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DEVELOPMENT OF NOVEL ANTIMICROBIAL STRATEGIES AND SURVEILLANCE PROGRAM TO FIGHT ANTIMICROBIAL RESISTANCE

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PART I

BACKGROUND

1

ANTIMICROBIAL DRUG RESISTANCE: A BRIEF HISTORY

Penicillin, discovered by Alexander Fleming in 1928, was the first antibiotic used successfully to control bacterial infections in soldiers during World War II. However, even before the use of penicillin, in 1940, the first penicillin resistant *Staphylococcus* strains had already been described. Methicillin was introduced in 1959 and one year later, in 1960, a methicillin resistant *Staphylococcus* strain was reported (Sengupta, Chattopadhyay and Grossart, 2013). Later on, vancomycin was introduced in 1958 for the treatment of methicillin-resistant staphylococci. However, a couple of decades later, in 1979, coagulase-negative staphylococci resistant to vancomycin were reported and ten years later resistance in enterococci was described (Courvalin, 2006), followed by, in 1997, the report of less-susceptible *S. aureus* (vancomycin- intermediate *S. aureus*, VISA) strains in Japan (Levine, 2006). Another historical example is tetracycline, which was introduced in 1950 followed by tetracycline resistant *Shigella* strains being reported in 1959.

For two decades, between 1960 and 1980, there was a seemingly adequate production of new antimicrobials by the pharmaceutical industry. However, after the 1980s, the rate of discovery of new antibiotic classes had dramatically decreased (Parmar *et al.*, 2018). As a consequence of increasing antimicrobial resistance and a thin new antimicrobial pipeline, bacterial infections due to multidrug-resistant (MDR) or extensively drug-resistant (XDR) pathogens have become a major concern in clinical practice worldwide.

Nowadays, epidemiological surveillance networks in Europe (European Antimicrobial Resistance Surveillance Network -EARS-Net) and in Asia, (Central Asia and Eastern European Surveillance of Antimicrobial Resistance-(CAESAR)) have documented that antibiotic-resistant bacteria have become much more prevalent during the last decade ((Report, 2014), (WHO, 2020). Also, the high levels of antimicrobial resistance (AMR) for several important bacterial species-antimicrobial group combinations reported to EARS-Net for 2020 show that AMR remains a serious challenge in the European Union/European Area (EEU/EEA) countries. Indeed, AMR is a considerable threat to public health, both in the EU/EEA and worldwide (WHO, 2020).

AMR will be an increasing concern unless governments respond more robustly to the threat. Further investment in public health interventions is urgently needed to tackle it. This would have a significant positive impact on population health and future healthcare expenditure in the world.

Since the drivers of AMR are multifactorial, including the widespread use and misuse of antibiotics in humans, agriculture, animal farming, and industry (Harbarth *et al.*, 2015) there is a urgent need to adopt a One-Health approach, as human health is inextricably linked to the health of animals and the viability of ecosystems.

2

GENETIC BASES OF ANTIMICROBIAL RESISTANCE

Antimicrobials are small molecules that can inhibit or kill bacteria. These small molecules are commonly used as therapeutics for bacterial infections, but some bacteria can grow and survive despite antimicrobial pressures, a property known as antimicrobial resistance. Resistance is observed against nearly all antimicrobials (Figure 5), including so-called last-resort drugs used in life-threatening infections.

Based on its developmental paths, antibiotic resistance can classified as an adaptative, intrinsic, or acquired trait (Lee, 2019).

Intrinsic resistance is defined as the resistance exhibited due to the inherent properties of the bacterium. Examples of intrinsic resistance include the glycopeptide resistance exhibited by Gram-negative bacteria due to the impermeability of the outer membrane present in the Gram-negative bacterial cell envelope.

Adaptive resistance is defined as the resistance to one or more antibiotics induced by a specific environmental signal (e.g., stress, growth state, pH, concentrations of ions, nutrient conditions, sub-inhibitory levels of antibiotics). In contrast to intrinsic and acquired resistance, adaptive resistance is transient, indeed, bacteria generally revert to their original state once the inducing signal is removed (Motta, Cluzel and Aldana, 2015; Salimiyan Rizi, Ghazvini and Noghondar, 2018; Lee, 2019). Adaptive resistance seems to be the result of modulations in gene expression as a response to environmental changes. In particular, modulation of the expression of efflux pumps and porins have been implicated in the emergence of adaptive resistance (Motta, Cluzel and Aldana, 2015). The phenomenon of adaptive resistance may be responsible for the differences observed when comparing the *in vitro* and *in vivo* effectiveness of an antibiotic and could be involved in the clinical failure of antibiotic treatments.

Acquired resistance is defined as the resistance exhibited when a previously sensitive bacterium acquires a resistance mechanism by either A) a mutation in chromosomal genes or B) the acquisition of new genetic material from an exogenous source (horizontal gene transfer).

2.1. MUTATIONS IN CHROMOSOMAL GENES

Resistance to antibiotics is typically the result of a variety of strategies such as: *i)* Modification of the antibiotic molecule, *ii)* Target alterations (changes and/or bypass of target sites) and *iii)* prevention to reach the antibiotic target (by decreasing penetration or actively extruding the antimicrobial compound).

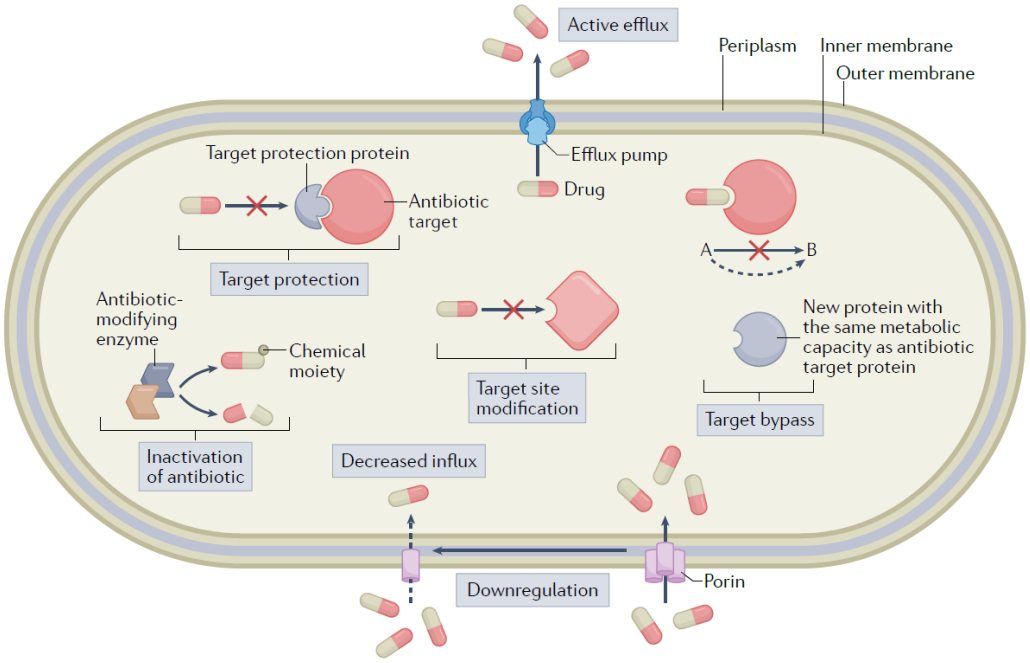


Figure 5. Overview of antibiotic resistance mechanisms. (**Baylay, Piddock and Webber, 2019**)

I) MODIFICATIONS OF THE ANTIBIOTIC MOLECULE

One of the most successful bacterial strategies to cope with the presence of antibiotics is to produce enzymes that inactivate the drug by adding specific chemical moieties to the compound or that destroy the molecule itself, rendering the antibiotic unable to interact with its target.

CHEMICAL ALTERATIONS OF THE ANTIBIOTIC

The production of enzymes capable of introducing chemical changes to the antimicrobial molecule is a well-known mechanism of acquired antibiotic resistance in both Gram-negative and Gram-positive bacteria. Many types of modifying enzymes have been described, and the most frequent biochemical reactions they catalyze include *i)* acetylation (aminoglycosides, chloramphenicol), *ii)* phosphorylation (aminoglycosides, chloramphenicol), and *iii)* adenylation (aminoglycosides) (Figure 6). Regardless of the biochemical reaction, the resulting effect is often related to steric hindrance that decreases the avidity of the drug for its target, which, in turn, is reflected in higher bacterial resistance.

DESTRUCTION OF THE ANTIBIOTIC MOLECULE

The main mechanism of β-lactam resistance relies on the destruction of these compounds by the action of β-lactamases (Figure 6). These enzymes destroy the amide bond of the β-lactam ring essentially rendering the antimicrobial ineffective. The first β-lactamases were described in the early 1940s (Abraham and Chain, 1940), one year before penicillin was introduced into clinical practice. More than 1000 β-lactamases have been reported up to date produced by diverse bacteria and many more are likely to continue to be reported, as part of the normal process of bacterial evolution. Genes encoding for β-lactamases are generally termed *bla*, followed by the name of the specific enzyme (e.g., $bla_{CTX\text{-}M}$) and they have been found in the chromosome or in mobile genetic elements (MGEs) as part of the accessory genome, which has facilitated their dissemination among bacteria. These genes can also be found forming part of integrons.

β-LACTAMSES

β-lactamases represent the major defense mechanism against β-lactam-based drugs in clinically important Gram-negative bacteria. These enzymes have the ability to hydrolyze the β-lactam chemical bond, thus rendering the molecules incapable of killing bacteria. Prevalence and variety of these enzymes have undergone a dramatic increase with the therapeutic use of β-lactam antibiotics; up to date, more than 2,000 uniquely occurring β-lactamases variants have been identified.

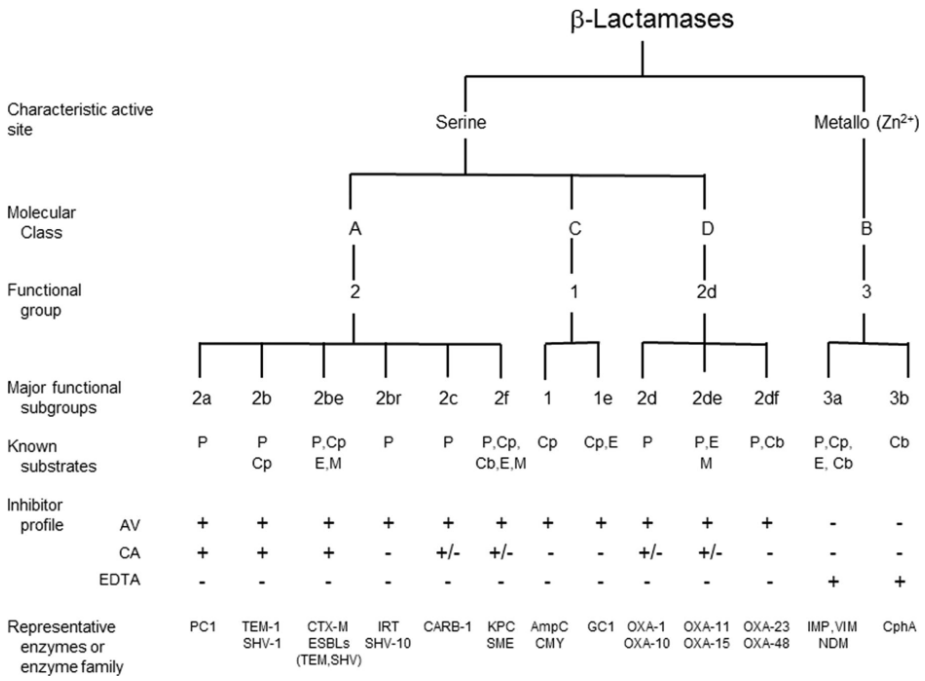


Figure 4. Molecular and functional relationships among β-lactamases**.** AV, avibactam; CA, clavulanic acid; Cb, carbapenem; Cp, cephalosporin; E, expanded-spectrum cephalosporin; M, monobactam; P, penicillin

The classifications of β-lactamases were traditionally based on either the primary amino acid sequence (Ambler *et al.*, 1991) or on functional aspects of the enzymes (Bush, Jacoby and Medeiros, 1995). Ambler classification divide the β-Lactamases into four classes (A-D) (Figure 4): the active-site serine β-lactamases (classes A, C and D) and the zinc-dependent or metallo-β-lactamases (MBLs; class B).

Ambler class A β-lactamases includes enzymes present in Enterobacterales, such as TEM and SHV that can be inactivated by clavulanate, sulbactam, and tazobactam-extended spectrum β-lactamases (ESBL) and *K. pneumoniae* carbapenemases (KPC and GES).

Class B β-lactamases includes the metallo-β-lactamases (MBLs), such as New Delhi MBL (NDM), Verona integrin-encoded MBL (VIM), and imipenemases (IMP). At present, these β-lactamases can be fought by a very limited number of antimicrobial agents.

Class C includes the AmpC β-lactamases group, which can be encoded by either chromosomal or plasmid genes and can therefore confer a variable degree of resistance to antimicrobial agents.

Ambler Class D β-lactamases mostly includes the oxacillinases (OXA), such as OXA-48, which has a broad spectrum of inhibition, including carbapenems, and is a common resistance mechanism among Enterobacterales, and OXA-23 and OXA-51-like enzymes, which are mostly found in *A. baumannii*.

EXTENDED-SPECTRUM B-LACTAMASES (ESBLs)

Extended-spectrum β-lactamases (ESBLs) are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third-generation cephalosporins and aztreonam (but not the carbapenems) yet are inhibited by clavulanic acid. Typically, they derive from genes for TEM-1 or SHV-1 by mutations that alter the amino acid configuration around the active site of these β-lactamases.

However, most of ESBLs originated from naturally occurring (chromosomal) enzymes present in some species of genus *Kluyvera*, a member of the *Eterobacteriaceae* family (D'Andrea *et al.*, 2013), promoting a large replacement of the early SHV and TEM variants with the CTX-M family of ESBLs.

The CTX-M β-lactamases are the most widespread enzymes. They were initially reported in the second half of the 1980s, and their rate of dissemination among bacteria and in most parts of the world has increased dramatically since 1995. CTX-M-type β-lactamase enzymes were initially reported in the late 1980s, emerging concomitantly in several locations. The worldwide expansion of isolates carrying ESBLs later would be referred as the 'CTX-M pandemic'. Since the early 2000s, CTX-M-types enzymes has been recognized as the most common ESBL group, replacing TEM and SHV as the dominant ESBL enzyme (D'Andrea *et al.*, 2013).

The CTX-M β-lactamases are a quite heterogeneous enzyme class, including five major subgroups according to their different progenitor species and sequence homologies: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Each group, in turn, includes a number of minor allelic variants which differ from each other by one or two amino acid substitutions.

The dissemination of these enzymes is a problem of global magnitude, with rates of ESBL production being particularly high in some enterobacterial species such as *K. pneumoniae* and *E. coli* (Paterson and Bonomo, 2005). Of note, *E. coli* sequence type 131 (ST131) (causes of a wide variety of infections, mostly UTIs) have reported to carry a variety of β-lactamases and several CTX-M types, mostly commonly CTX-M-15. Recently, *E. coli* sequence type 1193 (ST1193) was described as a novel pandemic clone of fluoroquinolone resistance isolates that frequently, carries $bla_{\text{CTX-M}}$ genes.

II) TARGET ALTERATIONS

The drug target can be modified (quantitatively or qualitatively) to prevent the action of the antibiotic. Spontaneous mutations in the drug target chromosomal gene, production of modifying enzymes altering the target, target protection systems, and production of alternative target lead to a decreased or a loss of affinity for the antibiotics. Paradigmatic example are represented by mutations in *rpoB* gene, encoding the beta-subunit of RNA polymerase, preventing rifampicin binding; expression of *rmt* and *arm* genes encoding methylases conferring resistance to aminoglycosides by altering 16S ribosomal RNA; expression of *qnr* genes encoding proteins that reduce susceptibility to quinolones by protecting the complex of DNA-DNA topoisomerase II enzymes from the inhibitory effect of quinolones; expression of *mec* genes encoding alternative penicillin-binding proteins (PBPs), not inhibited by conventional β-lactams (Figure 6).

III) DECREASED ANTIBIOTIC PENETRATION AND EFFLUX

Decrease Permeability

Many of the antibiotics used in clinical practice have intracellular bacterial targets or targets located in the inner membrane (in the case of Gram-negative bacteria). Therefore, the compound must penetrate the outer and/or cytoplasmic membrane to exert its antimicrobial effect.

The outer membrane acts as the first line of defence against the penetration of multiple toxic compounds, including several antimicrobial agents. The low permeability of the bacterial outer membrane to specific antibiotic agents is responsible for the intrinsic resistance of some Gram-negative bacteria to those antibiotics. The prime example of the efficiency of this natural barrier is the fact that vancomycin, a glycopeptide antibiotic, is not active against Gram-negative organisms due to the lack of penetration through the outer membrane (Figure 6). Moreover, changes in the outer membrane permeability can

contribute to the development of acquired resistance. The innate low susceptibility of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* to β-lactams (compared to *Enterobacteriaceae*) can be explained, at least in part, to a reduced number and/or differential expression of porins (Figure 6).

The number and type of porins expressed on the outer membrane will affect the entry of hydrophilic antibiotics and, therefore, the susceptibility of the bacterial cell to them (Fernández and Hancock, 2012). Moreover, mutations affecting the expression or the function of porins can lead to acquired antibiotic resistance. These mutations can have different effects: such as *i)* impairment of the porin function, *ii)* a shift in the type of porins expressed, or *iii)* the reduced expression of porins (changes in porin expression generally lead to low-level antibiotic resistance).

Several types of porins have been described, and they can be classified according to their structure, their selectivity, and the regulation of their expression. Among the best-characterized porins, the three major proteins produced by *Escherichia coli* (known as OmpF, OmpC, and PhoE) and the *P. aeruginosa* OprD are classical examples of porin-mediated antibiotic resistance. One classic example of porin-mediated resistance is the aberrant production of OprD in *P. aeruginosa*, which is normally used for the uptake of basic amino acids and antibiotics (i.e., imipenem).

Increased drug efflux

Efflux pumps are energy-dependent complex bacterial systems present on the cytoplasmic membrane which are capable of extruding toxic compound out of the cell. The first efflux pump pumping tetracycline out of the bacterial cell was described in *E. coli* in 1980 and was plasmid encoded (Figure 6) (Ball, Shales and Chopra, 1980). Since then, many classes of efflux systems involved in antibiotic resistance have been identified in both Gram-positive and Gram-negative bacteria. The genes encoding efflux pumps can be located in MGEs or in the chromosome. There are five major families of efflux pumps: *i)* the major facilitator superfamily, *ii)* the small multidrug resistance family (SMR), *iii)* the resistance-nodulation-cell-division family (RND), *iv)* the ATP-binding cassette family, and *v)* the multidrug and toxic compound extrusion family. These families differ in terms of structural conformation, energy source, range of substrates they are able to extrude, and the type of bacterial organisms in which they are distributed.

Clinical resistance is usually the result of mutational events leading to increased pump expression or improved pump effectiveness (Piddock, 2006). Examples of substrate-specific efflux pumps include those specific for tetracyclines, macrolides, and chloramphenicol (Figure 6) (Poole, 2005). Tetracycline resistance is one of the classic examples of efflux-mediated resistance, where the Tet efflux pumps extrude tetracyclines using proton exchange as the source of energy. Currently, more than 20 *tet* genes have been described, most of which are harbored in MGEs. In addition to the tetracycline-specific transport systems,

several MDR efflux pumps such as AcrAB-TolC in *Enterobacteriaceae* (classically found in *E. coli*, which is composed of a transporter protein located in the inner membrane (AcrB), a linker protein located in the periplasmic space (AcrA), and a protein channel located in the outer membrane (TolC)) and MexAB-OprM in *P. aeruginosa* (both belonging to the RND family) are able to extrude tetracyclines (including tigecycline) as part of their contribution to multidrug resistance.

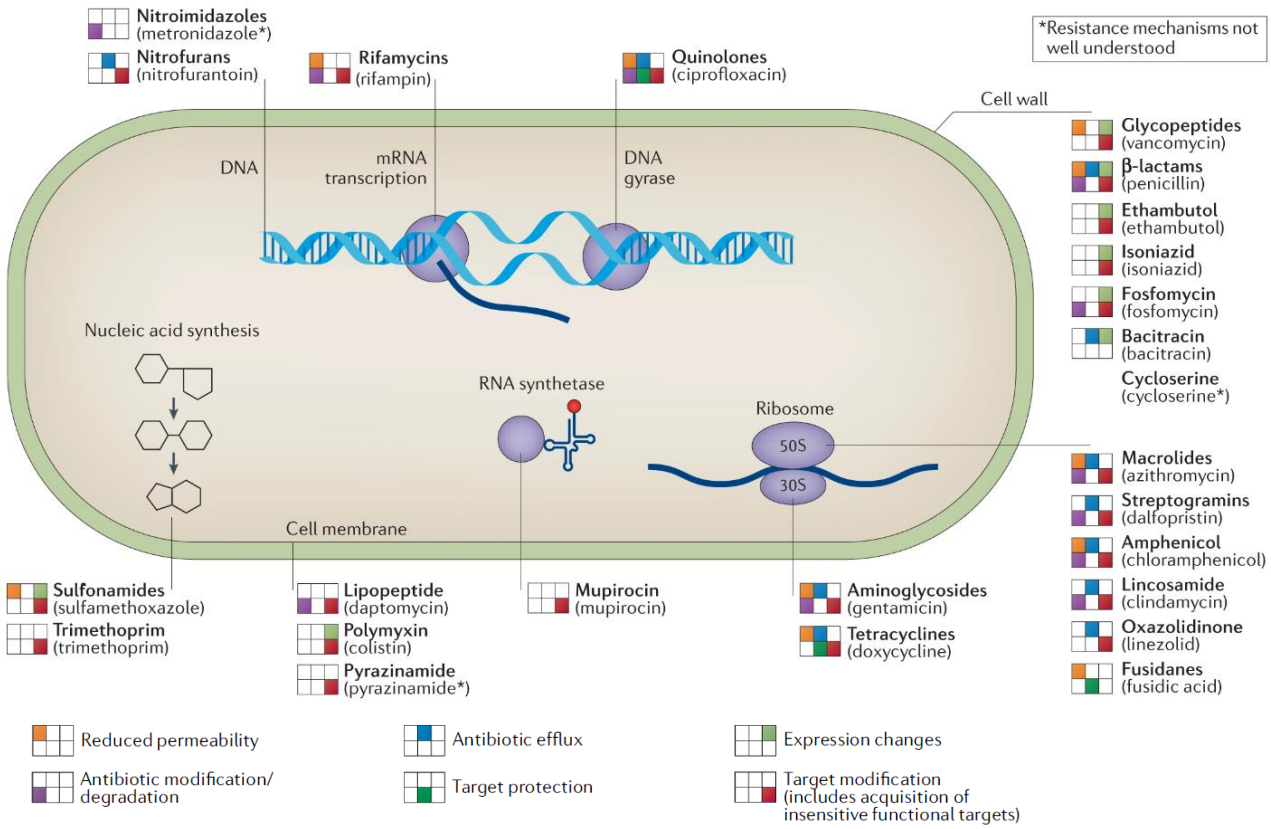


Figure 6. Antibiotic targets in bacterial cells. Different mechanisms of antibiotic resistance are represented by different colour: orange indicates the reduced permeability; blue indicates antibiotic efflux; light green indicates the expression changes; purple indicates antibiotics modification/degradation; dark green indicates target protection and red indicate target modification (Adapted from Boolchandani, D'Souza and Dantas, 2019).

2.2. HORIZONTAL GENE TRASFER

Horizontal gene transfer can occur through three main mechanisms (Holmes *et al.*, 2016) (Figure 7):

i) Transformation: a form of genetic recombination in which free DNA fragments from a dead bacterium enter a recipient bacterium and are incorporated into its chromosome. Only a few bacteria are naturally transformable.

ii) Transduction: Transduction involves the transfer of genetic material between a donor and a recipient bacterium by a bacteriophage.

iii) Conjugation: probably the most important mechanism of horizontal gene transfer. It involves the transfer of genetic material from one bacterial cell to another by direct physical contact between the cells. Multiple resistance genes are often present on a single plasmid enabling the transfer of MDR in a single conjugation event. The assembly of multiple resistance genes on a single plasmid is mediated by mobile genetic elements (transposons, integrons, and Insertion Sequence Common Region- ISCR-elements).

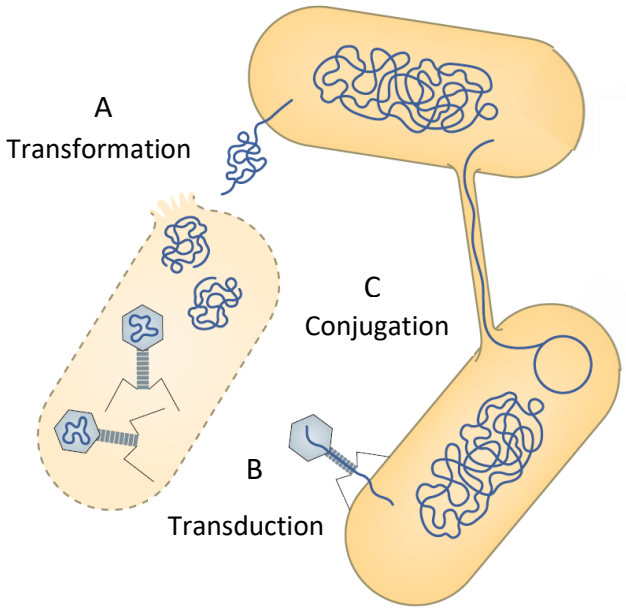


Figure 7. General routes of horizontal gene transfer. The schematic shows the different mechanisms of horizontal gene transfer. A| Transformation involves the uptake of naked DNA from lysed cells in the environment. B| During transduction, genetic material is introduced from a phage into bacterial genomes. C| Conjugation involves the transfer of DNA through conjugative pili and is the predominant mechanisms by which DNA is transferred between bacteria (Adapted from Brito, 2021).

3

BACTERIAL BIOFILM: A COMPLEX ECOSYSTEM

In addition to the above-mentioned antibiotic resistance mechanisms, persistent bacterial infection and increased antibiotic resistance can often be attributed to biofilm formation on host tissues and implants.

Biofilms are microbial communities that adhere to biotic or abiotic surfaces, and the cells within a biofilm are encased in self-produced matrix (Extracellular polymeric substances, EPS) (Table 1). The biofilm matrix contains polysaccharides, proteins and extracellular DNA, and the bacterial consortium can consist of one or more species living in a socio-microbiological way. The EPS is important since it provides structural stability and protection to the biofilm (Table 1). Biofilm formation contributes towards the development of antibiotic resistance and the formation of persistent cells which are responsible for the unmanageable persistence of microbial infections (Costerton, Stewart and Greenberg, 1999). Many clinically important species can produce biofilm, for instance, *Pseudomonas aeruginosa* biofilms cause chronic lung infections in cystic fibrosis (CF) patients (Singh *et al.*, 2000), while *S. aureus* biofilms can colonise indwelling medical devices such as pacemakers (Marrie, Nelligan and Costerton, 1982).

Components	Percentage (%)
Microbial cells	2-5
Water	Up to 97
Polysaccharides	1-2
Proteins*	<1-2
DNA and RNA	<1-2

Table 1. Composition of biofilm. Proteins including enzymes (Rather, Gupta and Mandal, 2021).

3.1. BIOFILM FORMATION

Biofilm formation is a multi-step and complex process that involves the transition of bacteria from free-swimming planktonic form to biofilm-making sessile form. The whole process of formation is influenced by external conditions like temperature, pH, gravitational forces, hydrodynamic forces, Brownian movements, nature of the inhabiting surfaces, quorum sensing, secondary messengers, and other signalling molecules as well. Different stages of biofilm formation can be divided into four major steps: *i)* attachment of planktonic microorganisms to the surfaces which is sub-divided into reversible and irreversible attachment followed by *ii)* formation of microcolonies. Microcolonies undergo *iii)* maturation characterized by specific composition, shape, and architecture followed by *iv)* dispersion of biofilm to repeat the cycle (Rather, Gupta and Mandal, 2021) (Figure 8).

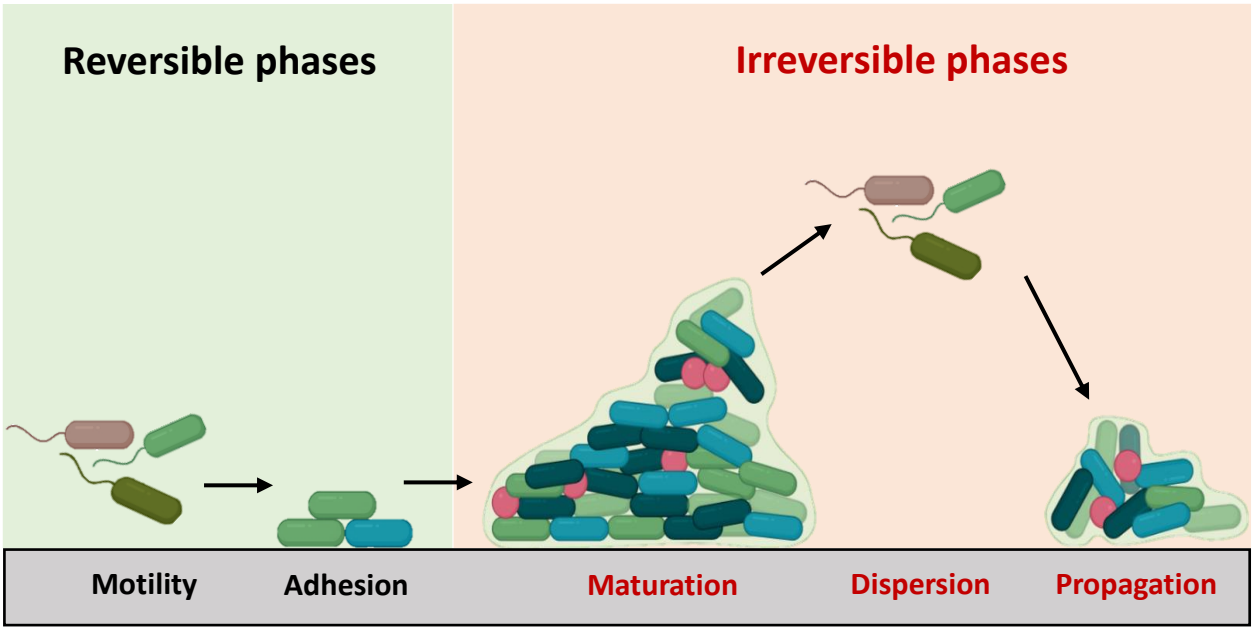


Figure 8. Steps of biofilm formation. A mature biofilm is a heterogeneous mixture of planktonic, sessile, persistent, dead cells, water channels, and different types of signaling and stabilizing molecules like lipids, polysaccharides, proteins and extracellular DNA (eDNA) (Adapted from https://biorender.com/).

There are several key characteristics that differentiate biofilm cells from planktonic cells. While the solitary planktonic cultures are exposed to relatively uniform environmental conditions, cells in a biofilm experience a gradient of nutrients, oxygen (the concentration of oxygen near the surface of biofilm is highest and declines towards the center, creating almost anaerobic conditions in the center) and waste products. There is a similar stratification in metabolic activity, growth, and protein synthesis in biofilms with a high rate at the surface and no or very less rate in the center. Subpopulations in biofilms are, as a result, physiologically heterogeneous, and this is characterised by differences in gene expression, metabolic activity and phenotype, including antimicrobial tolerance, of cells located in different geographical areas of a biofilm.

Moreover, the extracellular biofilm matrix serves as a scaffold that has an essential cell-to-cell connecting and structural function in biofilms and plays a role in several processes including cell attachment, cell-to-cell interactions, and antimicrobial tolerance (Rather, Gupta and Mandal, 2021).

3.2. BIOFILM RESISTANCE AND TOLERANCE

Biofims are resistant to antibiotics and disinfectants and impervious to phagocytosis, and tolerate the body's immune system mainly because of self-produced EPS (Høiby *et al.*, 2010). It is generally accepted that the basis for biofilm-specific antibiotic resistance and tolerance is multifactorial, and mechanisms of resistance and tolerance vary depending on the particular antimicrobial agent, the bacterial strain and species, the age and developmental stage of the biofilm, and the biofilm growth conditions (Figure 9).

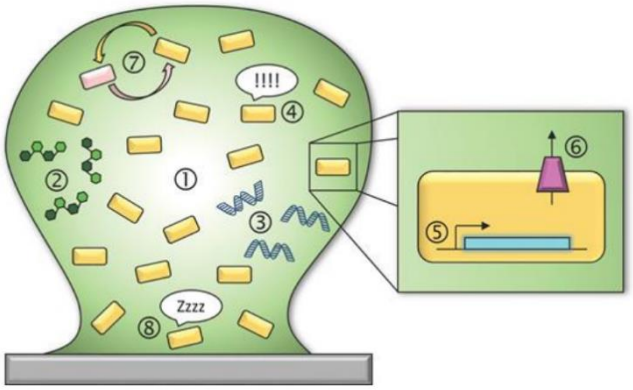


Figure 9. The major antimicrobial resistance and tolerance mechanisms employed by bacterial biofilms. Biofilm cells (yellow rectangles) are embedded in a mushroom-shaped matrix (shown in green). The biofilm is attached to a surface (grey rectangle), which can be biotic or abiotic. Pictorial representations of the resistance mechanisms are numbered as follows: (1) nutrient gradient (demonstrated here as a colour-intensity gradient) with less nutrient availability in the core of the biofilm, (2) matrix exopolysaccharides, (3) extracellular DNA, (4) stress responses (oxidative stress response, stringent response, etc.), (5) discrete genetic determinants that are specifically expressed in biofilms and whose gene products act to reduce biofilm susceptibility via diverse mechanisms (*ndvB*, *brlR*, etc.), (6) multidrug efflux pumps, (7) intercellular interactions (horizontal gene transfer, quorum sensing, multispecies communication, etc.) and (8) persister cells (Hall and Mah, 2017).

A multi-layer defense system is constituted in biofilm by the formation of persister cells, development of adaptive stress responses, very less antibiotic penetration, limited nutrition, less growth and metabolic activity (Stewart, 2002), and inactivation of antimicrobials within the components of the EPS matrix . Indeed, the factors which contribute towards antibiotic resistance of biofilm include restricted antimicrobial penetration, antibiotic-modifying enzymes in matrix, eDNA, hypoxia, reduced growth, variability in physiology, oxidative stress and amino acid starvation responses, efflux pumps, quorum sensing, persister cells, high mutation rate, and colony variants (Figure 9) (Hall and Mah, 2017).

4

THE MAIN ANTIMICROBIAL OPTIONS FOR GRAM-NEGATIVE BACTERIA

The emergence of multidrug resistance Gram-negative bacteria remains one of the major public health issues of global concern. Furthermore, WHO published its "priority list of antibiotic-resistant bacteria" (WHO, 2017). They include carbapenem-resistant *Acinetobacter*, carbapenem-resistant *Pseudomonas* and carbapenem-resistant extended spectrum β-lactamase (ESBL)-producing-Enterobacterales ("Priority 1: critical"). These bacteria can cause severe and often deadly infections such as bloodstream infections and pneumonia, demonstrating that strong countermeasures must be taken. This condition prompted the reintroduction in clinical practice of the old antibiotic such as colistin and fosfomycin.

4.1. COLISTIN

Colistin is a polypeptide antibiotic of the polymyxin family consisting of a cyclic heptapeptide with a tripeptide side chain acylated at the N terminus by a fatty acid tail.

Historically, colistin was first used in the 1950s as an intravenous formulation (Talbot *et al.*, 2006; Kaye *et al.*, 2016) and it exhibits a narrow spectrum of activity, mostly against Gram-negative bacteria, including common or important non-fermentative Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii.* Moreover, the polymyxins have bactericidal activity against most members of the Enterobacteriaceae family such as *E. coli, Enterobacter, Salmonella, Shigella* and *Klebsiella* (Tan and Ng, 2006).

Because of the reported adverse events of polymyxins mainly nephrotoxicity and neurotoxicity, alongside to the discovery and approval of new and effective antibiotics, the clinical use of polymyxins was largely abandoned by the mid-1970s. By the mid-1990s the polymyxins had re-emerged as a last-resort treatment against MDR and XDR Gram-negatives, not because of an improved safety profile, but rather due to the emergence of XDR Gram-negative superbugs, particularly *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, which are resistant against all other available antibiotics, besides the lack of novel antimicrobials available to treat MDR bacterial infections (Kaye *et al.*, 2016). Unfortunately, the overuse and misuse of colistin among humans and animals medicine have led to the global emergence of colistin-resistant pathogens (Giani *et al.*, 2018).

MECHANISM OF ACTION

The precise mechanism of antibacterial activity of polymyxins is not completely understood. However, the general current view is that polymyxins kill bacteria by disrupting the bacterial outer and inner membranes through the "self-promoted uptake" pathway (Sherry and Howden, 2018).

The interaction of polymyxins with lipopolysaccharides (LPS) is essential for their antimicrobial activity. It binds with the anionic LPS molecules by displacing divalent cations (Mg^{2+} and Ca^{2+}, that bridge the lipid A phosphoester) from the outer cell membrane of Gram-negative bacteria, leading successively to permeability changes in the cell envelope, leakage of cell contents, and cell death (Figure 1).

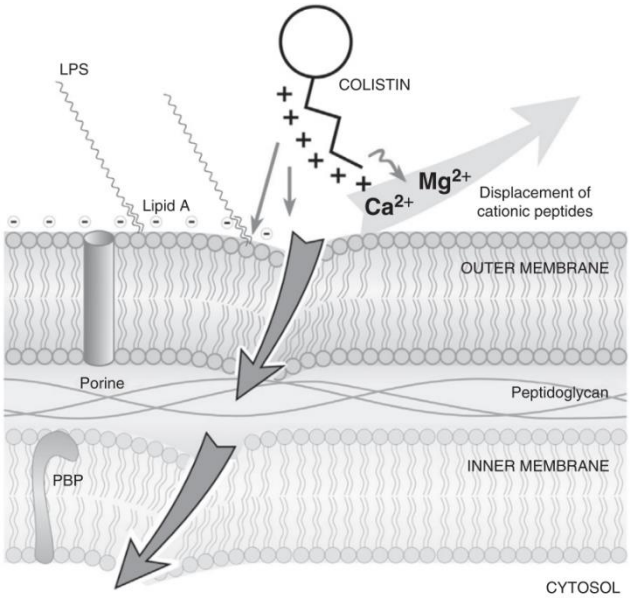


Figure1. Action of colistin on bacterial membrane. (Martis, Leroy and Blanc, 2014)

Generally, Gram-negative bacteria can develop resistance to polymyxins through intrinsic, mutation or adaptation mechanisms, besides the horizontally acquired resistance mediated via the *mcr-1* gene and its variants. The main polymyxins resistance mechanisms were adaptative mechanisms and can be summarized as follows: (i) modifications of the LPS moiety via the addition of cationic groups to the LPS (e.g., mutation in two component-system :PmrA/PmrB and PhoP/PhoQ); (ii) mutations that lead to the loss of the LPS (*e.g. A. baumannii*); (iii) porin mutations and overexpression of efflux pump systems (e.g., efflux pump AcrAB-TolC in *K. penumoniae*); (iv) overproduction of capsular polysaccharide (CPS) in some Gram-negative bacteria that hide the polymyxin binding sites and the release of CPS trapping polymyxins; and (v) enzymatic inactivation of colistin.

4.2. FOSFOMYCIN

Fosfomycin is a phosphonic acid antibiotic discovered in 1969 in Spain from cultures of *Streptomyces* spp. The structure of fosfomycin has two key features: an epoxide group, which is essential for its biological activity and a phosphonic acid moiety (Figure 2) (Falagas *et al.*, 2019). Fosfomycin remains one of the first-line agents for the treatment of acute uncomplicated urinary tract infections (UTI) mainly caused by *E. coli*, *Klebsiella* spp., *Proteus mirabilis*, *Enterococcus* spp. and *Streptococcus agalactiae* (López-Montesinos and Horcajada, 2019).

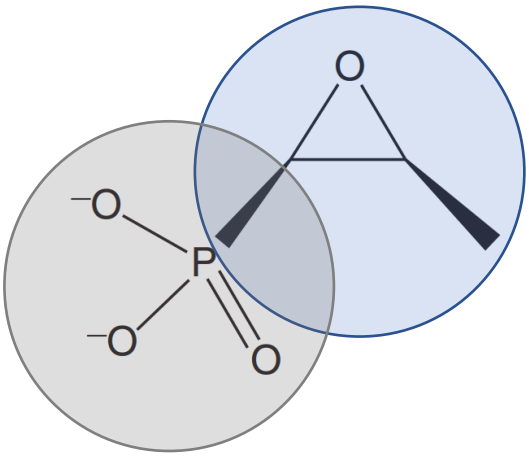


Figure 2. Molecular structure of Fosfomycin. The shadow blue identifies an epoxide group. The shadow grey identifies a phosphonic acid moiety.

Moreover, there is a global interest to further investigate fosfomycin as monotherapy and in combination with other antibiotics for the treatment of serious system infections due to MDR Gram-negative bacteria (López-Montesinos and Horcajada, 2019).

MECHANISM OF ACTION

Fosfomycin invades the bacteria through two different membrane transportation systems L-alpha glycerol-3-phosphate and the glucose-6-phosphate transporter (G6P) (GlpT and UhpT, respectively) (Silver, 2017). The chemical structure of fosfomycin imitates both glycerol-3-phosphate and G6P, which are normally transferred trough GlpT and UhpT and induce their expression. Fosfomycin has a bactericidal action that inhibits the biosynthesis of peptidoglycan in both Gram-positive and Gram-negative bacteria during the first step, leading to bacterial cell lysis and death. Specifically, fosfomycin inhibits the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (or MurA), which takes part in the transportation of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 3'-hydroxyl group of UDP-N-acetylglucosamine (UNAG) that is required for the biosynthesis of peptidoglycan.

The mechanisms of resistance to fosfomycin can be either chromosomal or plasmid mediated. Most chromosomally resistant mutants that do not easily transfer to other organisms, whereas plasmid-resistant mutants can transfer their resistance to other organisms through conjugation or transformation.

Bacterial resistance to fosfomycin occurs via three mechanisms, two of which are based on chromosomal genes and the third on plasmids. The main mechanism for acquisition of fosfomycin resistance is an inactivation in phosphonate transport or uptake pathways (e.g., GlpT, UhpT). Another common mechanism is the modification of the antibiotic MurA target, which inactivate the enzyme by irreversibly binding to the protein. The third mechanism is an antibiotic modification: several enzymes that inactivate fosfomycin by covalent modification cleave the carbon–oxygen bond of the epoxide moiety (e.g., FosA, FosB, FosX).

4.3. AMYNOGLICOSIDES

Since the first aminoglycoside, streptomycin (introduced in 1944), this class of antibiotic has played a vital role in the treatment of serious Gram-negative infections. Streptomycin, neomycin, kanamycin, tobramycin, and gentamicin are naturally occurring aminoglycosides, whereas amikacin is a semisynthetic derivative of kanamycin. Structurally, each of these aminoglycosides contains two or more amino sugars linked by glycosidic bonds to an aminocyclitol ring nucleus. The aminoglycosides are particularly potent against the *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp., but they are not active against anaerobes, making necessary an aerobic energy-dependent process to enable successful penetration of the bacterial inner cell membrane by the aminoglycosides.

MECHANISM OF ACTION

Aminoglycosides are bactericidal agents that inhibit bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosomal subunit. The aminoglycoside-bound bacterial ribosomes then become unavailable for translation of mRNA during protein synthesis, thereby leading to cell death. Moreover, the aminoglycosides also cause misreading of the genetic code, with resultant production of nonsense proteins. Bacterial uptake of these agents is facilitated by inhibitors of bacterial cell wall synthesis, such as β-lactams and vancomycin.

The main mechanisms of bacterial resistance to aminoglycosides are: *i)* decreased intracellular accumulation of the antibiotic by alteration of the outer membrane's permeability, decreasing inner membrane transport, or active efflux; *ii)* modification of the target site by mutation of the ribosomal proteins or 16S RNA or post-transcriptional methylation of 16S RNA; and *iii)* enzymatic modification of the drug (the most common resistance mechanism).

4.4. β-LACTAMS

β-Lactams are the most widely used class of antibacterial agents worldwide. This family of antibiotics is a bactericidal drug structurally-related containing the β-lactam ring in their chemical structure (Figure 3).

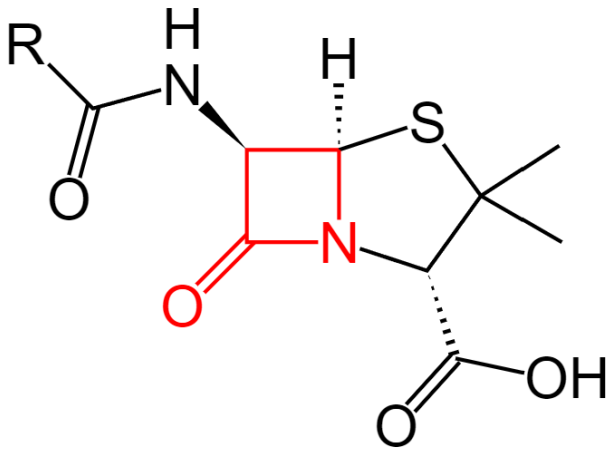


Figure 3. Structure of penicillins. β-lactam ring in red.

The discovery and the market of first β-lactam antibiotic (Penicillin G) is a symbolic landmark of modern chemotherapy. Since then, several other β-lactam antibiotics have been introduced in the therapy, revolutionizing the treatment of bacterial infections.

Clinically important β-lactams include the penicillins, cephalosporins, carbapenems, and monobactams. Their antibacterial efficacy has been kept in check by the emergence of bacterial resistance. Among the resistance mechanisms, the expression of β-lactamase enzymes is one of the most studied and prevalent.

MECHANISM OF ACTION

β-Lactam antibiotics are bactericidal agents that interrupt bacterial cell-wall formation as a result of covalent binding to essential penicillin-binding proteins (PBPs), enzymes that are involved in the terminal steps of peptidoglycan cross-linking in both Gram-negative and Gram-positive bacteria. The β-lactam antibiotics effectively inhibit the catalytic activity of bacterial transpeptidases (able to restabilize the peptide bonds in the last step of the peptidoglycan biosynthesis).

5

NOVEL STRATEGY TO COUNTERACT ANTIMICROBIAL RESISTANCE

Development of antibacterial resistance is considered one of the leading public health problems, since it has a significant impact on the medical costs, prolonger hospital stays and increased mortality. Since therapeutic options to treat infections are increasingly being limited due to antibacterial resistance, this escalates the morbidity and mortality associated with infectious diseases caused by bacteria (WHO), 2020). Therefore, research and development of a new generation of antimicrobials to mitigate the spread of antibiotic resistance has become imperative.

Current research and technology developments have promoted the improvement of antimicrobial agents that can selectively interact with a target site (e.g., a gene or a cellular process) or a specific pathogen (Jackson, Czaplewski and Piddock, 2018). Furthermore, antimicrobial peptides, metal nanoparticles, phage therapy, antibiotic resistance inhibitors and RNA therapy exemplify a novel approach to treat infectious diseases (Figure 10). Nonetheless, combinatorial treatments have been recently considered as an excellent platform to design and develop the next generation of antibacterial agents (Figure 10).

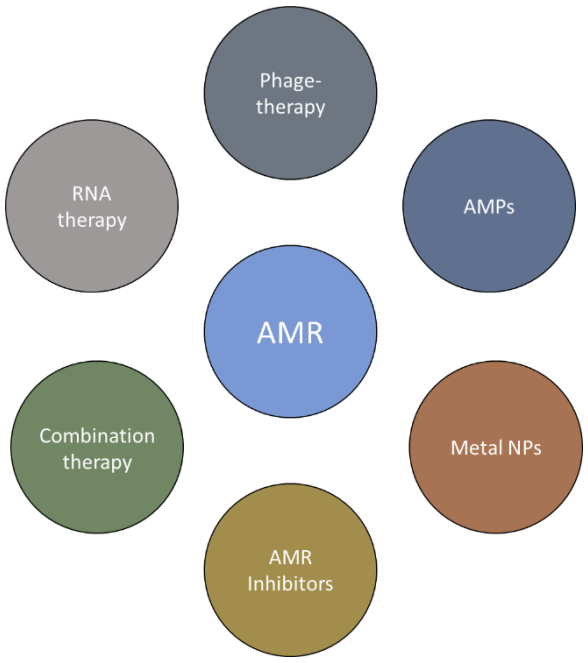


Figure 10. Different strategies for tackling antimicrobial resistance.

5.1. ANTIMICROBIAL PEPTIDE

Antimicrobial peptides (AMPs) are a highly diverse family of small proteins with a varying number of amino acids and they are divided into many subgroups on the basis of their amino acid composition and structure and other properties (Boparai and Sharma, 2020). AMPs interact with bacterial cell membrane through electrostatic interactions thus making it difficult for bacteria to develop resistance unlike conventional antibiotics (Pfalzgraff, Brandenburg and Weindl, 2018). Based on their mode of action, these peptides are classified into membrane acting and non-membrane acting peptides. Membrane acting peptides mainly harbour cationic peptides causing membrane disruptions, whereas non-membrane peptides are capable of translocation across the membrane without damaging it (Hancock and Patrzykat, 2002) and take part in the development of different intracellular processes, such as inhibition of transcription, translation, protein synthesis, and bacterial cell wall formation (Lohner, 2016).

AMPs represent a potential alternative to replace a wide variety of commonly used drugs. AMPs are promising potential candidates to counteract MDR pathogens since they possess many advantages: high potency in the micromolar range, efficacy selectivity, low resistance selection, potentially low toxicity and low accumulation in tissues.

5.2. METAL NANOPARTICLES

An additional alternative to fighting infections caused by AMR bacteria is the development of nanoparticles (NPs) that are between 0.1 and 100 nm (Wang, Hu and Shao, 2017; Lee, Ko and Hsueh, 2019). Several types of inorganic and organic NPs, including various metals, such silver NPs (AgNPs) that act by disturbing cell membrane permeability and gold (Au) NPs (RubenMorones-Ramirez *et al.*, 2013; Tiwari *et al.*, 2018) have been developed and evaluated *in vitro*. However, because the mechanisms of action of NPs are not well understood and this field of research is in its infancy, the potential risks of toxicity associated with using NPs in health care are not known yet (Shaikh *et al.*, 2019).

5.3. BACTERIOPHAGES AND ENDOLYSINS

Several studies demonstrated that bacteriophages can be successfully used in the therapy of animal and human bacterial infections. Phages are already used in the agricultural, food processing and fishery industries, and for the treatment of human bacterial infections in Georgia and Eastern Europe (Housby and Mann, 2009). Whereas phages are harmless to eukaryotic cells in patients, there are several obstacles to their use clinically: they are difficult to standardize and there is a need to employ cocktails of multiple phages due to their narrow spectra; there are differences in biological, physical, and pharmacological

properties of phages compared to conventional antimicrobials; and there is a relative lack of regulatory approval processes. In addition, the emergence of bacterial resistance to phages could be a problem in chronic conditions (e.g., CF) that require long-term therapy (Gordillo Altamirano and Barr, 2019).

Bacteriophage-derived enzymes, such as endolysins and other peptidoglycan hydrolases that disrupt bacterial cell walls, represent interesting alternatives to conventional antibiotics (Fischetti, 2018). Indeed, endolysins are enzymes used by bacteriophages at the end of their replication cycle to degrade the peptidoglycan layer of their bacterial hosts triggering lysis. Molecular engineering of endolysins has been employed to develop novel antimicrobials. These lytic enzymes, exhibiting a high degree of host specificity, could potentially replace or be utilized in combination with antibiotics.

5.4. ANTIBIOTIC RESISTANCE INHIBITORS

One of the prominent groups of new antibiotics with broad spectrum activity is the β-lactam–β-lactamase inhibitor combinations (BLBLIs). The development of β-lactamase inhibitors has contributed to the preservation of the efficacy of β-lactams against β-lactamase-producing pathogens.

There are many examples of antimicrobial resistance inhibitors already used in clinical practice or under development, such as β-lactamase and efflux pump inhibitors (Bush and Bradford, 2016; Wang, Venter and Ma, 2016). Among new β-lactamase inhibitors, 3 novel non-β-lactam compounds (i.e., avibactam, vaborbactam, and relebactam) are clinically available or in clinical trials (Wong and van Duin, 2017).

Several efflux pump inhibitors have been designed but their development of most was stopped because their molecular scaffolds must be optimized for higher activity and less toxicity (Farrell *et al.*, 2018). No efflux pump inhibitors have been used in clinics up to now.

5.5. RNA THERAPY

Bacterial small regulatory RNAs (sRNAs), that are approximately 50-500 nucleotides long, participate in many regulatory events in bacterial pathogens, including virulence onset (Waters and Storz, 2009). They can also control biofilm formation, antibiotic resistance, and a variety of other bacterial stress responses, some of which can reduce the effectiveness of antibiotic treatments or are implicated in bacterial persistence, inducing antibiotic tolerance by reduced metabolism (Waters and Storz, 2009). Modulating sRNA functions by specific drugs could enhance the efficacy of existing antibiotics, especially if sRNAs impact bacterial growth.

Clustered regularly interspaced short palindromic repeat (CRISPR)-based antibacterial are potentially capable of targeting any bacterial pathogen (Greene, 2018). Bacterial CRISPR-Cas systems prevent foreign genetic invasions. CRISPR-Cas9 contains an RNA-guided endonuclease, inducing RNA-targeted DNA breaks with the help of a guide RNA containing complementary base pairs at specific locations within the foreign DNA target. CRISPR-derived guide RNAs could be designed to target pathogen-specific virulence or essential genes. Such technology will allow manipulation of complex bacterial populations in a sequence-specific manner that could lead to very specific antibiotics, a step forward for personalized medicine.

5.6. COMBINATION THERAPY

The strategies to reduce antibiotic resistance include the limited use of antibiotics and the application of more effective antibacterial therapies. An alternative option is the use of combination therapies, which can lead to a synergistic and more effective response.

The combination of different drugs offers many advantages over their use as individual chemical moieties; these include a reduction in dosage of the individual drugs, fewer side effects compared to the monotherapy, reduced risk for the development of drug resistance, a better combined response compared to the effect of the individual drugs (synergistic effects), wide-spectrum antibacterial action, and the ability to attack simultaneously multiple target sites, in many occasions leading to an increased antibacterial effect.

The selection of the appropriate combinatorial treatment is critical for the successful treatment of infections. Therefore, the design of combinatorial treatments provides a pathway to develop antimicrobial therapeutics with broad-spectrum antibacterial action, bactericidal instead of bacteriostatic mechanisms of action, and better efficacy against MDR bacteria.

6

MONITORING ANTIMICROBIAL RESISTANCE: ROLE OF SURVEILLANCE

The “One Health” approach is a collaborative, multisectoral, and trans-disciplinary approach with the goal of achieving optimal health outcomes by improving coordination, collaboration, and communication at the human-animal-environment interface to address shared health threats such as zoonotic diseases, antimicrobial resistance, food safety and others. The World Health Organization defines “One Health” as "an approach to designing and implementing programmes, policies, legislation and research in which multiple sectors communicate and work together to achieve better public health outcomes".

The global spread of AMR pathogens has been declared a global public health emergency (WHO, 2020). Indeed, AMR imposes a substantial cost on societies that endangers economic growth and balanced access to resources. For all these reasons surveillance of antibiotic resistance is a crucial element for the implementation of intervention strategies aimed at preserving the efficacy of antibiotics (WHO, 2020).

6.1 SURVEILLANCE PROGRAMS IN LOW AND MIDDLE-INCOME COUNTRIES (LMICs)

Low and middle-income countries (LMICs) are disproportionately impacted by AMR. An important driver of AMR in LMICs is the unregulated antimicrobial use and the sale of counterfeit products (Kelesidis and Falagas, 2015). Sanitation is frequently poor, and wastewater enters the environment untreated. Agriculture and livestock farming play a major role in the economies of most LMICs. The interactions between humans and farm animals in LMICs are complex and the use of antimicrobials in farming is largely uncontrolled (Morgan *et al.*, 2011). Consequently, the risk of AMR epidemics in food animals and transmission between humans and livestock along various points in the food production chain is high, but poorly understood (WHO, 2020) . In this respect, WGS studies can untangle the processes that lead to the emergence and spread of AMR organisms in human-livestock interactions and identify novel resistance mechanisms in animal pathogens. The benefits of comprehensive AMR surveillance in LMICs will extend to the animal health and agricultural sector. Improved surveillance can reduce unnecessary use of standard and reserve antimicrobials and allow the use of narrow spectrum drugs, all of which reduce selective pressure for resistance.

6.2 WGS AN MOLECULAR SURVEILLANCE: A VALUABLE ADDITION TO CONVENTIONAL STRATEGIES

Applied as part of a one-health approach, whole-genome sequencing (WGS) can be used to infer transmission events between humans and animals and trace the origin of foodborne diseases. Molecular surveillance data based on bacterial DNA sequence information can be a valuable addition to a national surveillance system, and a complement to phenotypic surveillance by providing more detailed insights into the epidemiology of pathogens, including AMR strains. Using WGS achieves superior reproducibility and resolution compared with other molecular surveillance methods allowing not only for the possible origin of the host bacteria to be determined but also the genetics of the loci responsible for resistance to be investigated. WGS has become a key technology for understanding pathogen evolution and population dynamics on different spatial and temporal scales (Baker *et al.*, 2018). Additionally, WGS can determine other pathogen characteristics of public health importance, such as virulence and transmissibility (Bentley and Parkhill, 2015). Knowledge of these characteristics can improve the management of disease outbreaks and epidemics and have a direct impact on the health of individuals within a region.

Moreover, constant monitoring of the population structure of known pathogens facilitates a targeted response to emerging high-risk clones. A high-risk clone is a genetically uniform group of bacteria that by common ancestry share the same critical resistance mutations and genes making them resistant to one or more standard treatments. High-risk clones can be identified from WGS data based on clonal relatedness and abundance and inferring virulence and resistance profiles from gene content (Aanensen *et al.*, 2016).

In combination with phenotypic surveillance and epidemiological data, evidence from WGS data can be used to strengthen programmes for infection prevention and control, inform emergency responses and refine clinical decision making by lending further evidence on the origin of resistant clones that are dominant within a geographical region or globally.

6.3 GLOBAL DISSEMINATION OF ANTIMICROBIAL RESISTANCE: ROLE OF HIGH-RISK CLONES

The term “clone” is used to described isolates that, although they may have been cultured independently from different sources in different locations and perhaps at different time, still retain so many identical phenotypic and genotypic similarities that the most likely explanation is a common origin. This wider explanation commonly applies to multi-resistant bacteria found in multiple locations.

To qualify as a global MDR high-risk clone, isolates must the following traits: they *i)* have acquired certain adaptive traits that increase their pathogenicity (e.g., show increased pathogenicity and/or fitness) and survival skills (e.g., be able to colonize and persist in hosts for long time intervals), *ii)* possess various AMR determinants, *iii)* be obtained from several geographical locations across the world, *iv)* be transferred effectively between different hosts and *v)* cause severe and/or recurrent infections. Bacterial MDR high-risk clones are "collectors and spreaders" of AMR genes through horizontal and vertical transmission (Baker *et al.*, 2018). These clones have the tenacity and flexibility to accumulate and then provide resistance and virulence genes that allow them to dominate within populations.

Among the most common methods currently used for bacterial genotyping (e.g., multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE)) MLST, which uses sequence variation in a number of housekeeping genes to define types, is excellent for evolutionary studies, and for readily comparing isolates but may lack the discrimination required for outbreak analysis (van Belkum *et al.*, 2007; Diancourt *et al.*, 2010).

To track individual transmissions, a highly discriminatory method is required and many typing techniques fall short of this. Overall, SNP and whole-genome approaches are more suitable for this.

MDR global high-risk clones are found among various bacterial pathogens, especially *S. aureus*, *P. aeruginosa*, and the Enterobacterales (i.e., such as *K. pneumoniae*, *E. coli*, and the *Enterobacter cloacae* complex) (Woodford, Turton and Livermore, 2011). Particularly, the pandemics caused by MDR *E. coli* are due mostly to the global dissemination of certain high-risk clones, namely, *E. coli* sequence type 131 (ST131) and more recently, *E. coli* sequence type 1193 (ST1193) (Pitout *et al.*, 2022).

I) ESCHERICHIA COLI HIGH-RISK CLONES

Escherichia coli is the most common agent of urinary tract infections and the leading Gram-negative cause of bacteraemia. Since the mid-1990s or early 2000s, according to the country, there has been a worldwide increase in the prevalence of isolates that are resistant to oxyimino-cephalosporins and produce ESBLs, particularly CTX-M type enzyme (Livermore *et al.*, 2007). Considered globally, CTX-M-15 and CTX-M-14 are the most prevalent members of this family of >110 ESBL variants. As a consequence of this increase, research interest in the biology of multiresistant, extraintestinal pathogenic *E. coli* strains have increased dramatically, and has included studies to elucidate the epidemiology of the current 'CTX-M ESBL pandemic'.

Escherichia coli ST131 emerged during the early to mid-2000s as an important human pathogen, has spread extensively throughout the world, and is responsible for the rapid increase in antimicrobial resistance among *E. coli*.

ST131 is known to cause extraintestinal infections, to be fluoroquinolone resistant associated with ESBL production most often due to CTX-M-15 variant and to belong to B2 phylogenetic group. Moreover, all isolates of multidrug-resistant *E. coli* ST131 belonging to serotype O25b:H4 are likely to harbor IncF types of plasmids containing $bla_{\text{CTX-M-15}}$ gene.

Recent reports from diverse areas of the world describe a new such clone, ST1193, which likewise is from phylogroup B2 but, unlike ST131, represents sequence type complex 14 (STc14) and is associated with *fimH* allele 64, (i.e., ST1193-*H*64).

The earliest report regarding ST1193 documented its important contribution to the fluoroquinolone (FQ)-resistant (FQ-R) clinical *E. coli* population among humans in eastern Australia in 2008, with spillover into dogs (Joanne L Platell *et al.*, 2012).

Subsequent reports from China (Wu *et al.*, 2017a; Xia *et al.*, 2017), Korea (Kim *et al.*, 2017), Germany (Valenza *et al.*, 2019), and the United States (Tchesnokova *et al.*, 2019) documented ST1193 as a progressively emerging FQ-R human pathogen in these regions and sometimes associated with CTX-M ESBLs.

Gut colonization with multiresistant clones, such as ST131-*H*30 and ST1193-*H*64, may underlie these clones' epidemic spread and ability to cause extraintestinal disease. As such, the intestinal reservoir deserves attention, along with clinical isolates. To date, however, gut colonization with ST1193 has been reported only in healthy student in United States (Stephens *et al.*, 2020).

PART II

AIM OF THE PHD PROJECT

Antimicrobial resistance has emerged as one of the leading public health threats of the 21st century, being able to cross borders and human and animal species, and requiring concerted action at global, regional, and national levels. Nowadays, there is a considerable awareness of the actions needed to combat antimicrobial resistance. In this perspective, the surveillance of antibiotic resistance is a crucial element for the implementation of intervention strategies aimed at preserving the efficacy of currently available antimicrobial agents, including last-resource antibiotics. Beside conventional pathogens, there is increasing agreement about the importance of monitoring commensal bacteria, which can constitute a natural reservoir not only of resistance strains, but also of resistance genes that are potentially transferable among members of the resident microbiota. As such, there is an urgent need to develop alternatives to conventional antimicrobial strategies against resistant organisms, with a considerable attention to MDR gram-negative bacteria, that could play a major role in development of difficult-to-treat biofilm-related infections.

This PhD project aimed at providing novel insights about alternative therapeutic approaches against Gram-negative MDR bacteria and at investigating their dissemination pathways in healthy humans living in remote communities of Bolivian Chaco.

In the context of the present PhD research program, an in-depth discussion is provided for the following specific topics:

- Investigation the *in vitro* activity of fosfomycin, colistin and combinations thereof against planktonic and biofilm cultures of Gram-negative pathogens.

- *In vitro* antibiofilm activities of NAC alone and in combination with colistin against *Pseudomonas aeruginosa.*

- Epidemiological characterization of commensal CTX-M-producing *E. coli* isolated from healthy children living in a rural community of Bolivian Chaco.

- Phenotypic and genotypic Characterization of the E. coli high-risk clone ST1193 in the dissemination of antibiotic resistance among healthy children living in a rural community of Bolivian Chaco.

PART III

RESULTS AND DISCUSSION

1

COMBINATION OF FOSFOMYCIN AND COLISTIN AGAINST PLANKTONIC AND BIOFILM CULTURE OF GRAM-NEGATIVE PATHOGENS

RELATED PUBLICATION

Activity of fosfomycin/colistin combinations against planktonic and biofilm Gram-negative pathogens. Boncompagni SR, Micieli M, Di Maggio T, Aiezza N, Antonelli A, Giani T, Padoani G, Vailati S, Pallecchi L, Rossolini GM. *J Antimicrob Chemother*. **2022**; Jul 28;77(8):2199-2208.

Antimicrobial therapy has positively impacted on life expectancy in the last century, but the recent surge of infections caused by MDR pathogens represents a challenge to global health, reducing the available therapeutic options (Hutchings, Truman and Wilkinson, 2019). One of the most problematic infections are those caused by microbial biofilms, which play a crucial role in chronic lung colonization/infection of patients affected by cystic fibrosis (CF) and other chronic airway conditions such as chronic obstructive pulmonary disease (COPD) and non-CF bronchiectasis (Ciofu *et al.*, 2015). Treatment of such infections poses a remarkable clinical challenge, due to the inherent antibiotic tolerance of biofilms and the frequent involvement of bacterial strains expressing MDR phenotypes (Ciofu *et al.*, 2015). In this perspective, treatment regimens based on inhaled antibiotic combinations have been extensively investigated in recent years (Falagas, Trigkidis and Vardakas, 2015; Wenzler *et al.*, 2016; Vardakas *et al.*, 2018). In fact, this route of administration can achieve high antibiotic concentrations in the epithelial lining fluid (ELF), while minimizing systemic toxicity (Wenzler *et al.*, 2016).

Fosfomycin and colistin are “old” antibiotics that have recently regained attention due to the dearth of new compounds to treat infections caused by MDR pathogens (Karaiskos *et al.*, 2019). Recently, a synergistic effect of fosfomycin/colistin combinations has been reported against planktonic cultures of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae* (Corvec *et al.*, 2013; Khawcharoenporn *et al.*, 2018; Ku *et al.*, 2019; Erturk Sengel *et al.*, 2020). In addition, Corvec *et al*.

demonstrated the synergistic activity of fosfomycin and colistin against *E. coli* biofilm in an *in vivo* foreign-body infection model (Corvec *et al.*, 2013).

A collection of 130 clinical strains collected from different clinical specimens (49 blood-stream infections, 42 lower respiratory tract infections (of which 6 were from CF patients), 32 urinary tract infections and 7 from other sources), representative of different species was investigated, including enterobacteria and Gram-negative non-fermenting pathogens. The collection included 52 *P. aeruginosa* (of which 40 carbapenemase-producers), 47 carbapenemase-producing *Klebsiella pneumoniae*, 19 *Escherichia coli* (of which 8 carbapenemase-producers), 7 *Stenotrophomonas maltophilia* (of which 1 resistant to trimethoprim-sulfamethoxazole – SXT) and 5 carbapenemase-producing *Acinetobacter baumannii* (Table 2).

			MIC range (mg/L)		Number of strains (%)	
Species (no. of strains)	Relevant acquired resistant phenotypes/genotypes (*n*)	ST (*n*)	FOF	CST	Synergy (FICI ≤0.5)	No interaction (FICI >0.5–4)
A. baumannii complex (5)	Carba-R, MDR (5) Carbapenemase+ (5; OXA-23 and/or OXA-58, OXA-72) Colistin-R (2; mechanism unknown)	ST2 (3), ST78 (2)	64 to >1024	0.5–8	None	5 (100)
E. coli (19)	Carba-R, MDR (7) ESC-R, Carba-S, MDR (11) Carbapenemase+ (7; NDM, KPC, OXA-48-like, VIM) ESBL+ (10; CTX-M and/or TEM) CMY-2 (4) Colistin-R (3; MCR-1-like)	ST39 (1), ST43 (2), ST73 (2), ST117 (1), ST131 (1), ST167 (1), ST404 (1), ST405 (2), ST479 (2), ST648 (2), ST681 (1), ST2076 (1) ND (2)	0.25 to >128	0.25–8	None	19 (100)
K. pneumoniae (48)	Carba-R, MDR (48) Carbapenemase+ (46; NDM, KPC, OXA-48-like, VIM) ESBL+ (4; CTX-M), Colistin-R (1; MCR-1-like)	ST11 (1), ST35 (1), ST101 (6), ST147 (1), ST258 (5), ST307 (6), ST395 (1), ST512 (17), ST2217 (1), ST2502 (1), ND (8)	≤0.25 to >128	0.25–128	8 (16.7)	40 (83.3)
P. aeruginosa (51)	Carba-R, MDR (48) Carbapenemase+ (40; VIM, IMP, GES-5) ESBL+ (4; PER-1)	ST17 (2), ST111 (6), ST175 (8), ST179 (1), ST235 (11), ST260 (2), ST308 (4), ST532 (2), ST621 (12), ST646 (1), ST654 (1), ND (1)	4 to >1024	0.5–512	5 (9.8)	46 (90.2)
S. maltophilia (7)	Trimethoprim/sulfamethoxazole-R (1)	ST34 (1), ST87 (1), ST300 (1), ST335 (1), ND (3)	32–128	2–128	None	7 (100)

Table 2. Main features of the 130 Gram-negative clinical strains investigated, and the result of the chequerboard assays for fosfomycin and colistin combinations with planktonic cultures. ND, not determined; Carba-R, carbapenem resistant; Carba-S, carbapenem susceptible; ESC-R, expanded-spectrum cephalosporin resistant.

Most strains were carbapenemase producers, exhibited MDR phenotypes (N =121) and presented different antimicrobial susceptibility profiles to fosfomycin and colistin tested in accordance with internationally recognized standard ISO 20776:1-2019 (ISO 20776-1:2019) (Table 2).

Activity of fosfomycin/colistin combinations against planktonic cells was analysed by checkerboard assays in two independent experiments and the data obtained were analyzed in terms of fractional inhibitory concentration index (FICI). Broth microdilution fosfomycin/colistin checkerboard assays showed synergism in a low percentage (10%) of strains (e.g., with 13 out of 130 isolates). Indeed, the *in vitro* synergism of fosfomycin/colistin combination with checkerboard assays on planktonic cells was observed only with a minority of tested *P. aeruginosa* (5/52; 9.6%) and *K. pneumoniae* (8/47; 17%) strains, and with these appeared to be strain-related. All other strains except for one *E. coli* (not interpretable), showed a FICI indicative of no interaction to tested combination. No clear relationship was found between synergism and clonal lineage or specific resistance phenotypes/mechanisms (Table 2).

Previous studies reported overall higher rates of synergy with the same species on planktonic cells compared to this study, but these differences might be related with the diversity of the strains collection and/or experimental conditions.

The antibiofilm activity of fosfomycin, colistin and fosfomycin/colistin combinations was tested against a subset of 20 selected representative MDR Gram-negative pathogens of different species, different fosfomycin and colistin susceptibility profiles and different response to fosfomycin/colistin combinations in chequerboard assays performed with planktonic cultures (Table 3).

As shown in Table 3, MBEC values of fosfomycin and colistin alone could be determined for 3 and 10 strains, respectively, while for all other strains MBECs were higher than the maximum drug concentration tested. As expected, MBEC values of fosfomycin and colistin were consistently higher than the respective MICs with all tested strains: i.e. MIC_{median} = 64 (≤0.25 to >1024 μg/ml) *vs* $MBEC_{median}$ = 1024 μg/ml (4 to >1024 μg/ml) for fosfomycin; MIC_{median} = 4 (≤0.5 to 512 μg/ml) *vs* $MBEC_{median}$ = 640 μg/ml (8 to >1024 μg/ml) for colistin (Table 3).

Biofilm checkerboard assays showed a synergism of fosfomycin/colistin combinations with the majority of tested strains (i.e., 16 of 20 strains), with no straightforward association with checkerboard assays performed with planktonic cultures (Table 3). The synergistic antibiofilm activity of fosfomycin/colistin combinations did not appear to be species-related or dependent on fosfomycin and colistin MICs (e.g., *P. aeruginosa* FZ34 and FZ45 strains showed identical fosfomycin and colistin MICs, but discordant response to drug combinations exposure). No antagonism was observed with any strain.

Isolate	Species	Origin	MLST type[a]	Resistance profile	Carbapenemase/ ESBL	Resistance determinants		FOF		CST			
						FOF	CST	MIC (mg/L)	MBEC (mg/L)	MIC (mg/L)	MBEC (mg/L)	FICI	FBECI
FZ1	*A. baumannii*	CVC-BSI	ST2	MEM, IPM, CIP	OXA-72	—	S	512	>1024	0.5	64	1	**0.4**
FZ2	*A. baumannii*	BSI	ST78	MEM, IPM, CIP	OXA-23; OXA-58	—	S	>1024	>1024	1	>1024	0.8	**0.5**
FZ83	*A. baumannii*	LRTI	ST2	MEM, IPM, CIP, CST	OXA-23	—	PmrA (F105L); PmrB (E185K); PmrC (F166L, R348K, A370S, K531T)	64	>1024	8	128	0.8	**0.3**
FZ18	*P. aeruginosa*	LRTI	ST235	C/T, CAZ, FEP, MEM, IPM, AMK, CIP	—	S	S	64	>1024	4	>1024	**0.4**	**0.5**
FZ34	*P. aeruginosa*	BSI	ST175	TZP, CAZ, FEP, CIP, CST	PER-1	GlpT (F81Y)*	S	>1024	>1024	4	>1024	**0.3**	**0.5**
FZ45	*P. aeruginosa*	BSI	ST111	C/T, TZP, CAZ, FEP, MEM, IPM, CIP	VIM-1	GlpT (premature stop codon at nt 513)	S	>1024	>1024	4	>1024	**0.4**	1
FZ98	*P. aeruginosa*	CF	ST646	CIP, CST	—	S	ParS (D380N); CprS (R295H)*	64	>1024	512	>1024	**0.3**	**0.1**
FZ139	*P. aeruginosa*	LRTI	ST111	C/T, MEM, IPM, AMK, CIP	VIM-2	S	S	8	>1024	4	>1024	**0.3**	0.8
FZ6	*S. maltophilia*	BSI	ST300	—	—	—	—	64	>1024	4	>1024	0.8	**0.1**
FZ8	*S. maltophilia*	CF	ST34	—	—	—	—	128	>1024	4	>1024	0.6	**0.1**
FZ85	*S. maltophilia*	LRTI	ST87	—	—	—	—	32	>1024	16	>1024	0.8	0.8
FZ11	*E. coli*	UTI	ST73	—	—	GlpT (premature stop codon at nt 765)	S	64	>1024	0.5	32	0.9	**0.4**
FZ123	*E. coli*	UTI	ST405	TZP, CAZ, FEP, MEM, IPM, AMK, CIP	NDM-5; CTX-M-15	S	S	0.25	256	≤0.5	8	0.8	**0.4**
FZ128	*E. coli*	RS	ST167	TZP, CAZ, FEP, MEM, IPM, AMK, CIP	NDM-5; CTX-M-15	S	S	1	256	1	16	0.9	**0.04**
FZ80	*K. pneumoniae*	UTI	ST512	TZP, CAZ, FEP, MEM, IPM, CIP	KPC-3	GlpT (premature stop codon at nt 1176)	S	128	>1024	0.5	64	0.6	0.6
FZ103	*K. pneumoniae*	LRTI	ST512	TZP, CAZ, FEP, MEM, IPM, CIP, CST	KPC-3	S	unk	32	>1024	8	64	**0.4**	**0.1**
FZ105	*K. pneumoniae*	BSI	ST258	TZP, CAZ, FEP, MEM, IPM, CIP, CST	KPC-3	S	MgrB (interrupted at nt 129 by ISKpn25)	16	>1024	128	256	**0.3**	**0.1**
FZ106	*K. pneumoniae*	BSI	ST258	TZP, CAZ, FEP, MEM, IPM, CIP, CST	KPC-3	unk	MgrB (interrupted at nt 129 by ISKpn25)	64	>1024	32	256	**0.4**	**0.2**
FZ108	*K. pneumoniae*	UTI	ST258	TZP, CAZ, FEP, MEM, IPM, CIP, CST	KPC-3	UhpB (T140A); PtsI (N174K)	MgrB (interrupted at nt 129 by ISKpn25)	128	>1024	32	>1024	**0.3**	**0.1**
FZ141	*K. pneumoniae*	UTI	ST2502	TZP, CAZ, FEP, MEM, IPM, AMK, CIP	KPC-3	S	S	≤0.25	4	2	8	**0.4**	**0.4**

Table 3. Main features of the 20 strains studied and results of biofilm chequerboard assay. BSI, bloodstream infectious; CVC-BSI, central venous catheter-related BSI; LRTI, low respiratory tract infection; UTI, urinary tract infection; RS, rectal swab; AMK, amikacin; C/T, ceftolozane/tazobactam (tazobactam at fixed concentration of 4 mg/L); CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; FEP, cefepime; IPM, imipenem; MEM, meropenem; TZP, piperacillin/tazobactam (tazobactam at fixed concentration of 4 mg/L); S, susceptible; unk, unknown. Asterisks indicate a putative mutation. Previously described alterations are underlined. FICI and FBECI values were interpreted as follows: FICI/FBECI ≤0.5, synergy; FICI/FBECI >0.5-4.0, no interaction; FICI/FBECI >4.0, antagonism. Values in bold indicate synergy. [a]According to Pasteur and Achtman MLST scheme (http://pubmlst.org/mlst)

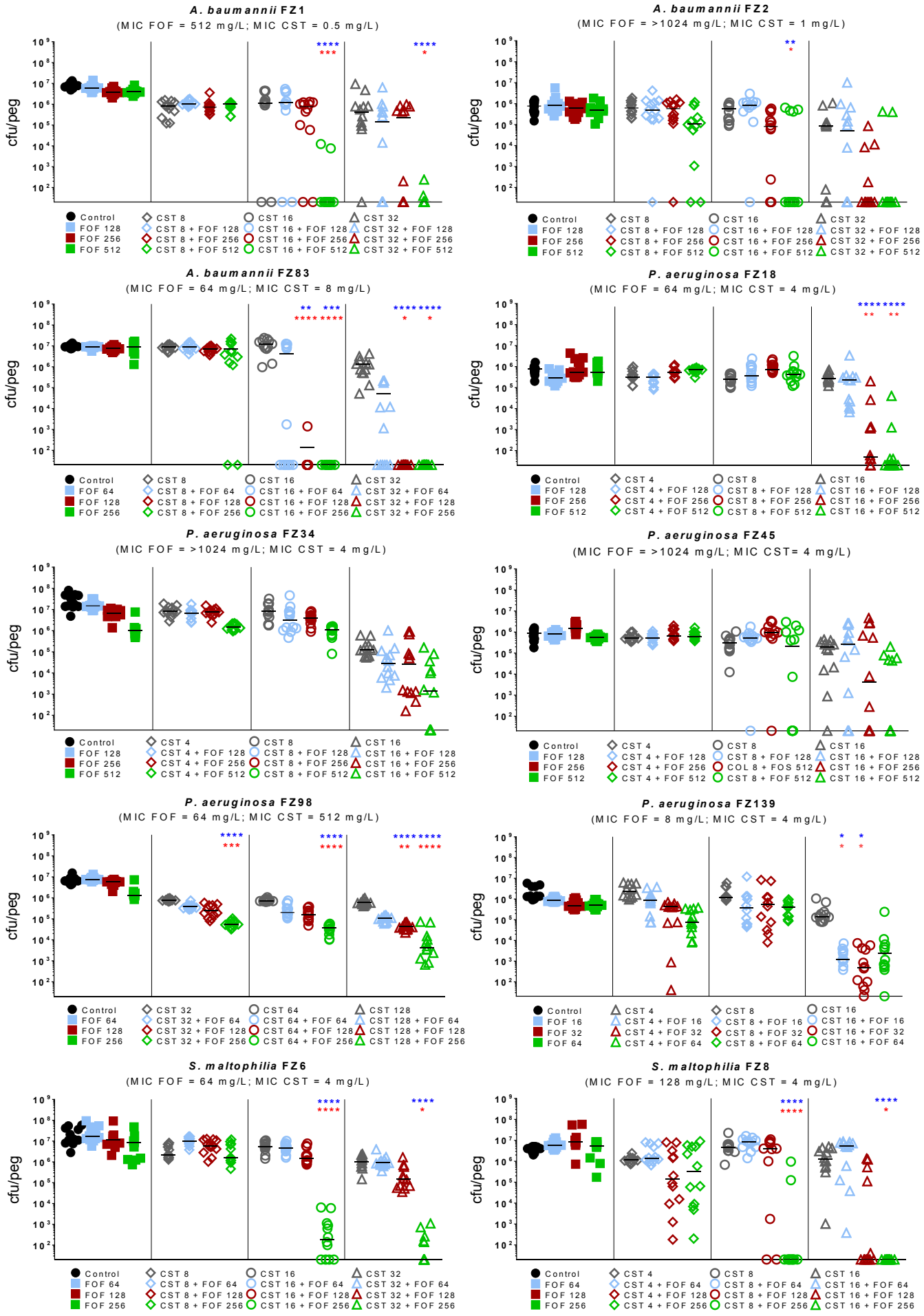


Figure 11A. Antibiofilm activity of fosfomycin and colistin combinations. Data from at least two independent experiments, with six replicates per condition per experiment. Median values are plotted. The x-axes are set at the limit of detection (20 CFU/peg). Fosfomycin and colistin concentration are expressed in mg/L. Significant differences compared with drug controls are indicated with asterisks (blue for fosfomycin, red for colistin). * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

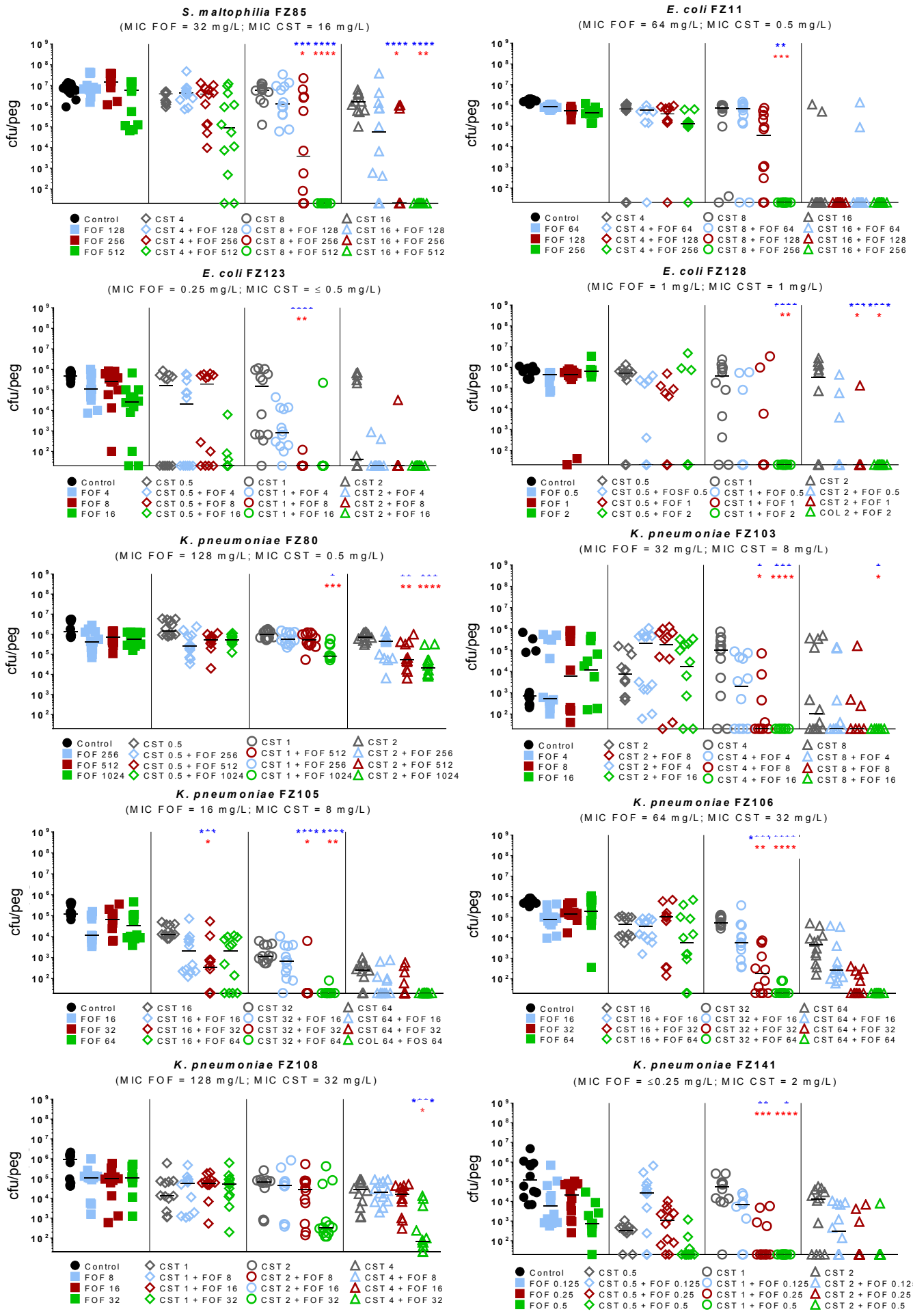


Figure 11B. (continued) Antibiofilm activity of fosfomycin and colistin combinations. Data from at least two independent experiments, with six replicates per condition per experiment. Median values are plotted. The x-axes are set at the limit of detection (20 CFU/peg). Fosfomycin and colistin concentration are expressed in mg/L. Significant differences compared with drug controls are indicated with asterisks (blue for fosfomycin, red for colistin). * *P*<0.05, ** *P*<0.01, *** *P*<0.001, **** *P*<0.0001.

Quantitative antibiofilm assays, using the high drugs concentrations achievable in ELF after inhalation, demonstrated a significant antibiofilm synergism of fosfomycin/colistin combinations against all tested strains, with the exception of two *P. aeruginosa* strains (i.e., *P. aeruginosa* FZ34 and FZ45), for which a trend suggesting a synergism was observed but statistical significance was not achieved (Figure 11A and 11B). In all strains investigated fosfomycin and colistin alone had no or little antibiofilm effect (Figure 11A and 11B).

Mutant prevention concentration (MPC) was determined for fosfomycin and colistin alone and in combinations. MPC data were used to calculate the mutant selection window (MSW = MPC – MIC) for fosfomycin and colistin alone and in combination which correspond to an antibiotic concentration range extending from the minimal concentration required to block the growth of wild-type bacteria (i.e., MIC) up to that required to inhibit the growth of the least susceptible mutant (i.e., MPC) (Table 4, Figure 12).

MPCs of fosfomycin, colistin, and fosfomycin/colistin combinations were determined for ten selected strains representative of different species and different fosfomycin and colistin susceptibility profiles (Table 4).

MPCs of fosfomycin and colistin in all tested strains were consistently lower when tested in combination (i.e., reduction corresponding to 1 to 6 log2 dilutions and 1 to 5 log2 dilutions for fosfomycin and colistin, respectively), which determined a relevant narrowing of the MSWs in all tested strains without clear relationship between species and concentrations used (Table 4).

			MIC (mg/L)				MPC (mg/L)			
Isolate	Species	ST	FOF	CST	FICI	FBECI	FOF	CST	FOF with [CST]	CST with [FOF]
FZ83	*A. baumannii*	2	64	8	0.8	**0.3**	>1024	128	512 [8]	32 [64]
FZ34	*P. aeruginosa*	175	>1024	4	**0.3**	**0.5**	>1024	16	1024 [4]	8 [512]
FZ45	*P. aeruginosa*	111	>1024	4	**0.4**	1	>1024	64	1024 [4]	16 [256]
FZ139	*P. aeruginosa*	111	8	4	**0.3**	0.8	>1024	32	64 [4]	8 [32]
FZ8	*S. maltophilia*	34	128	4	0.6	**0.1**	512	256	64 [64]	32 [128]
FZ85	*S. maltophilia*	87	32	16	0.8	0.8	1024	512	64 [128]	32 [128]
FZ11	*E. coli*	73	64	0.5	0.9	**0.4**	>1024	16	512 [4]	8 [128]
FZ103	*K. pneumoniae*	258	32	8	**0.4**	**0.1**	1024	512	128 [64]	64 [256]
FZ105	*K. pneumoniae*	258	16	128	**0.3**	**0.1**	>1024	1024	32 [128]	32 [256]
FZ106	*K. pneumoniae*	258	64	32	**0.4**	**0.2**	>1024	1024	64 [128]	64 [256]

Table 4. MPCs for fosfomycin (FOF) and colistin (CST) alone and in combination. FICI and FBECI values were interpretated as follows: FICI/FBECI ≤0.5, synergy; FICI/FBECI >0.5-4.0, no interaction; FICI/FBECI >4.0, antagonism. Values in bold type indicate synergy. Square brackets indicate antibiotic used in fixed concentration.

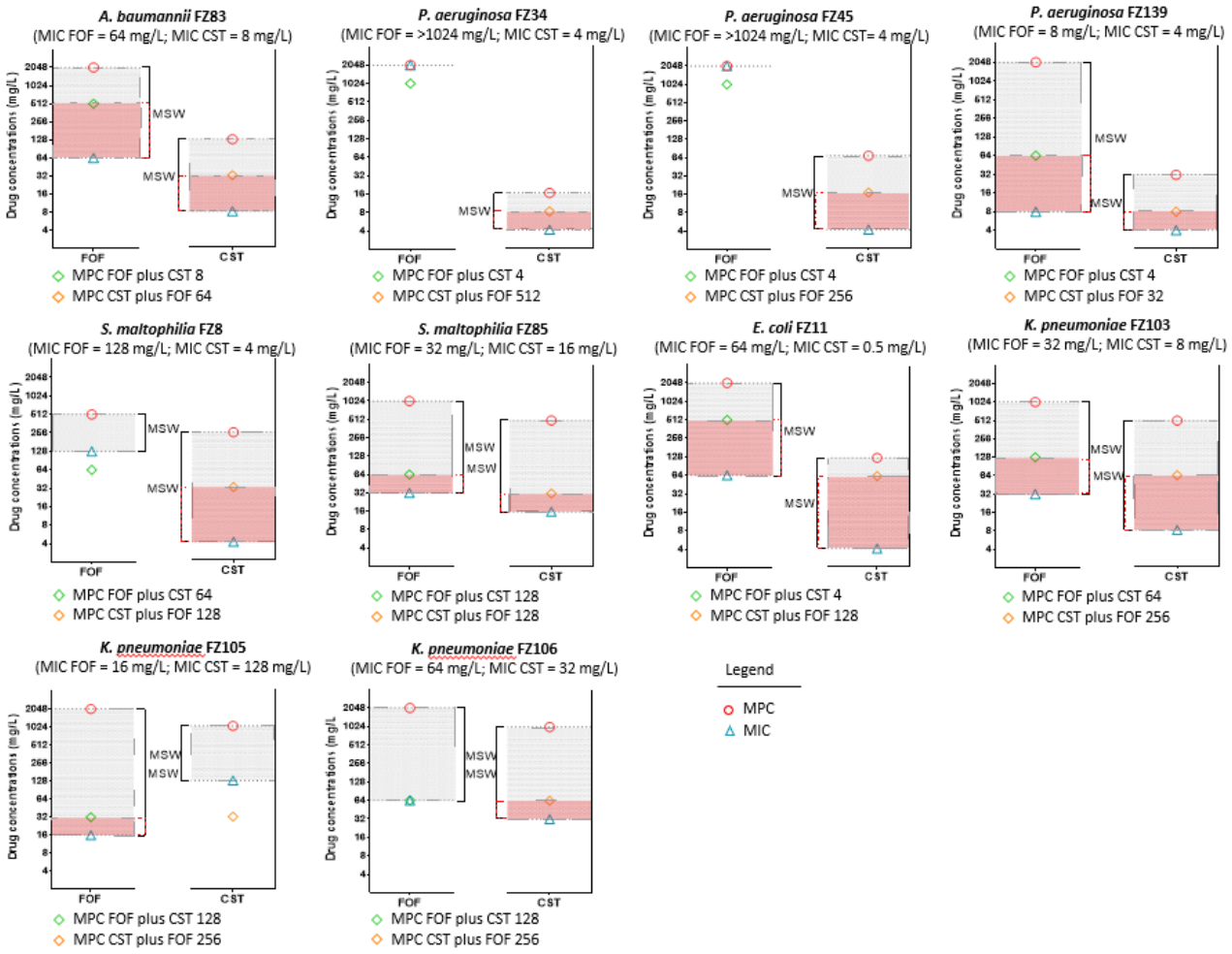


Figure 12. MPCs and MSWs of fosfomycin (FOF) and colistin (CST) combinations of 10 selected strains. Red shading indicates the MSW of fosfomycin/colistin combinations. FOF and CST concentrations are expressed in mg/L. MPC values > 1024 mg/L were reported as 2048 mg/L.

Although the few numbers of strains tested/per species on antibiofilm assays represents a limitation of this study, the observation of a synergism of fosfomycin/colistin combinations against strains representative of diverse clonal lineages and resistance phenotypes would suggest that this phenomenon is not strain-specific.

These results provided original data concerning antibiofilm activity of fosfomycin/colistin combinations, at concentrations achievable in the ELF after inhalation, against *A. baumannii, P. aeruginosa, S. maltophilia* and *K. pneumoniae in vitro* biofilm models and were found that fosfomycin plus colistin, were able to reduce the emergence of fosfomycin- and colistin-resistant subpopulations.

2

ANTIBIOFILM ACTIVITY OF N-ACETYLCYSTEINE ALONE AND IN COMBINATION WITH COLISTIN AGAINST *PSEUDOMONAS AERUGINOSA*

RELATED PUBLICATION

Activity of *N*-acetylcysteine alone and in combination with colistin against *Pseudomonas aeruginosa* biofilms and transcriptomic response to *N*-acetylcysteine exposure. Valzano F, Boncompagni SR, Micieli M, Di Maggio T, Di Pilato V, Colombini L, Santoro F, Pozzi G, Rossolini GM, Pallecchi L. *Microbiol Spectr*. **2022**; Aug 31;10(4):e0100622.

Pseudomonas aeruginosa is an opportunistic pathogen that leading cause of a variety of acute infections, including ventilator-associated pneumonia (VAT). In addition, *P. aeruginosa* can cause chronic lung infections in patients with cystic fibrosis (CF) and non CF-bronchiectasis.

CF is a genetic disorder that is caused by mutations in both copies of the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR), leading to formation of a thick mucus layer on the airway surfaces of CF patients, which hinders mucociliary clearance. Thus, the lung of a CF patient is a favourable environment for bacterial growth and colonization such as *P. aeruginosa.* Moreover, excessive use of antibiotics during treatment accelerates development of MDR *P. aeruginosa* strains, leading to the ineffectiveness of the empirical antibiotic therapy against this microorganism (Sadikot *et al.*, 2005).

Of note, the capacity to form biofilms provides the bacteria an enormous advantage to establish infections, including VAP and CF lung infections, within susceptible hosts.

Colistin is among the last-resort agents for the treatment of infections caused by MDR Gram-negative bacteria, and inhaled colistin (alone or in combination with intravenous colistin) has been increasingly used for the treatment of difficult-to-treat respiratory tract infections, especially in CF (Matthieu *et al.*, 2014; Vardakas *et al.*, 2018).

N-acetylcysteine (NAC) is a mucolytic agent commonly administered together with antibiotics for the management of lower respiratory tract infections, especially in patients with chronic respiratory diseases

characterized by abundant and/or thick mucus production (i.e., CF, COPD, and bronchiectasis) (Blasi *et al.*, 2016). Moreover, NAC commonly used as antioxidant and free-radical scavenging, because it increases cellular production of glutathione (Blasi *et al.*, 2016).

The antibiofilm activity of NAC alone and in combination with colistin was tested against a total of 17 *P. aeruginosa* clinical isolates, of which 15 were from CF patients, with different phenotypic and genotypic feature (e.g., ST and resistance profile) (Table 5).

Strain	yr of isolation	Phenotype	Origin[a]	ST[b]	O type	Resistance pattern[c]	MIC (mg/L)[d]	
							CST	NAC
PAO1	1954	Nonmucoid	Wound	ST549	O5	Wild type	2	64,000
Z33	2005	Nonmucoid	CF	ST235	O11	CP^r, FQ^r, AG^r	1	16,000
Z34	2006	Nonmucoid	CF	ST17	O1	CB^r, CP^r, FQ^r, AG^r	2	64,000
Z35	2006	Nonmucoid	CF	ST235	O11		1	16,000
Z152	2013	Mucoid	CF	ST155	O6	CB^r, FQ^r, AG^r	2	8,000
Z154	2016	Mucoid	CF	ST412	O6	CP^r, FQ^r, AG^r	2	16,000
M1	2002	Mucoid	CF	ST155	O6	CB^r, CP^r, FQ^r, AG^r	2	16,000
M4	2005	Mucoid	CF	ST155	O6	CB^r, CP^r, FQ^r, AG^r	2	32,000
M7	2005	Mucoid	CF	ST253	O10	AG^r	2	64,000
M13	2000	Mucoid	CF	ST274	O3	CB^r, CP^r, AG^r	1	32,000
M19	2006	Mucoid	CF	ST3509	O7		1	64,000
M25	2002	Mucoid	CF	ST235	O11		2	16,000
M32	2006	Mucoid	CF	ST235	O11		2	16,000
M42	2007	Mucoid	CF	ST2437	O6	CB^r, CP^r, FQ^r, AG^r	2	32,000
FC237	2007	Nonmucoid	CF	ST365	O3	CB^r, FQ^r, AG^r, CST^r	512	64,000
FC238	2007	Nonmucoid	CF	ST910	O6	CB^r, CST^r	8	64,000
FZ99	2018	Nonmucoid	RTI_{ICU}	ST111	O12	CB^r, CP^r, FQ^r, AG^r, CST^r	4	64,000

Table 5. Features of 17 *P. aeruginosa* strains included in the study. [a]CF, cystic fibrosis; RTI_{ICU}, respiratory infection in intensive care unit. [b] According with MLST Pasteur scheme. [c]CB^r, resistance to carbapenem (imipenem and meropenem); CP^r, resistance to cephems (ceftazidime and cefepime); FQ^r, resistance to fluoroquinolones (ciprofloxacin); AG^r, resistance to aminoglycosides (amikacin and gentamicin); CST^r, resistance to colistin. [d]CST, colistin; NAC, N-acetylcysteine.

Briefly, all experiments were performed using the Nunc-TSP lid system and evaluating the biofilm mass by viable cell count (i.e., by disruption of biofilms by sonication, and determination of colony forming units per biofilm, CFU/biolfim).

For biofilm eradication experiments, biofilms grown on Nunc-TSP lid system for 24 hours were challenged with NAC and colistin alone and in combinations. Interestingly, the NAC and colistin concentrations used in this study were selected taking into account the high colistin and NAC concentrations potentially achievable by inhalation.

NAC alone showed little or no effect on disruption or formation of *P. aeruginosa* biofilms. Indeed, the differences in terms of CFU/peg (i.e., CFU/biofilm) were in the great majority of cases within half log CFU, except for *P. aeruginosa* Z154, for which was observed a decrease of >1 log CFU/peg compared to the control, and for *P. aeruginosa* PAO1, for which was observed an increase of >1 log CFU/peg compared with

the control. These results indicated that inhaled NAC alone might not have major effects on *P. aeruginosa* biofilms already established in the lung.

Activity of NAC-colistin combinations against all the 17 studied strains, with a fixed concentration of NAC at 8,000 mg/L plus colistin at 8 mg/L, showed a relevant synergism in all tested strains, except for two *P. aeruginosa* (i.e., M4 and M32) that showed a reduction of viable cells up to 2 log CFU/peg after 24 hours, although statistical significance was not achieved (Table 6).

Strains	CONTROL	NAC8	CST8	NAC8+CST8	p value NAC8+COL8 vs NAC8	p value NAC8+COL8 vs COL8
PAO1	1,18E+06	1,40E+06	3,70E+07	1,16E+05	****	***
Z33	1,41E+06	8,30E+05	8,20E+05	1,56E+05	**	****
Z34	2,43E+06	8,40E+06	4,20E+06	4,80E+05	****	****
Z35	1,20E+06	9,50E+05	3,00E+05	7,70E+03	**	****
Z152	9,90E+05	8,30E+05	4,00E+05	1,25E+05	***	****
Z154	1,40E+06	6,50E+05	6,00E+04	2,00E+01	****	****
M1	6,90E+06	1,23E+06	7,30E+06	4,50E+04	****	***
M4	5,70E+06	4,10E+02	1,80E+06	2,00E+01	****	-
M7	2,06E+06	8,00E+03	2,17E+06	2,00E+01	****	-
M13	4,90E+05	1,27E+05	1,20E+05	2,00E+01	****	****
M19	1,91E+06	6,40E+05	3,60E+05	1,31E+03	****	***
M25	1,26E+06	1,27E+06	6,50E+05	2,00E+01	***	****
M32	6,60E+05	1,12E+04	4,40E+05	2,00E+01	****	-
M42	5,00E+05	7,10E+05	1,27E+06	9,00E+01	****	****
FC237	1,13E+06	1,02E+05	3,60E+06	1,04E+04	****	**
FC238	6,10E+06	2,56E+06	5,10E+06	2,00E+01	****	****
FZ99	1,56E+06	1,01E+05	3,60E+06	3,50E+02	****	**

Table 6. Antibiofilm results of NAC at 8,000 mg/L (NAC8), colistin (CST8) at 8 mg/L, and NAC8-CST8 combinations against all 17 *P. aeruginosa* studied. Median values are reported. All data were obtained in at least three independent experiments, with at least 12 replicates per condition per experiment. **P* <0.05, ** *P*<0.01, *** *P*<0.001, **** *P*<0.00001

Moreover, *P. aeruginosa* Z34 and *P. aeruginosa* Z154, both isolated from CF patients, were selected for susceptibility assays with the artificial sputum medium (ASM) biofilm model in order to mimic the *P. aeruginosa* biofilm environmental conditions experienced in CF mucus. These strains were selected because they exhibited different phenotypes: non-mucoid (i.e., *P. aeruginosa* Z34) and mucoid (i.e., *P. aeruginosa* Z154).

In ASM model the concentrations used of colistin that allowed observation of a synergism was much higher compared to the experiment performed with the Nunc-TSP lid system. Indeed, both strains showed a clear synergy with NAC at 8,000 mg/L in combination with colistin at 64 mg/L (Figure 13).

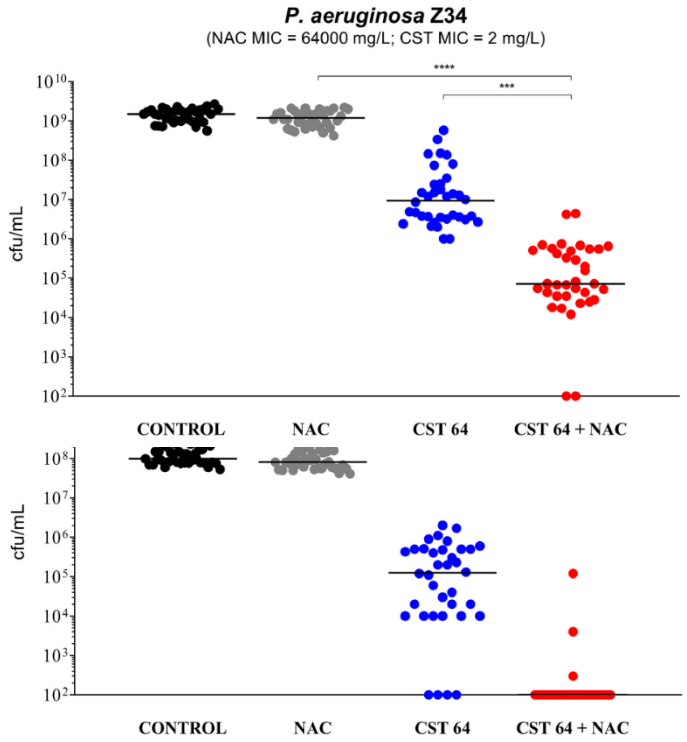


Figure 13. Antibiofilm activity of NAC at 8,000 mg/L (NAC8), colistin at 64 mg/L (CST 64) and the NAC-CST combination against *P. aeruginosa* Z154 and *P. aeruginosa* Z34 in the ASM biofilm model. **P* <0.05, ** *P*<0.01, *** *P*<0.001, **** *P*<0.00001

Moreover, transcriptome analysis was performed in *P. aeruginosa* Z154 for investigating the transcriptome response of planktonic cultures to NAC exposure (i.e., NAC at 8,000 mg/L). A total of 66 differentially expressed genes (DEGs) were identified (adjusted *P* value of <0.05 with 99% confidence interval [CI]), of which 46 were upregulated and 20 downregulated compared to the control. DEGs can be classified into several categories, which include the Zn^{2+} chelator as well as genes involved in oxidative stress and copper and iron uptake.

Most of these genes were upregulated and belonged to the *zur* regulon that included i) genes involved in splitting of septal peptidoglycan during cell division, ii) reversible hydration of carbon dioxide and important for growth under low-CO2 conditions and iii) modulators of the membrane FtsH protease.

These latter genes are required for the expression of diverse unrelated phenotypes such as swimming and twitching motility and biofilm formation.

The downregulated DEGs identified genes involved in denitrification (i.e., *norB*, *norS* and *norZ*). The nitric oxide reductase NorBC and the regulatory protein NosR were found to constitute the nucleus of the denitrification protein network.

Moreover, in order to confirm the results obtained by transcriptomic investigation, the inhibition of *P. aeruginosa* swimming and swarming motility and the inhibition of denitrification pathway of NAC were studied deeply by swimming and swarming tests (i.e., with the reference strain *P. aeruginosa* PAO1 and the CF strain *P. aeruginosa* Z154) and by measuring NO_3^- and NO_2^- concentrations in anaerobiosis (i.e., with the CF strain *P. aeruginosa* Z154).

Interestingly, the data obtained by swimming and swarming tests showed a clear inhibition of both mechanisms in presence of NAC at 8,000 mg/L. Indeed, the downregulation of genes of the flagellar apparatus and the induction of a zinc starvation response observed in transcriptomic data, could be related to the inhibition of this mechanisms (Figure 14).

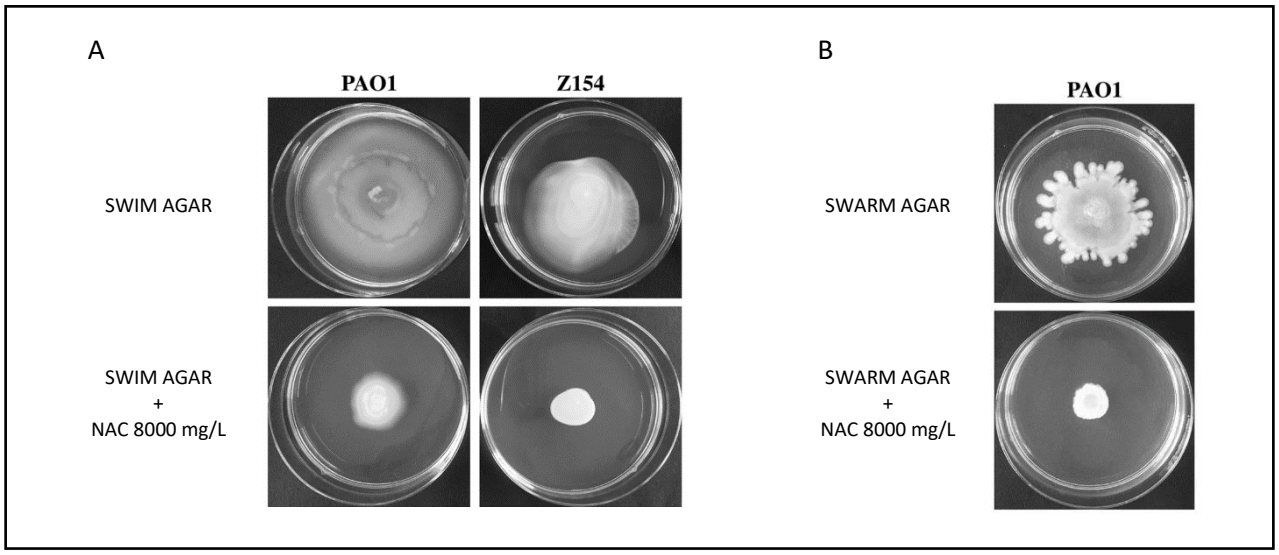


Figure 14. Swimming and swarming test in presence of NAC 8,000 mg/L. A) Swimming test was performed in at least three independent experiments with two *P. aeruginosa* (i.e., PAO1 and Z154). B) Swarming test was performed in in at least three independent experiments with *P. aeruginosa* Z154.

Moreover, the results obtained by measuring NO_3^- and NO_2^- concentrations in anaerobiosis in the presence of NAC at 8,000 mg/L were consistent with the transcriptomic data and showed that NAC was able to inhibit the denitrification pathway in anaerobic conditions (Figure 15).

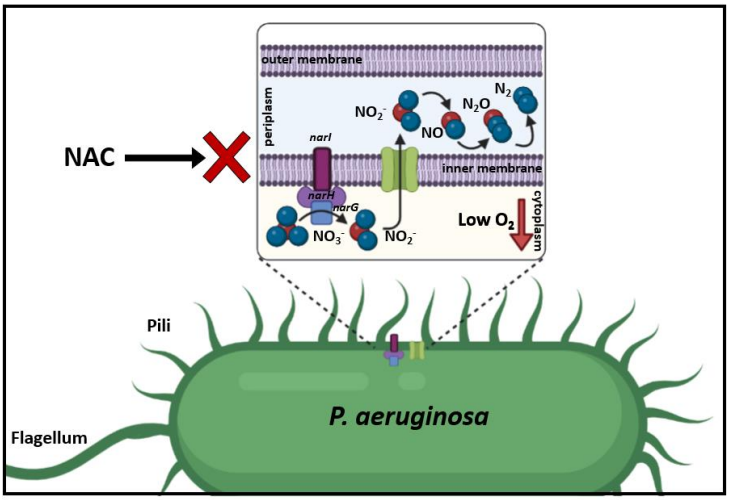


Figure 15. Schematic representation of denitrification pathway. *P. aeruginosa* is a facultative anaerobe, able to respire under anaerobic conditions in the presence of the alternative electron acceptors nitrate and nitrite, employing denitrification. In this metabolic pathway, nitrate is reduced to NO and then to N2 in four reaction steps, each catalyzed by a specific reductase. NAC was able to inhibit the denitrification pathway in anaerobic condition.

Previously studies have suggested that colistin in anaerobic conditions exerted increased antibiofilm activity against *P. aeruginosa*, probably due to a lower ability to implement the tolerance mechanisms (e.g., LPS modifications). In this perspective, time-kill assays of the NAC-colistin combination were performed with *P. aeruginosa* Z154 planktonic cultures under aerobic and anaerobic conditions.

As expected, anaerobic cultures were more susceptible to killing by colistin than the aerobic cultures indeed, no synergism was observed in aerobic conditions. On the contrary, colistin at 0.25 mg/L in combination with NAC at 8,000 mg/L exerted a clear synergy in planktonic cultures grown under anaerobic conditions, with eradication achieved after 24 hours of exposure.

These results showed that in anoxic conditions (e.g., the deeper biofilm layers), NAC-mediated inhibition of anaerobic respiration would prevent the adaptive response of *P. aeruginosa* to prevent from colistin toxicity.

The few numbers of strains tested on antibiofilm assays represents a limitation of this study as well as transcriptomic analysis performed in only one *P. aeruginosa* strain (i.e., Z154). However, further studies are encouraging to confirm these findings and understand the potential use of NAC, at the high concentrations achievable by inhalation, that might have beneficial effects in preventing biofilm formation and the establishment of a chronic colonization.

3

EPIDEMIOLOGICAL CHARACTERIZATION OF COMMENSAL CTX-M-PRODUCING *E. COLI* ISOLATED FROM HEALTHY CHILDREN LIVING IN A RURAL COMMUNITY OF BOLIVIAN CHACO

RELATED PUBLICATION

Relevant increase of CTX-M-producing *Escherichia coli* carriage in school-aged children from rural areas of the Bolivian Chaco in a three-year period. Boncompagni SR, Micieli M, Di Maggio T, Mantella A, Villagrán AL, Briggesth Miranda T, Revollo C, Poma V, Gamboa H, Spinicci M, Strohmeyer M, Bartoloni A, Rossolini GM, Pallecchi L. *Int J Infect Dis*. **2022**. Aug;121:126-129.

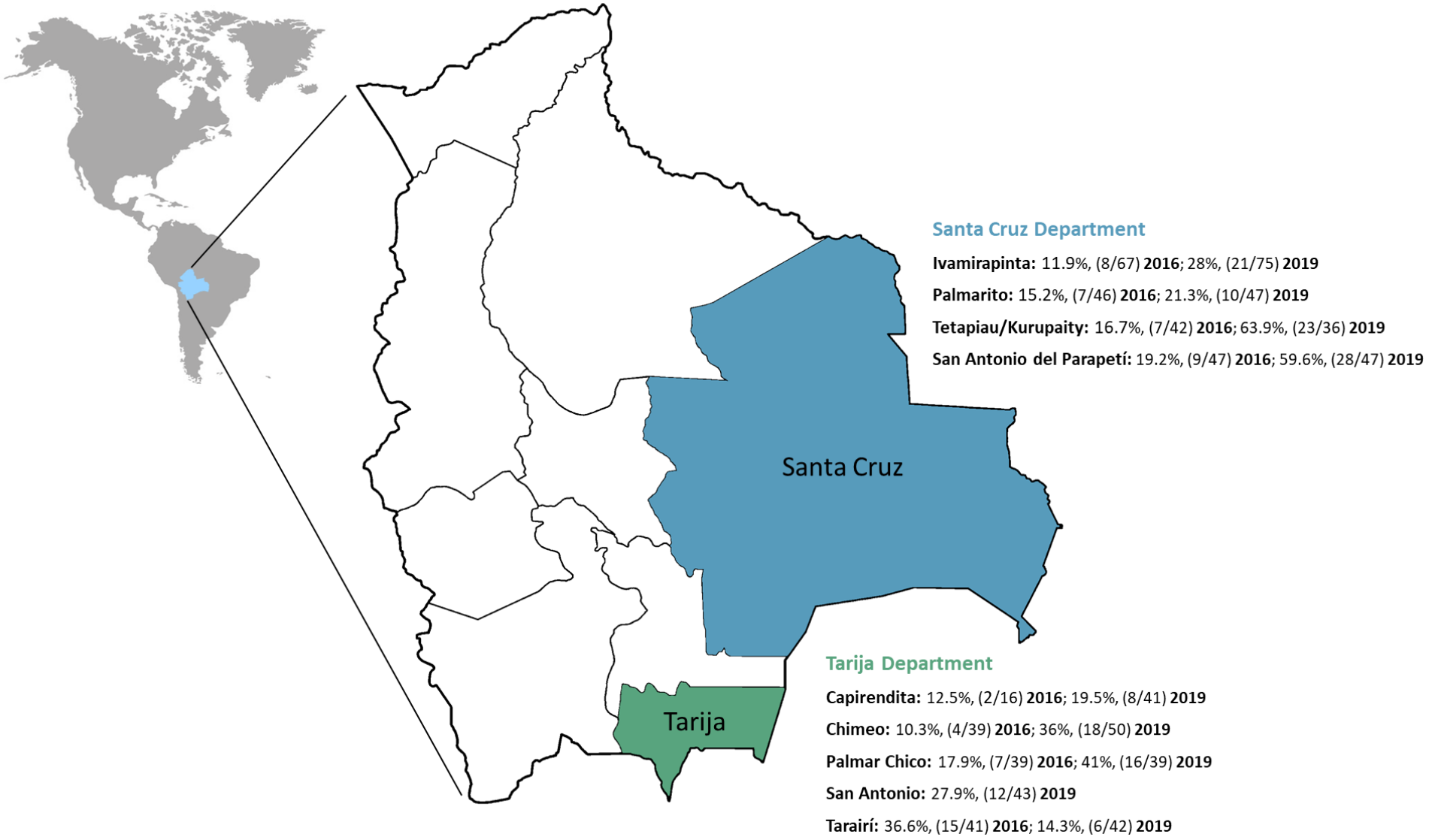
In the last 10 years, extended-spectrum- β-lactamase (ESBL)-producing enterobacteria, particularly *E. coli*, have become one of the main challenges for antibiotic treatment of enterobacterial infections, largely because of the current CTX-M enzyme pandemic. However, most of studies have focused on hospitalized patients, thought today it appears that the community is strongly affected as well (Woerther *et al.*, 2013).

In resource-limited countries, such as Bolivia, antimicrobial resistance rates have been demonstrated to be even higher than in the so named industrialized countries, for complex factors mainly related to poverty (e.g., poor access to healthcare, poor sanitation, unreliable water supplies), with a relevant impact on morbidity and mortality rates, especially in childhood (WHO, 2020).

In September of three years (i.e., 2016-2019), 757 school-aged (i.e., 6-14 years) children from nine indigenous community in rural areas of the Bolivian Chaco, were enrolled in a longitudinal study (Figure 16). Previous use of antibiotics during the last 15 days was investigated by a questionnaire administered to parents showing only 2 (0.6%) children in 2016 and 21 (5%) children in 2019 who had used antibiotics.

A total of 757 fecal samples (337 in 2016 and 420 in 2019) were collected, stored in faecal swabs (Copan, Brescia, Italy) and were shipped to Italy. Afterwards, fecal samples were streaked onto MacConkey agar supplemented with cefotaxime 2 μg/mL (MCA-CTX2), for the selection of expanded-spectrum cephalosporins-resistant *Enterobacteriaceae*.

Figure 16. Geographical area of the ten communities included in the study and percentages of children with CTX-M positive *E. coli*.



Some samples (N =19, in 2016; N =40, in 2019) showed the presence of more than one CTX-M-positive bacterial isolate, for a total of 274 CTX-M-positive isolates (N =83, 2016; N =191, 2019) collected (Table 7).

All isolates found positive were subjected to identification of species by MALDI-TOF mass spectrometry, which revealed that all positive samples were *E. coli* (N =272), except for one isolate in 2016 that were *Enterobacter cloacae* and one isolate in 2019 that was *Raoultella ornithinolytica*.

In 2016, of the 337 samples, 62 grew on MCA-CTX (18.4%). Of these, 59 (95.2%) were found positive for bla_{CTXM} genes. In 2019, of the 420 samples, 146 grew on MCA-CTX (34.8%) and 142 (97.3%) of these were found positive for $bla_{CTX.M}$ genes (Table 7).

Among CTX-M-groups, in both 2016 and 2019 CTX-M-1 group variant was the most prevalent (63.9% in 2016 and 61.5% in 2019), followed by CTX-M-9 group (28.9% in 2016 and 31.8% in 2019) and CTX-M-8/25 group (7.2% in 2016 and 6.7% in 2019), with five isolates harbouring variants from two groups (five strains with $bla_{CTX\text{-}M\text{-}1}$ plus $bla_{CTX\text{-}M\text{-}9}$ and one strain with $bla_{CTX\text{-}M\text{-}1}$ plus $bla_{CTX\text{-}M\text{-}8/25}$) in 2016 and one isolate harbouring variants from two groups (with $bla_{CTX\text{-}M\text{-}1}$ plus $bla_{CTX\text{-}M\text{-}9}$) in 2019. Group 2 was not detected (Table 7).

The last survey in two small urban area of the same region was conducted in 2011 and reported very high rates of resistance to expanded spectrum cephalosporins among commensal *E. coli* from healthy children (Bartoloni *et al.*, 2013). Interestingly, in less than 10 years the prevalence of CTX-M has almost tripled, suggesting a radical change in the epidemiology of ESBL profile in Bolivia.

Moreover, previous studies in South America reported wide dissemination of CTX-M type enzymes in clinical and commensal isolates from people living in urban areas (Bartoloni *et al.*, 2013; Rocha, Pinto and Barbosa, 2016; Saba Villarroel *et al.*, 2017; Pavez *et al.*, 2019) and although only few studies were conducted in rural communities worldwide, results are consistent with our study (Araque *et al*., 2018; Purohit *et al*., 2017).

In rural communities settings, where selective pressure from human antibiotic consumption is not intense, factors related to poverty may explain the emergence, dissemination, and persistence of AMR. Understanding the changing epidemiology of $bla_{CTX\text{-}M}$ genotypes will help in the development of hypotheses as to why these changes occur and how to implement strategies for reducing the spread of AMR.

Table 7. Features of children studies and features of CTX-M-producing *E. coli* studied in this study**.** [a], number (%), [b], during the 15 days preceding the survey, c, over the total CTX-M.

	Community	Year	No. of studied children			Antibiotic consumption [a,b]	Tot children with CTX-M[a]	*p* value	Children with multiple CTX-M producing *E. coli*	CTX-M producing *E. coli*	Tot CTX-M	CTX-M-group				
			M[a]	F[a]	Total							1[a,c]	9 n(%)[a,c]	8/25 n(%)[a,c]	1+9	1+8/25
Tarija Department	**Capirendita**	2016	9 (56.3)	7 (43.8)	16	-	2 (12.5)	0.8	-	1	2	-	2 (100)	-	-	-
		2019	23 (56.1)	18 (43.9)	41	-	8 (19.5)		3	11	12	7 (58.3)	5 (41.7)	-	1	-
	Chimeo	2016	18 (46.2)	21 (53.8)	39	-	4 (10.3)	0.01	-	4	4	3 (75)	1 (25)	-	-	
		2019	25 (50)	25 (50)	50	5 (10)	18 (36)		3	21	21	9 (42.9)	12 (57.1)	-	-	-
	Palmar Chico	2016	18 (46.2)	21 (53.8)	39	-	7 (17.9)	0.05	3	8	10	6 (60)	4 (40)	-	-	-
		2019	21 (53.8)	18 (46.2)	39	1 (2.6)	16 (41)		3	19	19	13 (68.4)	6 (31.6)	-	-	-
	San Antonio	2016	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		2019	24 (55.8)	19 (44.2)	43	-	12 (27.9)		2	14	15	12 (80)	2 (13.3)	1 (6.7)	-	1
	Tarairí	2016	18 (43.9)	23 (56.1)	41	-	15 (36.6)	0.03	4	21	22	18 (81.8)	4 (18.2)	-	1	-
		2019	19 (45.2)	23 (54.8)	42	-	6 (14.3)		1	7	7	3 (42.9)	3 (42.9)	1 (14.3)	-	-
Santa Cruz Department	**Ivamirapinta**	2016	35 (52.2)	32 (47.8)	67	-	8 (11.9)	0.03	3	12	12	9 (75)	3 (25)	-	-	-
		2019	41 (54.7)	34 (45.3)	75	4 (5.3)	21 (28)		10	35	35	26 (74.3)	6 (17.1)	3 (8.6)	-	-
	Palmarito	2016	19 (41.3)	27 (58.7)	46	1 (2.2)	7 (15.2)	0.6	2	9	9	-	3 (33.3)	6 (66.7)		-
		2019	21 (44.7)	26 (55.3)	47	5 (10.6)	10 (21.3)		3	14	15	9 (60)	5 (33.3)	1 (6.7)	1	-
	Tetapiau/	2016	18 (42.9)	24 (57.1)	42	1 (2.4)	7 (16.7)	<0.0001	2	9	10	8 (80)	2 (20)	-	-	-
	Kurupaity	2019	20 (55.6)	16 (44.4)	36	6 (16.7)	23 (63.9)		6	30	30	19 (63.3)	8 (26.7)	3 (10)	-	-
	San Antonio del Parapetí	2016	23 (48.9)	24 (51.1)	47	-	9 (19.2)	0.0001	5	13	14	9 (64.3)	5 (35.7)	-	-	-
		2019	24 (51.1)	23 (48.9)	47	-	28 (59.6)		9	39	41	22 (53.7)	15 (36.6)	4 (9.8)	2	-
	Total	2016	158 (46.9)	179 (53.1)	337 [b]	2 (0.6)	59 [e] (17.5)	<0.0001	19 (32.2)	82	83	53 (63.9)	24 (28.9)	6 (7.2)	1	-
		2019	218 (51.9)	202 (48.1)	420 [b]	21 (5)	142[f] (33.8)		40 (28.2)	190	195	120 (61.5)	62 (31.8)	13 (6.7)	4	1

4

DEFINING THE ROLE OF THE *E. COLI* ST1193 HIGH-RISK CLONE IN THE DISSEMINATION OF ANTIBIOTIC RESISTANCE IN HEALTHY CHILDREN LIVING IN A RURAL COMMUNITY OF BOLIVIAN CHACO

RELATED PUBLICATION

First report of the multidrug-resistant pandemic clone 1193 *Escherichia coli* among commensal isolates from Bolivia. Boncompagni SR, Di Pilato V, Micieli M, Di Maggio T, Mantella A, Villagrán AL, Revollo C, Poma V, Spinicci M, Strohmeyer M, Bartoloni A, Pallecchi L, Rossolini GM. *Manuscript in preparation (a draft version is supplied in the Annexes section)*

Escherichia coli sequence type 1193 (ST1193) was recently described as a fluoroquinolone-resistance (FQ^r) pandemic clone. Features of this clone are the inability of ferment lactose and the production of extended-spectrum β-lactamases (ESBLs), in particular CTX-M enzymes (T. J. Johnson *et al.*, 2019).

In Bolivia, a previous study reported a significant rise of CTX-M enzymes in commensal *E. coli* isolated from healthy children living in rural areas of Bolivian Chaco showing an increase from 17.5% to 33.8% over the total number of ESBL-producing *E. coli*. (Boncompagni *et al.*, 2022) However, data on clonal lineages driving such epidemiological change, and associated resistance features, are lacking.

The study population consisted of school-aged (6-14 years) children living in five rural communities in Tarija Department (i.e., Capirendita, Chimeo, Palmar Chico, San Antonio and Tarairí), in south-eastern Bolivia (between longitude 62°16' and 64°18' east and latitude 21°00' and 22°17' south) for a total of 800 children studied (135 in 2016, 247 in 2017, 203 in 2018 and 215 in 2019). The rural communities are located about 3 to 14 km from the Villa Montes town (Figure 17).

The use of antibiotics was found very limited, with only two (0.3%) children in 2016, one in 2017 (0.1%), and six (0.8%) in 2019 reporting antibiotic consumption during the 15 days preceding the survey.

Figure 17. Geographical area of the five communities near Villa Montes included in the study

One faecal sample for each child was collected during a two-month period from September to October, (from 2016 to 2019) for a total of 800 samples. All faecal swabs were then streaked onto MCA plates supplemented with cefotaxime 2 µg/ml (CTX2) showing that 28% (n = 224) grew on MCA-CTX2. The isolation of all different colonies showed that some children (n = 43, 19.2%) were colonized by more than one isolate for a total of 271 strains grew on MCA-CTX2.

The prevalence of ESBL-producing bacteria was 97% (N =263/271) and among these isolates the 99.2% (N =261/263) was found to carry bla_{CTXM} genes (Table 8). Species identification showed that all were *E. coli* (N = 257), except for two isolates in 2017 that were *Enterobacter cloaceae*, one isolate in 2018 that was *Klebsiella pneumoniae* and one isolate in 2019 that was *Raoultella ornithinolytica*.

The variants of bla_{CTXM} genes were investigated by PCR in all ESBL positive *E. coli* showed the prevalence of CTX-M-1 group variant (66.9%), followed by CTX-M-9 group (29.7%) CTX-M-8/25 group (2.3%) and CTX-M-2 group (0.8%) (Table 8). These results are consistent with previous study where high prevalence and rapid spread of CTX-M-producing *E. coli* were observed in a rural setting of Bolivian Chaco (Boncompagni *et al.*, 2022).

	2016[a]	2017[a]	2018[a]	2019[a]	Total[a]
Total of strains	39[b]	103[c]	55[d]	74[e]	**271**
ESBL positive strains	38 (97.4)	101 (98.1)	51 (92.7)	73 (98.6)	**263 (97)**
CTX-M producing strains:	38 (100)	99 (98)	51 (100)	73 (100)	**261 (99.2)**
E. coli	38 (100)	97 (98)	50 (98)	72 (98.6)	**257 (98.5)**
E. cloacae	0	2 (2)	0	0	**2 (0.8)**
K. pneumoniae	0	0	1 (2)	0	**1 (0.4)**
R. ornitolitica	0	0	0	1 (1.4)	**1 (0.4)**
CTX-M producing *E. coli*	38	97[d]	50[e]	72[d]	**257**
Total CTX-M detected:	39	98	52	74	**263**
CTX-M-1	28 (71.8)	78 (79.6)	26 (50)	44 (59.5)	**176**
CTX-M-2	0	1 (1)	1 (1.9)	0	**2**
CTX-M-8/25	0	3 (3.1)	1 (1.9)	2 (2.7)	**6**
CTX-M-9	11 (28.2)	15 (15.3)	24 (46.2)	28 (37.8)	**78**

Table 8. Distribution of strains per years and features of CTX-M-positive *E. coli* isolates. [a], number (%), [b], In 2016, one child carried one *Enterobacter* spp., [c], In 2017, two children carried one *Citrobacter* spp., [d], In 2018, one child carried one *Enterobacter* spp. and another child carried one *Klebsiella pneumoniae*., [e], In 2019, one child carried one *Serratia* spp. and another child carried one *Roultella* spp., [f], one *E. coli* carrying two $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$), [g], two *E. coli* carrying two $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$),

Phylogenetic group distribution were investigated in all *E. coli* isolated with established PCR-based methods (Clermont *et al.*, 2013a) showing a wide diversity amongst the CTX-M-producing *E. coli*. Indeed, the phylogenetic groups found can be divided into seven main phylogenetic groups designated A, B1, B2, C, D, E and F, with groups A and B being the predominant ones (Figure 18).

The extra-intestinal pathogenic strains usually contained more virulence factors and belong to groups B2 or D. On the contrary, the commensal strains belonged to phylogenetic groups A and B1. Interestingly, a total of 40 E. coli was found to belong to group B2 with uneven distribution showed a ten-fold increase over the years, from 3% in 2016 to 32% in 2019 (Figure 18).

Moreover, the B2 isolates showed that $bla_{\text{CTX-M-1}}$ gene variant has been found predominant in 2016. On the contrary, $bla_{\text{CTX-M-9}}$ gene variant has been found predominant since 2018, significantly reducing the contribution of $bla_{\text{CTX-M-1}}$ gene variant (from 100%, in 2016 to 17%, in 2019) (Figure 19A).

For this reason, molecular detection of *E. coli* pandemic clones B2-O25b-ST131 and ST1193 was investigated in B2 isolates.

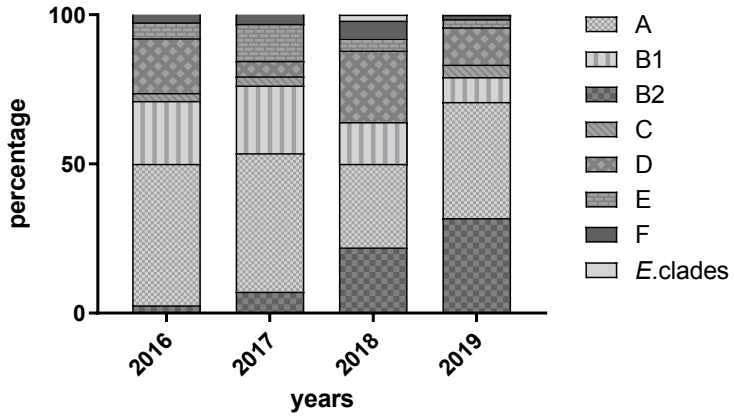


Figure 18. Percentage of phylogenetic groups amongst the 257 CTX-M-producing *E. coli* per years

Clonal PCR analysis revealed that the O25b-ST131 has been found predominant in 2017, carrying $bla_{\text{CTX-M-1}}$ (n=6/7, 85.7%) (**Figure 19B and 19C**). Further, in the 2018, the rates of clones changed deeply showing an emerged of Ec-ST1193 carried $bla_{\text{CTX-M-9}}$ gene (n=9/11, 81.8%) and a decreased of O25b-ST131 carried $bla_{\text{CTX-M-1}}$ gene (n=2/11, 18.2%) (**Figure 19B, Figure 19C, Figure 19D**). In the 2019 the ratio between clones remains constant with that observed in 2018 (Ec-ST131-H30 n=6/23, 26% and Ec-ST1193 n=13/23, 56.5%) with the difference that the Ec-ST131-H30 carrying $bla_{\text{CTX-M-9}}$ gene (**Figure 19B, Figure 19D**).

Overall, these results showed that CTX-M-9-producing *E. coli* mostly belonged to ST1193, and this clone may be emerged in this setting in 2018, contributing to the rapid spread of CTX-M-producing *E. coli* were observed in this study.

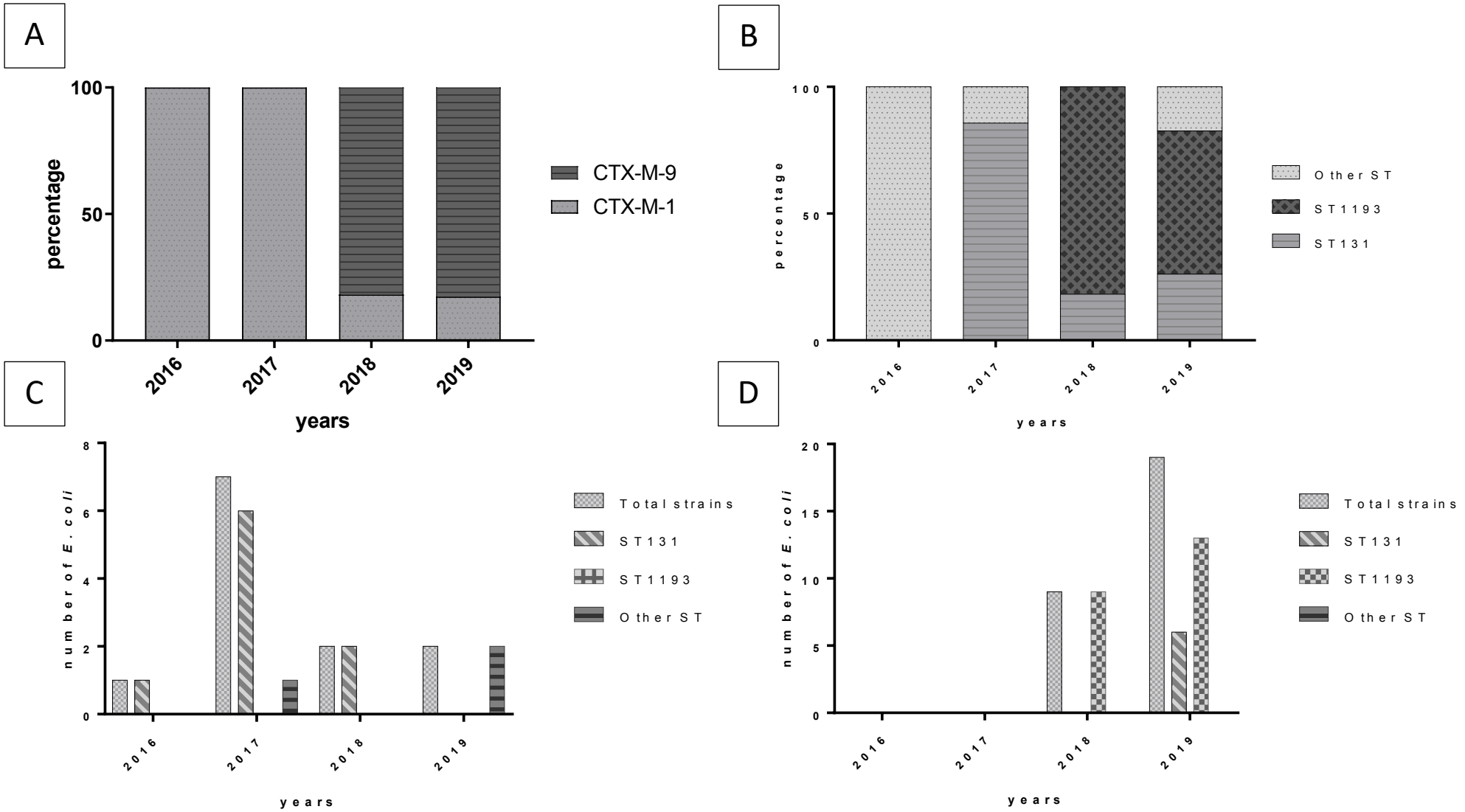


Figure 19. Analysis of CTX-M-producing *E. coli* belonging to the phylotype B2. (A) Prevalence of CTX-M groups in CTX-M-producing *E. coli* over the years. (B) Rates of ST131 and ST1193 clones carrying $bla_{\text{CTX-M}}$ genes over the years. (C) Clones distribution among CTX-M-1 producing *E.coli* over the years. (D) Clones distribution among CTX-M-9 producing *E. coli* over the years.

In order to investigate the presence of pandemic clone ST1193 in this setting all Ec-ST1193 (N = 22) were subjected to WGS using the Illumina NextSeq (2x150bp) platform (Illumina, Inc., San Diego, CA, USA).

The analysis of the distribution of antibiotic resistance genes among the Bolivian Ec-ST1193 isolates reveled a homogeneous distribution, except for 226 and 242 strains which carrying only $bla_{CTX\text{-}M\text{-}27}$ gene (Table 8). The other twenty Bolivian Ec-ST1193 strains showed a MDR phenotype and carried an extensive array of antimicrobial resistance (AMR) genes such as *sul*1 and *sul2* (sulfonamide resistance), *dfr*A17 (trimethoprim resistance), *mph*A (macrolide resistance), *aadA5* (streptomycin resistance), *aph*(3'')-Ib, *aph*(6)-Id (streptomycin phosphotransferase) and *tet*A (tetracycline efflux pump) (Table 8). Furthermore, all Bolivian Ec-ST1193 isolates had the same four nonsynonymous mutations in *gyrA* (S83L, D87N), *parC* (S80I), *parE* (L254Q) housekeeping genes that are known to confer fluoroquinolone resistance confirmed was previously described by Johnson *et al* (T. J. Johnson *et al.*, 2019a). Characterization of plasmids revealed that all Ec-ST1193 isolates belonged to the F plasmid family. In particular, the replicon types of IncF plasmids were FIA (n = 21) and FIB (n = 22) with F-:A1:B10 as one of the prevalent subgroup (21/22, 95.5%). About the virulence factor (VF), the most frequent genes were uropathogenic specific protein (*usp*), autotransporter toxin (*sat*), outer membrane protease (*ompT*), tellurium ion resistance protein (*terC*) genes encoding metal acquisition systems (*chuA*, *fyuA*, *irp*2, *iucC*, *iutA*) and adherence (*yfcV*, *pap*), each of which was detected in 100% of the isolates. Moreover, all Ec-ST1193 isolates possessed the K1 capsular genotype. Two genes were identified in 95.5% (21/22 *E. coli*) of the isolates, including toxins gees such as *senB* and *vat*. The 25 strain was the only showed the presence of *fimH* gene (Table 8 and Table 9).

Table 8. Features of twenty-two Bolivian Ec-ST1193 genomes. Plus, and minus indicated the presence or absence of the genes, respectively.

Strain	Collection Date	Phylogenetic group	capsule	O and H type	fimH	ST plasmid	Antibiotic resistance genes										QRDR mutations		
							aadA5	*aph(6)-Id*	*aph(3'')-Ib*	*bla* $_{CTX-M}$	*sul1*	*sul2*	*dfrA17*	*tet*	*erm(B)*	*mph(A)*	*gyrA*	*parC*	*parE*
25	2018	B2	K1	O75:H5	+	F2:A-:B10	+	+	+	+	+	+	+	+	-	+	S83L; D87N	S80I	L416F
41	2018	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
81	2018	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
199	2018	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
104	2018	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	-	+	S83L; D87N	S80I	L416F
115	2018	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
204	2018	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	-	+	S83L; D87N	S80I	L416F
207	2018	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
222B	2018	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
226	2019	B2	K1	O75:H5	-	F-:A1:B10	+	-	-	+	-	-	-	-	-	-	S83L; D87N	S80I	L416F
322	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
332	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
242	2019	B2	K1	O75:H5	-	F-:A1:B10	-	-	-	+	-	-	-	-	-	-	S83L; D87N	S80I	L416F
358	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
362	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
364B	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
366	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	-	+	S83L; D87N	S80I	L416F
371	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	-	+	S83L; D87N	S80I	L416F
375	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
421	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	-	S83L; D87N	S80I	L416F
387	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F
363	2019	B2	K1	O75:H5	-	F-:A1:B10	+	+	+	+	+	+	+	+	+	+	S83L; D87N	S80I	L416F

Global comparative genomic analysis was carried out including all the Ec-ST1193 genomes available at the NCBI-NHI database (https://www.ncbi.nlm.nih.gov/genome/; last accessed in May 2022), (n = 193) and the twenty-two described in this manuscript, for a total of 215 genomes.

Phylogeny was conducted in all 215 genomes after filtering recombinant regions using Gubbins (Croucher et al., 2015). The SNP-based tree of the entire collection was built using 97-88% (5,193,182 bp/4,739,571 bp) of the reference genome SCU-204 and resulting in an alignment with 6,526 SNPs. The SNPs matrix was uniform among all the Ec-ST1193 strains, showing a median of 49 SNPs with a range from a minimum 1 to a maximum 187, respectively. As a results, the phylogenetic analysis showed two distinct clusters (A-B) associated with different capsular type (K1 and K5) and it is consistent with the Ec-ST1193 evolution previously described (Johnson, 2019) (Figure 20). All the Ec-ST1193 isolates from Bolivia clustered together phylogenetically and showed a small range of SNPs, from 1 to 13, among them, except for 25 Bolivian strain that clustered with other Ec-ST1193 without a clear correlation with geographic distribution, K-type or other characteristics.

Among the 193 no-Bolivian Ec-ST1193, most of which were isolated from humans (Figure 21), 169 strains showed a MDR phenotype (87.6%) (Table 10). Screening for acquired resistance determinants revealed genes for aminoglycoside-modifying enzymes, sulfonamide, trimethoprim, macrolide and tetracycline resistance; and several β-lactamases, including TEM-1 (56.5%), OXA-1 (4.7%) and CTX-M (34.7%) (Table 10). More precisely, the majority of CTX-M-positive Ec-ST1193 carrying $bla_{\text{CTX-M-27}}$ (27/67, 40.3%) followed by $bla_{\text{CTX-M-55}}$ (16/67; 23.9%), $bla_{\text{CTX-M-14}}$ (10/67; 14.9%) and $bla_{\text{CTX-M-15}}$ (12/67; 17.9%).

Moreover, all Ec-ST1193 studied harbored the same four non-synonymous mutations in quinolone-resistance determining region (QRDR) (gyrA S83L, D87N; parC S80I; parE L416F) except for UPEC61 that shown only three mutations (gyrA S83L, D87N; parE L416F).

Using pMLST based on F plasmid alleles, the RepFIA (A1) allele was highly prevalent across Ec-ST1193 isolates. However, three different main alleles of RepFIB were identified (B1, B10, and B20), and these partially segregated according to the phylogeny (Figure 20).

Interestingly, the isolates with F1:A1:B20 and F-:A1:B20 were consistently associated with K5 capsular types. On the contrary those with F-:A1:B10 replicons were associated with the K1 capsular type.

The Ec-ST1193 strains also contained small plasmids. The small-plasmid replicons, which included Col156, Col(BS512), ColpVC, Col(MG828), Col828 and Col(phAD28), did not mimic the phylogenetic distribution of the F-type plasmids.

Eighteen virulence factor encoding genes were significantly associated with Ec-ST1193 included the outer membrane hemin receptor gene *chuA* (193/193, 100%), yersiniabactin siderophore receptor gene *fyuA*

(193/193, 100%), siderophore yersiniabactin gene *irp2* (193/193, 100%), the glutamate decarboxylase gene *gad* (167/193, 86.5%), adherence protein gene *iha* (188/193, 97.4%), aerobactin synthetase gene *iucC* (187/193, 96.9%), aerobactin receptor gene *iutA* (186/193, 96.4%), capsule polysaccharide export inner-membrane protein gene *kpsT* (156/193, 80.8%), group II capsule with K1 and K5 variants *KpsMII-K1/K5* (190/193, 98.4%), polysialic acid capsule biosynthesis protein *neuC* (156/193, 80.8%), outer membrane protease T gene *ompT* (193/193, 100%), secreted autotransporter toxin gene *sat* (177/193, 91.7%), plasmid-carried enterotoxin gene *senB* (164/193, 85%), iron transport protein *sitA* (191/193, 99%), tellurium ion resistance protein *terC* (191/193, 99%), uropathogenicspecific protein *usp* (191/193, 99%), vacuolating toxin gene *vat* (176/193, 91.2%) and YfC fimbria gene *yfcV* (189/193, 97.9%) (Table S1).

The majority of Ec-ST1193 were positive for the K1 capsular type (n = 162/193; 83.9%), on the contrary only 28 *E. coli* were positive for the K2 capsular type (14.5%).

The high prevalence of *senB* among ST1193 isolates was of interest, because this gene is on a plasmid that codes for a toxin associated with highly virulent or dominant clonal groups, such as ST131 and ST95. Moreover, Ec-ST1193 was always described as a clone carrying the *fimH64* allele. *fimH* is highly conserved and extremely common among *E. coli* isolates because induces *E. coli* adherence to the urothelium cells, and helps the formation of intracellular bacterial biofilms. Interestingly, this gene was not found in Bolivian ST1193 strains, except for one strain (e.g., 25).

Figure 20. Phylogenetic analysis and main features of 215 Ec-ST1193 studied. The two clusters associated with different capsular type, K1 and K5, are highlighted by blue and green shadows, respectively. The red shadow identifies the Bolivian Ec-ST1193. Squares, colored by trait category, represent the presence of a trait examined. The different country is indicated by colored circle.

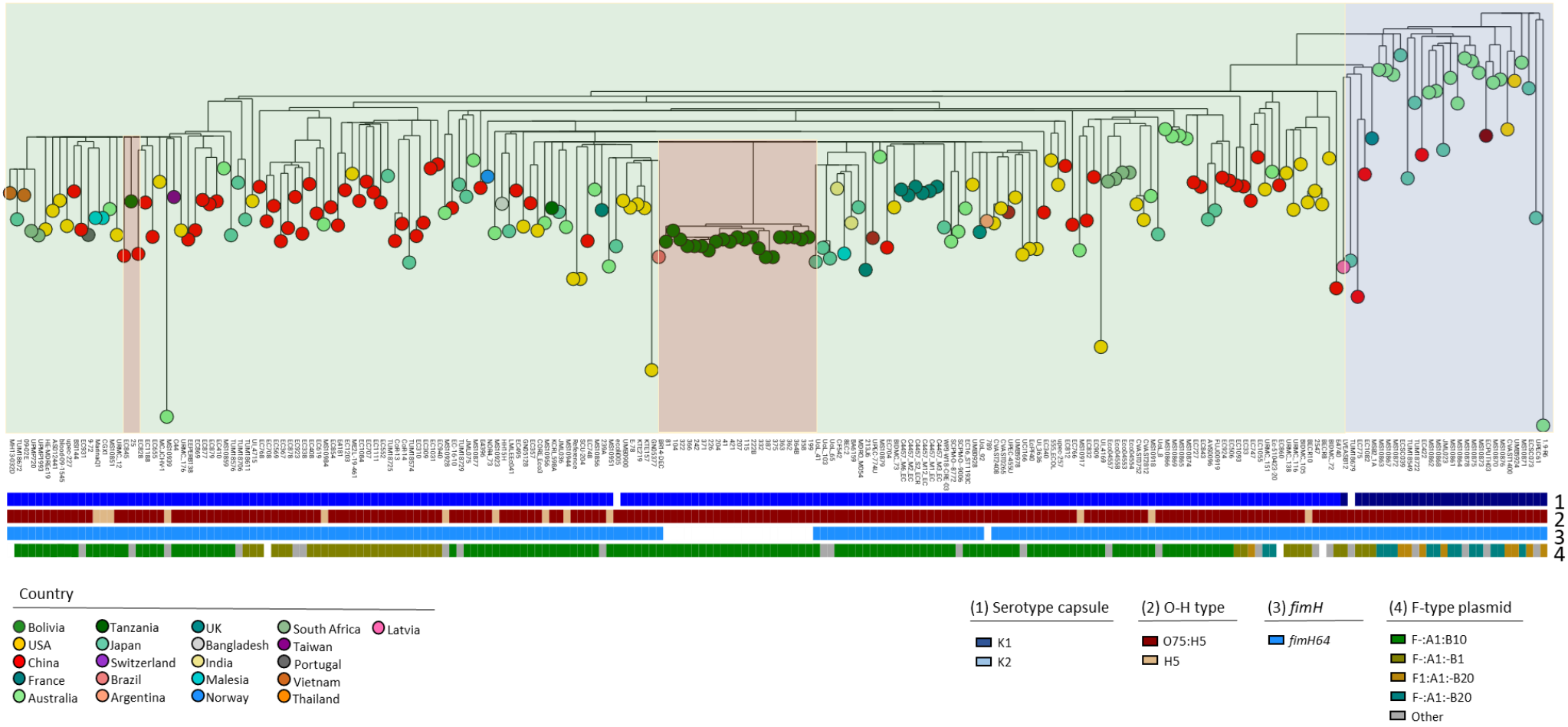


Figure 21. World distribution and host types of Ec-ST1193 genomes obtained by NCBI database

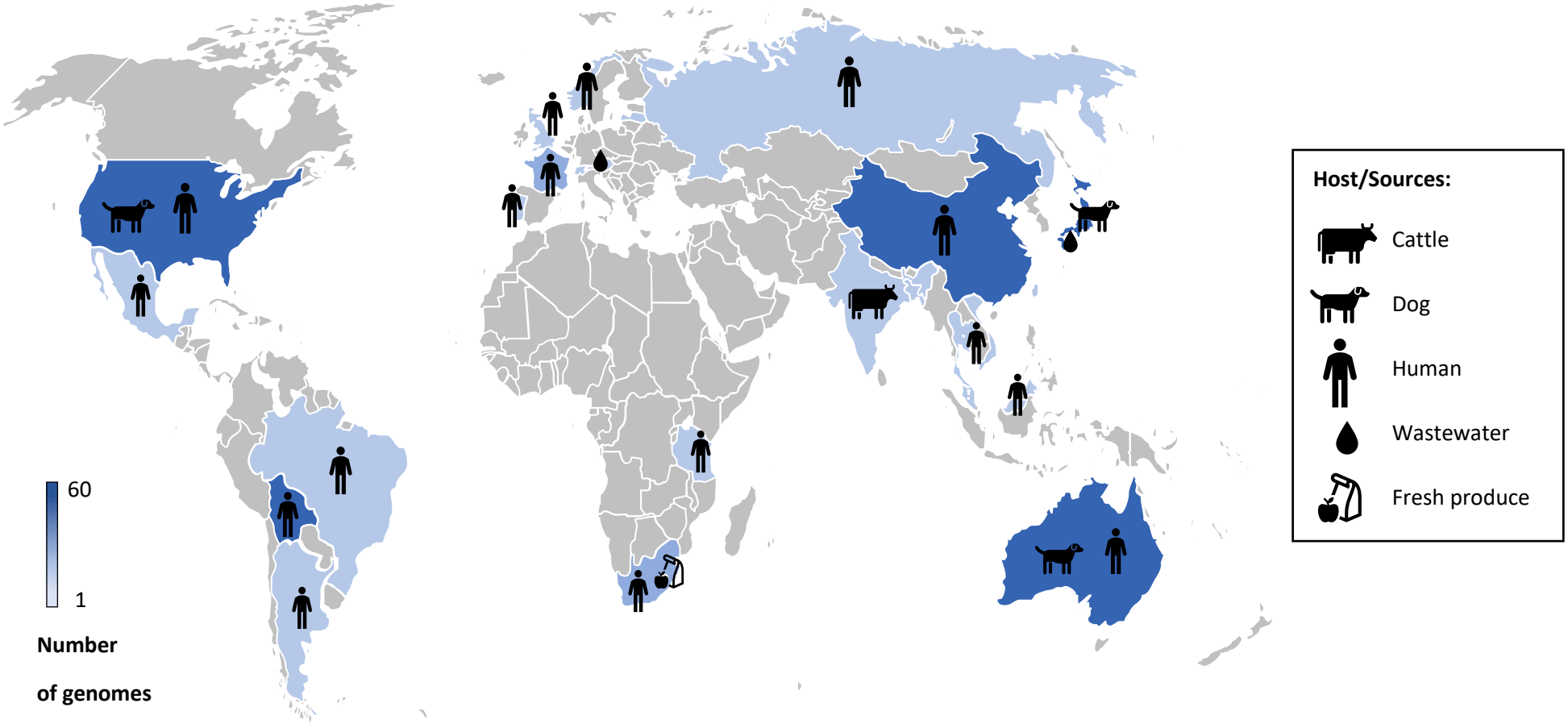


Table 9. Virulence-associated traits of Ec-ST1193 genomes include in the study

Category	Gene(s) or operon	Product/Function	%
Adhesins	*csgA*	Curli	99
	ecp	iron transport related genes	100
	fim	D-mannose-specific adhesin, type-1 fimbiae	97
	yfcV	Fimbial protein	99
	iha	Iron-regulated-gene-homologue adhesin	97
	pap	Pilus associated with pyelonephritis (P fimbriae)	99
Toxins	*cdtB*	Cytolethal distending toxin, CDT	2
	sat	Secreted autotransporter toxin (serine proteasi)	95
	vat	Vacuolating autotransporter toxin	97
	senB	plasmid-encoded enterotoxin	84
Nutrition	*chuA*	Heme receptor	99
	entF	Enterobactin synthesis	95
	fyuA, irp	Yersiniabactin (siderophore) receptor, synthesis	99/97
Iron uptake	*iuc*	Aerobactin (siderophore) syntesis	96
	iutA	Aerobactin receptor	96
	sitA	Iron/manganese transport protein, periplasmatic-binding protei	99
	sitBCD	Iron/manganese transport protein	99
Protectins	*kpsMTII*	Group II capsule synthesis (e.g., K1, K5, K10, K12)	97
	kpsE	Capsule polysaccharide export inner membrane protein	97
	neuC	Polysialic acid biosinthesis of the K1 capsule	90
	traT	Conjugal transfer surface exclusion protein; serum resistance-as	5
Invasins	*ibeB-C*	Invasion of brain endothelium IbeB and C	94
	ompA	Outher membrane protein A (cellular invasion)	100
	traJ	Cellular invasion (F-like plasmid transfer region homologue)	1
Misc.	*ompT*	outer membrane (protease)	99
	usp	putative bacteriocin	99
	gad	glutammate decarboxylase	82
	terC	Tellurium ion resistance protein	99

Misc., miscellaneous.

Table 10. Antibiotic resistance genes in all No-Ec-ST1193 in comparison with Ec-St1193 Bolivian isolates.

		BOLIVIAN ISOLATES N (%)	NO-BOLIVIAN ISOLATES N (%)	TOTAL N (%)
Aminoglycosides	*aadA5*	20 (90.9)	84 (43,5)	104 (48.4)
	aph(6)-Id	20 (90.9)	147 (76.2)	167 (77.7)
	aph(3'')-Ib	20 (90.9)	144 (74.6)	164 (76.3)
	aac(3)-IIa	0	8 (4.1)	8 (3.7)
	aac(3)-IId	0	48 (24.9)	48 (22.3)
	aac(6')-Ib	0	10 (5.2)	10 (4.7)
	rmtB	0	2 (1)	2 (0.9)
β-lactams	bla_{TEM}	0	109 (56.5)	109 (50.7)
	bla_{CTX-M}	22 (100)	67 (34.7)	89 (41.4)
	bla_{OXA}	0	9 (4.7)	9 (4.2)
	bla_{CMY}	0	3 (1.6)	3 (1.4)
Sulfonamides	*sul1*	20 (90.9)	90 (46.6)	110 (51.2)
	sul2	20 (90.9)	147 (76.2)	167 (77.7)
Trimethoprim	*dfrA*	20 (90.9)	130 (67.4)	150 (69.8)
Tetracyclines	*tetA/B*	20 (90.9)	135 (70)	155 (72.1)
Macrolides	*mph(A)*	19 (86.4)	111 (57.5)	130 (60.5)
	erm(B)	14 (63.6)	11 (5.7)	25 (11.1)
Fenicols	*floR*	0	2 (1)	2 (0.9)
	cat	0	7 (3.6)	7 (3.3)
Fosfomycin	*fosA3*	0	1 (0.5)	1 (0.5)
Quiolones	*qnrB*	0	1 (0.5)	1 (0.5)
MDR phenotype		20 (90.9)	169 (87.6)	187 (87)

The pan-genome analysis revealed a total of 10089 of which the 34.3% composed the core genome. Twenty-one Ec-ST1193 Bolivian strains showed the presence of 73 specific genes, with the exception of 25 strain. 35 of them were identified as hypothetical proteins and 38 were known coding-genes, including metabolic proteins (e.g., *nanS*, *era1*), membrane proteins (e.g. *yjgN*, *yehB*), IS-family transposases (e.g. IS4, IS66), siderophore-iron reductase (e.g., *fhuF*), Ag43-encoding gene (*flu*) and a toxin component (*cbtA*) (Kim, Nagore and Nikaido, 2010; Ravan and Amandadi, 2015; Heller, Tavag and Hochschild, 2017). On the contrary, in 99% of the other Ec-ST1193 no-Bolivian strains plus the 25 Bolivian Ec-ST1193 strain, 48 specific-genes (13 hypothetical proteins and 35 coding-genes) were detected. Of these, 25 encoded metabolic proteins (e.g., *idnT*, *deoR*), transcriptional regulators (e.g., *kdgR*), transporter family (e.g., *dctM*) and the remaining genes were virulence determinants or toxin-antitoxin system, including the *fimH* operon and toxic proteins *symE* and *higB*.

Interestingly, the Parsnp tool analysis identified one highly recombinogenic (HR) region similar among all Ec-ST1193 Bolivian strains where most of SNPs were localized. This genomic region has been estimated to vary from 200 to 290 kb on the chromosomes and it was located just downstream of the *treC* locus. Interestingly, all specific-genes were located in the HR-region including *fim*H gene.

Further insights into this region are needed to better understand the differences between Ec-ST1193 Bolivian isolated with other Ec-ST1193.

In the present study, Ec-ST1193 strains were detected for the first time in healthy children living in a small rural community in Bolivia. Gut colonization with multiresistant clones, such as ST131 and ST1193, may favor the epidemic spread of these clones with high pathogenic potential. For this reason, the intestinal reservoir deserves attention, along with clinical isolates. Noteworthy, the role of the intestinal microbiota as reservoir of resistance determinants is an important concern, particularly in limited resource countries like those of Latin America, where the combination of factors (i.e., inefficient health systems, poor sanitation and uncontrolled use of antimicrobials) provide conditions to develop and maintain resistant strains in the hospital settings, at the community level and in the environment.

All Ec-ST1193 isolates studied showed the same characteristics: they were resistant to fluoroquinolones, showed serogroup O75:H5, phylogroup B2, frequently harboured IncFIA and IncFIB plasmids and carried ESBL genes such as $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-27}}$.

In addition, the occurrence of a similar virulence gene profile in the ST1193 strains together with the results of serotyping and phylotyping confirm that Ec-ST1193 strains have a high level of homogeneity independently from the geographic region, thus suggesting global clonal dissemination.

Nevertheless, it is interesting to note how the Bolivian ST1193 strains showed some differences compared to all the others ST1193 strains, particularly, the lack of *fim* operon, which was demonstrated to promotes antigen-specific immune activation. This evidence may be the result of an evolutionary event that could contributed to its epidemiological success.

5

CONCLUSIONS AND PERSPECTIVES

Bacterial resistance to antibiotics represents a major public health problem and measures to prevent the increase in antibiotic resistant organisms require knowledge of both the dissemination of antibiotic resistance using the surveillance approach and alternative strategies for tackling its.

An early and leading priority to do this is to strengthen AMR surveillance, particularly in low-income countries where the burden of infectious diseases is highest and where data are most limited. Moreover, the role of commensal microbiota as a potential reservoir of resistance determinants is often underestimated, even if, the investigation of the resistance rate observed in the microbiota of healthy individuals could play a crucial role as an indicator in predicting the dissemination of resistance among pathogens.

The expansion of genome sequencing capabilities and data availability over the last decade has led to an improved understanding of the epidemiological dynamics of the resistance determinants and could provide a more comprehensive view about the mechanisms by which antibiotic resistance is spread among bacteria.

In this study, the epidemiological investigation of CTX-M-producing *E. coli* among healthy children living in rural community of the Bolivian Chaco, where antibiotic usage remains scarce, provided for the first time a description of an emerging clone *E. coli* ST1193 in this setting. In combination with phenotypic surveillance and epidemiological data, evidence from WGS data can be used to provided more detailed into the epidemiology pathogens and this integrative approach has been increasingly essential. These findings underscore that surveillance of antimicrobial resistance on commensal microbiota can be useful to guide management of infections, especially in settings where microbiological diagnosis is not available.

In the other hand, due to rapidly growing antimicrobial resistance, there is an urgent need to develop alternative strategies. Antibiotics can be strategically utilized in combination therapy to yield more potent (synergistic) outcomes. There is good evidence that combinations of antibiotics have much lower development costs and could be sold at a lower price which may be more acceptable in the present market conditions. Moreover, the use of combination of antibiotics makes the reuse of many of our old antibiotics in large numbers of different combinations possible. Indeed, one of the alternative antibiotic therapies studied in this thesis, colistin and fosfomycin combinations, may be provide an alternative strategy to prevent the increase in antibiotic resistance.

Overall, these findings suggest that, although crucial for reducing the emergence and dissemination of resistance, strategies based only on antibiotic restriction policies are unlikely to fully succeed. It is now clear

that antibiotic resistance should be viewed as a complex ecological problem and also addressed by applying a wide-ranging approach, which takes into account the multitude of factors affecting this phenomenon.

PART IV

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PART V

ANNEXES

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Activity of fosfomycin/colistin combinations against planktonic and biofilm Gram-negative pathogens

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Objectives: To investigate the *in vitro* activity of fosfomycin, colistin and combinations thereof against planktonic and biofilm cultures of Gram-negative pathogens, mostly showing MDR phenotypes, at concentrations achievable via inhalation of aerosolized drugs.

Methods: Activity against planktonic cultures was tested by the chequerboard assay with 130 strains, including 52 *Pseudomonas aeruginosa*, 47 *Klebsiella pneumoniae*, 19 *Escherichia coli*, 7 *Stenotrophomonas maltophilia* and 5 *Acinetobacter baumannii*. Activity against biofilm cultures was tested by biofilm chequerboard and quantitative antibiofilm assays with a subset of 20 strains. In addition, 10 of these strains were tested in mutant prevention concentration (MPC) assays.

Results: Against planktonic cultures, synergism between fosfomycin and colistin was detected with a minority (10%) of strains (eight *K. pneumoniae* and five *P. aeruginosa*), while antagonism was never observed. Synergism between fosfomycin and colistin against biofilms was observed with the majority of tested strains (16/20 in biofilm chequerboard assays, and 18/20 in the quantitative antibiofilm assays), including representatives of each species and regardless of their resistance genotype or phenotype. Furthermore, combination of fosfomycin and colistin was found to significantly reduce the MPC of individual drugs.

Conclusions: Fosfomycin and colistin in combination, at concentrations achievable via inhalation of nebulized drugs, showed notable synergy against MDR Gram-negative pathogens grown in biofilm, and were able to reduce the emergence of fosfomycin- and colistin-resistant subpopulations.

Introduction

Antimicrobial chemotherapy has positively impacted life expectancy, and the recent surge of infections caused by MDR pathogens represents a major challenge to global health, reducing the available therapeutic options. Among the most problematic infections are those caused by microbial biofilms, which play a crucial role in chronic lung colonization/infection of patients affected by cystic fibrosis (CF) and other chronic airway conditions such as COPD and non-CF bronchiectasis. In addition, the growth of biofilms in endotracheal tubes is implicated in infection-related ventilation-associated complications (IVACs), which have a relevant impact on morbidity and mortality rates in ICUs. Treatment of such infections poses a clinical challenge, due to the inherent antibiotic tolerance of biofilms and the frequent involvement of bacterial strains expressing MDR phenotypes.

In this scenario, treatment regimens based on inhaled antibiotics have been extensively investigated in recent years.[1–3] In fact, this route of administration can achieve high antibiotic concentrations in the epithelial lining fluid (ELF), overcoming antibiotic resistance while minimizing systemic toxicity.[2]

Fosfomycin and colistin are 'old' antibiotics that recently regained interest due to the dearth of new compounds to treat infections caused by MDR pathogens.[4] Aerosolized fosfomycin, administered via inhalation, was estimated to reach concentrations higher than 1200 mg/L in ELF,[5] and promising results were obtained in a Phase II study evaluating inhaled fosfomycin in combination with tobramycin in CF patients.[1,6] Aerosolized

colistin, administered by inhalation, has been increasingly used for the treatment of some acute and chronic respiratory tract infections, especially in patients affected by CF.[7] Colistin ELF concentrations after aerosol delivery are heterogeneous (i.e. range 9.5–1137 mg/L), but higher than those achievable after IV administration (i.e. range 1.5–28.9 mg/L).[7]

Recently, a synergistic effect of fosfomycin/colistin combinations has been reported against planktonic cultures of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumoniae*.[8–12] In addition, synergistic activity of fosfomycin and colistin was reported against *E. coli* biofilm in an *in vivo* foreign-body infection model,[10] and against *P. aeruginosa* biofilm in an *in vitro* model.[12] However, knowledge in this area remains scarce overall. In this work, we investigated the *in vitro* activity of fosfomycin/colistin combinations (tested at the high concentrations potentially achievable in ELF, after inhalation) on planktonic and biofilm cultures of several Gram-negative pathogens, including strains expressing clinically relevant MDR phenotypes.

Materials and methods

Bacterial strains

A collection of 130 clinical strains of different Gram-negative species (52 *P. aeruginosa*, 47 *K. pneumoniae*, 19 *E. coli*, 7 *Stenotrophomonas maltophilia* and 5 *A. baumannii*) was investigated in this work. The strains were from the laboratory repositories at the universities of Florence and Siena, and had mostly been collected during previous national and international surveys.[13–22] The strains were from different sources (49 bloodstream infections, 42 lower respiratory tract infections (of which 6 were from CF patients), 32 urinary tract infections and 7 from other sources). Most strains were carbapenemase producers and exhibited MDR phenotypes (i.e. exhibited acquired resistance to at least one agent in three antibiotic classes, according to the EUCAST clinical breakpoints).[23] The investigated strains exhibited different susceptibility profiles to fosfomycin and colistin, tested in accordance with international standard ISO 20776-1:2019.[24] Susceptibility to fosfomycin and colistin was defined according to the available EUCAST clinical breakpoints.[23] WGS data were available for most strains. A summary of the main features of the investigated strains is reported in Table 1. A detailed description of the features of each strain is reported in Table S1, available as Supplementary data at *JAC* Online.

Genomic analysis of fosfomycin- and colistin-resistant strains

WGS data of colistin-resistant strains were investigated by bioinformatic analysis for the presence of known acquired colistin resistance genes (*mcr*) and chromosomal alterations known to be associated with colistin resistance, including (i) *pmrAB*, *phoPQ*, *parRS* and *cprRS* for *P. aeruginosa*;[25] (ii) *pmrCAB* for *A. baumannii*;[26] and (iii) *mgrB*, *pmrB* and *phoPQ* for *K. pneumoniae*.[27] WGS data of fosfomycin-resistant strains were investigated by bioinformatic analysis for the presence of acquired *fosA/C* genes and chromosomal alterations known to be associated with fosfomycin resistance, including *glpT* and *uhpT* for *K. pneumoniae* and *E. coli* (Table S1).[28]

Chequerboard assays with planktonic cultures

Chequerboard assays to assess synergism of fosfomycin and colistin combinations with planktonic cultures were carried out in CAMHB (Becton Dickinson, Milan, Italy) supplemented with 25 mg/L glucose-6-phosphate (Sigma–Aldrich, St Louis, USA) (CAMHBG6P).[29,30] The ranges of fosfomycin (Sigma–Aldrich) and colistin (AppliChem, Darmstadt, Germany) concentrations tested were 0.015–1024 and 0.03–1024 mg/L, respectively. The data produced by the chequerboard assays were analysed in terms of FIC index (FICI) and interpretated as follows: FICI $\leq$0.5, synergy; FICI >0.5–4.0, no interaction; FICI >4.0, antagonism.[30] All strains were tested in two independent experiments and discrepancies in FICI values were adjudicated by a third chequerboard assay.

Activity against biofilms

Activity of fosfomycin, colistin and combinations thereof against biofilms was determined by a standardized *in vitro* biofilm model in which biofilm is formed on plastic pegs on a modified 96-well microtitre plate (Innovotech, Edmonton, Alberta, Canada), as previously described.[31] Biofilms were grown in CAMHBG6P (static conditions, 35°C) for 24 h, except for *A. baumannii*, which required 7 days of growth in daily refreshed medium.[30] Under these conditions, the quantity of preformed biofilms, assayed by viable cell counting, was homogeneous overall among different strains. Preformed biofilms were then exposed to different concentrations of fosfomycin, colistin and fosfomycin/colistin combinations in CAMHBG6P for 24 h (static conditions, 35°C). After antibiotic exposure, biofilms were washed twice with PBS (Sigma–Aldrich) to remove loosely adherent bacteria, and sessile cells were removed from pegs by sonication for 30 min (peak ultrasonic power 260 W) (Soltec, Sonica Ultrasonic Cleaner 2400 ETH, Milan, Italy) in 200 μL of recovery medium made of tryptic soy broth (Oxoid, Milan, Italy) supplemented with 0.1% Tween 20 (Sigma–Aldrich). For determination of minimum biofilm eradication concentrations (MBECs) and biofilm chequerboard assays,[32] visible bacterial growth was evaluated after subsequent incubation of the recovery medium for 24 h (static conditions, 35°C). In chequerboard assays, the synergistic activity of fosfomycin/colistin combinations was evaluated by calculation of the fractional biofilm eradication concentration index (FBECI), where an FBECI value of $\leq$0.5 indicates a synergistic effect, as previously described.[32]

Data were obtained in at least two independent experiments, with six replicates per condition for each drug combination in each experiment. Median values were used for data analysis. With selected fosfomycin and colistin concentrations (alone and in combination), quantitative antibiofilm activity was evaluated in terms of cfu/peg, by counting viable cells in the recovery medium after biofilm disruption, as previously described.[30,33] Preliminary experiments were performed in order to determine the optimal range of antibiotic concentrations to be tested for each strain. Data were obtained in at least two independent experiments, with six replicates per condition for each drug combination in each experiment. Median values were used for data analysis.

Determination of mutant prevention concentrations (MPCs)

MPCs were determined as described by Wei *et al.*,[34] with minor modifications. Briefly, overnight cultures in CAMHBG6P at 35°C were diluted 1:10 into pre-warmed CAMHBG6P, incubated at 35°C until late-exponential phase (OD_{600} ~1.5–2) to achieve a suspension of ~10^9 cfu/mL. This suspension (0.1 mL) was then spread onto Mueller–Hinton agar (MHA) plates supplemented with 25 mg/L glucose-6-phosphate (Sigma–Aldrich) (MHAG6P) containing fosfomycin or colistin at concentrations of 1×, 2×, 4×, 8×, 16×, 32×, 64× and 128× the respective MICs for each strain. MPC was recorded as the lowest antibiotic concentration at which no colonies grew on the agar plate after 48 h of incubation at 35°C. MPCs were also determined for fosfomycin/colistin combinations. For this purpose, scalar concentrations of fosfomycin were tested in combination with a fixed colistin concentration, corresponding to the highest colistin concentration at which confluent/subconfluent growth was observed. The same procedure was adopted for testing scalar colistin concentrations in

Table 1. Main features of the 130 Gram-negative clinical strains investigated in this work, and results of the chequerboard assays for fosfomycin (FOF)/colistin (CST) combinations with planktonic cultures

Species (no. of strains)	Relevant acquired resistant phenotypes/genotypes (*n*)	ST (*n*)	MIC range (mg/L)		Number of strains (%)	
			FOF	CST	Synergy (FICI ≤0.5)	No interaction (FICI >0.5–4)
A. baumannii complex (5)	Carba-R, MDR (5) Carbapenemase+ (5; OXA-23 and/or OXA-58, OXA-72) Colistin-R (2; mechanism unknown)	ST2 (3), ST78 (2)	64 to >1024	0.5–8	None	5 (100)
E. coli (19)	Carba-R, MDR (7) ESC-R, Carba-S, MDR (11) Carbapenemase+ (7; NDM, KPC, OXA-48-like, VIM) ESBL+ (10; CTX-M and/or TEM) CMY-2 (4) Colistin-R (3; MCR-1-like)	ST39 (1), ST43 (2), ST73 (2), ST117 (1), ST131 (1), ST167 (1), ST404 (1), ST405 (2), ST479 (2), ST648 (2), ST681 (1), ST2076 (1) ND (2)	0.25 to >128	0.25–8	None	19 (100)
K. pneumoniae (48)	Carba-R, MDR (48) Carbapenemase+ (46; NDM, KPC, OXA-48-like, VIM) ESBL+ (4; CTX-M), Colistin-R (1; MCR-1-like)	ST11 (1), ST35 (1), ST101 (6), ST147 (1), ST258 (5), ST307 (6), ST395 (1), ST512 (17), ST2217 (1), ST2502 (1), ND (8)	≤0.25 to >128	0.25–128	8 (16.7)	40 (83.3)
P. aeruginosa (51)	Carba-R, MDR (48) Carbapenemase+ (40; VIM, IMP, GES-5) ESBL+ (4; PER-1)	ST17 (2), ST111 (6), ST175 (8), ST179 (1), ST235 (11), ST260 (2), ST308 (4), ST532 (2), ST621 (12), ST646 (1), ST654 (1), ND (1)	4 to >1024	0.5–512	5 (9.8)	46 (90.2)
S. maltophilia (7)	Trimethoprim/sulfamethoxazole-R (1)	ST34 (1), ST87 (1), ST300 (1), ST335 (1), ND (3)	32–128	2–128	None	7 (100)

ND, not determined; Carba-R, carbapenem resistant; Carba-S, carbapenem susceptible; ESC-R, expanded-spectrum cephalosporin resistant.

combination with a fixed fosfomycin concentration. MPCs were determined in at least two independent experiments, with a third experiment performed in case of discordant results (more than 2-fold dilution). MPC data were used to calculate the mutant selection window (MSW), which represents the antibiotic concentration range between the MIC and the MPC,[34] for fosfomycin and colistin alone and in combination.

Statistical analysis

For comparison of the antibiofilm activity of fosfomycin/colistin combinations versus single drugs in quantitative anti-biofilm assays, statistical analysis was performed using the unpaired *t*-test with Welch's correction (GraphPad Prism version 7.0, San Diego, CA, USA).

Results

Activity of fosfomycin/colistin combinations against Gram-negative pathogens in planktonic cultures

Activity of fosfomycin/colistin combinations was tested against a collection of 130 Gram-negative strains of clinical origin, representative of different species (including *A. baumannii*, *P. aeruginosa*, *S. maltophilia*, *E. coli* and *K. pneumoniae*) and different clonal lineages, by chequerboard assays with planktonic cultures. The collection included several strains exhibiting MDR phenotypes and producing various carbapenemases, and also fosfomycin- and/or colistin-resistant strains with different resistance mechanisms (Table 1 and Table S1).

Overall, synergism was observed with 13 of 130 strains (10%), including a minority of *P. aeruginosa* (5/51; 9.8%) and *K. pneumoniae* (8/48; 16.7%). All other strains showed an FICI indicative of no interaction, while antagonism was never observed. Interestingly, a substantial proportion of the strains for which no interaction was observed showed $0.5 < \text{FICI} \leq 1$ values (3/5 *A. baumannii*, 60%; 38/51 *P. aeruginosa*, 74.5%; 7/7 *S. maltophilia*, 100%; 8/19 *E. coli*, 42.1% and 26/48 *K. pneumoniae*, 54.2%) (Table 1 and Table S1). No clear relationship was apparent between synergism and clonal lineage or specific resistance phenotypes/mechanisms (Table S1).

Activity of fosfomycin/colistin combinations against biofilms

Activity of fosfomycin, colistin and combinations thereof was then tested against a subset of 20 selected strains, representative of different species, different fosfomycin and colistin susceptibility profiles, and different response to fosfomycin/colistin combinations in chequerboard assays performed with planktonic cultures (Table 2).

Table 2. Main features of the 20 strains included in the biofilm susceptibility testing and results of biofilm chequerboard assays

						Resistance determinants		FOF		CST			
Isolate	Species	Origin	MLST type[a]	Resistance profile	Carbapenemase/ ESBL	FOF	CST	MIC (mg/L)	MBEC (mg/L)	MIC (mg/L)	MBEC (mg/L)	FICI	FBECI
FZ1	*A. baumannii*	CVC-BSI	ST2	MEM, IPM, CIP	OXA-72	—	S	512	>1024	0.5	64	1	**0.4**
FZ2	*A. baumannii*	BSI	ST78	MEM, IPM, CIP	OXA-23; OXA-58	—	S	>1024	>1024	1	>1024	0.8	**0.5**
FZ83	*A. baumannii*	LRTI	ST2	MEM, IPM, CIP, CST	OXA-23	—	PmrA (F105L); PmrB (E185K); PmrC (F166L, R348K, A370S, K531T)	64	>1024	8	128	0.8	**0.3**
FZ18	*P. aeruginosa*	LRTI	ST235	C/T, CAZ, FEP, MEM, IPM, AMK, CIP	—	S	S	64	>1024	4	>1024	**0.4**	**0.5**
FZ34	*P. aeruginosa*	BSI	ST175	TZP, CAZ, FEP, CIP, CST	PER-1	GlpT (F81Y)*	S	>1024	>1024	4	>1024	**0.3**	**0.5**
FZ45	*P. aeruginosa*	BSI	ST111	C/T, TZP, CAZ, FEP, MEM, IPM, CIP	VIM-1	GlpT (premature stop codon at nt 513)	S	>1024	>1024	4	>1024	**0.4**	1
FZ98	*P. aeruginosa*	CF	ST646	CIP, CST	—	S	ParS (D380N); CprS (R295H)*	64	>1024	512	>1024	**0.3**	**0.1**
FZ139	*P. aeruginosa*	LRTI	ST111	C/T, MEM, IPM, AMK, CIP	VIM-2	S	S	8	>1024	4	>1024	**0.3**	0.8
FZ6	*S. maltophilia*	BSI	ST300	—	—	—	—	64	>1024	4	>1024	0.8	**0.1**
FZ8	*S. maltophilia*	CF	ST34	—	—	—	—	128	>1024	4	>1024	0.6	**0.1**
FZ85	*S. maltophilia*	LRTI	ST87	—	—	—	—	32	>1024	16	>1024	0.8	0.8
FZ11	*E. coli*	UTI	ST73	—	—	GlpT (premature stop codon at nt 765)	S	64	>1024	0.5	32	0.9	**0.4**
FZ123	*E. coli*	UTI	ST405	TZP, CAZ, FEP, MEM, IPM, AMK, CIP	NDM-5; CTX-M-15	S	S	0.25	256	≤0.5	8	0.8	**0.4**
FZ128	*E. coli*	RS	ST167	TZP, CAZ, FEP, MEM, IPM, AMK, CIP	NDM-5; CTX-M-15	S	S	1	256	1	16	0.9	**0.04**
FZ80	*K. pneumoniae*	UTI	ST512	TZP, CAZ, FEP, MEM, IPM, CIP	KPC-3	GlpT (premature stop codon at nt 1176)	S	128	>1024	0.5	64	0.6	0.6
FZ103	*K. pneumoniae*	LRTI	ST512	TZP, CAZ, FEP, MEM, IPM, CIP, CST	KPC-3	S	unk	32	>1024	8	64	**0.4**	**0.1**
FZ105	*K. pneumoniae*	BSI	ST258	TZP, CAZ, FEP, MEM, IPM, CIP, CST	KPC-3	S	MgrB (interrupted at nt 129 by IS*Kpn25*)	16	>1024	128	256	**0.3**	**0.1**
FZ106	*K. pneumoniae*	BSI	ST258	TZP, CAZ, FEP, MEM, IPM, CIP, CST	KPC-3	unk	MgrB (interrupted at nt 129 by IS*Kpn25*)	64	>1024	32	256	**0.4**	**0.2**
FZ108	*K. pneumoniae*	UTI	ST258	TZP, CAZ, FEP, MEM, IPM, CIP, CST	KPC-3	UhpB (T140A); PtsI (N174K)	MgrB (interrupted at nt 129 by IS*Kpn25*)	128	>1024	32	>1024	**0.3**	**0.1**
FZ141	*K. pneumoniae*	UTI	ST2502	TZP, CAZ, FEP, MEM, IPM, AMK, CIP	KPC-3	S	S	≤0.25	4	2	8	**0.4**	**0.4**

BSI, bloodstream infection; CVC-BSI, central venous catheter-related BSI; LRTI, low respiratory tract infection; UTI, urinary tract infection; RS, rectal swab; AMK, amikacin; C/T, ceftolozane/tazobactam (tazobactam at fixed concentration of 4 mg/L); CAZ, ceftazidime; CIP, ciprofloxacin; CST, colistin; FEP, cefepime; IPM, imipenem; MEM, meropenem; TZP, piperacillin/tazobactam (tazobactam at fixed concentration of 4 mg/L); S, susceptible; unk, unknown. Asterisks indicate putative mutations. Previously described alterations are underlined. FICI and FBECI values were interpreted as follows: FICI/FBECI ≤0.5, synergy; FICI/FBECI >0.5–4.0, no interaction; FICI/FBECI >4.0, antagonism. Values in bold type indicate synergy.

[a]According to Pasteur and Achtman MLST scheme (https://pubmlst.org/mlst).

As expected, MBECs of fosfomycin and colistin were consistently higher than the respective MICs with all tested strains: for fosfomycin, MIC_{median} = 64 mg/L (range ≤0.25 to >1024 mg/L) versus $MBEC_{median}$ = 1024 mg/L (range 4 to >1024 mg/L); for colistin, MIC_{median} = 4 mg/L (range ≤0.5 to 512 mg/L) versus $MBEC_{median}$ = 512 mg/L (range 8 to >1024 mg/L) (Table 2).

Biofilm chequerboard assays showed synergism of fosfomycin/colistin combinations with the majority of tested strains (16/20; 80%) with no clear association with results of chequerboard assays performed with planktonic cultures, while antagonism was never observed (Table 2).

Quantitative antibiofilm assays, using the high drug concentrations achievable in ELF after inhalation of aerosolized drugs, demonstrated significant antibiofilm synergism of fosfomycin/colistin combinations against all tested strains with the exception of two *P. aeruginosa* (*P. aeruginosa* FZ34 and FZ45), for which a trend suggesting synergism was observed but statistical significance was not achieved (Table 2, Figure 1).

MPCs and MSWs

MPCs of fosfomycin, colistin and fosfomycin/colistin combinations were determined for 10 selected strains, representative of different species, different fosfomycin and colistin MICs, and different response to fosfomycin/colistin combinations shown in quantitative antibiofilm assays (Table 3).

Overall, fosfomycin presented higher MPC values (range 512 to >1024 mg/L; median >1024 mg/L) than colistin (range 16 to 1024 mg/L; median 128 mg/L). When tested in combination, with one drug at fixed subMPC concentration, the MPCs of fosfomycin and colistin were consistently lowered for all tested strains, regardless of their fosfomycin and colistin MICs (Table 3), although narrowing of the MSWs was variable with different strains (Figure 2).

Discussion

Polymyxins and fosfomycin are old antibiotics that recently regained interest for treating infections caused by MDR Gram-negative pathogens,[35] with the advantage of possible administration also as inhaled formulations.[1,7]

Data pointing towards synergistic activity of fosfomycin/colistin combinations have previously been reported, but mostly against planktonic cultures,[11,12,36] while experience with biofilms remains very limited.[10,12]

In this study, we investigated the *in vitro* activity of fosfomycin/colistin combinations against planktonic and biofilm cultures of clinically relevant Gram-negative pathogens of several different species, including strains from CF patients and/or those expressing MDR phenotypes. High drug concentrations, potentially achievable in ELF after inhalation, were used to treat preformed biofilms, and also to investigate the ability of the combined drugs to prevent or limit the emergence of resistant subpopulations.

Activity of fosfomycin/colistin combinations against planktonic cultures

Our data showed that in chequerboard experiments with planktonic cells, carried out with a large and diverse collection of Gram-negative strains of clinical origin, fosfomycin/colistin combinations exerted synergistic activity only against a minority of strains of *K. pneumoniae* and *P. aeruginosa*, and never against strains of the other tested species (*E. coli*, *A. baumannii* and *S. maltophilia*).

Previous studies reported higher rates of synergism with *K. pneumoniae* and *P. aeruginosa* planktonic cells.[11,36,37] These differences might be related to differences in the strain collections and/or experimental conditions (in some cases synergism was evaluated with different methods or interpreted with different criteria). However, the relatively high number of strains showing 0.5 < FICI ≤ 1 values in chequerboard assays (84/130; 64.6%), values that have been considered in some studies as partially synergistic (even if this definition is controversial),[29,36,38] might be of interest.

Activity of fosfomycin/colistin combinations against biofilms

Our results consistently showed synergistic activity of fosfomycin/colistin combinations against biofilms of Gram-negative strains of different species. Although the number of tested strains per species was relatively low in these experiments, the synergistic antibiofilm activity of fosfomycin/colistin combinations did not appear to be species-related or dependent on fosfomycin and colistin MICs, resistance mechanisms or clonal lineage. Moreover, the results of biofilm chequerboard assays were in accordance overall with results obtained from quantitative antibiofilm assays, carried out with drug concentrations achievable via inhalation of aerosolized drugs. Interestingly, synergism against biofilms was observed regardless of results obtained with planktonic cells, emphasizing the complexity of biofilm response to antibiotic exposure and underscoring the poor predictivity of planktonic models for biofilm infections.

Our results, therefore, were consistent with those previously reported by Corvec *et al.*[10] against *E. coli* biofilms, and with those reported by Memar *et al.*[12] against *P. aeruginosa*, and expand current knowledge in this area. To the best of our knowledge, our results are original in showing synergistic activity of fosfomycin/colistin combinations against biofilms of *A. baumannii*, *S. maltophilia* and *K. pneumoniae*.

MPCs and MSWs of fosfomycin, colistin and fosfomycin/colistin combinations

The combination of fosfomycin and colistin, at concentrations achievable by inhalations, was also found to notably reduce the MPCs of both fosfomycin and colistin with Gram-negative strains of different species, narrowing the MSWs and limiting the probability of the bacteria further mutating and developing drug resistance. Interestingly, lowering of the MPC values closer to MIC values by combination of fosfomycin and colistin was also observed with strains of species causing difficult-to-treat infections, such as *S. maltophilia* and *P. aeruginosa*. Altogether, these results reinforce the notion that these antibiotics should not be used in monotherapy, and that their combinations could represent a valid alternative to prevent the emergence of resistant subpopulations.

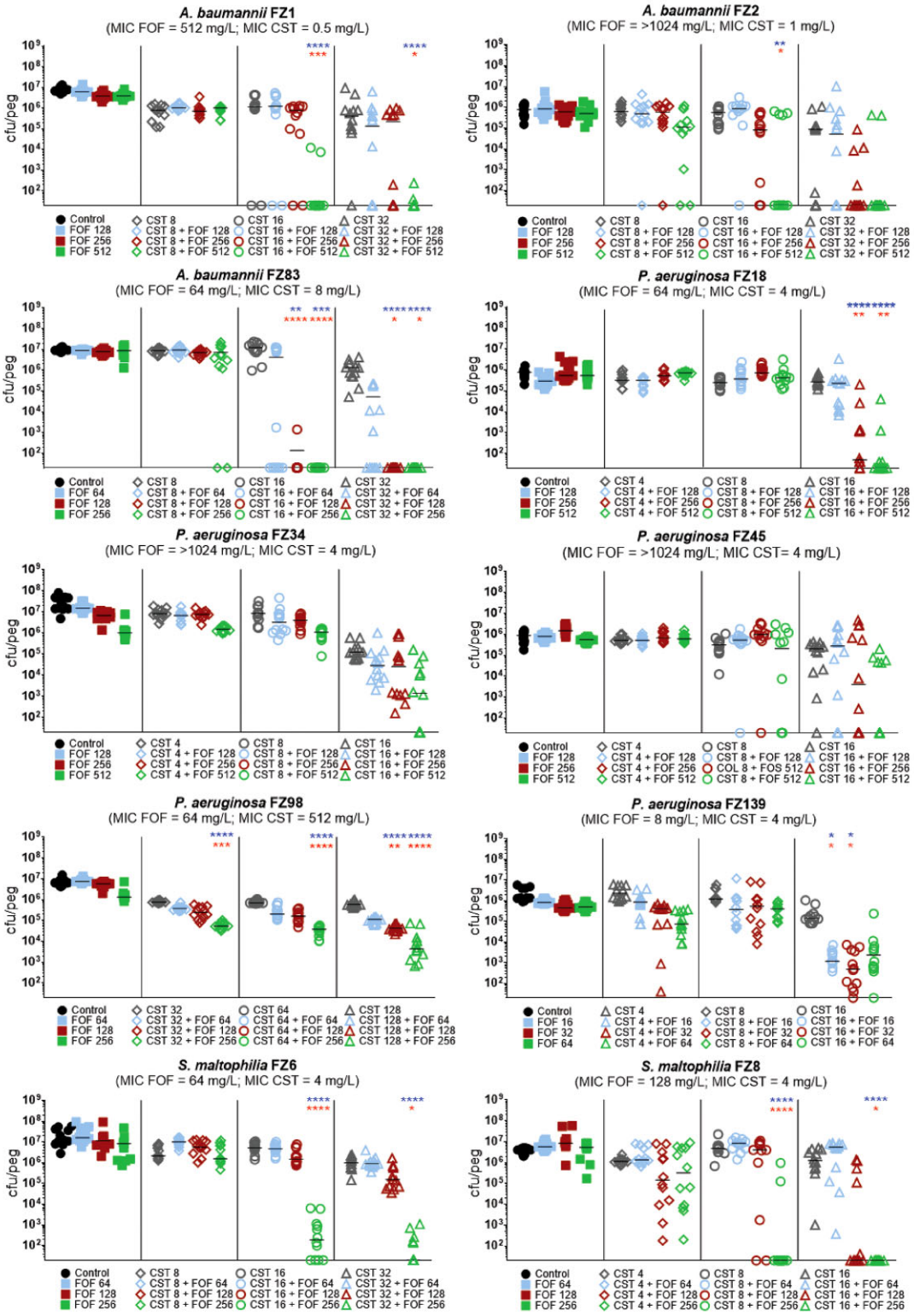

Figure 1. Antibiofilm activity of fosfomycin (FOF)/colistin (CST) combinations. Data from at least two independent experiments, with six replicates per condition per experiment. Median values are plotted. The *x*-axes are set at the limit of detection (i.e. 20 cfu/peg). In graphic legends, FOF and CST concentrations are expressed in mg/L. Significant differences compared with drug controls are indicated with asterisks (blue for fosfomycin, red for colistin).* $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.00001$. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

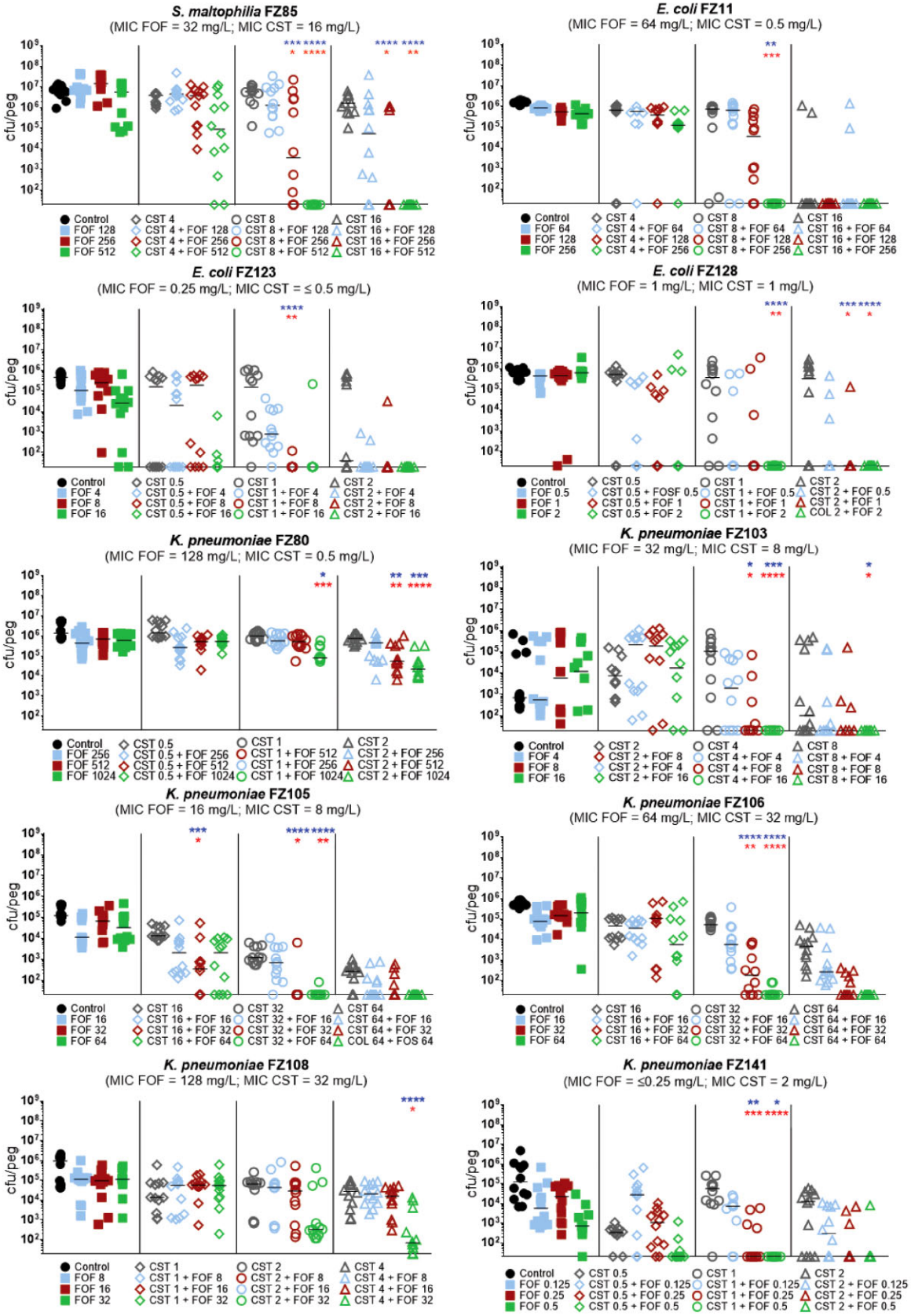

Figure 1. Continued

Table 3. MPCs for fosfomycin (FOF) and colistin (CST) alone and in combination

			MIC (mg/L)				MPC (mg/L)			
Isolate	Species	ST	FOF	CST	FICI	FBECI	FOF	CST	FOF with [CST]	CST with [FOF]
FZ83	*A. baumannii*	2	64	8	0.8	**0.3**	>1024	128	512 [8]	32 [64]
FZ34	*P. aeruginosa*	175	>1024	4	**0.3**	**0.5**	>1024	16	1024 [4]	8 [512]
FZ45	*P. aeruginosa*	111	>1024	4	**0.4**	1	>1024	64	1024 [4]	16 [256]
FZ139	*P. aeruginosa*	111	8	4	**0.3**	0.8	>1024	32	64 [4]	8 [32]
FZ8	*S. maltophilia*	34	128	4	0.6	**0.1**	512	256	64 [64]	32 [128]
FZ85	*S. maltophilia*	87	32	16	0.8	0.8	1024	512	64 [128]	32 [128]
FZ11	*E. coli*	73	64	0.5	0.9	**0.4**	>1024	16	512 [4]	8 [128]
FZ103	*K. pneumoniae*	258	32	8	**0.4**	**0.1**	1024	512	128 [64]	64 [256]
FZ105	*K. pneumoniae*	258	16	128	**0.3**	**0.1**	>1024	1024	32 [128]	32 [256]
FZ106	*K. pneumoniae*	258	64	32	**0.4**	**0.2**	>1024	1024	64 [128]	64 [256]

FICI and FBECI values were interpreted as follows: FICI/FBECI ≤0.5, synergy; FICI/FBECI >0.5–4.0, no interaction; FICI/FBECI >4.0, antagonism. Values in bold type indicate synergy. Square brackets indicate antibiotics used at fixed concentrations (selected based on the highest drug concentration that achieved a confluent/subconfluent growth on MHAG6P plates).

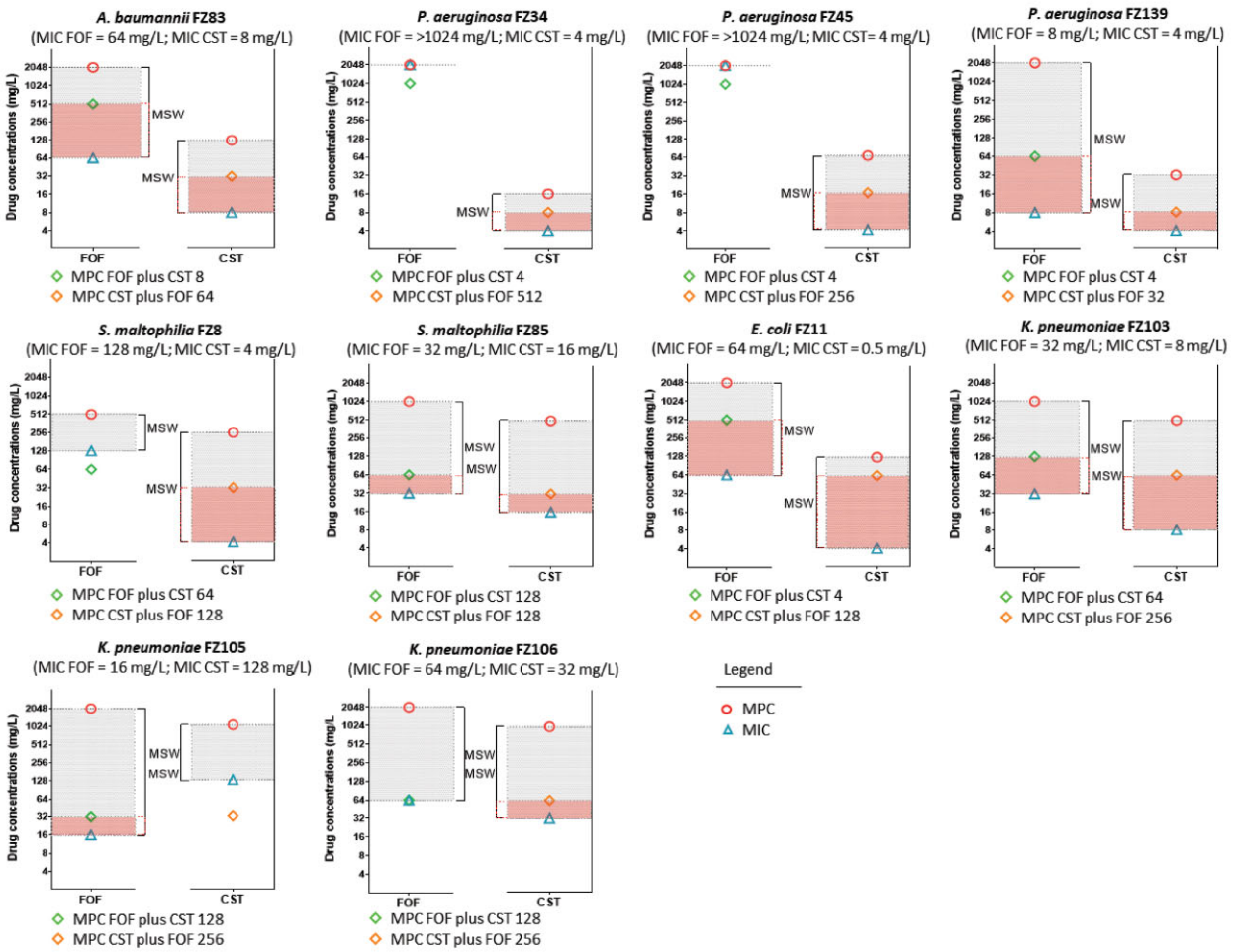

Figure 2. MPCs and MSWs of fosfomycin (FOF)/colistin (CST) combinations of 10 selected strains. Red shading indicates the MSW of fosfomycin/colistin combinations. In graphic legends, FOF and CST concentrations are expressed in mg/L. MPC values >1024 mg/L were reported as 2048 mg/L. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Conclusions

In conclusion, we found that fosfomycin plus colistin, at concentrations achievable in the ELF after inhalation, showed remarkable *in vitro* antibiofilm synergism against clinically relevant MDR Gram-negative bacteria, and were able to reduce the emergence of fosfomycin- and colistin-resistant subpopulations.

The relatively small number of strains tested on antibiofilm assays may be a limitation in this study. However, the synergism of fosfomycin/colistin observed against strains of diverse clonal lineages and resistance phenotypes and genotypes would suggest that this phenomenon is general rather than strain-specific. In order to consolidate these findings, further *in vitro* studies on a higher number of strains and *in vivo* animal models are warranted.

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Transparency declarations

Gloria Padoani and Silvia Vailati are employed by Zambon S.p.A. All other authors: none to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online.

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RESEARCH ARTICLE

Activity of *N*-Acetylcysteine Alone and in Combination with Colistin against *Pseudomonas aeruginosa* Biofilms and Transcriptomic Response to *N*-Acetylcysteine Exposure

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ABSTRACT Chronic colonization by *Pseudomonas aeruginosa* is critical in cystic fibrosis (CF) and other chronic lung diseases, contributing to disease progression. Biofilm growth and a propensity to evolve multidrug resistance phenotypes drastically limit the available therapeutic options. In this perspective, there has been growing interest in evaluating combination therapies, especially for drugs that can be administered by nebulization, which allows high drug concentrations to be reached at the site of infections while limiting systemic toxicity. Here, we investigated the potential antibiofilm activity of *N*-acetylcysteine (NAC) alone and in combination with colistin against a panel of *P. aeruginosa* strains (most of which are from CF patients) and the transcriptomic response of a *P. aeruginosa* CF strain to NAC exposure. NAC alone (8,000 mg/L) showed a limited and strain-dependent antibiofilm activity. Nonetheless, a relevant antibiofilm synergism of NAC-colistin combinations (NAC at 8,000 mg/L plus colistin at 2 to 32 mg/L) was observed with all strains. Synergism was also confirmed with the artificial sputum medium model. RNA sequencing of NAC-exposed planktonic cultures revealed that NAC (8,000 mg/L) mainly induced (i) a Zn^{2+} starvation response (known to induce attenuation of *P. aeruginosa* virulence), (ii) downregulation of genes of the denitrification apparatus, and (iii) downregulation of flagellar biosynthesis pathway. NAC-mediated inhibition of *P. aeruginosa* denitrification pathway and flagellum-mediated motility were confirmed experimentally. These findings suggested that NAC-colistin combinations might contribute to the management of biofilm-associated *P. aeruginosa* lung infections. NAC might also have a role in reducing *P. aeruginosa* virulence, which could be relevant in the very early stages of lung colonization.

IMPORTANCE *Pseudomonas aeruginosa* biofilm-related chronic lung colonization contributes to cystic fibrosis (CF) disease progression. Colistin is often a last-resort antibiotic for the treatment of such *P. aeruginosa* infections, and it has been increasingly used in CF, especially by nebulization. *N*-acetylcysteine (NAC) is a mucolytic agent with antioxidant activity, commonly administered with antibiotics for the treatment of lower respiratory tract infections. Here, we show that NAC potentiated colistin activity against *in vitro* biofilms models of *P. aeruginosa* strains, with both drugs tested at the high concentrations achievable after nebulization. In addition, we report the first transcriptomic data on the *P. aeruginosa* response to NAC exposure.

KEYWORDS *N*-acetylcysteine, *Pseudomonas aeruginosa*, biofilms, colistin, cystic fibrosis, synergism, transcriptomic response

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Pseudomonas aeruginosa is a leading pathogen infecting the airways of patients affected by cystic fibrosis (CF) and other chronic lung diseases (e.g., chronic obstructive pulmonary disease and non-CF bronchiectasis) (1). Once established in the CF airways, *P. aeruginosa* develops into chronic infections and generally persists indefinitely, contributing to frequent exacerbations, decline of pulmonary function, and higher rates of mortality (1, 2). Chronic infections by *P. aeruginosa* in CF lungs are associated with adaptive changes of the pathogen, such as conversion to a mucoid phenotype, switching to the biofilm mode of growth, and acquisition of antibiotic resistance (3). Cumulative exposure to antibiotics during treatment causes dissemination of multidrug-resistant (MDR) *P. aeruginosa* strains, leading to the ineffectiveness of the antibiotic therapy and consequently worse clinical outcomes (3).

Colistin is among the last-resort agents for the treatment of *P. aeruginosa* infections caused by MDR strains, with the advantage of being also administrable by nebulization, which allows the achieving of high lung concentrations while reducing systemic toxicity (4). In this perspective, inhaled colistin has been increasingly used for the treatment of difficult-to-treat respiratory tract infections, especially those related to biofilm formation (5).

N-acetylcysteine (NAC) is a mucolytic agent commonly administered with antibiotics for the treatment of lower respiratory tract infections, which has been demonstrated to exert also antimicrobial and antibiofilm activity against relevant respiratory pathogens (6–8). Recently, a potent *in vitro* antibiofilm synergism of NAC-colistin combinations was demonstrated against colistin-susceptible and colistin-resistant *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* strains (9, 10).

NAC has been demonstrated to exert several heterogeneous biological activities (whose molecular bases have not always been clearly elucidated) and has recently been under extensive investigation for potential clinical applications beyond the approved therapeutic usage as an antidote in acetaminophen (paracetamol) overdose and as a mucolytic (11). Overall, NAC can act as a direct or indirect antioxidant, due to the ability of the free thiol group to react with reactive oxygen and nitrogen species and by constituting a precursor of intracellular glutathione (11). In addition, NAC can bind transition and heavy metal ions and act as a reducing agent of protein sulfhydryl groups involved in intracellular redox homeostasis (11). Despite several studies that have addressed the biological effects of NAC on planktonic and biofilm bacterial cultures (8), to the best of our knowledge, no data on bacterial transcriptomic response to NAC exposure have been reported so far.

In this study, we investigated the *in vitro* antibiofilm activities of NAC alone and in combination with colistin (at the high concentrations achievable by the inhalation route of administration) (8, 12) against a panel of *P. aeruginosa* strains (most of which are from CF patients) representative of different phenotypes (in terms of mucoidy, antimicrobial susceptibility pattern, and O type) and multilocus sequence type (MLST) genotypes. In addition, we provided original data on the transcriptomic response of *P. aeruginosa* planktonic cultures to NAC exposure.

RESULTS AND DISCUSSION

Activity of NAC alone against preformed biofilm. The antibiofilm activity of NAC alone was tested with 17 *P. aeruginosa* strains (Table 1), of which 15 were from CF patients, using the Nunc-TSP lid system.

NAC at 8,000 mg/L (i.e., a high concentration achievable after inhalation) showed limited and strain-dependent activity (Fig. 1 to 4). In particular, major effects were observed with *P. aeruginosa* Z154 (i.e., decrease of $>$1 log CFU/peg compared to the control) (Fig. 1) and *P. aeruginosa* PAO1 (i.e., increase of $>$1 log CFU/peg compared to the control) (Fig. 2). With an additional 7 strains, a very slight but statistically significant activity was observed (i.e., $<$0.5 log CFU/peg compared to the control), resulting in biofilm reduction in six cases (i.e., *P. aeruginosa* Z33, Z35, Z152, M13, M19, and M25) and biofilm increase in the remaining one (i.e., *P. aeruginosa* M42) (Fig. 2 and 3).

Overall, these results indicated that inhaled NAC alone might not have major effects on

TABLE 1 Features of the 17 *P. aeruginosa* strains included in this study

Strain	yr of isolation	Phenotype	Origin[a]	ST[b]	O type	Resistance pattern[c]	MIC (mg/L)[d]	
							CST	NAC
PAO1	1954	Nonmucoid	Wound	ST549	O5	Wild type	2	64,000
Z33	2005	Nonmucoid	CF	ST235	O11	CP^r, FQ^r, AG^r	1	16,000
Z34	2006	Nonmucoid	CF	ST17	O1	CB^r, CP^r, FQ^r, AG^r	2	64,000
Z35	2006	Nonmucoid	CF	ST235	O11		1	16,000
Z152	2013	Mucoid	CF	ST155	O6	CB^r, FQ^r, AG^r	2	8,000
Z154	2016	Mucoid	CF	ST412	O6	CP^r, FQ^r, AG^r	2	16,000
M1	2002	Mucoid	CF	ST155	O6	CB^r, CP^r, FQ^r, AG^r	2	16,000
M4	2005	Mucoid	CF	ST155	O6	CB^r, CP^r, FQ^r, AG^r	2	32,000
M7	2005	Mucoid	CF	ST253	O10	AG^r	2	64,000
M13	2000	Mucoid	CF	ST274	O3	CB^r, CP^r, AG^r	1	32,000
M19	2006	Mucoid	CF	ST3509	O7		1	64,000
M25	2002	Mucoid	CF	ST235	O11		2	16,000
M32	2006	Mucoid	CF	ST235	O11		2	16,000
M42	2007	Mucoid	CF	ST2437	O6	CB^r, CP^r, FQ^r, AG^r	2	32,000
FC237	2007	Nonmucoid	CF	ST365	O3	CB^r, FQ^r, AG^r, CST^r	512	64,000
FC238	2007	Nonmucoid	CF	ST910	O6	CB^r, CST^r	8	64,000
FZ99	2018	Nonmucoid	RTI_{ICU}	ST111	O12	CB^r, CP^r, FQ^r, AG^r, CST^r	4	64,000

[a]CF, cystic fibrosis; RTI_{ICU}, respiratory tract infection in intensive care unit.
[b]According to the MLST Pasteur scheme.
[c]CB^r, resistance to carbapenems (imipenem and meropenem); CP^r, resistance to cephems (ceftazidime and cefepime); FQ^r, resistance to fluoroquinolones (ciprofloxacin); AG^r, resistance to aminoglycosides (amikacin and gentamicin); CST^r, resistance to colistin.
[d]CST, colistin; NAC, *N*-acetylcysteine.

P. aeruginosa biofilms already established in the lung and that the response to NAC was not related to phenotypic or genotypic features. The few previous studies that have addressed the activity of NAC against preformed *P. aeruginosa* biofilms have reported similar results (i.e., usually limited and strain-dependent effects), although a direct comparison of data is not straightforward due to different methodological approaches (e.g., different biofilm models and different NAC concentrations tested) and the low number of strains often tested in such studies (i.e., usually reference strains) (8, 13, 14). This study provided a wider picture on this topic by investigating a panel of characterized *P. aeruginosa* strains using a standardized *in vitro* biofilm model and *in vivo* achievable NAC concentrations. Interestingly, NAC alone (at the concentration used in this study and the same biofilm

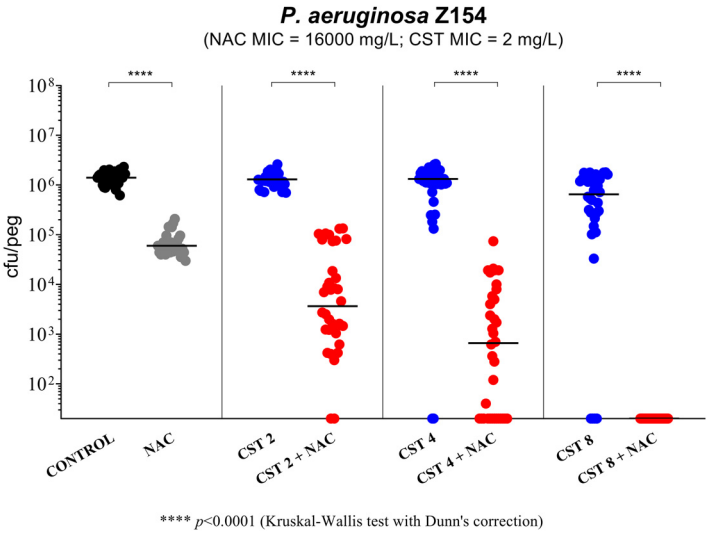
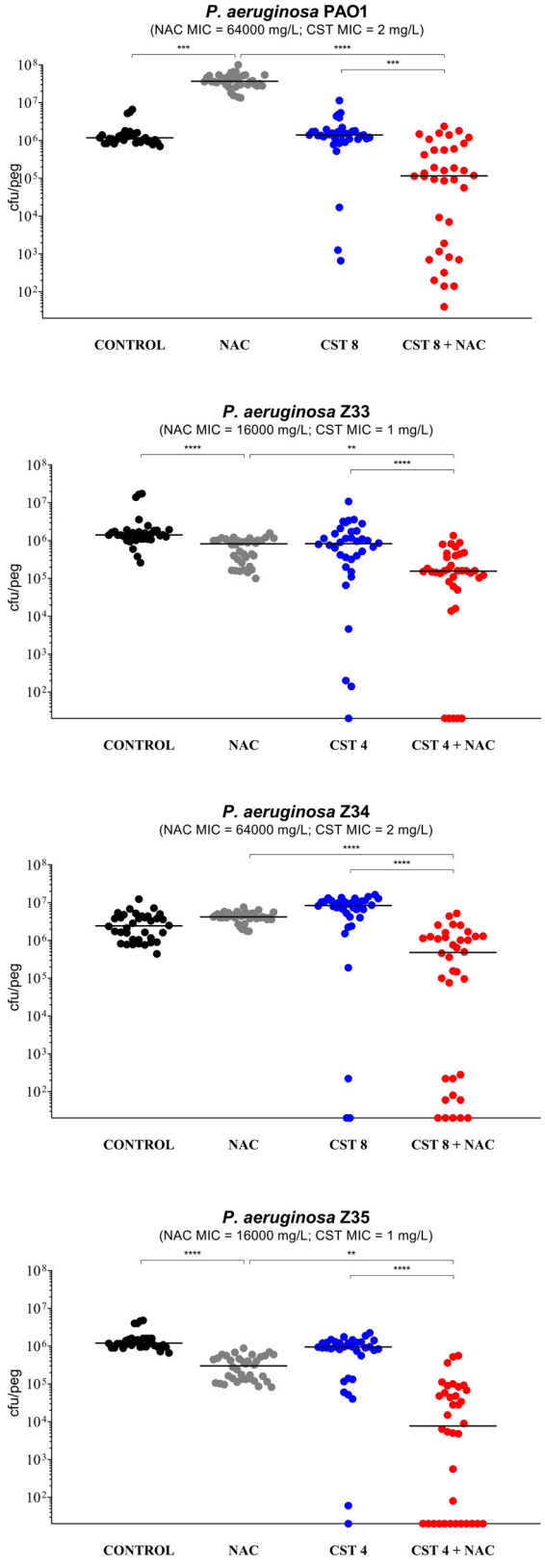


FIG 1 Antibiofilm activity of *N*-acetylcysteine (NAC) at 8,000 mg/L, colistin (CST), and NAC-CST combinations against *P. aeruginosa* Z154 in the Nunc-TSP lid system. A relevant potentiation of colistin antibiofilm activity was observed with all NAC-CST combinations tested. CST 2, colistin at 2 mg/L; CST 4, colistin at 4 mg/L; CST 8, colistin at 8 mg/L. Biofilms not exposed to NAC or CST represent the control. Black lines indicate median values. The *x* axis is set at the limit of detection (20 CFU/peg).



** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (Kruskal-Wallis test with Dunn's correction)

FIG 2 Antibiofilm activity of *N*-acetylcysteine (NAC) at 8,000 mg/L, colistin (CST), and NAC-CST combinations against *P. aeruginosa* PAO1 and three colistin-susceptible nonmucoid strains in the

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model) was recently shown to exert relevant activity against preformed biofilms of two relevant CF pathogens, namely, *S. maltophilia* and *Burkholderia cepacia* complex (BCC) (7). The reasons for such a diverse response of *P. aeruginosa* compared to *S. maltophilia* and BCC should deserve further attention, because they could possibly help identifying critical targets in the complex biofilm environments, to be used for the implementation of new antibiofilm strategies.

Activity of NAC-colistin combinations against preformed biofilms. *P. aeruginosa* Z154 (a mucoid, MDR, colistin-susceptible CF strain) was first used to test the potential antibiofilm synergism of NAC at 8,000 mg/L plus diverse colistin concentrations. As shown in Fig. 1, a relevant synergism was observed already with colistin at 2 mg/L (i.e., the colistin MIC for the tested strain), with a dose-dependent effect at increasing colistin concentrations, and complete biofilm eradication was achieved with the combination of NAC at 8,000 mg/L plus colistin at 8 mg/L (Fig. 1).

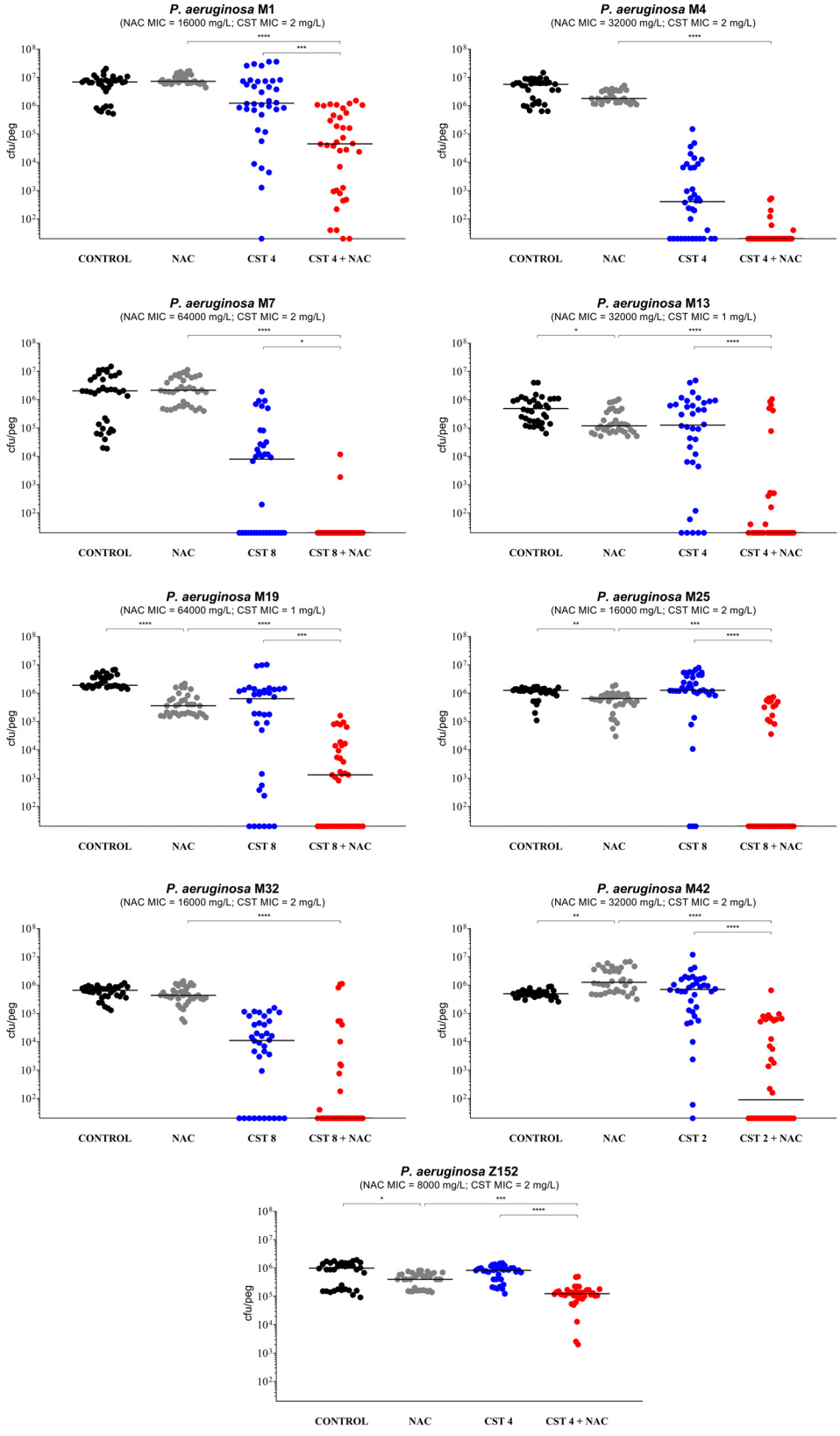
The remaining 16 strains were initially tested with the combination of NAC at 8,000 mg/L plus colistin at 8 mg/L. In order to detect a potential synergism, the concentration of colistin was then modified for strains forming biofilms highly susceptible to colistin ($n = 7$) or particularly resistant ($n = 2$) (Fig. 2 to 4). Overall, a relevant synergism of NAC-colistin combinations was observed with all tested strains (including the three colistin-resistant ones), although in two cases (i.e., *P. aeruginosa* M4 and M32), statistical significance was not achieved (Fig. 2 to 4). These latter strains were also tested with lower colistin concentrations (i.e., 2 and 4 mg/L, respectively), but synergism was not observed (data not shown). Concerning the synergism observed with the three colistin-resistant strains (Fig. 4), it is interesting to note that with strain FC237 (nonmucoid, MDR), an important decrease in viable biofilm cells was observed with a combination including a colistin concentration much lower than the colistin MIC for this strain (i.e., 1/64 MIC) (Fig. 4).

Overall, these data demonstrated that NAC could potentiate colistin activity against preformed biofilms of colistin-susceptible and colistin-resistant *P. aeruginosa* strains, regardless of the mucoid/nonmucoid phenotype, the resistance pattern, and the ST and O type. Present findings are consistent with the previously observed antibiofilm synergism of NAC-colistin combinations against colistin-susceptible and colistin-resistant strains of *A. baumannii* and *S. maltophilia* (9, 10). Further studies with a higher number of *P. aeruginosa* clinical isolates, especially with a colistin-resistant phenotype, are encouraged.

Activity of NAC-colistin combinations in the ASM biofilm model. Two *P. aeruginosa* CF strains exhibiting different phenotypes were selected for susceptibility assays with the artificial sputum medium (ASM) biofilm model: *P. aeruginosa* Z34 (nonmucoid, MDR, ST17, O1) and *P. aeruginosa* Z154 (mucoid, MDR, ST412, O6). Biofilms were grown in ASM, in order to mimic the *P. aeruginosa* biofilm environmental conditions experienced in the CF mucus. Preformed biofilms were then challenged in the same medium with NAC-colistin combinations.

As shown in Fig. 5, a clear synergism of NAC at 8,000 mg/L in combination with colistin at 64 mg/L was observed with both strains (Fig. 5). Compared to the experiments performed with the Nunc-TSP lid system, the concentration of colistin that allowed observation of a synergism was much higher (i.e., 32× the MIC), possibly due to colistin strong ionic interactions with ASM components (e.g., extracellular DNA and mucin) (15). Indeed, preliminary experiments carried out with lower colistin concentrations did not show either colistin antibiofilm activity or synergism with NAC (data not shown). In addition, the antibiofilm activity of NAC alone observed against *P. aeruginosa* Z154 in the Nunc-TSP lid system was not observed in the ASM model (Fig. 5), confirming that

FIG 2 Legend (Continued)
Nunc-TSP lid system. A potentiation by NAC of colistin antibiofilm activity was observed with all tested strains. CST 4, colistin 4 mg/L; CST 8, colistin 8 mg/L. Biofilms not exposed to NAC or CST represented the control. Black lines indicate median values. The *x* axis is set at the limit of detection (20 CFU/peg).



* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (Kruskal-Wallis test with Dunn's correction)

FIG 3 Antibiofilm activity of *N*-acetylcysteine (NAC) at 8,000 mg/L, colistin (CST), and NAC-CST combinations against nine colistin-susceptible mucoid *P. aeruginosa* strains in the Nunc-TSP lid system. A potentiation by

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the efficacy of NAC alone against preformed *P. aeruginosa* biofilms could be limited *in vivo*.

Overall, these data demonstrated that the antibiofilm synergism of NAC-colistin combinations against *P. aeruginosa* strains is preserved also under the environmental conditions mimicking the CF mucus, which is promising for clinical applications. Furthermore, the lower susceptibility to colistin of *P. aeruginosa* biofilms in the ASM model compared to biofilm susceptibility in standard media observed in this study is consistent with what was previously reported with *P. aeruginosa* (16).

Transcriptomic response of *P. aeruginosa* Z154 to NAC exposure. *P. aeruginosa* Z154 (i.e., colistin-susceptible CF strain, mucoid, MDR, ST412, O6) was selected for investigating the transcriptome response of planktonic cultures to NAC exposure (i.e., NAC at 8,000 mg/L). A total of 66 differentially expressed genes (DEGs) were identified (adjusted *P* value of <0.05 with 99% confidence interval [CI]), of which 46 were upregulated and 20 downregulated compared to the control (Table 2).

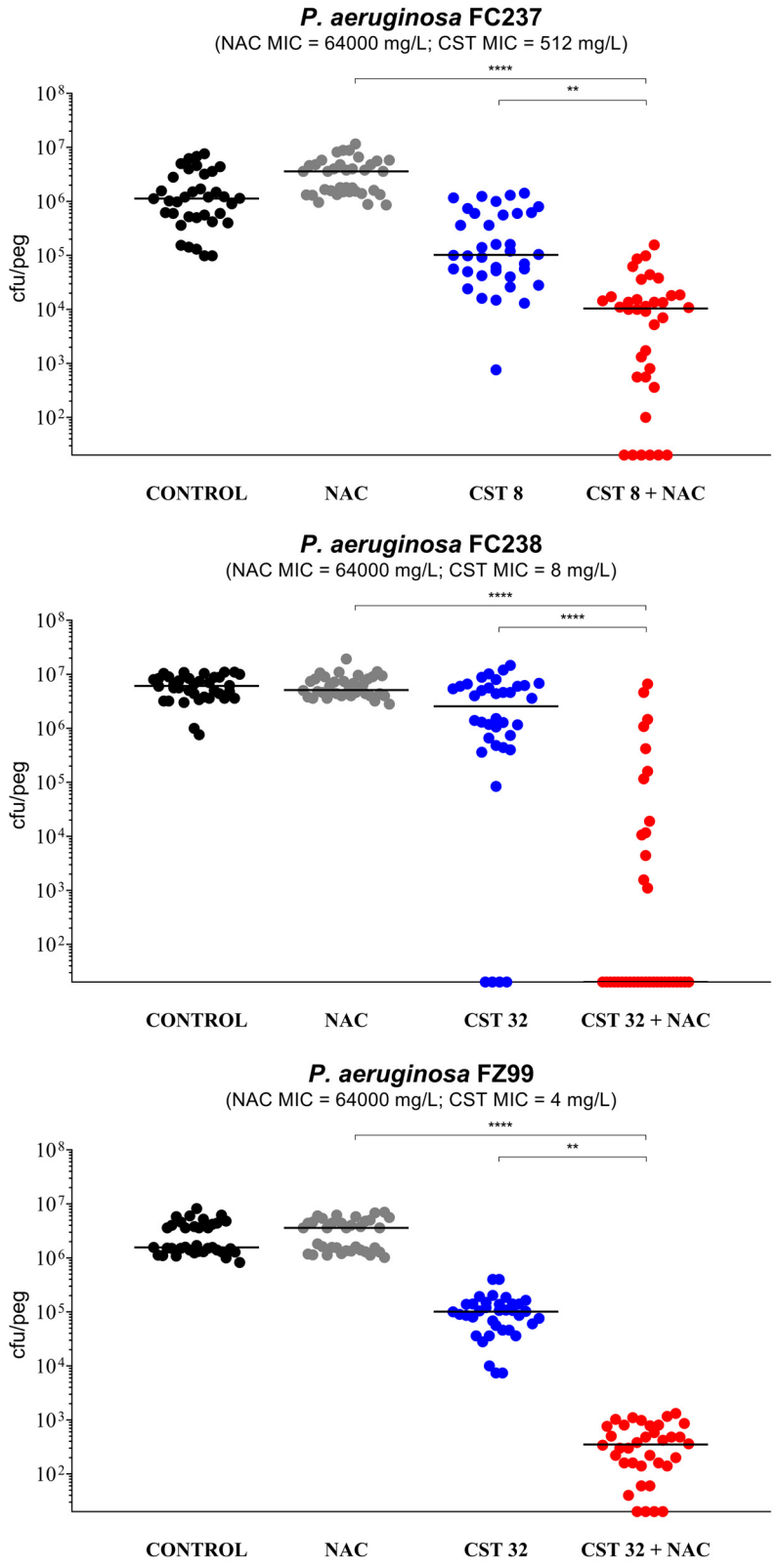
Analysis of DEGs revealed that NAC mainly acted as Zn^{2+} chelator, inducing a strong Zn^{2+} starvation response. DEGs associated with such response were consistent with data reported in previous studies addressing zinc homeostasis in *P. aeruginosa* and other bacteria (Table 2) (17–22). In particular, 31 of the 46 upregulated DEGs belonged to the *zur* regulon and are known to be activated in response to Zn^{2+} starvation (Table 2) (17–22). Such genes mainly included operons involved in zinc uptake (e.g., the PA4063-PA4064-PA4065-PA4066 operon, *cntOLMI* operon, and *znuABC* operon) and genes encoding zinc-independent paralogs of cellular proteins (i.e., type B 50S ribosomal proteins L31 and L36, RNA polymerase-binding protein DksA2, adn GTP-cyclohydrolase FolE2) (Table 2) (17–23). Upregulated DEGs belonging to the *zur* regulon also included genes encoding an *N*-acetylmuramoyl-L-alanine amidase (AmiA, involved in splitting of septal peptidoglycan during cell division), a γ-carbonic anhydrase (Cam, involved in reversible hydration of carbon dioxide and important for growth under low-CO_2 conditions), and three modulators of the membrane FtsH protease (i.e., HflC and HflK family modulators) (Table 2). The membrane FtsH zinc-dependent protease is required for the expression of diverse unrelated phenotypes (e.g., swimming and twitching motility, biofilm formation, autolysis, production of secondary metabolites, maintenance of plasma membrane integrity by degrading misfolded proteins), and it has been recently demonstrated to represent an important virulence factor in *P. aeruginosa* clone C (23). HflC and HflK family modulators interact with FtsH at the level of the plasma membrane, usually with an inhibitory effect (23). The NAC-mediated effects on the phenotypes related to FtsH would deserve further attention.

The remaining 15 upregulated DEGs included genes encoding a recently described transcriptional regulator, PA2100 (also named MdrR2) (24), an AhpC-like alkyl hydroperoxide reductase (involved in protection from oxidative stress) (25), and proteins possibly involved in copper and iron uptake (Table 2).

MdrR2, together with MdrR1, has been demonstrated to repress the *mexAB-oprM* operon (independently from the MexR repressor), activate the EmrAB efflux pump, and indirectly inhibit biofilm formation (Table 2) (24). The effect of NAC on the MdrR1-MdrR2 dual-regulation system should be further investigated. Nonetheless, a previous study aimed at investigating the potential antagonism of high NAC concentrations (i.e., as those tested in this study) on the activity of the major classes of antibiotics used in the clinical practice, did not show major effects (with the exception of carbapenems, due to a chemical instability of carbapenems in the presence of NAC) (26), suggesting that the activation of the EmrAB efflux could not be relevant or circumvented by compensatory mechanisms.

Analysis of downregulated DEGs identified genes involved in denitrification, in particular *norB* (encoding the nitric oxide reductase subunit NorB), *nosR* (encoding the

FIG 3 Legend (Continued)
NAC of colistin antibiofilm activity was observed with all tested strains, although in two cases, statistical significance was not achieved (i.e., strains M4 and M32). CST 2, colistin at 2 mg/L; CST 4, colistin at 4 mg/L; CST 8, colistin at 8 mg/L. Biofilms not exposed to NAC or CST represent the control. Black lines indicate median values. The *x* axis is set at the limit of detection (20 CFU/peg).



** $p<0.01$, **** $p<0.0001$ (Kruskal-Wallis test with Dunn's correction)

FIG 4 Antibiofilm activity of *N*-acetylcysteine (NAC) at 8,000 mg/L, colistin (CST), and NAC-CST combinations against three colistin-resistant nonmucoid *P. aeruginosa* strains in the Nunc-TSP lid

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regulatory protein NosR), and *nosZ* (encoding the nitrous oxide reductase NosZ) (Table 2). These data suggested that NAC might affect *P. aeruginosa* anaerobic respiration (which is crucial in the deeper biofilm layers and in the CF mucus) (27), because the nitric oxide reductase NorBC and the regulatory protein NosR have been recently demonstrated to constitute the nucleus of the denitrification protein network (28). NAC-mediated inhibition of the *P. aeruginosa* denitrification pathway might be implicated in the observed antibiofilm synergism of the NAC-colistin combination. Indeed, colistin has been demonstrated to exert increased antibiofilm activity against *P. aeruginosa* under anaerobic conditions, possibly due to a lower ability to implement the tolerance mechanism (e.g., lipopolysaccharide [LPS] modification) because of the low metabolism accompanying anaerobic growth (29). In this perspective, the inhibition of anaerobic respiration by NAC would further inhibit a *P. aeruginosa* adaptive response to colistin toxicity. This could be particularly relevant in *P. aeruginosa* biofilm in the CF mucus, where the anoxic conditions of biofilm cells are related not only to the position of the bacteria within the biofilm (i.e., anoxic conditions in the deeper layers), but also to the intense O_2 depletion caused by polymorphonuclear leukocytes (PMNs), determining entire biofilm growth without aerobic respiration (29).

Downregulated DEGs also included the following: (i) two genes involved in flagellar biosynthesis (i.e., *fliF*, encoding the flagellar M-ring protein FliF, and *flhF*, encoding the flagellar biosynthesis protein FlhF); (ii) a NAD(P)H-quinone oxidoreductase protecting against ROS-induced oxidative stress, which was recently demonstrated to be part of the core biofilm transcriptome (PA1137) (30); and (iii) *nalD*, encoding a second repressor of the *mexAB-oprM* operon (31). Finally, consistent with previous studies on *Pseudomonas* response to zinc starvation, downregulation of *copA* and *copZ*, involved in copper efflux, was observed, suggesting interplay between zinc and copper homeostasis (Table 2) (32).

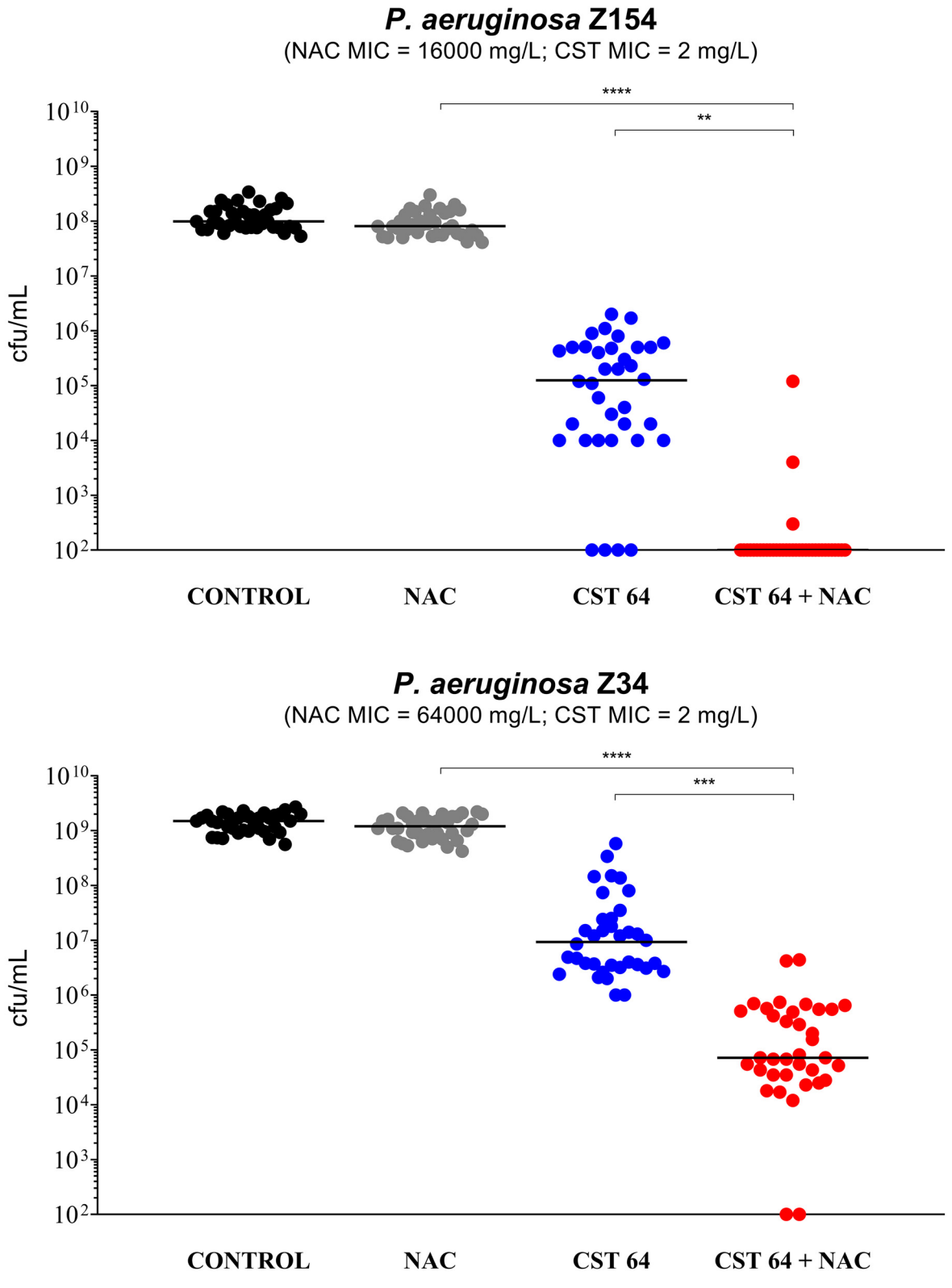
NAC-mediated inhibition of *P. aeruginosa* denitrification pathway. The role of NAC in the inhibition of the denitrification pathway was confirmed by measuring NO_3^- and NO_2^- concentrations during anaerobic growth of the *P. aeruginosa* Z154 strain (i.e., the strain used for transcriptomic analysis) in culture media supplemented with 10 mM $NaNO_3$ or KNO_2, in the presence or absence of NAC at 8,000 mg/L.

As expected from previous studies (33), in $NaNO_3$-containing medium, the levels of NO_3^- and its reduction product, NO_2^-, fell below the detection limit after 24 h, in the absence of NAC (Fig. 6A). However, in the presence of NAC at 8,000 mg/L, the depletion of NO_3^- was followed by an accumulation of NO_2^- (evident at both 24 and 48 h), indicating that further reduction of NO_2^- was inhibited in the presence of NAC (Fig. 6A). In order to consolidate these data, the experiments were repeated using a medium supplemented with KNO_2. In the absence on NAC, complete reduction of NO_2 was observed after 48 h (Fig. 6B), as expected (33). On the contrary, in the presence of NAC at 8,000 mg/L, NO_2 levels did not decrease (Fig. 6B).

These results were consistent with the transcriptomic data and showed that NAC was able to inhibit the denitrification pathway in anaerobic environments, such as those encountered in endobronchial CF mucus. This feature might contribute to the observed antibiofilm synergism of NAC-colistin combinations, as previously discussed.

Time-kill assays of the NAC-colistin combination against planktonic cultures grown under anaerobic and aerobic conditions. Transcriptomic and biological data from this study suggested a role of NAC in inhibiting the *P. aeruginosa* denitrification apparatus, which could contribute to the observed antibiofilm synergy of NAC-colistin combinations. In order to further investigate this issue, time-kill assays of the NAC-colistin combination were performed with *P. aeruginosa* Z154 (i.e., the strain used for transcriptomic analysis) planktonic cultures, under both anaerobic and aerobic conditions.

FIG 4 Legend (Continued)
system. A potentiation by NAC of colistin antibiofilm activity was observed with all tested strains. CST 8, colistin at 8 mg/L; CST 32, colistin at 32 mg/L. Biofilms not exposed to NAC or CST represent the control. Black lines indicate median values. The *x* axis is set at the limit of detection (20 CFU/peg).



** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (Kruskal-Wallis test with Dunn's correction)

FIG 5 Antibiofilm activity of *N*-acetylcysteine (NAC) at 8,000 mg/L, colistin at 64 mg/L (CST 64), and the NAC-CST combination against *P. aeruginosa* Z154 and *P. aeruginosa* Z34 in the ASM biofilm model. A potentiation by NAC of colistin antibiofilm activity was observed with both strains. Biofilms not exposed to NAC or CST represent the control. Black lines indicate median values. The *x* axis is set at the limit of detection (100 CFU/mL).

TABLE 2 DEGs in *P. aeruginosa* Z154 planktonic cultures exposed to 8,000 mg/L NAC compared to control

DEG	Locus tag in *P. aeruginosa* strain			Gene	Product (function)[a]	Zur regulon	Adjusted *P* value	Log_2 fold change
	Z154	PAO1	UCBPP-PA14					
Upregulated	IS492_10415	PA0781	PA14_54180	*znuD*	TBDR ZnuD (zinc uptake)	+	4.6E−36	1.9
	IS492_17070	PA1922	PA14_39650	*cirA*	TBDR CirA (iron and zinc uptake)	+	0.0E+00	2.4
	IS492_17075	PA1923	PA14_39640		Cobaltochelatase subunit CobN-like (cobalamin biosynthesis)	+	7.9E−36	1.9
	IS492_17080	PA1924	PA14_39630	*exbD*	ExbD proton channel family protein (energy support for TBDR, cotranscribed with PA1922)	+	1.7E−03	0.6
	IS492_17085	PA1925	PA14_39620		Hypothetical protein (unknown function, DUF2149 domain-containing protein)	+	7.5E−06	0.8
	IS492_19940	PA2437	PA14_33110		HflC family modulator of membrane FtsH protease	+	5.1E−06	0.8
	IS492_19945	PA2438	PA14_33080		HflC modulator of membrane FtsH protease	+	7.0E−03	0.6
	IS492_19950	PA2439	PA14_33070	*hflK*	HflK family modulator of membrane FtsH protease	+	6.5E−03	0.6
	IS492_23615	PA2911	PA14_26420		TBDR (possibly involved in zinc uptake)	+	7.6E−03	0.6
	IS492_27310	PA3600	PA14_17710	*rpmJ2*	Zinc-independent paralog type B 50S ribosomal protein L36	+	2.0E−16	1.3
	IS492_27315	PA3601	PA14_17700	*rpmE2*	Zinc-independent paralog type B 50S ribosomal protein L31	+	1.2E−04	0.7
	IS492_29825	PA4063	PA14_11320		Zinc SBP (zinc uptake)	+	7.0E−41	2.0
	IS492_29830	PA4064	PA14_11310		Zinc ABC transporter, ATP-binding protein (zinc uptake)	+	4.2E−08	0.9
	IS492_29835	PA4065	PA14_11290		Zinc ABC transporter, permease (zinc uptake)	+	4.9E−13	1.2
	IS492_29840	PA4066	PA14_11280		Zinc SBP (zinc uptake)	+	8.5E−05	0.7
	IS492_06220	PA4834	PA14_63910	*cntI*	Pseudopaline transport plasma membrane protein CntI (zinc uptake)	+	6.1E−05	0.7
	IS492_06215	PA4835	PA14_63920	*cntM*	Pseudopaline biosynthesis dehydrogenase CntM (zinc uptake)	+	8.1E−26	1.7
	IS492_06210	PA4836	PA14_63940	*cntL*	Pseudopaline biosynthesis enzyme CntL (zinc uptake)	+	9.3E−39	2.0
	IS492_06205	PA4837	PA14_63960	*cntO*	Pseudopaline transport outer membrane protein CntO (zinc uptake)	+	0.0E+00	2.5
	IS492_06200	PA4838	PA14_63970		Hypothetical membrane protein	+	8.0E−04	0.7
	IS492_31595	PA5498	PA14_72550	*znuA*	Zinc soluble binding protein ZnuA (zinc uptake)	+	9.0E−08	0.9
	IS492_31600	PA5499	PA14_72560	*zur*	Transcriptional regulator for zinc homeostasis	+	5.3E−10	1.0
	IS492_31605	PA5500	PA14_72580	*znuC*	Zinc ABC transporter, ATP-binding protein ZnuC (zinc uptake)	+	1.2E−07	0.9
	IS492_31610	PA5501	PA14_72590	*znuB*	Zinc ABC transporter, ZnuB permease (zinc uptake)	+	1.9E−03	0.6
	IS492_31780	PA5534	PA14_73000		Hypothetical protein (unknown function, DUF1826 domain-containing protein)	+	9.8E−23	1.5
	IS492_31785	PA5535	PA14_73010	*zigA*	Zinc metallochaperone GTPase ZigA	+	5.9E−42	2.1
	IS492_31790	PA5536	PA14_73020	*dksA2*	Zinc-independent paralog of RNA polymerase-binding protein DksA	+	2.4E−23	1.5
	IS492_31800	PA5538	PA14_73040	*amiA*	*N*-acetylmuramoyl-L-alanine amidase (splitting of septal peptidoglycan during cell division)	+	1.3E−08	1.0
	IS492_31805	PA5539	PA14_73050	*folE2*	Zinc-independent paralog of GTP-cyclohydrolase FolE (folate biosynthesis)	+	4.5E−28	1.7
	IS492_31810	PA5540	PA14_73060	*cam*	γ-Carbonic anhydrase (reversible hydration of carbon dioxide)	+	1.5E−24	1.6
	IS492_31815	PA5541	PA14_73070	*pyrC2*	Zinc-independent paralog of dihydroorotase PyrC (pyrimidine biosynthesis)	+	3.1E−09	1.0
	IS492_02205	PA0433	PA14_05630		Hypothetical protein (unknown function, DUF2946 domain-containing protein)		1.3E−03	0.7
	IS492_02210	PA0434	PA14_05640		TBDR for which the siderophore has not been identified		1.5E−28	1.7
	IS492_02430	PA0478	PA14_06250	*fiuC*	GNAT family *N*-acetyltransferase (release of iron from desferrichrome in the cytoplasm)		3.9E−06	0.8
	IS492_10765	PA0848	PA14_53300	*ahpB*	AhpC-like alkylhydroperoxide reductase (oxidative stress response and cell redox homeostasis)		3.9E−16	1.3
	IS492_17945	PA2100	ND[b]	*mdrR2*	Transcriptional regulator, regulatory partner of MdrR1 (regulator of efflux systems)		6.3E−05	0.7
	IS492_17950	PA2101	ND		Conserved hypothetical protein (EamA-like transporter family)		1.7E−26	1.7
	IS492_17955	PA2102	ND		Hypothetical protein (unknown function, Mov34/MPN/PAD-1 family protein)		5.7E−13	1.2
	IS492_17960	PA2103	ND	*moeB*	Probable molybdopterin biosynthesis protein MoeB (ubiquitin-like modifier-activating activity)		7.5E−06	0.8
	IS492_25770	PA3287	PA14_21530		Ankyrin repeat domain-containing protein (unknown function)		1.9E−04	0.7
	IS492_27305	PA3599	PA14_17720		Probable transcriptional regulator		5.2E−12	1.1
	IS492_28275	PA3784	PA14_15130		Hypothetical protein (unknown function)		1.4E−05	0.8
	IS492_28280	PA3785	PA14_15120		Copper chaperone PCu(A)C		8.6E−07	0.9
	IS492_28305	PA3790	PA14_15070		TBDR copper receptor OprC (copper uptake)		1.0E−03	0.6
	IS492_06715	PA4739	PA14_62690		Hypothetical protein (unknown function, BON domain-containing protein)		9.8E−03	0.6

(Continued on next page)

TABLE 2 (Continued)

DEG	Locus tag in *P. aeruginosa* strain			Gene	Product (function)[a]	Zur regulon	Adjusted *P* value	Log_2 fold change
	Z154	PAO1	UCBPP-PA14					
	IS492_31510	PA5481	PA14_72360		Hypothetical periplasmic protein (inhibitor of vertebrate lysozyme)		3.9E−04	0.7
Downregulated	IS492_00850	PA0164	PA14_02050		γ-Glutamyltransferase family protein		8.0E−04	−0.6
	IS492_02660	PA0524	PA14_06830	*norB*	Nitric oxide reductase subunit NorB (denitrification)		3.9E−03	−0.6
	IS492_02685	PA0529	PA14_06890		Hypothetical protein (unknown function, MOSC domain-containing protein)		2.0E−05	−0.7
	IS492_02690	PA0530	PA14_06900		Probable class III pyridoxal phosphate-dependent aminotransferase (diverse metabolic pathways)		5.7E−05	−0.8
	IS492_02695	PA0531	PA14_06920		Aspartate aminotransferase family protein		4.7E−03	−0.6
	IS492_12670	PA1101	PA14_50140	*fliF*	Flagellar M-ring protein FliF (motility)		5.7E−05	−0.7
	IS492_12855	PA1136	PA14_49700		Probable transcriptional regulator		1.5E−12	−1.1
	IS492_12860	PA1137	PA14_49690		Oxidoreductase zinc-binding dehydrogenase family protein (protection from oxidative stress)		0.0E+00	−2.3
	IS492_14625	PA1453	PA14_45660	*flhF*	Flagellar biosynthesis protein FlhF (motility)		7.6E−03	−0.6
	IS492_19230	PA2298	PA14_34900		Probable oxidoreductase		4.9E−05	−0.7
	IS492_19235	PA2299	PA14_34880		Probable transcriptional regulator		3.2E−04	−0.7
	IS492_26340	PA3391	PA14_20230	*nosR*	Regulatory protein NosR (denitrification)		3.2E−04	−0.6
	IS492_26345	PA3392	PA14_20200	*nosZ*	Nitrous oxide reductase (denitrification)		4.1E−05	−0.8
	IS492_26895	PA3519	PA14_18810		Iron-containing redox enzyme family protein		2.8E−05	−0.3
	IS492_26920	PA3523	PA14_18760	*mexP*	Resistance-nodulation-cell division (RND) efflux membrane fusion protein		3.2E−03	−0.2
	IS492_27180	PA3574	PA14_18080	*nalD*	Transcriptional regulator NalD (second repressor of MexAB-OprM)		1.5E−19	−1.3
	IS492_27185	PA3574a	PA14_18070	*copZ*	Copper chaperone CopZ (copper efflux)		9.1E−11	−1.0
	IS492_27760	PA3690	PA14_16660		Heavy metal-translocating P-type ATPase (efflux)		1.1E−08	−1.0
	IS492_28975	PA3920	PA14_13170	*copA*	Copper-translocating P-type ATPase CopA1 (copper efflux)		1.2E−27	−1.2
	IS492_04870	PA5100	PA14_67350	*hutU*	Urocanate hydratase (histidine catabolic process)		4.0E−04	−0.6

[a]TBDR, TonB-dependent receptor; SBP, soluble binding protein; ABC, ATP-binding cassette. Protein functions were inferred from the literature and PseudoCAP (https://www.Pseudomonas.com/pseudocap).
[b]ND, not determined.

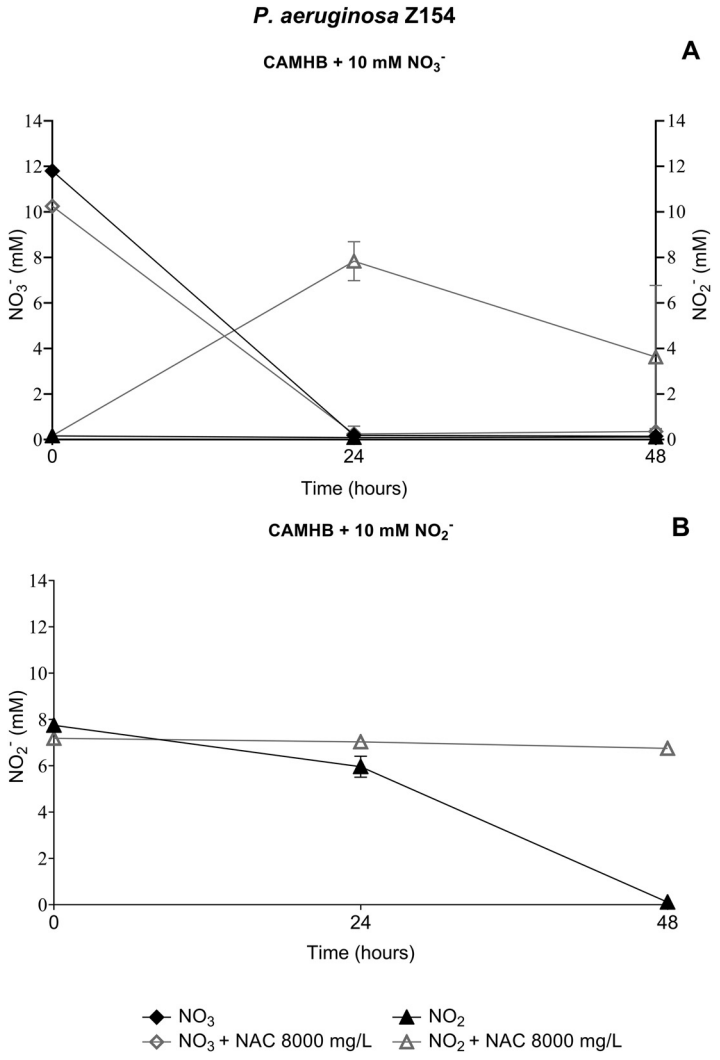


FIG 6 NAC-mediated inhibition of *P. aeruginosa* Z154 denitrification pathway. (A) NO_3^- and NO_2^- concentrations in anaerobic CAMHB supplemented with 10 mM NO_3^-, with or without NAC at 8,000 mg/L; (B) NO_2^- concentration in anaerobic CAMHB supplemented with 10 mM NO_2^-, with or without NAC at 8,000 mg/L. Data are plotted as the mean values of NO_3^- and/or NO_2^- levels detected at each time point.

Consistent with previous studies, anaerobic cultures were more susceptible to killing by colistin than aerobic cultures (34, 35) (Fig. 7A and B). Interestingly, a clear bactericidal effect of colistin at 0.25 mg/L (i.e., 1/8 MIC) in combination with NAC at 8,000 mg/L was observed in planktonic cultures grown under anaerobic conditions, with eradication achieved after 24 h of exposure (Fig. 7A). The wide error bars were due to the fact that in 2 out of 8 replicates (related to two independent experiments), no synergism was observed (Fig. 7A). This discrepancy was probably related to the low colistin concentration tested and the possible presence of heteroresistant subpopulations. On the contrary, cultures grown in the presence of oxygen were not affected by the NAC-colistin combination, demonstrating the influence of the growth conditions on the susceptibility of *P. aeruginosa* to such combination (Fig. 7B).

These results supported the hypothesis that, under anoxic conditions like those present in the deeper biofilm layers and in CF mucus, NAC-mediated inhibition of anaerobic respiration would prevent an adaptive response of *P. aeruginosa* to protect from colistin toxicity.

NAC-mediated inhibition of *P. aeruginosa* swimming and swarming motility. Transcriptomic results indicated that NAC downregulated two genes belonging to *P. aeruginosa* flagellar apparatus (i.e., *fliF* and *flhF*), which are necessary for the first step

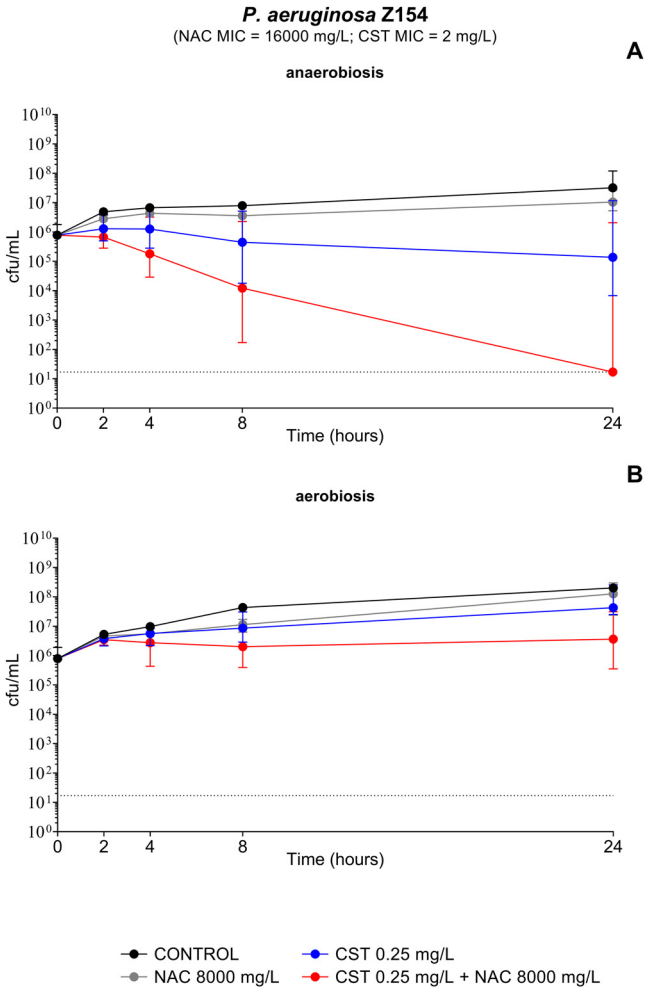


FIG 7 Time-kill curves of *P. aeruginosa* Z154 planktonic cultures exposed to *N*-acetylcysteine (NAC) at 8,000 mg/L, colistin (CST) at 0.25 mg/L, and the NAC-CST combination under anaerobic (A) and aerobic (B) conditions. NAC potentiated the bactericidal activity of colistin only under anaerobic conditions. Data are plotted as the median values of CFU per milliliter for each time point. Dotted lines indicate the detection limit (17 CFU/mL).

of flagellum assembly (36). In order to confirm the potential NAC-induced inhibition of flagellum-mediated motility, we performed classical swimming and swarming tests with the reference strain *P. aeruginosa* PAO1 and the CF strain *P. aeruginosa* Z154 (i.e., the strain used for transcriptomic analysis). *P. aeruginosa* Z154 was not capable of swarming motility under our laboratory conditions, so only the effect of NAC on swimming motility could be tested with this strain.

Overall, the results showed a clear inhibition of both swimming and swarming motility in the presence of NAC at 8,000 mg/L (Fig. 8 and 9). Such inhibition could be related to the downregulation of crucial genes of the flagellar apparatus and/or the induction of a zinc starvation response. Indeed, zinc starvation has been demonstrated to affect the ability of *P. aeruginosa* to express several virulence phenotypes, crucial for the ability of this pathogen to colonize CF lung, including motility, biofilm formation and siderophore synthesis (37).

Conclusions. In conclusion, the results of this study demonstrated a relevant antibiofilm synergism of NAC-colistin combinations (at the high concentrations achievable by inhalation) against *P. aeruginosa*, which would deserve further investigation for potential clinical applications of inhaled formulations. Transcriptomic and biological experiments suggested that NAC inhibited *P. aeruginosa* anaerobic respiration, which could be relevant for the observed antibiofilm synergism with colistin.

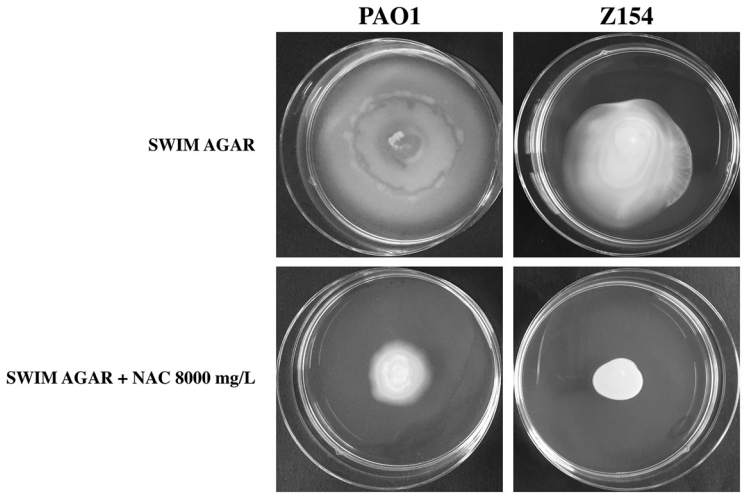


FIG 8 NAC-mediated inhibition of *P. aeruginosa* PAO1 and Z154 swimming motility. Assays were performed in at least three independent experiments (with three replicates per condition per experiment), and representative data are shown.

In addition, although NAC alone was not demonstrated to be effective against preformed *P. aeruginosa* biofilms, transcriptomic analysis of NAC-exposed planktonic cultures revealed that NAC could attenuate *P. aeruginosa* virulence, mainly by inducing a zinc starvation response, affecting anaerobic respiration and inhibiting flagellum-mediated motility (with the last two features confirmed experimentally). In this perspective, NAC, at the high concentrations achievable by inhalation, might have beneficial effects in the very first steps of lung infection, possibly preventing biofilm formation and the establishment of a chronic colonization, which should be further investigated.

MATERIALS AND METHODS

Bacterial strains. Seventeen strains were investigated, including 15 clinical isolates from CF patients, an MDR clinical isolate from a respiratory tract infection (RTI) from an intensive care unit (ICU), and the reference strain, *P. aeruginosa* PAO1 (Table 1). Identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Shimadzu). Antimicrobial susceptibility was determined using the reference broth microdilution method (38). Whole-genome sequencing of clinical isolates was performed with the Illumina (San Diego, CA, USA) MiSeq platform, using a 2× 150-bp paired-end approach. Raw reads were assembled using SPAdes (39), and draft genomes were used to determine multilocus sequence types (MLSTs) and O types at the Oxford PubMLST site (https://pubmlst.org/) (40) and at the

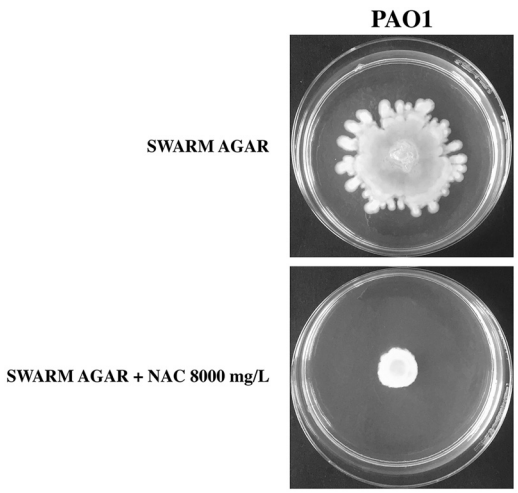


FIG 9 NAC-mediated inhibition of *P. aeruginosa* PAO1 swarming motility. Assays were performed in at least three independent experiments (with three replicates per condition per experiment), and representative data are shown.

Center for Genomic Epidemiology site (https://cge.food.dtu.dk/services/PAst/) (41), respectively. The complete genome of *P. aeruginosa* Z154 was obtained by combining results from Illumina with those obtained using the Oxford Nanopore Technologies (Oxford, United Kingdom) MinION platform, and *de novo* assembly was generated using Unicycler v0.4.4 as previously described (42).

Preparation of culture media. NAC stock solutions (100 g/L) were prepared immediately before use. NAC powder (Zambon, Bresso, Italy) was dissolved in sterile distilled water, the pH was adjusted to 6.5 to 6.8 with NaOH at 10 M, and the solution was filtered through a 0.22-μm-pore membrane filter. All experiments were performed in cation-adjusted Mueller-Hinton broth (CAMHB) (Becton Dickinson, Milan, Italy), unless otherwise specified, starting from an appropriately concentrated medium to avoid broth dilution when NAC solution was used. The artificial sputum medium (ASM) was also used in selected experiments and was prepared as previously described by Kirchner et al. (43).

***In vitro* biofilm susceptibility testing.** Biofilm susceptibility testing was first performed using the Nunc-TSP lid system (Thermo Fisher Scientific, Waltham, MA, USA), as described previously (44). Briefly, biofilms were grown for 24 h in CAMHB at 35°C under static conditions. Preformed biofilms were then exposed to NAC at 8,000 mg/L and colistin (colistin sulfate; Applichem, Darmstadt, Germany) at 2 to 32 mg/L, alone and in combination. The colistin concentration was selected according to preliminary results of antibiofilm susceptibility testing and the colistin MIC for each strain. After 24 h of exposure (i.e., 35°C, static conditions), biofilms were washed twice with 200 μL of phosphate-buffered saline (PBS) (Sigma-Aldrich, Milan, Italy) to remove loosely adherent bacteria, and sessile cells were removed from pegs by sonication for 30 min (Elma Transsonic T 460; Elma, Singen, Germany) in 200 μL of tryptic soy broth (TSB) (Oxoid, Milan, Italy) supplemented with 1% Tween 20 (Sigma-Aldrich) (i.e., the recovery medium). The median number of CFU per peg was then determined by plating 10 μL of appropriate dilutions of the recovery medium onto tryptic soy agar (TSA) (Oxoid) and incubating for 24 h at 35°C (detection limit, 20 CFU/peg). The colony count was also double-checked after 48 h of incubation.

The potential antibiofilm synergism of NAC-colistin combinations was further investigated using an *in vitro* ASM biofilm model (43) in order to mimic *P. aeruginosa* biofilm conditions within the CF mucus. The study was carried out with two selected CF strains (*P. aeruginosa* Z154 and Z34), exhibiting different features (i.e., mucoid/nonmucoid phenotype, antimicrobial susceptibility pattern, MLST, and O type) (Table 1). In brief, biofilms were grown in 2 mL ASM in 24-well plates (Sarstedt, Nümbrecht, Germany), for 72 h at 35°C under static conditions. Preformed biofilms were then exposed to NAC at 8,000 mg/L and colistin at 64 mg/L, alone and in combination. Preliminary experiments carried out with lower colistin concentrations (i.e., 2 to 32 mg/L) did not show evident synergistic antibiofilm activity, while higher colistin concentrations (i.e., >64 mg/L) led to eradication of the biofilm cultures even in the absence of NAC (data not shown). After 24 h of exposure (i.e., 35°C, static conditions), bacterial biofilms were disrupted by 30 min of sonication followed by manual pipetting, and the median number of CFU per milliliter was determined following the same protocol described for the Nunc-TSP lid assay.

Data from both biofilm models were obtained in at least three independent experiments, with at least 12 replicates per condition per experiment.

RNA-seq and transcriptomic analysis. *P. aeruginosa* Z154 (i.e., colistin-susceptible CF strain, mucoid, MDR, ST412, O6) (Table 1) was selected for studies aimed at investigating the transcriptomic response of *P. aeruginosa* to NAC exposure. A CF strain, rather than a reference strain (such as *P. aeruginosa* PAO1), was selected for this analysis because of the known adaptive diversification of *P. aeruginosa* into "specialized" types during chronic/recurrent infections in CF patients (3).

Because these represented the first data on the transcriptomic response of *P. aeruginosa* to NAC exposure, and considering the complex and still largely unknown effects of NAC on microbial physiology, we decided to perform the experiments with planktonic cultures, which represent a more homogenous and better standardized model for transcriptomic studies.

Overnight cultures in CAMHB were diluted at 1:50 in the same medium and incubated at 35°C with agitation to achieve an optical density at 600 nm (OD_{600}) of 1.0. The cells were then exposed to NAC at 8,000 mg/L for 30 min at 35°C under static conditions. Cultures treated in the same way but not exposed to NAC represented the control. Total RNA extraction was performed using the SV total RNA isolation system (Promega, Madison, WI, USA) following the manufacturer's instructions. rRNA depletion, cDNA library construction, and Illumina HiSeq 4000 platform-based transcriptome sequencing (RNA-seq) were performed by Eurofins Genomics Europe Sequencing (Constance, Germany). The transcriptome libraries were single-end sequenced with 50-bp reads for a total of 10 million reads per sample. Bioinformatic analysis was performed using the SeqMan NGen v17.3 software tool (DNASTAR Lasergene, Madison, WI, USA), with default parameters. Reads were aligned using *P. aeruginosa* Z154 complete genome (n = 6,344 coding DNA sequences [CDSs]) as a reference. Differentially expressed genes (DEGs) of the NAC-exposed cultures compared to the control were analyzed considering false-discovery rate (FDR) adjusted P values of <0.05 from DeSeq2. DEGs with a 99% confidence interval (CI) were discussed. Results were obtained from two independent experiments. In order to favor comparison with data present in the literature, genes without a univocal name have been indicated as *P. aeruginosa* PAO1 locus tags throughout the text and reported in Table 2 also as *P. aeruginosa* UCBPP-PA14 locus tags.

NO_3^- and NO_2^- quantification. NAC-mediated inhibition of the denitrification pathway was investigated by measuring the concentration of NO_3^- and NO_2^- in anaerobic cultures of *P. aeruginosa* Z154 (i.e., the strain used for transcriptomic analysis). For this purpose, the Griess nitrite/nitrate colorimetric assay (Cayman Chemicals, Ann Arbor, MI, USA) was used according to the manufacturer's recommendations and as previously described, with some modification (33). CAMHB was supplemented with 10 mM $NaNO_3$ or KNO_2 and allowed to equilibrate for 3 days at 35°C in an anaerobic atmosphere by using the AnaeroGen kit (Oxoid). Overnight cultures were then diluted in 20 mL of each anoxic culture medium to reach a concentration of 10^6 CFU/mL and challenged with NAC at 8,000 mg/L. At times 0, 24, and 48 h of incubation under

anoxic conditions at 35°C, supernatants were harvested and subjected to Griess colorimetric reaction in order to detect NO_3^- and NO_2^- levels. NAC-free cultures represented the control. Experiments were carried out in triplicate with one replicate per time point per condition.

Time-kill assays. Time-kill assays were performed according to CLSI guidelines (45) with the colistin-susceptible strain *P. aeruginosa* Z154 (i.e., the strain used for transcriptomic analysis). Colistin at 0.25 mg/L was tested alone and in combination with NAC at 8,000 mg/L under both aerobic and anaerobic conditions. We decided to use this colistin concentration since a higher concentration led to eradication of the planktonic cultures (data not shown). The medium (CAMHB) used to obtain anoxic cultures was placed under an anaerobic atmosphere by using the AnaeroGen kit (Oxoid) for 3 days prior to use and during the whole experiment. The killing curves were carried out in borosilicate glass bottles with a final volume of 20 mL of CAMHB. At 0, 2, 4, 8, and 24 h of exposure, CFU per milliliter were determined by plating 60 μL of appropriate dilutions of each condition onto TSA and incubating for 24 h at 35°C (detection limit, 17 CFU/mL). Data were obtained from at least four independent experiments with two replicates per condition per experiment.

Motility tests. NAC-induced inhibition of flagellum-mediated motility (i.e., both swimming and swarming motility) was investigated with the reference strain *P. aeruginosa* PAO1, which has been used for similar motility experiments in several previous studies (46), and *P. aeruginosa* Z154 (i.e., the strain used for transcriptomic analysis). *P. aeruginosa* Z154 was not capable of swarming motility under our laboratory conditions (perhaps due to the known reduction of flagellar expression in mucoid CF-adapted strains) (47), so only the effect of NAC on swimming motility could be tested with this strain. Swim plates consisted of Luria-Bertani (LB) broth (Oxoid) containing 0.3% agar (46). Swarm plates consisted of nutrient broth (Oxoid) with 0.5% glucose and 0.5% agar (46). Overnight cultures in CAMHB were diluted in the same medium to a final OD_{600} of 3.0, and 5 μL was spotted onto swim and swarm plates, with or without NAC at 8,000 mg/L. Results were observed after incubation at 35°C for 48 h. Assays were performed in at least three independent experiments with three replicates per condition per experiment.

Statistical analysis. Statistical analysis of biofilm susceptibility assays was performed using GraphPad Prism version 8.0 (San Diego, CA, USA). Multiple-comparison tests were performed by the Kruskal-Wallis test with Dunn's correction. A P value of ≤ 0.05 was considered significant. RNA-seq statistical analysis was performed using the SeqMan NGen v17.3 software tool.

Data availability. The complete genome sequence of *P. aeruginosa* Z154 was deposited in GenBank under accession no. CP069177. RNA-seq data were also deposited in the NCBI Gene Expression Omnibus (GEO) database under accession no. GSE190946.

ACKNOWLEDGMENTS

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Relevant increase of CTX-M-producing *Escherichia coli* carriage in school-aged children from rural areas of the Bolivian Chaco in a three-year period

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ABSTRACT

Objectives: The aim of this study was to perform two cross-sectional surveys on the fecal carriage of CTX-M–producing Enterobacterales in school-aged children from rural areas of the Bolivian Chaco (2016 vs 2019).

Methods: A total of 757 fecal samples were collected from school-aged children living in nine indigenous communities (n=337, 2016; n=420, 2019). After a first passage onto MacConkey agar (MCA), samples were plated onto MCA plus cefotaxime 2 µg/mL (MCA-CTX), and a loopful of the bacterial growth was used as a template for the detection of group 1, 2, 8/25, and 9 bla_{CTX-M} variants by multiplex reverse transcriptase polymerase chain reaction . Positive samples were tested again for detecting, identifying, and characterizing CTX-M-positive isolates.

Results: Growth onto MCA-CTX was obtained with 208 samples (27.5%; 62/337, 2016; 146/420, 2019), of which 201 (96.6%) were positive for bla_{CTX-M} genes. Overall, a relevant increase of fecal carriage of CTX-M–producing Enterobacterales was observed in the study period: 17,5% (59/337) in 2016 compared with 33,8% (142/420) in 2019, $p<0.01$. Nonetheless, the relative group distribution of CTX-M groups remained stable, with group 1 being the prevalent, followed by group 9 and group 8/25. Group 2 was not detected.

Conclusions: The present study demonstrated an alarming spread of CTX-M enzymes in rural areas of the Bolivian Chaco, where antibiotics consumption is limited. Further studies are encouraged to better understand the dissemination dynamics of such relevant resistance determinants.

Background

Extended-spectrum β-lactamases (ESBLs) have become endemic in Enterobacterales, in both hospital and community settings. CTX-M-type ESBLs have rapidly disseminated since the early 1990s and currently represent the most prevalent ESBLs among Enterobacterales worldwide (Peirano and Pitout, 2019). In particular, *Escherichia coli* has become the species most frequently associated with CTX-Ms, with some clones showing a pandemic dissemination (i.e., ST131 and ST1193 clonal groups) (Peirano and Pitout, 2019). The role of commensal *E. coli* as a reservoir of genes encoding ESBL has been recognized globally

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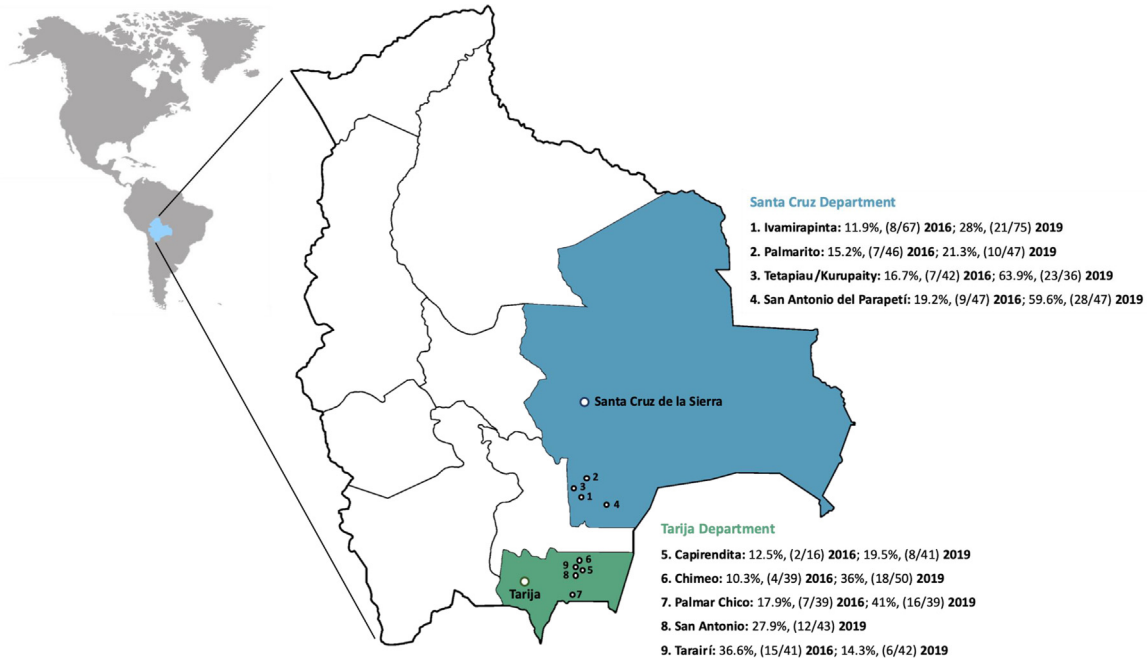

Fig. 1. Geographical area of the nine communities included in the study and percentages of children with CTX-M–positive *Escherichia coli*.

(World Health Organization WHO and GLASS, 2020), and several studies have reported high prevalence of CTX-M-type ESBLs in commensal isolates from healthy adults and children in the community setting (Woerther *et al.*, 2013).

In low/medium-income (LMI) settings, antimicrobial resistance rates have been demonstrated to be even higher than in higher-income countries, for complex factors mainly related to poverty (e.g., poor access to healthcare, poor sanitation, and unreliable water supplies), with a relevant impact on morbidity and mortality rates, especially in childhood (World Health Organization WHO and GLASS, 2020; Murray *et al.*, 2022).

In this study, we performed two cross-sectional surveys (i.e., 2016 vs 2019) to investigate the fecal carriage of CTX-M-producing Enterobacterales in school-aged children living in nine indigenous communities in rural areas of the Bolivian Chaco.

Methods

The study population was represented by school-aged children (i.e., aged 6–14 years) living in nine indigenous communities in rural areas of the Bolivian Chaco (Fig 1). Administration of antibiotics during the 15 days preceding the survey was investigated by a questionnaire administered to parents.

A total of 757 fecal samples (337 in 2016; 420 in 2019) were collected and transferred to the Laboratories of Camiri or Villa Montes Hospitals within six hours, for immediate plating onto MacConkey agar (MCA; Oxoid LTD, UK). After incubation at 35 °C for 18 hours, the bacterial growth (representative of the total enterobacterial microbiota) was collected using fecal swabs (Copan, Brescia, Italy), shipped to Italy, and preserved at 4°C until processed (within 30 days) (Giani *et al.*, 2018). For detection of CTX-M–producing enterobacteria, fecal swabs were plated onto MCA plates plus cefotaxime 2 µg/ml (MCA-CTX). After incubation at 35 °C for 18 hours, a loopful of the bacterial growth (taken either from confluent growth or from isolated colonies of different morphologies) was used as a template for the detection of group 1, 2, 8/25, and 9 $bla_{\text{CTX-M}}$ by mRT-PCR, as previously described (Giani *et al.*, 2017). To identify CTX-M–positive isolates, CTX-M–positive samples were again streaked onto MCA-CTX, and all colonies with a different appearance were re-isolated and subjected to i) a phenotypic test for ESBL production (using the double disk method with amoxicillin-clavulanate and cefotaxime), ii) characterization of $bla_{\text{CTX-M}}$ group by multiplex reverse transcriptase polymerase chain reaction (mRT-PCR), and iii) identification by the Bruker MS system (Bruker Daltonics, Germany; MBT reference library, version 2021) (Giani *et al.*, 2017).

Statistical analyses were performed using Pearson's Chi-square test with Yates' continuity correction with R version 4.0.5 for Windows. A *P*-value <0.05 was considered significant.

Results and discussion

In 2016, 337 children (mean age = 9.2 years, SD = 1.25; median age = 9 years; male:female ratio = 1:1.13) were included in the study (Table 1). Of the 337 samples, 61 grew on MCA-CTX (18.1%). Of these, 59 (96.7%) were found positive for $bla_{\text{CTX-M}}$ genes. The remaining two were found negative for ESBL production through phenotypic tests. Identification of CTX-M-producing isolates showed that all were *E. coli*, except for one *Enterobacter cloacae* complex. Some children (n=19, 32.2%) were found to be infected by more than one CTX-M-producing *E. coli,* for a total of 82 *E. coli* isolates, with one isolate carrying two $bla_{\text{CTX-M}}$ variants (i.e., $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$ groups) (Table 1).

In 2019, 420 children were included in the study (mean age = 9.6 years, SD = 1.4; median age = 10 years; male:female ratio = 1.08:1). Of the 420 fecal samples collected, 146 (34.8%) grew on MCA-CTX, and 142 (97.3%) of these were found positive for $bla_{\text{CTX-M}}$ genes (Table 1). Only one of four CTX-M negative isolates showed a result of ESBL-producer through phenotypic testing. CTX-M–producing isolates were identified as *E. coli,* except for one *Raoultella ornithinolytica*. A totoal of 40 children (28.2%) were infected by multiple CTX-M–producing *E. coli*, for a total of 190 CTX-M–producing isolates, with five isolates carrying two $bla_{\text{CTX-M}}$ variants (i.e., n=4, $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$ groups, and n=1, $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-8/25}}$ groups) (Table 1).

Usage of antibiotics was found to be very limited, with only two children (0.6%) in 2016 and 21 children (5%) in 2019 report-

Table 1
Features of the study population sorted by communities and by year with features of CTX-M-positive *Escherichia coli* isolates.

Community		Year	No. of studied children			Antibiotic consumption[a,b]	Tot children with CTX-M[a]	*p* value	CTX-M producing *E. coli*	Tot CTX-M detected	CTX-M-groups		
			M[a]	F[a]	Total						1[a,c]	9[a,c]	8/25[a,c]
Tarija Department	**Capirendita**	2016	9 (56.3)	7 (43.8)	16	-	2 (12.5)	0.8	2	2	-	2 (100)	-
		2019	23 (56.1)	18 (43.9)	41	-	8 (19.5)		11	12	7 (58.3)	5 (41.7)	-
	Chimeo	2016	18 (46.2)	21 (53.8)	39	-	4 (10.3)	0.01	4	4	3 (75)	1 (25)	-
		2019	25 (50)	25 (50)	50	5 (10)	18 (36)		21	21	9 (42.9)	12 (57.1)	-
	Palmar Chico	2016	18 (46.2)	21 (53.8)	39	-	7 (17.9)	0.05	8	10	6 (60)	4 (40)	-
		2019	21 (53.8)	18 (46.2)	39	1 (2.6)	16 (41)		19	19	13 (68.4)	6 (31.6)	-
	San Antonio	2016	-	-	-	-	-	-	-	-	-	-	-
		2019	24 (55.8)	19 (44.2)	43	-	12 (27.9)		14	15	12 (80)	2 (13.3)	1 (6.7)
	Tarairí	2016	18 (43.9)	23 (56.1)	41	-	15 (36.6)	0.03	21	22	18 (81.8)	4 (18.2)	-
		2019	19 (45.2)	23 (54.8)	42	-	6 (14.3)		7	7	3 (42.9)	3 (42.9)	1 (14.3)
Santa Cruz Department	**Ivamirapinta**	2016	35 (52.2)	32 (47.8)	67	-	8 (11.9)	0.03	12	12	9 (75)	3 (25)	-
		2019	41 (54.7)	34 (45.3)	75	4 (5.3)	21 (28)		35	35	26 (74.3)	6 (17.1)	3 (8.6)
	Palmarito	2016	19 (41.3)	27 (58.7)	46	1 (2.2)	7 (15.2)	0.6	9	9	-	3 (33.3)	6 (66.7)
		2019	21 (44.7)	26 (55.3)	47	5 (10.6)	10 (21.3)		14	15	9 (60)	5 (33.3)	1 (6.7)
	Tetapiau/ Kurupaity	2016	18 (42.9)	24 (57.1)	42	1 (2.4)	7 (16.7)	<0.0001	9	10	8 (80)	2 (20)	-
		2019	20 (55.6)	16 (44.4)	36	6 (16.7)	23 (63.9)		30	30	19 (63.3)	8 (26.7)	3 (10)
	San Antonio del Parapetí	2016	23 (48.9)	24 (51.1)	47	-	9 (19.2)	0.0001	13	14	9 (64.3)	5 (35.7)	-
		2019	24 (51.1)	23 (48.9)	47	-	28 (59.6)		39	41	22 (53.7)	15 (36.6)	4 (9.8)
	Total	2016	158 (46.9)	179 (53.1)	337	2 (0.6)	59 (17.5)	<0.0001	82	83	53 (63.9)	24 (28.9)	6 (7.2)
		2019	218 (51.9)	202 (48.1)	420	21 (5)	142 (33.8)		190	195	120 (61.5)	62 (31.8)	13 (6.7)

[a] number (%)
[b] during the 15 days preceding the survey
[c] over the total CTX-M

ing antibiotic consumption during the 15 days preceding the survey (Table 1).

Overall, despite the relevant spread of CTX-M enzymes over the study period (17.5%, 2016 vs 33.8%, 2019, $p<0.001$), the relative prevalence of each CTX-M group remained stable. The CTX-M-1 group represented the most prevalent one (n=53, 63.9% in 2016; n=120, 61.5% in 2019), followed by the CTX-M-9 group (n=24, 28.9% in 2016; n=62, 31.8% in 2019) and the CTX-M-8 group (n=6, 7.2% in 2016; n=13, 6.7% in 2019) (Table 1). Group 2 was not detected.

This study reported a notable increase of carriage of CTX-M–producing *E. coli* among healthy children living in rural communities of the Bolivian Chaco, where antibiotic usage remains scarce. Discordant data were only found in one community, where a decreasing trend was observed (Table 1).

The results of the present survey were consistent with the few similar studies that have been performed so far in rural communities from other LMI settings (Araque and Labrador, 2018; Purohit *et al.*, 2017).

Our study has some limitations. The number of children in each community was not representative of the total number of the population. Indeed, only children aged between six and 14 years were included. Moreover, it would be interesting to investigate the allelic variants of CTX-M ESBLs to better understand the dissemination dynamics of these enzymes.

Previous large-scale surveys conducted by our group in small urban areas of the Bolivian Chaco had demonstrated a dramatic increase of fecal carriage of CTX-M–producing *E. coli* in healthy children during the last two decades, from 0.1% in 2002 to 12% in 2011, with a change in the molecular epidemiology of CTX-M enzymes characterized by the CTX-M-1 group outcompeting the initially prevalent CTX-M-2 group (Bartoloni *et al.*, 2013). Data from the present study are overall consistent with such scenario and demonstrate the rapid spread and maintenance of CTX-M–producing Enterobacterales even in indigenous communities with poor access to conventional medicine and antibiotics. Further studies are encouraged to better understand the dissemination dynamics of these resistance determinants.

Declaration of Competing Interest

The authors have no competing interests to declare.

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Ethical approval

Fecal samples were obtained from enrolled children, after informed consent was obtained from parents or legal guardians. Full ethical clearance was obtained from the qualified local authorities (Convenio de Salud, Ministerio de Salud—Vicariato de Camiri) who reviewed and approved the study design.

Authors'contributions

SRB and MM analyzed the data and drafted the manuscript; SRB, MM, TDM and AM produced phenotypic data, molecular detection and handled the samples; SRB, TDM, ALV, TBM, CR, VP, HG,

MS and MS collected the samples and participated in the coordination of the survey; AB, GMR and LP coordinated the survey, analyzed the data and produced the final version of the manuscript.

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First report of the multidrug-resistant pandemic clone 1193 *Escherichia coli* among commensal isolates from Bolivia.

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Introduction

CTX-Ms are the most common extended-spectrum β-lactamase (ESBL) among Enterobacteriaceae, especially in *Escherichia coli*. Among clinical isolates of ESBL-producing *E. coli*, sequence type 131 (ST131) has emerged and globally disseminated as multidrug-resistance (MDR) pandemic clone responsible for community and hospital-acquired urinary tract and bloodstream infections.[1] Nowadays, the CTX-M-27-producing *Escherichia coli* ST131 cluster has been rapidly disseminating in Asia, Europe, and north America.[2–5]

Simultaneously, ST1193 has been reported in several countries, since 2012, as a new, virulent and resistant clone that commonly causes extraintestinal infections, in particular is generally associated with bloodstream infection, septicemia, urinary tract infections and meningitis.[6–14] *E. coli* ST1193 (Ec-ST1193) showed many features, including fluoroquinolone resistant (FQ^r), lactose non-fermenting and phylogenetic group B2.[6,7] Additionally, ESBL-producers *E. coli* belonged to ST1193 were found in China carrying $bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}123}$, and in Germany with $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$.[3,5] While most of ST1193 isolates was identified among human-source clinical *E. coli* isolates, ST1193 has also been described as a commonly occurring variant from faecal samples of healthy student between 2014 to 2019 in a study in the United States.[15]

In Bolivia, a previous study reported a significant rise of CTX-M enzymes in commensal *E. coli* isolated from healthy children living in rural areas of Bolivian Chaco showing an increase from 17.5% to 33.8% over the total number of ESBL-producing *E. coli*.[16] However, data on clonal lineages driving such epidemiological change, and associated resistance features, are lacking.

The aim of this study was to characterize CTX-M-producing *E. coli* strains, isolated from school-aged children living in rural communities in Tarija Department, a setting with a low antibiotic selective pressure.

Materials and methods

Study population

The study population consisted of school-aged (6-14 years) children living in five rural communities in Tarija Department (i.e., Capirendita, Chimeo, Palmar Chico, San Antonio and Tarairí), in south-eastern Bolivia (between longitude 62°16' and 64°18' east and latitude 21°00' and 22°17' south). The rural communities are located about 3 to 14 km from the Villa Montes town (**Figure S1**). The study population consist of Guaraní Indians and mestizos living in poor dwellings

with walls of sticks, straw and clay and thatched roofs. Information about previous drug use (in the 15 days preceding the survey) was obtained from each children participant by a questionnaire administrated to parents or legal guardians.

Strains isolation

One faecal sample for each child was collected during a two-month period from September to October over a three-years period (from 2016 to 2019). The samples were transferred to the Laboratories of Villa Montes Hospitals within 5-6 hours and were plated onto MacConkey agar (Oxoid LTD, UK) (MCA). After incubation at 35 °C for 24 hours, the bacterial growth was collected, stored in faecal swabs (Copan, Brescia, Italy) and was shipped to Italy. All faecal swabs were then streaked onto MCA plates supplemented with cefotaxime 2 µg/ml (CTX2) and all colonies with different morphologies were screened for extended-spectrum β-lactamases (ESBL) production by a combination disk test according to CLSI (ceftazidime and cefotaxime as substrates and clavulanic acid as an inhibitor)[17]. All ESBL positive colonies were then screened for the detection of $bla_{\text{CTX-M}}$ 1, 2, 8/25 and 9 variants by mRT-PCR and identified by Bruker MS system (Bruker Daltonics, German). Established PCR-based methods were used to define *E. coli* phylogenetic group (A, B1, B2, C, D, E and F),[18] ST131 and its H30-Rx subclone [19] and ST1193-specific clones using the primers described by Johnson *et al*.[20–23]

DNA extraction, sequencing and bioinformatics analyses

Twenty-two phylogenetic group B2-EC-ST1193 isolates were subjected to WGS, after DNA extraction by phenol-chloroform methods, using the Illumina NextSeq (2x150bp) platform (Illumina, Inc., San Diego, CA, USA) and *de novo* assemblies were performed using SPAdes v3.14.1 genome assembler.

Generation and *in silico* characterization of a global Ec-ST1193 database

Global comparative genomic analysis was carried out including a total of 215 ST1193 *E. coli* genomes. Among the 29218 genomes, 193 *E. coli* were selected among all the *E. coli* genomes available at the NCBI-NHI database (https://www.ncbi.nlm.nih.gov/genome/; last accessed in May 2022; N=29218, selected by the following inclusion criteria: "*Escherichia coli*" [Organism name]; presence of RefSeq accession number) for which MLST were performed using mlst v2.11 tool (https://github.com/tseemann/mlst) and twenty-two were ST1193 genomes described in this manuscript. Clinically important virulence factors and antimicrobial resistance were detected by ABRIcate (https://github.com/tseemann/abricate). Mutations in quinolone resistance determining regions (QRDR) of *gyrA*

(Accession number WP_074153749.1), *parC* (Accession number AML00471.1) and *parE* (Accession number NP_417502.1) genes in all the Ec-ST1193 were detected using Protein BLAST tool (https://blast.ncbi.nlm.nih.gov/).

Phylogenetic analysis

To investigate the phylogenetic structure of Ec-ST1193 isolates from our study in a global context, we combined genomic data from our Bolivian Ec-ST1193 isolates together with Ec-ST1193 genomes downloaded from NCBI. Each sample were mapped against the reference complete genome, SCU-204 (accession number: GCA_013085905.3) to generate the core genome SNP alignment files using Snippy v4.4.3 (https://github.com/tseemann/snippy). Gubbins v2.3.4 was used to remove recombinant regions from the snippy resulting alignment file, and this were used to infer maximum likelihood trees with IQ-TREE v1.6.12 using a general time reversible model. Finally, Microreact (https://microreact.org) was used for adding Metadata.

Pangenome analysis

Roary was used to define the pangenome of all isolates studied, with a 100% BLAST identity threshold using the MAFFT setting. Before implementing the Roary pipeline, all the genome sequences were annotated with Prokka (https://github.com/tseemann/prokka).[24] The genes contained in all strains were called core genes, and the genes contained only in a subset of strains were called specific genes.

Data availability

Raw reads from isolates sequenced (n= XXXXX) in this study are available at the NCBI short read archive (SRA) under BioProject PRJNAXXXXX. Individual accession numbers for *de novo* assemblies and genetic features of the sequenced strains are detailed in **Table S1** (**available as Supplementary data**).

Results

Faecal specimens were obtained from 800 school-age children, 135 in 2016, 247 in 2017, 203 in 2018 and 215 in 2019. Children (male:female ratio = 1:1), were aged from 6 to 14 years (mean age = 9.6, SD = 1.6; median age = 9). In 2016, samples from a rural community of Tarija Department (i.e., San Antonio) could not be collected.

The use of antibiotics was found very limited, with only two (0.3%) children in 2016, one in 2017 (0.1%), and six (0.8%) in 2019 reporting antibiotic consumption during the 15 days preceding the survey.

Prevalence of ESBL-positive bacteria and CTX-M-producing *E. coli*

Of the 800 samples, 224 grew on MCA-CTX2 (28%). The isolation of all different colonies showed that some children (n = 43, 19.2%) were colonized by more than one isolate for a total of 271 strains grew on MCA-CTX.

The prevalence of ESBL-producing bacteria was 97% (n=263/271) and among these isolates the 99.2% (n=261/263) was found to carry *bla*$_{CTXM}$ genes (**Table 1**).

Species identification showed that all were *E. coli*, except for two isolates in 2017 that were *Enterobacter cloaceae*, one isolate in 2018 that was *Klebsiella pneumoniae* and one isolate in 2019 that was *Raoultella ornithinolytica* (**Table 1**).

A total of 257 *bla*$_{CTXM}$-carrying *E. coli* was found, with six isolates harbouring variants from two groups: *bla*$_{CTX\text{-}M\text{-}1}$ and *bla*$_{CTX\text{-}M\text{-}9}$ (one *E. coli* in 2016, in 2017 and in 2019 and two *E. coli* in 2018) or *bla*$_{CTX\text{-}M\text{-}1}$ and *bla*$_{CTX\text{-}M\text{-}8}$ (one *E. coli* in 2019). Among the CTX-M-positive *E. coli*, the CTX-M-1 group variant represented the most prevalent (66.9%), followed by CTX-M-9 group (29.7%) CTX-M-8/25 group (2.3%) and CTX-M-2 group (0.8%), this latter was found in two *E. coli*, one in 2017 and the other in 2018, respectively (**Table1**).[16]

Phylogenetic grouping of CTX-M-producing *E. coli*

The **Figure 1** shows a wide diversity of phylogenetic groups distribution amongst the CTX-M-producing *E. coli*. Indeed, *E. coli* can be divided into seven main phylogenetic groups designated A, B1, B2, C, D, E and F, with groups A and B being the predominant ones. Interestingly, a total of 40 *E. coli* was found to belong to group B2 with uneven distribution showed a ten-fold increase over the years, from 3% in 2016 to 32% in 2019 (**Figure 1**).

Distribution Prevalence of ST1193 and ST131-H30 clones in CTX-M-producing *E. coli* B2.

The CTX-M groups distribution in *E. coli* B2 over the years showed that *bla*$_{CTX\text{-}M\text{-}9}$ gene variant has been found predominant since 2018, significantly reducing the contribution of *bla*$_{CTX\text{-}M\text{-}1}$ gene variant (from 100%, in 2016 to 17%, in 2019) (**Figure 2A**).

Moreover, clonal PCR analysis revealed that the Ec-ST131-H30 has been found predominant in 2017, carrying *bla*$_{CTX\text{-}M\text{-}1}$ (n=6/7, 85.7%) (**Figure 2B and 2C**). In the 2018, the rates of clones changed deeply showing an emerged of Ec-ST1193 carried *bla*$_{CTX\text{-}M\text{-}9}$ gene (n=9/11, 81.8%) and a decreased of Ec-ST131-H30 carried *bla*$_{CTX\text{-}M\text{-}1}$ gene (n=2/11, 18.2%) (**Figure 2B, Figure 2C, Figure 2D**). In the 2019 the ratio between clones remains constant with that observed in 2018 (Ec-ST131-

H30 n=6/23, 26% and Ec-ST1193 n=13/23, 56.5%) with the difference that the Ec-ST131-H30 carrying *bla*$_{CTX-M-9}$ gene (**Figure 2B, Figure 2D**).

Bioinformatic analysis of ST1193 Bolivian isolates

The analysis of the distribution of antibiotic resistance genes among the Bolivian Ec-ST1193 isolates reveled a homogeneous distribution, except for 226 and 242 strains which carrying only *bla*$_{CTX-M-27}$ gene (**Table 2**). The other twenty Bolivian Ec-ST1193 strains showed a MDR phenotype and carried an extensive array of antimicrobial resistance (AMR) genes such as *sul*1 and *sul2* (sulfonamide resistance), *dfr*A17 (trimethoprim resistance), *mph*A (macrolide resistance), *aadA5* (streptomycin resistance), *aph*(3'')-Ib, *aph*(6)-Id (streptomycin phosphotransferase) and *tet*A (tetracycline efflux pump) (**Table 2**). Furthermore, all Bolivian Ec-ST1193 isolates had the same five nonsynonymous mutations in *gyrA* (S83L, D87N), *parC* (S80I), *parE* (L254Q, L416F) housekeeping genes that are known to confer fluoroquinolone resistance confirmed was previously described by Johnson *et al*[6].

Characterization of plasmids revealed that all Ec-ST1193 isolates belonged to the F plasmid family. The replicon types of IncF plasmids were FIA (n = 21) and FIB (n = 22). Further typing of the IncF plasmids identified one prevalent subgroup (21/22, 95.5%), F-:A1:B10, which as one of the most common associated with Ec-ST1193.[6,25] In addition, It has been shown a variable presence of the small-plasmid replicons which included Col156 (n = 21) and Col(BS512) (n = 22) (**Table S1**).

About the virulence factor (VF), the most frequent genes were uropathogenic specific protein (*usp*), autotransporter toxin (*sat*), outer membrane protease (*ompT*), tellurium ion resistance protein (*terC*) genes encoding metal acquisition systems (*chuA*, *fyuA*, *irp*2, *iucC*, *iutA*) and adherence (*yfcV*, *pap*), each of which was detected in 100% of the isolates. Moreover, all Ec-ST1193 isolates possessed the K1 capsular genotype. Two genes were identified in 95.5% (21/22 *E. coli*) of the isolates, including toxins gees such as *senB* and *vat* (**Table 2**). The 25 strain was the only showed the presence of *fimH* gene.

Generation and *in silico* characterization of a global *E. coli* ST1193 database

Among the 29218 isolates only 22785 were confirmed *E. coli* species. Of the 22785 *E. coli* most belonged to ST131 (7%) and ST10 (7%) followed by ST11 (6%). Only a small fraction of the thousands of *E. coli* genomes in the NCBI Genomes database were Ec-ST1193 (0,85%, n = 193).

Phylogenetic analysis

Phylogeny was conducted after filtering recombinant regions using Gubbins[26]. The SNP-based tree of the entire collection was built using 97-88% (5,193,182 bp/4,739,571 bp) of the reference genome SCU-204 and resulting in an alignment with 6,526 SNPs. The SNPs matrix was uniform among all the Ec-ST1193 strains, showing a median of 49 SNPs with a range from a minimum 1 to a maximum 187, respectively. Overall, we identified two distinct clusters (A-B) associated with different capsular type (K1 and K5) (**Figure 3**).

All the Ec-ST1193 isolates from Bolivia clustered together phylogenetically, except for 25 Bolivian strain, and showed a small range of SNPs, from 1 to 13, among them. In contrast, the Bolivian 25 strain clustered with other Ec-ST1193 without a clear correlation with geographic distribution, K-type or other characteristics.

Bioinformatic analysis of 193 Ec-ST1193 isolates

Resistome

Among the 193 no-Bolivian Ec-ST1193, most of which were isolated from humans (**Figure S2**), 169 strains showed a MDR phenotype (87.6%) (**Table 3**, **Table S1**). Screening for acquired resistance determinants revealed genes for aminoglycoside-modifying enzymes (e.g. *aph(6)-Id*, 76,2%; and *aph(3'')-Ib*, 74.6%), sulfonamide (e.g. *sul1*, 46.6%; and/or *sul2*, 76.2%), trimethoprim (e.g. *dfrA*, 67.4%), macrolide (e.g. *mphA*, 57.5%) and tetracycline (e.g. *tet(A/B)*, 70%) resistance; and several β-lactamases, including TEM-1 (56.5%), OXA-1 (4.7%) and CTX-M (34.7%) (**Table 3, Table S1**). More precisely, the majority of CTX-M-positive Ec-ST1193 carrying $bla_{\text{CTX-M-27}}$ (27/67, 40.3%) followed by $bla_{\text{CTX-M-55}}$ (16/67; 23.9%), $bla_{\text{CTX-M-14}}$ (10/67; 14.9%) and $bla_{\text{CTX-M-15}}$ (12/67; 17.9%).

Moreover, all Ec-ST1193 studied harbored the same four non-synonymous mutations in quinolone-resistance determining region (QRDR) (*gyrA* S83L, D87N; *parC* S80I; *parE* L416F) except for UPEC61 that shown only three mutations (*gyrA* S83L, D87N; *parE* L416F) (**Table S1**).

Plasmidome

Using pMLST based on F plasmid alleles, the RepFIA (A1) allele was highly prevalent across Ec-ST1193 isolates. However, three different main alleles of RepFIB were identified (B1, B10, and B20), and these partially segregated according to the phylogeny (**Figure 3**).

Interestingly, the isolates with F1:A1:B20 and F-:A1:B20 were consistently associated with K5 capsular types. On the contrary those with F-:A1:B10 replicons were associated with the K1 capsular type.

The Ec-ST1193 strains also contained small plasmids. The small-plasmid replicons, which included Col156, Col(BS512), ColpVC, Col(MG828), Col828 and Col(phAD28), did not mimic the phylogenetic distribution of the F-type plasmids (**Table S1**).

Virulome

Eighteen virulence factor encoding genes were significantly associated with Ec-ST1193 included the outer membrane hemin receptor gene *chuA* (193/193, 100%), yersiniabactin siderophore receptor gene *fyuA* (193/193, 100%), siderophore yersiniabactin gene *irp2* (193/193, 100%), the glutamate decarboxylase gene *gad* (167/193, 86.5%), adherence protein gene *iha* (188/193, 97.4%), aerobactin synthetase gene *iucC* (187/193, 96.9%), aerobactin receptor gene *iutA* (186/193, 96.4%), capsule polysaccharide export inner-membrane protein gene *kpsT* (156/193, 80.8%), group II capsule with K1 and K5 variants *KpsMII-K1/K5* (190/193, 98.4%), polysialic acid capsule biosynthesis protein *neuC* (156/193, 80.8%), outer membrane protease T gene *ompT* (193/193, 100%), secreted autotransporter toxin gene *sat* (177/193, 91.7%), plasmid-carried enterotoxin gene *senB* (164/193, 85%), iron transport protein *sitA* (191/193, 99%), tellurium ion resistance protein *terC* (191/193, 99%), uropathogenicspecific protein *usp* (191/193, 99%), vacuolating toxin gene *vat* (176/193, 91.2%) and YfC fimbria gene *yfcV* (189/193, 97.9%) (**Table S1, Table 4**).

The majority of Ec-ST1193 were positive for the K1 capsular type (n = 162/193; 83.9%), on the contrary only 28 *E. coli* were positive for the K2 capsular type (14.5%).

Pan-genome analysis

The pan-genome was analyzed by Roary in all strains considered the 100% gene-identity. A total of 10089 genes were found. Of these, 3464 genes (34.3%) composed the core genome. Comparison of pan-genome showed the presence of 73 specific-genes on the twenty-one Ec-ST1193 Bolivian strains, with the exception of 25 strain. 35 of them were identified as hypothetical proteins and 38 were known coding-genes, including metabolic proteins (e.g. *nanS*, *era1*), membrane proteins (e.g. *yjgN*, *yehB*), IS-family transposases (*e.g.* IS4, IS66), siderophore-iron reductase (e.g *fhuF*), Ag43-encoding gene (*flu*) and a toxin component (*cbtA*)[27–29].

On the contrary, in 99% of the other Ec-ST1193 no-Bolivian strains plus the 25 Bolivian Ec-ST1193 strain, 48 specific-genes (13 hypothetical proteins and 35 coding-genes) were detected. Of these, 25 encoded metabolic proteins (e.g., *idnT*, *deoR*), transcriptional regulators (*e.g. kdgR*), transporter family (*e.g. dctM*) and the remaining genes were virulence determinants or toxin-antitoxin system, including the *fimH* operon and toxic proteins *symE* and *higB*.

Discussion

CTX-M-producing *E. coli*

The majority of ESBL-producing bacteria were found carry $bla_{CTX\text{-}M}$ genes (e.g., 99.2%) and among the CTX-M-positive *E. coli* the prevalence of group variant is consistent with previous study where a notable increase of carriage of CTX-M–producing *E. coli* among healthy children living in rural communities of the Bolivian Chaco, was described [16].

Phylogenetic group and distribution of clones

The distribution of phylogenetic groups amongst CTX-M-producing *E. coli* showed that there was a curious increase in the B2 phylogenetic group from 2016 to 2019. Indeed, the results presented here show that the increased prevalence of phylogenetic group B2 in commensal *E. coli* strains isolated from fecal samples in Bolivian Chaco is concomitant with the clonal transition. I particular, until 2017 the O25b-ST131 clone has been found predominant carried $bla_{CTX\text{-}M\text{-}1}$, but, in the 2018 the rate of clones changed deeply showing an emerged of Ec-ST1193 carried $bla_{CTX\text{-}M\text{-}9}$.

This change is likely to have a major clinical impact and underlining the importance of studying epidemiological phenomena in depth.

However, the contribution of the ST131 clone in the spread of CTX-M remains fundamental. Notably, in 2019 ST131 was found carrying both, $bla_{CTX\text{-}M\text{-}1}$, and $bla_{CTX\text{-}M\text{-}9}$.

Bioinformatic analysis

The global comparative genomic analysis shows the presence of two clusters associated with different capsular type (K1 and K5) and this is consistent with the Ec-ST1193 evolution previously described (**Johnson, 2019**). Moreover, all the Ec-ST1193 Bolivian isolates clustered together phylogenetically showing a small range of SNPs (e.g., from 1 to 13) among them, with the exception of one Bolivian strain (e.g., 25) that clustered with other Ec-ST1193 without a clear correlation with geographic distribution, K-type or other characteristics.

The most prevalent Ec-ST1193 studied were coresistant to aminoglycosides, β-lattams, sulfonamides, trimethoprim, tetracyclines, macrolides and clearly to fluoroquinolones showing a MRD profile. Moreover, the virulence factors found among Ec-ST1193 showing a homogeneous distribution. Indeed, no correlation was observed between the presence of resistance genes or virulence factors with the phylogenetic distribution except for the two aspects: capsular type (e.g., K1 or K5) and the presence/absence of *fim* operon that usually highly conserved and extremely common among Ec-

ST1193 isolates (induces *E. coli* adherence to the urothelium cells, and helps the formation of intracellular bacterial biofilms). Interestingly, this latter operon was not found complete in Ec-ST1193 Bolivian strains that clustered together, except for the 25 strain which clusters separately.

These findings showed that the Ec-ST1193 analyzed have a high level of homogeneity independently from the geographic region or different source, in the other hand, the Ec-ST1193 Bolivian strains seems to belong to a new sub-clade which was characterized by lack of *fim*H gene.

In this study we described the clonal distribution among CTX-M-producing *E. coli* in a rural communities of Bolivian Chaco, but the spread of this clone may have been underestimated because the information deriving from the global survey of the ST1193 clone highlights the presence of the latter clone even without CTX-M enzyme.

These findings highlighted the role of the intestinal microbiota as reservoir of emerging MDR clone and suggested the importance of studying epidemiological phenomena.

Further studies of current and historical FQ^r *E. coli* are required to control the evolution of this clone and the spread of resistance determinants within species and within the microbiome providing critical insights into trends in drug resistance among pathogens. Indeed, commensal *E. coli* can convert into opportunists causing extraintestinal infections (e.g., UTIs or sepsis).

Ethics

The study was approved by Ethic Committee (Colegio Médico de Santa Cruz, TDEM CITE No. 005/2016). Written informed consent was obtained by a parent or a legal guardian of each enrolled child.

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Transparency declarations

The authors have no competing interests to declare.

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Table 1. Features of total isolates and CTX-M-positive *E. coli* isolates

	2016[a]	2017[a]	2018[a]	2019[a]	Total[a]
Total of strains	39[b]	103[c]	55[d]	74[e]	**271**
ESBL positive strains	38 (97.4)	101 (98.1)	51 (92.7)	73 (98.6)	**263 (97)**
CTX-M producing strains:	38 (100)	99 (98)	51 (100)	73 (100)	**261 (99.2)**
E. coli	38 (100)	97 (98)	50 (98)	72 (98.6)	**257 (98.5)**
E. cloacae	0	2 (2)	0	0	**2 (0.8)**
K. pneumoniae	0	0	1 (2)	0	**1 (0.4)**
R. ornitolitica	0	0	0	1 (1.4)	**1 (0.4)**
CTX-M producing *E. coli*	38	97[d]	50[e]	72[d]	**257**
Total CTX-M detected:	39	98	52	74	**263**
CTX-M-1	28 (71.8)	78 (79.6)	26 (50)	44 (59.5)	**176**
CTX-M-2	0	1 (1)	1 (1.9)	0	**2**
CTX-M-8/25	0	3 (3.1)	1 (1.9)	2 (2.7)	**6**
CTX-M-9	11 (28.2)	15 (15.3)	24 (46.2)	28 (37.8)	**78**

[a], number (%)

[b], In 2016, one child carried one *Enterobacter* spp.

[c], In 2017, two children carried one *Citrobacter* spp.

[d], In 2018, one child carried one *Enterobacter* spp. and another child carried one *Klebsiella pneumoniae.*

[e], In 2019, one child carried one *Serratia* spp. and another child carried one *Roultella* spp.

[f], one *E. coli* carrying two $bla_{CTX\text{-}M}$ ($bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$)

[g], two *E. coli* carrying two $bla_{CTX\text{-}M}$ ($bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$)

[h], two *E. coli* carrying two $bla_{CTX\text{-}M}$ (one $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$ and the other one $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}8}$)

Figure 1. Phylogenetic groups distribution amongst the 257 CTX-M-producing *E. coli* per years

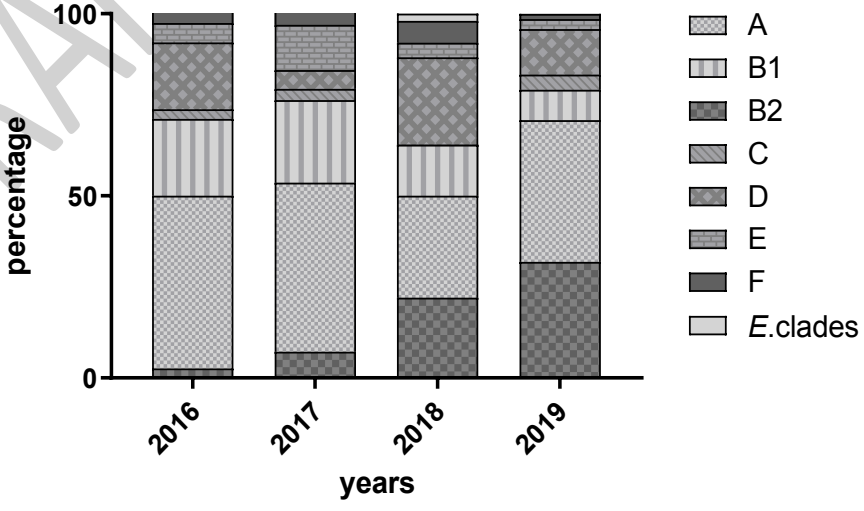
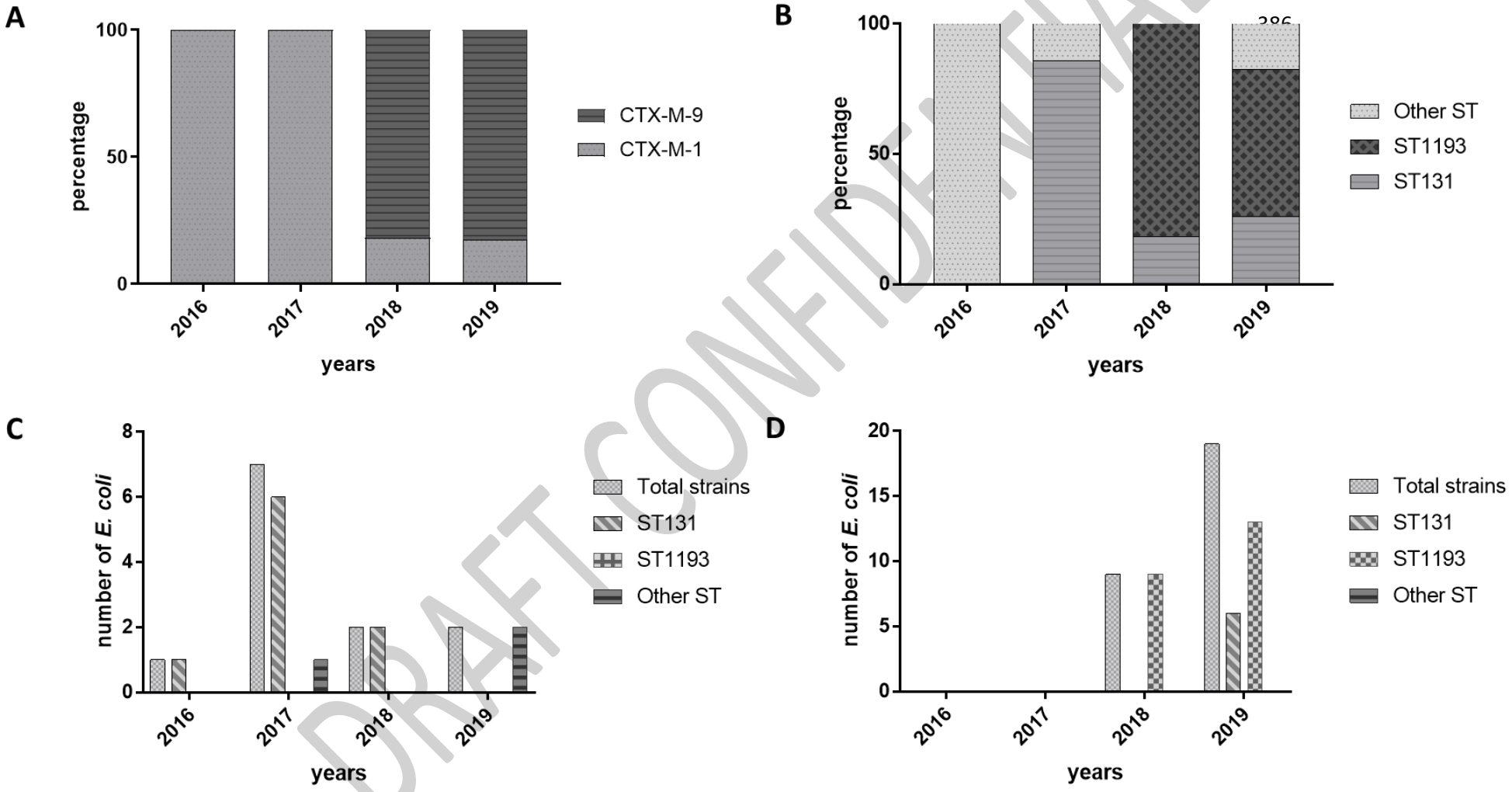


Figure 2. Analysis of CTX-M-producing *E. coli* belonging to the phylotype B2. (A) Prevalence of CTX-M groups in CTX-M-producing *E. coli* over the years. (B) Rates of ST131 and ST1193 clones carrying $bla_{CTX\text{-}M}$ genes over the years. (C) Clones distribution among CTX-M-1 producing *E.coli* over the years. (D) Clones distribution among CTX-M-9 producing *E. coli* over the years.



387 **Table 2 Features of twenty-two Bolivian Ec-ST1193 genomes.**

Strains	Source	Serotype	FimH type	ESBL type	Phylogroup	Chromosomal mutations defining quinolone resistance	ST_Plasmid	Resistant genes	Virulence factor genes
25	feces	O75:H5	*fimH64*	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F2:A-:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fimH, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
41	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
81	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
104	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
115	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
199	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
204	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
207	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
222B	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
226	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	-	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
242	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	-	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
322	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
332	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
358	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
362	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*

388 **Table 2 (continued)**

363	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
364B	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
366	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
371	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
375	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
387	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*
421	feces	O75:H5	–	CTX-M-27	B2	*gyrA* S83L, D87N; *parC* S80I; *parE* L416F	F-:A1:B10	*sul1* , *sul2* , *dfr* A17, *mph* A, *aadA5* , *aph* (3'')-Ib, *aph* (6)-Id, *tetA, ermB,*	*chuA, fyuA, irp2, iucC, iutA, neuC, ompT, papB-I, sat, senB, sitA, terC, usp, vat, yfcV*

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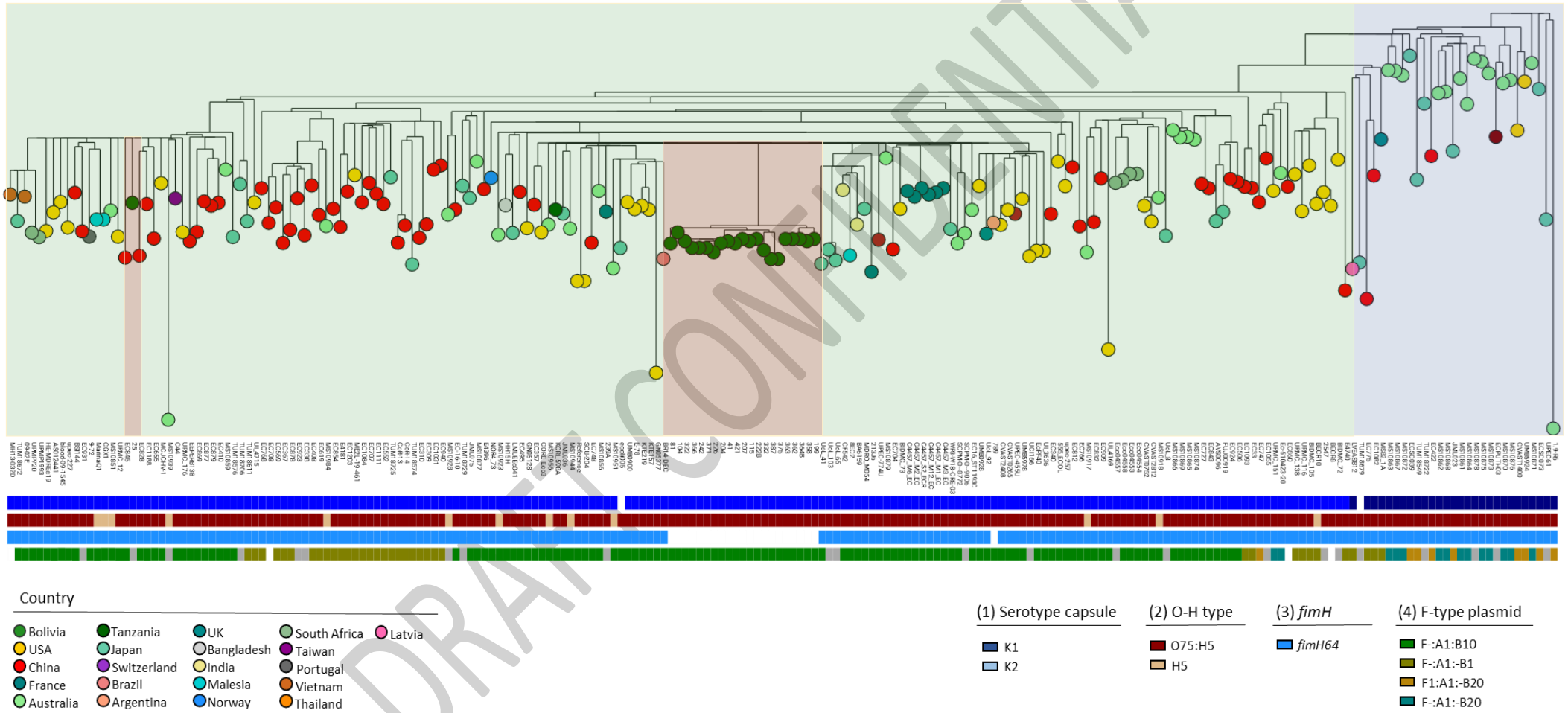
Figure 3. Phylogenetic analysis and main features of 215 Ec-ST1193 studied. The two clusters associated with different capsular type, K1 and K5, are highlighted by blue and green shadows, respectively. The red shadow identifies the Bolivian Ec-ST1193. Squares, colored by trait category, represent the presence of a trait examined. The different country is indicated by colored circle.



405 **Table 3. Percentage of antimicrobial resistance genes in Bolivian Ec-ST1193 stains in comparison with other no-**
406 **Bolivian Ec-ST1193 strains.**

		BOLIVIAN ISOLATES N (%)	NO-BOLIVIAN ISOLATES N (%)	TOTAL N (%)
Aminoglycosides	*aadA5*	20 (90.9)	84 (43,5)	104 (48.4)
	aph(6)-Id	20 (90.9)	147 (76.2)	167 (77.7)
	aph(3'')-Ib	20 (90.9)	144 (74.6)	164 (76.3)
	aac(3)-IIa	0	8 (4.1)	8 (3.7)
	aac(3)-IId	0	48 (24.9)	48 (22.3)
	aac(6')-Ib	0	10 (5.2)	10 (4.7)
	rmtB	0	2 (1)	2 (0.9)
β-lactams	bla_{TEM}	0	109 (56.5)	109 (50.7)
	bla_{CTX-M}	22 (100)	67 (34.7)	89 (41.4)
	bla_{OXA}	0	9 (4.7)	9 (4.2)
	bla_{CMY}	0	3 (1.6)	3 (1.4)
Sulfonamides	*sul1*	20 (90.9)	90 (46.6)	110 (51.2)
	sul2	20 (90.9)	147 (76.2)	167 (77.7)
Trimethoprim	*dfrA*	20 (90.9)	130 (67.4)	150 (69.8)
Tetracyclines	*tetA/B*	20 (90.9)	135 (70)	155 (72.1)
Macrolides	*mph(A)*	19 (86.4)	111 (57.5)	130 (60.5)
	erm(B)	14 (63.6)	11 (5.7)	25 (11.1)
Fenicols	*floR*	0	2 (1)	2 (0.9)
	cat	0	7 (3.6)	7 (3.3)
Fosfomycin	*fosA3*	0	1 (0.5)	1 (0.5)
Quiolones	*qnrB*	0	1 (0.5)	1 (0.5)
MDR phenotype		20 (90.9)	169 (87.6)	187 (87)

407 **Table 4. Virulence-associated traits of *E. coli* ST1193 genomes include in the study**

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Category	Gene(s) or operon	Product/Function	%
Adhesins	*csgB-G*	curli	99.5
	fim	D-mannose-specific adhesin, type-1 fimbiae	90.2
	yfcV	fimbrial protein	98.1
	iha	Iron-regulated-gene-homologue adhesin	97.7
	pap	Pilus associated with pyelonephritis (P fimbriae)	95.8
Toxins	*sat*	Secreted autotransporter toxin (serine proteasi)	92.6
	vat	Vacuolating autotransporter toxin	90.7
	senB	plasmid-encoded enterotoxin	84.6
Nutrition	*chuA*	Heme receptor	100
	fyuA, irp	Yersinabactin (siderophore) receptor, synthesis	99.5
Iron uptake	*iuc*	Aerobactin (siderophore) synthesis	97.2
	iutA	Aerobactin receptor	96.7
	sitA	Iron/manganese transport protein, periplasm atic-binding protein	99.1
Protectins	*kpsMTII*	Group II capsule synthesis (e.g., K1, K5, K10, K12)	100
	neuC	Polysialic acid biosinthesis of the K1 capsule	82.8
	traT	Conjugal transfer surface exclusion protein, serum resistance --	14.9
Invasins	*ompA*	Outher membrane protein A (cellular invasion)	100
Misc.	*ompT*	outer membrane (protease)	100
	usp	putative bacteriocin	99.1
	gad	glutammate decarboxylase	77.7
	terC	Tellurium iron resistance protein	98.6

Mic., miscellaneous

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