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ANTIMICROBIAL RESISTANCE IN COMMENSAL AND PATHOGENIC BACTERIA FROM RESOURCE-LIMITED SETTINGS

PHD STUDENT
Maria Micieli

SUPEVISOR
Prof. Gian Maria Rossolini

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Last but certainly not least, I wish to express my deep appreciation to all my colleagues and friends, particularly Selene and Tiziana. I'm profoundly grateful to them for never ceasing to believe in me, even for a single moment.

FOREWORD

This thesis is divided in six sections: general introduction, aim of the PhD project, results and discussion, conclusions and perspectives, selected bibliography and annexes.

The annexes include the related material that has been published to international scientific journals in relation to the thesis work and also two papers published in PhD period, but not discussed in the present work.

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PART I GENERAL INTRODUCTION

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PART I

GENERAL INTRODUCTION

1
ANTIBIOTIC RESISTANCE

Antibiotics are a class of drugs that can bind to specific bacterial targets, inducing a reversible (bacteriostatic) or irreversible (bactericidal) inhibition of bacterial growth. These compounds selectively target vital bacterial functions or growth processes, primarily aiming at disrupting or eliminating the bacterial cell wall, the cell membrane, or critical bacterial enzymes responsible for nucleic acid and protein synthesis (Monserrat-Martinez et al., 2019).

Since Alexander Fleming's discovery of penicillin in 1929 and its widespread use, in 1940, Abraham and Chain reported the first case of penicillin resistance in *Escherichia coli* due to the production of an enzyme called penicillinase (Abraham and Chain, 1940). Up to now, no class of antibiotics has been exempt from this persistent phenomenon (Figure 1). For several decades, the issue of antibiotic resistance has been mitigated by the continuous introduction of new antibiotics. However, this progress has significantly slowed in recent years, leading to a rise in the prevalence of antibiotic-resistant bacterial pathogens (WHO and World Health Organization, 2020).

Antibiotic treatments have improved clinical outcomes for infections, reducing morbidity and mortality in surgical, transplant, cancer, and critical care patients. With the use of powerful broad-spectrum antibiotics, selective pressures have turned antibiotic resistance into a pressing global concern (Kon and Rai, 2016). In previous times, resistant infections were mainly connected to hospitals and care settings. However, in the last decade, the world has witnessed the emergence of difficult-to-treat infections within the general community as well (O'Neill, 2016). Indeed, while obtaining precise estimates of drug resistance is challenging, projections indicate that antimicrobial-resistant infections could result in nearly 10 million annual deaths by 2050 unless appropriate measures are implemented (O'Neill, 2016).

The use of these molecules, not only linked to overuse in the clinical field, but also in the agricultural and zootechnical field, has contributed to increasing phenomenon of resistance, even in potentially pathogenic bacteria for humans. Increasing numbers of hospital-acquired infections are now caused by multidrug-resistant pathogens, making treatment progressively difficult and antibiotic choice increasingly limited.

The spread of multidrug-resistant microorganisms (MDR), which are resistant to more than one antimicrobial agent, extensively drug-resistant (XDR) strains, which are only sensitive to one or two classes of antibiotics, and pan drug-resistant (PDR) strains, which are resistant to all known categories of antimicrobials, combined with the lack of development of new antibiotics, has resulted in the so-called "Antibiotic resistance crisis", a serious global health issue (Michael et al., 2014). Another exacerbating factor in this problem is the proliferation of highly virulent strains, known as "high-risk clones", such as *E. coli* ST131 and *Klebsiella pneumoniae* ST258. These clones, due to their ability to reproduce rapidly and survive for extended periods, play a significant role in the diffusion of antibiotic resistance (Mathers et al., 2015).

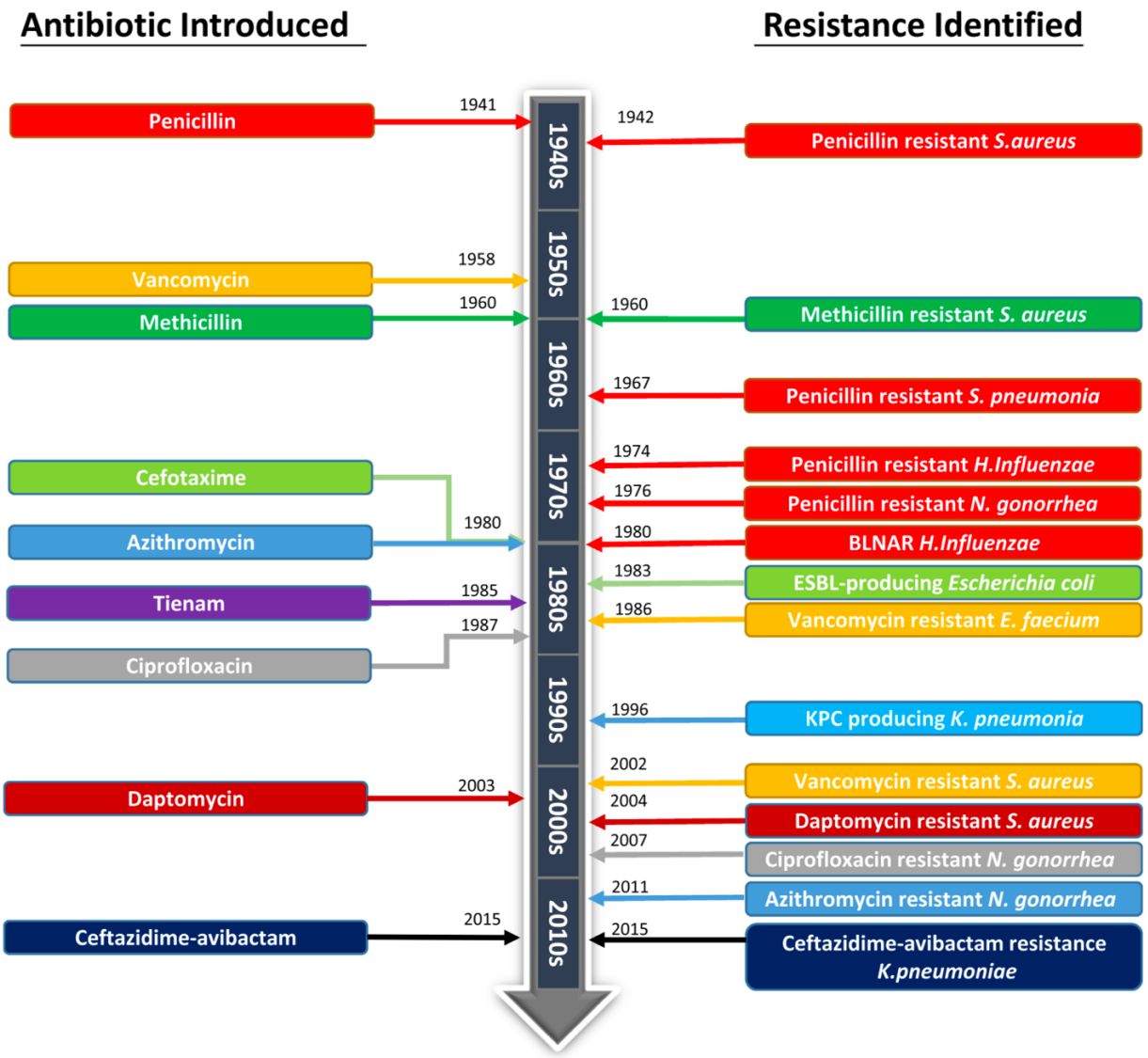

Figure 1. Abbreviated timeline by year of antibiotic introduction and organism with specific resistance identified. BLNAR – b-lactamase negative ampicillin resistant; ESBL, extended spectrum b-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase. (Lai et al., 2022)

2
ANTIBIOTIC RESISTANCE MECHANISMS

Antibiotic resistance in bacteria can result from two main mechanisms: vertical and horizontal transmission (Figure 2) (Dantas and Sommer, 2014). Vertical transmission involves the gradual accumulation of genetic changes as a part of the natural genome replication process, resulting in the inheritance of antibiotic resistance across generations. In contrast, horizontal transmission involves the exchange of resistant genes between different bacteria through several processes like transformation (where bacteria acquire resistance genes from deceased bacterial cells), transduction (the transfer of resistance genes by bacteriophages), and conjugation (gene transfer between bacterial cells via conjugative pili) (Dantas and Sommer, 2014).

When antibiotic resistance is inherent to a bacterial species, it is termed "intrinsic resistance" and is typically specific to that species, being passed down vertically to progeny. Intrinsic resistance mechanisms often involve factors such as low-affinity targets, the absence of specific targets, reduced drug uptake, or increased drug efflux (Arzanlou et al., 2017).

For instance, *Mycoplasma* spp. are intrinsically resistant to beta-lactams due to their lack of peptidoglycan, while gram-negative bacteria are intrinsically resistant to glycopeptides because these drugs cannot penetrate the external cell membrane.

Conversely, when a typically susceptible bacterial strain becomes resistant to an antimicrobial agent, it is referred to as "acquired resistance." Acquired resistance can result from mutations in indigenous genes or the acquisition of additional genes from other bacteria, potentially leading to lateral spread of resistance among bacterial populations (von Wintersdorff et al., 2016).

From a biochemical perspective, antibiotic resistance in both gram-positive and gram-negative bacteria can be attributed to various factors, including i) the loss of porins, which reduces the outer membrane's permeability to antibiotics (e.g., *K. pneumoniae* reduces expression of OmpK35 and OmpK36, limiting carbapenem uptake); ii) modification of antibiotic targets, resulting in reduced affinity (e.g., Methicillin-resistant *Staphylococcus aureus* alters penicillin-binding proteins like PBP2a, reducing beta-lactam antibiotic binding); iii) production of enzymes capable of hydrolyzing antibiotics, preventing their binding to their targets (e.g., Enterobacterales, including *E. coli*, commonly produce beta-lactamase enzymes, such as CTX-M, that cleave beta-lactam antibiotics, rendering them ineffective); and iv) increased export of antibiotics through efflux pumps (e.g. *Pseudomonas aeruginosa* employs efflux pump systems, such as MexAB-OprM, contributing to

resistance against various antibiotic classes (Figure 3) (D'Andrea et al., 2013; Ma et al., 2021; Makgotlho et al., 2009; Shi et al., 2013). These mechanisms may act independently or in combination, ultimately leading to resistance against multiple classes of antibiotics.

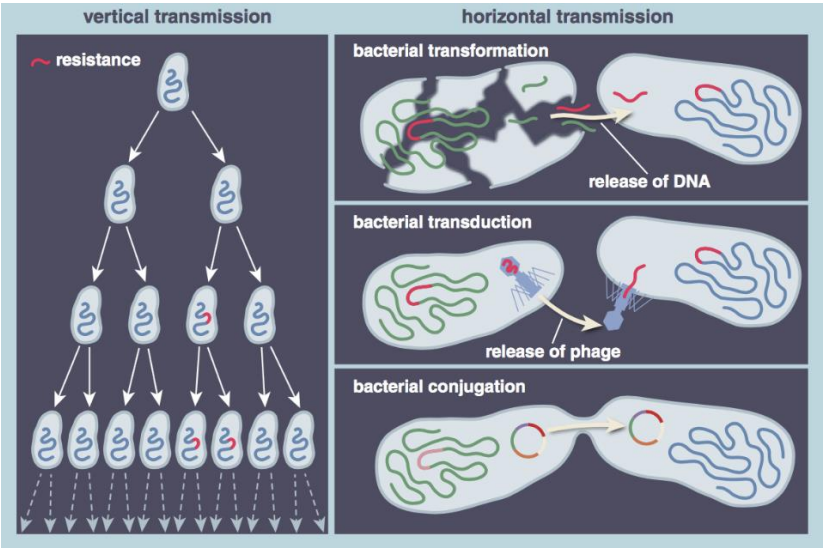

Figure 2. Antibiotic resistance can be acquired in two basic ways: vertical and horizontal transmissions. (Dantas and Sommer, 2014)

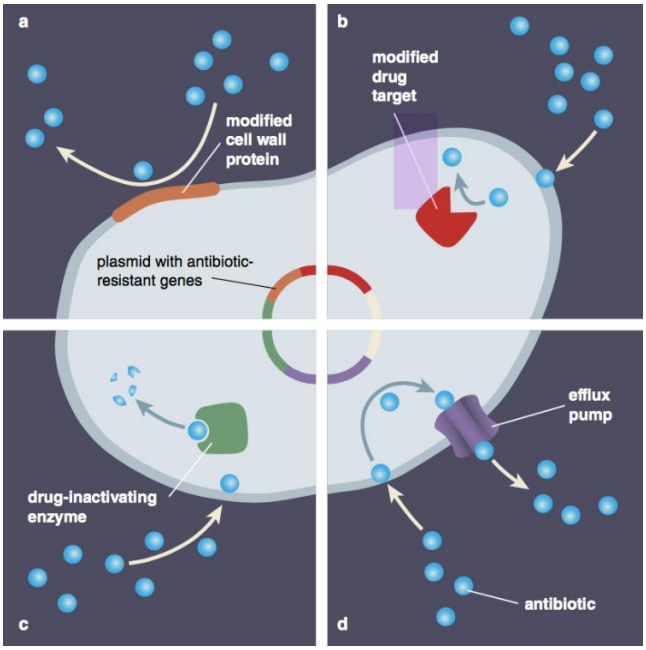

Figure 3. Four mechanisms of resistance: impermeable barrier (a); target modification (b); drug-inactivating enzyme (c); efflux pump (d). (Dantas and Sommer, 2014)

3
ANTIBIOTIC RESISTANCE IN RESOURCE-LIMITED COUNTRIES

Antibiotic resistance is a significant global public health concern, with particularly severe implications in resource-limited countries (RLCs). In these countries, bacterial infections remain a leading cause of illness and death, especially among children (WHO, 2022).

In 2019, according to the World Health Organization (WHO), infectious diseases accounted for five out of the top ten leading causes of death in RLCs, contributing to 55% of the total 55.4 million global deaths (WHO, 2020).

Bacterial pathogens are a major contributor to these infectious diseases, causing conditions such as acute respiratory infections, diarrheal diseases, tuberculosis, and other less common but serious infections (e.g., meningitis, typhoid fever, plague).

Considering the crucial role that antibiotics play in reducing illness and fatalities resulting from bacterial infections, antibiotic resistance represents a significant challenge in RLCs.

Murray et al. estimated that, in 2019, bacterial antibiotic resistance directly caused 1.27 million deaths globally, with an additional 4.95 million deaths associated with bacterial antibiotic resistance (more than HIV, TB and Malaria combined) (Figure 4) (Murray et al., 2022).

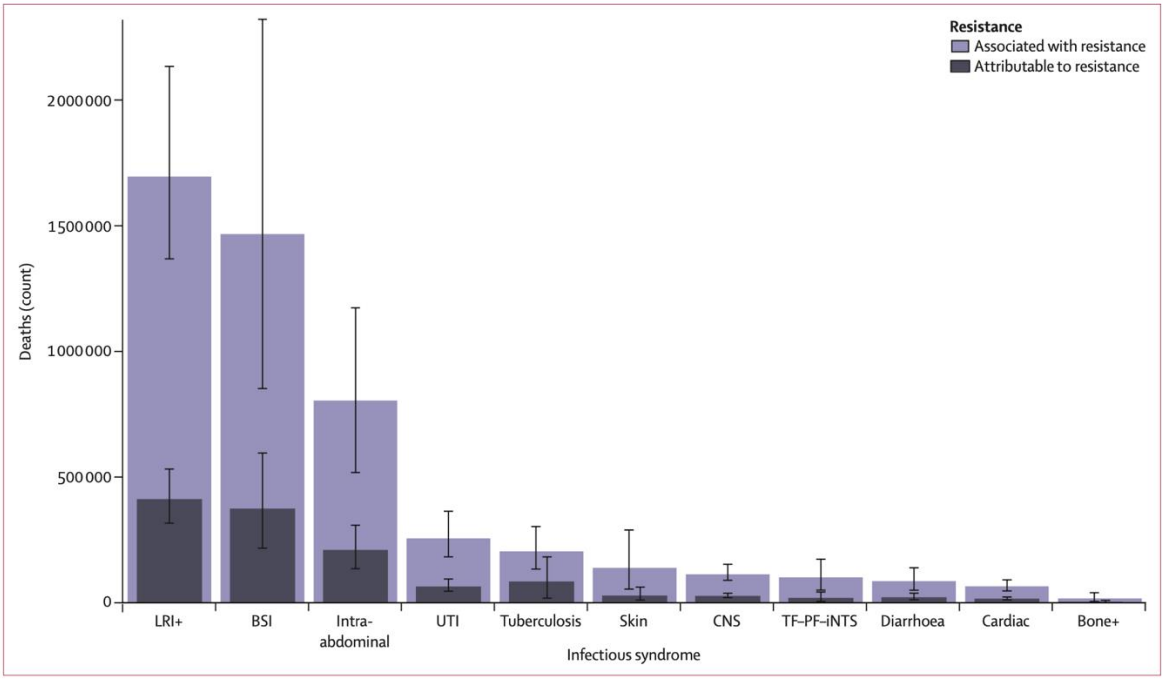

Figure 4. Global deaths (counts) attributable to and associated with bacterial antimicrobial resistance by infectious syndrome, 2019 (Murray et al., 2022)

Moreover, their analysis revealed that all-age death rates attributable to AMR were most pronounced in certain RLCs, underscoring that AMR is not only a significant global health concern but also a particularly grave issue for some of the poorest countries in the world (Murray et al., 2022).

The elevated prevalence of antibiotic resistance in RLCs can be attributed to various factors, including the inappropriate use of antibiotics and unique environmental conditions such as overcrowding and inadequate sanitation (Figure 5). Many of these contributing factors are closely linked to poverty, which encourages the misuse of antibiotics. For instance, there is often a lack of knowledge among healthcare professionals, insufficient financial resources to obtain the appropriate quantity of antibiotics, inadequate laboratory facilities, a healthcare system that doesn't meet standards, and the presence of substandard or counterfeit drugs (Batista et al., 2020; Collignon et al., 2018; Torres et al., 2021). Moreover, poverty-related conditions, such as limited access to hygienic facilities, crowded living environments, and the consumption of contaminated food and water, facilitate the proliferation of resistant bacteria (Collignon et al., 2018).

Furthermore, numerous studies have shown that antibiotic use in animal agriculture contributes to the presence of resistant organisms in both humans and the surrounding environment (Arcilla et al., 2016; Delahoy et al., 2018) (Figure 5). The spread of these drug-resistant bacteria through animal products and environmental discharge amplifies the development of drug resistance. Initially selected during animal production, these resistant organisms can come into contact with human hosts through direct interaction with animals, the distribution of animal waste in the surroundings, and the consumption of animal products (Delahoy et al., 2018; Graham et al., 2008).

While some high-income countries have introduced regulations to limit antibiotic use in animal farming, RLCs are expected to double their antibiotic usage in animal production by 2030 (Van Boeckel et al., 2015). This increase in industrial animal farming, driven by global demand for meat, relies on cost-saving measures and often lacks strict regulations (Sulis et al., 2022).

The consumption of antibiotics has experienced a significant surge since their introduction, and in most RLCs, they now constitute the largest category of pharmaceuticals purchased. Misuse of antibiotics is widespread in RLCs and can manifest as inappropriate prescriptions or incorrect dosages (Batista et al., 2020).

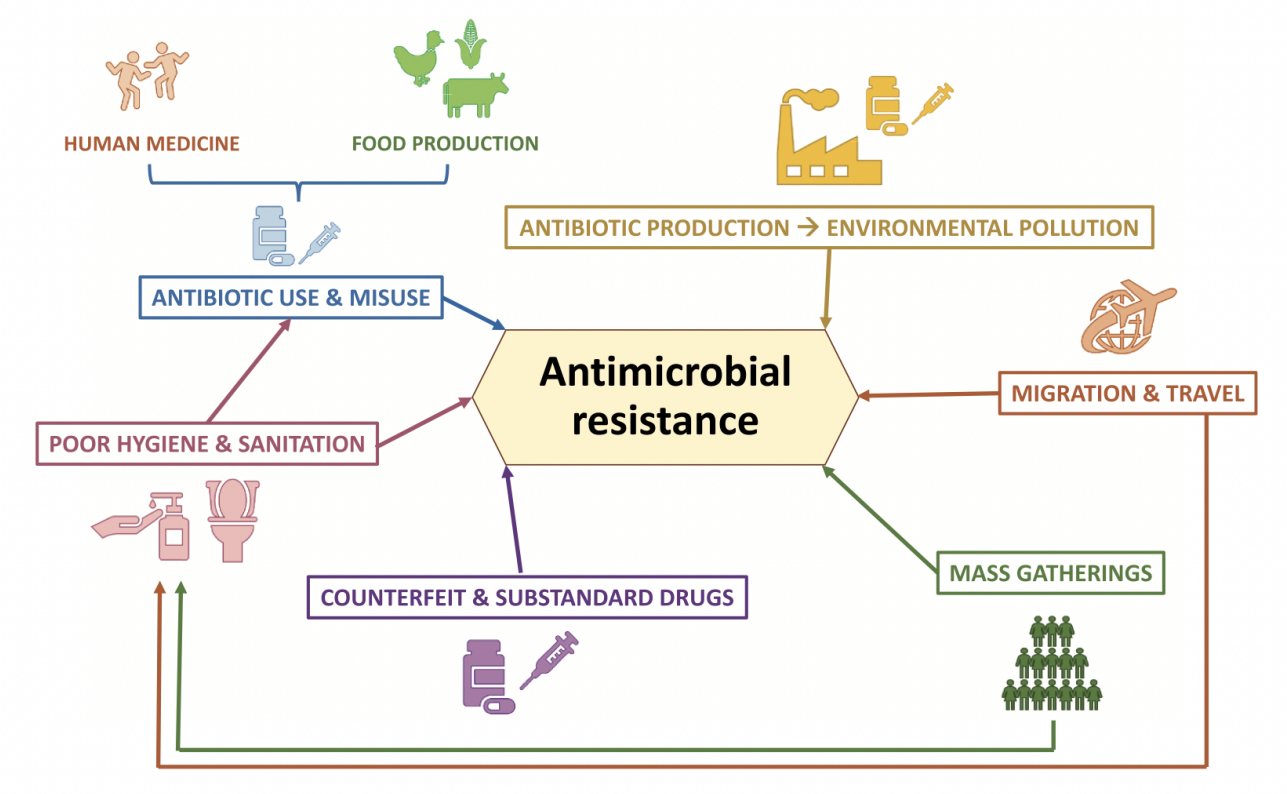

Figure 5. Key drivers of antimicrobial resistance in resource-limited countries (Sulis et al., 2022)

4
SURVEILLANCE AS A WEAPON TO FIGHT ANTIMICROBIAL RESITANCE

Monitoring microbial resistance is undeniably recognized as one of the initial measures to address the antimicrobial therapy crisis (Rossolini et al., 2014). Surveillance plays a pivotal role in furnishing data concerning the extent and patterns of resistance. This information can serve multiple purposes, including i) assisting clinicians in optimizing empirical treatment, ii) aiding policy makers in establishing or revising standard treatment protocols, intervention strategies, and the implementation of infection control measures, and iii) supporting researchers engaged in antimicrobial resistance studies.

In high-income countries, well-established surveillance systems offer a comprehensive view of drug resistance prevalence among common bacterial pathogens in various settings, facilitating the tracking of AMR trends. However, in most RLCs, such well-structured and comprehensive systems are notably absent (Sulis et al., 2022). This absence makes it even more challenging to estimate the true prevalence and burden of diseases caused by antimicrobial-resistant bacteria.

Surveillance trough commensal bacteria

There is a growing body of evidence suggesting that non-clinical isolates, including the commensal bacteria found in humans, animals, plants, and soil, may play a significant role in antibiotic resistance (Leekitcharoenphon et al., 2021; Munk et al., 2018; von Wintersdorff et al., 2016). While commensal bacteria are not the primary target, they are constantly exposed to the selective pressure created by antibiotic treatments, which can lead to the development of acquired resistance. Consequently, commensal bacteria can serve as a crucial reservoir for both i) resistant strains with the potential to become pathogenic and ii) resistance genes that could be transferred to pathogenic bacteria (Pallecchi et al., 2004; von Wintersdorff et al., 2016) (Figure 6).

It is well-documented that commensal bacteria can serve as a significant reservoir for resistance genes that can be transferred to more pathogenic hosts (Urban-Chmiel et al., 2022). For instance, the transfer of resistance genes has been observed from commensal enterobacteria to *Shigella* spp. and *Salmonella* spp. (Blake et al., 2003).

In light of this knowledge, acquired antibiotic resistance in the commensal microbiota is considered a valuable indicator for monitoring the phenomenon of microbial drug resistance. It serves multiple purposes, including i) measuring the selective pressure generated by antibiotic consumption, ii) assessing the impact of changes in antibiotic prescription policies, and iii) predicting the emergence of resistance in pathogens. The human gastrointestinal tract, particularly the distal ileum and colon, harbors an immense population of bacteria. Resistance patterns of certain members of the intestinal microbiota, such as *E. coli*, have been studied in various epidemiological contexts (Giani et al., 2018; Hesp et al., 2021; Liu et al., 2016; Nji et al., 2021; Pallecchi et al., 2012; Purohit et al., 2019).

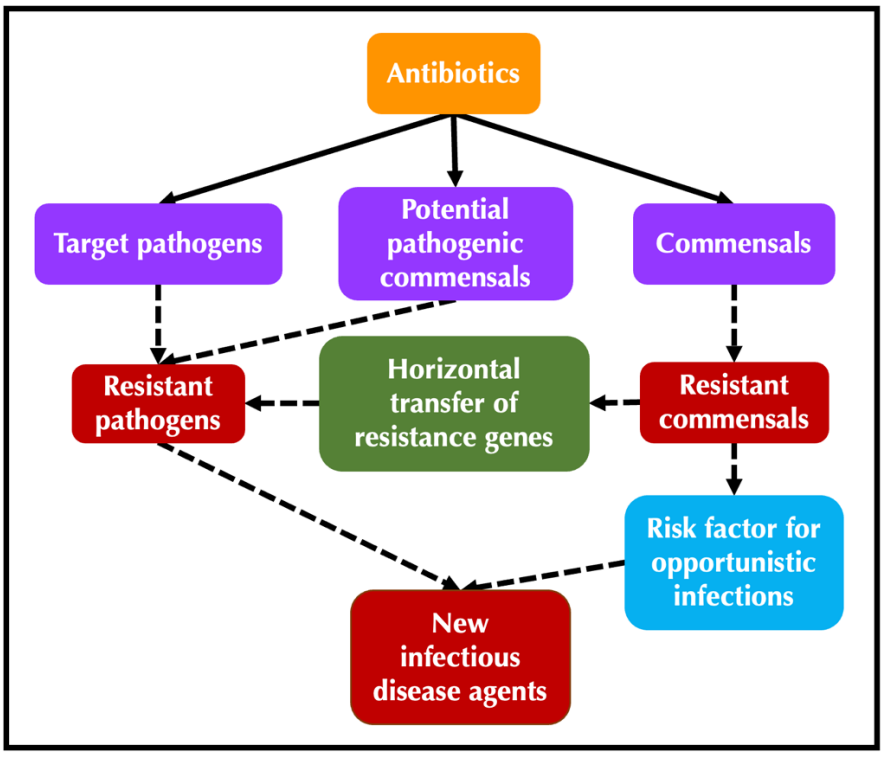

Figure 6. Commensal bacteria as important reservoir of resistant strains and resistance genes.

5
ENTEROBACTERALES AND THE ROLE OF COMMENSAL BACTERIA IN DISSEMINATING RESISTANCE GENES

Enterobacterales constitute the largest group of clinically significant gram-negative bacteria. They are responsible for a wide spectrum of infectious diseases, including bacteremia, pneumonia, urinary tract infections, intra-abdominal infections like gastroenteritis and enteritis, skin infections (particularly in wounds), and soft tissue infections. These bacteria can either be mobile or non-mobile, do not form spores, and can thrive in both aerobic and anaerobic environments.

Enterobacteria are omnipresent microorganisms that typically inhabit the human intestines and those of most animals. However, they can also exist as benign colonizers on the skin, in the female genital tract, and in the tracheobronchial system, where they serve various functions within the host's body, such as acting as a biological defense against potential pathogenic bacteria. Nonetheless, they can transform into opportunistic pathogens when they invade sites beyond their usual habitat, such as entering the bloodstream following abdominal surgeries.

Moreover, some commensal enterobacteria can shift into pathogens after acquiring particular virulence factors through horizontal gene transfer. For example, strains of *E. coli* can evolve into uropathogenic or diarrheagenic variants.

The use of antibiotics plays a pivotal role in the acquisition of resistance by commensal bacteria. Antibiotic therapy not only affects the targeted pathogens but also impacts the commensal microorganisms residing in the human host. This can lead to the development of antibiotic tolerance, which may subsequently be transferred from commensal bacteria to pathogenic microorganisms through horizontal gene transfer mechanisms. For instance, Figure 7 depicts the impact of antibiotic administration on the bacterial community of the colon. Following the initiation of treatment, an escalation in the population of resistant bacteria becomes evident. This rise can be attributed to either a previously susceptible bacterium acquiring resistance or the proliferation of pre-existing resistant bacteria, initially present in small quantities, due to their capacity to withstand the selective pressure imposed by the antibiotic (Jernberg et al., 2010) (Figure 7).

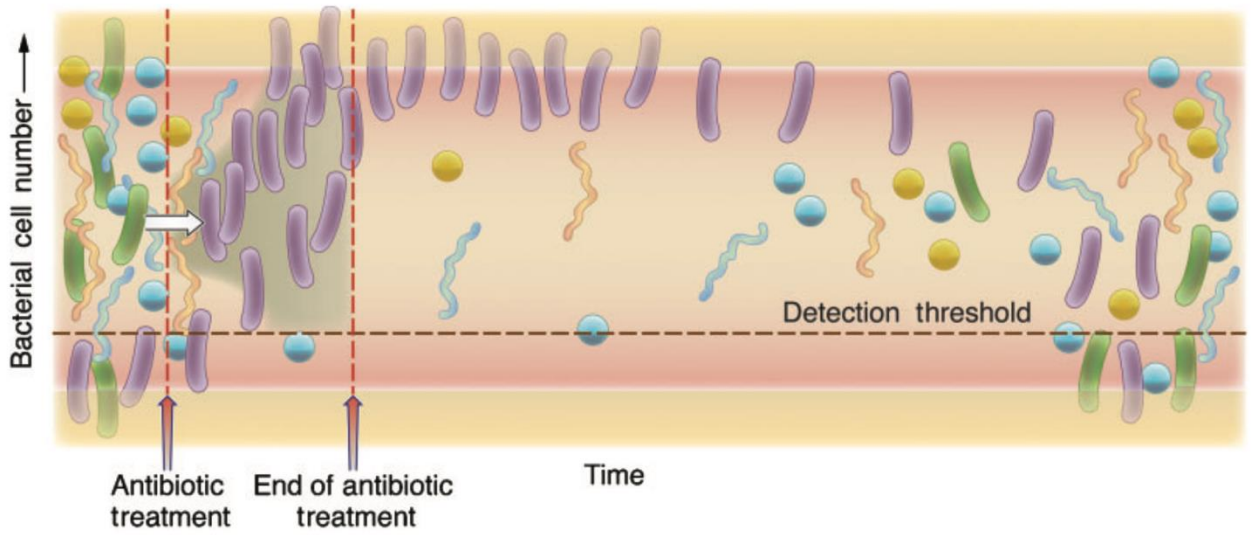

Figure 7. Representation of the impact of antibiotic administration on the bacterial community of the colon. Purple rods, resistant bacteria; green rods, susceptible bacterium becoming resistant; white arrow, horizontal gene transfer or mutation events. (Jernberg et al., 2010)

6
THE ROLE OF HIGH-RISK CLONES IN THE SPREAD OF MULTIFRUG RESISTANCE

In order to be classified as a 'successful' high-risk MDR clone, isolates must exhibit specific distinguishing attributes. These encompass the following key features: (1) global distribution across diverse geographical regions, (2) possession of a wide array of antimicrobial resistance determinants, (3) capability to establish and maintain prolonged colonization within host organisms, (4) efficient transmission potential among diverse host populations, (5) demonstrated elevated pathogenicity and adaptability, and (6) direct association with severe and recurring infections (Mathers et al., 2015).

High-risk clones have had a profound impact on the worldwide dissemination of multidrug resistance, serving as crucial vehicles for the transfer of various genetic elements, including plasmids and resistance genes (Woodford et al., 2011). These globally distributed high-risk clones can be recognized in a variety of bacterial pathogens. Particularly, they are frequently found in species like *S. aureus*, *P. aeruginosa*, and various members of the Enterobacterales group, including *E. coli*, *K. pneumoniae* and the *Enterobacter cloacae* complex (Woodford et al., 2011).

The global spread of multidrug-resistant *E. coli* pandemics can be attributed primarily to specific high-risk clones, such as *E. coli* sequence type 131 (ST131) (Figure 8) (Mathers et al., 2015). Furthermore, a novel MDR high-risk clone of *E. coli*, ST1193 has recently emerged rapidly across the globe, mimicking the most successful high-risk clone of *E. coli*, ST131 (Figure 9) (Kocsis et al., 2022; Pitout et al., 2022).

Figure 8. Global dissemination of *E. coli* ST131 clone. Red stars indicate isolates producing ESBL enzymes, and blue stars indicate fluoroquinolone-resistant, non-ESBL-producing isolates (Nicolas-Chanoine et al., 2014).

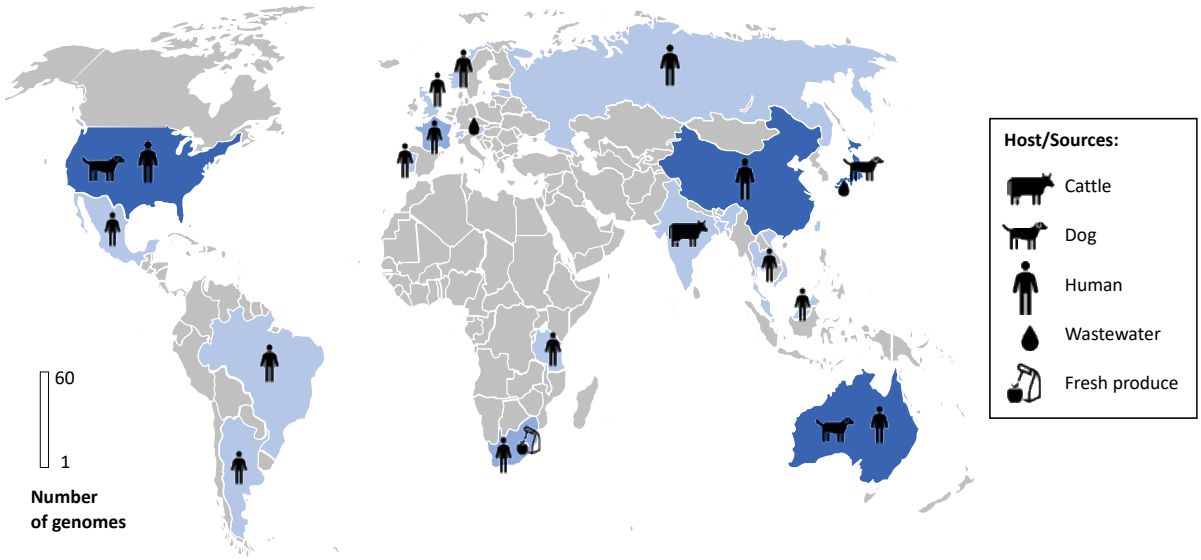

Figure 9. Global dissemination and host associations of *E. coli* ST1193 based on genomic data from NCBI database.

7
EMERGING RESISTANCE TO EXPANDED-SPECTRUM CEPHALOSPORINS IN ENTEROBACTERALES

Resistance to expanded-spectrum cephalosporins in Enterobacterales is primarily conferred by the production of extended-spectrum beta-lactamases (ESBLs), plasmid-mediated AmpC-type enzymes, and carbapenemases (De Angelis et al., 2020). ESBLs have the ability to hydrolyze penicillins, narrow- and expanded-spectrum cephalosporins, and monobactams, but not carbapenems and cephamycins (Castanheira et al., 2021).

ESBL-producing strains are a major clinical concern due to their rapid, widespread dissemination and frequent multidrug resistance, resulting in limited treatment options. Recognized by the WHO as "priority pathogens" for their substantial threat to human health and the urgent demand for new antibiotics, these infections also contribute to elevated morbidity, mortality, and healthcare costs (Ling et al., 2021; Tacconelli et al., 2018).

Many ESBLs have evolved from TEM-1 and SHV-1 beta-lactamases, initially found in Gram-negative bacteria and capable of conferring resistance to penicillins and narrow-spectrum cephalosporins (Castanheira et al., 2021). However, a new lineage of beta-lactamases, the CTX-M-type ESBLs, emerged in the early 2000s and rapidly spread in *Enterobacteriaceae*, and actually being recognized as the most common ESBL group. These CTX-M enzymes exhibit significant sequence diversity, with about 200 variants categorized into six major phylogenetic groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25, and KLUC (Figure 10) (Mendonça et al., 2022). Among these groups, CTX-M group 1 (e.g., CTX-M-1 and CTX-M-15) and 9 (e.g., CTX-M-14 and CTX-M-27) are the most frequently observed, with CTX-M-15 representing the most widely distributed variant within clinical Enterobacterales worldwide (Castanheira et al., 2021).

The dissemination of CTX-M-type ESBLs has been further facilitated by epidemic plasmids associated with highly successful clonal lineages of *E. coli* and *K. pneumoniae*, referred to as high-risk multiresistant and virulent clones circulating worldwide (Castanheira et al., 2021). The paradigmatic example is the pandemic *E. coli* ST131 clone (phylogenetic group B2), which significantly contributed to the global spread of CTX-M-15, one of the most widely distributed CTX-M allelic variants globally (Kocsis et al., 2022).

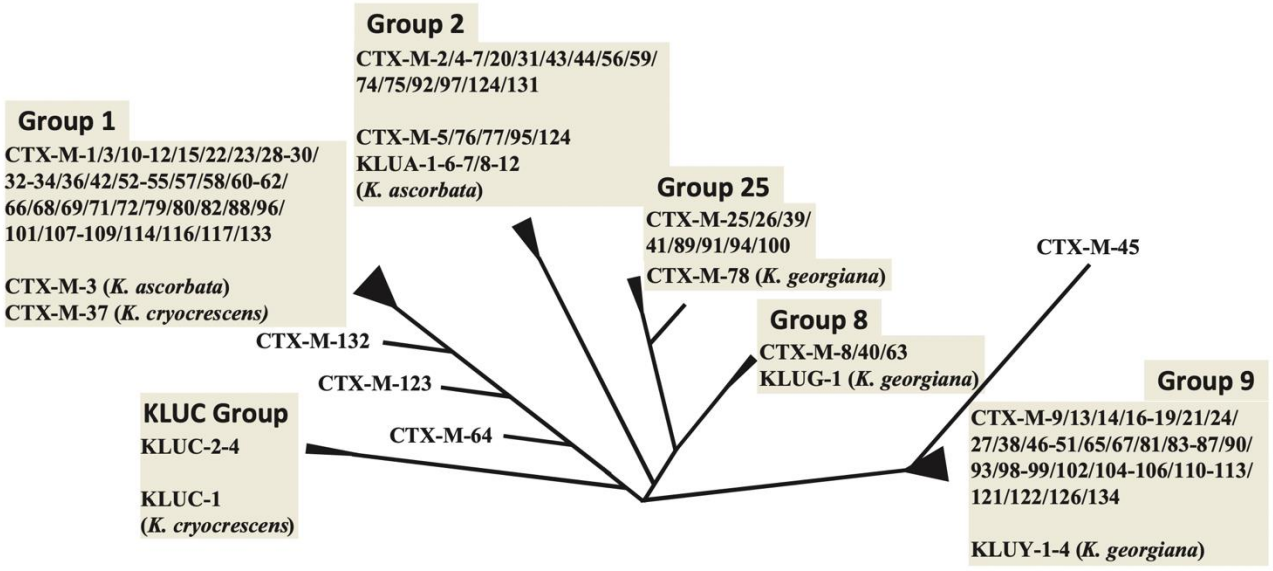

Figure 10. Tree diagram showing the similarity among enzymes of the CTX-M lineage and clustering of members of different CTX-M groups (D'Andrea et al., 2013) .

8
RESEARCH ACTIVITIES OF THE UNIVERSITY OF FLORENCE IN THE BOLIVIAN CHACO

In the entire Bolivian Chaco region and its neighboring territories in Argentina and Paraguay, Franciscan missionaries have been actively promoting human development for many years, with a particular focus on public health and prevention. In 1982, the Vicariate of Cuevo entered a 'convention' with the Bolivian Ministry of Health, known as the *Convenio Ministerio de Salud – Vicariato de Cuevo*, aimed at developing basic healthcare services throughout the region in accordance with the principles outlined in the 1978 Alma-Ata Declaration. Additionally, the Convention oversees a technical school named *Tekove Katu*, which was established to address the immediate needs of the region. *Tekove Katu* has been offering vocational courses since 1985 and is an integral part of the Bolivian Pluralistic System for Education and Health. In 2009, it was designated as a model school by the WHO.

The University of Florence, specifically the Unit of Infectious and Tropical Diseases, has been collaborating with the Ministry of Health of the Plurinational State of Bolivia for over 30 years in clinical and epidemiological research activities in this region. This collaboration is based on an official agreement signed in 1987 and renewed every 5 years (*Convenio de salud/Ministerio de Salud y Deportes, Estado Plurinacional de Bolivia*/Department of Experimental and Clinical Medicine, University of Florence). The project has been supported by the Guaraní political organization (Asamblea del Pueblo Guaraní, APG) and the Pan American Health Organization (PAHO) (Università degli studi di Firenze, 2010).

The ongoing work over the years has been made possible thanks to numerous funding organizations and national and international partners, including:

- Ministry of Health of Bolivia, La Paz, Plurinational State of Bolivia
- PAHO/WHO
- Departmental Health Service (SEDES) of Santa Cruz, Plurinational State of Bolivia
- Departmental Health Service (SEDES) of Tarija, Plurinational State of Bolivia
- Convenio Ministerio de Salud – Vicariato de Cuevo, Camiri, Plurinational State of Bolivia
- Universidad Autonoma Gabriel René Moreno, Faculty of Integral Studies of the Chaco, Camiri, Plurinational State of Bolivia
- Teko Guaraní, Camiri, Plurinational State of Bolivia

- University of Florence, Italy
- University of Siena, Italy
- University of Rome "La Sapienza", Italy

PART II

AIM OF THE PHD PROJECT

The general objective of the PhD research was to investigate the molecular epidemiology of acquired antibiotic resistance in commensal and uropathogenic bacteria from humans living in urban areas and very remote communities of Bolivia.

The research study was conducted within the framework of a larger project funded by the Tuscany Region, Italy, with the overall goal of supporting the health development of the population in the Bolivian Chaco (Regional Initiative Projects - PIR - 'Support for the improvement of the health condition of the population in the Bolivian Chaco'). Among the project's objectives are the study of antibiotic use and resistance in the commensal microbiota of healthy individuals, as well as the investigation into controlling the spread of antibiotic-resistant bacterial infections and preventing both hospital- and community-acquired infections in Bolivia.

In particular, the following specific topics are discussed in the PhD research:

1. Carriage rates and molecular epidemiology of acquired resistance genes in expanded-spectrum cephalosporin-resistant enterobacteria present in the commensal microbiota of healthy children living in rural areas of the Bolivian Chaco.
2. Analysis of antimicrobial susceptibility and molecular epidemiology of expanded-spectrum cephalosporins resistant-enterobacteria responsible for urinary tract infections in hospitalized patients in a small urban community in the Bolivian Chaco.
3. Analysis of the antimicrobial susceptibility and emerging resistance determinants in bacteria responsible for uncomplicated and complicated community-acquired urinary tract infections in the Bolivian Chaco.

PART III

RESULTS AND DISCUSSION

1

CARRIAGE RATES AND MOLECULAR EPIDEMIOLOGY OF ESBL-PRODUCING ENTEROBACTERIA IN HEALTHY CHILDREN LIVING IN RURAL COMMUNITIES OF THE 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.

Over the years, extensive surveillance studies in the Bolivian Chaco have monitored antibiotic resistance in commensal *E. coli* from healthy children, yielding notable findings. Since the early 1990s, these investigations have identified high levels of resistance to older antibiotics like ampicillin, tetracycline, trimethoprim-sulfamethoxazole, and chloramphenicol (Bartoloni et al., 1998). In the 2000s, a concerning trend emerged, with resistance observed against newer drugs, including fluoroquinolones and expanded-spectrum cephalosporins (Bartoloni et al., 2008, 2006, 1998).

Subsequent research confirmed that resistance to expanded-spectrum cephalosporins in commensal *E. coli* in this region was primarily linked to the emergence of CTX-M-type extended-spectrum beta-lactamase (ESBL) determinants, most notably those in the CTX-M-2 group (Pallecchi et al., 2007, 2004).

In a follow-up study in 2011, fecal samples from 482 healthy children, aged 6-72 months, living in three urban areas of the Bolivian Chaco (Camiri, Monteagudo, and Villa Montes) were analyzed (Bartoloni et al., 2013). This study pointed out a dramatic increase of resistance to expanded-spectrum cephalosporins with a prevalence of 12% and an overall similar distribution in the three urban areas (Bartoloni et al., 2013).

Five cross-sectional surveys were conducted on the fecal carriage of CTX-M–producing Enterobacterales in school-age children living in rural areas of the Bolivian Chaco from 2016 to 2022 (Boncompagni et al., 2022). A total of 938 fecal samples were collected from 2016 to 2022 from

healthy school age children (6-12 years) living in five indigenous communities of the Bolivian Chaco (Tarija Department, Plurinational State of Bolivia) (Table 1, Figure 11) and plated onto MacConkey agar supplemented with cefotaxime 2 µg/mL (MCA-CTX), for the selection of expanded-spectrum cephalosporins-resistant Enterobacterales.

A loopful of the bacterial growth (taken either from confluent growth or from isolated colonies of different morphologies) representative of each sample was taken from the MCA+CTX plates and used as a template for the search of *bla*$_{CTX-M}$ genes multiplex real time polymerase chain reaction (mRT-PCR) (Giani et al., 2017). To identify CTX-M–positive isolates, CTX-M–positive samples were again streaked onto MCA-CTX, and all colonies with 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*$_{CTX-M}$ groups by mRT-PCR, and iii) identification by the Bruker MS system (Bruker Daltonics, Germany; MBT reference library, version 2021) (Giani et al., 2017).

Table 1. Distribution by community of fecal samples included in the study obtained from children of five rural communities of the Bolivian Chaco (Tarija Department, Plurinational State of Bolivia) in 2016-2022.

Community	No. of Fecal Sample				
	2016	**2017**	**2018**	**2019**	**2022**
Tarairí (1)	41	50	48	42	11
Chimeo (2)	39	43	46	50	47
Capirendita (3)	16	42	37	41	9
Palmar Chico (5)	39	54	35	39	38
San Antonio (4)	-	58	37	43	33
Total	135	247	203	215	138

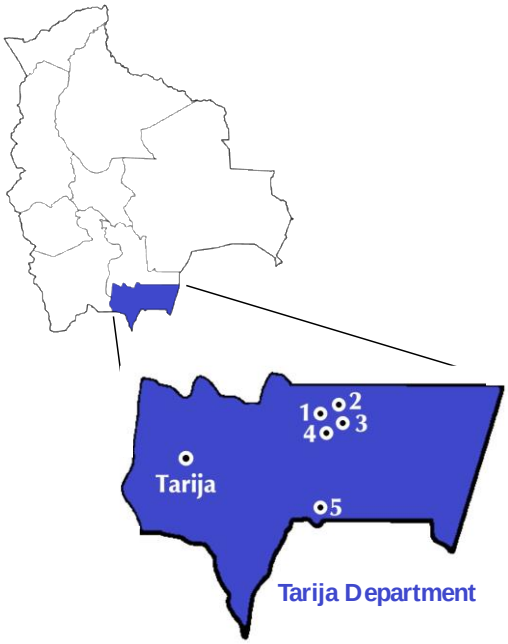

Figure 11. Geographical area of the five communities included in the study. 1, Tarairí; 2, Chimeo; 3, Capirendita; 4, Palmar Chico; 5, San Antonio de Villamontes.

Between 2016 and 2022, a total of 938 children participated in the study. Their mean ages ranged from 9.2 to 9.7 years (standard deviations between 1.4 and 1.7). Median ages varied between 9 and 10 years. The male-to-female ratio approached or closely approximated 1:1 in all years (see Table 2 for details). Usage of antibiotics was found to be very limited, with only one child (0.5%) in 2018, 10 children (0.5%) in 2019 and 11 (8%) in reporting antibiotic consumption during the 15 days preceding the surveys (Table 2).

Sample analysis across these years revealed a relevant increase in the prevalence of CTX-M-producing *E. coli* in commensal microbiota of healthy children, ranging from 19.3% in 2016 to 60.1% in 2022 (p value < 0.0001) (Table 2).

Almost all samples that grew on MCA-CTX (96 to 100%) were confirmed as ESBL producers via phenotypic tests and tested positive for $bla_{\text{CTX-M}}$ genes. These CTX-M-producing isolates were primarily *E. coli*, although there were exceptions (i.e. n=2, *Enterobacter cloacae* in 2017, n=1, *K. pneumoniae* in 2018; n= 1, *Raoultella ornithinolytica* in 2019; and n=1, *Citrobacter freundii*, n=1, *Morganella morganii* in 2022).

Furthermore, a subset of children in each year was colonized by multiple CTX-M-producing *E. coli* strains, resulting in varying numbers of total CTX-M-producing *E. coli* isolates across the years (Table 2). In addition, some isolates carried two different $bla_{\text{CTX-M}}$ variants. Overall, throughout the 6-year

study, the increase was noted across all communities, albeit with fluctuations in some of them, likely due to a limited sample size (Table 2).

Despite the notable increase of CTX-M enzymes prevalence over the study period, the relative prevalence of each CTX-M group remained fairly stable (Table 2). The CTX-M-1 group was the most prevalent (47% to 79.6%), followed by the CTX-M-9 group (15.3% to 49%), the CTX-M-8 group (2% to 3.1%), and the CTX-M-2 group (0.9% to 2%) (Table 2). On note, in 2018, the CTX-M-9 group briefly became the most prevalent, reaching almost 50% of all CTX-Ms (Table 2).

Table 2. Features of the study population sorted by communities and by year with features of CTX-M-positive *Escherichia coli* isolates in 2016-2022.

Community	Years	No. of studied children			Antibiotic Consumption [b]	CTX-M-positive children	*p* value[c] 2016 vs 2022	CTX-M-positive *E. coli*	Total CTX-M	CTX-M groups			
		M	F	Total						CTX-M-1	CTX-M-2	CTX-M-9	CTX-M-8/25
Tarairì	2016	18	23	41	-	15 (36.6)	ns	21	22	18 (81.8)	0	4 (18.2)	0
	2017	28	22	50	-	15 (30)		18	18	16 (88.9)	0	2 (11.1)	0
	2018	24	24	48	-	5 (10.4)		4	4	3 (75)	0	1 (25)	0
	2019	19	23	42	-	6 (14.3)		7	7	3 (42.9)	0	3 (42.9)	1 (14.3)
	2022	4	7	11	1 (9.1)	7 (63.6)		9	10	9 (90)	1 (10)	0	0
Chimeo	2016	18	21	39	-	4 (10.3)	< 0.0001	4	4	3 (75)	0	1 (25)	0
	2017	22	21	43	-	13 (30.2)		15	15	12 (80)	1 (6.7)	2 (13.3)	-
	2018	22	24	46	1 (2.2)	10 (21.7)		10	10	3 (30)	1 (10)	5 (50)	1 (10)
	2019	25	25	50	5 (10)	18 (36)		20	20	9 (45)	0	11 (55)	0
	2022	14	33	47	2 (4.3)	29 (61.7)		31	33	23 (69.7)	0	6 (18.2)	2 (6.1)
Capirendita	2016	9	7	16	-	2 (12.5)	< 0.05	1	1	0	0	1 (100)	0
	2017	19	23	42	-	9 (21.4)		8	8	7 (87.5)	1 (12.5)	0	0
	2018	19	18	37	-	6 (16.2)		7	7	3 (42.9)	0	4 (57.1)	0
	2019	23	18	41	4 (9.8)	8 (19.5)		11	11	6 (54.5)	0	5 (45.5)	0
	2022	4	5	9	-	5 (55.6)		5	5	3 (60)	0	2 (40)	0
Palmar Chico	2016	18	21	39	-	5 (12.8)	< 0.0001	7	7	4 (57.1)	0	3 (42.9)	0
	2017	27	27	54	-	29 (53.7)		35	36	26 (72.2)	0	8 (22.2)	2 (5.6)
	2018	20	15	35	-	10 (28.6)		13	14	5 (35.7)	0	9 (64.3)	0
	2019	21	18	39	1 (2.6)	16 (41)		17	17	12 (70.6)	0	5 (29.4)	0
	2022	20	18	38	8 (21.1)	26 (68.4)		37	38	26 (68.4)	0	10 (26.3)	1 (2.6)
San Antonio[a]	2016	-	-	-	-	-	ns	-	-	-	-	-	-
	2017	27	31	58	-	18 (31)		21	21	17 (80.9)	0	3 (14.3)	1 (4.8)
	2018	17	20	37	-	12 (32.4)		16	16	10 (62.5)	0	6 (37.5)	0
	2019	24	19	43	-	12 (27.9)		15	16	12 (75)	0	3 (18.6)	1 (6.3)
	2022	25	8	33	3 (9.1)	16 (48.5)		19	19	14 (73.7)	0	5 (26.3)	0
Total	2016	63	72	135	-	26 (**19.3**)	< 0.0001	33	34	25 (73.5)	0	9 (26.5)	0
	2017	123	124	247	-	85 (**34.4**)		97	98	78 (79.6)	2 (2)	15 (15.3)	3 (3.1)
	2018	102	101	203	1 (0.5)	44 (**21.7**)		50	51	24 (47)	1 (2)	25 (49)	1 (2)
	2019	112	103	215	10 (0.5)	60 (**27.9**)		70	71	42 (59.2)	0	27 (38)	2 (2.8)
	2022	67	71	138	11 (8)	83 (**60.1**)		101	105	78 (74.3)	1 (0.9)	23 (21.9)	3 (2.9)

[a], *p* value calculated 2017 vs 2022 due to lack of data for 2016; [b], during 15 days preceding the survey; [c], calculated by Chi-square test (with Yates' correction), $p < 0.05$, significant; ns, non-significant

To better understand the rapid spread of CTX-Ms during the study period, phylogenetic groups were investigated by PCR for all CTX-M-producing *E. coli* collected from 2016 to 2022, as previously described (Clermont et al., 2009; Johnson et al., 2019).

As expected, the most prevalent phylogenetic groups were A and B1, often associated with commensal strains (Figure 12). However, starting from 2017, the prevalence of the B2 phylogenetic group, mostly linked to virulence and antibiotic resistance in pathogens, increased, reaching up to 30% in 2019 and displaying a rate of 21.7% in 2022 (Figure 12). It's worth noting that until 2017, all B2 isolates were exclusively associated with CTX-M-1, while from 2018, the CTX-M-9 group became the most prevalent among B2 isolates (Figure 13).

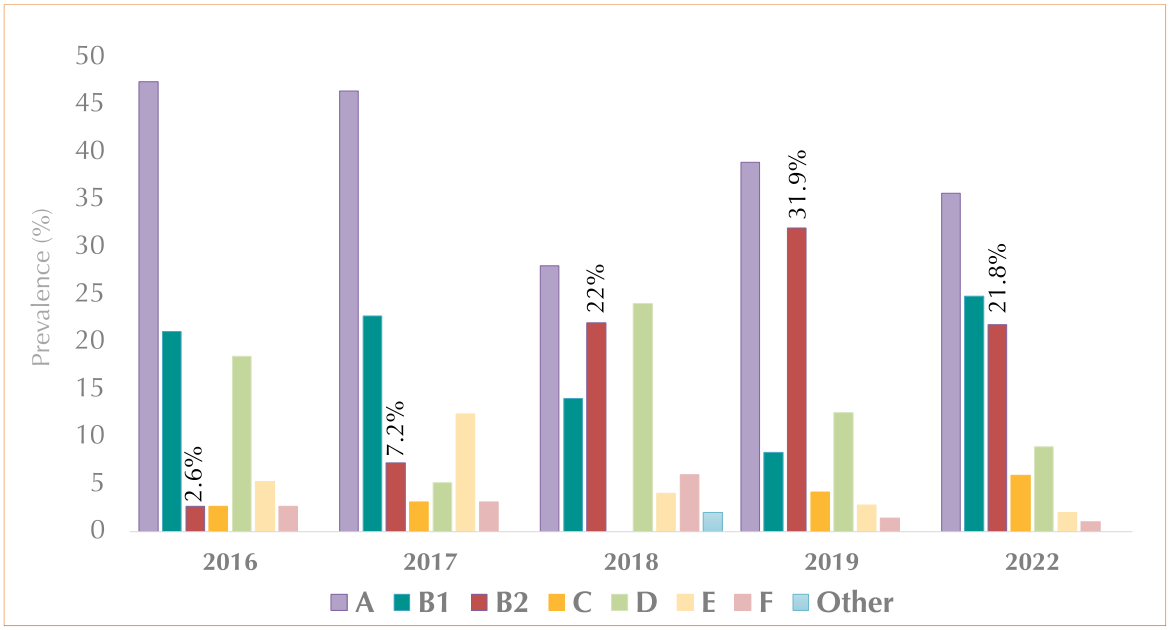

Figure 12. Phylogenetic groups distribution among commensal CTX-M-producing *E. coli* per year.

To further investigate, molecular detection of *E. coli* pandemic clones ST131 and ST1193 was conducted in all B2 isolates. The results revealed that the majority of B2 CTX-M-producing *E. coli* were attributed to these two pandemic clones (63.6% to 100%). Specifically, B2-CTX-M-1-producing *E. coli* were predominantly linked to ST131, whereas the majority of B2-CTX-M-9-producing *E. coli* were associated with ST1193 (Figure 13).

Notably, since 2018, when CTX-M-9 became the most prevalent group, 36% (n=9), 48% (n=11), and 47.8% (n=13) of CTX-M-9-producing *E. coli* were attributed to ST1193 in 2018, 2019, and 2022,

respectively. This underscores the significant role of the ST1193 pandemic clone in the dissemination of CTX-M-9 enzymes in this area.

The findings of this study underscore a rapid spread of CTX-M determinants, contributing to the increased prevalence of resistance to extended-spectrum cephalosporins in commensal *E. coli* among healthy children living in rural communities of the Bolivian Chaco, where antibiotic usage remains scarce. This spread is also attributed to the dissemination of successful clones such as *E. coli* ST131 and ST1193.

This is a matter of concern, as intestinal colonization by ESBL-producing isolates can potentially serve as a reservoir for the introduction of these determinants into hospital settings, thereby posing a risk factor for subsequent infections caused by ESBL-producing strains in hospitalized patients.

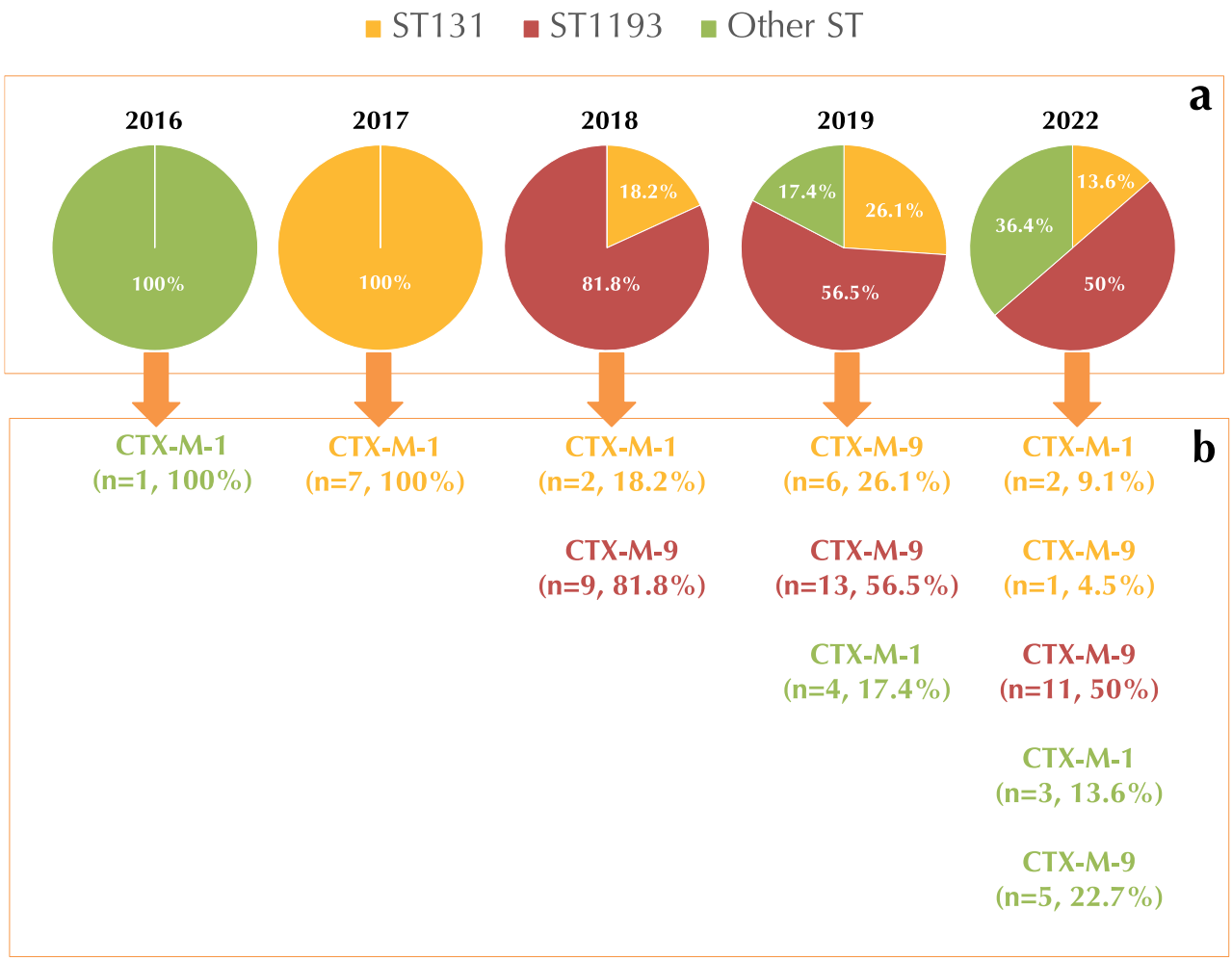

Figure 13. Distribution of pandemic high-risk clones ST131 and ST1193 (a) and CTX-M-group (b) among B2 CTX-M-producing *E. coli* per year.

2
ANALYSIS OF THE ANTIMICROBIAL SUSCEPTIBILITY AND MOLECULAR EPIDEMIOLOGY OF CLINICAL ISOLATES RESPONSIBLE FOR URINARY TRACT INFECTIONS IN HOSPITALIZED PATIENTS OF THE BOLIVIAN CHACO

To investigate the correlation between resistance rates observed in the microbiota of healthy individuals and those in pathogens circulating in the same environment, we conducted a study on the antimicrobial susceptibility and resistance determinants of bacterial pathogens responsible for urinary tract infections (UTIs) in hospitalized patients at the Hospital Básico of Villamontes in the Tarija Department, Plurinational State of Bolivia.

This hospital houses one of the initial clinical microbiology laboratories established in the Bolivian Chaco region. This region's healthcare system, comprising numerous rural areas and indigenous communities, relies on small hospitals with limited access to clinical microbiology facilities. As a result, systematic collection of antimicrobial susceptibility data from routine microbiological analyses of clinical samples is challenging.

The most recent study in the same region, focusing on the molecular epidemiology and resistance rates in hospital-acquired pathogens responsible for UTIs, dates back to 2015 and includes data up to 2014) (Bartoloni et al., 2016). In this earlier study, high resistance rates to several antibiotics were documented.

A total of 709 non-replicate clinical isolates were collected between 2015 and 2021 from patients clinically diagnosed with UTIs at the Hospital Básico in Villamontes, Tarija Department, Plurinational State of Bolivia.

Bacterial identification, performed by MALDI-TOF, showed that 694 (97.9%) were Enterobacterales, while the remaining 15 were non-fermenter pathogens, including *Acinetobacter* spp., *Pseudomonas* spp. and *Stenotrophomonas maltophilia*) (Table 3).

Antimicrobial susceptibility testing, performed using the disk diffusion method (with the exception of fosfomycin susceptibility testing, which used the agar dilution method) in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2022, 2018) demonstrated high resistance rates among UTI isolates (Table 4).

When considering *E. coli*, which was the most prevalent pathogen, the drugs most affected by resistance were trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin, tetracycline, and

amoxicillin-clavulanic acid (Table 4). Expanded-spectrum cephalosporins remained effective against the majority of isolates, with 73.1% and 78.3% susceptibility to cefotaxime and ceftazidime, respectively (Table 4). Multidrug resistance (i. e. co-resistance to three or more antimicrobial classes) occurred in 56.6% of *E. coli* isolates (N=305). Overall, the most effective drugs were meropenem, nitrofurantoin, fosfomycin and amikacin (Table 4).

The present results confirmed the very high resistance rates observed in the previous study for trimethoprim-sulphamethoxazole, tetracycline, amoxicillin-clavulanic acid, and gentamicin and showed an alarmingly relentless increase of resistance to quinolones (including fluoroquinolones) and expanded-spectrum cephalosporins (*p* value < 0.001) (Bartoloni et al., 2016)(Table 4).

Table 3. Etiology of urinary tract infections in hospitalized patients in the Bolivian Chaco (2015-2021).

Species	No. of isolates	%
Escherichia coli	539	76
Klebsiella pneumoniae	55	7.8
Enterobacter cloacae complex	37	5.2
Morganella morganii	18	2.5
Proteus mirabilis	18	2.5
Citrobacter freundii	11	1.6
Citrobacter koseri	3	0.4
Klebsiella oxytoca	3	0.4
Providencia spp.	3	0.4
Pseudomonas aeruginosa	3	0.4
Pseudomonas stutzeri	3	0.4
Stenotrophomonas maltophilia	3	0.4
Pseudomonas putida	2	0.3
Serratia marcescens	2	0.3
Acinetobacter baumannii	1	0.1
Acinetobacter pitti	1	0.1
Acinetobacter nosocomialis	1	0.1
Citrobacter brakii	1	0.1
Escherichia vulneris	1	0.1
Pseudomonas oleovorans	1	0.1
Pantoea agglomerans	1	0.1
Raoultella ornithinolytica	1	0.1
Raoultella planticola	1	0.1

Table 4. Antibiotic susceptibility rates (%) of *E. coli* urinary isolates (2010-2021).

Drug	2010-2014 (n=170)[a]	2015-2021 (n=539)[b]	*p* value[c]
Trimethoprim-Sulphamethoxazole	26.5	32.8	ns
Nalidixic acid	51.2	33.2	< 0.001
Ciprofloxacin	61.8	42.1	< 0.001
Tetracycline	47.1	42.9	ns
Amoxicillin-Clavulanic acid	56.5	53.2	ns
Gentamicin	72.4	69.6	ns
Cefotaxime	89.4	73.1	< 0.001
Ceftazidime	92.3	78.3	< 0.001
Amikacin	95.3	90.7	ns
Fosfomycin	98.2	94.8	ns
Nitrofurantoin	91.8	95.4	ns
Meropenem	100	100	ns

[a], data from (Bartoloni et al., 2016); [b], data from this study; [c], calculated by Chi-square test (with Yates' correction), $p < 0.05$, significant; ns, non-significant

Out of 146 isolates resistant to expanded-spectrum cephalosporins, 144 were identified as ESBL producers through phenotypic testing. All ESBL producers were found to carry $bla_{\text{CTX-M}}$ genes. CTX-M-1 group was the most prevalent (119 isolates, 82.6%), followed by the CTX-M-9 group (26 isolates, 18.1%). One isolate carried variants from two different groups, i.e., $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-9}}$. CTX-M-2 and the CTX-M-8/25 group were not detected.

Since the elevated resistance rates to expanded-spectrum cephalosporins observed in commensal strains from the microbiota of healthy individuals were partly attributed to the dissemination of two high-risk clones of *E. coli*, namely ST131 and ST1193, phylogenetic groups and molecular detection of ST131 and ST1193 were investigated.

The most prevalent phylogenetic group was B2 (229/539, 42.5%), followed by group A (105/539, 19.5%), B1 (69/539, 12.8%), E (58/539, 10.8%), F (32/539, 5.9%), D (21/539, 3.9%), and C (8/539, 1.5%). Additionally, 17 isolates belonged to Escherichia cryptic clades (3.2%) (Figure 14a).

Molecular detection of *E. coli* pandemic clones ST131 and ST1193 in B2 isolates revealed that the majority of these were assigned to the two pandemic clones (143/229, 62.4%), with ST131 being the most prevalent (112/229, 48.9%) (Figure 14b). Notably, all ST131 and ST1193 *E. coli* isolates exhibited high resistance rates to almost all the tested antibiotics and displayed a multidrug-

resistant (MDR) profile (Table 5). Interestingly, ST131 and ST1193 accounted for 46.9% (143/305) of all detected MDR isolates. Furthermore, the majority of ST131 isolates were ESBL producers and carried *bla*$_{CTX-M}$ genes (84/112, 75%), with *bla*$_{CTX-M-1}$ being the most prevalent, in contrast to those assigned to ST1193, which carried only *bla*$_{CTX-M-9}$ (8/31, 25.8%) (Figure 15).

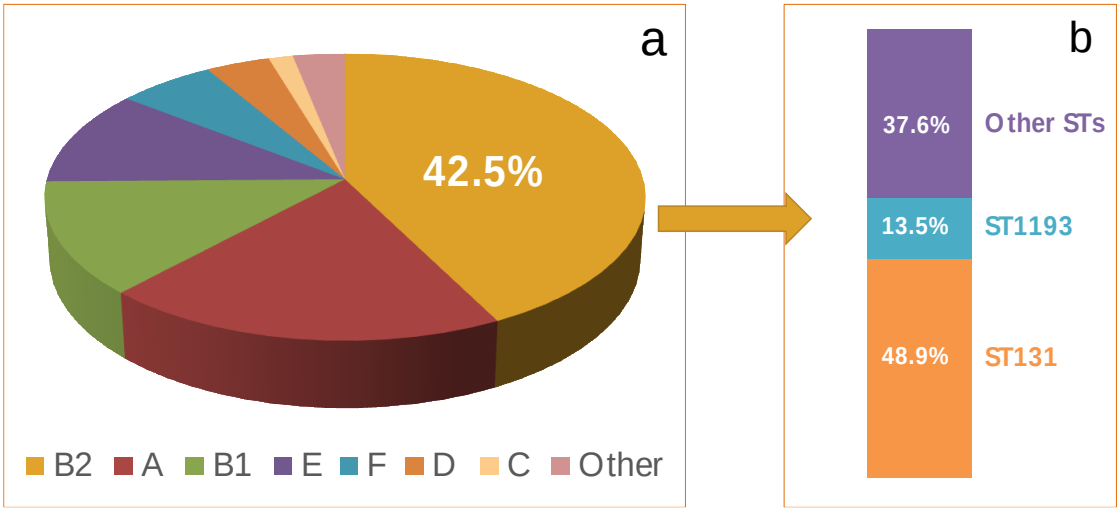

Figure 14. (a) Distribution of phylogenetic groups among uropathogenic *E. coli* from patients of the Bolivian Chaco; (b) Clonal distribution among B2 isolates.

Table 5. Antibiotic resistance rates (%) of ST131 and ST1193 clones.

Drug	ST131 (n=112)	ST1193 (n=31)
Nalidixic acid	100	100
Ciprofloxacin	100	100
Trimethoprim-Sulphamethoxazole	90	74.2
Amoxicillin-Clavulanic acid	78.6	29
Tetracycline	77.7	70.9
Cefotaxime	75.9	25.8
Gentamicin	68.7	38.7
Ceftazidime	64.3	19.4
Amikacin	54.5	32.3
Nitrofurantoin	4.5	0
Fosfomycin	0	0
Meropenem	0	0

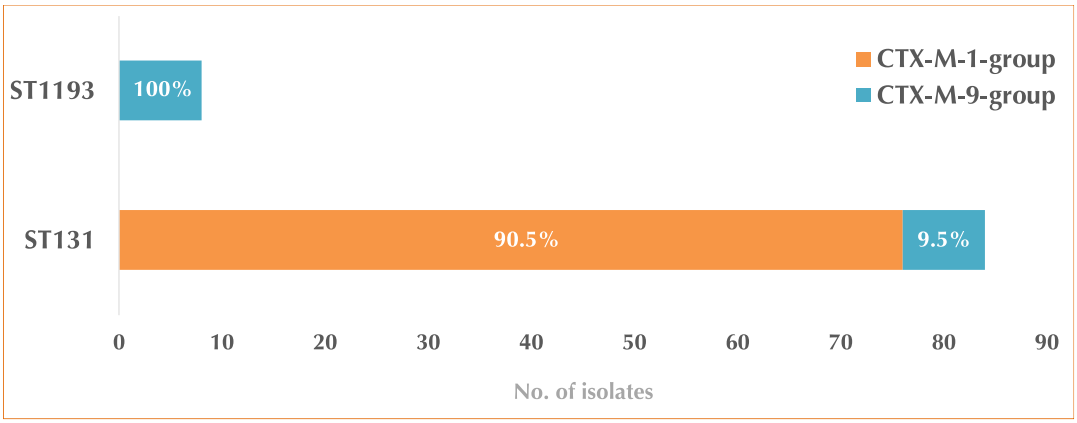

Figure 15. Distribution of CTX-M groups among ST131 and ST1193 clones.

Interestingly, the urinary *E. coli* isolates examined in this study demonstrated a comparable prevalence of CTX-M producers (26.7% vs. 27.9%; $p = 0.9$) and a similar distribution of CTX-M groups (with the CTX-M-1 group being the most prevalent, followed by the CTX-M-9 group) as those found in commensal *E. coli* collected in 2019 from healthy children residing in the same area (Boncompagni et al., 2022).

Regarding the prevalence of pandemic high-risk clones among B2 isolates, we observed a substantial dissemination of these clones in both commensal and pathogenic strains. The average prevalence of ST131 and ST1193 among commensal B2 *E. coli* from 2016 to 2022 was 77.5%, while the prevalence in uropathogenic *E. coli* from 2015 to 2021 was 62.4%. Additionally, we observed a similar distribution of CTX-M enzymes among these clones, with ST131 likely carrying $bla_{\text{CTX-M-1}}$ and ST1193 carrying $bla_{\text{CTX-M-9}}$ (Figure 13, 15).

This underscores the significant role of commensal enterobacteria as a reservoir for clinically relevant resistance determinants.

3

ANALYSIS OF THE ANTIMICROBIAL SUSCEPTIBILITY AND EMERGING RESISTANCE DETERMINANTS IN CLINICAL ISOLATES RESPONSIBLE FOR COMMUNITY-ACQUIRED URINARY TRACT INFECTIONS OF THE BOLIVIAN CHACO

RELATED PUBLICATION:

Etiology and antimicrobial susceptibility of community-acquired urinary tract infections in Bolivia: insights from a two-year study in the Bolivian Chaco region. Micieli M, *et al.*

(Manuscript in preparation)

UTIs are prevalent in both community and hospital settings, categorized as uncomplicated UTIs (uUTIs), affecting non-pregnant women without anatomical abnormalities or comorbidities, and complicated UTIs (cUTIs) linked to factors like catheterization, urinary obstruction, immunosuppression, renal issues, and pregnancy (European Association of Urology (EAU), 2022).

Diagnosing uUTIs relies on typical symptoms (i. e., pain during urination, frequent urination, and urgency), but a urine culture is recommended when symptoms are atypical or initial antibiotic treatment fails (European Association of Urology (EAU), 2022). Consequently, treatment for uUTIs is often empirical, based on general guidelines that consider local AMR data. However, in countries with limited resources like Bolivia, access to local pathogen resistance data can be challenging.

A recent Latin American consensus panel, following 2018 EAU guidelines, suggests nitrofurantoin and fosfomycin as primary treatments for recurrent uUTIs, with cephalosporins or co-trimoxazole considered when local resistance rates are below 20% (Haddad et al., 2020; Wagenlehner et al., 2022). Nevertheless, Latin America has reported significantly high resistance rates in recent years (Bartoloni et al., 2016, 2013; Bours et al., 2010; Li et al., 2022), underscoring the importance of considering regional susceptibility data when choosing appropriate empirical antibiotics.

Due to limited data on antibiotic resistance and molecular epidemiology of bacterial pathogens causing community-acquired UTIs in Bolivia, we conducted an observational study between

February 2020 and November 2021, to assess antimicrobial susceptibility and resistance determinants.

A total of 731 adult individuals (mean age of 44.9 (SD = 18.5); median age of 41 (IQR = 28); male:female ratio of approx. 1:4), all experiencing urinary tract symptoms, were included in this study. They reside in the urban area of Villamontes within the Tarija Department of the Plurinational State of Bolivia and sought medical care at six primary health centers in Villamontes or requested external consultations at three hospitals in the area (Figure 16). UTIs were classified as complicated or uncomplicated according to European Association of Urology (EAU) (European Association of Urology (EAU), 2022).

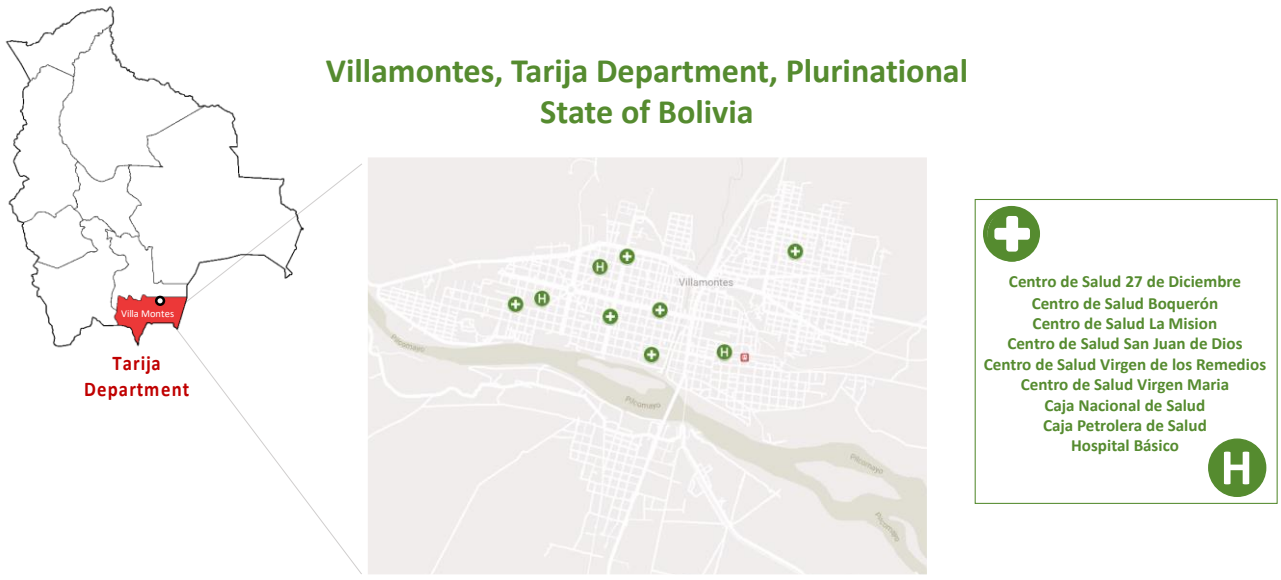

Figure 16. Geographical area (Villamontes, Tarija Department, Plurinational State of Bolivia) and list of hospital and primary health centers included in the study.

Overall, out of 731 enrolled subjects, 45.6% (333/731) tested positive for UTI, with females accounting for the majority (288/333, 86.5%) (Table 6). Among the 333 positive urine cultures, 194 cases (41.1%) were diagnosed in females with uUTIs, while the remaining 139 cases were classified as cUTIs. Within these 139 cases, 56 (40.3%) were females with cUTIs, 38 (27.3%) were females in pregnancy, and 45 (32.4%) were males.

A subset of subjects were infected by two or more different microorganisms (i.e., n=14 with cUTIs and n=4 with uUTIs) or provided more than two samples during the study period (i.e. n=12 with cUTIs and n=2 with uUTIs). Only urine samples provided after at least 6 months and after a negative

urine sample following the initial positive sample were included. This resulted in varying total microorganisms isolated, with a total of 213 and 181 isolates for uUTI and cUTIs, respectively.

Table 6. Characteristics of enrolled subjects.

		UTIs		
	No UTIs (n = 398)	**Total UTIs (n = 333)**	**uUTIs (n = 194)**	**cUTIs (n = 139)**
Age: mean (SD); median (IQR)	41.5 (17.6); 37 (24)	47.7 (19.1); 47 (30)	45.5 (17.9); 41.5 (25)	50.8 (20.3); 52 (34)
Age groups, years				
18-50	335 (84.2)	191 (57.4%)	130 (67%)	61 (43.9%)
>51	51 (12.8)	142 (42.6%)	64 (32.9%)	78 (56.1%)
Females	296 (74.4)	288 (86.5%)	194 (100%)	94 (67.6%)
Underlying diseases				
Renal failure	5 (1.3%)	9 (2.7%)	0	9 (6.5%)
Liver cirrhosis	0	1 (0.3%)	0	1 (0.7%)
Diabetes mellitus	28 (7%)	53 (15.95)	0	53 (38.1%)
Predisposing factors				
Previous UTI	104 (26.1)	63 (18.9%)	58 (29.9%)	32 (23%)
Recurrent UTI	46 (11.6)	88 (26.4%)	29 (14.9%)	59 (42.4%)
Catheter	0	16 (4.8%)	0	16 (11.5%)
Pregnancy	61 (15.2)	38 (11.4%)	0	38 (27.3%)
Prostatitis	7 (1.8)	7 (2.1%)	0	7 (5%)
Antibiotic assumption				
Up to 30 days before sampling	78 (19.6)	66 (18.3)	36 (17.1)	30 (19.9)

SD, standard deviation; IQR, interquartile range; results are n (%) unless otherwise specified.

The most frequently isolated microorganisms for uncomplicated and complicated UTI were *E. coli* (uUTIs, n=184/213, 86.4%; cUTIs, n=156/181, 86.2%), *Klebsiella* spp. (uUTIs, n=11/213, 5.2%; cUTIs, n=12/181, 6.6%), and *Proteus* spp. (uUTIs, n=5/213, 2.3%; cUTIs, n=4/181, 2.2%), (Table 7). Considering *E. coli*, which was by far the most prevalent pathogen, very high resistance rates were observed for both cUTI and uUTI (statistically significant differences were not observed for all antibiotics tested). Specifically, the most affected antibiotics were ampicillin, nalidixic acid, trimethoprim-sulfamethoxazole, ciprofloxacin, and cefotaxime (Table 8). The only drugs with a susceptibility rate against *E. coli* higher than 80% were nitrofurantoin (uUTIs, 95.7%; cUTIs, 96.2%), fosfomycin (uUTIs, 95.7%; cUTIs, 96.8%), colistin (uUTIs, 96.2%; cUTIs, 98.7%), imipenem (uUTIs and cUTIs, 100%), and meropenem (uUTIs and cUTIs, 100%) (Table 8).

Table 7. Etiology of uncomplicated (a) and complicated (b) community-acquired urinary tract infections in the Bolivian Chaco (2020-2021).

a Uncomplicated UTIs			**b** Complicated UTIs		
Species	**No.**	**%**	**Species**	**No.**	**%**
Escherichia coli	184	86.4	*Escherichia coli*	156	86.2
Klebsiella spp.	11	5.2	*Klebsiella spp.*	12	6.6
Proteus spp.	5	2.3	*Proteus spp.*	4	2.2
Citrobacter koseri	2	0.9	*Morganella morganii*	2	1.1
Acinetobacter spp.	2	0.9	*Citrobacter freundii*	1	0.6
Morganella morganii	2	0.9	*Enterococcus faecalis*	1	0.6
Pseudomonas spp.	2	0.9	*Enterobacter asburiae*	1	0.6
Staphylococcus saprophyticus	2	0.9	*Enterobacter bugandensis*	1	0.6
Enterobacter cloacae	1	0.5	*Escherichia vulneris*	1	0.6
Salmonella spp.	1	0.5	*Pseudomonas putida*	1	0.6
Stenotrophomonas maltophilia	1	0.5	*Serratia marcescens*	1	0.6

Table 8. Antibiotic susceptibility rates (%) of *E. co*li from uncomplicated (uUTIs) and complicated (cUTIs) community-acquired urinary tract infections.

Antibiotic	**uUTIs**	**cUTIs**	**Total UTIs**
	***E. coli* (n=184)**	***E. coli* (n=156)**	***E. coli* (n=340)**
Ampicillin	18.5	17.7	16.8
Nalidixic acid	37.6	26.9	32.6
Trimethoprim-Sulphamethoxazole	38	34.0	36.2
Ciprofloxacin	47.3	39.1	43.5
Cefotaxime	71.7	67.9	70
Amoxicillin-Clavulanic acid	73.4	67.3	70.6
Ceftazidime	79.3	72.4	76.2
Gentamicin	79.3	75.6	77.6
Nitrofurantoin	95.7	96.2	95.9
Fosfomycin	95.7	96.8	96.2
Colistin	96.2	98.7	97.1
Imipenem	100	100	100
Meropenem	100	100	100

To investigate the resistance mechanisms responsible for resistance to fosfomycin (a first-line antibiotic in UTIs) and colistin (a last-resort antibiotic), molecular detection of acquired colistin resistance genes (*mcr*) and acquired fosfomycin resistance genes (*fosA/C*, *fosL1*) was investigated in resistant strains using mRT-PCR, as previously described (Coppi et al., 2018).

In total, among the 10 colistin-resistant *E. coli* strains, 9 (90%) were found to carry the *mcr-1* gene. In the case of fosfomycin-resistant isolates (n=11), nearly all except one isolate (90.9%) carried *fos* genes (i.e. n=6, *fosA3/4*; n=3, *fosA5/6*; n=1 *fosL1*). Despite the relatively low prevalence of colistin and fosfomycin resistance among uropathogenic *E. coli*, these findings are noteworthy, given that these genes are often harbored on plasmids and can be horizontally transferred to other strains (Falagas et al., 2019; Kieffer et al., 2020; Poirel et al., 2017).

Since very high resistance rates to expanded-spectrum cephalosporins were detected, ESBL phenotypic confirmatory tests and molecular detection of $bla_{\text{CTX-M}}$ genes were investigated.

Overall, 102/340 (30%) *E. coli* were resistant to cefotaxime. Of these all were confirmed to be ESBL producers by phenotypic test and the majority (97/102, 95.1%) were found to carry $bla_{\text{CTX-M}}$ genes. CTX-M-1-group was the most prevalent (n=59/97, 60.8%), followed by CTX-M-9-group (n=38/97,39.2%).

Interestingly, resistance rates in CTX-M-producing *E. coli* were significantly higher to almost all tested antibiotic, except for nitrofurantoin and colistin compared to pathogens that did not produce CTX-Ms (Table 9).

Table 9. Antibiotic susceptibility rates (%) of CTX-M-producing and CTX-M-negative *E. coli* from uncomplicated and complicated community-acquired urinary tract infections.

Drug	CTX-M-producing *E. coli* (n=97)	CTX-M-negative *E. coli* (n=243)	*p* value[a]
Nalidixic acid	0	46.7	< 0.0001
Ciprofloxacin	1	58.8	< 0.0001
Trimethoprim-Sulphamethoxazole	13.4	44.6	< 0.0001
Amoxicillin-Clavulanic acid	50.5	75.8	< 0.0001
Gentamicin	59.8	83.8	< 0.0001
Nitrofurantoin	91.8	96.7	ns
Colistin	94.8	97.5	ns
Fosfomycin	90.7	98.8	< 0.001

[a], calculated by Chi-square test (with Yates' correction), $p < 0.05$, significant; ns, non-significant

The current data unveils a substantial overall resistance burden among enterobacteria responsible for community-acquired urinary tract infections, aligning with rates observed in hospitalized patients. This resistance is marked by exceptionally high levels of resistance to ampicillin, trimethoprim-sulfamethoxazole, and fluoroquinolones. Furthermore, there's a notable presence of ESBL determinants within *Enterobacteriaceae*, in which even higher resistance rates are observed compared to non-ESBL strains. This emphasizes that nitrofurantoin and fosfomycin could be the primary treatment choices for uncomplicated UTIs in this region. However, it's important to note that the dissemination of resistance genes carried on plasmids, which can be horizontally transferred, is a cause for concern.

PART IV

CONCLUSIONS AND PERSPERCTIVES

While antibiotic use remains a significant factor driving the emergence of resistance in pathogenic bacteria, there is now substantial evidence indicating the presence of acquired antibiotic resistance, even at noteworthy levels, in the commensal bacteria of individuals living in remote areas with limited prior exposure to antibiotics for clinical or other purposes. This phenomenon highlights the complex mechanisms involved in the dissemination of antibiotic resistance, with significant implications for resistance control strategies. In addition, in RLCs, antimicrobial resistance rates are notably higher than in high-income countries (Murray et al., 2022). This increase in resistance is largely due to factors associated with poverty, such as limited access to healthcare, inadequate sanitation, and unreliable water sources. These issues have a substantial impact on disease rates, especially among children (WHO, 2022). Effective surveillance is crucial for developing interventions that help maintain the effectiveness of antimicrobial treatments.

In this study, the molecular epidemiology of emerging antibiotic resistance mechanisms in both commensal and pathogenic bacteria in rural and urban area of Bolivian Chaco was analyzed.

The study of commensal microbiota revealed a rapid increase in the prevalence of CTX-M determinants. This has significantly contributed to the rise in resistance to extended-spectrum cephalosporins in commensal *E. coli* among healthy children residing in rural communities of the Bolivian Chaco over a six-years period. This trend persisted despite limited antibiotic usage in the studied area. While the findings suggest that clonal expansion may not be the primary factor driving the spread of CTX-M ESBLs among enterobacteria, a substantial prevalence of two pandemic *E. coli* clones (i.e. ST131 and ST1193) was observed among CTX-M-producing commensal *E. coli*. Notably, the ST1193 clone, a new MDR pandemic clone, has emerged as a significant contributor to the dissemination of CTX-M-9-group enzymes, particularly from 2018 onward.

The study results concerning uropathogenic bacteria isolated from hospitalized patients residing in the same region revealed significantly elevated resistance rates, with the majority of strains displaying a MDR profile. Furthermore, we observed a similar prevalence of CTX-M enzymes as that found in commensal *E. coli*, as well as a substantial dissemination of the pandemic ST131 and ST1193 clones among uropathogenic enterobacteria, which contributed to a significant portion of MDR isolates, emphasizing the role of commensal enterobacteria as a reservoir for clinically relevant resistance determinants.

Finally, finding of the study of community-acquired urinary tract infections, revealed high resistance rates against various antibiotics, mirroring resistance rates observed in hospitalized patients. In particular, the study also highlighted the emergence of resistance to first-line antibiotic for UTIs,

fosfomycin and last-resort antibiotic, colistin, due to presence of acquired resistance genes, which are often carried on plasmids, posing a risk of horizontal gene transfer. Additionally, the dissemination of CTX-M enzymes was notably high and associated with increased resistance to multiple antibiotics, emphasizing the complexity of antibiotic resistance mechanisms. In light of these findings, it is evident that UTIs in the region are associated with high resistance rates, raising concerns about the choice of empirical antibiotic treatment. Nitrofurantoin and fosfomycin appear to be primary treatment options for uncomplicated UTIs, considering the substantial resistance to other antibiotics. However, the dissemination of resistance genes, particularly those carried on plasmids, presents a challenge that requires continued monitoring and research to develop effective strategies for resistance control.

In summary, these results suggest that relying solely on antibiotic restriction policies may not be sufficiently effective in addressing the emergence and spread of resistance. They emphasize the need for adopting a multi-faceted approach to combat antibiotic resistance, recognizing it as a complex ecological challenge. This approach should consider a variety of influential factors, including the type and extent of selective pressure, transmission pathways, and mechanisms of persistence. To tackle this challenge, it is essential to promote multidisciplinary and collaborative efforts at local, national, and international levels, working towards the goal of achieving "One Health" that encompasses the well-being of humans, animals, and the surrounding environment. Furthermore, the ongoing surveillance of settings with limited antibiotic exposure is crucial for gaining insights into the evolution of antibiotic resistance.

PART V

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PART VI

ANNEXES

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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

Selene Rebecca Boncompagni[a], Maria Micieli[b], Tiziana Di Maggio[a], Antonia Mantella[b], Anna Liz Villagrán[c], Tatiana Briggesth Miranda[d], Carmen Revollo[e], Veronica Poma[f], Herlan Gamboa[g], Michele Spinicci[b,h], Marianne Strohmeyer[b], Alessandro Bartoloni[b,h], Gian Maria Rossolini[b,i], Lucia Pallecchi[a,*]

[a] Department of Medical Biotechnologies, University of Siena, Santa Maria alle Scotte University Hospital, Siena, Italy
[b] Department of Experimental and Clinical Medicine, University of Florence, Careggi University Hospital, Florence, Italy
[c] Hospital Básico Villa Montes, Villa Montes, Plurinational State of Bolivia
[d] Hospital Municipal de Camiri, Camiri, Plurinational State of Bolivia
[e] Instituto Nacional de Laboratorios de Salud (INLASA), La Paz, Plurinational State of Bolivia
[f] Escuela de Salud del Chaco Tekove Katu, Gutierrez, Plurinational State of Bolivia
[g] Facultad Integral del Chaco, Universidad Autónoma Gabriel René Moreno, Camiri, Plurinational State of Bolivia
[h] Infectious and Tropical Diseases Unit, Careggi University Hospital, Florence, Italy
[i] Clinical Microbiology and Virology Unit, Careggi University Hospital, Florence, Italy

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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

* Corresponding author: Lucia Pallecchi Department of Medical Biotechnologies, University of Siena, Santa Maria alle Scotte University Hospital, 53100, Siena, Italy.
E-mail address: lucia.pallecchi@unisi.it (L. Pallecchi).

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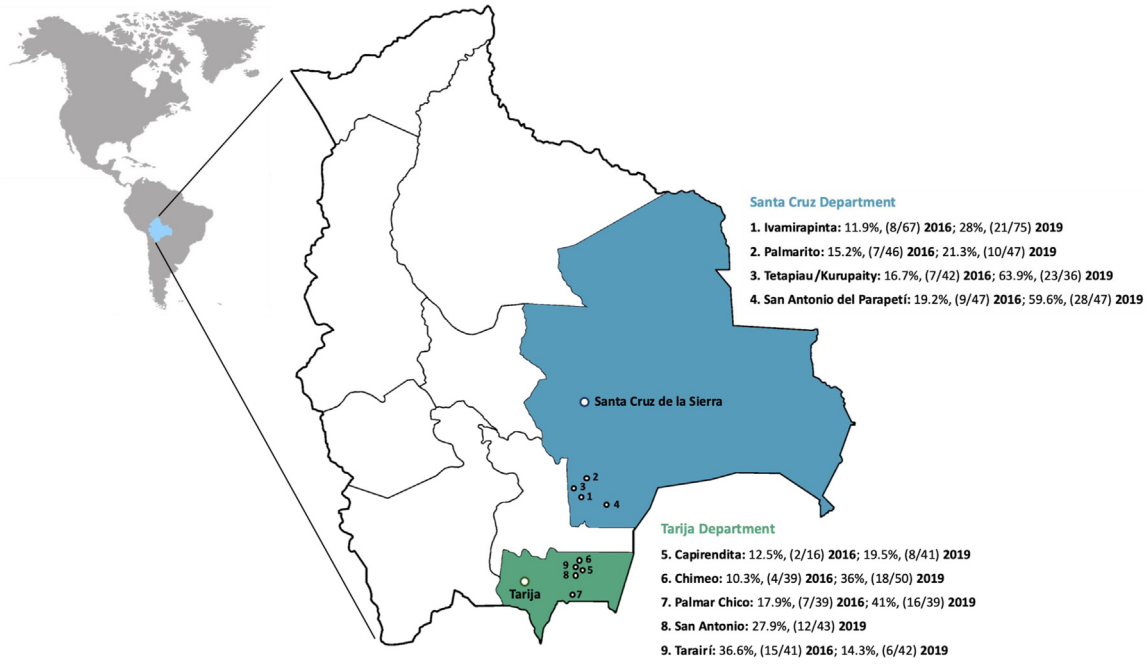

Fig. 1. Geographical area of the nine communities included in the study and percentages of children with CTX-M–positive *Escherichia coli*.

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

Selene Rebecca Boncompagni[1]†, Maria Micieli[2]†, Tiziana Di Maggio[1], Noemi Aiezza[2], Alberto Antonelli[2,3], Tommaso Giani[2,3], Gloria Padoani[4], Silvia Vailati[4], Lucia Pallecchi[1] and Gian Maria Rossolini[2,3]*

[1]*Department of Medical Biotechnologies, University of Siena, Santa Maria alle Scotte University Hospital, Viale Bracci, 53100 Siena, Italy;* [2]*Department of Experimental and Clinical Medicine, University of Florence, Careggi University Hospital, Largo Brambilla, Florence, Italy;* [3]*Microbiology and Virology Unit, Careggi University Hospital, Largo Brambilla, Florence, Italy;* [4]*Zambon S.p.A. via Lillo del Duca 10, 20091 Bresso (Milan), Italy*

*Corresponding author. E-mail: gianmaria.rossolini@unifi.it
†These authors contributed equally to this work.

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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.

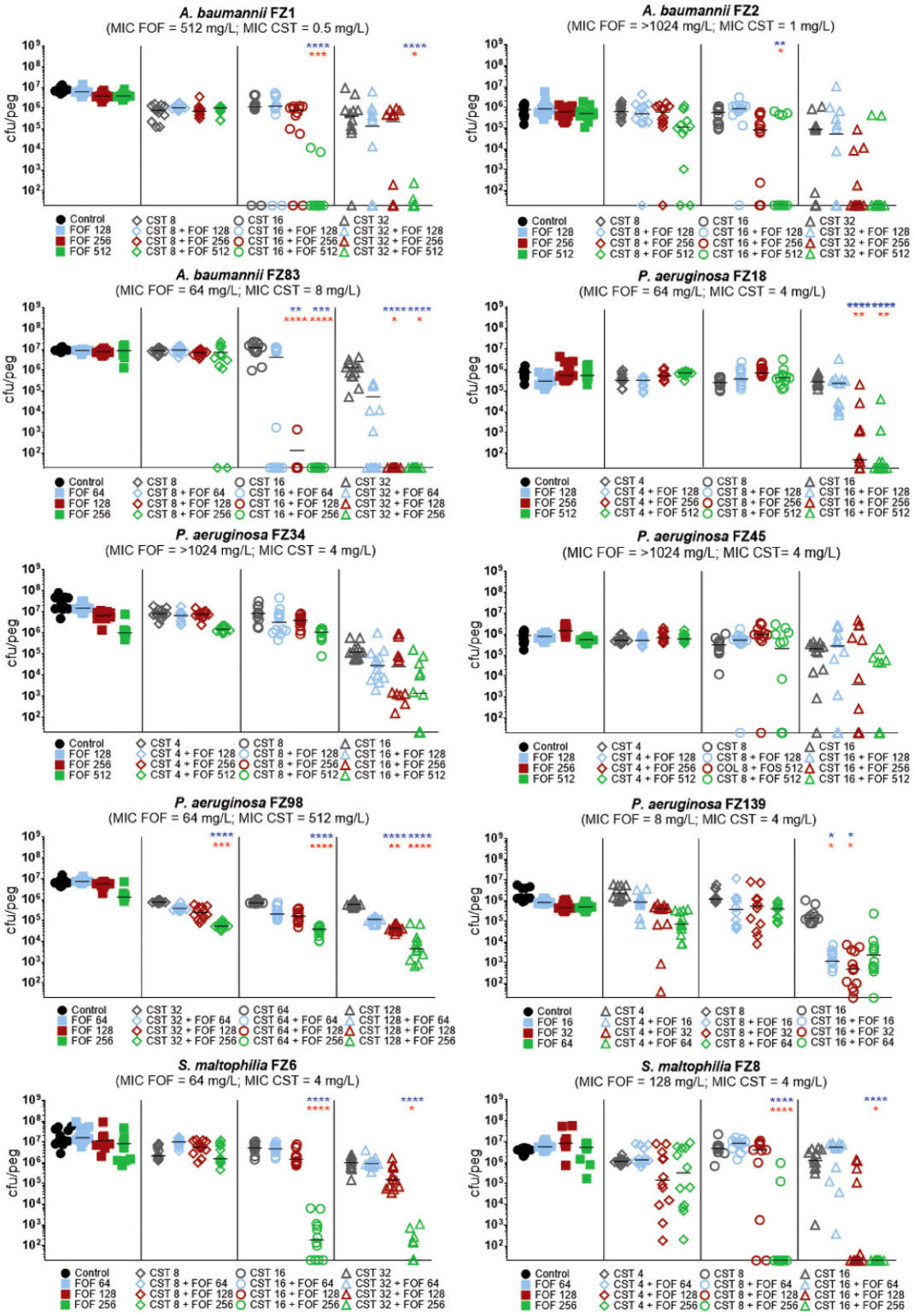

Figure 1. Antibiofilm activity of fosfomycin (FOF)/colistin (CST) combinations. Data from at least two independent experiments, with six replicates per condition per experiment. Median values are plotted. The *x*-axes are set at the limit of detection (i.e. 20 cfu/peg). In graphic legends, FOF and CST 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*.

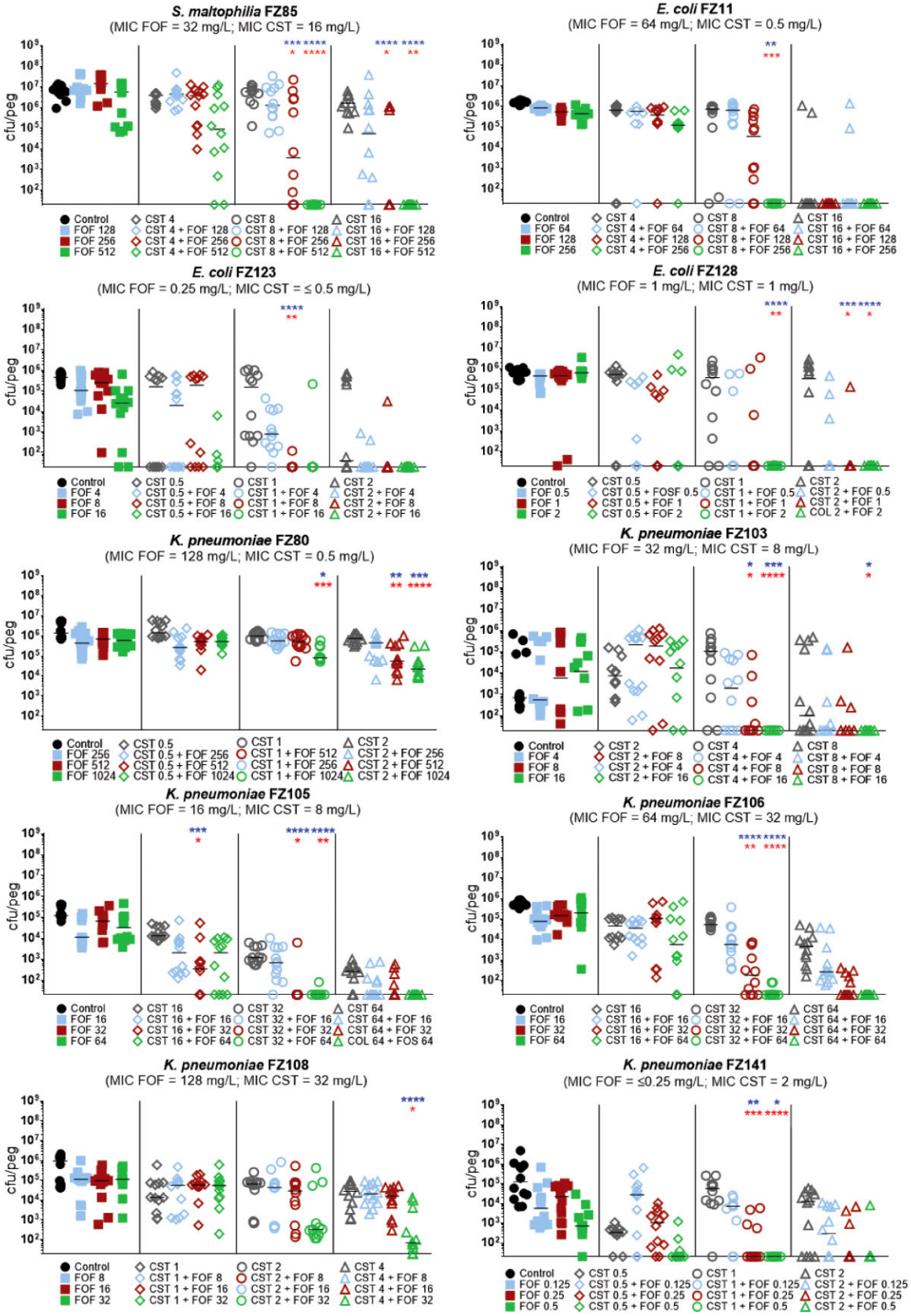

Figure 1. Continued

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).

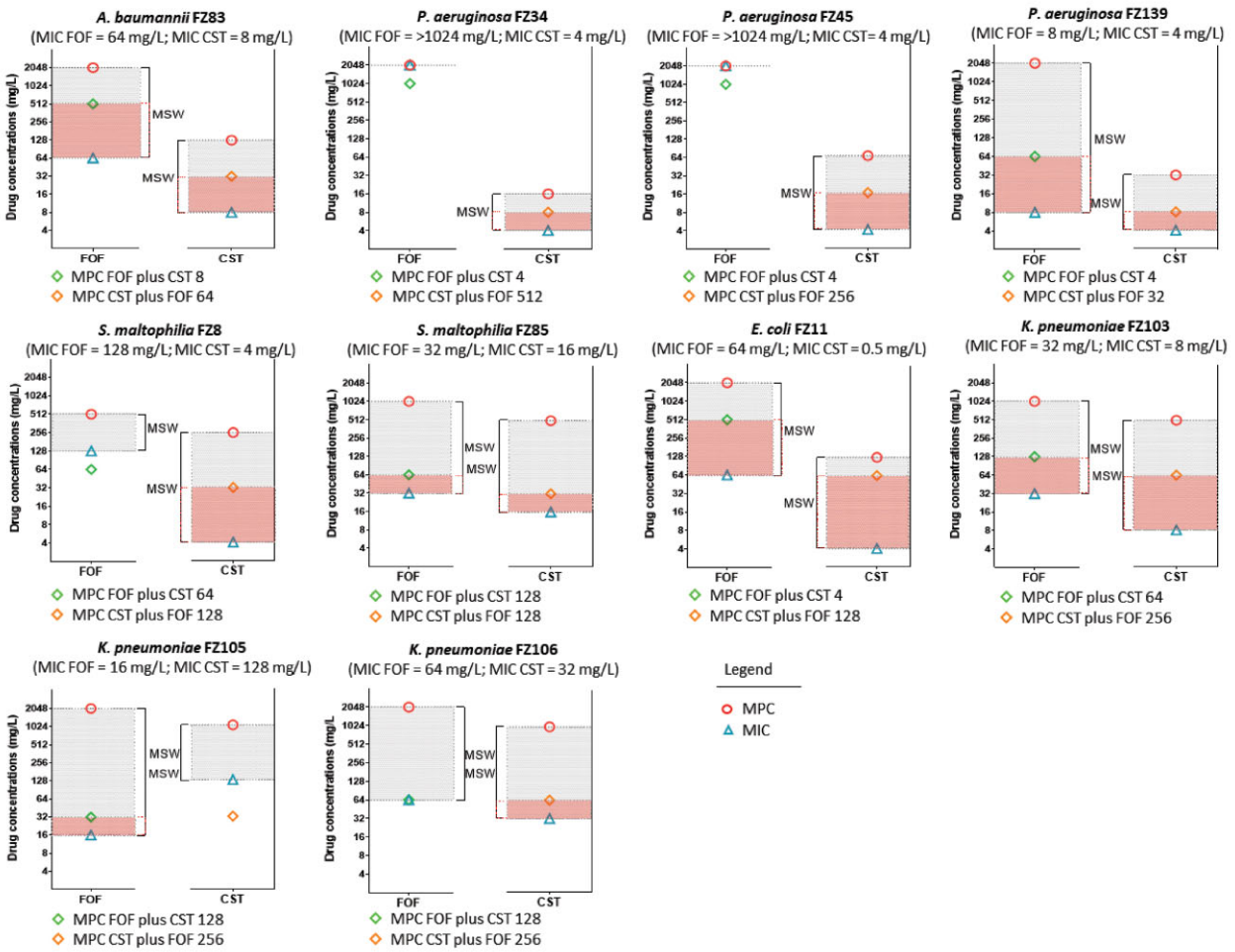

Figure 2. MPCs and MSWs of fosfomycin (FOF)/colistin (CST) combinations of 10 selected strains. Red shading indicates the MSW of fosfomycin/colistin combinations. In graphic legends, FOF and CST concentrations are expressed in mg/L. MPC values >1024 mg/L were reported as 2048 mg/L. This figure appears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

Felice Valzano,[a] Selene Rebecca Boncompagni,[a] Maria Micieli,[b] Tiziana Di Maggio,[a] Vincenzo Di Pilato,[c] Lorenzo Colombini,[d] Francesco Santoro,[d] Gianni Pozzi,[d] Gian Maria Rossolini,[b,e] Lucia Pallecchi[a]

[a]Department of Medical Biotechnologies, University of Siena, Siena, Italy
[b]Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy
[c]Department of Surgical Sciences and Integrated Diagnostics, University of Genoa, Genoa, Italy
[d]Laboratory of Molecular Microbiology and Biotechnology, Department of Medical Biotechnologies, University of Siena, Siena, Italy
[e]Clinical Microbiology and Virology Unit, Careggi University Hospital, Florence, Italy

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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Address correspondence to Lucia Pallecchi, lucia.pallecchi@unisi.it.

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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

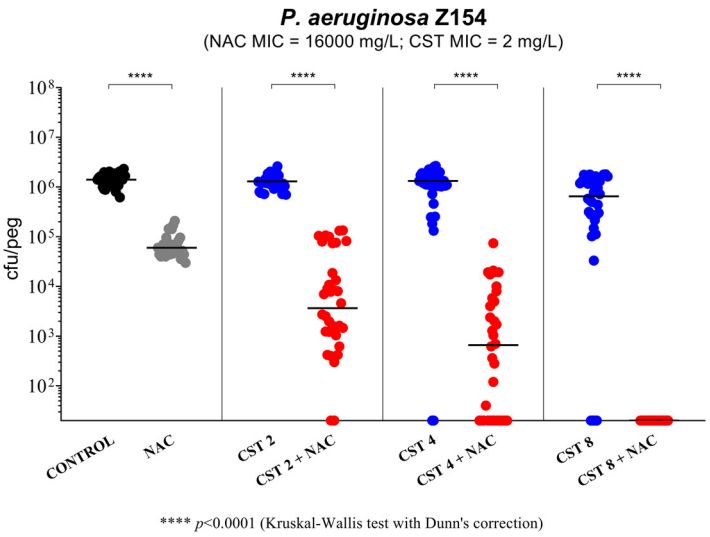
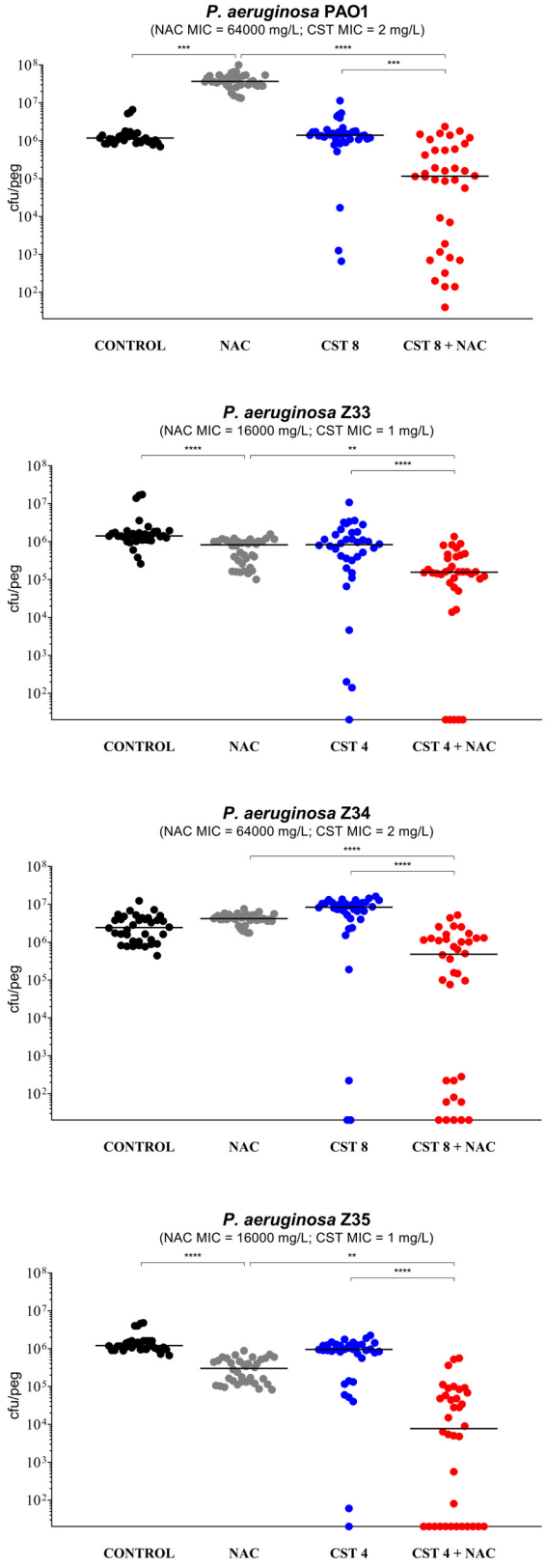


FIG 1 Antibiofilm activity of *N*-acetylcysteine (NAC) at 8,000 mg/L, colistin (CST), and NAC-CST combinations against *P. aeruginosa* Z154 in the Nunc-TSP lid system. A relevant potentiation of colistin antibiofilm activity was observed with all NAC-CST combinations tested. CST 2, colistin at 2 mg/L; 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).

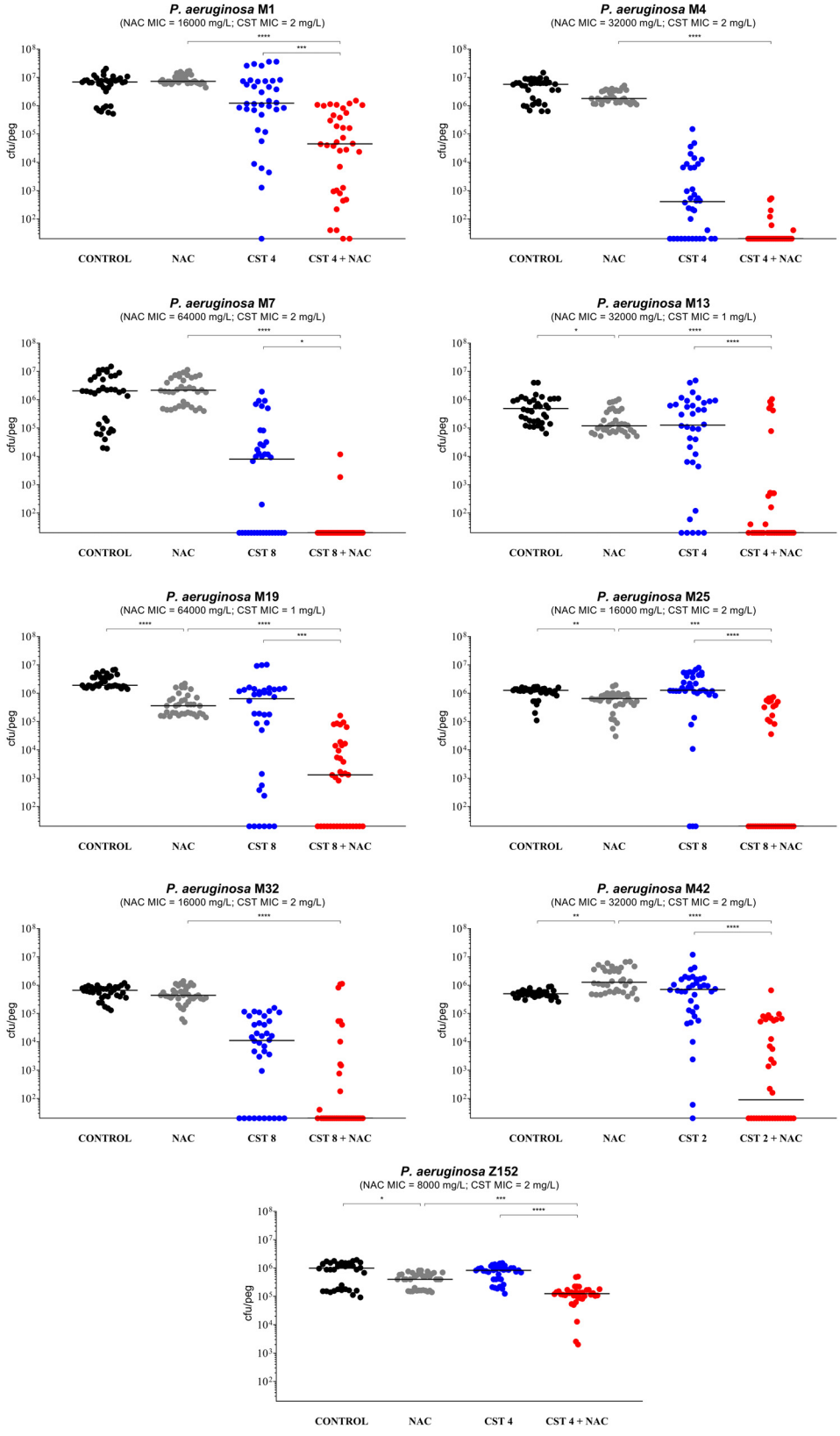
The remaining 16 strains were initially tested with the combination of NAC at 8,000 mg/L plus colistin at 8 mg/L. In order to detect a potential synergism, the concentration of colistin was then modified for strains forming biofilms highly susceptible to colistin ($n = 7$) or particularly resistant ($n = 2$) (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).

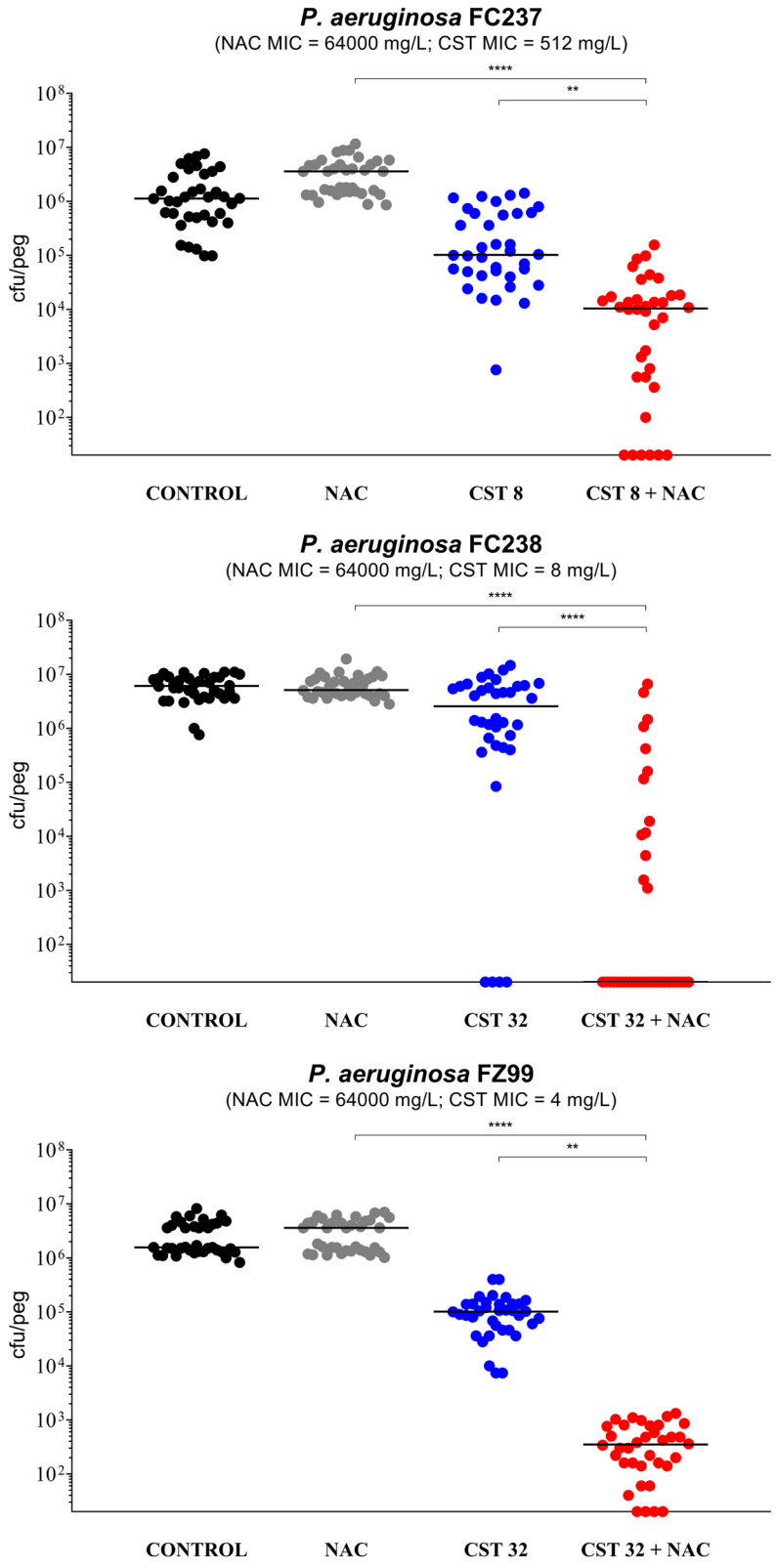
Analysis of DEGs revealed that NAC mainly acted as Zn^{2+} chelator, inducing a strong Zn^{2+} starvation response. DEGs associated with such response were consistent with data reported in previous studies addressing zinc homeostasis in *P. aeruginosa* and other bacteria (Table 2) (17–22). In particular, 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).

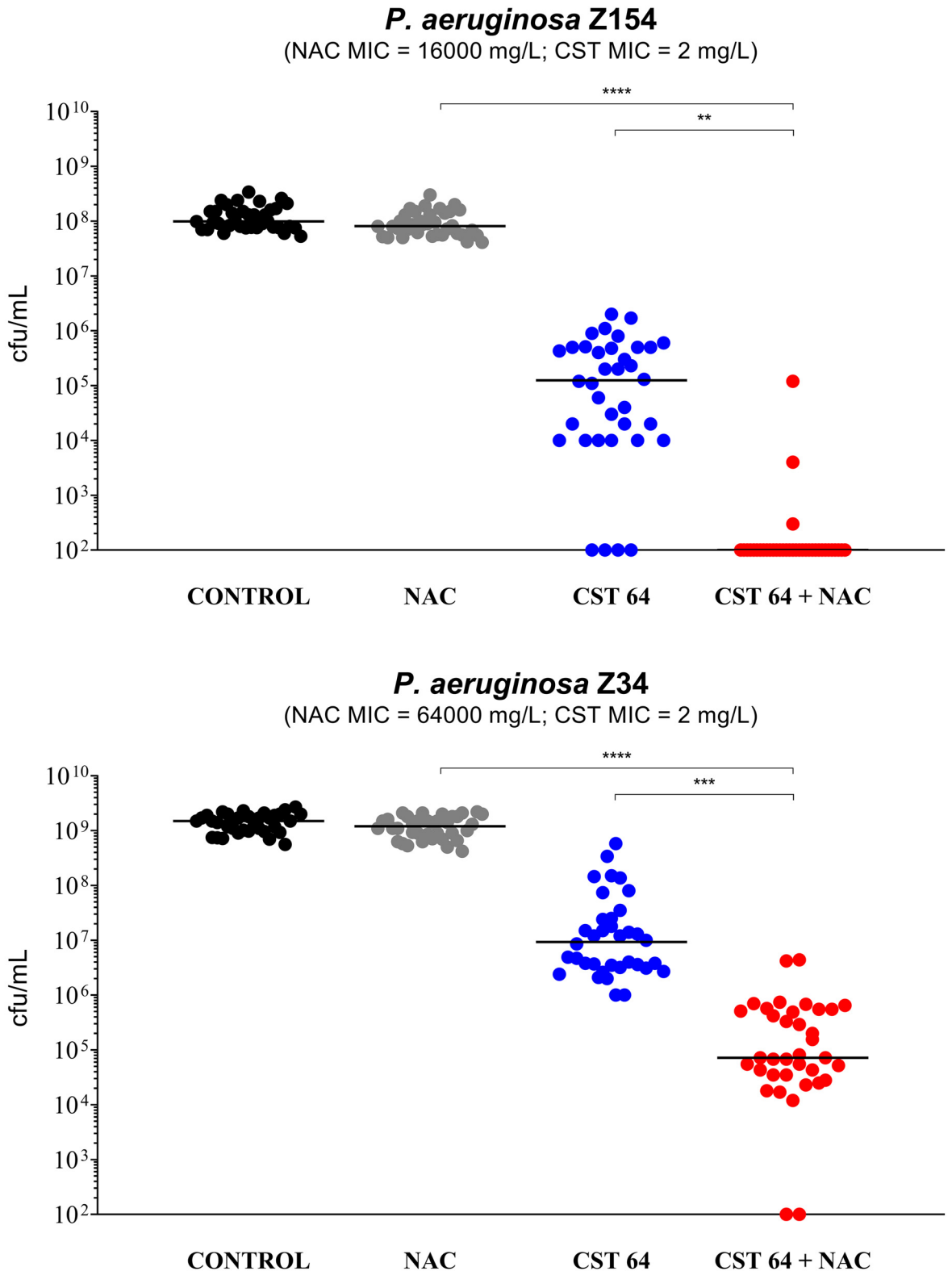
NAC-mediated inhibition of *P. aeruginosa* denitrification pathway. The role of NAC in the inhibition of the denitrification pathway was confirmed by measuring NO_3^- and NO_2^- concentrations during anaerobic growth of the *P. aeruginosa* Z154 strain (i.e., the strain used for transcriptomic analysis) 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.

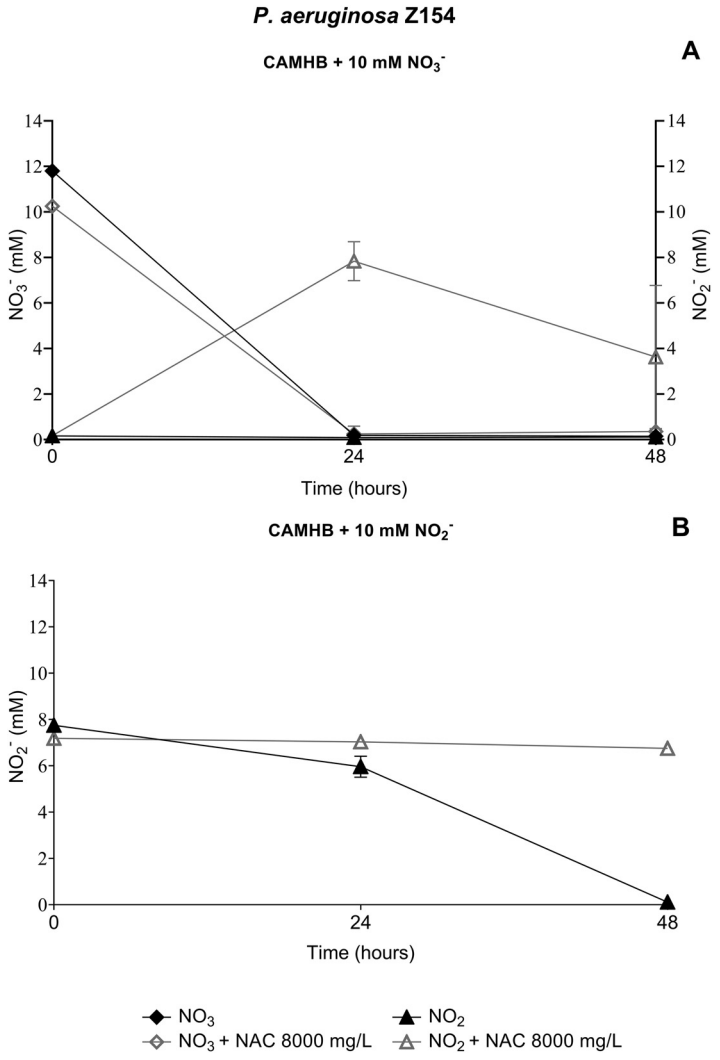


FIG 6 NAC-mediated inhibition of *P. aeruginosa* Z154 denitrification pathway. (A) NO_3^- and NO_2^- concentrations in anaerobic CAMHB supplemented with 10 mM NO_3^-, with or without NAC at 8,000 mg/L; (B) NO_2^- concentration in anaerobic CAMHB supplemented with 10 mM NO_2^-, with or without NAC at 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

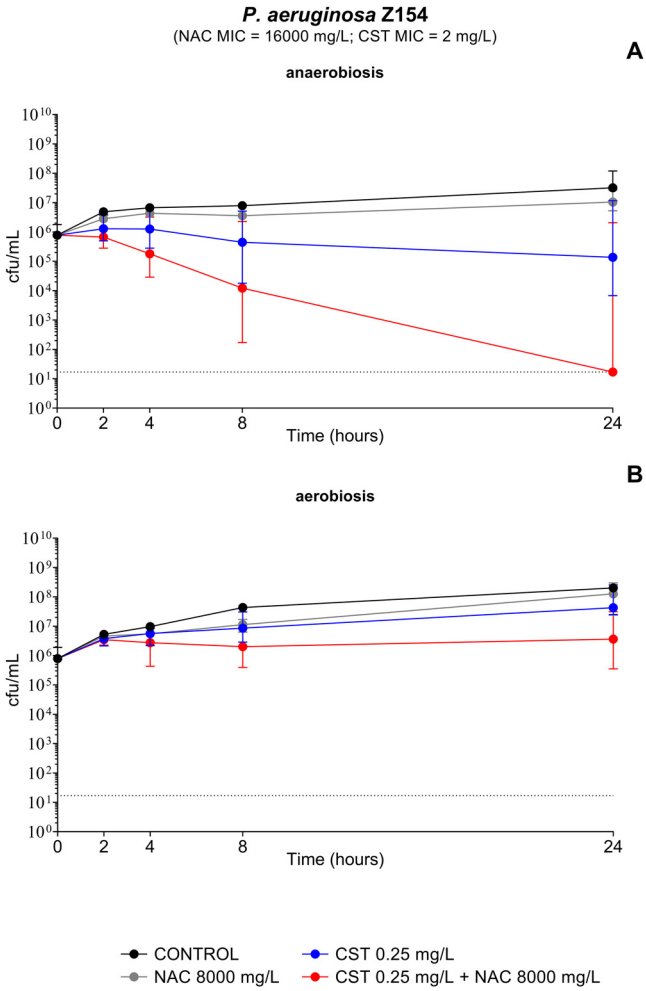


FIG 7 Time-kill curves of *P. aeruginosa* Z154 planktonic cultures exposed to *N*-acetylcysteine (NAC) at 8,000 mg/L, colistin (CST) at 0.25 mg/L, and the NAC-CST combination under anaerobic (A) and aerobic (B) conditions. NAC potentiated the bactericidal activity of colistin only under anaerobic conditions. 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.

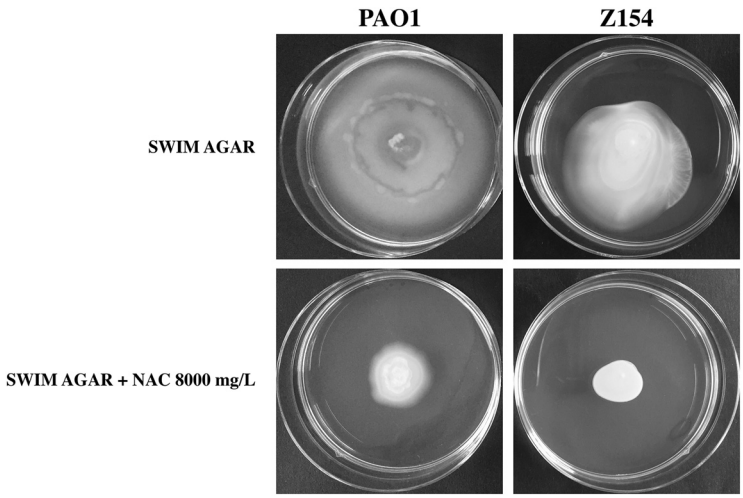


FIG 8 NAC-mediated inhibition of *P. aeruginosa* PAO1 and Z154 swimming motility. Assays were performed in at least three independent experiments (with three replicates per condition per experiment), and representative data are shown.

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

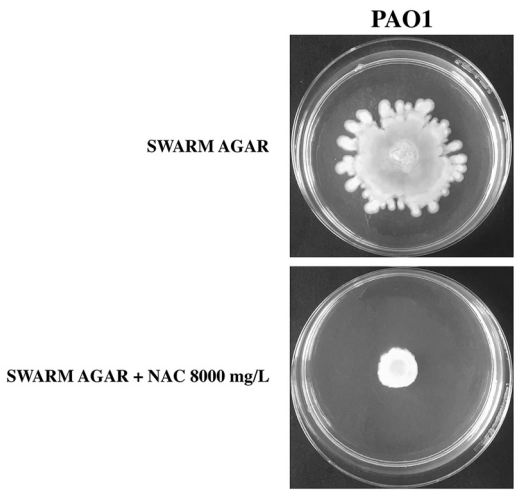


FIG 9 NAC-mediated inhibition of *P. aeruginosa* PAO1 swarming motility. Assays were performed in at least three independent experiments (with three replicates per condition per experiment), and representative data are shown.

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.

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