mean (95% confidence interval) fecal titers of ESBL-Ec according to the AUC group. (C) Distribution of AUCs according to the treatment.

Assessment of taxa titers in the feces DNAs by qPCR

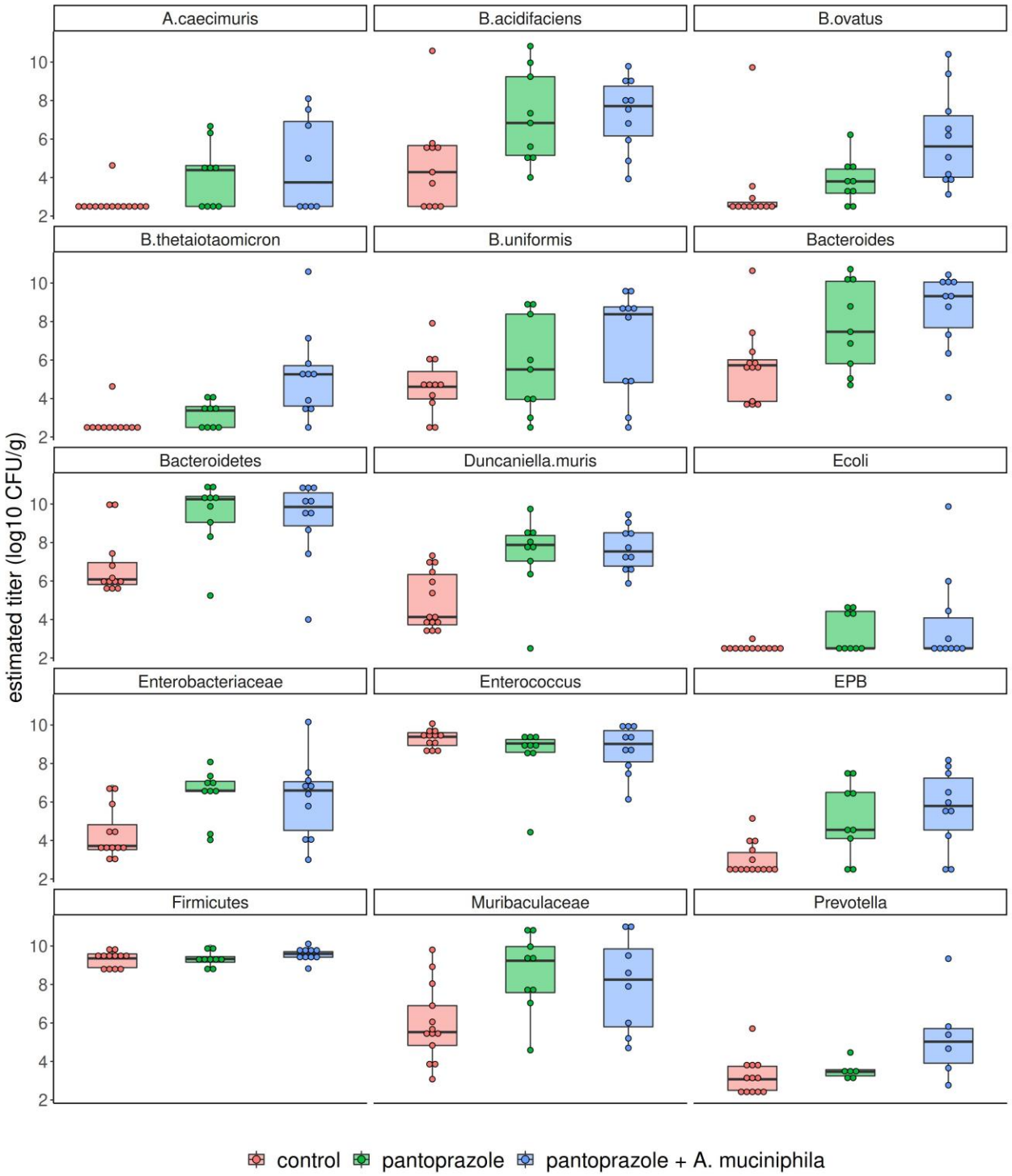
DNA fecal concentrations before ESBL-Ec inoculation were significantly higher with pantoprazole, and pantoprazole + *A. muciniphila* groups than in control group (**Figure 10**).

Figure 10. Fecal DNA concentrations according to treatments



We further determined the titers of different taxa by qPCR to assess the influence of *A. muciniphila* + pantoprazole treatment on microbiota changes, in comparison with control group. The combination of pantoprazole and *A. muciniphila* increased significantly titers of *Bacteroidetes, Prevotella, Enterobacteriaceae, A. caecimuris, Akkermansia, Bacteroides, B. acidifaciens, B. ovatus, B. thetaiotaomicron, B. uniformis, Duncaniella muris, E. coli,* and Equol producing bacteria (EPB). Moreover, pantoprazole alone increased the abundance of Bacteroidetes, *Muribaculaceae*, *Duncaniella muris* and EPBs (**Figure 11; Table 3**).

Figure 11. Comparison of fecal titers of selected taxa, using quantitative PCR, in control, pantoprazole, and *A. muciniphila* + pantoprazole treated mice



Note: Primers that were used for *Enterobacteriaceae* at qPCR can anneal to DNA sequences that are specific for *Salmonella*, *E. coli*, *Shigella* at least). *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

Table 3. Quantitative PCR analysis of fecal taxa in control, pantoprazole, and *A. muciniphila* with pantoprazole treated mice.

	control	***A. muciniphila* with pantoprazole**		**pantoprazole**	
	median titer (log10cfu/g)	median titer (log10cfu/g)	P value vs control	median titer (log10cfu/g)	P value vs control
Bacteroidetes	6.1[5.8-7]	9.8[8.9-10.6]	0.006	10.3 (9-10.4)	0.002
Firmicutes	9.4[8.9-9.6]	9.6[9.4-9.7]	0.12	9.3 (9.2-9.4)	1.00
Enterobacteriaceae	3.7[3.5-4.8]	6.6[4.5-7.1]	0.02	6.6 (6.5-7.1)	0.050
A. caecimuris	2.5[2.5-2.5]	3.5(2.5-6.9)	0.02	4.4 (2.5-4.6)	0.23
Akkermansia	3.5[3.5-3.6]	9.5[9.2-9.8]	< 00001	3.1 (2.5-4.3)	1.00
Bacteroides	5.7[3.9-6]	9.3[7.7-10.0]	0.003	7.5 (5.8-10.1)	0.11
B. acidifaciens	4.3[2.5-5.7]	7.7[6.2-8.7]	0.01	6.8 (5.2-9.2)	0.087
B. ovatus	2.5[2.5-2.7]	5.6[4-7.2]	0.01	3.8 (3.2-4.4)	0.96
B. thetaiotaomicron	2.5[2.5-2.5]	5.3[3.6-5.7]	0.002	3.4 (2.5-3.6)	0.93
B. uniformis	4.6[4-5.4]	8.4[4.8-8.8]	0.03	5.5 (4-8.4)	0.80
Duncaniella muris	4.1[3.7-6.3]	7.5[6.8-8.5]	0.0002	7.9 (7-8.4)	0.014
E. coli	2.5[2.5-2.5]	2.5[2.5-4.1]	0.07	2.5 (2.5-4.4)	0.60
Enterococcus	9.4[8.9-9.6]	9.0[8.1-9.7]	0.16	9 (8.6-9.2)	0.68
EPB	2.5[2.5-2.9]	5.8[4.5-7.2]	0.0001	4.6 (4.1-6.5)	0.020
Prevotella	3.1[2.5-3.7]	5[3.9-5.7]	0.016	3.5 (3.3-3.6)	0.48

Note. Median fecal titers (1st and 3rd quartile) just before ESBL-Ec inoculation (0 dpi), P-values comparison with control group (Dunnett test). Primers that were used for *Enterobacteriaceae* at qPCR can anneal to DNA sequences that are specific for *Salmonella*, *E. coli*, *Shigella* at least). *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

We also conducted qPCR analysis of 0 dpi feces to find an association between microbiota composition and *A. muciniphila* + pantoprazole treatment efficacy. This analysis was not applied to mice treated with *A. muciniphila* alone, since too few mice were effectively treated with this treatment. Thereby, we compared effectively treated mice with both ineffectively treated and control (**Figure 12, Table 4**). Compared to control mice, mice effectively treated with *A. muciniphila* combined with pantoprazole (low ESBL-Ec titers) had significantly increased titers of *Akkermansia, Bacteroidetes, Prevotella, EPB* and *Duncaniella muris* at 0 dpi. Only *Prevotella* had significantly higher titers in mice effectively treated by the pantoprazole-*Akkermansia* combination in comparison with mice ineffectively treated. Hence, fecal titers of *Prevotella* at 0 dpi are predictive of the efficacy of the pantoprazole-*Akkermansia* combination.

Figure 12. Comparison of fecal titers of *Prevotella*, using quantitative PCR, in control mice, pantoprazole, and mice treated effectively and ineffectively with *A. muciniphila* + pantoprazole

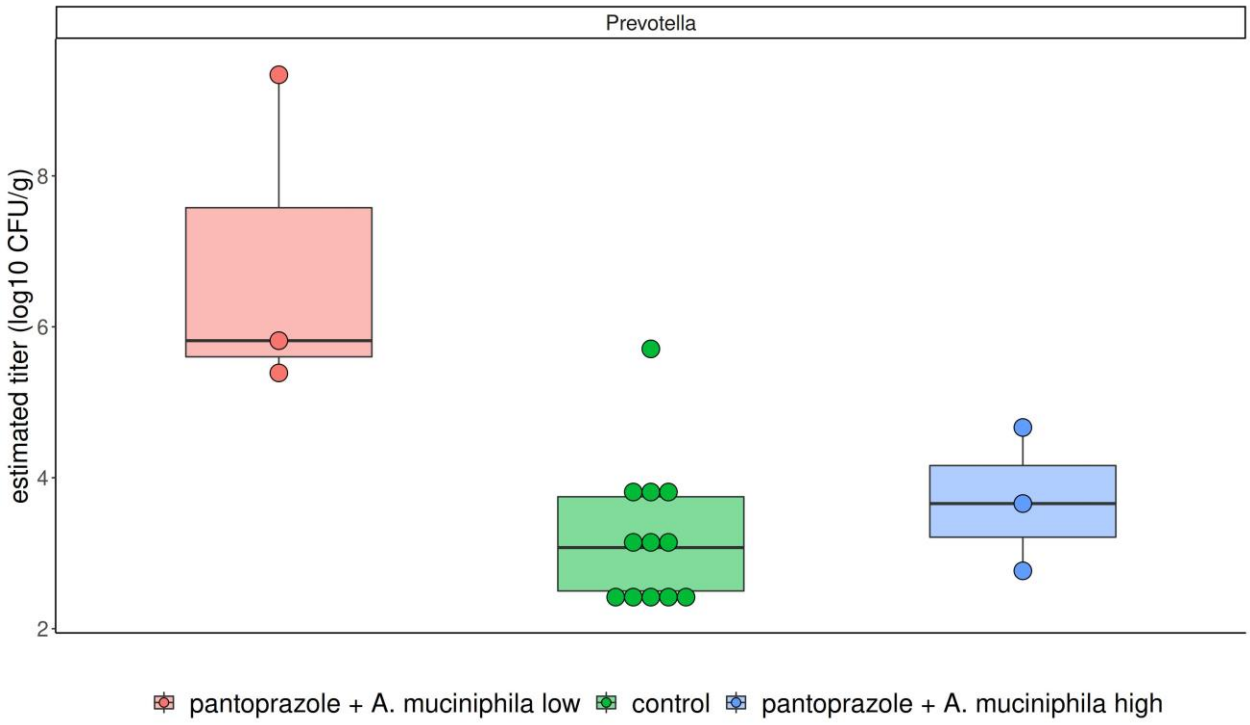


Table 4. Search of taxa predictive of the efficacy of the combination of pantoprazole and *A. muciniphila* on ESBL-Ec colonization

Taxa	Control	*A. muciniphila*+ Pantoprazole		
	Median titer log10 CFU/g	Treated low, Median titer log10 CFU/g	Treated low vs control, P value	Treated low vs High, P value
Bacteroidetes	6.1[5.8-7.1]	10.7[9.7-10.8]	0.018165824	0.438305189
Firmicutes	9.4 [8.9-9.6]	9.6[9.5-9.7]	0.399182495	0.986526476
Enterobacteriaceae	3.7[3.5-5.2]	6.4[6.1-6.6]	0.151795812	0.970212771
A. caecimuris	2.5[2.5-2.5]	5[3.8-6.3]	0.092241002	0.873626743
Akkermansia	3.5[3.5-3.6]	9.3[9.2-9.4]	< 0.000001	0.377281098
Bacteroides	5.7[3.8-6.1]	9.3[7.8-9.7]	0.06733863	0.999834443
B. acidifaciens	4[2.5-5]	6.8[5.8-7.3]	0.311372801	0.650961135
B. ovatus	2.5[2.5-2.5]	6.2[5-7.8]	0.085339457	0.864565749
B. thetaiotaomicron	2.5[2.5-2.5]	5.4[4.4-5.6]	0.096022368	0.830731351
B.uniformis	4.6[3.9-5.7]	8.2[6.6-8.7]	0.134544876	0.828184031
Duncaniella.muris	4.1[3.7-6.6]	8.4 7.9-8.7[]	0.001965861	0.545475611
E. coli	2.5[2.5-2.5]	2.5[2.5-2.5]	0.99833174	0.141205635
Enterococcus	9.3[8.9-9.6]	9.3[8.4-9.4]	0.51424977	0.999959772
EBP	2.5 [2.5-3.2]	7.5 [5.9-7.7]	0.001524371	0.30190253
Prevotella	3.1[2.5-3.8]	5.8[5.6-7.6]	0.000565369	0.010128257
Muribaculaceae	5.5[4.6-6.3]	8.7[8.3-9.1]	0.180099857	0.77196686

Note. Median fecal titers (1st and 3rd quartile) just before ESBL-Ec inoculation (0 dpi). Treated low, effectively treated mice (i.e., low level of ESBL-Ec colonization); treated high: ineffectively treated mice (i.e., high level of ESBL-Ec colonization). Dunnett tests were used to compare effectively treated mice (set as the reference group) with control and ineffectively treated mice. A taxa was considered as predictive of treatment efficacy if its titer at 0 dpi in effectively treated mice was significantly different from both control and ineffectively treated mice. Primers that were used for *Enterobacteriaceae* at qPCR can anneal to DNA sequences that are specific for *Salmonella*, *E. coli*, *Shigella* at least). *Enterobacteriaceae* is used here instead of *Enterobacterales* because it is a conserved data.

DISCUSSION

We have shown a significant activity of *A. muciniphila* combined with pantoprazole to decrease ESBL-Ec fecal titers. This treatment was effective in 36 % of mice. Therefore, we studied microbiota composition between effectively, ineffectively treated and control mice by using qPCR analysis. These analyses allowed also to check the increase of *A. muciniphila* after its administration. Hence, for *A. muciniphila* + pantoprazole treatment, qPCR analysis revealed a strong *A. muciniphila* colonization at dpi0 with or without pantoprazole (> 9 log10 CFU/g). However only *A. muciniphila* with pantoprazole had a consistent effect over time to decrease significantly ESBL-Ec fecal titers. This could be explained by the potential role of pantoprazole in attenuating the effect of amoxicillin to microbiota-induced dysbiosis, as illustrated by the higher DNA feces concentration found with pantoprazole treatment versus control. Since pantoprazole alone was poorly

effective, and only at the beginning of the colonization, *A. muciniphila* seems to play a role in the ESBL-Ec decolonization in effectively treated mice. The production of SCFA (acetate, propionate) from dieterary polysaccharides (Parada Venegas et al., 2019), may explain - at least partially - the activity of *A. muciniphila* against ESBL-Ec. Alternatively, SCFAs could stimulate other antagonist bacteria through cross-feeding (Belzer et al., 2017). Surprisingly, there was very few differences between taxa in effectively and ineffectively treated mice. Among the tested taxa, only *Prevotella* were predictive of the efficacy of the pantoprazole-*A. muciniphila* combination. Indeed, nothing is known concern the efficacy of *Prevotella* Spp on MDR-EB. Therefore, it could be interesting to isolate new *Prevotella Spp* from feces of effectively treated mice, and test their activity on ESBL-Ec intestinal colonization. Moreover, using species like a human gut commensal *Prevotella histicola*, could be also promising, especially since it has shown an important role to mediate gut microbiota restoration in mice (Shahi et al., 2019).

However, the number of taxa assessed by qPCR is limited and we need deeper metagenomic analysis to have a better overview of taxa that may be predictive of the efficacy of the combination of pantoprazole and *Akkermansia*. Another limitation of this study is that we only assessed microbiota architecture at 0 dpi. Microbiota analysis at other time points may lead to identify other taxa involved in the efficacy of the combined treatment.

Conclusion

The combination of pantoprazole and A. muciniphila proved effective to decrease the level of ESBL-Ec fecal colonization, and was more effective than a treatment with *A. muciniphila* alone. This result suggests the role of pantoprazole to attenuate amoxicillin-induced dysbiosis and perform better activity overtime for *Akkermansia* treatment. It also suggests that *A. muciniphila* needs other taxa to reduce ESBL-Ec titer. As higher titers of *Prevotella* were predictive of the efficacy of the combined therapy, it may be interesting to try to increase *Prevotella* fecal titers in mice treated with the combination that have low *Prevotella* titers just before ESBL-Ec inoculation. The combination of comEc and *Akkermansia* would be also interesting to test since some activity was found with each separated treatment.

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2.5 Treatment of ESBL-Ec colonization with *Cutibacterium acnes* and *Prevotella spp*: preliminary results

1) Preventive activity of *Cutibacterium acnes* on ESBL-Ec intestinal colonization

One of the main aims of our study was to assess the preventive activity of bacteria isolated from feces of effectively treated mice. Fecal samples were cultivated on a rich medium designed to isolate *Adlercreutzia* (see manuscript n °1 for details). A colony was isolated, identified through its 16S rRNA sequence as *Cutibacterium acnes* and named *C. acnes* 1552G.

First, we observed a strong *in vitro* growth inhibition of ESBL-Ec with *C. acnes* 1552G on BHI medium + cysteine 0,05%, + glucose 0,2%. Therefore, we assessed the potential efficacy of this bacterium on ESBL-Ec colonization in our murine model (see manuscript1): amoxicillin-dysbiosed mice were inoculated with ESBL-Ec (10^6 CFU per mouse) two days after amoxicillin discontinuation (0 dpi). *C. acnes* 1552G was intragastrically administered for each mouse (10^7 CFU) the day before the ESBL-Ec inoculation (-1 dpi). Compared to control group, preventive treatments with *C. acnes* decreased significantly ESBL-Ec titers by 2 $\log_{10}$ CFU/g. Between 1 and 8 dpi, ESBL-Ec fecal titer in the control group decreased from 7.8 ± 2.2 to 6.76 ± 2.9 $\log_{10}$ CFU/g. Meanwhile, in the *C. acnes* 1552G groups, it decreased from 5.8 ± 2.4 to 3.6 ±0.7 $\log_{10}$ CFU/g (Figure 1).

Indeed, *C. acnes* produces SCFAs, mainly acetate and propionate (148), which has proved to be deleterious for ESBL-Ec (149). Moreover, as we reported for previous treatments, the treatment efficacy of *C. acnes* 1552G was highly variable among mice, which could suggest the involvement of other taxa on ESBL-Ec colonization. To this respect, metagenomic analyses were carried out on the fecal DNAs from *C. acnes* 1552G treated mice: it revealed a strong positive correlation with the presence of *B. acidifaciens* in effectively treated mice (3/5 mice, data not shown), a bacterium which is also known to produce SCFAs (150).

In addition, we found high variability of ESBL-Ec titer among control mice, which gave a spontaneous decrease of mean ESBL-Ec titers in control group along the time. This may have led to underestimate the activity of *C. acnes* on ESBL-Ec.

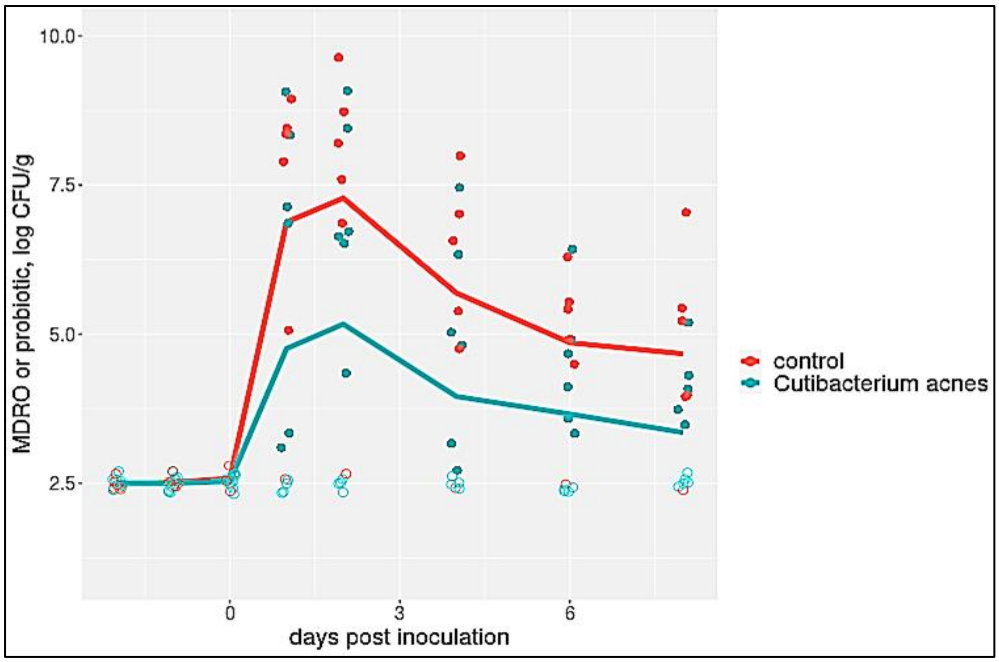

Figure1: mean effect of *C. acnes* on ESBL-Ec in comparison with control

2) Preventive activity of *Prevotella denticola* and *Prevotella bivia* mixture on ESBL-Ec intestinal colonization

As we found that *Prevotella* was predictive of the efficacy of treatment with pantoprazole and *Akkermansia*, we assessed the potential efficacy of *P. denticola*, and *P. bivia* strains mixture on ESBL-Ec in our murine model. These clinical isolates of *Prevotella* (CHU of nantes) were grown on *S*chaedler agar medium for two days in anaerobic conditions. Their inoculum titers were determined by colonies counting on this medium after incubation at 37°C for 48 hr in anaerobic conditions, and fecal titers were assessed by qPCR as previously described in manuscript 1. Then amoxicillin-dysbiosed mice were inoculated with ESBL-Ec (10^6 CFU per mouse) after two days of amoxicillin cessation (0 dpi). *Prevotella* mix (*P. denticola* + *P. bivia*) were intragastrically administered for each mouse (10^7 CFU) the day before the ESBL-Ec inoculation (-1 dpi).

A preventive treatment with *Prevotella* mix had no effect on ESBL-Ec titers in comparison with control mice (Figure 2). We also tested if *Prevotella* mix could provide an additive effect to the comEc mix on ESBL-Ec, but it also showed no efficacy (Figure 2). Indeed, *P. denticola* and *P. bivia* strains are inhabitants of the oral and vaginal microbiota respectively(151,152), and there is no clear evidence of their ability to colonize the gut microbiota. Therefore, isolation of representative isolates from the mouse gut microbiome could be more suitable to obtain an activity in a murine model.

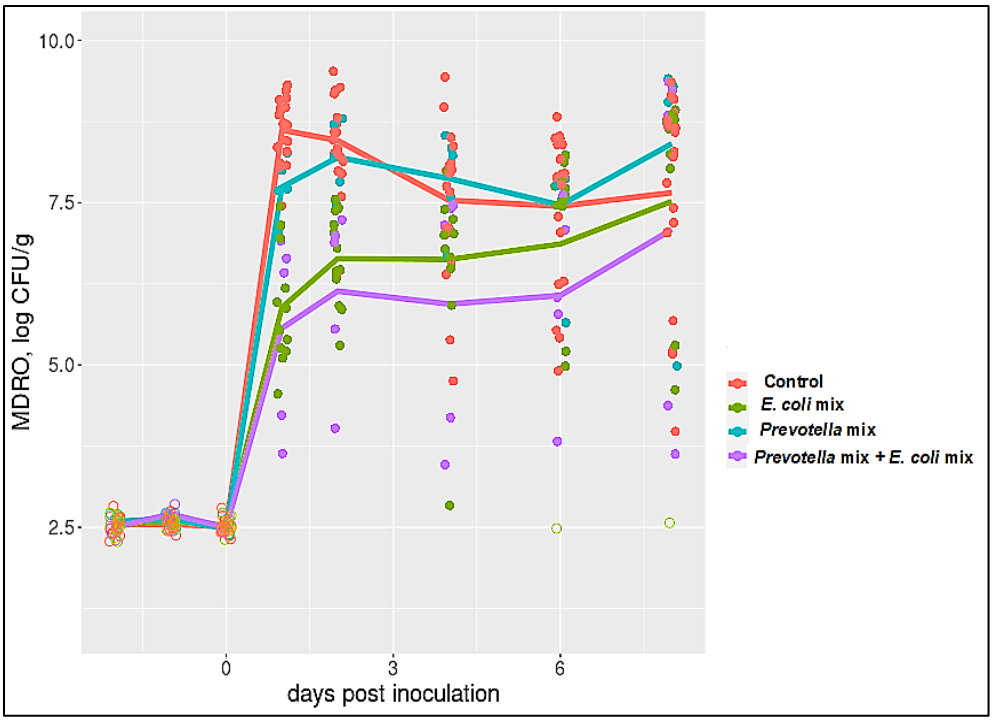

Figure 2: Mean effect of *Prevotella* mix with/without comEc mix, and comEc mix, on ESBL-Ec in comparison with control.

3. General discussion

3.1. The murine model of MDRE intestinal colonization

Different animal models have been used to explore the efficacy of treatments against intestinal colonization by MDR, but mammalian models in particular murine, remain the most used. However, Ren et al, 2019, and Saliu et al 2020 tested the effects of probiotics and food additives on the intestinal carriage of ESBL-EB in broilers model (153,154). Also, pigs were used to assessed probiotics efficacy on the gut carriage of ESBL-Ec (155). Moreover, mice, sheeps, rabbits, calfs, and pigs have been used to test phage therapy on intestinal carriage with MDR *E. coli* strains (76).

Nevertheless, as we previously described, using live bacteria (probiotics) to treat intestinal colonization by MDRO after antimicrobial therapy is becoming increasingly plausible with the growing knowledge of the structure and functions of the intestinal microbiota (68,69,97,120).

During our work, we tested innovative treatments of digestive colonization by ESBL-Ec in a murine experimental model. This model is suitable as the gut physiology of mouse is near from that of human and it is cost and time effective. However, it is estimated that only up to 4 % of bacterial species are shared between human and mice gut (156,157). The Firmicutes / Bacteroidetes ratio is higher in mice than in humans (156). In mice, Firmicutes are dominated by Clostridiales, and Bacteroidetes by *Muribaculaceae*. By contrast, human Firmicutes are mainly represented by *Ruminococcaceae*, and Bacteroidetes by *Bacteroidaceae* and *Prevotellaceae*. The *Muribaculaceae* family is 30 times higher in mice than in humans, and *Bacteroidaceae* are 14 times more abundant in humans than in mice (158,159). Moreover, nearly 95% of taxa ensure the same functions in mice and humans (96,116). However, mice gut microbiota produce higher concentrations of lactate, while human's microbiota produce higher levels of SCFAs than mice ones, such as acetate, butyrate and propionate (160).

In our murine model, we used Swiss mice, and we learned how to set up several parameters, including the choice of antibiotic to alter the microbiota, the duration and dose of antibiotic therapy, the titer of the ESBL-Ec inoculum, the delay between the cessation of antibiotic therapy and the inoculation of ESBL-E. coli, the delay between the start of treatment and the inoculation of ESBL-E. coli, the dose and duration of treatment (probiotic/prebiotic/pantoprazole). We used amoxicillin, to alter the intestinal microbiota and to facilitate colonization of a ESBL-Ec isolate. We also added pantoprazole in some

groups in order to avoid gastric acidity that might kill part of the inoculum. Thereby we were able to render the mice susceptible to ESBL-Ec colonization. Thus, after establishing this robust model, we were able to assess the efficacy of various probiotic or prebiotic treatments.

Hence, we tested the activity of potential probiotics with our murine model from isolates of murine origin. Therefore, the transferability of these potential treatments to human could not be necessarily directly, but instead we can look for relevant strains of human origin and test first their efficacy against ESBL-EB intestinal colonization in a humanized murine model. Then we can test their efficacy along clinical trials. Meanwhile, the prebiotic inulin is already used for gut health in humans, therefore we can assess its efficacy against intestinal colonization by ESBL-EB directly in human, and use fecal metagenomic analysis to identify taxa with predictive efficacy.

3.2. General strategy: choice of probiotics / prebiotics and microbiota analyses

Literature review revealed that all probiotic-based treatments have shown to date modest activity against MDRE infections. Hence, there is an urgent need to identify new appropriate probiotics toward intestinal colonization by MDRE.

Our project strategy was as follows : (i) start with a prebiotic or a probiotic considered to have beneficial effect on digestive infections or on intestinal microbiota ; (ii) assess their activity on MDRE intestinal carriage in our murine model ; (iii) in some cases, assess the composition of microbiota (either by shotgun sequencing or by qPCR) to understand why the treatment was effective, and (iv) to identify the relevant over-represented taxa associated with treatment efficacy; (v) in some cases isolate and cultivate these taxa and assess their own therapeutic activity.

Accordingly, we isolated new probiotics that have never been tested against MDR-EB. Our first choice was *Bacillus* species since some of them already exhibited antimicrobial activities against various human pathogens (102). Moreover, it is also a spore-forming soil bacteria that is naturally ingested by humans and animals, and whose spores could tolerate gastric acidity (161). We initially tested the curative treatment with our probiotics *Bacillus*, since there is a need for curative treatments in medicine. Although we inoculated the *Bacillus* strains 4 times, we could not find any curative activity against intestinal colonization by ESBL- Ec(162).

Then we tested prebiotic inulin since it is known to promotes the growth of genera reported to be lower in mice that are susceptible to colonization by MDR-EB, including SCFA

producing bacteria (96,111,139,140). It also attenuated the severity of several intestinal infections in human and mice (130,135). We found that the combination of inulin and pantoprazole reduced ESBL-Ec colonization in some mice, and was ineffective in other mice. These results prompted us to consider the combination of inulin and probiotics.

Therefore, we tried to find other potential probiotics that we defined as taxa with titers just before ESBL-Ec inoculation being higher in effectively treated mice than in control and ineffectively treated mice. We also considered taxa reportedly promoted by inulin (163,143).

First, we used qPCR analysis to identify the fecal abundance of chosen taxa based on availability of primers and standards. Such taxa were selected from literature review based on:

- Their ability to be promoted by inulin (135,163,139).
- The correlation between their titers and the level of colonization by MDR-EB (96, 139,111).
- Their ability to produce of SCFA or equol (146,164).

Indeed, qPCR was first used since it is a cost-effective and simple to perform. However, it was limited to determine all dynamic dissimilarities of bacterial abundance between different treatments. Therefore, we conducted shotgun metagenomic sequencing to compare feces of effectively treated, ineffectively treated, and control mice. Moreover, analysis of metagenomic data allowed us to assess the functional and structural microbiota components able to reduce ESBL-Ec colonization (165).

Thus, we isolated three comEc strains and one *Adlercreutzia* from feces of mice that had a low level of ESBL-Ec colonization, and tested their preventive activity against ESBL-E. coli. We also tested *A. muciniphila* since it has been reported to be associated with a significant decrease of ESBL-producing *K. pneumoniae* in mice (111). All these bacterial species naturally colonize the mammalian gastrointestinal tract, may contribute to the maintenance of gut health and could be altered in a disrupted gut (103,116).

3.3. Efficacy of our probiotic/ prebiotic combined or not with pantoprazole in comparison with other studies

The ability of probiotics to exert their activity is dependent of many factors including the ability to colonize the intestinal tract, in relation to the host microbiota architecture (68). We used fecal analysis to judge the success of their colonization as well as the gut

microbiota composition. Although the fecal titers may be different of colonic titers, they were easier to determine and it allowed us to obtain large data (166).

Bacillus

The few probiotic trials conducted to date, have revealed significant in-vitro activity for probiotics against MDR-EB (167,168). However, in-vivo and clinical probiotic effects either failed or partially eradicated MDR-EB (69,86).

Bacillus fecal titers rapidly increased after inoculation, but also rapidly decreased if it was not repeatedly inoculated. This result suggests that our *Bacillus* isolates might be adversely affected by colonization resistance of intestinal microbiota (162). Piewngam et al reported that *Bacillus* spores eliminated methicillin resistant *S. aureus* intestinal colonization in mice. They have precisely inoculated *Bacillus subtilis* strain ZK3814 spores in C57BL/6J female mice, which were pretreated for 7 days by ampicillin, metronidazole, neomycin and vancomycin (169). We made the hypothesis that the *Bacillus* therapy was effective in this study because the 4-antibiotic regimen reduced more dramatically the abundance of endogenous microbiota than the amoxicillin regimen that we used, providing less resistance to colonization by the inoculated *Bacillus*. Comparing the ability of *Bacillus* to colonize feces and to decrease ESBL-Ec colonization after different antibacterial treatments would allow to support or infirm this assumption.

We found subsequently from shotgun sequencing detected *Bacillus* in feces of only 7 among 47 mice (15%), including *Bacillus thuringiensis* in 3 mice and *Bacillus* AC_sp001076885 in 7 mice. In mice with detected *Bacillus*, median relative abundancy of *Bacillus* was 2.10^{-5} (5.10^{-6} - 3.10^{-5}) (data not shown). This result suggests that these strains or species may be more adapted than *B. subtilis* to the murine microbiota.

Furthermore, although *Bacillus* could not reduce ESBL-Ec colonization as a curative treatment, it may be efficacious as a preventive one. Indeed, a preventive activity were obtained with *B. subtilis* strain PY79 spores in mice, against *C. rodentium* entero-pathogen suggesting that a preventive treatment may be more effective than a curative one (170).

Commensal *E. coli*

Considering that it may less easy to achieve efficacy with a curative treatment, we therefore focused our analyses on preventive treatments of MDRE colonization. Administered preventively, probiotics could early exploit available nutrients and niches hence competing more efficiently with MDRE.

Accordingly, we tested the preventive activity of comEc isolates against ESBL-Ec colonization. We initially checked that comEc reached a high titer in the feces. Hence, the treatments with comEc significantly reduced temporarily ESBL-Ec colonization just after inoculation with either single strains or with strains mixture. Such temporary activity had previously been reported by Maltby et al, who found that a treatment with *E. coli* HS and *E. coli* Nissle 1917 in streptomycin-dysbiosed mice eradicated *E. coli* EDL933 for only a limited period (116). This phenomenon could be related to the competition for nutrients or specific niches.

Moreover, after a few days, we found that comEc titers decreased simultaneously with ESBL-Ec in effectively treated mice. This shows that prophylactic comEc are not the only cause responsible for ESBL-Ec titer decrease. It also could indicate that both comEc, and ESBL-Ec might be competed by other taxa. Moreover, the high variability of treatment effect among mice suggests – as for other treatments – the involvement of mice microbiota in the efficacy of treatment with comEc (171). To this respect considering the predictive effect of *E. coli* described in the manuscript n°1, it was surprising not to obtain a more significant effect of comEc administration. The reason could be that previously detected comEc at dpi0 were amoxicillin resistant and likely present during the amoxicillin treatment. Consequently, they early decreased the gut amoxicillin concentration which is less the case with the late comEc administration at dpi-1. Thereby, the resulting microbiota could be less rich and less resistant to exogenous ESBL-Ec colonization.

Finally, changing some experimental conditions might confer a better efficacy of treatment with our comEc strains. For instance, a 10-day treatment with E. coli was effective against Enterohemorrhagic E. coli EDL933 colonization in streptomycin-dysbiosed mice (171). This result leads us to hypothesize that different efficacies of E. coli treatment may be linked to differences in dysbiosis induced by streptomycin or amoxicillin.

Akkermansia muciniphila

We then tested the preventive activity of *A. muciniphila* on ESBL-Ec intestinal colonization. Hence, despite *A. muciniphila* efficiently colonized intestinal microbiota with and without pantoprazole and is known to *produce* SCFA (91), *A. muciniphila* had no effect on ESBL-Ec colonization without pantoprazole, but was active in mice exposed to pantoprazole. This result suggest a critical synergism between pantoprazole and *A. muciniphila*. *Akkermansia* stimulates various taxa by cross-feeding, especially *Anaerostipes caccae, Eubacterium hallii*, and *Faecalibacterium prausnitzii.* These taxa produce butyrate that attenuates MDRE colonization (108,172). In our model, amoxicillin might reduce the

abundance of these taxa, and then prevent any effect of *Akkermansia* on ESBL-Ec colonization.

We found that pantoprazole prevented amoxicillin-induced decrease of total bacterial DNA per gram of feces, and various taxa of fecal microbiota. This result seems contradictory with results of studies of the effect of PPI on human microbiota, although one study showed that PPI increased microbial-diversity in older inpatients treated with antibiotics (173,174). One mechanism of action of pantoprazole may be the stimulation of antibiotic efflux pump/s as already shown for Tigecycline (175). PPI could also act by raising intragastric pH, and decreasing the gastric acidity, or through a direct inhibition of bacteria proton pumps, or by altering nutrients absorption in intestinal lumen (160). Hence, pantoprazole may prevent amoxicillin-induced decrease of *Akkermansia*-promoted butyrate-producing taxa, hence acting in synergy with *Akkermansia* to decrease ESBL-Ec colonization. If confirmed, this hypothetical mechanism of action of the combination of pantoprazole and *Akkermansia* may help to identify other potential probiotics able to decrease ESBL-EC colonization.

Finally, *A. muciniphila* and pantoprazole combination effectively reduced ESBL-Ec colonization only in a subgroup of mice. This result suggests that no single treatment is effective in all individuals, and that other gut microbiota taxa have a significant capacity to influence the effect of *A. muciniphila* on ESBL-Ec. As previously indicated, this taxa could be Prevotella and that's led us to test 2 human strains of this genus in our mouse model. Finally, they did not display preventive effect on the ESBL-Ec colonization, with or without additional strains of comEc. However, we must consider this result with caution because the relevant murine strains were not isolated and thus not tested.

Adlercreutzia

As we found that *Adlercreutzia* titers were predictive of efficacy of the combination of inulin and pantoprazole, we assessed the efficacy of *Adlercreutzia* species on colonizing the mice intestine, and it was able to reduce ESBL-Ec colonization. However, *Adlercreutzia* fecal titers were rather low one day after inoculation (our preliminary results with 10 mice found 6-7 $\log_{10}$ CFU/g as estimated by qPCR of the *tdr* gene) but significantly higher than what we detected in the control mice (2,5 $\log_{10}$CFU/g, that is the detection limit). Of note, the abundance of *Adlercreutzia* in 10 naïve mice feces reached 8 $\log_{10}$ CFU/g in average (data not shown). *Adlercreutzia* is known to use only rhamnose sugar among carbohydrates (143). This poor ability to degrade carbohydrates probably limits their ability to colonize gut content at high levels. We cannot exclude that the number of *Adlercreutzia*

cells effectively administered to mice was lower than we estimated from the optical density of suspension. Indeed, it's possible that part of the inoculum was killed in the stomach since pantoprazole was not administered in these experiments. Identifying ways to increase *Adlercreutzia* titers in intestinal microbiota may help to increase its efficacy to treat MDRE colonization.

Inulin

A major effect of inulin on intestinal microbiota is the stimulation of SCFA-producing bacteria. SCFA can acidify the intestinal environment, and boost the gut barrier function to prevent intestinal colonization by *Enterobacterales* (91,176).

Indeed, stimulation of SCFA bacteria by inulin is known to be dose dependent and duration dependent. Hence, in our model, we showed that low dose inulin (0.2%, or 0.012 g/mouse/day) increased neither total fecal bacterial DNA content nor titers of taxa reported to be promoted by inulin, including *Muribaculaceae*, *Akkermansia*, Bacteroides, *Lactobacillus* and *Bifidobacterium* in amoxicillin-dysbiosed mice. This lack of effect on SCFA producers may partly explain the lack of efficacy on ESBL-Ec colonization. Consequently, we wondered if our inulin dose was sufficient: we thus tested higher doses of inulin (5%, or 0.3g/mouse/day), in combination with pantoprazole, on amoxicillin-dysbiosed mice (n=4), and we found that they have similar effect to low dose (data not shown).

Then, another parameter is the duration of treatment. Indeed, positive effect was obtained in the following experiments: medium dose inulin (0.5 % / Dose 5g kg−1 day−1) appended for 6 weeks for C57BL/6J mice, significantly elevated the abundance of main SCFA producing bacteria including *Muribaculaceae*, *Ruminococcaceae*, *Lactobacillaceae*, *Bifidobacteriaceae*, *Bacteroides vulgatus*, and *A. muciniphila* (176).

Similarly, Weitkunat et al. reported a significant increase of SCFAs concentrations, and SCFA-producing bacteria including *B. thetaiotaomicron*, *B. longum*, and *C. butyricum* in SIHUMI mice, beyond 6 weeks of inulin 10% supplementation (177).

On other hand, we found using qPCR that the combination of early inulin and pantoprazole increased several taxa that were not increased by early inulin alone or pantoprazole alone. In comparison with pantoprazole alone, the early inulin/pantoprazole combination increased *B. thetaiotaomicron* and *B. ovatus*, and late inulin and pantoprazole increased *Akkermansia*, all being SCFA producers. However, titers of these taxa were not predictive of treatment efficacy. By contrast, shotgun sequencing identified 2 SCFA producers to be

predictive of treatment efficacy, i.e., *Duncaniella muris* and *Alistipes*. qPCR confirmed the predictive value of *D. muris* titers for EIP efficacy.

3.4. Factors that might be associated with ESBL-Ec decolonization

In addition to the potential activity of probiotics and/or inulin, with or without pantoprazole, we tried to figure out other possible factors that might be associated with the ESBL-Ec decolonization in effectively treated mice. Such findings could give new insights to improve the efficacy of our future treatments, through predicting new effective combinations that could eradicate MDR-EB intestinal colonization.

Normally, the gut microbiota of healthy individuals and animals provides colonization resistance against pathogen invasions. Colonization resistance (CR) is the ability of microbiota to resist to the invading pathogens, through several direct and indirect mechanisms (178,179). Any disruption of the typical microbiota composition may facilitate MDR-EB colonization. We therefore have analyzed the microbiota composition prior ESBL-Ec inoculation, and found specific taxa that could induce CR, and predict the efficacy of our treatments.

qPCR and metagenomic analysis of feces prior ESBL-Ec inoculation has shown that the intestinal microbiota of mice effectively treated by EIP were characterized by higher abundance of *Enterobacteriaceae,* EPB and *Adlercreutzia*. We also detected the increase of these taxa in mice treated with the combination of *Akkermansia* and pantoprazole. Thus, we have confirmed this predictive model, and we succeeded to obtain a preventive activity of *Adlercreutzia* strains against ESBL-Ec colonization.

Moreover, despite the alterations of intestinal microbiota induced by the amoxicillin treatment, addition of pantoprazole could maintain a significant richness of intestinal microbiota, including amoxicillin-resistant taxa.

We could show that β-lactamases producing bacteria (in particular *Enterobacteriaceae*) decreased amoxicillin fecal concentration which allowed the development of potential beneficial, amoxicillin-sensible bacteria. These amoxicillin-resistant *Enterobacteriaceae* would be effective if they grow and reduce the amoxicillin concentration before the inoculation of ESBL-Ec. The interest of increasing the β-lactamase activity of microbiota has been recently shown in a mouse model, where an engineered β-lactamase-producing *Lactococcus lactis* probiotic attenuated the ampicillin-induced gut dysbiosis (180). In another study, Connelly et al showed that ribaxamase, a β-lactamase, protected the gut microbiota of pigs from amoxicillin disruption (181).

3.5. Multimodal treatments of intestinal colonization by ESBL-Ec

Identifying taxa involved in treatment efficacy should lead to the design of new combinations of treatments among prebiotics, probiotics and pantoprazole, to effectively treat ESBL-Ec intestinal colonization.

Based on our findings, we observed that all treatments were active only in some mice, and that this inter-individual susceptibility may be explained by the differential composition of intestinal microbiota. Hence, we aimed to predict the efficacy of tested treatments from the pre-treated microbiota composition. Finally, these microbiota analyses allowed us to identify taxa displaying higher abundances in effectively treated mice: such taxa are potential new probiotic for ESBL-Ec colonization treatments to be tested in future studies.

Moreover, monotherapies have limited efficacy, i.e., they modestly reduce the average MDRE fecal titer (e.g., by one or two log CFU/g), depending of the microbiota composition. However, from the analysis of this microbiota composition before treatment it becomes possible to predict which taxa is lacking and therefore to adapt the treatment to each host. More realistically, combinations of probiotics could be more successful than monotherapies. To this respect, some taxa like EPB and *Muribaculaceae* seem to have a great potential. Alternatively, since these taxa are difficult to cultivate, the finding of new prebiotic like chitooligosaccharides able to stimulate their growth would be of great interest (182). We assume that taxa that are predictive of the efficacy of inulin and pantoprazole combination, including *A. caecimuris*, that we have showed to be active as a monotherapy, could provide an effective synbiotic mixture with inulin to eradicate intestinal ESBL-Ec. Combining *Adlercreutzia* with *Muribaculaceae* (or *Barnesiella*, the equivalent genus of *Muribaculaceae* in human gut) may also be interesting.

4. Conclusions

We described new strains of *Bacillus subtilis* exhibiting in vitro activities against ESBL-Ec. We also described interesting in-vivo preventive activities of potential probiotics *A. muciniphila*, commensal *E. coli*, and *Adlercreutzia*, as well of prebiotic inulin, to decrease ESBL-Ec intestinal carriage in amoxicillin-dysbiosed mice. Through metagenomic and qPCR analyses of fecal microbiota, we identified taxa predictive of treatment efficacy.

The treatments with comEc were only temporarily effective on ESBL-Ec colonization and were not modulated by pantoprazole. It suggests the role of another bacteria that remain to be identified. Moreover, comEc activity may potentially be improved by using another antibiotic treatment not deleterious for beneficial taxa.

By contrast, the treatment with *A. muciniphila* or inulin was only efficient when combined with pantoprazole. Pantoprazole improves the richness of amoxicillin treated microbiota which could explain its preventive activity in combination with other treatments.

Metagenomic and qPCR analysis enabled us to identify several taxa that could be associated with the treatment efficacy in mice effectively treated with the combination of inulin and pantoprazole. *Adlercreutzia caecimuris, A. muris* and *E. coli* are of particular interest. Indeed, we found a promising preventive activity of *Adlercreutzia* strains against ESBL-Ec colonisation in a murine model. However, our tested probiotics displayed only partial activity. Therefore, new combinations of potential probiotic should be now tested, especially with *Adlercreutzia* to improve its efficacy for inhibiting ESBL-Ec. Such combinations could be *Adlercreutzia* with *Akkermansia* or with species of *Muribaculaceae*. Synbiotic combination of inulin with these taxa could also be effective. Our overall findings could open new insight for the future use of prebiotics and probiotics for the prevention of ESBL-Ec colonization. Further studies are still needed to determine the ultimate applicability of these treatments to decrease the intestinal carriage of ESBL-Ec in humans.

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DOCTORAT BIOLOGIE BRETAGNE SANTE LOIRE

Nantes Université

Titre : titre (en français) : Traitement multimodal du portage intestinal de bactéries multirésistantes avec des probiotiques et des prébiotiques..... .....

Mots clés : probiotiques, prébiotiques, *Enterobacterales* , *Escherichia coli* Oxa-48, BLSE (beta-lactamase à spectre élargi), résistance aux antibiotiques, portage intestinal

Résumé : L'intestin est le principal réservoir d'entérobactéries multirésistantes (MDRE), et il est urgent d'identifier des traitements de la colonisation intestinale par MDRE. Nos objectifs étaient de tester des traitements innovants de la colonisation digestive par un *Escherichia coli* producteur de BLSE (EcBLSE) dans un modèle expérimental murin de dysbiose intestinale induite par l'amoxicilline, et de prédire l'efficacité des traitements à partir de la composition du microbiote avant traitement. Dans ce travail, nous avons obtenu une forte activité in vitro de nouvelles souches de *B. subtilis* contre EcBLSE. Cependant ces souches se sont révélées inactives in vivo. Nous avons ensuite montré une activité temporaire in vivo de souches d'*E. coli* commensales. En outre, les traitements par *Akkermansia muciniphila* et inuline étaient efficaces chez la souris, mais uniquement lorsqu'ils étaient associés au pantoprazole. Le pantoprazole seul n'a montré aucun effet sur la colonisation par notre EcBLSE, mais il réduisait la dysbiose induite par l'amoxicilline. Les analyses par qPCR et shotgun sequencing ont identifié *Adlercreutzia caecimuris* et *A. muris* parmi d'autres taxons comme prédicteurs de l'efficacité de la combinaison inuline/pantoprazole. Nous avons pu montrer qu'utilisés comme traitement préventif, ils réduisaient significativement les titres fécaux d'EcBLSE Ces taxons pourraient avoir un effet additif sur nos traitements. Il sera donc intéressant de tester de nouveaux mélanges symbiotiques pour diminuer la colonisation intestinale par les EcBLSE. De plus, la forte activité bêta-lactamase détectée dans les fèces avant inoculation d'EcBLSE était remarquablement corrélée à la décolonisation ultérieure, probablement en diminuant la concentration fécale d'amoxicilline et en réduisant la dysbiose induite par l'amoxicilline. Cette étude ouvre donc de nouvelles voies pour le traitement de la colonisation intestinale par les entérobactéries multirésistantes.

Title: titre (en anglais): Multimodal treatment of intestinal carriage of multi-drug resistant bacteria with probiotics and prebiotics ....

Keywords: Probiotics, prebiotics, *Enterobacterales*, OXA-48-producing *Escherichia coli,* Extended-Spectrum Beta-lactamase (ESBL), Antimicrobial resistance.

Abstract: The intestine is the main reservoir of multidrug-resistant *Enterobacterales* (MDRE), and there is an urgent need to identify treatments for intestinal colonization by MDRE. Our objectives were to test innovative treatments for digestive colonization by ESBL *Escherichia coli* (ESBL-Ec) in an experimental mouse model of intestinal dysbiosis induced by amoxicillin, and to predict the efficacy of treatments from the composition of the microbiota before treatment. We obtained a strong in vitro activity of new strains of *B. subtilis* against ESBL-Ec. However, these strains were not effective in vivo. We then showed a temporary in vivo activity of commensal *E. coli*. In addition, treatments with *Akkermansia muciniphila* and inulin were significantly effective in mice, but only when combined with pantoprazole. Pantoprazole alone showed no effect on ESBL-Ec colonization, but alleviated the effect of amoxicillin on the intestinal microbiota. qPCR and metagenomic analysis identified *Adlercreutzia caecimuris* and *A. muris* among other taxa as predictors of efficacy of the inulin/pantoprazole combination. We showed that, administered as a treatment, they significantly reduced fecal titers of ESBL-Ec. These taxa could have an additive effect with other tested treatments. It would be interesting to test such new symbiotic mixtures to reduce intestinal colonization by ESBL-Ec. In addition, the strong beta-lactamase activity detected in feces before ESBL-Ec inoculation was remarkably associated with subsequent decolonization, likely by decreasing amoxicillin fecal concentration and promoting rich gut microbiota. This study therefore opens up new avenues for the treatment of intestinal colonization by multi-drug resistant *Enterobacterales* .