Impact of commonly used antimicrobial biocides on resistance and cross-resistance in carbapenemase-producing Enterobacteriaceae

Thesis presented for the Degree of Doctor of Philosophy by

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Summary

Infections due to multi-drug resistant organisms (MDROs) are a major concern worldwide. With very few new antibiotics on the market, infection prevention and control measures, including the use of biocides, are paramount to limit the spread of MDROs. Little is known about the impact of biocide overuse and the selective pressure they exert on adaptive mechanisms (co-selection) within bacteria. This study evaluated the effects of biocides commonly used in the UK on antibiotic susceptibility and on the mechanisms they might trigger within carbapenemase-producing Enterobacteriaceae (CPE).

The minimum inhibitory concentrations (MICs) of a wide range of antibiotics and four biocides (benzalkonium chloride, chlorhexidine digluconate [CHX], copper sulphate and silver nitrate) were determined for multi-drug resistant, carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates collected from UK hospitals. Regarding biocides, MICs were found to be much lower than in-use concentrations found in most commercial products (e.g. CHX MICs: 0.5-16 μg/mL; CHX concentration in a skin decontamination product: 40,000 μg/mL). Correlation were detected between antibiotic and biocide MICs, especially between CHX and carbapenems, cephalosporins, ciprofloxacin and tetracycline in *K. pneumoniae*. Exposure to CHX for 50 strains led to increased lag phase duration and changes in growth rate. Addition of efflux-pump inhibitor reduced the CHX MICs. These findings seemed to indicate the involvement of efflux in reduced susceptibility to CHX and antibiotics. Overexpression of efflux-related genes (*acrB*, extruding a wide range of antibiotics; *smvA*, for cationic compounds) was observed following exposure to sub-MIC CHX concentrations for two *K. pneumoniae* isolates. However, the molecular changes did not necessarily translate at the phenotypical level, with unchanged susceptibility towards CHX and several antibiotics.

This work highlighted the involvement of efflux pumps following CHX exposure in CPE; while it did not result in reduced susceptibility to antimicrobials, precautions should still be taken, considering the extensive use of biocides.

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ABC	ATP-binding cassette
ADP	Adenosine disphosphate
AK	Amikacin
AMP	Ampicillin
AmpC	Ampicillin-hydrolysing enzyme
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
ATM	Aztreonam
ATP	Adenosine triphosphate
BMD	Broth microdilution
BSAC	British Society for Antimicrobial Chemotherapy
BZC	Benzalkonium chloride
CAMHA	Cation-adjusted Mueller-Hinton agar
CAMHB	Cation-adjusted Mueller-Hinton broth
CAZ	Ceftazidime
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
CFU	Colony forming unit
CHX	Chlorhexidine digluconate
CIP	Ciprofloxacin
CLED	Cystine-lactose-electrolyte-deficient
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamycin
COL	Colistin
CPE	Carbapenemase-producing Enterobacteriaceae
CPM	Cefepime
CRE	Carbapenem-resistant Enterobacteriaceae
CS	Copper sulphate
CTX	Cefotaxime
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECOFF	Epidemiologic cut-off value
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum β-lactamase
ETP	Ertapenem
EUCAST	European Union Committee on Antimicrobial Susceptibility Testing
FOX	Cefoxitin
GES	Guiana extended-spectrum β-lactamase
HGT	Horizontal gene transfer
IM	Inner membrane
IMI	Imipenem-hydrolysing β-lactamase
IMP	Active on imipenem β-lactamase
IPM	Imipenem
ISA	Iso-Sensitest agar

ISB	Iso-Sensitest broth
KPC	*Klebsiella pneumoniae* carbapenemase
LB	Luria-Bertani broth
LPS	Lipopolysaccharides
MATE	Multridrug and toxic compound extrusion
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm eradication concentration
MBL	Metallo-β-lactamase
MDRO	Multi-drug resistant organism
MEM	Meropenem
MFS	Major facilitator family
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MIN	Minocycline
MLST	Multi-locus sequence typing
mRNA	Messenger RNA
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSC	Minimum selective concentration
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
NDARO	National Database of Antibiotic Resistant Organisms
NDM	New Delhi metallo-β-lactamase
NMC	Non-metallo carbapenemase
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OXA	Oxacillinase
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PIP	Piperacillin
PRE	Post re-exposure
QAC	Quaternary ammonium compound
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
rRNA	Ribosomal RNA
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SD	Standard deviation
sdH_2O	Sterile distilled water
SME	*Serratia marcescens* enzyme
SMR	Small multi-drug resistance
SN	Silver nitrate
ST	Sequence type
TBE	Tris-borate EDTA

TE	Tris-EDTA
TEMO	Temocillin
TIG	Tigecycline
TOB	Tobramycin
tRNA	Transfer RNA
TSA	Tryptone soya agar
TSB	Tryptone soya broth
TSC	Tryptone sodium chloride
TYE	Tryptone yeast extract broth
UTI	Urinary tract infection
VIM	Verona integron-encoded metallo-β-lactamase
WGS	Whole-genome sequencing
WHO	World Health Organization

Chapter 1. General introduction

1.1 Definitions

The term '**antimicrobial**' encompasses both antibiotics and biocides (Scientific Committee on Emerging and Newly Identified Health Risks, SCENIHR, 2009; Harbarth *et al.*, 2014). On one hand, **antibiotics** are defined as active substances of synthetic or natural origin harbouring anti-infective activity against bacteria in human or animal tissues (SCENIHR, 2009; Wales & Davies, 2015). On the other hand, **biocides** are active chemical substances able to destroy, render harmless, prevent the action of, or exert a controlling effect on harmful organisms (SCENIHR, 2009; Wales & Davies, 2015).

Biocides targeting bacteria include disinfectants, antiseptics and preservatives, and can also be referred to as microbicides (Maillard *et al.*, 2013); in this study, the term 'biocide' will be used in its narrow meaning, i.e. microbicide. **Disinfectants** are used on inanimate objects to reduce their amounts of micro-organisms; high-level disinfectants are referred to as **sterilants** (Wales & Davies, 2015; Beier *et al.*, 2016). **Antiseptics** are used for skin decolonisation or to treat infections in surface wounds but cannot be used within the body (SCENIHR, 2009; Harbarth *et al.*, 2014). **Preservatives** are chemicals added to products such as cosmetics or food to inhibit the growth of micro-organisms within the products (SCENIHR, 2009; Wales & Davies, 2015).

1.2 Rise of antibiotic resistance: a global public health threat

1.2.1 Current status and international guidelines

Since the discovery of penicillin in the late 1920s (Fleming, 1929), antibiotics have been heavily used and relied upon, especially for the treatment of severe infections (World Health Organization, WHO, 2018a). However, resistance has now been found to emerge all around the world for almost all the currently available classes of antibiotics (Ventola, 2015; WHO, 2018a).

The development of antibiotic resistance naturally occurs, as most antibiotics derive directly or indirectly from microbial products; genes similar to the ones involved in antibiotic resistance have been found in samples aged several millions of years. The ability of bacteria to develop adaptation mechanisms existed well before the introduction of antibiotics in human health (Iredell *et al.*, 2016; Alav *et al.*, 2018). However, the rise of antibiotic

resistance has been accelerated by the extended use and misuse of these compounds, and seems to be mainly linked to the selective pressure applied by both human and animal medicines, as reported by the World Health Organization (WHO) in 2014 (WHO, 2014). In veterinary medicine, antibiotics belonging to the same classes as the ones prescribed in human medicine are used to prevent infections, but also as growth factors (Chattopadhyay, 2014). The United Kingdom (UK) Department of Health estimated however that the antibiotic consumption in human health had a more important impact on antibiotic resistance than veterinary medicine (UK Department of Health, 2013).

Without regards to the factors involved in the emergence and spread of resistance among bacteria, the increasing number of non-treatable infections due to multi-drug resistant organisms (MDROs) is now a public health threat and a major challenge to overcome (WHO, 2018a). Infections caused by MDROs engender adverse impacts on clinical outcomes such as higher morbidity and mortality rates (MacVane, 2017; WHO, 2018b), and are estimated to account for up to 10 million deaths by 2050 (Review on Antimicrobial Resistance, 2014). International (WHO, United Nations) and national (Review on Antimicrobial Resistance, 2016; UK Department of Health, 2019) commissions, in addition of groups of experts (Wilson *et al.*, 2016), highlighted key aspects and edited guidelines for the current and future management of resistance to antibiotics. The UK Department of Health's 2019 to 2024 plan of action developed three main courses of actions (UK Department of Health, 2019), including the reduction of the need for and unintentional exposure to antibiotics (where infection prevention and control measures are paramount, among other measures), the optimisation of the use of the current antibiotics in both human and animal health (highlighting the importance of antibiotic stewardships and data surveillance), and the need for innovation (including new antibiotics, new diagnostic tools and vaccines).

In addition to the human perspective, resistance to antibiotics involves higher costs due to consumption of healthcare resources. This cost is estimated at 1.5 billion euros per year in Europe (UK Department of Health, 2013; WHO, 2014), and could reach more than 100 trillion US dollars in the whole world by 2050 (Review on Antimicrobial Resistance, 2014). Few antibiotics have been developed over the last decades (corresponding to the so-called 'discovery void'), mostly based on existing molecules or antibiotic-inhibitor combinations, and a minority of them target the MDROs involved in the most severe infections such as carbapenemase-producing bacteria (Review on Antimicrobial Resistance, 2015; WHO, 2020). Most of the current antibiotics have been discovered during the 1940s and 1960s (Coates *et al.*, 2002). Nowadays, the antibiotic market is not the most attractive

for economic reasons: antibiotic therapy is a short-term process, lasting several days, and resistance is likely develop, reducing the lifetime of treatments (Lewis, 2013; Review on Antimicrobial Resistance, 2015; Laws *et al.*, 2019).

1.2.2 Multi-drug resistant organisms on priority list for the development of new antibiotics

The WHO reported in its communication in 2014 several MDROs of particular concern (WHO, 2014), and more recently classified them according to their importance (level three being medium priority and level one being critical priority; WHO, 2017; Tacconelli *et al.*, 2018). Medium priority organisms include penicillin-resistant *Streptococcus pneumoniae* (worldwide leading cause of community-acquired pneumonia), fluoroquinolones-resistant *Shigella* spp. (major cause of diarrhoea and dysentery) and ampicillin-resistant *Haemophilus influenzae*. MDROs in high need for new antibiotics (level two) include, among others, non-typhoidal *Salmonella* species (involved in foodborne illness through the ingestion of contaminated water and/or food but also in enteric fever) and *Neisseria gonorrhoea* with reduced susceptibility to cephalosporins and fluoroquinolone (involved in sexually transmitted infections able to lead to severe complications related to the reproductive tract), alongside some members of the 'ESKAPE' group (Boucher *et al.*, 2009; Laws *et al.*, 2019). The high priority ESKAPE organisms include vancomycin-resistant *Enterococcus faecium* ('E'), which is a major organism involved in bloodstream infections, and methicillin-resistant *Staphylococcus aureus* (MRSA, 'S') with reduced susceptibility to vancomycin. MRSA treatment options become limited, due to the combination of toxicities outcomes and resistance (WHO, 2014). The following MDROs are identified as critical priority for the need of new antibiotics (level one): *Acinetobacter* species ('A'), including carbapenem-resistant *Acinetobacter baumannii*; *Pseudomonas aeruginosa* ('P'), associated with an increasing incidence of resistances to quinolones, carbapenems but also aminoglycosides and polymyxins (important threat especially for ventilator dependent patients and individuals suffering from cystic fibrosis); carbapenem-resistant Enterobacteriaceae (CRE), including extended-spectrum β-lactamase (ESBL)-producing *E. coli* and *Klebsiella* species ('K', but also stands for KPC [*Klebsiella pneumoniae* carbapenemase]-producing Enterobacteriaceae) and *Enterobacter* species ('E'), especially found in healthcare-associated infections (Boucher *et al.*, 2009; Lewis, 2013; WHO, 2014; WHO, 2017).

The present work will focus on Enterobacteriaceae species, especially *E. coli* and *K. pneumoniae*.

1.2.3 Focus on Enterobacteriaceae

Multi-drug resistant Gram-negative bacteria were defined as resistant to at least three different classes of antibiotics (Magiorakos *et al.*, 2012). CRE are increasingly reported, especially in urine samples (Fitzpatrick *et al.*, 2014; Wilson *et al.*, 2016). Both the UK Department of Health and the WHO expressed a high level of concern about *E. coli* and *K. pneumoniae* harbouring important resistances in their reports, as major contributors for healthcare-associated and community-acquired infections (UK Department of Health, 2013; WHO, 2014).

Infections due to those particular organisms are mainly bloodstream infections, followed by respiratory and urinary tracts infections (Lee & Burgess, 2012; Drekonja *et al.*, 2014; Pitout *et al.*, 2015). Several major clones, identified by multilocus sequence typing (MLST) are reported in the literature worldwide. Regarding *E. coli*, the most successful isolates produce ESBLs and/or belong to the sequence type (ST) ST131 often associated to fluoroquinolone resistance (Woodford *et al.*, 2011; Wilson *et al.*, 2016). For *K. pneumoniae*, the predominant ST is ST258, its variants (ST11, ST340, ST512) being less common. The *K. pneumoniae* ST258 clone is strongly associated with KPC production (Naparstek *et al.*, 2012; Pitout *et al.*, 2015; David *et al.*, 2019), especially KPC-2 (clade I) and KPC-3 (clade II) (Peirano *et al.*, 2017).

1.3 Antibiotics

1.3.1 Mechanisms of action

Antibiotics exert their antimicrobial properties through different mechanisms, as illustrated in Figure 1-1. However, most molecules seem to interfere with three main pathways: cell wall synthesis (essential to maintain the integrity of the organism and for osmoregulation), ribosome sub-units (translation step for protein syntheses) and DNA (deoxyribonucleic acid) gyrases and topoisomerases (involved in the chromosomal replication; Lewis, 2013). The β-lactams, including penicillins (e.g. penicillin, amoxicillin), cephalosporins (e.g. cefepime, ceftazidime), carbapenems (e.g. imipenem, ertapenem) and monobactams (e.g. aztreonam) are part of the most often prescribed antibiotics (Iredell *et*

al., 2016) and target the cell wall synthesis through inhibition of the penicillin-binding proteins (PBPs) (Hazra *et al.*, 2014).

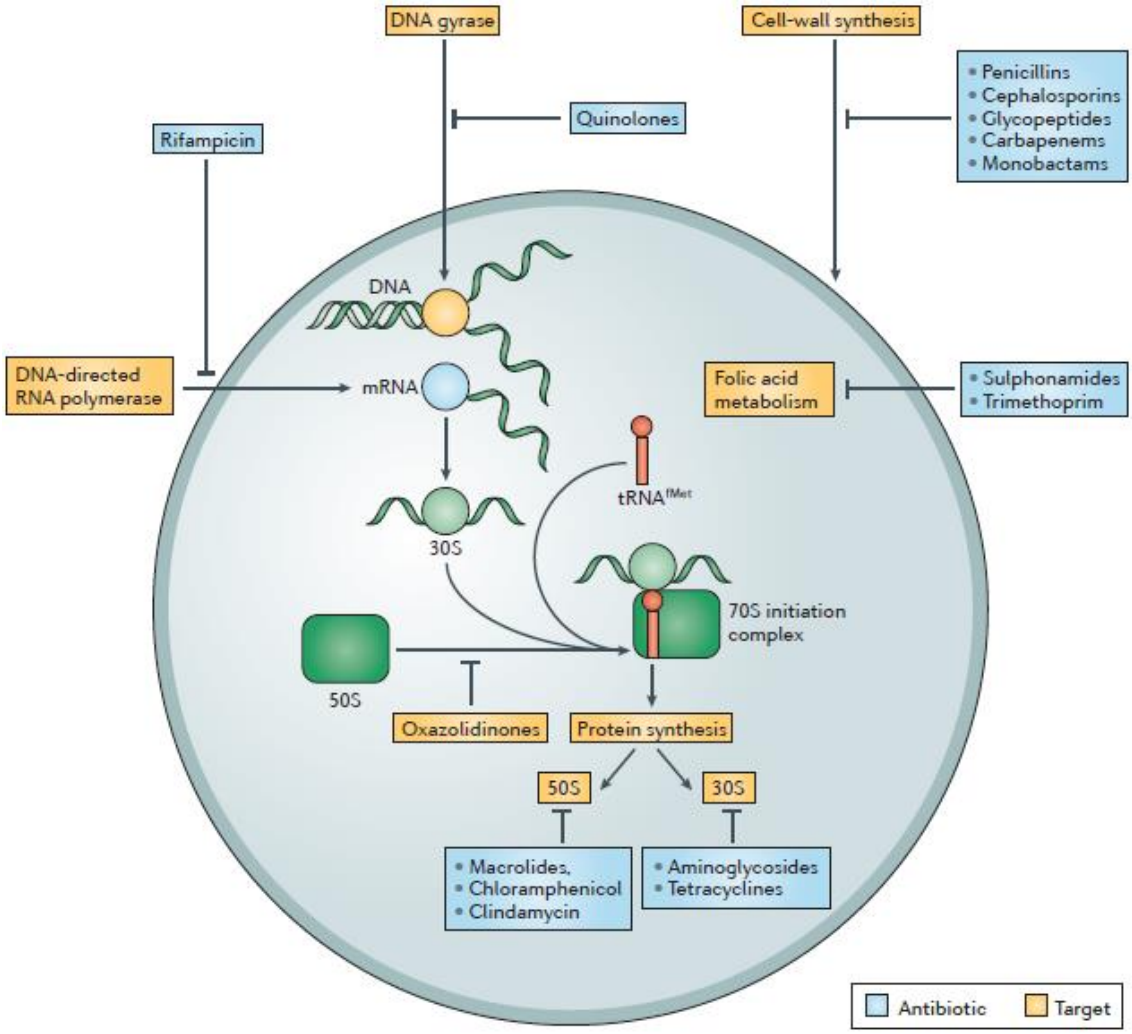

Figure 1-1. Bacterial targets for antibiotics.

Figure from Lewis, 2013. Most antibiotics target major bacterial functions, such as protein synthesis (e.g. by targeting either the 50S or 30S sub-units of the ribosomes), cell-wall synthesis (e.g. by inhibiting penicillin-binding proteins [PBPs], which is the case for β-lactam antibiotics), or chromosomal replication (e.g. through the inhibition of DNA gyrases or topoisomerases).

1.3.2 Resistance to antibiotics

Some bacterial species are intrinsically less susceptible than others due to specific properties, e.g. mycobacteria and Gram-negative bacteria are less susceptible than Gram-positive bacteria due to their cell wall structures (Denyer & Maillard, 2002; Wales & Davies,

2015; Nasiri *et al.*, 2017). Antibiotic resistance mechanisms can also be acquired, through horizontal gene transfer (HGT) or mutation (Andersson & Hughes, 2012; Lewis, 2013), to counteract the deleterious effects of antibiotics. They can result in a decreased entry of the molecules, an increased efflux, the alteration and/or overproduction of the target or the enzymatic modification or destruction of the antibiotic, as illustrated in Figure 1-2 (Poole, 2002; Lewis, 2013); these mechanisms will be further detailed in the following sections. Additional mechanisms include metabolic changes leading to the formation of persister cells. Persisters are non-growing (dormant) but still viable bacteria with importantly reduced susceptibility to antibiotics, and are found to be a frequent cause of chronic infections (Lewis, 2013; Wood *et al.*, 2013). Persisters can be found within biofilms; bacteria living within a biofilm are also less susceptible to antibiotics than their planktonic-growing counterparts. Indeed, the biofilm matrix acts as an additional barrier, limiting the amount of antibiotic able to reach its target (Laws *et al.*, 2019), and bacterial growth rates are usually lower within biofilms, rendering antibiotics of which the mechanism of action affects an active target (e.g. cell wall synthesis for β-lactams) inefficient (Das *et al.*, 1998; Lewis, 2013; Alav *et al.*, 2018).

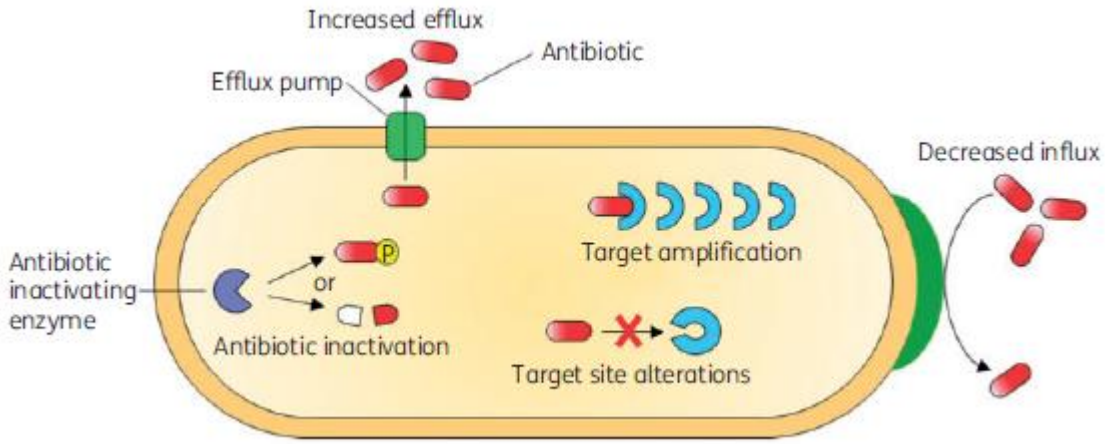

Figure 1-2. General mechanisms of resistance to antibiotics within bacteria.

Figure from Alav *et al.*, 2018. Main antibiotic resistance mechanisms result in the limitation of the antibiotic impact on its target (target alteration or overproduction), the decrease in intracellular antibiotic concentration (decreased intake or increased efflux), or the antibiotic inactivation (modification or destruction).

1.3.2.1 Resistance mechanisms limiting the impact of antibiotics on their target

Target modification or overproduction are involved in resistance to several antibiotics as a way to limit their impact on the bacterial target (Denyer & Maillard, 2002). Indeed, modification of lipid A has been reported to reduce the affinity of polymyxins to their target (Liu *et al.*, 2016). Target modification has also been seen to affect susceptibility to aminoglycosides and fluoroquinolones, with modified 16S ribosomal RNA (ribonucleic acid) methylases and DNA gyrase/topoisomerase, respectively (Iredell *et al.*, 2016).

1.3.2.2 Resistance mechanisms decreasing the intracellular antibiotic concentration

In order to decrease the intracellular concentration of antibiotics, bacteria can either reduce the amount of antibiotics entering the cell, increase the amount expelled from the cell, or a combination of both. Changes in membrane permeability (especially outer membrane for Gram-negative bacteria) include changes in lipopolysaccharide (LPS), fatty acid or protein compositions and changes in hydrophobicity (Denyer & Maillard, 2002; Ortega Morente *et al.*, 2013; Wales & Davies, 2015). However, altered membrane permeability does not seem to confer resistance on its own but can become significant when additional resistance mechanisms are involved (Denyer & Maillard, 2002; Poole, 2002).

Porins (also referred to as outer membrane proteins; OMPs) are hydrophilic channels spanning the outer membrane of Gram-negative bacteria enabling the specific or non-specific entry of several compounds depending on their size and charge (Fernandez & Hancock, 2012; Choi & Lee, 2019). Several porins have been described in *E. coli*, including OmpC, OmpF and PhoE (Denyer & Maillard, 2002; Choi & Lee, 2019). A mutation in the sequence or a downregulation of the expression of porin-encoding genes can lead to a decrease in porin availability (loss of porins); alone, or in combination with an alteration of the porin's channel size, this can result in a decreased entry of antibiotics. Examples of both phenomena can be found among Enterobacteriaceae, with the loss of OmpF involved in β-lactam resistance in *E. coli* (Denyer & Maillard, 2002; Choi & Lee, 2019), or a decreased susceptibility to cefoxitin in *K. pneumoniae* after inactivation of the *ompK36* gene following insertion in the sequence (Denyer & Maillard, 2002; Iredell *et al.*, 2016).

Bacterial efflux pumps are able to expel a wide range of substrates, from antibiotics (e.g. fluoroquinolones, tetracyclines) to biocides and dyes (Nikaido & Pages, 2012; Venter *et al.*, 2015). They can be classified into five superfamilies: the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), the ATP (adenosine triphosphate)-binding cassette

(ABC) superfamily and the resistance-nodulation-division (RND) family (only found in Gram-negative bacteria) (Marquez, 2005; Alav *et al.*, 2018), as illustrated in Figure 1-3. In Gram-negative bacteria, the RND family is of particular importance, with the example of AcrA-AcrB-TolC in *E. coli*, as they are associated with high levels of resistance to antibiotics (Poole, 2007; Sun *et al.*, 2014; Slipski *et al.*, 2018).

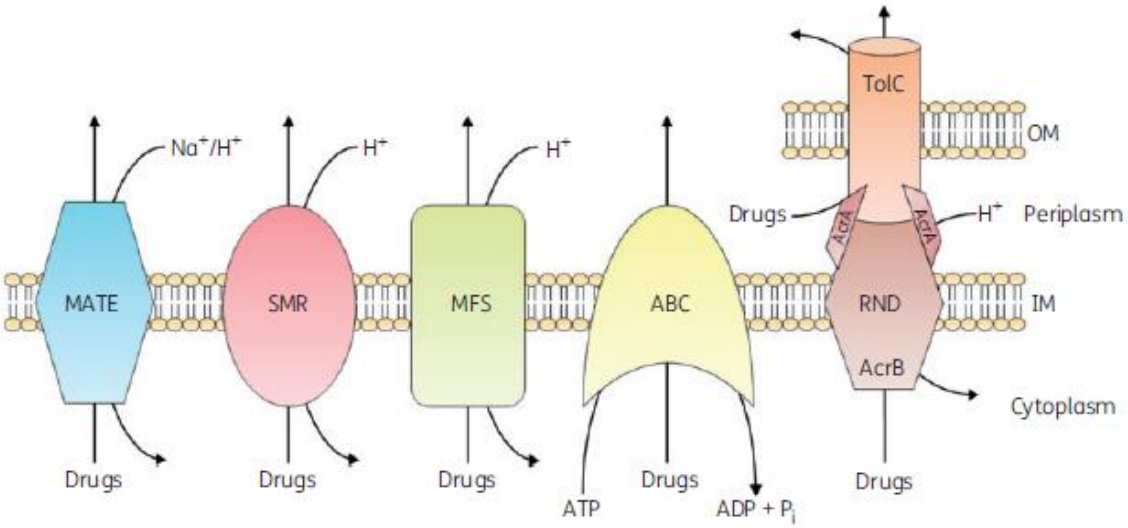

Figure 1-3. Bacterial efflux pump superfamilies.

Figure from Alav *et al.*, 2018. Efflux pumps expel their substrates to the extra-cellular environment through antiport mechanisms (mostly protons but also sodium ions) or using ATP. ADP: adenosine diphosphate; OM: outer membrane; IM: inner membrane.

1.3.2.3 Resistance mechanisms resulting in antibiotic inactivation

The modification or destruction of antibiotics by bacterial enzymes have been reported for several antibiotics and aim to avoid their binding to their target. For instance, the predominant mechanism of resistance to β-lactams is the hydrolysation of the β-lactam ring of these compounds by β-lactamases (Poole, 2002; Lewis, 2013), and modifications of aminoglycosides (acetylation, phosphorylation) has been found to impair their ability to bind to their target (ribosomes), rendering these antibiotics inefficient (Poole, 2002; Iredell *et al.*, 2016).

1.3.3 Focus on carbapenems and carbapenemases

1.3.3.1 Carbapenems

Carbapenems belong to the superfamily of β-lactam antibiotics. Their pioneer molecule, thienamycin, produced by *Streptomyces cattleya*, was first reported in 1976. Due to low rates for the purification of this natural product and stability issues, synthesis of derivatives was investigated (Queenan & Bush, 2007; Papp-Wallace *et al.*, 2011). The first developed was imipenem, released on the market in 1985 for the treatment of severe bacterial infections (Ventola, 2015). Imipenem was however administered with an inhibitor of a human renal enzyme which was able to degrade it. Nevertheless, this led to the development of more stable compounds, including meropenem, ertapenem and doripenem, the main carbapenems referred to in the literature (Martinez *et al.*, 2010; Papp-Wallace *et al.*, 2011).

The mechanisms of action of carbapenems are similar to the ones of the other β-lactams; they inhibit PBPs, stopping cell wall synthesis and resulting in the weakening of peptidoglycan, which is not able to protect the cell from osmotic pressure anymore (Papp-Wallace *et al.*, 2011; Hazra *et al.*, 2014). Nonetheless, they exert a broader antimicrobial spectrum than β-lactams and their combinations due to their ability to bind several different PBP subtypes (Sauvage & Terrak, 2016), while being stable to hydrolysis by almost all ESBLs and AmpC (ampicillin-hydrolysing enzymes), which are other β-lactamases (Thomson, 2010). Indeed, they are used as 'last-resort treatments' for severe infections including the ones caused by ESBL-producing Enterobacteriaceae (Martinez *et al.*, 2010; Djahmi *et al.*, 2014; Morrill *et al.*, 2015). Some of them are more active against Gram-negative bacteria (ertapenem and meropenem) or against Gram-positive ones (e.g. imipenem), while doripenem shows good antimicrobial properties against both. Their properties also differ in terms of potency and stability, with doripenem less susceptible to degradation by carbapenemases, or a narrower spectrum for ertapenem, which is not effective for the treatment of infections caused by *P. aeruginosa* (Martinez *et al.*, 2010; Papp-Wallace *et al.*, 2011; Bassetti & Righi, 2015).

1.3.3.2 Carbapenemases

Now, carbapenemase-producing Enterobacteriaceae (CPE), among other MDROs, constitute a major threat, compromising the efficacy of these last-line treatments (David *et al.*, 2019). The resistance mechanisms involved include a reduced permeability through loss of porins and/or expression of efflux pumps, alteration, reduction and/or elimination of the

target (common mechanisms with other β-lactams) but also production of carbapenemases. Combinations of these mechanisms can result in synergistic activities against carbapenems (Martinez-Martinez & Gonzalez-Lopez, 2014; MacVane, 2017). Carbapenemases can belong to Ambler classes A, B and D β-lactamases (Thomson, 2010; Canton *et al.*, 2012); a brief overview is presented in Table 1-1. They will be further detailed in the following sections, albeit they will not constitute an exhaustive list of all the existing carbapenemases.

Table 1-1. Key carbapenemases mediating resistance in Enterobacteriaceae.

Table adapted from MacVane, 2017. Not all existing carbapenemases are listed in this table. GES: Guiana extended-spectrum β-lactamase; IMI: imipenem-hydrolyzing β-lactamase; IMP: active on imipenem β-lactamase; KPC: *Klebsiella pneumoniae* carbapenemase; NDM: New-Delhi metallo-β-lactamase; OXA: oxacillinase; SME: *Serratia marcescens* enzyme; VIM: Verona integron-encoded metallo-β-lactamase.

Ambler class	**A**	**B**	**D**
Active site	Serine	Bivalent metal ion (Zn^{2+})	Serine
Resistance conferred	Monobactams, carbapenems and 3rd-generation cephalosporins	Penicillins, cephalosporins, carbapenems	May demonstrate activity against carbapenems
Genetics	Highly transmissible on mobile genetic elements (transposons, plasmids) often carrying other multiple resistance determinants	Highly transmissible on plasmids carrying multiple other resistance determinants	Possible acquisition or occurrence on chromosome, may be co-located on plasmids with other β-lactamases
Common species	*E. coli*, *K. pneumoniae*	*E. coli*, *K. pneumoniae*, many Enterobacteriaceae	*K. pneumoniae*
Carbapenemases involved	GES, IMI, KPC, SME	IMP, NDM, VIM	OXA types (mainly OXA-48 and derivatives)

1.3.3.2.1 Class A carbapenemases: penicillinases

The class A carbapenemases became of major importance with the discovery and subsequent massive spread of KPC-producing bacteria around the world. KPC enzymes

were first reported in 1996 in North America (Yigit *et al.*, 2001) and the *bla*$_{KPC}$ gene family includes now more than 40 variants according to the National Database of Antibiotic Resistant Organisms (NDARO), a tool published by the National Center for Biotechnology Information (NCBI), with *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ being the most commonly found variants (Martinez-Martinez & Gonzalez-Lopez, 2014). However, after re-sequencing *bla*$_{KPC-1}$, it was found that its firstly established sequence was wrong, making *bla*$_{KPC-1}$ and *bla*$_{KPC-2}$ the same variant (Nordmann *et al.*, 2009; Pitout *et al.*, 2015). KPC enzymes confer low to moderate resistance to carbapenems, while they are highly active against penicillins and cephalosporins. The genetic determinants coding for KPC enzymes are mainly found in Tn*4401*, a transposon found in a wide range of transferrable plasmids, allowing it to quickly spread among other strains and species (Djahmi *et al.*, 2014; Nordmann, 2014).

Other members of the class A carbapenemases are the *Serratia marcescens* enzymes (SME) and imipenem-hydrolysing β-lactamases (IMI). They are able to hydrolyse a wide range of substrates like penicillins, some cephalosporins and carbapenems (Djahmi *et al.*, 2014; Martinez-Martinez & Gonzalez-Lopez, 2014). According to the NDARO, five variants of *bla*$_{SME}$ have been described to date, as well as 18 variants of *bla*$_{IMI}$ (including *bla*$_{NMC-A}$; non-metallo carbapenemase).

Guiana extended-spectrum β-lactamases (GES) were first considered as ESBLs. About 40 variants of *bla*$_{GES}$ have been identified to date (according to the NDARO), but only some GES enzymes, including GES-2, GES-4, GES-5, GES-6, GES-11, GES-14 and GES-18 are categorised as carbapenemases. The genes coding these enzymes are mainly present as genes cassettes on class 1 integrons but have also been found on some Enterobacteriaceae chromosomes (Martinez-Martinez & Gonzalez-Lopez, 2014). They confer resistance to broad-spectrum cephalosporins and, for several variants, to carbapenems, as mentioned earlier (Nordmann & Poirel, 2014).

1.3.3.2.2 Class B carbapenemases: metallo-β-lactamases

Metallo-β-lactamases (MBLs) are able to hydrolyse almost all β-lactams, with the exception of monobactams. Their activity relies on the presence of zinc ions in their core.

The first plasmid-encoded MBL, IMP-1 (for 'active on imipenem β-lactamase'), was detected in *P. aeruginosa* in the late 1980s (Watanabe *et al.*, 1991). Since then, IMP enzymes have spread worldwide and more than 70 variants of *bla*$_{IMP}$ have been described (NDARO). Their corresponding genes are mainly present within class 1 integrons gene cassettes, but also on class 3 integrons and, less commonly, on chromosomes. Another subclass of MBL,

VIM (Verona integron-encoded metallo-β-lactamase), is also known to be disseminated through the spread of class 1 integrons (Nordmann & Poirel, 2014). To date, about 60 different *bla*$_{VIM}$ variants have been identified (NDARO).

The most recently discovered MBLs are NDM enzymes (New-Delhi metallo-β-lactamase), in the late 2000s (Yong *et al.*, 2009). Since then, NDM enzymes have been progressively identified on all continents, with a dissemination especially associated with travel (including medical tourism) in Asia (Zmarlicka *et al.*, 2015). Twenty-six variants have been reported so far according to the NDARO. Genes coding for these enzymes can be found either on chromosomes (mainly for *Acinetobacter* species) or plasmids belonging to different incompatibility groups, mediating an efficient spread among bacteria (Djahmi *et al.*, 2014; Martinez-Martinez & Gonzalez-Lopez, 2014).

1.3.3.2.3 Class D carbapenemases: oxacillinases

OXA (oxacillinase) enzymes are narrow-spectrum β-lactamases. Among the approximately 800 enzymes (according to the NDARO), not all of them are carbapenemases, and other members of this family are ESBLs (Nordmann & Poirel, 2014). They harbour a weaker activity against carbapenems than the previously described carbapenemases. However, resistant phenotypes can be observed when OXA are present along with another resistance mechanism like porin loss, expression of efflux pumps or ESBLs (Djahmi *et al.*, 2014).

The main OXA enzymes with carbapenemase activity are OXA-48 and its derivatives (OXA-48-like); these variants differ by a small number of amino acid substitutions (Mathers *et al.*, 2015). The corresponding genes are flanked by two copies of the same insertion sequence within the transposon Tn*1999*. This genetic context favours both the expression and the mobilization of the genetic determinants (Martinez-Martinez & Gonzalez-Lopez, 2014; Pitout *et al.*, 2015).

1.4 Biocides

1.4.1 General

Biocides englobe a wide range of unrelated chemical families, offering various possible uses, from food preservatives to high-level disinfection compounds. Examples of biocide families, biocidal compounds and possible uses are listed in Table 1-2.

Table 1-2. Biocide families and their associated uses.

Data extracted from Fraise *et al.*, 2013 (chapter 2). Not all existing families of biocides, biocidal compounds or uses are listed in this table. QAC: quaternary ammonium compound.

Chemical families	Examples of compounds	Examples of uses
Alcohols	Ethanol, phenoxy ethanol	Surface disinfection, skin decolonization
Aldehydes	Glutaraldehyde, formaldehyde	High-level disinfection
Biguanides	Chlorhexidine, alexidine	Surface disinfection, skin decolonization, dental care
Halogens	Iodine compounds, chlorine compounds	Wound treatment, skin decolonization, sanitizing agents in the food industry
Heavy metal derivatives	Copper, silver	Fungicide/algicide and other microbicidal properties, burn wound treatment
Microbicidal dyes	Acridines, triphenylmethane dyes	Wound treatment, local antiseptics
Organic and inorganic acids	Acetic acid, salicylic acid	Food preservatives, insecticides, fungicides
Peroxygens	Hydrogen peroxide, peracetic acid	High-level disinfection
Phenols	Triclosan	Preservatives, disinfectants for manufactured products
Surface-active agents	QACs	Surface disinfection, preservation of pharmaceutical and cosmetic products

While being around for decades, biocides were put aside during the antibiotic golden era; however, there is a revival of interest for these compounds with the rise of antibiotic resistance and the subsequent need for improved infection control and prevention practices (Maillard *et al.*, 2013). The extensive use and misuse of biocides, now found in hospitals settings but also in home-cleaning products, cosmetics and textiles among others, is now questionable regarding the hypothetic detrimental effect on human health of these compounds and their possible involvement in driving antibiotic resistance (Maillard, 2005; SCENIHR, 2009; Morrissey *et al.*, 2014).

The present work will focus mainly on four biocidal compounds. Chlorhexidine digluconate (CHX) and benzalkonium chloride (BZC; a quaternary ammonium compound [QAC]), both cationic compounds, are amongst the most commonly used biocides in healthcare settings (Russell, 1986; Buffet-Bataillon *et al.*, 2012b; Ortega Morente *et al.*, 2013; Kampf, 2016). Copper and silver, two heavy metals, were also included as they seem to harbour less toxicity to both humans and the environment and are involved in more recent

technologies through the development of antimicrobial surfaces and nanoparticles, respectively (O'Gorman & Humphreys, 2012; SCENIHR, 2014; Cavassin *et al.*, 2015; Hans *et al.*, 2015).

1.4.2 Mechanisms of action

As opposed to antibiotics, biocides usually possess multiple targets within the bacterial cell (Figure 1-4), which is thought to limit the possibility to develop mechanisms resulting in reduced susceptibility to these compounds (Bloomfield, 2002; Yazdankhah *et al.*, 2006).

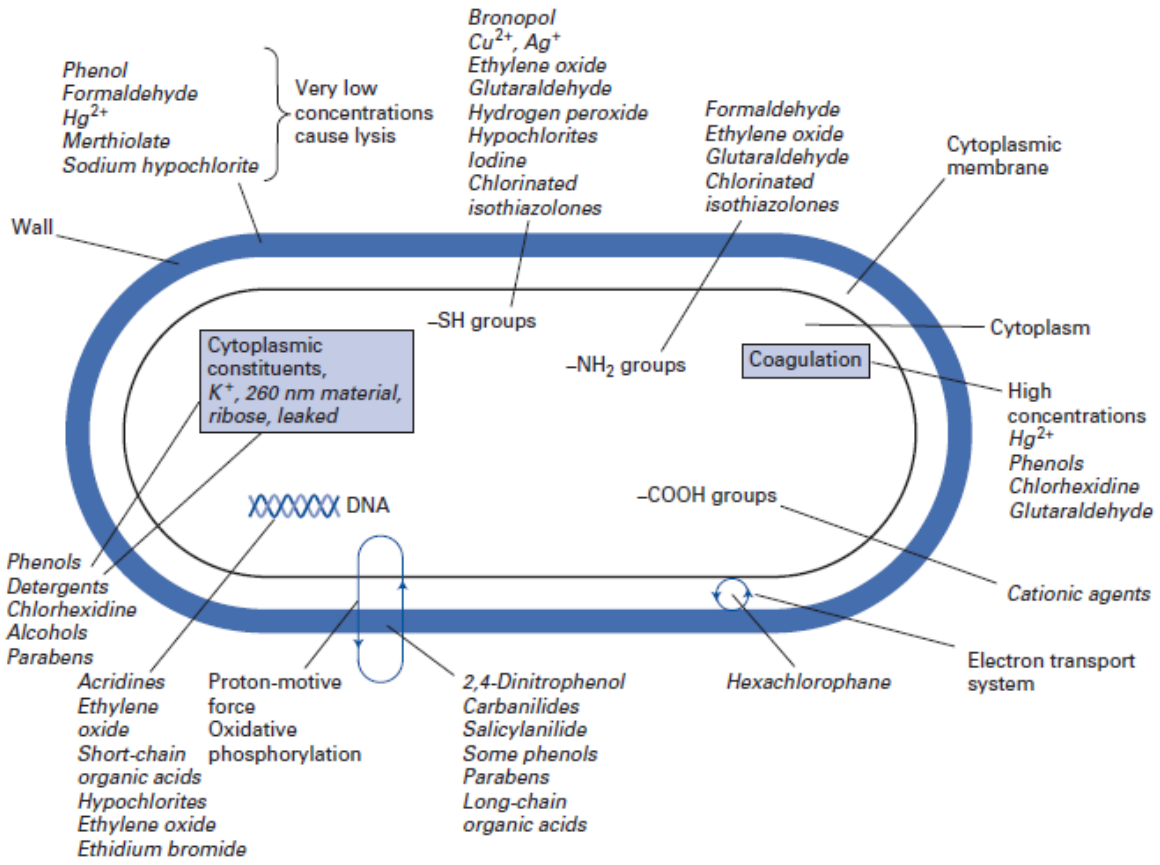

Figure 1-4. Different biocides' mechanisms of action in bacteria.
Figure from Fraise *et al.*, 2013 (chapter 5, page 96). Biocides possess several targets within bacteria, and their mechanisms of action can depend on their concentrations.

BZC and CHX, both cationic compounds, share some similarities in their mechanisms of action. Indeed, they are attracted by the negative charges on the bacterial membrane and displace the metal cations (Mg^{2+}, Ca^{2+}) ensuring its stability (Maillard, 2002;

Gilbert & Moore, 2005). QACs are then able to integrate into the membrane thanks to their hydrophobic tail, disturbing the lipid bilayer, resulting into a progressive leakage of cellular content (Buffet-Bataillon *et al.*, 2012b). The activity of QACs, including BZC, depends on their concentrations: low concentrations would result in the loss of proton and sodium gradients and of the subsequent ability to regulate osmosis; indeed, the proton-motive force, relying on proton gradient, is a cytoplasmic membrane-located process upon which several major cell functions rely (Maloney *et al.*, 1974; Kashket, 1985). At medium concentrations, more severe effects are observed on membrane-located processes, including respiration, cell wall synthesis and solute transport; high concentrations of QACs are able to solubilise the membranes and release the entire cell content, resulting in bacterial death (Gilbert & Moore, 2005; Buffet-Bataillon *et al.*, 2012b).

The mechanism of action of CHX differs to the one of QACs in the fact that CHX does not integrate the membrane but bridges pairs of adjacent polar heads groups in the membrane to displace the metallic cations (Gilbert & Moore, 2005). Like QACs, the activity of CHX is dose-dependent: at lower concentrations, a reduction in membrane fluidity, as well leakage of cellular constituents (including potassium ions and protons) are observed. Low concentrations of CHX also affect respiratory functions and solute transport. At high concentrations of CHX, the cytoplasmic constituents are coagulated associated with the loss of the membrane's structural integrity, resulting in its bactericidal effect (Gilbert & Moore, 2005; Fraise *et al.*, 2013 page 20).

Silver and copper ions interact especially with thiol groups, which are essential for the activity of various enzymes (Fraise *et al.*, 2013 page 42). Reaction with these groups can lead to cell inhibition or inactivation through interference with electron transport (then inhibiting cellular respiration) for example (Maillard, 2002; Glasser *et al.*, 2010). Silver salts also cause membrane damage as can be indicated by potassium leakage, and inhibit cell division mechanisms (Fraise *et al.*, 2013 page 42).

1.4.3 Mechanisms involved in reduced susceptibility to biocides

Mechanisms leading to reduced susceptibility to biocides are similar to the ones involved in antibiotic resistance. Firstly, it can be driven by degradation, dilution or modification of the biocide; examples include the enzymatic degradation of triclosan in *Pseudomonas putida* (Schweizer, 2001; Yazdankhah *et al.*, 2006), or the presence of catalase or superoxide dismutase for oxidizing agents (Maillard, 2018). Target alteration has also

been reported for triclosan, with mutations in *fabI*, rendering the biocide inefficient (Schweizer, 2001; Yazdankhah *et al.*, 2006).

Changes in membrane permeability, including in the cell wall composition and the loss of porins can be involved in reduced susceptibility to biocides, while usually not being the sole mechanisms (Maillard, 2018). For example, deficiency in OmpF and/or OmpC has been described to contribute to the reduced susceptibility to silver in *E. coli* (Li *et al.*, 1997). Efflux has also been reported for biocides, e.g. QAC efflux can be mediated by the Qac efflux pumps belonging to the SMR family, with the importance of the *qacE* and *qacΔE* genes in Enterobacteriaceae; these determinants are commonly found on class 1 integrons, allowing their wide dissemination (Tezel & Pavlostathis, 2015). Another efflux pump, CepA, confers CHX protection in *K. pneumoniae* (Ortega Morente *et al.*, 2013). Copper and silver efflux can be mediated through the Cus and Sil systems in *E. coli* (Nies, 2003).

Physiological and metabolic changes, including biofilm formation, can be involved in the development of reduced susceptibility to biocides as well, as bacteria harbouring a low to null metabolism are less susceptible than the ones with an important metabolic activity (Ortega Morente *et al.*, 2013; Maillard, 2018).

1.4.4 Role of biocides in antibiotic resistance

1.4.4.1 Co-selection mechanisms

Reduced susceptibility to different classes of antimicrobials can be due to several mechanisms including co-resistance, cross-resistance and co-regulation; they constitute the different co-selection mechanisms between biocides and antibiotics found in bacteria (Baker-Austin *et al.*, 2006; Buffet-Bataillon *et al.*, 2012a).

Co-resistance mechanisms depend on the presence of several genetic determinants responsible for the reduced susceptibility to several antimicrobials on the same mobile genetic element, like a plasmid or a transposon; the selective pressure exerted by one compound would select for both genetic determinants to be expressed or transferred (Baker-Austin *et al.*, 2006). In Gram-negative bacteria, the *qacΔE* gene, involved in QAC efflux, is widely found on class 1 integrons, along with other genetic determinants such as *sulI*, conferring resistance to sulphonamides (Fluit & Schmitz, 1999; Domingues *et al.*, 2012). Co-resistance mechanisms are also thought to be involved between susceptibility to antibiotics and heavy metals (Baker-Austin *et al.*, 2006; Jakobsen *et al.*, 2011; Pal *et al.*, 2015).

Cross-resistance happens when several antimicrobials share the same target, initiate a common pathway leading bacterial inhibition and/or death, or share a common access route (Fernández-Fuentes *et al.*, 2012). Such mechanisms depend on the presence and the ability of a common resistance mechanism to counteract the different antimicrobials; as a consequence, if a cross-resistance mechanism is developed following exposure to one compound, resistance or reduced susceptibility to the other compound will arise as well (Buffet-Bataillon *et al.*, 2012a). As previously highlighted in both 'antibiotic resistance' and 'biocide resistance' sections, efflux pumps are widely distributed and can expel an important range of substrates. The efflux pump AcrA-AcraB-TolC, found in *E. coli* for example, can extrude different antibiotics and biocides, especially QACs (Poole, 2007; Sun *et al.*, 2014).

Co-regulation mechanisms would occur when the exposure to an antimicrobial impacts one or several regulatory responses, themselves impacting bacterial susceptibility to other unrelated compounds (Baker-Austin *et al.*, 2006); they can result in the induction of stress response or an increased efflux among others (Bore *et al.*, 2007; Osei Sekyere & Amoako, 2017). For example, the *mar* operon can be expressed following copper exposure, resulting, among other consequences, in an alteration of the outer membrane permeability (Hao *et al.*, 2014).

1.4.4.2 Current controversy and clinical relevance

It is to date not clear whether or not the use of biocides has an influence on the maintenance and spread of antibiotic resistance genes. Indeed, a long-term exposure to sub-inhibitory concentrations of biocides has been shown to impact several regulation pathways, including the SOS response within bacteria, boosting gene transfer and facilitating mutations (Jutkina *et al.*, 2018). This is exacerbated with bacteria growing in biofilms, where exchanges are enhanced within the populations (Tezel & Pavlostathis, 2015; Buffet-Bataillon *et al.*, 2016). The scientific community is divided, with several studies claiming the linkage between antibiotic and biocide susceptibility, mainly due to efflux pumps, while others report no correlation between reduced susceptibility between these antimicrobials (Oggioni *et al.*, 2013; Harbarth *et al.*, 2014; Kampf, 2016; Kampf, 2018). Without consensus, it seems reasonable to assume that the overuse and misuse of biocides could present a risk for resistance selection, even if none has been observed to date; in order to keep biocides efficient at controlling infections, precaution principles should be applied (SCENIHR, 2010; Oggioni *et al.*, 2013; Ortega Morente *et al.*, 2013; Harbarth *et al.*, 2014; Gupta *et al.*, 2018).

1.5 Scope of the study

This study, entitled 'Impact of commonly used antimicrobial biocides on resistance and cross-resistance in carbapenemase-producing Enterobacteriaceae', aimed to understand the role played by several biocides frequently used within healthcare settings, including in infection control and prevention regimens (BZC, CHX, copper and silver ions) in the maintenance of antibiotic resistance genes among clinical, carbapenemase-producing *E. coli* and *K. pneumoniae*. The subsequent objectives were:

- To establish the baseline susceptibility to the four biocides investigated and to a wide range of antibiotics
- To look for potential links between susceptibility to biocides and antibiotics and select representative isolates for further experimental work
- To measure the impact of exposure to sub-inhibitory concentrations of biocide on the phenotypic and molecular levels

Chapter 2. Bacterial susceptibility profiles to antimicrobials

2.1 Introduction

2.1.1 Bacterial susceptibility profiles to chemotherapeutic antibiotics

The determinations of the minimum inhibiting concentration (MIC; the lowest concentration able to inhibit bacterial growth) and of the minimum bactericidal concentration (MBC; the lowest concentration able to kill a micro-organism) are routinely used, easy to implement (including high-throughput testing) techniques to estimate the *in vitro* effect of antimicrobials (Turnidge & Paterson, 2007; Wiegand *et al.*, 2008). Establishing susceptibility profiles to antibiotics is of particular importance to define the susceptible ('high likelihood of therapeutic success using a standard dosing of the agent'), intermediate ('high likelihood of therapeutic success when exposure to the agent is increased either by adjusting the dosing regimen or by its concentration at the site of infection') or resistant ('high likelihood of therapeutic failure even when there is increased exposure') status of a bacterium (European Committee on Antimicrobial Susceptibility Testing, EUCAST, 2019d) and therefore to choose the appropriate treatment for a bacterial infection (Turnidge & Paterson, 2007; Iredell *et al.*, 2016). However, these assays do not reflect clinical conditions: they are usually performed with rapidly growing bacteria belonging to a single bacterial species with a standardised inoculum, in rich media and controlled conditions, while in reality bacteria usually grow slower than in the testing conditions (Mouton, 2018); in addition, as examples, bacteria could also be growing in biofilms with other species instead of their planktonic counterparts, or form an abscess with higher bacterial density and deleterious conditions for some antibiotics (pH variations or additional barriers limiting the antibiotic penetration among others) (Brook, 1989; Iredell *et al.*, 2016).

National and international commissions, including the Clinical and Laboratory Standards Institute (CLSI), the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) and the British Society for Antimicrobial Chemotherapy (BSAC), propose standard protocols for antibiotic susceptibility testing (Andrews, 2001; CLSI, 2012; EUCAST, 2019c) and set breakpoints, which are cut-off values enabling to decipher between susceptible, intermediate and resistant isolates (BSAC, 2015; CLSI, 2019; EUCAST, 2019b). MIC values indicated for a reference strain (e.g. *E. coli* American Type Culture Collection [ATCC] 25922) are usually in accordance between the different standards. This

is partly due to wide ranges of concentrations the MIC obtained for the quality control strain can fall into: despite the target MIC for few antibiotics being different between standards, the authorised ranges of concentrations overlap (Table 2-1). However, when considering the clinical breakpoints, more discrepancies are observed between the different standards' recommendations, which could have an impact on the subsequent susceptibility profile of the tested organism (Table 2-2). In order to facilitate international standardisation of susceptibility testing, BSAC ceased to develop its own recommendations in January 2016 and redirected laboratories towards EUCAST standards. However, as this work started before this event, antibiotic susceptibility testing had already been performed following BSAC's protocol, hence the need for highlighting the differences between the various commissions.

Table 2-1. Target MICs and authorised ranges of concentrations for several antibiotics given by BSAC (2015), EUCAST (2019) and CLSI (2019) guidelines for *E. coli* ATCC 25922.

Target values and ranges were included when available. Antibiotics for which MIC target/range values were not indicated for the three standards, and antibiotics presenting the same MIC target/range for the three standards were not included in this table. The fold-change observed between the different standards is indicated as a range, from the minimum to the maximum fold-change observed. BMD: broth microdilution; ISA: Iso-Sensitest agar; CAMHB: cation-adjusted Mueller-Hinton broth.

Standard Method Media	**BSAC** Agar-dilution ISA Target µg/mL	**EUCAST** BMD CAMHB Target	Min	Max µg/mL	**CLSI** BMD CAMHB Min	Max µg/mL	**Fold-change between different standards**
Aztreonam	0.25	0.125	0.06	0.25	0.06	0.25	1-4
Cefixime	0.25	0.5	0.25	1	0.25	1	1-4
Cefpodoxime	0.25	0.5	0.25	1	0.25	1	1-4
Ciprofloxacin	0.015	0.008	0.004	0.016	0.004	0.016	1-4
Ertapenem	0.015	0.008	0.004	0.016	0.004	0.016	1-4
Meropenem	0.008	0.016-0.03	0.008	0.06	0.008	0.06	1-8
Trimethoprim	0.25	1	0.5	2	0.5	2	1-4

Table 2-2. Breakpoints for several antibiotics given by BSAC (2015), EUCAST (2019) and CLSI (2019) commissions for Enterobacteriaceae.

Antibiotics for which breakpoints were not indicated for the three standards and antibiotics presenting the same breakpoints between the three standards were not included in this table The fold-change observed between the different standards is indicated as a range, from the minimum to the maximum fold-change observed. S: susceptible; R: resistant.

Standard Method Media	**BSAC** Agar-dilution ISA S ≤ R > µg/mL		**EUCAST** BMD CAMHB S ≤ R > µg/mL		**CLSI** BMD CAMHB S ≤ R > µg/mL		**Fold-change between different standards**
Amikacin	8	16	8	16	16	32	1-2
Ampicillin	8	8	8	8	8	16	1-2
Aztreonam	1	4	1	4	4	8	1-2
Cefepime	1	4	1	4	2	8	1-2
Cefixime	1	1	1	1	1	2	1-2
Cefpodoxime	1	1	1	1	2	4	1-4
Ceftazidime	1	4	1	4	4	8	1-4
Chloramphenicol	8	8	8	8	8	16	1-2
Ciprofloxacin	0.5	1	0.25	0.5	0.25	0.5	1-2
Ertapenem	0.5	1	0.5	0.5	0.5	1	1-2
Fosfomycin	32	32	32	32	64	128	1-4
Gentamicin	2	4	2	4	4	8	1-2
Imipenem	2	8	2	4	1	2	1-4
Mecillinam	8	8	8	8	8	16	1-2
Meropenem	2	8	2	8	1	2	1-4
Nitrofurantoin	64	64	64	64	32	64	1-2
Ofloxacin	0.5	1	0.25	0.5	2	4	1-4
Piperacillin	8	16	8	16	16	64	1-4
Tobramycin	2	4	2	4	4	8	1-2
Trimethoprim	2	4	2	4	8	8	1-4

Several factors are known to influence MIC and MBC values, as well as disk diffusion methods. The protocol used, as highlighted previously, is of importance. For example, regarding colistin susceptibility testing, broth dilutions methods are recommended by the CLSI and EUCAST over agar-based (agar-dilution or disk-/gradient-diffusion) protocols due to the poor diffusion of colistin in agar (Giske & Kahlmeter, 2018; Matuschek *et al.*, 2018). The broth composition, including the amount of nutrients available and the pH, can impact the MIC (Green, 1978; Buchta & Otcenasek, 1996; Wiegand *et al.*, 2015; Mouton, 2018); the cation concentrations seem to be particularly relevant when testing

cationic compounds (Chow *et al.*, 1989; Bock *et al.*, 2018). The material of the plates and their coating can impact the availability of certain compounds like polymyxins, whose binding onto the surface depends on the plastic used (Wiegand *et al.*, 2008; Bock *et al.*, 2018). The MIC values obtained can also depend on the initial inoculum used for the antimicrobial susceptibility testing: this phenomenon is referred to as the inoculum effect, and is well described for several antibiotic/micro-organism combinations, including for example β-lactams and β-lactamase-producing isolates (Brook, 1989; Argemi *et al.*, 2013; Smith & Kirby, 2018). The growth phase the micro-organisms are in when the test is carried out can also have an impact on the kill rate of some agents (Argemi *et al.*, 2013). The incubation conditions (temperature, duration of incubation, agitation; Buchta & Otcenasek, 1996; Sawer *et al.*, 1997; Argemi *et al.*, 2013) and the precision in the preparation of the assay (Turnidge & Paterson, 2007) are other factors influencing MIC determination and impacting inter-laboratory variability in measurements (Mouton *et al.*, 2018).

It is important to note that MICs and MBCs represent a range of concentrations rather than discrete values: for two-fold dilution series, if a MIC value is indicated as 1 μg/mL, it corresponds in reality to a concentration strictly greater than 0.5 μg/mL and lower or equal to 1 μg/mL (Turnidge & Paterson, 2007). In addition, it seems commonly admitted that MIC determinations within 'plus or minus one two-fold dilution' from each other are within the acceptable error range (Turnidge & Paterson, 2007; Wesgate *et al.*, 2016; Mouton *et al.*, 2018).

2.1.2 Bacterial susceptibility profiles to biocides

Breakpoints are well-defined for a wide range of antibiotics (as shown previously), however this is not the case for biocides (Morrissey *et al.*, 2014). Indeed, biocides are used at concentrations much higher than the ones investigated *in vitro* for MIC determination, as shown with a few examples in Table 2-3 below. The definition of a breakpoint as a cut-off value able to distinguish between susceptible and resistant isolates and, therefore, indicating a success or failure of treatment is then inadequate in the case of biocides (Horner *et al.*, 2012; Maillard *et al.*, 2013; Harbarth *et al.*, 2014).

Table 2-3. Examples of MICs obtained for several *E. coli* reference strains in the literature for different biocides and corresponding in-use concentrations of biocides.

Non-exhaustive lists of commercial products and their corresponding biocide concentrations (when available) are indicated for different biocides. BZC: benzalkonium chloride; CHX: chlorhexidine digluconate; CS: copper sulphate; ISB: Iso-Sensitest broth; MHB: Mueller-Hinton broth; SN: silver nitrate; TSB: tryptone soya broth.

Reference	Design of the assay	Biocide	MIC	Strain tested	Examples of in-use concentrations
Hammond *et al.*, 1987	Agar-dilution with ISA	BZC	20-50 µg/mL	*E. coli* ATCC 10536 and ATCC 11776	Dettol Cleansing Surface Wipes (Dettol) for surface disinfection: 0.4 % w/w Dermax Therapeutic Shampoo (Dermal Laboratories) for scalp conditions: 5,000 µg/mL Beechams Max Strength Sore Throat (GSK): 1,200 µg/lozenge
Bock *et al.*, 2018	Microdilution with different broth/plates	BZC	8-16 µg/mL	*E. coli* ATCC 10536	
Bore *et al.*, 2007	Microdilution with TSB	BZC	13 µg/mL	*E. coli* ATCC 47076	
Biagi *et al.*, 2014	Microdilution with CAMHB	CS	12,500 µg/mL	*E. coli* CCUG 50175	Copper and copper alloys surfaces (≥ 60 % copper) as antibacterial surfaces (Hans *et al.*, 2015)
Sutterlin *et al.*, 2018	Microdilution with ISB	CS	1,024-2,048 µg/mL	*E. coli* ATCC 25922	
Sutterlin *et al.*, 2018	Microdilution with ISB	SN	16-32 µg/mL	*E. coli* ATCC 25922	Avoca Silver Nitrate Applicator (Bray) for wart treatment: 75 % w/w (up to 22.5 mg SN released on treatment site)

Table 2-3 – *Continued*.

Reference	Design of the assay	Biocide	MIC	Strain tested	Examples of in-use concentrations
Naparstek *et al.*, 2012	Agar-dilution with CAMHA	CHX	2 µg/mL	*E. coli* ATCC 25922	HiBiScrub® (Mölnlycke) for skin disinfection: 40,000 µg/mL Medipal® Chlorhexidine Surface Wipes (Pal) for surface disinfection: 2 %/wipe Corsodyl Treatment Mouthwash (Corsodyl) for gingivitis: 2,000 µg/mL
Hammond *et al.*, 1987	Agar-dilution with ISA	CHX	1.5 µg/mL	*E. coli* ATCC 10536 and ATCC 11776	
Tetz & Tetz, 2015	Macrodilution (broth?)	CHX	2 µg/mL	*E. coli* ATCC 25922	
Braoudaki & Hilton, 2004	Macrodilution with nutrient broth	CHX	4 µg/mL	*E. coli* ATCC 700728	
O'Driscoll *et al.*, 2014	Macrodilution with nutrient broth	CHX	0.6 µg/mL	*E. coli* NCTC 4174	
Thomas *et al.*, 2000	Macrodilution with nutrient broth	CHX	0.2 µg/mL	*E. coli* ATCC 10536	
Wesgate *et al.*, 2016	Microdilution (broth?)	CHX	7 µg/mL	*E. coli* ATCC 8739	
Grare *et al.*, 2010	Microdilution with CAMHB	CHX	< 1 µg/mL	*E. coli* ATCC 25922	
Bock *et al.*, 2018	Microdilution with different broth/plates	CHX	0.125-2 µg/mL	*E. coli* ATCC 10536	
Koburger *et al.*, 2010	Microdilution with MHB	CHX	8 µg/mL	*E. coli* ATCC 35218	
McBain *et al.*, 2004	Microdilution with nutrient broth	CHX	13 µg/mL	*E. coli* ATCC 8729	

In order to decipher between wild-type and isolates exhibiting reduced susceptibility to a biocide, researchers have established epidemiological cut-off values (ECOFF) based on the distribution of MIC of wild-type isolates for a given biocide/organism combination (Turnidge & Paterson, 2007; Iredell *et al.*, 2016); isolates presenting MIC values higher than the ECOFF (corresponding to the concentration at which 95 to 99.9 % of the population would be inhibited or killed for unimodal distributions, or the concentration between the two sub-populations for bi-modal distributions) would then be considered as tolerant (Morrissey *et al.*, 2014; Lavilla Lerma *et al.*, 2015). However, with the lack of a standardised method for biocide susceptibility testing (Maillard *et al.*, 2013), and the different factors impacting MIC determination (mentioned in the previous section for antibiotic susceptibility testing),

the variability of MIC values for biocides found in the literature is important, as highlighted in Table 2-3. In spite of this, MIC determination for biocides seems to be preferred to other biocide-testing protocols such as time-kill studies or suspension tests for example (British Standards Institution, 1997; British Standards Institution, 2005; Maillard *et al.*, 2013) in the literature, especially with the possibility of high-throughput testing (Maillard *et al.*, 2013; Wales & Davies, 2015).

2.1.3 Aim

In this work, MICs for a wide range of antibiotics and MICs and MBCs for four commonly used biocides were determined for a collection of 210 isolates. The aim was to establish a susceptibility baseline enabling the selection of representative organisms for further investigations.

2.2 Material and methods

2.2.1 Bacterial isolates and cultivation

2.2.1.1 Bacterial isolates

A total of 210 carbapenemase-producing isolates (160 *Klebsiella pneumoniae* and 50 *Escherichia coli*) provided by Public Health England, were considered for this study. Their detailed information is presented in appendix I. Three collection strains were also included as elements of comparison for susceptibility profiles and/or as quality controls when required: *K. pneumoniae* ATCC 13883 (used in a similar study; Naparstek *et al.*, 2012), *E. coli* ATCC 25922 and *E. coli* NCTC (National Collection of Type Cultures) 13846 (recommended by EUCAST and CLSI for antibiotic susceptibility testing).

The 210 isolates were collected from 69 different hospitals across the United Kingdom between July 2010 and August 2015. They were mainly isolated from rectal swabs (27.1 %), urine (20.5 %), faeces (10.5 %) and blood cultures (9.0 %). Four isolates were collected from the hospital environment and the isolation sites of 10 isolates were not indicated. Whole-genome sequencing (WGS; Illumina) was performed by Public Health England prior to this study, enabling the identification of carbapenemase-encoding genes and multi-locus sequence types (MLSTs) of the different strains.

The selection of these isolates by Katie Hopkins from Public Health England was based on several criteria indicated in appendix I. Firstly, representative strains from recent

outbreaks (notably in North-West England) were included. Moreover, the sequence types (STs) and their combinations with carbapenemase-encoding genes were of particular interest in this process to ensure a wide variety of isolates for this study. 'High risk' clones, like *K. pneumoniae* ST258 and *E. coli* ST131, are widely distributed internationally and show enhanced abilities to spread, colonize and persist (Chen *et al.*, 2014; Mathers *et al.*, 2015; Pitout *et al.*, 2015). 'International' clones corresponded to clones which were also identified outside the UK in the literature. Strains indicated as 'successful clones' were labelled as such when they were referred by multiple laboratories and associated with different carbapenemases, while not being described as 'high risk' in the literature at the time. Frequently found ST/carbapenemase combinations among carbapenemase-producing Enterobacteriaceae in the United Kingdom were tagged as 'common clones' by Public Health England. Finally, several isolates with unusual MLST profiles, infrequently identified, indicated as 'outlier clones', were included as additional comparison elements.

Among the selected *E. coli* isolates, $bla_{\text{NDM-5}}$ (excluding combination with others, 24.0 %) and $bla_{\text{KPC-2}}$ (22.0 %) were the most commonly encountered carbapenemase-encoding genes. One isolate possessed both $bla_{\text{NDM-4}}$ and $bla_{\text{OXA-181}}$ and another one harboured $bla_{\text{NDM-5}}$ and $bla_{\text{OXA-181}}$. Out of 24 STs, the most frequent ones were ST636 (12.0 %) and ST131, ST38, ST405 and ST410 (8.0 % each). This information was unknown for three isolates. Regarding the *K. pneumoniae* isolates, amongst the 11 carbapenemase-encoding genes or combinations present, $bla_{\text{KPC-2}}$ (31.9 %), $bla_{\text{NDM-1}}$ (23.8 %) and $bla_{\text{OXA-48}}$ (19.4 %) were the most represented. Two isolates presented a combination of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-232}}$ and another one possessed both $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-181}}$. The *K. pneumoniae* population covered 57 different STs (the ST of four isolates was unknown), the most encountered ones being ST14 and ST15 (7.5 % each), ST231 (6.3 %), and ST11, ST258 and ST307 (5.6 % each).

2.2.1.2 Storage and cultivation

The different isolates were stored frozen at -80ºC in cryopreservative tubes (Protect, Technical Service Consultants Ltd, Heywood, UK). A single colony grown on tryptone soya agar (TSA, E&O Laboratories Ltd, Bonnybridge, UK) was transferred into a tube containing ceramic beads in a cryopreservative fluid according to the manufacturer's protocol. After vortexing, the suspension was left at room temperature for 5 to 10 min. The liquid was removed and the tube placed at -80ºC. When needed, strains were defrosted by taking one

bead out of the cryopreservative tube and used to inoculate a TSA plate and incubated at 37 ± 1ºC overnight.

Liquid cultures were inoculated by transferring several colonies from a TSA plate into 25 mL of tryptone soya broth (TSB, Oxoid, Basingtoke, UK) and incubated overnight at 37 ± 1ºC. The cultivation of several colonies was chosen to avoid picking a single mutant colony which could have affected a strain's behaviour.

2.2.2 Biocides

Four biocides were investigated in this study: benzalkonium chloride (BZC), chlorhexidine digluconate (CHX; both from Sigma-Aldrich, Gillingham, UK), copper(II) sulphate pentahydrate (CS) and silver nitrate (SN; both from Acros Organics, Geel, Belgium). Stock solutions were prepared from powder for BZC, CS and SN and from a liquid solution (20 % w/v) for CHX in deionized water, sterilized by filtration through 0.20 µm cellulose acetate sterile filters and kept at room temperature in the dark for a maximum of two weeks or less if any visible degradation was observed (precipitation or change in coloration). Stock solution concentrations corresponded to 10 times the working solution concentrations, the latter being obtained by dilution in R2A broth (LabM, Heywood, UK).

2.2.3 Minimum inhibitory concentration determination for antibiotics

Determination of antibiotic MICs was performed prior to this study by Public Health England (Colindale). The protocol was based on the BSAC agar dilution method (Andrews, 2001). The investigated antibiotics were ampicillin (AMP), piperacillin (PIP), temocillin (TEMO), imipenem (IPM), meropenem (MEM), ertapenem (ETP), cefoxitin (FOX), cefotaxime (CTX), ceftazidime (CAZ), cefepime (CPM), aztreonam (ATM), amikacin (AK), gentamicin (CN), tobramycin (TOB), ciprofloxacin (CIP), tigecycline (TIG), minocycline (MIN) and colistin (COL). Agar plates containing serial doubling dilutions of antibiotics (Sigma-Aldrich, Gillingham, UK) were prepared in Iso-Sensitest agar (Oxoid, Basingstoke, UK).

2.2.3.1 Preparation of inoculum by direct colony suspension

A minimum of five individual, well-isolated colonies growing on a non-inhibitory agar plate were touched with a swab and resuspended in Iso-Sensitest broth (ISB; Oxoid, Basingstoke, UK) until reaching a turbidity equivalent to a 0.5 McFarland standard,

corresponding to a bacterial density of approximately 1.5×10^8 CFU (colony forming unit)/mL. Suspensions were stored at 4ºC until used.

2.2.3.2 Plate inoculation

Suspensions were diluted 1/3 in Iso-Sensitest broth in microtitre plates. The microtitre plate was then placed on a multipoint inoculator (Mast Group Ltd, Merseyside, UK). Pins inoculated 0.33 µL of the suspensions in the microtitre plates onto the antibiotic-containing agar plates in ascending concentrations. Control plates containing no antibiotics were also inoculated as controls and a 1 µL loopful from each microtitre well was streaked on a cystine-lactose-electrolyte-deficient (CLED; Oxoid, Basingstoke, UK) agar plate to control the purity of the suspensions. Each spot resulting from the inoculation corresponded to approximately 1.5×10^4 CFU. Plates were incubated at 36 ± 2ºC for 18-20 hours.

2.2.3.3 MIC determination for antibiotics

Plates were read by the Sorcerer™ Colony Counter (Perceptive Instruments Ltd., Suffolk, UK) with the Sorcerer MIC software and the MICs calculated were double-checked by eye. The MIC was defined as the lowest concentration of antibiotic inhibiting visible growth. One or two colonies, as well as hazes of inhibited growth, were not taken into account.

2.2.4 Minimum inhibitory concentration determination for biocides

The protocol detailed in the following sections was adapted from the ISO 20776-1:2006 microdilution broth protocol (International Organization for Standardization, 2006), recommended by EUCAST (EUCAST, 2019c) and the CLSI M07-A9 standard (CLSI, 2012). The assay was performed in polystyrene, tissue-culture treated, round bottom 96-well microtitre plates (Corning, Ewloe, UK) using the Viaflo Assist pipetting assistant (Integra, Zizers, Switzerland) in order to allow high-throughput testing. The experiments were conducted in triplicate.

The cation-adjusted Mueller-Hinton broth (CAMHB) recommended in the standard protocol was replaced by R2A broth for both broth and diluent uses. The CAMHB possessed a high content in salts, which was not compatible with the range of CHX concentrations tested here, causing precipitation. The biocide working solutions pH were between 7.2 and 7.4, except for CS. The pH measured for the different CS solutions are listed in Table 2-4. They could not be increased due to precipitation issues. The effects of this acidic

environment on bacterial growth were assessed by performing growth curves in R2A broth adjusted to different pH values. Fifty µL of bacterial suspension (*K. pneumoniae* ATCC 13883 or *E. coli* ATCC 25922) containing approximately 10^6 CFU/mL (as described further in section 3.2.3.1 page 66) was added to 50 µL of a pH-adjusted R2A solution in a Bioscreen Honeycomb microplate (Fisher Scientific, Loughborough, UK). The plate was incubated in a Bioscreen C Analyzer (Oy Growth Curves Ab Ltd, Helsinki, Finland) for 24 hours at 37ºC with continuous shaking. The optical density (OD) was measured at 600 nm every 4 min.

Table 2-4. pH values measured for different concentrations of copper sulphate solutions.

CS solutions were prepared in R2A broth to reflect the experimental conditions of the assay.

CS concentrations (µg/mL)	pH
15,979.5	3.56
7,989.8	3.67
3,994.9	3.81
1,997.4	3.98
998.7	4.22
499.4	4.63
249.7	5.37
124.8	6.25
62.4	6.70
31.2	6.91
15.6	7.01
7.8	7.06

2.2.4.1 Bacterial suspension wash and optical density adjustment

Bacteria were grown overnight in 25 mL TSB at 37 ± 1ºC. The suspensions were centrifuged at 5,000 g for 10 min at 20ºC. Each pellet was resuspended in 10 mL R2A broth and subsequently diluted to reach an OD_{625nm} value between 0.150 and 0.175. This range was higher than the one recommended in the ISO 20776-1:2006 (0.080 to 0.130) in order to ensure a high enough bacterial density for the 96-well plate inoculation.

The OD_{625nm}-adjusted suspension was diluted at 1/100 in R2A broth, corresponding to approximately 10^6 CFU/mL. This suspension, referred to as the inoculum test suspension, was kept at 4ºC until needed.

2.2.4.2 Viable counts

Viable counts were performed to ensure a high enough bacterial concentration in the inoculum suspensions. According to the ISO 7218:2007, viable counts are considered reliable when comprised between 10 and 300 CFU (International Organization for Standardization, 2007). Below 10 CFU, the number would not be statistically significant (not representative of the sample), while above 300 CFU colonies would be too close to be able to distinguish different colonies.

A 1/500 dilution from each inoculum suspension was performed in R2A broth. Two volumes, 40 µL and 100 µL, were spread on TSA plates. With an inoculum suspension of 10^6 CFU/mL, these volumes would result in 80 and 200 CFU on the plates, respectively. Colonies were enumerated after an overnight incubation of the TSA plates at 37 ± 1ºC. The means of counts within 10 and 300 CFU were used to determine the real bacterial concentrations.

2.2.4.3 96-well plate inoculation

The layout of the 96-well microtitre plates is presented in Figure 2-1. The plates were first filled with 50 µL of R2A broth. Two-fold serial dilutions of the biocides, one over the top four rows, another over the bottom four rows, were performed across the plates, except in the last column where no biocide was added. Fifty µL of the inoculum suspension was then added to each well, resulting in a final bacterial concentration of approximately 5.5 x10^5 CFU/mL, except for the top and bottom rows where 50 µL of R2A broth was added as negative controls for each biocide. Two plates were necessary in order to determine the MIC of each strain for the four tested biocides. Additional plates were included to enable the testing of both *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922, used as quality controls. Microtitre plates were incubated for 16 to 24 hours at 37 ± 1ºC.

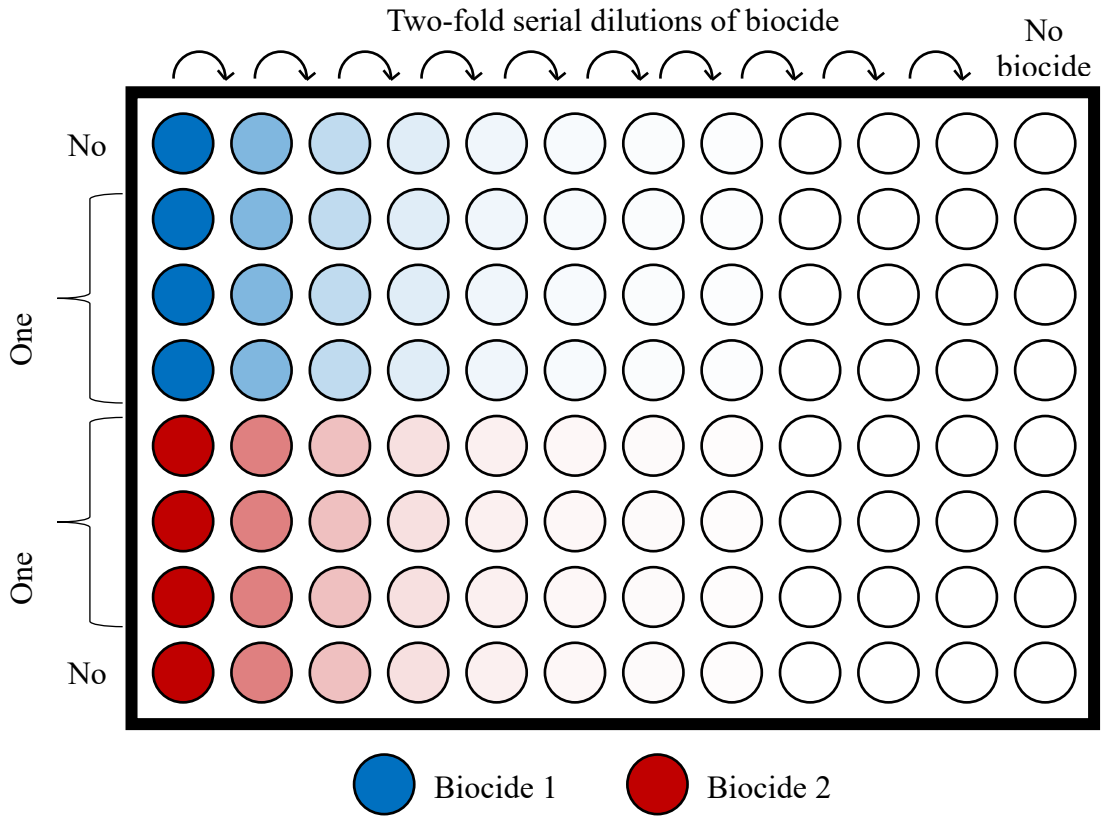

Figure 2-1. 96-well microtitre plate layout.
Testing for one strain and two biocides with three technical replicates each was performed on one 96-well plate.

Due to the design of this protocol, biocides underwent a ¼ dilution in the first column. Four-times more concentrated solutions were then needed to fill in the plates. The highest tested concentration for BZC, CHX and SN being 128 µg/mL (concentrations range: 128 to 0.125 µg/mL), each solution used to fill in the 96-well plates had to contain 512 µg/mL of the corresponding biocide, except when stated otherwise. In a similar way, two CS solutions reaching 15,979.5 µg/mL and 3,994.9 µg/mL were used for the MIC testing of the studied strains (concentration range: 3,994.9 to 3.9 µg/mL) and the ATCC strains (concentration range: 998.7 to 1.0 µg/mL), respectively.

2.2.4.4 MIC determination for biocides

The MIC was defined as the lowest concentration of the active agent able to inhibit growth of the considered organism. The MIC therefore corresponded to the lowest concentration of biocide where neither turbidity nor growth at the bottom of the well was observed with a naked eye.

2.2.5 Minimum bactericidal concentration determination for biocides

The MBC was defined as the lowest concentration of an active agent able to kill the considered organism. In order to determine the MBC, the biocides needed to be neutralised to allow the recovery of any viable bacteria which growth could be inhibited.

2.2.5.1 Neutraliser composition

Biocides were neutralised both by dilution and by the use of a universal neutraliser recommended by the standard BS EN 1276:1997 (British Standards Institution, 1997). Its composition was as follow: 30 g/L polysorbate 80, 30 g/L saponin, 3 g/L lecithin (all three from Acros Organics, Geel, Belgium), 5 g/L sodium thiosulfate (Fisher Scientific, Loughborough, UK) and 1 g/L L-Histidine (Sigma-Aldrich, Gillingham, UK) in tryptone sodium chloride (TSC) buffer. Sodium thiosulfate was used to quench the effects of the heavy metals. Lecithin was used to counteract the activity of CHX, while both lecithin and polysorbate 80 neutralised BZC, a QAC (Johnston *et al.*, 2002; Sutton *et al.*, 2002; Koburger *et al.*, 2010). The TSC was prepared by mixing 8.5 g/L sodium chloride (Fisher Scientific, Loughborough, UK) and 1 g/L tryptone (Oxoid, Basingtoke, UK) in deionized water. The neutraliser was sterilized by autoclaving at 121 ± 3ºC for a minimum holding time of 15 min.

2.2.5.2 Neutraliser efficacy and toxicity assessments

The efficacy of the neutraliser to quench the activity of the tested biocides was tested by adding 10 µL of the highest concentration of the biocide used (512 µg/mL for BZC, CHX and SN, 15,797.5 µg/mL for CS) to 180 µL of neutraliser, except when stated otherwise. In a similar way, the neutraliser toxicity was evaluated by mixing 10 µL of R2A broth to 180 µL of neutraliser. After brief mixing and 5-30 min contact, 10 µL of an inoculum test suspension (as described in section 2.2.4.1 page 31; either *K. pneumoniae* ATCC 13883 or *E. coli* ATCC 25922) was added. The suspension was briefly mixed and, after 5 min, a 1/50 dilution in R2A broth was performed and 200 µL and 80 µL were spread on two distinct TSA plates for viable counts. Colonies were enumerated after an overnight incubation at 37 ± 1ºC. A viable count was also performed on the inoculum suspensions without any contact with a biocide (as described previously in section 2.2.4.2 page 32) to determine the original bacterial concentration.

The neutraliser was considered efficient if less than one $\log_{10}$ reduction was observed when comparing the bacterial concentration in the neutralised biocide solutions to the

untreated control. It was considered toxic if more than one $\log_{10}$ reduction was observed compared to the untreated control (Knapp *et al.*, 2013).

2.2.5.3 MBC determination for biocides

The MBC determination was based and adapted from a previous study performed by Knapp and colleagues (2015). After reading the MIC, the wells corresponding to the three concentrations immediately above the MIC and the one corresponding to the MIC were neutralised as described below. The two wells corresponding to the concentrations immediately below the MIC were also neutralised as controls. After neutralisation, the microtitre plates used for MIC determination were kept at 4ºC for a maximum of 24 hours in case further testing was needed.

Twenty µL from each selected well was removed and transferred to 180 µL of neutraliser. After brief mixing and 5-30 min contact, 20 µL from each suspension was removed and dropped on a TSA plate (except stated otherwise), as shown in Figure 2-2. TSA plates were incubated overnight at 37 ± 1ºC and the MBC corresponded to the lowest concentration of biocide where no growth was observed on TSA. If growth was observed on TSA for all the tested concentrations, the wells containing higher concentrations of biocides were neutralised following the same protocol.

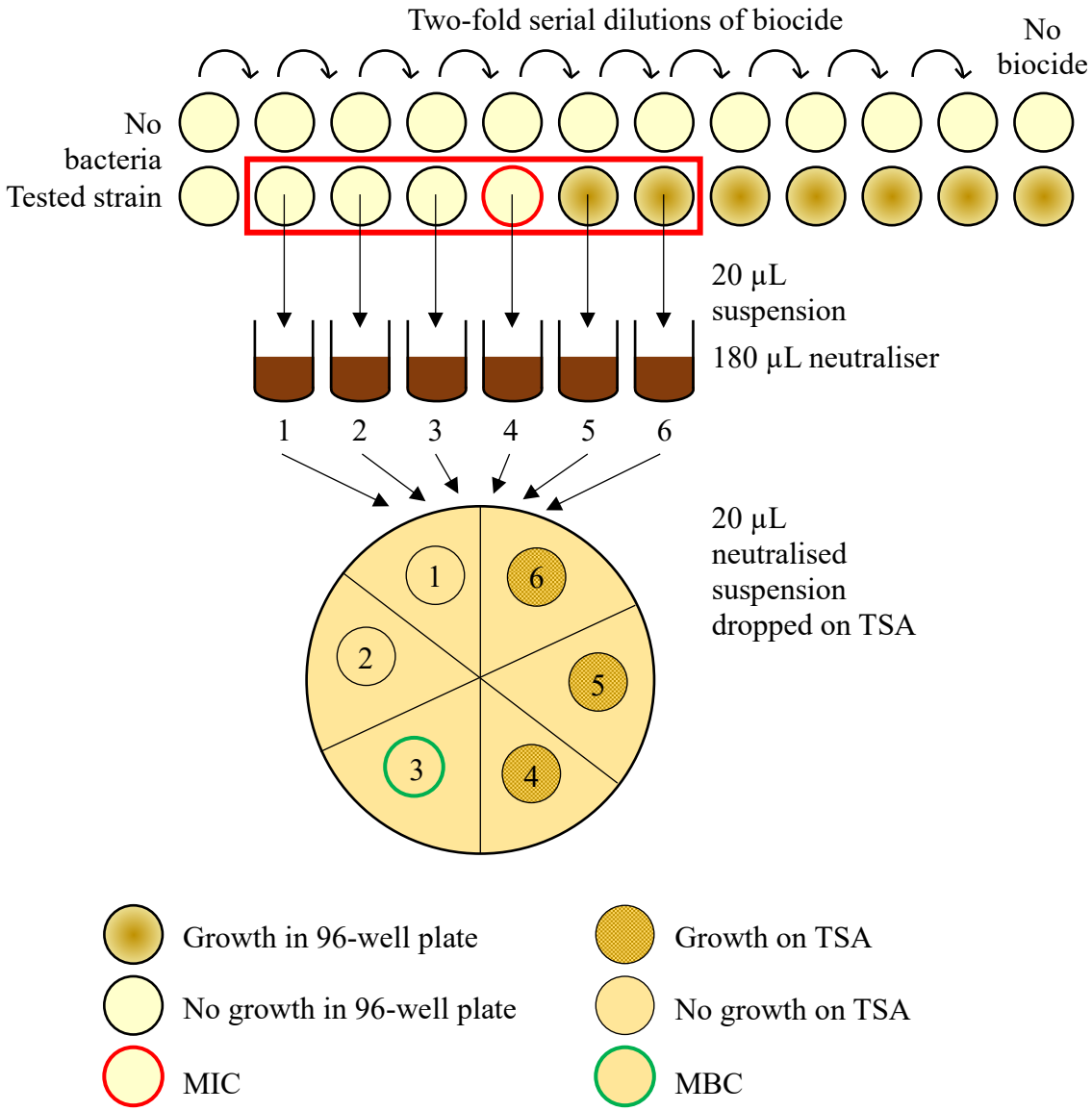

Figure 2-2. MBC determination protocol steps.

After determining the MIC as the lowest concentration of biocide where no growth was observed (red circle), six wells (three above the MIC, the one corresponding to the MIC and two below the MIC; red rectangle) were neutralised to determine whether the bacteria were killed or if their growth was inhibited by the biocide. The MBC was read as the lowest concentration of biocide where no growth was observed on TSA (green circle).

2.2.6 Statistical analysis

Statistical analysis was performed using Prism GraphPad 5 for Windows. Calculations of mean and minimum to maximum values were realised for each species and each biocide, separately for MICs and MBCs. The lowest concentrations able to inhibit 50% (MIC_{50}) or 90% (MIC_{90}) of the total number of tested isolates were also determined.

Similarly, the lowest concentrations able to kill 50% (MBC_{50}) or 90% (MBC_{90}) were calculated. D'Agostino and Pearson normality test was used to verify the nature of the distribution of the values. Comparison between MIC and MBC distributions of the four biocides for both species were performed using Kruskal-Wallis test followed by Dunn's multiple comparison post-test.

2.3 Results

2.3.1 Impact of pH on bacterial growth

Due to low pH issues encountered with CS at high concentrations, the growth of both *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922 was assessed by performing growth curves in pH-adjusted R2A solutions (Figure 2-3). Mean of three replicates are presented in the figure below. For both species no growth was observed at pH 3.24 and below, while growth was obtained in all cases for pH 6.09 and above. At pH 4.53, *E. coli* ATCC 25922 was not able to grow over a 24-hour period, while in 2/3 repeats growth was observed for *K. pneumoniae* ATCC 13883. Solutions harbouring pH values between 4.53 and 6.09 were not obtained probably due to the buffering capacity of the broth. Two CS solutions in R2A harboured pH values within that range: 499.4 μg/mL (pH=4.63) and 249.7 μg/mL (pH=5.37). Solutions containing 124.8 μg/mL CS (pH=6.25) or less corresponded to pH values able to support growth for *K. pneumoniae* and *E. coli* isolates.

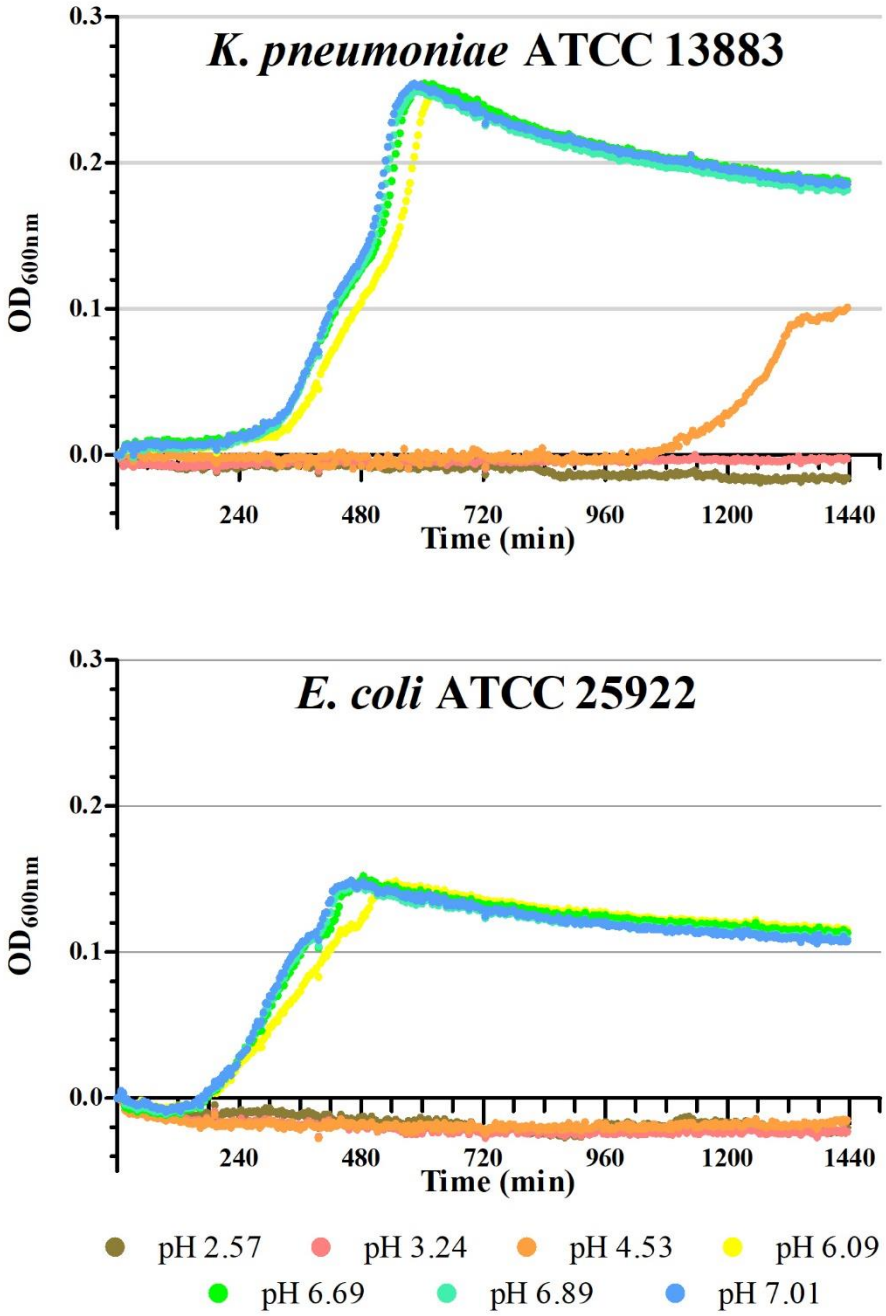

Figure 2-3. Growth curves performed in different pH-adjusted R2A solutions for both *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922.

The y axis corresponds to blanked OD_{600nm} values. Each point represented the mean of three values (n=3). In the case of *K. pneumoniae* ATCC 13883, growth in R2A at pH 4.53 was obtained in 2/3 repeats.

2.3.2 Neutraliser efficacy and toxicity assessments

The neutraliser was efficient in quenching the activities of each biocide at the concentrations tested (0 to 0.3 $\log_{10}$ reduction for both species for the four biocides). However, for one isolate (Kp-94), the SN concentration range had to be increased to 1,024 µg/mL. This concentration was not neutralised (1.0 $\log_{10}$ reduction for both reference strains) in the described conditions and the protocol had to be altered. In this case, 20 µL from the selected wells was added to 980 µL of neutraliser. Forty µL of the obtained suspensions was dropped on TSA plates. Neutralisation of 1,024 µg/mL SN was obtained in these conditions (0.3 $\log_{10}$ reduction).

Regarding the toxicity assessment, 0 to 0.2 $\log_{10}$ reduction was observed for either *K. pneumoniae* or *E. coli* ATCC strains. The neutraliser was not toxic for both reference strains. By extent, the neutraliser was considered as not toxic for the studied species, as these strains were susceptible strains, more likely to be inhibited by low concentrations of compounds than resistant isolates.

2.3.3 Susceptibility profiles to antibiotics

Regarding the antibiotic susceptibility testing performed by Public Health England, MIC values are presented in appendix II for *K. pneumoniae* isolates and in appendix III for *E. coli* isolates. The resistant / intermediate / susceptible status of each isolate for each antibiotic were based on breakpoints established by BSAC for Enterobacteriaceae, when available (BSAC, 2015). The breakpoint for FOX and a ECOFF for MIN were obtained from EUCAST recommendations (EUCAST, 2009; EUCAST, 2019b).

A summary of the susceptibility status of *K. pneumoniae* and *E. coli* isolates for the different antibiotics tested in presented in Table 2-5. Regarding *K. pneumoniae* isolates, 12/160 strains were not tested against any of the investigated antibiotics. Eighty-four to 93 % of the 160 *K. pneumoniae* isolates have been tested for each antibiotic. Regarding the *E. coli* strains, 6/50 did not possess any antibiotic susceptibility data. Except for MIN, 76 to 88 % of the 50 *E. coli* isolates have been tested for each antibiotic.

Table 2-5. Susceptibility profiles to antibiotics for the overall *K. pneumoniae* and *E. coli* populations.

The percentage of the tested strains corresponding to each category (R: resistant; I: intermediate; S: susceptible) is indicated for each antibiotic tested, for both species. Isolates without MIC values for specific antibiotics were not included in the percentage calculations for the corresponding antibiotics. AMP: ampicillin; PIP: piperacillin; TEMO: temocillin; TOB: tobramycin; AK: amikacin; CN: gentamycin; CTX: cefotaxime; CAZ: ceftazidime; CPM: cefepime; FOX: cefoxitin; IPM: imipenem; MEM: meropenem; ETP: ertapenem; ATM: aztreonam; CIP: ciprofloxacin; TIG: tigecycline; MIN: minocycline; COL: colistin; N/A: not available.

		K. pneumoniae			*E. coli*		
		R	**I**	**S**	**R**	**I**	**S**
Penicillins	**AMP**	100	0	0	100	0	0
	PIP	100	0	0	100	0	0
	TEMO	85	0	15	82	0	18
Amino-glycosides	**TOB**	70	1	28	57	9	34
	AK	32	11	57	32	0	68
	CN	57	1	42	48	2	50
Cephalo-sporins	**CTX**	91	5	4	95	2	2
	CAZ	84	5	10	80	14	7
	CPM	73	14	13	70	16	14
	FOX	84	0	16	80	0	20
Carba-penems	**IPM**	56	32	12	43	36	20
	MEM	61	22	16	43	20	36
	ETP	100	0	0	98	2	0
Other antibiotics	**ATM**	88	1	11	81	0	19
	CIP	76	3	21	52	7	41
	TIG	18	27	56	0	0	100
	MIN	46	0	54	N/A	N/A	N/A
	COL	8	0	92	0	0	100

All of the tested isolates were resistant to AMP, AUG, PIP and ETP (with the exception of Ec-181 for ETP, showing an intermediate status). In addition, resistance to TEMO, TOB (*K. pneumoniae* only), cephalosporins, ATM and CIP (*K. pneumoniae* only) was observed for at least two thirds of the tested population for both species, except stated otherwise. Approximately half of the tested isolates showed resistance for CN, IPM, MEM and MIN (*K. pneumoniae* only). Intermediate profiles were not commonly detected (0 to

32 % of the tested population depending on the antibiotic). Susceptibility for more than 50 % of the tested population was observed only for AK, CN (*E. coli* only), TIG (56 % for *K. pneumoniae*, 100 % of the tested isolates for *E. coli*) and COL (92 % for *K. pneumoniae* and 100 % for *E. coli*).

2.3.4 Susceptibility profiles to biocides

2.3.4.1 *Klebsiella pneumoniae* isolates

Results obtained for the reference strain *K. pneumoniae* ATCC 13883 are presented in Table 2-6. The data obtained for the overall population are grouped in appendix IV and summarised in Table 2-7. The reference strain MICs and MBCs were below the mean and the MIC_{50} of the overall population for each biocide. Regarding the clinical isolates, data obtained for BZC, CHX, and to some extent SN, were similar, as opposed to CS where higher values were obtained. According to D'Agostino and Pearson normality test, MIC and MBC values were not normally distributed for any of the biocides tested ($p<0.05$).

Table 2-6. Mean biocide MIC and MBC for the reference strain *K. pneumoniae* ATCC 13883.

MICs and MBCs were determined at least in triplicate for each biocide (n≥3). SD: standard deviation.

		Mean	SD
BZC µg/mL	**MIC**	3.2	0.7
	MBC	3.6	0.4
CHX µg/mL	**MIC**	1.4	0.4
	MBC	1.6	0.5
CS µg/mL	**MIC**	38.1	6.0
	MBC	59.0	6.0
SN µg/mL	**MIC**	1.6	0.5
	MBC	1.7	0.3

Table 2-7. Summary of the MICs and MBCs obtained for the four investigated biocides on the overall *K. pneumoniae* population.

For each isolate, MICs and MBCs were determined at least in triplicate for each biocide (n≥3).

	BZC µg/mL		CHX µg/mL		CS µg/mL		SN µg/mL	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Mean	7.3	8.8	6.2	7.2	157.0	171.4	4.9	5.0
Minimum	2.0	3.3	2.0	2.7	7.8	15.6	2.0	2.0
Maximum	16.0	32.0	16.0	16.0	417.0	417.0	256.0	256.0
MIC_{50}/MBC_{50}	8.0	8.0	6.7	8.0	167.3	207.2	4.0	4.0
MIC_{90}/MBC_{90}	10.7	13.3	8.0	10.7	249.7	249.7	4.0	4.0

The distributions of the overall population of *K. pneumoniae* isolates according to their MIC and MBC for the different investigated biocides are presented in Figure 2-4. Regarding BZC and CHX, MIC and MBC values followed similar uni-modal distributions (Dunn's test; $p>0.05$), while a wide-range, bi-modal distribution was observed for CS. With the exception of Kp-94 exhibiting a MIC of 256 µg/mL, the repartitions of MICs and MBCs for SN were narrow (2-4 µg/mL). Distributions of MICs and MBCs for both CS and SN were significantly different when compared to the other investigated biocides (Dunn's test; $p<0.05$). MBC were usually equivalent or double the MIC for each biocide (no statistically significant difference between their distributions according to Dunn's test). An MBC/MIC ratio higher than two was obtained for Kp-5 (MBC/MIC ratio: 3.2), Kp-78 (2.5), Kp-102 (2.7) with BZC and Kp-8 (2.7), Kp-19 (4.0), Kp-107 (4.0), Kp-126 (3.3) with CS.

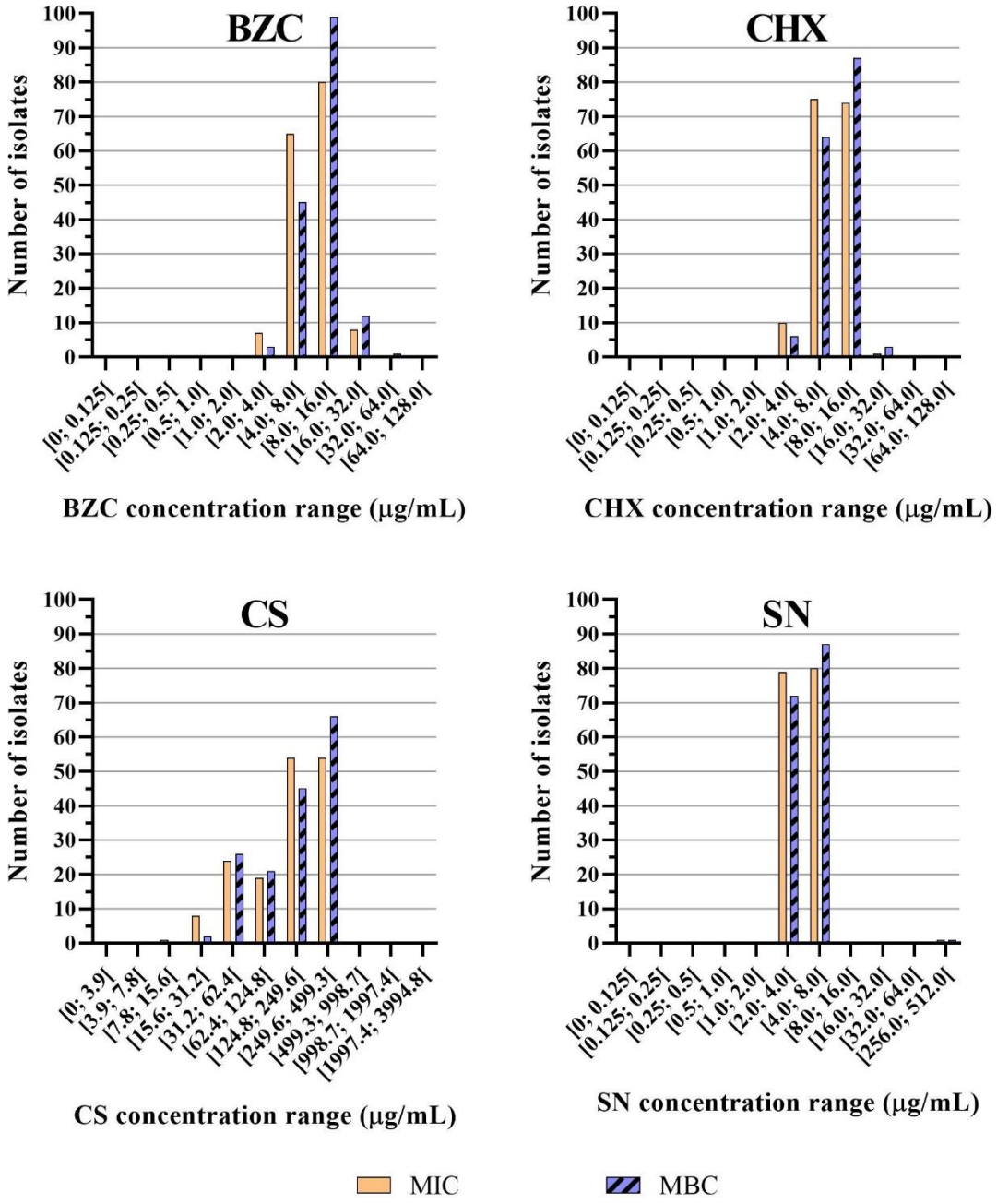

Figure 2-4. Repartition of MICs and MBCs for four biocides among *K. pneumoniae* isolates.

The intervals of concentrations (in µg/mL) are indicated on the x axis as [A; B[, corresponding to concentrations higher or equal to A but strictly lower than B. MIC and MBC repartitions among the 160 *K. pneumoniae* isolates are indicated for BZC, CHX, CS and SN.

The impact of the carbapenemase produced by the isolates on the MIC repartitions was investigated (Figure 2-5). When considering each biocide separately, NDM-, VIM-,

OXA-48-like- and KPC-producing isolates presented similar MIC distributions (Dunn's test; $p>0.05$). The outlier Kp-94 with an elevated SN MIC possessed both bla_{NDM-1} and $bla_{OXA-232}$.

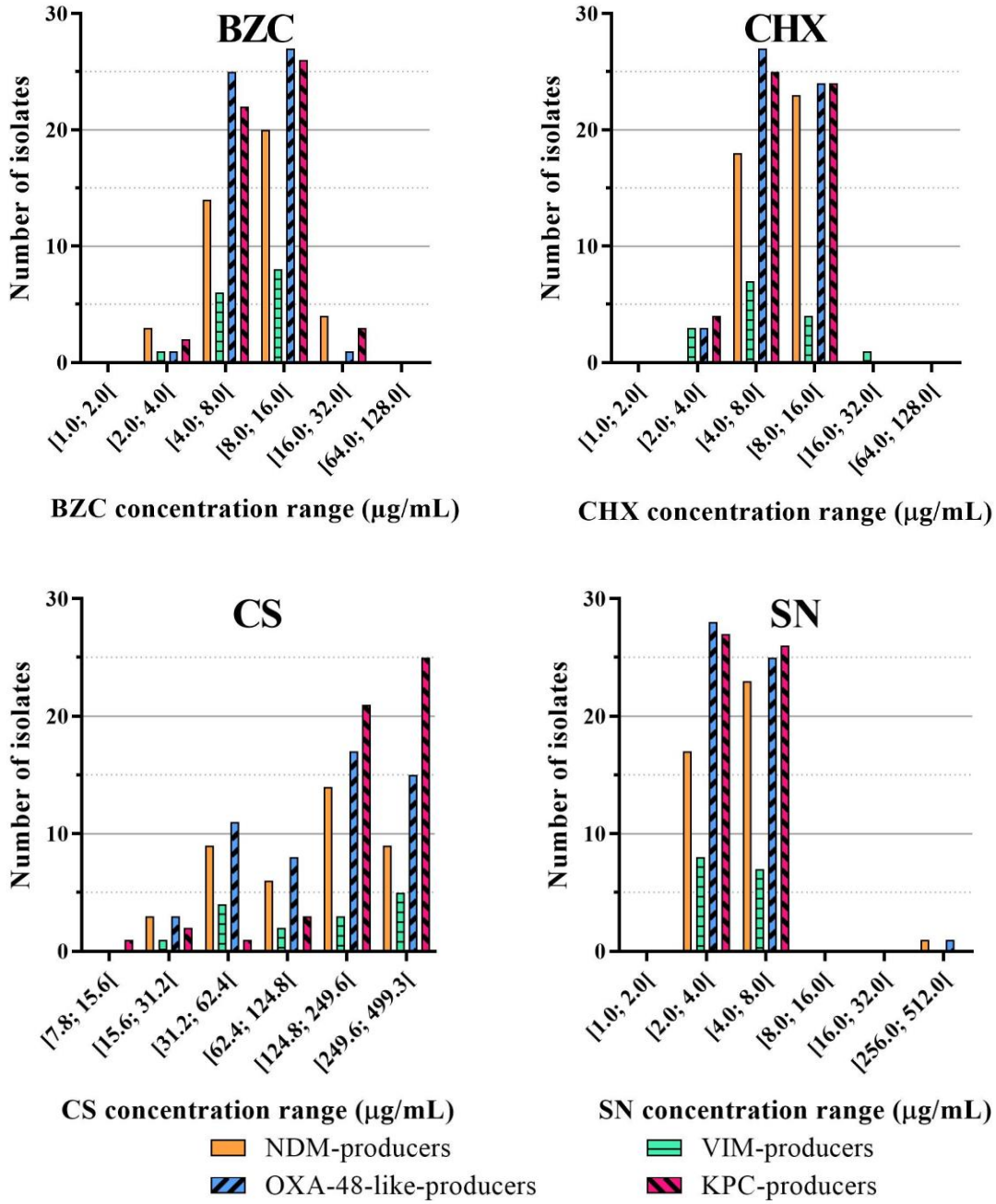

Figure 2-5. Repartition of MICs among *K. pneumoniae* isolates according to their produced carbapenemases.

The intervals of concentrations (in µg/mL) are indicated on the x axis as [A; B[, corresponding to concentrations higher or equal to A but strictly lower than B. MIC repartitions among the NDM- (n=41), VIM- (n=15), OXA-48-like- (n=54) and

KPC-producing (n=53) *K. pneumoniae* isolates are indicated for BZC, CHX, CS and SN. Isolates producing a combination of two different carbapenemases were represented in both categories.

2.3.4.2 *Escherichia coli* isolates

The MICs and MBCs obtained for *E. coli* ATCC 25922 are grouped in Table 2-8. The data obtained for the clinical isolates are summarised in Table 2-9 and detailed values are included in appendix V. MICs and MBCs obtained for the reference strain were in accordance with the 50% most susceptible fraction of the clinical isolates investigated, except with BZC where the reference strain MIC was higher than the MIC_{50} obtained for the overall population. BZC susceptibility data seemed higher than CHX in *E. coli* while, despite a narrow range of values, SN MIC and MBC were comparable to those of CHX. CS showed higher values for both MIC and MBC when compared to the other biocides. Data were not normally distributed ($p<0.05$) for any of the biocides according to D'Agostino and Pearson normality test.

Table 2-8. Mean biocide MICs and MBCs for the reference strain *E. coli* ATCC 25922. MICs and MBCs were determined at least in triplicate for each biocide (n≥3). SD: standard deviation.

		Mean	SD
BZC µg/mL	**MIC**	5.7	1.7
	MBC	5.8	0.8
CHX µg/mL	**MIC**	1.7	0.3
	MBC	1.7	0.3
CS µg/mL	**MIC**	31.2	0.0
	MBC	38.1	12.0
SN µg/mL	**MIC**	1.1	0.3
	MBC	1.2	0.2

Table 2-9. Summary of the MICs and MBCs obtained for the four investigated biocides on the overall *E. coli* population.

For each isolate, MICs and MBCs were determined at least in triplicate for each biocide (n≥3).

	BZC µg/mL		CHX µg/mL		CS µg/mL		SN µg/mL	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Mean	5.2	6.6	2.0	2.8	61.8	72.8	2.6	2.8
Minimum	2.7	2.7	0.5	1.7	7.8	15.6	1.7	1.7
Maximum	8.0	16.0	4.0	5.3	249.7	249.7	4.0	5.4
MIC_{50}/MBC_{50}	4.0	6.7	2.0	2.7	31.2	31.2	2.0	2.7
MIC_{90}/MBC_{90}	8.0	10.7	3.9	4.0	124.8	203.2	4.0	4.0

The overall repartitions of MICs and MBCs for BZC, CHX, CS and SN obtained for the *E. coli* isolates are presented in Figure 2-6. Uni-modal distributions of MICs and MBCs were observed for BZC, CHX and SN, while a double-bell shape with a wider range of concentrations was obtained for CS. MIC and MBC distributions of both BZC and CS were significantly different than the ones obtained for the other investigated biocides (Dunn's test; $p<0.05$). No significant difference between the distributions obtained for CHX and SN was detected (Dunn's test; $p>0.05$). No statistically significant differences were observed between MIC and MBC distributions when considering each biocide separately (Dunn's test; $p>0.05$), with MBC being usually double or equivalent to the MIC. MBC/MIC ratios higher than two were however obtained for Ec-187 (MBC/MIC ratio: 3.3) and Ec-210 (2.4) with BZC, Ec-161 (2.7) and Ec-206 (4.0) with CHX, and Ec-158 (2.7), Ec-162 (3.2), Ec-187 (2.7), Ec-199 (2.7) and Ec-206 (16.0) with CS. With regards to Ec-206, however, MICs were difficult to evaluate due to the low turbidity obtained after incubation when growth was obtained, which could influence the ratio value.

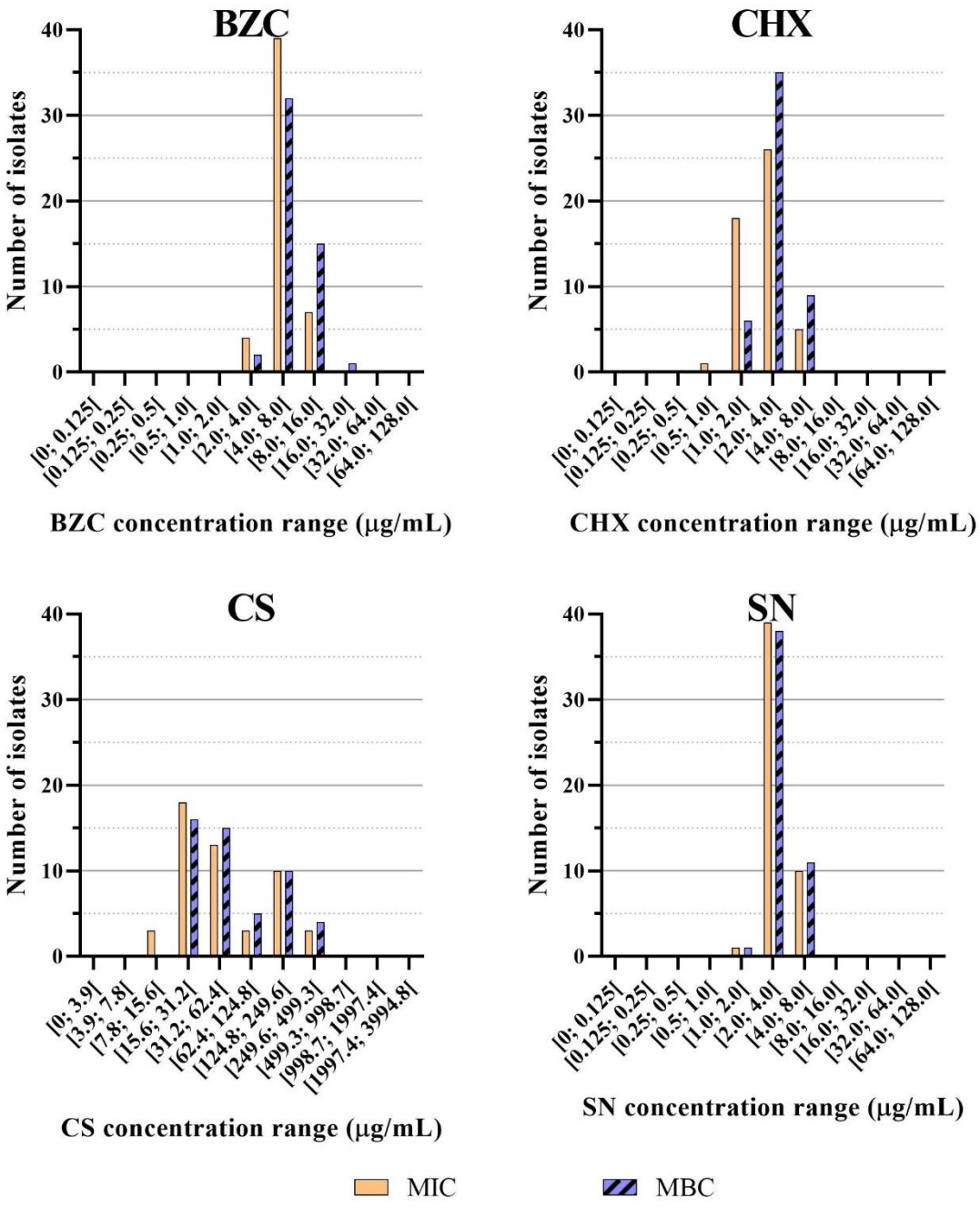

Figure 2-6. Repartition of MICs and MBCs for four biocides among *E. coli* isolates.
The intervals of concentrations (in µg/mL) are indicated on the x axis as [A; B[, corresponding to concentrations higher or equal to A but strictly lower than B. MIC and MBC repartitions among the 50 *E. coli* isolates are indicated for BZC, CHX, CS and SN.

The repartitions of MIC and MBC according to the carbapenemase-encoding genes possessed by the different isolates are shown in Figure 2-7. The type of carbapenemase produced (NDM, VIM, OXA-48-like and KPC) did not have an impact on the MIC and MBC distribution when considering each biocide separately (Dunn's test; $p>0.05$).

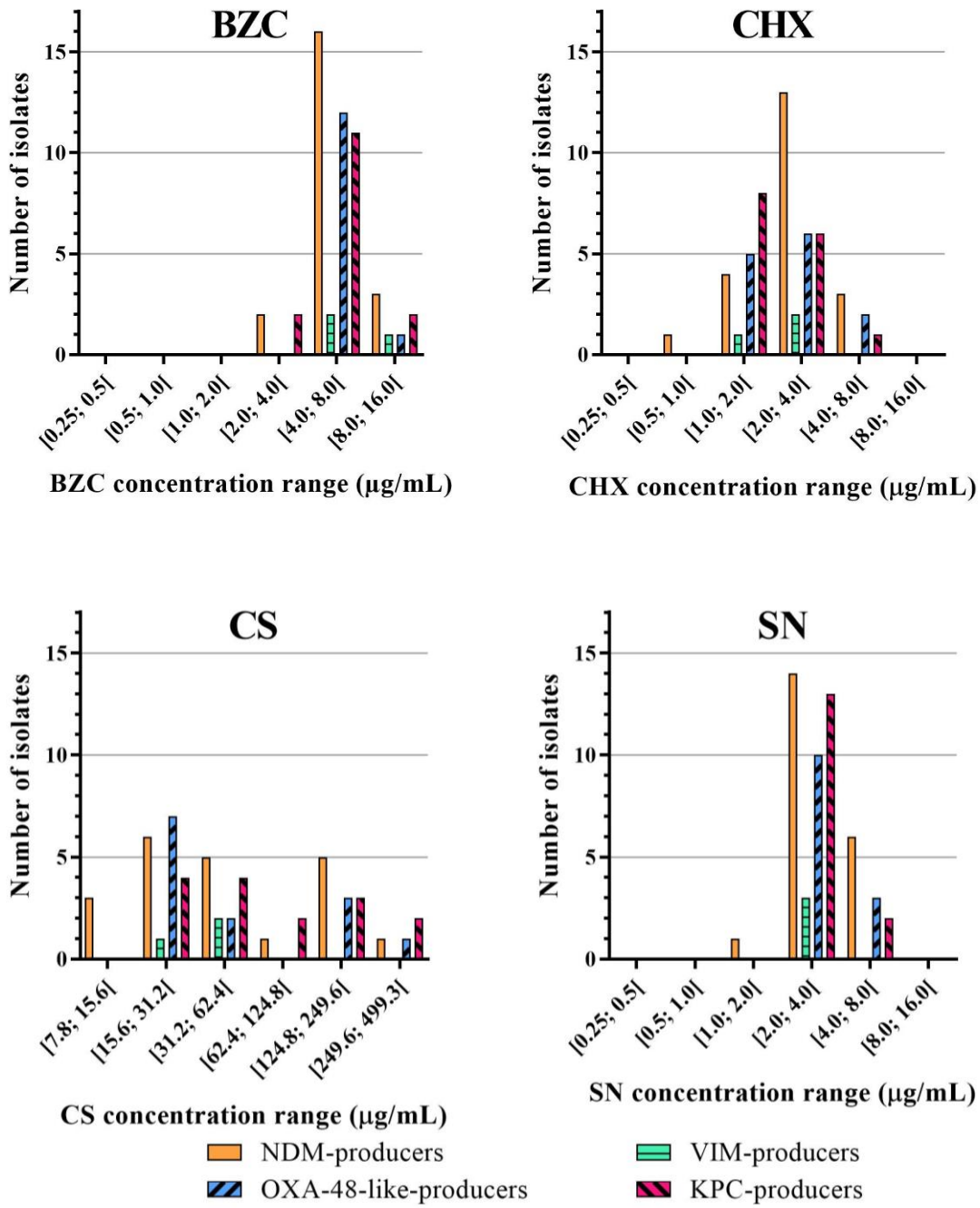

Figure 2-7. Repartition of MICs for four biocides among *E. coli* isolates according to their produced carbapenemases.

The intervals of concentrations (in µg/mL) are indicated on the x axis as [A; B[, corresponding to concentrations higher or equal to A but strictly lower than B. MIC repartitions among the NDM- (n=21), VIM- (n=3), OXA-48-like- (n=13) and KPC-producing (n=15) *E. coli* isolates are indicated for BZC, CHX, CS and SN. The isolates producing a combination of different carbapenemases were represented in both categories.

When comparing both species, MIC and MBC distributions for each biocide were similar (Dunn's test; $p>0.05$), except for CHX where statistically significant higher values were obtained for the *K. pneumoniae* population (Dunn's test; $p<0.05$).

2.4 Discussion

Regarding antibiotic susceptibility profiles, all isolates in this study were categorised as multi-drug resistant according to a harmonised definition established by international experts: 'multi-drug resistance is defined as non-susceptibility to at least one agent in three or more antimicrobial categories' (Magiorakos *et al.*, 2012; Mathers *et al.*, 2015). Extensive drug resistance corresponds to 'non-susceptibility to at least one agent in all but two or fewer antimicrobial categories' (corresponding to the list of recommended antibiotics and antibiotic categories to test for resistance by Magiorakos and colleagues). As not every recommended category of antibiotics was tested for this work, it was not possible to define any isolate as extensively-drug resistant. No pan-drug resistant isolate was identified, as no 'non-susceptibility to all agents in all antimicrobial categories' was detected; isolates were susceptible to at least one of the tested antibiotics (Magiorakos *et al.*, 2012). It is important to note however that MICs were determined by agar-dilution method, including for colistin, for which testing is now recommended to be performed by broth dilution methods; this could lead to false estimates of MICs and, thus, incorrect susceptibility profiles to colistin (Giske & Kahlmeter, 2018; Matuschek *et al.*, 2018).

As opposed to antibiotics, MIC determination for biocides is not a standardised procedure, preventing from being able to compare data with the existing literature; moreover, a similar lack of standard protocol is also observed when considering biocide susceptibility testing in general, mostly due to the differences in regulations between countries and/or agencies (some more stringent than others) and factors inherent to the test protocol itself such as the biocide concentration and formulation or the contact time for example (SCENIHR, 2009; Knapp *et al.*, 2015). When focusing on MIC determination, the method (agar-diffusion, macrodilution and microdilution methods), the media and its composition, the nature and coating of the microtitre plates (for microdilution method), among other parameters, can have a major impact on the MICs obtained (Maillard *et al.*, 2013; Wessels & Ingmer, 2013; Bock *et al.*, 2018). This can be illustrated in Table 2-10 (*K. pneumoniae*) and Table 2-11 (*E. coli*), regrouping MIC values collected from different studies using various methods and media combinations. Even when the same protocol was followed, e.g. the broth microdilution method detailed in the ISO 20776-1:2006, any

modification would impact the results. Indeed, in this study, the recommended CAMHB had to be replaced by R2A broth due to precipitation outcomes with CHX. The cation content, especially Mg^{2+} and Ca^{2+}, is lower in R2A than in CAMHB. Divalent cations are known to increase the susceptibility towards QACs, including BZC, and to impact some antibiotic MICs (Chow *et al.*, 1989). When comparing MIC data obtained in this study for *E. coli* ATCC 25922, values similar to the ones found in the literature were obtained for CHX (Grare *et al.*, 2010; Naparstek *et al.*, 2012; Tetz & Tetz, 2015), but they were very different for CS and SN (Sutterlin *et al.*, 2018). *K. pneumoniae* ATCC 13883 was used in only two of the studies grouped in Table 2-10 below, and a 2- to 8-fold difference in MIC was obtained for CHX (Naparstek *et al.*, 2012; Hashemi *et al.*, 2019). Due to this variability, the MIC values found in the literature were used as indicators of general trends rather than strictly being compared to the results generated in this study.

Table 2-10. MICs reported in the literature and from this study determined by different protocols for *K. pneumoniae*.

*Individual MICs were not reported; the indicated values correspond to the MIC_{90}. **Data were available only on graphics and not mentioned in the text and are therefore an estimation in this table. CAMHA: cation-adjusted Mueller-Hinton agar; ISB: Iso-Sensitest broth; MHA: Mueller-Hinton agar; MHB: Mueller-Hinton broth; TYE: tryptone yeast extract broth.

Reference	Design of the assay	Biocide	MIC (µg/mL)	Isolates tested
This study	Microdilution with R2A	BZC	2-4	*K. pneumoniae* ATCC 13883
This study	Microdilution with R2A	BZC	2-16	Clinical isolates
Pastrana-Carrasco *et al.*, 2012	Agar-dilution (media?)	BZC	32-128	Clinical isolates
Abuzaid *et al.*, 2012	Agar-dilution with ISA	BZC	16-64	Clinical isolates
Hammond *et al.*, 1987	Agar-dilution with ISA	BZC	15	*K. pneumoniae* ATCC 10273
Curiao *et al.*, 2015	Microdilution (broth?)	BZC	16	Clinical isolate
Guo *et al.*, 2015	Microdilution (broth?)	BZC	8-32	Clinical isolates
Morrissey *et al.*, 2014	Microdilution (broth?)	BZC	16*	Not specified
Wand *et al.*, 2015	Microdilution (broth?)	BZC	1-32	Pre-antibiotic era and modern isolates
Vijayakumar *et al.*, 2018	Microdilution with MHB	BZC	8-32	Clinical isolates
Houari & Di Martino, 2007	Microdilution with TSB	BZC	125	Clinical strain
Knapp *et al.*, 2015	Microdilution with TSB	BZC	≈ 20**	Unilever collection isolate
This study	Microdilution with R2A	CHX	1-2	*K. pneumoniae* ATCC 13883
This study	Microdilution with R2A	CHX	2-16	Clinical isolates
Pastrana-Carrasco *et al.*, 2012	Agar-dilution (media?)	CHX	16-64	Clinical isolates
Naparstek *et al.*, 2012	Agar-dilution with CAMHA	CHX	8-256	Clinical isolates

Table 2-10 – *Continued*.

Reference	Design of the assay	Biocide	MIC (µg/mL)	Isolates tested
Naparstek *et al.*, 2012	Agar-dilution with CAMHA	CHX	16	*K. pneumoniae* ATCC 13883
Abuzaid *et al.*, 2012	Agar-dilution with ISA	CHX	4-128	Clinical isolates
Hammond *et al.*, 1987	Agar-dilution with ISA	CHX	40	*K. pneumoniae* ATCC 10273
Block & Furman, 2002	Agar-dilution with MHA	CHX	2-128	Clinical isolates
Tetz & Tetz, 2015	Macrodilution (broth?)	CHX	32	Private collection isolate
Koljalg *et al.*, 2002	Macrodilution with CAMHB	CHX	16	Clinical isolates
Bock *et al.*, 2016	Microdilution (broth?)	CHX	128-512	Pre-chlorhexidine / modern era isolates
Curiao *et al.*, 2015	Microdilution (broth?)	CHX	32	Clinical isolate
Guo *et al.*, 2015	Microdilution (broth?)	CHX	32	Clinical isolates
Morrissey *et al.*, 2014	Microdilution (broth?)	CHX	32*	Not specified
Wand *et al.*, 2015	Microdilution (broth?)	CHX	0.25-32	Pre-antibiotic era and modern isolates
Wand *et al.*, 2017	Microdilution (broth?)	CHX	32-512	Clinical isolates
Grare *et al.*, 2010	Microdilution with CAMHB	CHX	2-4	Clinical isolates
Suwantarat *et al.*, 2014	Microdilution with CAMHB	CHX	2->32	Clinical isolates
Zhou *et al.*, 2015	Microdilution with CAMHB	CHX	16-32	Clinical isolates
Hashemi *et al.*, 2019	Microdilution with MHB	CHX	4	*K. pneumoniae* ATCC 13883
Vijayakumar *et al.*, 2018	Microdilution with MHB	CHX	16-64	Clinical isolates
Darouiche *et al.*, 2008	Microdilution with TSB	CHX	6.25	Clinical isolate
Houari & Di Martino, 2007	Microdilution with TSB	CHX	625	Clinical isolate

Table 2-10 – *Continued*.

Reference	Design of the assay	Biocide	MIC (µg/mL)	Isolates tested
Knapp *et al.*, 2015	Microdilution with TSB	CHX	≈ 4**	Unilever collection isolate
This study	Microdilution with R2A	SN	1-2	*K. pneumoniae* ATCC 13883
This study	Microdilution with R2A	SN	2-256	Clinical isolates
Elkrewi *et al.*, 2017	Agar-dilution (media?)	SN	1-8	Clinical isolates
Kapoor *et al.*, 1989	Macrodilution with TYE	SN	5-20	Clinical isolates
Cavassin *et al.*, 2015	Microdilution with both CAMHB and TSB	SN	3.4-13.5	Enterobacteriaceae (no species indication)

Table 2-11. MICs reported in the literature and from this study determined by different protocols for *E. coli*.

*Individual MIC were not reported; the indicated values correspond to the MIC_{90}. ISA: Iso-Sensitest agar; LB: Luria-Bertani broth; MHA: Mueller-Hinton agar; MHB: Mueller-Hinton broth.

Reference	Design of the assay	Biocide	MIC (µg/mL)	Isolates tested
This study	Microdilution with R2A	BZC	4-8	*E. coli* ATCC 25922
This study	Microdilution with R2A	BZC	2.7-8	Clinical isolates
Hammond *et al.*, 1987	Agar-dilution with ISA	BZC	20-50	*E. coli* ATCC 10536 and ATCC 11776
Hammond *et al.*, 1987	Agar-dilution with ISA	BZC	25-100	Clinical isolates
Soumet *et al.*, 2012	Macrodilution with MHB	BZC	16-32	Avian and porcine isolates
Curiao *et al.*, 2015	Microdilution (broth?)	BZC	16	Clinical isolate
Morrissey *et al.*, 2014	Microdilution (broth?)	BZC	32*	Not specified
Aarestrup & Hasman, 2004	Microdilution with CAMHB	BZC	16-128	Isolates from livestock animals
Beier *et al.*, 2016	Microdilution with CAMHB	BZC	4-32	Food animals and clinical isolates

Table 2-11 – *Continued*.

Reference	Design of the assay	Biocide	MIC (µg/mL)	Isolates tested
Bock *et al.*, 2018	Microdilution with different broth/plates	BZC	8-16	*E. coli* ATCC 10536
Deus *et al.*, 2017	Microdilution with MHB	BZC	4-32	Isolates from patients and broiler chickens
Henly *et al.*, 2019	Microdilution with MHB	BZC	15.6	Clinical isolate
Bore *et al.*, 2007	Microdilution with TSB	BZC	13	*E. coli* ATCC 47076
Houari & Di Martino, 2007	Microdilution with TSB	BZC	62.5	Clinical isolate
This study	Microdilution with R2A	CHX	1.3-2	*E. coli* ATCC 25922
This study	Microdilution with R2A	CHX	0.5-4	Clinical isolates
Naparstek *et al.*, 2012	Agar-dilution with CAMHA	CHX	2	*E. coli* ATCC 25922
Hammond *et al.*, 1987	Agar-dilution with ISA	CHX	1.5	*E. coli* ATCC 10536 and ATCC 11776
Hammond *et al.*, 1987	Agar-dilution with ISA	CHX	1-5	Clinical isolates
do Amorim *et al.*, 2004	Agar-dilution with MHA	CHX	2.67	Clinical isolate
Tetz & Tetz, 2015	Macrodilution (broth?)	CHX	2	*E. coli* ATCC 25922
Tetz & Tetz, 2015	Macrodilution (broth?)	CHX	16	Private collection isolate
Koljalg *et al.*, 2002	Macrodilution with CAMHB	CHX	2	Clinical isolates
Braoudaki & Hilton, 2004	Macrodilution with nutrient broth	CHX	4	*E. coli* ATCC 700728
O'Driscoll *et al.*, 2014	Macrodilution with nutrient broth	CHX	0.6	*E. coli* NCTC 4174
Thomas *et al.*, 2000	Macrodilution with nutrient broth	CHX	0.2	*E. coli* ATCC 10536
Curiao *et al.*, 2015	Microdilution (broth?)	CHX	8	Clinical isolate
Morrissey *et al.*, 2014	Microdilution (broth?)	CHX	16*	Not specified
Wesgate *et al.*, 2016	Microdilution (broth?)	CHX	7	*E. coli* ATCC 8739

Table 2-11 – *Continued*.

Reference	Design of the assay	Biocide	MIC (µg/mL)	Isolates tested
Aarestrup & Hasman, 2004	Microdilution with CAMHB	CHX	1-2	Isolates from livestock animals
Beier *et al.*, 2016	Microdilution with CAMHB	CHX	0.25-4	Food animals and clinical isolates
Grare *et al.*, 2010	Microdilution with CAMHB	CHX	< 1-2	*E. coli* ATCC 25922 and clinical isolates
Suwantarat *et al.*, 2014	Microdilution with CAMHB	CHX	2-4	Clinical isolates
Bock *et al.*, 2018	Microdilution with different broth/plates	CHX	0.125-2	*E. coli* ATCC 10536
Deus *et al.*, 2017	Microdilution with MHB	CHX	0.5-4	Isolates from patients and broiler chickens
Koburger *et al.*, 2010	Microdilution with MHB	CHX	8	*E. coli* ATCC 35218
McBain *et al.*, 2004	Microdilution with nutrient broth	CHX	13	*E. coli* ATCC 8729
Darouiche *et al.*, 2008	Microdilution with TSB	CHX	0.39	Clinical isolate
Houari & Di Martino, 2007	Microdilution with TSB	CHX	80	Clinical isolate
This study	Microdilution with R2A	CS	31.2-52.0	*E. coli* ATCC 25922
This study	Microdilution with R2A	CS	7.8-249.7	Clinical isolates
Aarestrup & Hasman, 2004	Microdilution with CAMHB	CS	1,997.4-5,992.3	Isolates from livestock animals
Biagi *et al.*, 2014	Microdilution with CAMHB	CS	12,500	*E. coli* CCUG 50175 and clinical isolates
Sutterlin *et al.*, 2018	Microdilution with ISB	CS	512-2,048	Clinical isolates
Sutterlin *et al.*, 2018	Microdilution with ISB	CS	1,024-2,048	*E. coli* ATCC 25922
Harrison *et al.*, 2005	Microdilution with LB broth	CS	1,124	Not specified
Deus *et al.*, 2017	Microdilution with MHB	CS	512-4,096	Isolates from patients and broiler chickens

Table 2-11 – *Continued*.

Reference	Design of the assay	Biocide	MIC (µg/mL)	Isolates tested
This study	Microdilution with R2A	SN	0.8-1.3	*E. coli* ATCC 25922
This study	Microdilution with R2A	SN	1.7-4	Clinical isolates
Elkrewi *et al.*, 2017	Agar-dilution (media?)	SN	1-8	Clinical isolates
Sutterlin *et al.*, 2014	Macro- or microdilution(?) with ISB	SN	8-32	Isolates from human and wild birds' faecal samples
Cavassin *et al.*, 2015	Microdilution with both CAMHB and TSB	SN	3.4-13.5	Enterobacteriaceae (no species indication)
Sutterlin *et al.*, 2018	Microdilution with ISB	SN	8-512	Clinical isolates
Sutterlin *et al.*, 2018	Microdilution with ISB	SN	16-32	*E. coli* ATCC 25922
Harrison *et al.*, 2005	Microdilution with LB broth	SN	10.2	Not specified
Deus *et al.*, 2017	Microdilution with MHB	SN	2-4	Isolates from patients and broiler chickens
Henly *et al.*, 2019	Microdilution with MHB	SN	31.3	Clinical isolate

Uni-modal distributions were obtained for BZC, CHX and SN in both species, being in accordance with previous work (Kumari *et al.*, 2011; Deus *et al.*, 2017), while CS MICs and MBCs followed a bi-modal distribution. This could indicate the presence of different subpopulations harbouring a wide range of tolerance thresholds within the collection of strains used for this study (Morrissey *et al.*, 2014). MBCs were usually found to be equivalent or two-fold higher than MICs for both species and for all the biocides investigated, apart from the few exceptions mentioned previously. This seemed to be in accordance with the literature, especially for cationic compounds like BZC and CHX (Biagi *et al.*, 2014; Morrissey *et al.*, 2014; Bock *et al.*, 2016).

Regarding CS susceptibility profiles, it is important to notice that the ranges of MICs and MBCs obtained might have been affected by the acidity of the solution containing the highest concentrations of CS. Growth curves in R2A broth adjusted to similar pH values than the ones presented in Table 2-4 (page 31) were performed for both *E. coli* and *K. pneumoniae* reference strains. It appeared that, at pH equivalent to a CS solution of

approximately 249.6 μg/mL, growth was not observed. As a consequence, for strains harbouring MICs and/or MBCs corresponding to this value, it was not possible to determine if the inhibitory/killing effect was due to the low pH, the concentration of CS, or a combination of both phenomena.

Despite available cut-off values to decipher between 'susceptible' and 'resistant' isolates to certain biocides (Morrissey *et al.*, 2014), the variability inherent to the design of the assay does not enable the use of such established thresholds. Moreover, the literature does not seem to agree on the notion of 'resistant' isolates to biocides. Isolates can be labelled as resistant when their MIC and MBC are higher than the ones obtained for a standard strain (Guo *et al.*, 2015), when they are not inactivated by an in-use concentration of a biocide or a concentration that inactivates other strains of that organism (Kampf, 2018), or when a change in susceptibility renders a biocide ineffective against a given organism to which it was previously susceptible (Maillard *et al.*, 2013). However, isolates showing a reduced susceptibility to a biocide, hence whose MIC would be higher than a reference strain or most of the population, could still be killed by the in-use concentration of the biocide, as highlighted previously (Table 2-3 page 25) (do Amorim *et al.*, 2004; Maillard *et al.*, 2013; Harbarth *et al.*, 2014; Wales & Davies, 2015), and with the example of this study where CHX MICs ranged from 0.5 to 16 μg/mL (when considering both species), while a skin disinfection solution, HiBiScrub®, contains 40,000 μg/mL CHX. As a consequence, the term 'resistance' was not used here for biocides, preferring 'reduced susceptibility' for isolates harbouring high MICs. In addition, MIC values do not necessarily correspond to an indicator of success or failure of disinfection procedures, as testing conditions for MIC determination do not reflect 'real-life', in-use conditions for biocides. Indeed, biocidal solutions usually consist in a mixture of one or more active compounds at high concentrations, formulated with excipients and applied for a specific amount of time (i.e. contact time) on different surface types, under different conditions (e.g. temperature, humidity, nutrient availability for microorganisms), among other parameters; when determining MICs, some important differences are that only one biocidal compound is investigated at a time, and put in contact with a specific bacterial density, incubated over 24 hours at 37ºC in rich media (SCENIHR, 2009; Maillard *et al.*, 2013; Morrissey *et al.*, 2014; Knapp *et al.*, 2015; Pelling *et al.*, 2019).

Regarding SN data, one strain, Kp-94, showed an important reduction in susceptibility, with an MIC and MBC of 256 μg/mL, while the other clinical isolates were inhibited and/or killed by concentrations between 2 and 4 μg/mL. It appears that Kp-94 was

isolated from a wound drain. Although no additional information regarding the potential treatments received by the patient was provided, it is known that silver can be used as an antimicrobial agent in wound treatments, including wound dressings (Gaisford *et al.*, 2009; Kumari *et al.*, 2011; Deus *et al.*, 2017). A hypothesis explaining this particularly high MIC for SN could be that Kp-94 was in contact with sub-lethal concentrations of silver ions, consequently reducing its susceptibility to this antimicrobial (McHugh *et al.*, 1975; Ip *et al.*, 2006).

When comparing both species, *K. pneumoniae* isolates seemed to be less susceptible to three out of four biocides than *E. coli*, while similar MIC and MBC values were observed for SN. This fact was consistent with previous studies where *K. pneumoniae* was found to harbour higher MICs for CHX than *E. coli* (Koljalg *et al.*, 2002; Guet-Revillet *et al.*, 2012). Isolates did not seem to be segregated according to their carbapenemase-encoding genes regarding their MIC and MBC distributions. This did not agree with the findings by Deus and colleagues, where differences in MICs were observed based on the produced ESBLs in *E. coli* (Deus *et al.*, 2017).

2.5 Conclusion

This work enabled the establishment of baseline data regarding the susceptibility profiles to four commonly used biocides of a wide collection of clinical isolates. Nonetheless, they could not be categorized as ‘susceptible’ or ‘resistant’ due to a lack of standardisation of the method leading to important variability within the results obtained, the absence of widely-accepted thresholds and the significance of ‘resistance’ when biocide concentrations within commercial products are found to be well above the MICs determined *in vitro*.

Chapter 3. Links between susceptibility to biocides and antibiotics

3.1 Introduction

3.1.1 Current status and associated risks

Several sectors rely heavily on the use of antibiotics, biocides and heavy metals, including healthcare but also farming and aquaculture, among others (SCENIHR, 2009; Ortega Morente *et al.*, 2013; Romero *et al.*, 2017). Low, sub-inhibitory concentrations of these antimicrobials can reach and persist in the environment, through inappropriate use, excretion after treatment, dilution or degradation in the environment among others (Andersson & Hughes, 2014; Wales & Davies, 2015; Martinez-Suarez *et al.*, 2016; Gadea *et al.*, 2017a).

A major concern is, with the importance of infection prevention and control measures relying especially on biocides to limit the spread of MDROs, that biocides may instead contribute to this (Maillard *et al.*, 2013; Harbarth *et al.*, 2014). Indeed, exposure to sub-inhibitory concentrations of antibiotics or biocides can trigger several mechanisms within bacteria for them to counteract the adverse effects of these compounds (Andersson & Hughes, 2014) and can result in reduced susceptibility to other unrelated antimicrobials; this is referred to as co-selection. Co-selection between different antimicrobials can occur through different mechanisms: co-resistance, cross-resistance and co-regulation (Baker-Austin *et al.*, 2006; Buffet-Bataillon *et al.*, 2012a). Co-resistance can happen when the genes responsible for resistance to each compound are located on the same mobile genetic element, e.g. *qacΔE*, involved in QACs efflux, and *sulI*, conferring resistance to sulphonamides, both present in class 1 integrons and widespread among Gram-negative bacteria (Fluit & Schmitz, 1999; Domingues *et al.*, 2012). The selective pressure exerted by one compound would select for both genetic determinants to be expressed or transferred. Cross-resistance results from the ability of a common resistance mechanism to counteract several unrelated antimicrobials; a development of resistance as a response to the selective pressure exerted by one compound would result in resistance to the other antimicrobial as well (Buffet-Bataillon *et al.*, 2012a). An example of cross-resistance would be the involvement of efflux pumps, such as AcrAB-TolC in *E. coli*, able to expel a wide range of substrates (Poole, 2007; Sun *et al.*, 2014). Exposure to antimicrobials could engender a cascade of regulatory responses impacting bacterial susceptibility to several compounds, e.g. copper provoking the dissociation of the transcriptional repressor MarR from its promoter region, resulting in the

expression of the *mar* operon involved in antibiotic resistance and virulence (with, among other consequences, an alteration of the outer membrane permeability; Hao *et al.*, 2014). This phenomenon can be referred to as co-regulation (Baker-Austin *et al.*, 2006).

Conflicting reports can be found in the literature regarding co-selection between biocides and antibiotics (Gnanadhas *et al.*, 2013; Harbarth *et al.*, 2014; Kampf, 2016; Kampf, 2018), and the clinical significance of such links between these antimicrobials is questioned (Wales & Davies, 2015). The impact of the use of biocides on the maintenance and spread of resistance genes among bacteria is controversial within the scientific community; nonetheless, the precaution principle, i.e. using biocides when appropriate and according to the manufacturers' recommendations, has to be kept in mind to ensure their efficacy (Oggioni *et al.*, 2013; Harbarth *et al.*, 2014; Wilson *et al.*, 2016).

3.1.2 Investigating potential links between biocide and antibiotic susceptibility

No international organisation (including the Food and Drug Administration in the United States or the European Chemicals Agency) indicates what tests to conduct when investigating bacterial resistance to biocide and, therefore, co-resistance mechanisms with antibiotics, to ensure the safety of these products (Maillard, 2018). A first step to investigate the presence of links between susceptibility to biocides and antibiotics is to perform correlation studies. Based on statistical analysis, correlation studies can indicate the presence of hypothetical relationships between two factors and give an insight about the next experimental work to perform (Chow *et al.*, 1989; Lavilla Lerma *et al.*, 2015).

The involvement of co-selection mechanism(s) is often associated with a fitness cost for the bacteria, especially cross-resistance such as efflux (Andersson & Hughes, 2010; Andersson & Hughes, 2012). Exposure to sub-inhibitory concentrations of biocides while monitoring bacterial growth can enable the evaluation of the associated fitness cost, through measurements of the lag phase duration or the growth rate, i.e. an extended lag phase could be linked to adaptation from the bacteria in order to respond to the selective pressure before resuming normal growth (Maillard *et al.*, 2013; Andersson & Hughes, 2014). Analyses of the outer membrane composition (proteins, fatty acids) and properties (hydrophobicity, permeability) is also important when considering cross-resistance mechanisms (Gnanadhas *et al.*, 2013). Re-evaluating antimicrobial susceptibility in the presence of additional compounds, such as ethylenediaminetetraacetic acid (EDTA) known to disturb the membrane structure by chelating the divalent cations within (Pelletier *et al.*, 1994; Sawer *et al.*, 1997; Denyer & Maillard, 2002), or in the presence of efflux pump inhibitors, e.g.

carbonyl cyanide 3-chorophenylhydrazone (CCCP) or reserpine (Marquez, 2005; Opperman & Nguyen, 2015; Alav *et al.*, 2018), could also provide insights regarding the involvement of an alteration of the membrane permeability or an increase in efflux, respectively. Expression analyses through transcriptomics studies (quantitative real-time polymerase chain reaction [PCR], RNA-sequencing) can help unveil the underlying mechanisms following exposure to sub-inhibitory concentrations of biocides (Gnanadhas *et al.*, 2013; Kim *et al.*, 2018a).

3.1.3 Aim

The aim of this chapter was to determine if correlations between biocide and antibiotic MIC were present for the 210 strains comprised within this study. Fifty representative isolates were then selected to pursue further work and investigate the presence and nature of potential links between susceptibility to biocides and antibiotics.

3.2 Methods

3.2.1 Investigation of links between susceptibility to biocides and antibiotics

3.2.1.1 Choice of test and interpretation criteria

The MIC and MBC values obtained for BZC, CHX, CS and SN did not follow a normal distribution (see section 2.3.4 page 41). Using Prism GraphPad 5 for Windows, correlation studies were therefore performed using Spearman's nonparametric test described to be more robust when extreme values are involved (Ferguson, 2009; Mukaka, 2012).

Correlations were considered significant when p-values were lower than 0.05. Positive r scores indicated positive correlations, i.e. when one variable increases, the other one increases too. Negative values highlighted inverted correlations, i.e. when one variable increases, the other one decreases. The strength of the correlations was measured based on criteria established within similar studies, including one performed on *S. aureus* using Spearman's test (Oggioni *et al.*, 2015), and two others where Pearson's test, the equivalent test used for normally distributed data, was used (Lavilla Lerma *et al.*, 2015; Romero *et al.*, 2017). Correlations were then categorized as weak when $0 < r < 0.4$, moderate when $0.4 \leq r < 0.7$, and strong when $0.7 \leq r \leq 1$ (considering the absolute value of r). The stronger the correlation, the more the variation of one variable is explained by the variation of the

other one, i.e. if A and B are strongly correlated, a variation in A will be mainly explained by a variation in B and vice-versa (Goodwin & Leech, 2006).

3.2.1.2 Data pre-treatment

Correlations studies were performed using MIC and MBC values obtained during the establishment of the susceptibility profiles to biocides (see section 2.3.4 page 41) and, when data were available, MICs determined for a wide-range of antibiotics prior to this study by Public Health England (see section 2.3.3 page 39). Regarding antibiotic MICs, when values were indicated as '> x' (x being the highest concentration tested for the MIC determination of a given antibiotic), they were changed into '2x', i.e. twice the concentration x. When values were indicated as '≤ y' (y being the lowest concentration tested for the MIC determination of a specific antibiotic), they were changed to 'y'. As Spearman's test is based on the rank of the values and not the values themselves, these changes were necessary to include all the available data in the correlation studies without having an impact on the scores obtained (Lambert, 2004).

3.2.2 Selection of representative strains for further studies

3.2.2.1 Rationale for the selection of strains

In order to proceed with further experiments, a more manageable amount of strains was needed. A target number of 50 strains was considered, instead of the initial 210 isolates. *K. pneumoniae* isolates represented 76.2 % (160/210) of the initial population, while *E. coli* represented 23.8 % (50/210 isolates). In order to keep the same ratio between both species, the target was to select 38 *K. pneumoniae* (76.0 %) and 12 *E. coli* (24.0 %) isolates. The aim was to choose isolates harbouring different categories of MIC values for BZC, CHX, CS and SN to represent the initial population.

The first step was to allocate a sub-group number to each strain and for each biocide according to their MIC. The sub-groups were designed as indicated in Table 3-1 (*K. pneumoniae*) and Table 3-2 (*E. coli*). The summary of the different allocated sub-groups for each strain is indicated in appendix VI for *K. pneumoniae* isolates and in appendix VII for *E. coli* isolates.

Table 3-1. Design of the different sub-groups for each biocide for *K. pneumoniae* isolates.

Sub-group	MIC range (µg/mL)	Number of strains within the sub-group
BZC1	[2; 4[	7
BZC2	[4; 8[	65
BZC3	[8; 16[	80
BZC4	[16; 32[	8
CHX1	[2; 4[	10
CHX2	[4; 8[	75
CHX3	[8; 16[	74
CHX4	[16; 32[	1
CS1	[7.8; 15.6[	1
CS2	[15.6; 31.2[	8
CS3	[31.2; 62.4[	24
CS4	[62.4; 124.8[	19
CS5	[124.8; 249.7[	54
CS6	[249.7; 499.4[	54
SN1	[2; 4[	79
SN2	[4; 8[	80
SN3	[256; 512[	1

Table 3-2. Design of the different sub-groups for each biocide for *E. coli* isolates.

Sub-group	MIC range (µg/mL)	Number of strains within the sub-group
BZC1	[2; 4[	4
BZC2	[4; 8[	39
BZC3	[8; 16[	7
CHX1	[0.5; 1[	1
CHX2	[1; 2[	18
CHX3	[2; 4[	26
CHX4	[4; 8[	5
CS1	[7.8; 15.6[	3
CS2	[15.6; 31.2[	18
CS3	[31.2; 62.4[	13
CS4	[62.4; 124.8[	3
CS5	[124.8; 249.7[	10
CS6	[249.7; 499.4[	3
SN1	[1; 2[	1
SN2	[2; 4[	39
SN3	[4; 8[	10

CS was the biocide harbouring the widest range of MICs for both species, therefore it was the biocide with the highest number of sub-groups. The second step consisted in

picking an equivalent number of strains within each CS sub-group. For example, regarding *E. coli*, the target was to select 12 isolates. As there were 6 CS sub-groups, 2 isolates from each sub-group had to be picked. The same rationale was then applied to the other biocides to have approximately the same number of strains within each sub-group of each biocide.

The last step consisted in choosing a limited number of isolates when several candidates were available within the same sub-group. In this case, the availability of WGS data (performed prior to this study by Public Health England), the availability of antibiotic susceptibility profiles, the sequence type and the carbapenemase-encoding genes harboured by the different isolates were considered. These criteria were decided in order to facilitate further experiments by avoiding completing pre-existing data (WGS, antibiotic susceptibility profiles) and to ensure representative and different isolates (in terms of molecular characterisation, provenance, susceptibility profiles) would be investigated.

3.2.2.2 Selected strains and characteristics

The 38 selected *K. pneumoniae* and the 12 chosen *E. coli* isolates, and the different sub-groups they belong to (indicated with a X), are listed in Table 3-3 and Table 3-4, respectively. The total number of strains within each sub-group is indicated at the bottom of the table. More detailed information about these strains (provenance, carbapenemase-encoding genes, susceptibility profiles to biocides) are available in appendix I (highlighted in blue).

Table 3-3. Selected *K. pneumoniae* isolates and their sub-group for each biocide.

The different sub-groups each of the selected isolates belong to (as determined previously, see Table 3-1 page 63), i.e. BZC1-4, CHX1-4, CS1-6 and SN1-3, are indicated with a X.

	BZC				CHX				CS						SN		
Strain	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**
Kp-1				X			X							X		X	
Kp-7				X			X						X		X		
Kp-13				X		X								X		X	
Kp-16			X			X				X					X		
Kp-18	X					X					X				X		
Kp-19	X				X				X						X		
Kp-20			X			X				X						X	
Kp-22		X			X							X				X	
Kp-26	X					X						X				X	
Kp-27		X					X			X					X		
Kp-28			X				X			X						X	
Kp-30			X		X							X				X	
Kp-45	X					X					X					X	
Kp-51	X					X								X		X	
Kp-58		X					X					X				X	
Kp-61			X		X									X		X	
Kp-63	X					X							X		X		
Kp-69				X		X					X				X		
Kp-79			X		X						X				X		
Kp-85		X			X									X	X		
Kp-88				X	X								X		X		
Kp-94		X				X							X				X
Kp-96			X				X			X					X		
Kp-99			X				X			X					X		
Kp-101		X				X						X			X		
Kp-106				X			X						X			X	
Kp-107			X				X			X					X		
Kp-109		X			X								X		X		
Kp-112	X					X						X				X	
Kp-114		X				X						X			X		
Kp-115		X				X						X			X		
Kp-118		X			X						X				X		
Kp-125				X			X						X			X	
Kp-126			X				X				X					X	
Kp-136			X			X					X				X		
Kp-147			X				X			X						X	
Kp-149				X			X							X		X	
Kp-155			X				X							X		X	
Total	7	10	13	8	9	15	14	0	1	8	7	8	7	7	19	18	1

Table 3-4. Selected *E. coli* isolates and their sub-group for each biocide.

The different sub-groups each of the selected isolates belong to (as determined previously, see Table 3-2 page 63), i.e. BZC1-3, CHX1-4, CS1-6 and SN1-3, are indicated with a X.

	BZC			CHX				CS						SN		
Strain	**1**	**2**	**3**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**
Ec-158			X			X		X							X	
Ec-161		X			X						X				X	
Ec-165		X					X						X			X
Ec-172		X				X				X						X
Ec-180			X				X			X						X
Ec-187		X				X				X						X
Ec-188			X				X		X						X	
Ec-191		X			X							X		X		
Ec-198	X				X						X				X	
Ec-199	X					X		X							X	
Ec-206	X			X					X						X	
Ec-207		X					X					X			X	
Total	3	6	3	1	3	4	4	2	2	3	2	2	1	1	7	4

3.2.3 Growth curves analysis

The protocol detailed is this section is adapted from a pioneer study in the exposure of bacteria to low concentrations of antimicrobials (Gullberg *et al.*, 2011). To ensure continuity with the MIC determinations performed in the previous chapter, considering the different factors able to impact MICs (inoculum size, temperature, volumes), the previously described adapted version of the ISO 20776-1:2006 was followed (International Organization for Standardization, 2006). Growth curves were performed on the 50 selected isolates and were exposed to CHX only. CHX was chosen for further experiments with regards to the correlations found and its important use as a biocide in healthcare settings. Experiments were carried at least in triplicate (n≥3).

3.2.3.1 Bioscreen plates inoculation and incubation

The bacterial suspensions were prepared, and bacterial counts were checked as described in sections 2.2.4.1 and 2.2.4.2 (page 31). The assay was performed using Honeycomb Bioscreen plates (Fisher Scientific, Loughborough, UK), whose layout is presented in Figure 3-1.

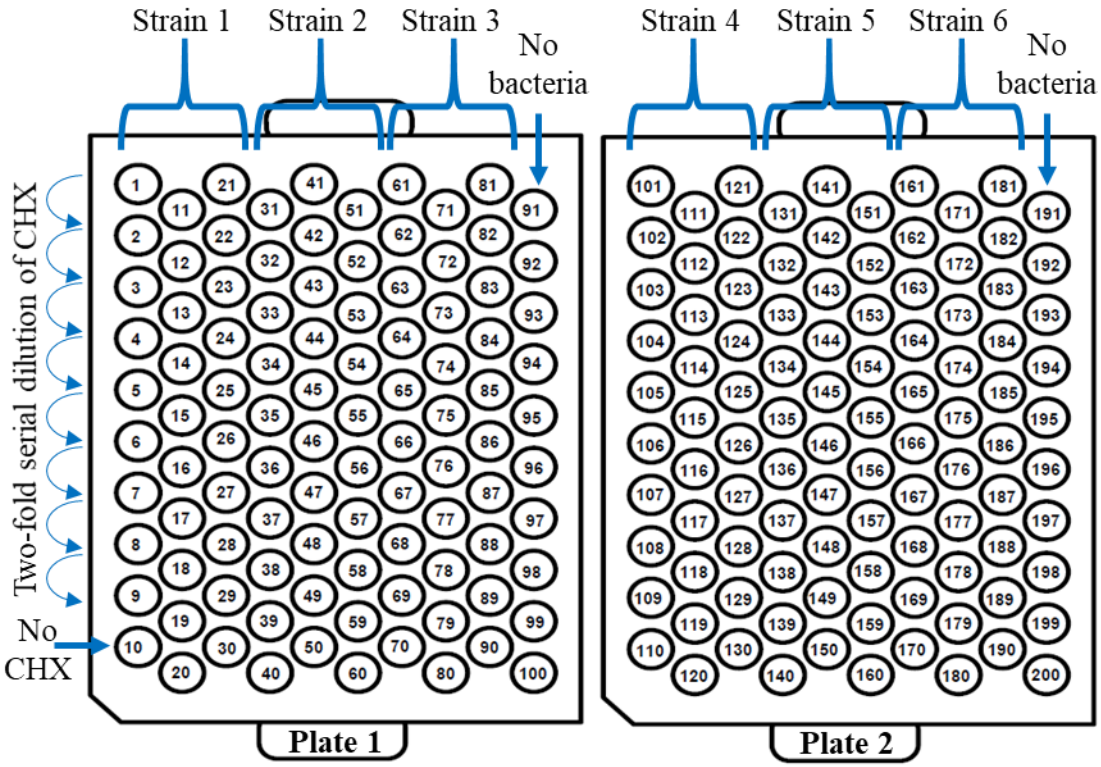

Figure 3-1. Bioscreen plates layout.

Testing for six strains with three technical replicates was performed using two Bioscreen plates simultaneously.

The CHX concentrations to test depended on the MBCs previously determined (see appendix IV and appendix V for CHX MBC values measured for *K. pneumoniae* and *E. coli* isolates, respectively) for the different isolates. Indeed, the highest concentration tested for each strain corresponded to twice its MBC in case variability was to be observed between both methods.

Two-fold serial dilutions of CHX were prepared in R2A, in centrifuge tubes. Each solution corresponded to twice the final tested concentration, i.e. if the concentration to test was 0.5 µg/mL CHX, the solution was prepared at 1 µg/mL CHX in the centrifuge tube. Fifty µL of the different CHX solutions in R2A was distributed to the corresponding wells. R2A broth only was used for the bottom row, as a CHX-free control. Fifty µL of the inoculum suspension (approximately 1.0 x10^6 CFU/mL) was then added to each well over three columns; R2A broth was used for the last column as a control without bacteria. *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922 were used as quality controls. Plates were incubated at 37ºC for 24 hours in a Bioscreen C Analyzer (Oy Growth Curves Ab Ltd,

Helsinki, Finland) with continuous agitation (medium amplitude, rotation speed not specified). The OD was measured every 4 min at 600 nm.

3.2.3.2 Data acquisition and pre-treatment

Bioscreen C was used in combination with the software EZExperiment for Windows (Oy Growth Curves Ab Ltd, Helsinki, Finland). The OD_{600nm} measured every 4 min for each well were automatically grouped into a spreadsheet.

The OD_{600nm} measured for R2A broth and the different solutions containing CHX was found to decrease over time and to vary between experiments, as illustrated in Figure 3-2. The OD_{600nm} measured for the first 40 to 50 min were especially high when compared to the following time points, and these values were higher than the OD_{600nm} measured in wells containing bacteria as well, leading to negative values when blanking against the bacteria-free well OD values. To avoid this, for each well, the OD_{600nm} value was blanked against its own value measured 4 min after the beginning on the incubation, corresponding to the first time point after the start of the agitation mechanism. After blanking, the OD_{600nm} values were converted into logarithms and plotted against time.

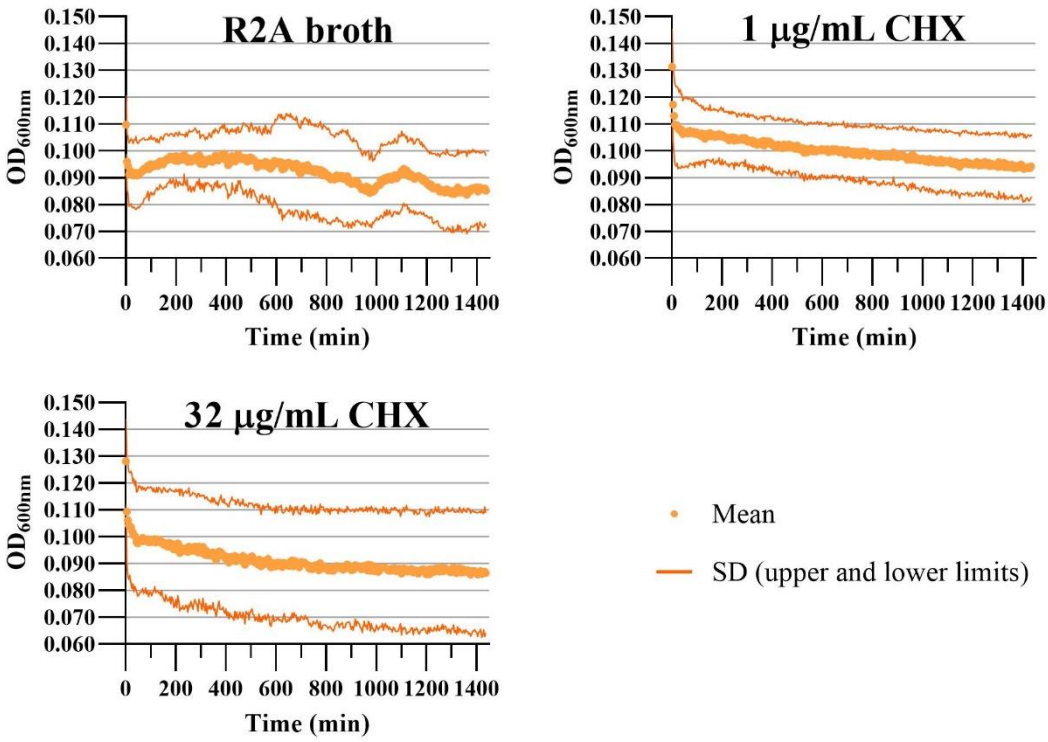

Figure 3-2. Variations in OD_{600nm} values over time for R2A only, R2A containing 1 µg/mL and 32 µg/mL CHX.

The OD_{600nm} was measured every 4 min over a 24-hour period. Data were obtained for 14 replicates for 32 µg/mL CHX, 24 replicates for 1 µg/mL CHX and 25 replicates for R2A broth only.

3.2.3.3 Determination of lag phase duration and growth rate

Due to very low blanked OD_{600nm} values at the early stages of growth, the lag phase duration and growth rate determinations were carried manually for each technical replicate after plotting the blanked OD_{600nm} and their logarithm values against time using Prism GraphPad 5 for Windows, respectively. The means obtained for the different parameters from the technical replicates constituted one biological replicate. Each parameter was determined over a minimum of three biological replicates.

The lag phase duration corresponded to the elapsed time until the point where an increase in the blanked OD_{600nm} values leading to the exponential growth phase was observed. Its determination is illustrated in Figure 3-3. The lag phase delay was obtained by subtracting the lag phase duration measured when the strain was grown in R2A only (0 µg/mL CHX) to the lag phase duration measured at a set concentration (x µg/mL CHX):

$$Delay_{(x\ \mu g/mL\ CHX)} = Duration_{(x\ \mu g/mL\ CHX)} - Duration_{(0\ \mu g/mL\ CHX)}$$

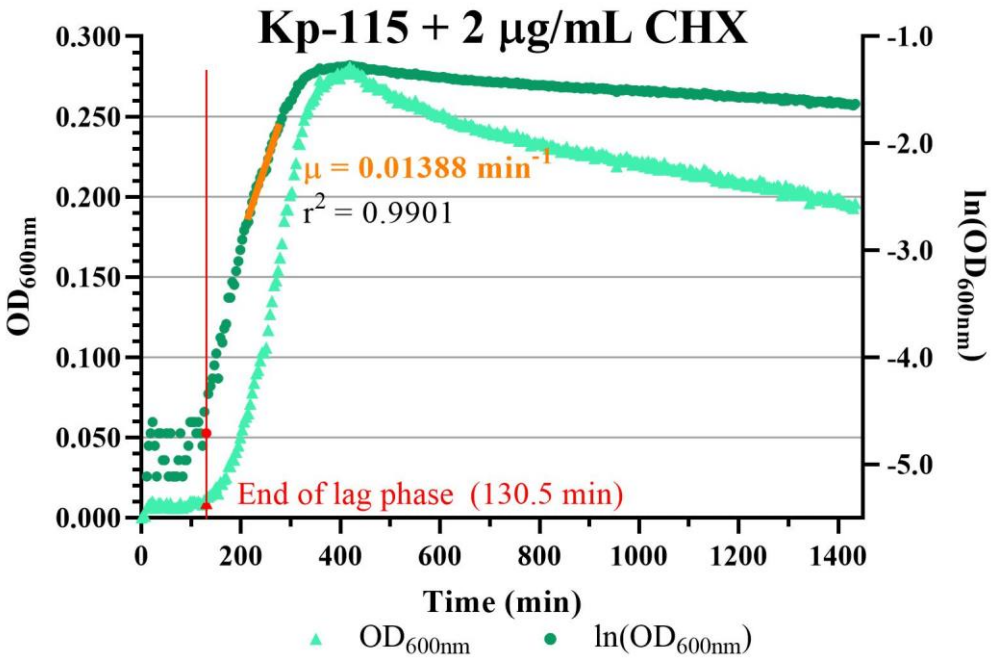

Figure 3-3. Lag phase duration and growth rate determinations.
Blanked OD_{600nm} and corresponding $\ln(OD_{600nm})$ values were obtained from one technical replicate where Kp-115 was exposed to 2 µg/mL CHX. The data point corresponding to the end of the lag phase is indicated in red. The lag phase duration corresponded to 130.5 min. A linear regression (orange line) was fitted on the exponential growth phase on the $\ln(OD_{600nm})$ against time graph. Its slope corresponded to the growth rate µ (0.01388 min^{-1}). The r^2 value showed that the regression fitted the curve (the closer to 1.0 the better the fit).

The growth rate was calculated based on the $\ln(OD_{600nm})$ *versus* time graph, as illustrated in Figure 3-3. A linear regression was fitted on the linear part of the curve corresponding to the exponential growth phase, so to obtain a coefficient correlation r^2 value the closest to 1 possible (representing a perfect correlation between the model and the curve). The slope of the linear regression corresponded to the growth rate µ.

The doubling time $t_{1/2}$, corresponding to the time needed for the bacterial population to double, was determined as follow (Hall *et al.*, 2014):

$$t_{1/2} = \frac{\ln(2)}{\mu}$$

3.2.3.4 Statistical analysis

Statistical analyses were carried using Prism GraphPad 8 for Windows. CHX MICs obtained with the Bioscreen assay were compared to the ones previously obtained in 96-well plates using two-way analysis of variance (ANOVA) followed by Sidak's multiple-comparison post-test. Differences were considered significant when $p<0.05$. For each strain, the different parameters measured at each CHX concentration tested were compared to the control condition, i.e. bacteria growing in CHX-free R2A broth (i.e. 0 µg/mL CHX) using the one-way ANOVA followed by Dunnett's post-test (significant differences when $p<0.05$). For the growth rate only, when CHX concentrations were tested for both a clinical isolate and the reference strain belonging to the same species, the difference between them was assessed using two-way ANOVA followed by Sidak's post-test (significant when $p<0.05$).

3.2.4 Investigation of possible cross-resistance mechanisms between biocides and antibiotics

3.2.4.1 Determination of minimum inhibitory concentrations of chlorhexidine in the presence of a chelator of cations

CHX MICs were determined in the presence of the ion chelator ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, Gillingham, UK). A stock solution containing 1,200 µg/mL EDTA (pH 8.0-8.5) was prepared in distilled water, filtered onto 0.20 µm cellulose acetate membrane filter and stored at 4ºC for up to a month. The solution was diluted in R2A when needed for testing.

MICs for CHX were determined in the presence of 60 µg/mL EDTA. The CHX concentrations tested ranged from 32 µg/mL to 0.03125 µg/mL for *K. pneumoniae* isolates, and from 4 µg/mL to 0.00781 µg/mL for *E. coli* isolates. This concentration of EDTA was chosen to ensure the growth of all the tested isolates after testing several concentrations (EDTA MIC for both reference strains corresponding to 125 µg/mL).

The inoculum was prepared as previously described (see section 2.2.4.1 page 31). The microtitre plates were first filled with 50 µL R2A containing twice the final appropriate concentration of EDTA (120 µg/mL EDTA, for a final concentration of 60 µg/mL). The serial dilution was performed by mixing 50 µL of a four-times concentrated solution of CHX (128 µg/mL CHX for *K. pneumoniae*, 32 µg/mL CHX for *E. coli*) in R2A containing 120 µg/mL EDTA across the microtitre plate, except in the final column (control without biocide). Fifty µL of the inoculum suspension (approximately 1.0×10^6 CFU/mL) was

finally added to the wells, except in the control wells without bacteria where R2A broth was used instead. Plates were incubated for 24 hours at 37ºC ± 1ºC. MICs were read as the lowest concentrations of CHX without growth as perceived by an un-aided eye.

3.2.4.2 Determination of minimum inhibitory concentrations of chlorhexidine in the presence of a proton-motive force inhibitor

The proton-motive force inhibitor carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was prepared at 2,000 µg/mL in 100 % dimethylsulfoxide (DMSO; both from Acros Organics, Geel, Belgium), sterilised by filtration on 0.20 µm nylon membrane filter and stored at 4ºC for up to a month. This concentration allowed to reach a final concentration of DMSO below toxic levels (DMSO MIC was 5 % v/v for both *K. pneumoniae* ATCC 13883 and *E. coli* ATCC 25922 when tested as previously described). When needed, the solution was diluted in R2A.

MICs for CHX were determined in the presence of 20 µg/mL CCCP (1 % v/v DMSO) for *K. pneumoniae* isolates (except for Kp-63, which needed the concentration to be reduced to 5 µg/mL to be able to grow) and 10 µg/mL CCCP (0.5 % v/v DMSO) for *E. coli* isolates (as they were not able to grow at higher concentrations of CCCP). The CHX concentrations tested ranged from 32 µg/mL to 0.03125 µg/mL for *K. pneumoniae* isolates, and from 4 µg/mL to 0.00781 µg/mL for *E. coli* isolates. These concentrations were below the CCCP MIC obtained for both *K. pneumoniae* and *E. coli* ATCC strains (40 µg/mL).

The inoculum was prepared as previously detailed (see section 2.2.4.1 page 31). The microtitre plates were first filled with 50 µL R2A containing twice the final desired concentration of CCCP (40 µg/mL CCCP for *K. pneumoniae*, 20 µg/mL CCCP for *E. coli*). The serial dilution was performed by mixing 50 µL of a four-times concentrated solution of CHX (128 µg/mL CHX for *K. pneumoniae*, 32 µg/mL CHX for *E. coli*) in R2A containing either 40 µg/mL or 20 µg/mL CCCP depending on the species across the microtitre plate, except in the final column (control without biocide). Finally, 50 µL of the inoculum suspension (approximately 1.0 x 10^6 CFU/mL) was added to the wells (R2A broth was used instead as a control without bacteria). Plates were incubated for 24 hours at 37ºC ± 1ºC. MICs were determined as the lowest concentrations of CHX without growth as seen by a naked eye as previously described.

3.2.4.3 Statistical analysis

CHX MICs measured in the presence of either EDTA or CCCP were compared to the MICs previously obtained (see appendix IV and appendix V for detailed values) using two-way ANOVA followed by Dunnett's post-test. Differences were considered significant when $p<0.05$. The test was performed with GraphPad Prism 8 for Windows.

3.3 Results

3.3.1 Correlation study

3.3.1.1 *Klebsiella pneumoniae* isolates

3.3.1.1.1 Correlations between biocides

The Spearman's r scores obtained after performing the correlations studies on MICs and MBCs between biocides are shown in Table 3-5. For each biocide, strong positive correlations were observed between MICs and MBCs, and weak to moderate positive correlations were obtained between BZC and CHX, for the overall *K. pneumoniae* population and the different carbapenemase-producing categories with the exception of the KPC-producers where no correlation between BZC and CHX was detected. A moderate to strong positive correlation was determined between CHX and SN for VIM-producers, while the relationship between these biocides corresponded to a weak inverted correlation for NDM-producers. Weak to moderate correlations between both heavy metals were obtained when considering the overall population and the KPC-producers.

Table 3-5. Spearman's r scores between biocide MICs and MBCs for *K. pneumoniae* isolates.

Correlations were considered as weak when r ranged from 0 to 0.4, moderate when r ranged from 0.4 to 0.7 and strong for values above 0.7 (when considering the absolute value of r). Only significant correlations (Spearman's test; $p<0.05$) are displayed in this table. Correlations were calculated for the whole *K. pneumoniae* population (n=160) and according to the carbapenemases produced (NDM-, VIM-, OXA-48-like- and KPC-producing *K. pneumoniae* isolates). When an isolate possessed a combination of carbapenemases, it was represented in both categories.

			CHX		BZC		CS		SN	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
All (n=160)	CHX	MIC								
		MBC	0.850							
	BZC	MIC	0.407	0.454						
		MBC	0.323	0.359	0.823					
	CS	MIC								
		MBC					0.936			
	SN	MIC					0.158			
		MBC							0.935	
NDM (n=41)	CHX	MIC								
		MBC	0.718							
	BZC	MIC	0.371	0.461						
		MBC	0.389	0.393	0.847					
	CS	MIC								
		MBC					0.959			
	SN	MIC								
		MBC	-0.317						0.927	
VIM (n=15)	CHX	MIC								
		MBC	0.908							
	BZC	MIC	0.593	0.620						
		MBC			0.744					
	CS	MIC								
		MBC					0.971			
	SN	MIC	0.721	0.655						
		MBC	0.630	0.521					0.875	

Table 3-5 – *Continued*.

			CHX		BZC		CS		SN	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
OXA (n=54)	CHX	MIC								
		MBC	0.869							
	BZC	MIC	0.542	0.535						
		MBC	0.394	0.473	0.863					
	CS	MIC								
		MBC					0.984			
	SN	MIC								
		MBC							0.973	
KPC (n=53)	CHX	MIC								
		MBC	0.794							
	BZC	MIC								
		MBC			0.794					
	CS	MIC								
		MBC					0.828			
	SN	MIC					0.561	0.414		
		MBC					0.436		0.910	

3.3.1.1.2 Correlations between biocides and antibiotics

The different correlations calculated between biocide and antibiotic MICs are summarised in Table 3-6. Regarding the overall *K. pneumoniae* population, weak to moderate positive correlations were obtained between CHX and carbapenems, cephalosporins, AK, ATM, CIP and TIG. Weak positive correlations were also calculated between BZC and AK, TIG and MIN. Weak inverted correlations were obtained between CS and MEM, cephalosporins and TEMO.

When considering the NDM-producers, moderate positive correlations were determined between CHX and TIG and MIN, and weak positive correlations between BZC and MIN. Weak to moderate inverted correlations were obtained between CS and carbapenems, CTX, CPM and CIP. Regarding the VIM-producers, moderate to strong positive correlations were determined between either one or both of the cationic biocides and carbapenems, FOX, CTX, aminoglycosides and TIG. OXA-48-like-producers harboured weak to moderate positive correlations between CHX and carbapenems, FOX, TIG, MIN and COL. Similarly, weak to moderate positive correlations were obtained between BZC and aminoglycosides, TIG and MIN. Weak to moderate inverted correlations were determined between CS and carbapenems. For KPC-producers, weak to moderate

positive correlations were calculated between CHX and FOX, CAZ and ATM, and weak inverted correlations were found between BZC and TOB.

Table 3-6. Spearman's r scores between biocide and antibiotic MICs for *K. pneumoniae* isolates.

A), correlations between biocide MICs and carbapenem and cephalosporin MICs. B), correlations between biocide MICs and aminoglycoside and other antibiotic MICs. Correlations were considered as weak when r ranged from 0 to 0.4, moderate when r ranged from 0.4 to 0.7 and strong for values above 0.7 (when considering the absolute value of r). Only significant correlations (Spearman's test; $p<0.05$) are displayed in this table. Correlations were calculated for the whole *K. pneumoniae* population (n=160) and according to the carbapenemases produced (NDM-, VIM-, OXA-48-like- and KPC-producing *K. pneumoniae* isolates). When an isolate possessed a combination of carbapenemases, it was represented in both categories. The pair number indicates on how many isolates the correlation was determined according to their antibiotic MIC availabilities.

A		**Carbapenems**			**Cephalosporins**			
		IPM	**MEM**	**ETP**	**FOX**	**CTX**	**CAZ**	**CPM**
All (n=160)	Pairs	148	148	147	147	147	148	139
	CHX	0.218	0.297	0.245	0.363		0.225	0.191
	BZC							
	CS		-0.193		-0.185	-0.178		-0.232
	SN							
NDM (n=41)	Pairs	41	41	41	41	41	41	37
	CHX							
	BZC							
	CS	-0.344	-0.331	-0.310		-0.317		-0.435
	SN							
VIM (n=15)	Pairs	14	14	14	14	14	14	12
	CHX		0.683	0.613				
	BZC	0.582	0.810	0.839	0.746	0.570		
	CS							
	SN							
OXA (n=54)	Pairs	48	48	47	47	47	48	45
	CHX	0.378	0.451	0.444	0.496			
	BZC							
	CS	-0.362	-0.424	-0.295				
	SN							
KPC (n=53)	Pairs	48	48	48	48	48	48	48
	CHX				0.469		0.412	
	BZC							
	CS							
	SN							

Table 3-6 – *Continued*.

B		Aminoglycosides			Other antibiotics					
		TOB	**AK**	**CN**	**TEMO**	**ATM**	**CIP**	**TIG**	**MIN**	**COL**
All (n=160)	Pairs	148	148	148	135	143	148	147	138	148
	CHX		0.209			0.165	0.163	0.370	0.434	
	BZC		0.166					0.285	0.263	
	CS				-0.233					
	SN									
NDM (n=41)	Pairs	41	41	41	36	40	41	41	39	41
	CHX							0.426	0.588	
	BZC								0.366	
	CS						-0.343			
	SN									
VIM (n=15)	Pairs	14	14	14	14	14	14	14	13	14
	CHX	0.591	0.534	0.585				0.650	0.740	
	BZC	0.768	0.783	0.652					0.638	
	CS									
	SN									
OXA (n=54)	Pairs	48	48	48	48	44	48	47	44	48
	CHX							0.406	0.363	0.290
	BZC	0.356	0.357	0.317				0.422	0.324	
	CS									
	SN									
KPC (n=53)	Pairs	48	48	48	40	48	48	48	45	48
	CHX					0.342				
	BZC	-0.293								
	CS									
	SN									

3.3.1.2 *Escherichia coli* isolates

3.3.1.2.1 Correlations between biocides

The results of the Spearman's correlation test between biocide MICs performed on the overall population of *E. coli* and after segregation according to the produced carbapenemases are presented in Table 3-7. Strong positive correlations between MICs and MBCs for each biocide were observed in all cases, except for NDM-producers (moderate correlation between CHX MICs and MBCs) and VIM-producers (no significant correlation). Weak to moderate positive correlations were obtained between both cationic compounds, with the exception of VIM- and KPC-producers (no significant correlation). Moderate to strong positive correlations were observed between BZC and SN for the overall population and for NDM- and OXA-48-like-producers. Weak positive correlations were calculated between heavy metals when considering the overall *E. coli* population only.

Table 3-7. Spearman's r scores between biocide MICs and MBCs for *E. coli* isolates.
Correlations were considered as weak when r ranged from 0 to 0.4, moderate when r ranged from 0.4 to 0.7 and strong for values above 0.7 (when considering the absolute value of r). Only significant correlations (Spearman's test; $p<0.05$) are displayed in this table. Correlations were calculated for the whole *E. coli* population (n=50) and according to the carbapenemases produced (NDM-, VIM-, OXA-48-like- and KPC-producing *E. coli* isolates). When an isolate possessed a combination of carbapenemases, it was represented in both categories. N/A: not available as isolates possessed the same MIC for at least one of the agents investigated.

			CHX		BZC		CS		SN	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
All (n=50)	CHX	MIC								
		MBC	0.715							
	BZC	MIC	0.370	0.422						
		MBC	0.418	0.464	0.790					
	CS	MIC								
		MBC					0.843			
	SN	MIC			0.345	0.500		0.283		
		MBC				0.411		0.295	0.873	
NDM (n=21)	CHX	MIC								
		MBC	0.567							
	BZC	MIC	0.608	0.590						
		MBC	0.549	0.648	0.750					
	CS	MIC								
		MBC					0.724			
	SN	MIC				0.485				
		MBC							0.907	
VIM (n=3)	CHX	MIC								
		MBC								
	BZC	MIC								
		MBC								
	CS	MIC								
		MBC				N/A				
	SN	MIC	N/A	N/A	N/A	N/A	N/A	N/A		
		MBC	N/A	N/A	N/A	N/A	N/A	N/A	N/A	

Table 3-7 – *Continued*.

			CHX		BZC		CS		SN	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
OXA (n=13)	CHX	MIC								
		MBC	0.769							
	BZC	MIC		0.740						
		MBC	0.562	0.635	0.844					
	CS	MIC	0.598							
		MBC	0.701				0.926			
	SN	MIC			0.608	0.800				
		MBC			0.615	0.815			0.995	
KPC (n=15)	CHX	MIC								
		MBC	0.779							
	BZC	MIC								
		MBC			0.837					
	CS	MIC								
		MBC					0.992			
	SN	MIC								
		MBC							0.731	

3.3.1.2.2 Correlations between biocides and antibiotics

The different Spearman's r scores calculated between biocide and antibiotic MICs are presented in Table 3-8. Regarding the overall *E. coli* population, a weak positive correlation was obtained between SN and TEMO. NDM-producers harboured moderate positive correlations between TEMO and both BZC and CS. No significant correlation was calculated for VIM-producers. Moderate positive correlations were obtained between CHX and ETP and AK, and between CS and IPM, ETP and CPM for OXA-48-like-producers. A strong positive correlation was also observed between SN and COL for this group. Regarding KPC-producers, a moderate positive correlation was determined between BZC and CTX, and moderate inverted correlations were obtained between CS and FOX and CPM.

Table 3-8. Spearman's r scores between biocide and antibiotic MICs for *E. coli* isolates. A), correlations between biocide MICs and carbapenem and cephalosporin MICs. B), correlations between biocide MICs and aminoglycoside and other antibiotic MICs. Correlations were considered as weak when r ranged from 0 to 0.4, moderate when r ranged from 0.4 to 0.7 and strong for values above 0.7 (when considering the absolute value of r). Only significant correlations (Spearman's test; $p<0.05$) are displayed in this table. Correlations were calculated for the whole *E. coli* population (n=50) and according to the carbapenemases produced (NDM-, VIM-, OXA-48-like- and KPC-producing *E. coli* isolates). When an isolate possessed a combination of carbapenemases, it was represented in both categories. The pair number indicates on how many isolates the correlation was determined according to their antibiotic MIC availabilities. N/A: not available as isolates possessed the same MIC for at least one of the agents investigated.

A

		Carbapenems			Cephalosporins			
		IPM	**MEM**	**ETP**	**FOX**	**CTX**	**CAZ**	**CPM**
All (n=50)	Pairs	44	44	44	44	44	44	43
	CHX							
	BZC							
	CS							
	SN							
NDM (n=21)	Pairs	21	21	21	N/A	21	N/A	20
	CHX				N/A		N/A	
	BZC				N/A		N/A	
	CS				N/A		N/A	
	SN				N/A		N/A	
VIM (n=3)	Pairs	3	3	3	N/A	3	3	3
	CHX				N/A			
	BZC				N/A			
	CS				N/A	N/A		
	SN	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OXA (n=13)	Pairs	11	11	11	11	11	11	11
	CHX			0.641				
	BZC							
	CS	0.630		0.678				0.656
	SN							
KPC (n=15)	Pairs	11	11	11	11	11	11	11
	CHX							
	BZC					0.660		
	CS				-0.696			-0.637
	SN							

Table 3-8 – *Continued*.

B		**Aminoglycosides**			**Other antibiotics**				
		TOB	**AK**	**CN**	**TEMO**	**ATM**	**CIP**	**TIG**	**COL**
All (n=50)	Pairs	44	44	44	38	43	44	44	44
	CHX								
	BZC								
	CS								
	SN				0.322				
NDM (n=21)	Pairs	21	21	21	16	20	21	21	21
	CHX								
	BZC				0.510				
	CS				0.573				
	SN								
VIM (n=3)	Pairs	3	3	3	N/A	3	3	3	3
	CHX				N/A				N/A
	BZC	N/A		N/A	N/A				
	CS				N/A				
	SN	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OXA (n=13)	Pairs	11	11	11	11	11	11	11	11
	CHX		0.603						
	BZC								
	CS								
	SN								0.857
KPC (n=15)	Pairs	11	11	11	10	11	11	11	11
	CHX								
	BZC								
	CS								
	SN								

3.3.2 Growth curves analysis

3.3.2.1 Unusual growth patterns

When performing the Bioscreen assay, it was decided not to pursue with Ec-206. Despite viable counts corresponding to expected bacterial concentration, very low and variable OD_{600nm} were measured for this isolate, rendering the data unreliable and difficult to analyse. As a consequence, Ec-206 was removed from any further experiment.

Several isolates showed variability in growth at some concentrations, i.e. growth was not observed for all the replicates under set conditions (Kp-7, Kp-45, Kp-51, Kp-94, Kp-99, Kp-106 and Ec-207). As this impacted the different analysis, and to facilitate the following explanations, this group of strains will further be referred to as the "variable-growth strains".

One isolate in particular, Kp-125, harboured an exponential growth phase separated in two distinct parts as illustrated in Figure 3-4. As a consequence, analysis, when needed, were performed on each part, labelled as "first phase" and "second phase" as *per* indicated on the figure below.

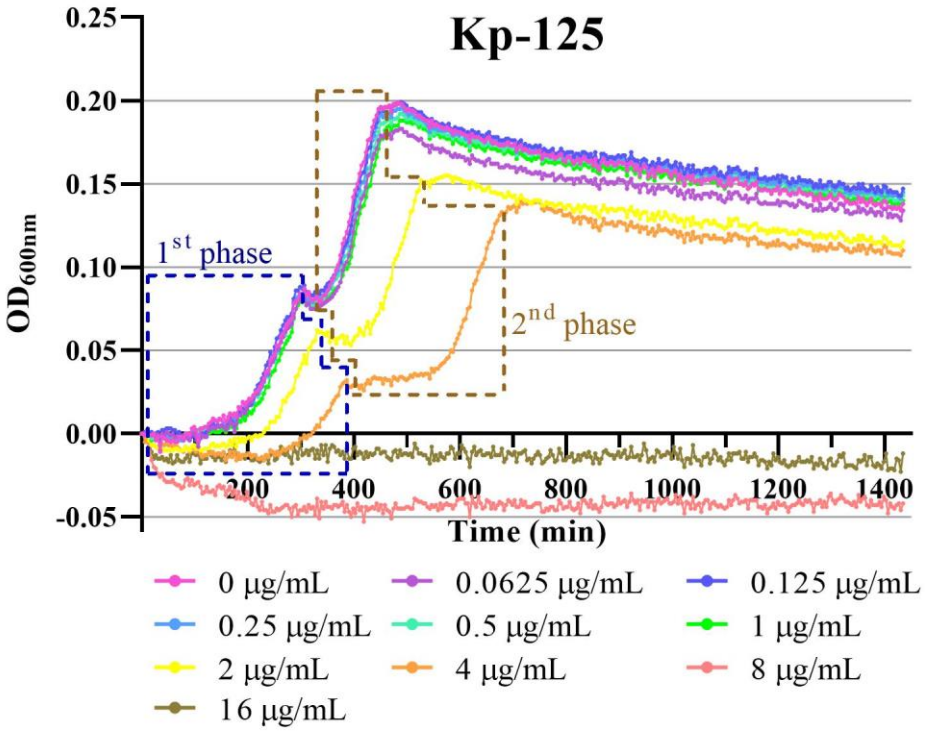

Figure 3-4. Growth pattern of Kp-125 when exposed to different concentrations of CHX.

The blanked OD_{600nm} is indicated on the y axis. Each curve corresponds to the growth of Kp-125 in R2A containing the indicated CHX concentration. The dark blue dashed box indicates the data points corresponding to the first growth phase of Kp-125, and the brown dashed box corresponds to its second growth phase. This figure corresponds to one replicate only.

3.3.2.2 *Klebsiella pneumoniae* isolates

The detailed results obtained for the *K. pneumoniae* reference strain and clinical isolates are presented in appendix VIII. Isolates for which statistically significant differences were observed when compared to the control condition are detailed in Table 3-9.

Table 3-9. Results obtained from the growth curves study for the *K. pneumoniae* isolates.

Experiments were carried at least in triplicate for each condition (n≥3). Values in bold and red corresponded to significant differences (one-way ANOVA followed by Dunnett's post-test; $p<0.05$) observed when compared to the CHX-free R2A growth condition (0 µg/mL CHX). Only isolates and concentrations at which significant differences for at least one of the investigated parameters are displayed, alongside with the control condition. For "variable-growth strains", the concentration at which variability in growth was observed was highlighted in orange. Statistical analyses were not performed on doubling times as they were inferred from the mean growth rate values.

Strain	CHX (µg/mL)	Lag phase duration (min)		Lag phase delay (min)		Growth rate µ (min^{-1})		Doubling time $t_{1/2}$ (min)	OD_{600nm} max	
		Mean	SD	Mean	SD	Mean	SD		Mean	SD
K. pneumoniae ATCC 13883	4	**1,436.1**	**0.0**	**1,218.0**	**42.8**	**0.0000**	**0.0000**		**0.002**	**0.003**
	2	**329.9**	**134.6**	**117.7**	**124.3**	0.0119	0.0018	58.3	0.216	0.010
	1	218.5	41.5	0.4	8.4	0.0122	0.0012	57.0	**0.230**	**0.008**
	0.5	213.0	36.9	-5.0	11.3	0.0124	0.0012	56.1	**0.235**	**0.008**
	0.25	209.3	38.2	-4.2	7.8	0.0123	0.0013	56.5	**0.238**	**0.009**
	0.125	210.0	36.6	-8.1	14.9	0.0124	0.0011	55.8	**0.240**	**0.007**
	0.0625	210.4	36.6	-7.7	14.1	0.0124	0.0012	56.1	**0.240**	**0.008**
	0.03125	211.5	37.4	-6.6	12.8	0.0121	0.0014	57.2	**0.239**	**0.009**
	0.01563	213.3	38.7	-4.8	9.7	0.0120	0.0014	57.7	**0.233**	**0.009**
	0	218.1	42.8	0.0	0.0	0.0124	0.0019	55.9	0.210	0.012
Kp-1	16	**1,436.1**	**0.0**	**1,316.4**	**10.1**	**0.0000**	**0.0000**		**0.005**	**0.006**
	8	**1,436.1**	**0.0**	**1,316.4**	**10.1**	**0.0000**	**0.0000**		**0.005**	**0.004**
	4	**535.9**	**115.9**	**414.9**	**120.4**	0.0204	0.0030	34.0	0.236	0.009
	0	119.7	10.1	0.0	0.0	0.0184	0.0022	37.7	0.263	0.018
Kp-7	32	**1,436.1**	**0.0**	**1,299.3**	**16.8**	**0.0000**	**0.0000**		**0.005**	**0.005**
	16	**1,436.1**	**0.0**	**1,299.3**	**16.8**	**0.0000**	**0.0000**		**0.004**	**0.005**
	8	**1,168.6**	**337.8**	**1,031.7**	**333.4**	**0.0061**	**0.0074**	114.0	**0.074**	**0.081**
	4	**314.1**	**144.1**	**177.7**	**136.6**	**0.0121**	**0.0018**	57.1	**0.195**	**0.017**
	0	136.8	16.8	0.0	0.0	0.0089	0.0011	78.3	0.245	0.014
Kp-13	16	**1,436.1**	**0.0**	**1,316.8**	**6.3**	**0.0000**	**0.0000**		**0.005**	**0.005**
	8	**1,436.1**	**0.0**	**1,316.8**	**6.3**	**0.0000**	**0.0000**		**0.002**	**0.002**
	4	**1,436.1**	**0.0**	**1,316.8**	**6.3**	**0.0000**	**0.0000**		**0.002**	**0.002**
	2	**171.8**	**64.3**	**52.5**	**65.0**	0.0174	0.0019	39.8	0.288	0.023
	0	119.3	6.3	0.0	0.0	0.0167	0.0011	41.4	0.308	0.024

Table 3-9 – *Continued*.

Strain	CHX (µg/mL)	Lag phase duration (min) Mean	Lag phase duration (min) SD	Lag phase delay (min) Mean	Lag phase delay (min) SD	Growth rate µ (min^{-1}) Mean	Growth rate µ (min^{-1}) SD	Doubling time $t_{1/2}$ (min)	OD_{600nm} max Mean	OD_{600nm} max SD
Kp-16	8	**1,436.1**	**0.0**	**1,311.3**	**6.6**	**0.0000**	**0.0000**		**0.002**	**0.002**
	4	**1,436.1**	**0.0**	**1,308.7**	**4.5**	**0.0000**	**0.0000**		**0.001**	**0.001**
	0.0625	125.0	6.8	0.2	0.5	0.0172	0.0030	40.3	**0.300**	**0.014**
	0.03125	125.2	7.0	0.4	1.1	0.0173	0.0028	40.1	**0.296**	**0.008**
	0	124.8	6.6	0.0	0.0	0.0175	0.0028	39.5	0.279	0.004
Kp-18	8	**1,436.1**	**0.0**	**1,329.2**	**5.6**	**0.0000**	**0.0000**		**0.001**	**0.002**
	4	**1,436.1**	**0.0**	**1,328.9**	**5.2**	**0.0000**	**0.0000**		**0.002**	**0.003**
	2	**139.3**	**36.9**	**32.3**	**34.5**	0.0137	0.0018	50.7	0.260	0.013
	0.03125	106.9	5.6	0.0	0.0	0.0131	0.0011	52.9	**0.288**	**0.009**
	0	106.9	5.6	0.0	0.0	0.0137	0.0010	50.7	0.274	0.012
Kp-19	8	**1,436.1**	**0.0**	**1,329.6**	**9.5**	**0.0000**	**0.0000**		**0.002**	**0.003**
	4	**1,436.1**	**0.0**	**1,329.6**	**9.5**	**0.0000**	**0.0000**		**0.003**	**0.002**
	2	**162.7**	**76.3**	**56.2**	**70.4**	0.0122	0.0022	57.0	0.246	0.016
	0	106.5	9.5	0.0	0.0	0.0114	0.0017	60.7	0.247	0.012
Kp-26	16	**1,436.1**	**0.0**	**1,329.9**	**4.7**	**0.0000**	**0.0000**		**0.005**	**0.009**
	8	**1,436.1**	**0.0**	**1,329.9**	**4.7**	**0.0000**	**0.0000**		**0.001**	**0.002**
	4	**585.0**	**64.5**	**477.7**	**61.9**	0.0155	0.0011	44.9	**0.244**	**0.013**
	0	106.2	4.7	0.0	0.0	0.0152	0.0012	45.5	0.297	0.012
Kp-27	16	**1,436.1**	**0.0**	**1,306.4**	**10.2**	**0.0000**	**0.0000**		**0.003**	**0.005**
	8	**1,436.1**	**0.0**	**1,306.4**	**10.2**	**0.0000**	**0.0000**		**0.002**	**0.003**
	4	**459.5**	**224.6**	**330.2**	**227.1**	0.0163	0.0017	42.5	**0.245**	**0.008**
	0	129.7	10.2	0.0	0.0	0.0158	0.0011	43.8	0.281	0.007
Kp-28	16	**1,436.1**	**0.0**	**1,296.0**	**32.6**	**0.0000**	**0.0000**		**0.004**	**0.005**
	8	**1,436.1**	**0.0**	**1,296.0**	**32.6**	**0.0000**	**0.0000**		**0.001**	**0.001**
	4	**492.5**	**299.6**	**352.4**	**267.0**	**0.0206**	**0.0014**	33.6	**0.228**	**0.010**
	2	140.1	32.6	0.0	0.0	0.0171	0.0009	40.6	**0.280**	**0.005**
	0.0625	140.1	32.6	0.0	0.0	0.0158	0.0001	43.9	**0.303**	**0.002**
	0	140.1	32.6	0.0	0.0	0.0163	0.0004	42.5	0.291	0.001
Kp-30	16	**1,436.1**	**0.0**	**1,311.1**	**10.8**	**0.0000**	**0.0000**		**0.002**	**0.002**
	8	**1,436.1**	**0.0**	**1,311.1**	**10.8**	**0.0000**	**0.0000**		**0.003**	**0.003**
	4	**1,436.1**	**0.0**	**1,311.0**	**11.0**	**0.0000**	**0.0000**		**0.003**	**0.001**
	2	**181.0**	**54.0**	**56.0**	**56.1**	0.0123	0.0006	56.4	0.273	0.016
	1	125.3	10.7	0.3	0.6	**0.0108**	**0.0006**	64.2	**0.280**	**0.012**
	0.5	125.0	10.8	0.0	0.0	**0.0105**	**0.0006**	65.8	**0.285**	**0.011**
	0.25	125.0	10.8	0.0	0.0	**0.0105**	**0.0007**	66.2	**0.286**	**0.011**
	0.125	125.0	10.8	0.0	0.0	**0.0106**	**0.0007**	65.2	**0.284**	**0.011**
	0.0625	125.0	10.8	0.0	0.0	**0.0112**	**0.0011**	61.9	0.277	0.011
	0	125.0	10.8	0.0	0.0	0.0129	0.0016	53.9	0.261	0.007

Table 3-9 – *Continued*.

Strain	CHX (µg/mL)	Lag phase duration (min) Mean	SD	Lag phase delay (min) Mean	SD	Growth rate µ (min^{-1}) Mean	SD	Doubling time $t_{1/2}$ (min)	OD_{600nm} max Mean	SD
Kp-45	16	**1,436.1**	**0.0**	**1,334.3**	**15.8**	**0.0000**	**0.0000**		**0.001**	**0.002**
	8	**1,436.1**	**0.0**	**1,334.3**	**15.8**	**0.0000**	**0.0000**		**0.001**	**0.002**
	4	**1,137.2**	**409.4**	**1,035.4**	**418.3**	**0.0064**	**0.0090**	107.7	**0.097**	**0.132**
	0	101.8	15.8	0.0	0.0	0.0181	0.0014	38.4	0.300	0.010
Kp-51	16	**1,436.1**	**0.0**	**1,326.4**	**13.9**	**0.0000**	**0.0000**		**0.003**	**0.004**
	8	**1,436.1**	**0.0**	**1,326.4**	**13.9**	**0.0000**	**0.0000**		**0.002**	**0.002**
	4	**891.4**	**499.6**	**781.6**	**503.0**	**0.0121**	**0.0096**	57.2	**0.142**	**0.115**
	0	109.7	13.9	0.0	0.0	0.0209	0.0008	33.2	0.288	0.008
Kp-58	16	**1,436.1**	**0.0**	**1,311.0**	**12.6**	**0.0000**	**0.0000**		**0.002**	**0.001**
	8	**1,436.1**	**0.0**	**1,311.0**	**12.6**	**0.0000**	**0.0000**		**0.001**	**0.002**
	4	**388.7**	**63.5**	**263.7**	**71.5**	0.0127	0.0015	54.8	**0.165**	**0.004**
	0	125.1	12.6	0.0	0.0	0.0122	0.0012	56.9	0.237	0.008
Kp-61	8	**1,436.1**	**0.0**	**1,311.3**	**20.4**	**0.0000**	**0.0000**		**0.000**	**0.000**
	4	**1,436.1**	**0.0**	**1,313.3**	**24.4**	**0.0000**	**0.0000**		**0.003**	**0.001**
	2	**166.4**	**30.3**	**41.7**	**19.4**	0.0133	0.0009	52.1	0.259	0.017
	0	124.8	20.4	0.0	0.0	0.0140	0.0011	49.7	0.279	0.018
Kp-63	16	**1,436.1**	**0.0**	**1,265.0**	**27.9**	**0.0000**	**0.0000**		**0.001**	**0.001**
	8	**1,436.1**	**0.0**	**1,265.0**	**27.9**	**0.0000**	**0.0000**		**0.003**	**0.004**
	4	**632.0**	**101.7**	**445.3**	**111.2**	0.0118	0.0010	58.5	0.185	0.008
	0	171.1	27.9	0.0	0.0	0.0101	0.0016	68.4	0.210	0.014
Kp-69	16	**1,436.1**	**0.0**	**1,308.6**	**18.7**	**0.0000**	**0.0000**		**0.003**	**0.003**
	8	**1,436.1**	**0.0**	**1,308.6**	**18.7**	**0.0000**	**0.0000**		**0.003**	**0.002**
	4	**825.4**	**247.2**	**694.7**	**257.4**	0.0178	0.0021	38.9	**0.220**	**0.011**
	0	127.5	18.7	0.0	0.0	0.0162	0.0013	42.8	0.270	0.005
Kp-79	8	**1,436.1**	**0.0**	**1,312.9**	**10.8**	**0.0000**	**0.0000**		**0.002**	**0.001**
	4	**1,436.1**	**0.0**	**1,312.0**	**11.6**	**0.0000**	**0.0000**		**0.001**	**0.000**
	2	127.6	4.3	4.4	6.6	0.0151	0.0002	45.9	**0.251**	**0.006**
	0.03125	123.2	10.8	0.0	0.0	0.0142	0.0009	49.0	**0.283**	**0.006**
	0	123.2	10.8	0.0	0.0	0.0153	0.0006	45.4	0.271	0.007
Kp-88	8	**1,436.1**	**0.0**	**1,319.3**	**19.6**	**0.0000**	**0.0000**		**0.001**	**0.001**
	4	**1,436.1**	**0.0**	**1,319.3**	**19.6**	**0.0000**	**0.0000**		**0.003**	**0.005**
	0.125	116.8	19.6	0.0	0.0	0.0182	0.0019	38.0	**0.279**	**0.005**
	0.0625	116.8	19.6	0.0	0.0	0.0181	0.0014	38.3	**0.281**	**0.004**
	0.03125	116.8	19.6	0.0	0.0	0.0183	0.0010	37.9	**0.280**	**0.005**
	0	116.8	19.6	0.0	0.0	0.0182	0.0018	38.0	0.269	0.004
Kp-94	16	**1,436.1**	**0.0**	**1,321.9**	**9.0**	**0.0000**	**0.0000**		**0.004**	**0.005**
	8	**1,436.1**	**0.0**	**1,321.9**	**9.0**	**0.0000**	**0.0000**		**0.003**	**0.002**
	4	**917.4**	**479.0**	**802.4**	**483.6**	**0.0074**	**0.0068**	93.6	**0.180**	**0.157**
	0	114.2	9.0	0.0	0.0	0.0115	0.0011	60.3	0.323	0.017

Table 3-9 – *Continued*.

Strain	CHX (µg/mL)	Lag phase duration (min) Mean	SD	Lag phase delay (min) Mean	SD	Growth rate µ (min^{-1}) Mean	SD	Doubling time $t_{1/2}$ (min)	OD_{600nm} max Mean	SD
Kp-96	16	**1,436.1**	**0.0**	**1,309.1**	**10.7**	**0.0000**	**0.0000**		**0.003**	**0.003**
	8	**1,436.1**	**0.0**	**1,307.1**	**11.0**	**0.0000**	**0.0000**		**0.006**	**0.007**
	4	**373.3**	**99.7**	**246.2**	**101.8**	**0.0139**	**0.0022**	49.9	**0.193**	**0.011**
	0	127.0	10.7	0.0	0.0	0.0102	0.0015	68.0	0.240	0.015
Kp-99	16	**1,436.1**	**0.0**	**1,308.8**	**10.3**	**0.0000**	**0.0000**		**0.006**	**0.005**
	8	**1,129.3**	**184.0**	**1,001.7**	**189.4**	0.0100	0.0060	69.4	**0.098**	**0.060**
	0	127.3	10.3	0.0	0.0	0.0113	0.0012	61.6	0.225	0.017
Kp-101	8	**1,436.1**	**0.0**	**1,305.3**	**2.3**	**0.0000**	**0.0000**		**0.002**	**0.003**
	4	**437.4**	**28.8**	**306.7**	**31.1**	0.0157	0.0027	44.1	**0.196**	**0.009**
	0	130.8	2.3	0.0	0.0	0.0126	0.0017	54.9	0.240	0.007
Kp-106	32	**1,436.1**	**0.0**	**1,319.8**	**6.1**	**0.0000**	**0.0000**		**0.016**	**0.006**
	16	**1,436.1**	**0.0**	**1,319.8**	**6.1**	**0.0000**	**0.0000**		**0.009**	**0.006**
	8	**1,220.3**	**256.5**	**1,104.0**	**259.8**	**0.0045**	**0.0049**	154.5	**0.102**	**0.106**
	4	**303.0**	**7.4**	**186.7**	**9.4**	0.0143	0.0009	48.3	0.251	0.005
	0	116.3	6.1	0.0	0.0	0.0118	0.0004	58.6	0.299	0.005
Kp-107	16	**1,436.1**	**0.0**	**1,322.4**	**0.8**	**0.0000**	**0.0000**		**0.008**	**0.009**
	8	**1,436.1**	**0.0**	**1,320.6**	**3.9**	**0.0000**	**0.0000**		**0.006**	**0.002**
	4	**333.7**	**62.9**	**220.0**	**63.3**	**0.0148**	**0.0028**	46.7	**0.165**	**0.019**
	0	113.7	0.8	0.0	0.0	0.0087	0.0011	79.4	0.103	0.014
Kp-109	8	**1,436.1**	**0.0**	**1,336.1**	**5.1**	**0.0000**	**0.0000**		**0.022**	**0.032**
	4	**1,436.1**	**0.0**	**1,335.0**	**5.7**	**0.0000**	**0.0000**		**0.020**	**0.029**
	2	120.0	24.1	**20.0**	**21.5**	0.0119	0.0024	58.2	0.284	0.010
	0	100.0	5.1	0.0	0.0	0.0119	0.0011	58.1	0.291	0.008
Kp-112	16	**1,436.1**	**0.0**	**1,324.6**	**6.2**	**0.0000**	**0.0000**		**0.006**	**0.006**
	8	**1,436.1**	**0.0**	**1,324.6**	**6.2**	**0.0000**	**0.0000**		**0.005**	**0.001**
	4	**458.6**	**52.3**	**347.1**	**50.3**	**0.0172**	**0.0009**	40.3	**0.226**	**0.005**
	0	111.5	6.2	0.0	0.0	0.0124	0.0018	55.8	0.271	0.003
Kp-114	16	**1,436.1**	**0.0**	**1,316.7**	**16.1**	**0.0000**	**0.0000**		**0.007**	**0.006**
	8	**1,436.1**	**0.0**	**1,316.7**	**16.1**	**0.0000**	**0.0000**		**0.006**	**0.003**
	4	**490.2**	**18.7**	**370.7**	**18.2**	**0.0192**	**0.0009**	36.1	**0.234**	**0.009**
	0	119.4	16.1	0.0	0.0	0.0122	0.0010	56.6	0.264	0.004
Kp-115	16	**1,436.1**	**0.0**	**1,336.4**	**6.3**	**0.0000**	**0.0000**		**0.003**	**0.003**
	8	**1,436.1**	**0.0**	**1,336.4**	**6.3**	**0.0000**	**0.0000**		**0.004**	**0.001**
	4	**1,436.1**	**0.0**	**1,336.4**	**6.3**	**0.0000**	**0.0000**		**0.008**	**0.002**
	2	**122.8**	**14.1**	**23.1**	**20.2**	0.0141	0.0019	49.2	0.269	0.012
	0	99.7	6.3	0.0	0.0	0.0127	0.0006	54.6	0.275	0.002

Table 3-9 – *Continued*.

Strain	CHX (µg/mL)	Lag phase duration (min) Mean	SD	Lag phase delay (min) Mean	SD	Growth rate µ (min^{-1}) Mean	SD	Doubling time $t_{1/2}$ (min)	OD_{600nm} max Mean	SD
Kp-125 (1st phase)	16	**1,436.1**	**0.0**	**1,283.9**	**18.6**	**0.0000**	**0.0000**		**0.006**	**0.008**
	8	**1,436.1**	**0.0**	**1,283.9**	**18.6**	**0.0000**	**0.0000**		**0.001**	**0.002**
	4	**322.3**	**20.6**	**170.0**	**35.5**	**0.0055**		127.1	**0.030**	**0.009**
	2	160.2	32.9	8.0	19.6	0.0136	0.0015	51.0	**0.071**	**0.006**
	1	152.2	18.6	0.0	0.0	0.0139	0.0015	49.9	**0.080**	**0.005**
	0	152.2	18.6	0.0	0.0	0.0127	0.0020	54.6	0.094	0.008
Kp-125 (2nd phase)	16	**1,436.1**	**0.0**	**1,103.8**	**6.3**	**0.0000**	**0.0000**		**0.007**	**0.009**
	8	**1,436.1**	**0.0**	**1,103.8**	**6.3**	**0.0000**	**0.0000**		**0.001**	**0.002**
	4	**624.3**	**88.5**	**292.0**	**92.4**	**0.0145**	**0.0020**	47.8	**0.146**	**0.012**
	2	**396.3**	**12.1**	**64.0**	**7.2**	0.0102	0.0014	67.8	**0.166**	**0.012**
	1	337.6	3.2	5.3	6.5	0.0107	0.0013	64.5	**0.182**	**0.007**
	0	332.3	6.3	0.0	0.0	0.0102	0.0004	68.1	0.201	0.008
Kp-126	16	**1,436.1**	**0.0**	**1,289.5**	**20.5**	**0.0000**	**0.0000**		**0.002**	**0.003**
	8	**1,436.1**	**0.0**	**1,294.0**	**19.5**	**0.0000**	**0.0000**		**0.004**	**0.004**
	4	**404.6**	**35.0**	**258.0**	**23.7**	**0.0153**	**0.0017**	45.4	**0.220**	**0.005**
	0	146.6	20.5	0.0	0.0	0.0123	0.0003	56.5	0.278	0.012
Kp-136	8	**1,436.1**	**0.0**	**1,334.3**	**4.0**	**0.0000**	**0.0000**		**0.030**	**0.030**
	4	**1,436.1**	**0.0**	**1,334.3**	**4.0**	**0.0000**	**0.0000**		**0.025**	**0.022**
	2	**113.8**	**17.3**	**12.0**	**13.9**	0.0188	0.0046	36.9	0.269	0.025
	0	101.8	4.0	0.0	0.0	0.0201	0.0019	34.5	0.278	0.008
Kp-147	16	**1,436.1**	**0.0**	**1,326.5**	**3.0**	**0.0000**	**0.0000**		**0.004**	**0.003**
	8	**1,436.1**	**0.0**	**1,326.5**	**3.0**	**0.0000**	**0.0000**		**0.003**	**0.002**
	4	**335.4**	**14.7**	**225.8**	**12.0**	**0.0155**	**0.0022**	44.9	**0.246**	**0.002**
	0	109.6	3.0	0.0	0.0	0.0127	0.0015	54.8	0.327	0.003
Kp-149	32	**1,436.1**	**0.0**	**1,331.8**	**6.0**	**0.0000**	**0.0000**		**0.005**	**0.008**
	16	**1,436.1**	**0.0**	**1,331.8**	**6.0**	**0.0000**	**0.0000**		**0.005**	**0.008**
	8	**1,436.1**	**0.0**	**1,331.0**	**6.3**	**0.0000**	**0.0000**		**0.001**	**0.001**
	4	**219.0**	**23.5**	**114.7**	**22.7**	**0.0192**	**0.0035**	36.0	**0.226**	**0.013**
	2	104.3	6.0	0.0	0.0	0.0170	0.0011	40.8	**0.261**	**0.006**
	1	104.3	6.0	0.0	0.0	0.0170	0.0011	40.8	**0.268**	**0.008**
	0	104.3	6.0	0.0	0.0	0.0160	0.0010	43.3	0.287	0.005
Kp-155	16	**1,436.1**	**0.0**	**1,296.2**	**12.7**	**0.0000**	**0.0000**		**0.000**	**0.000**
	8	**1,436.1**	**0.0**	**1,296.2**	**12.7**	**0.0000**	**0.0000**		**0.003**	**0.002**
	4	**397.2**	**85.3**	**257.3**	**86.1**	**0.0153**	**0.0020**	45.4	**0.167**	**0.007**
	2	147.9	20.6	8.0	9.8	0.0125	0.0011	55.5	**0.197**	**0.006**
	0.0625	139.9	12.7	0.0	0.0	0.0110	0.0008	63.1	**0.219**	**0.005**
	0	139.9	12.7	0.0	0.0	0.0111	0.0005	62.7	0.210	0.003

3.3.2.2.1 Differences in MICs between assays

The CHX MICs obtained with the Bioscreen assay are summarised in Table 3-10, alongside the values previously determined in 96-well plates. Significant differences between both methods (Sidak's post-test; $p<0.05$) were observed for *K. pneumoniae* ATCC 13883 (fold-change: 2.8), Kp-7 (1.5), Kp-26 (2.0), Kp-63 (2.0), Kp-69 (2.0), Kp-99 (1.8) and Kp-101 (2.0), with higher MICs obtained with the Bioscreen assay.

Table 3-10. Differences between CHX MICs obtained with the 96-well plate and the Bioscreen assays for the *K. pneumoniae* reference strain and clinical isolates.

MICs were determined at least in triplicates ($n\geq3$). Significant differences (two-way ANOVA followed by Sidak's post-test; $p<0.05$) are indicated in bold and red.

Strain	96-well plate assay CHX MIC µg/mL		Bioscreen assay CHX MIC µg/mL	
	Mean	SD	Mean	SD
***K. pneumoniae* ATCC 13883**	**1.42**	**0.41**	**4.00**	**0.00**
Kp-1	8.00	0.00	8.00	0.00
Kp-7	**8.00**	**0.00**	**12.36**	**4.18**
Kp-13	6.67	2.31	4.00	0.00
Kp-16	4.00	0.00	4.00	0.00
Kp-18	4.00	0.00	4.00	0.00
Kp-19	2.67	1.15	4.00	0.00
Kp-20	4.00	0.00	4.00	0.00
Kp-22	3.33	1.15	4.00	0.00
Kp-26	**4.00**	**0.00**	**8.00**	**0.00**
Kp-27	8.00	0.00	8.00	0.00
Kp-28	8.00	0.00	8.00	0.00
Kp-30	3.33	1.15	4.00	0.00
Kp-45	6.67	2.31	5.60	2.19
Kp-51	5.33	2.31	6.67	2.07
Kp-58	8.00	0.00	8.00	0.00
Kp-61	2.00	0.00	4.00	0.00
Kp-63	**4.00**	**0.00**	**8.00**	**0.00**
Kp-69	**4.00**	**0.00**	**8.00**	**0.00**
Kp-79	2.00	0.00	4.00	0.00
Kp-85	2.00	0.00	4.00	0.00
Kp-88	2.67	1.15	4.00	0.00
Kp-94	4.00	0.00	6.40	2.19
Kp-96	8.00	0.00	8.00	0.00

Table 3-10 – *Continued*.

Strain	96-well plate assay CHX MIC µg/mL Mean	SD	Bioscreen assay CHX MIC µg/mL Mean	SD
Kp-99	**8.00**	**0.00**	**14.40**	**3.58**
Kp-101	**4.00**	**0.00**	**8.00**	**0.00**
Kp-106	10.67	4.62	12.00	4.38
Kp-107	8.00	0.00	8.00	0.00
Kp-109	3.33	1.15	4.00	0.00
Kp-112	6.67	2.31	8.00	0.00
Kp-114	6.67	2.31	8.00	0.00
Kp-115	4.00	0.00	4.00	0.00
Kp-118	2.00	0.00	2.00	0.00
Kp-125	8.00	0.00	8.00	0.00
Kp-126	8.00	0.00	8.00	0.00
Kp-136	4.00	0.00	4.00	0.00
Kp-147	8.00	0.00	8.00	0.00
Kp-149	8.00	0.00	8.00	0.00
Kp-155	8.00	0.00	8.00	0.00

3.3.2.2.2 Impact on the maximum OD_{600nm}

The detailed results obtained for each isolate are grouped in appendix VIII, and statistically significant results presented in Table 3-9 (page 83). The highest OD_{600nm} measured during the growth was recorded and reported as OD_{600nm} max for the 38 selected clinical isolates and the reference strain. For all the strains, the OD_{600nm} max was obtained at the beginning of the plateau phase. Within each strain, the OD_{600nm} max values obtained when exposed to each tested concentration were compared to the control condition, i.e. the strain growing in R2A only (0 µg/mL CHX). All *K. pneumoniae* isolates reached OD_{600nm} max ranging from 0.200 to 0.340 approximately, except Kp-107 and Kp-125 (first growth phase only) for which the OD_{600nm} max was lower (approximately 0.100).

For 12/39 isolates (Kp-1, Kp-13, Kp-19, Kp-20, Kp-22, Kp-61, Kp-63, Kp-85, Kp-109, Kp-115, Kp-118 and Kp-136), the OD_{600nm} max was not impacted by the CHX concentrations they were exposed to (one-way ANOVA followed by Dunnett's test; $p>0.05$), except for concentrations corresponding to their MIC or above.

Regarding the "variable-growth strains", a significant decrease in OD_{600nm} max was observed at the concentration to which variability in growth was observed (Dunnett's test;

$p<0.05$). For Kp-26, Kp-27, Kp-58, Kp-69, Kp-79, Kp-96, Kp-101, Kp-112, Kp-114, Kp-126, Kp-147 and Kp-7, exposure to CHX concentrations corresponding to half their MIC significantly decreased their OD_{600nm} max. This observation was extended to 1/4 and 1/8 of their MICs as well for Kp-125 (for both phases) and Kp-149. On the contrary, the OD_{600nm} max observed when exposed to half its MIC significantly increased for Kp-107.

Regarding the other isolates, there seemed to be a tendency where the OD_{600nm} max measured in CHX-free media was significantly lower than when the bacteria were growing in CHX-containing R2A. The reference strain, *K. pneumoniae* ATCC 13883, harboured a significantly higher OD_{600nm} max when growing in R2A containing CHX at concentrations below half its MIC (2 μg/mL) when compared to CHX-free media. A similar phenomenon was observed for Kp-30, and, to a lesser extent, for Kp-16, Kp-18, Kp-28, Kp-79, Kp-88 and Kp-155.

3.3.2.2.3 Impact on the lag phase duration

The detailed results obtained for each strain are grouped in appendix VIII, and statistically significant differences in lag phase duration are highlighted in Table 3-9 (page 83). The lag phase durations measured for each *K. pneumoniae* isolate tested under different CHX exposure conditions (CHX concentrations expressed as a fraction of their CHX MIC) are presented in Figure 3-5. When no growth was observed, the lag phase duration was considered as equal to 1,436.1 min, corresponding to the last time point where the OD_{600nm} was measured. For each strain, the lag phase duration measured for each CHX concentration was compared to growth in the CHX-free R2A (0 μg/mL CHX).

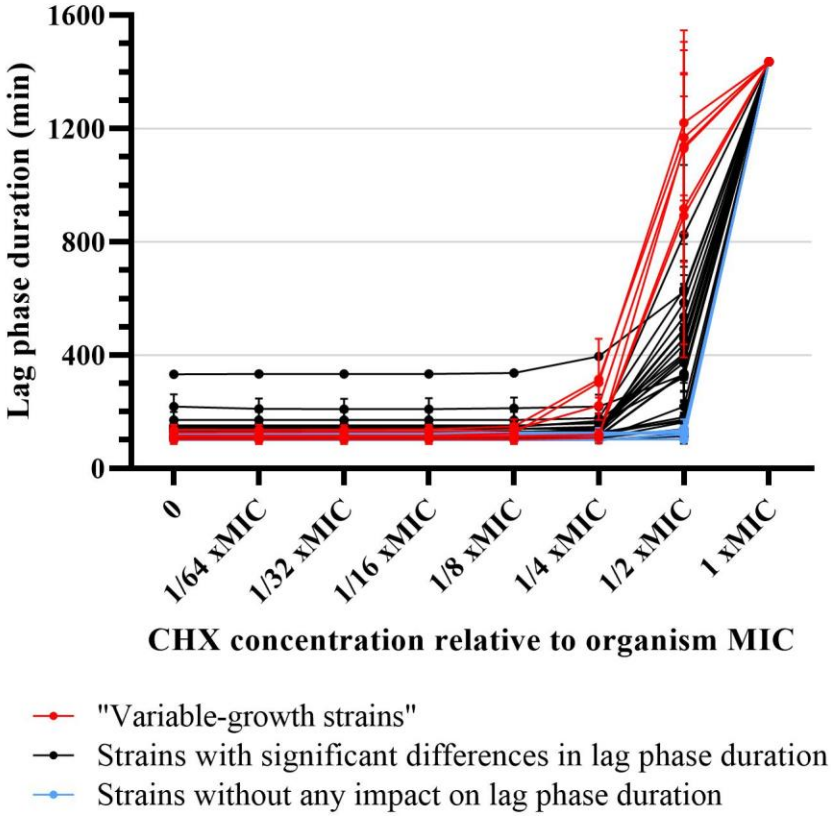

Figure 3-5. Changes in lag phase duration in *K. pneumoniae* isolates depending on CHX concentrations expressed as fractions of the organism's MIC.

Experiments were carried at least in triplicate (n≥3) and reported results correspond to mean values and standard deviations. *K. pneumoniae* isolates segregated into three different clusters: the "variable-growth strains" (red), for which variability in growth was observed at concentrations corresponding to approximately half of their MIC values; the strains for which significant changes in lag phase duration were obtained, mostly when exposed to concentrations corresponding to half their MICs (black); the strains for which no significant difference in lag phase duration was measured across the different CHX concentrations investigated (blue).

In the cases of Kp-16, Kp-20, Kp-22, Kp-79, Kp-85, Kp-88, Kp-109 and Kp-118 (blue isolates in Figure 3-5), the exposure to CHX did not have any impact on the lag phase duration (one-way ANOVA followed by Dunnett's post-test; $p>0.05$), except for concentrations corresponding to their MICs or above.

Regarding the "variable-growth strains" (red isolates in Figure 3-5), the lag phase duration was significantly extended (Dunnett's post-test; $p<0.05$) when exposed to the

concentration at which the variability in growth was observed (phenomenon observed as well at the following dilution for Kp-7 and Kp-106). For most strains (25/39; black isolates in Figure 3-5), including the reference strain *K. pneumoniae* ATCC 13883, the duration of the lag phase was significantly increased when exposed to concentrations corresponding to half their MICs, up to approximately 700 min delay when compared to CHX-free R2A for Kp-69. Regarding Kp-125, the lag phase was also significantly longer when exposed to a quarter of its MIC when considering the second growth phase only.

3.3.2.2.4 Impact on the growth rate

Growth rates measured for all the investigated *K. pneumoniae* isolates are presented in appendix VIII. For a majority of isolates (23/39), including the reference strain *K. pneumoniae* ATCC 13883, exposure to CHX concentrations lower than their MICs did not have any significant impact on their growth rate when compared to the CHX-free control (0 μg/mL CHX). Without CHX, the growth rates measured ranged from 0.0087 min^{-1} for Kp-7 (corresponding $t_{1/2}$: 79.4 min) to 0.0209 min^{-1} for Kp-51 ($t_{1/2}$: 33.2 min).

Regarding the "variable-growth strains", the growth rate when exposed to the CHX concentration at which variability was observed was significantly lower than in CHX-free R2A, with the exception of Kp-99 where no difference was observed. The results obtained for the following statistically significant differences observed are grouped in Table 3-9 (page 83). When exposed to half their MICs, Kp-7, Kp-28, Kp-96, Kp-107, Kp-112, Kp-114, Kp-125 (second growth phase), Kp-126, Kp-147, Kp-149 and Kp-155 harboured a significantly higher growth rate than in R2A only. However, it was significantly decreased when considering the first growth phase of Kp-125 (for which only one replicate was obtained). Kp-30 had a significantly higher growth rate when growing in CHX-free R2A than when exposed to CHX, except when exposed to half its MIC where there was no significant difference.

The comparisons between the growth rates measured for the clinical isolates and the reference strain *K. pneumoniae* ATCC 13883 are grouped in appendix IX; statistically significant differences observed are presented in Figure 3-6. When both a clinical strain and the reference strain were able to grow, there was no or almost no significant difference (Sidak's post-test; $p>0.05$) between their growth rates for 21/38 clinical strains. Regarding Kp-1, Kp-13, Kp-16, Kp-20, Kp-26, Kp-28, Kp-45, Kp-51, Kp-69, Kp-79, Kp-88, Kp-136 and Kp-149, these isolates harboured significantly higher growth rates than the reference strain when growing in CHX-containing R2A (Sidak's post-test; $p<0.05$). Few isolates

(Kp-7, Kp-63, Kp-96, Kp-107 and Kp-125 for its second growth phase only) displayed significantly lower growth rates than *K. pneumoniae* ATCC 13883 when growing in R2A containing different CHX concentrations (Sidak's post-test; $p<0.05$).

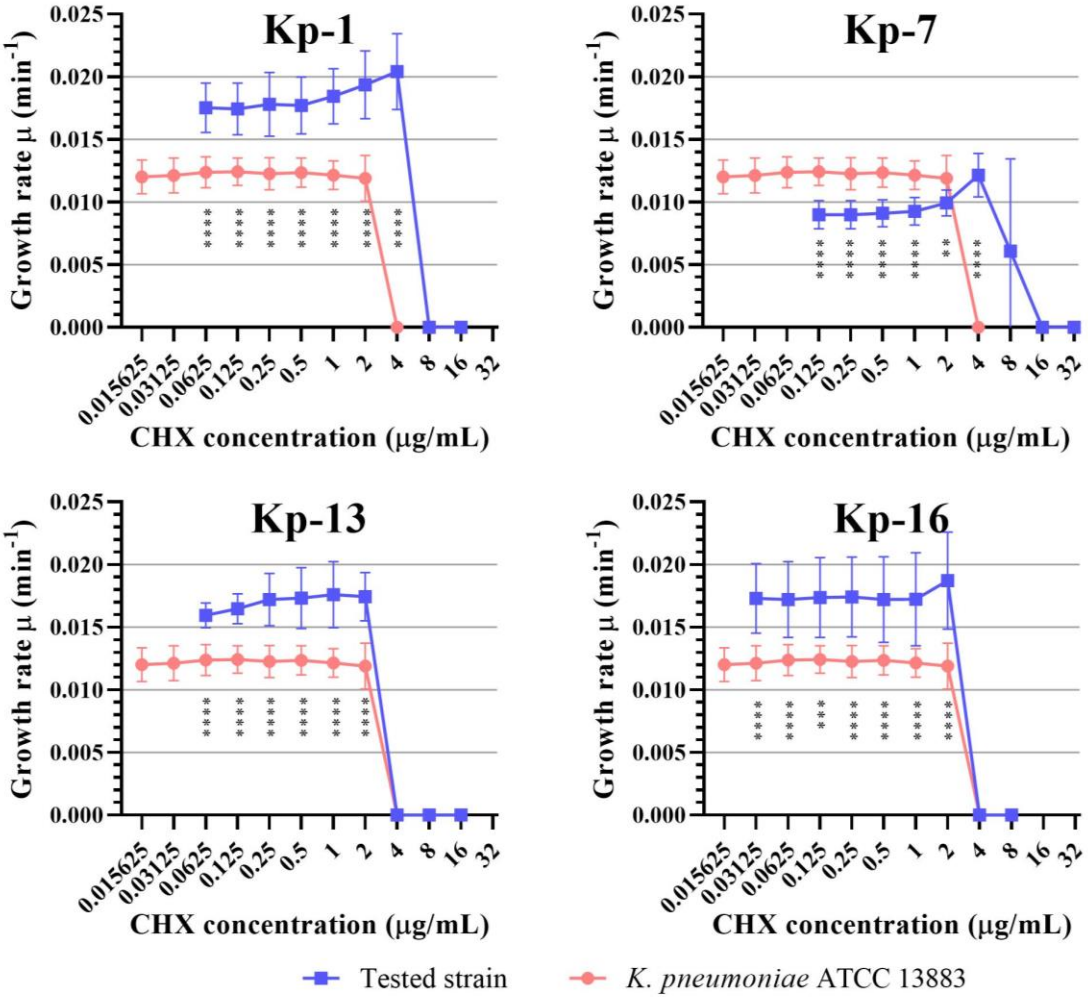

Figure 3-6. Comparison between the growth rates measured for the clinical isolates and the reference strain *K. pneumoniae* ATCC 13883 when exposed to different sub-inhibitory concentrations of CHX.

Experiments were carried at least in triplicates ($n\geq3$). Data points represent the mean of the different replicates and bars represent the corresponding SD. Only isolates presenting significant differences when compared to the reference strain (two-way ANOVA followed by Sidak's post-test) are displayed in this figure. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$.

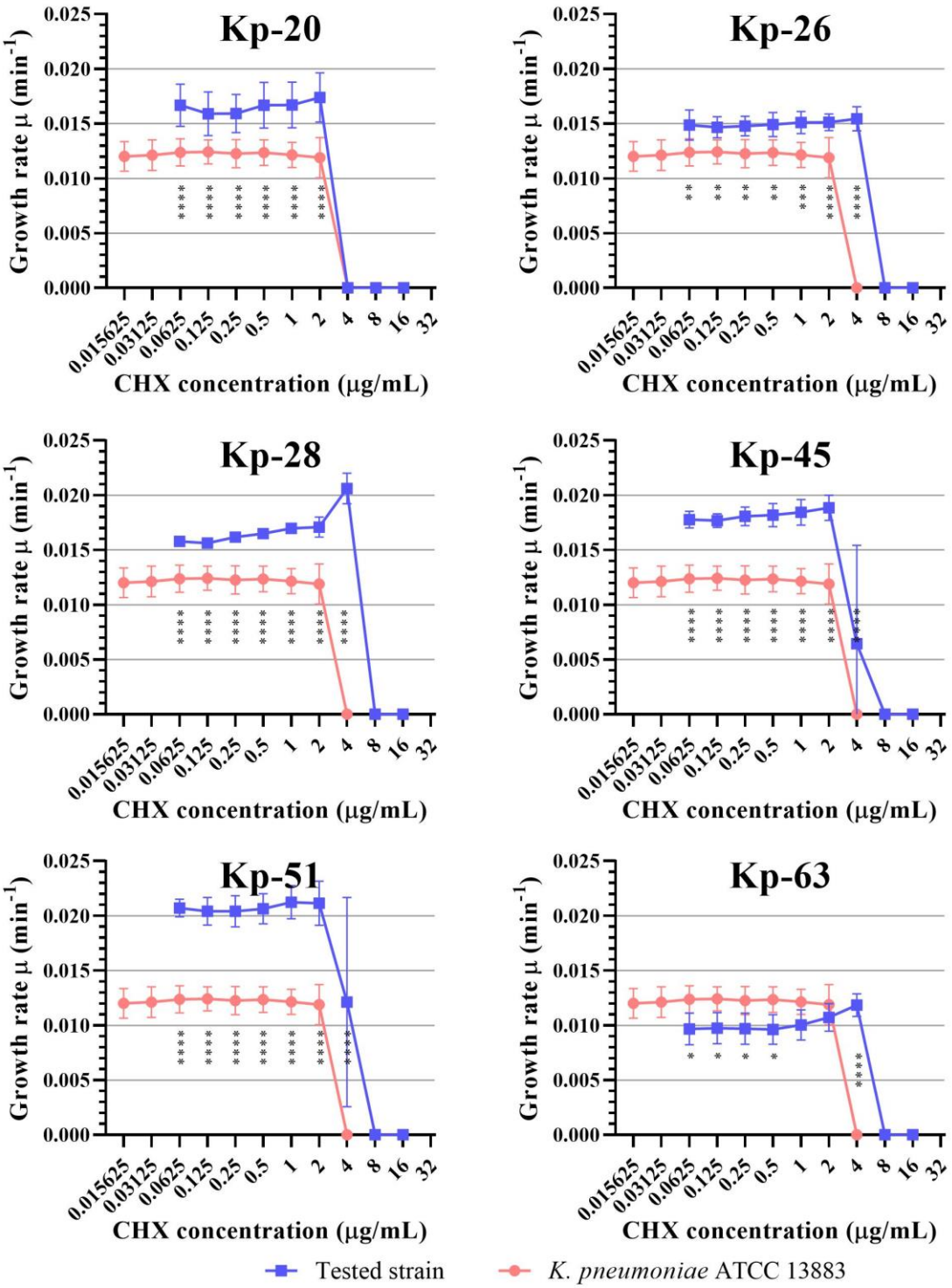

Figure 3-6 – *Continued*.

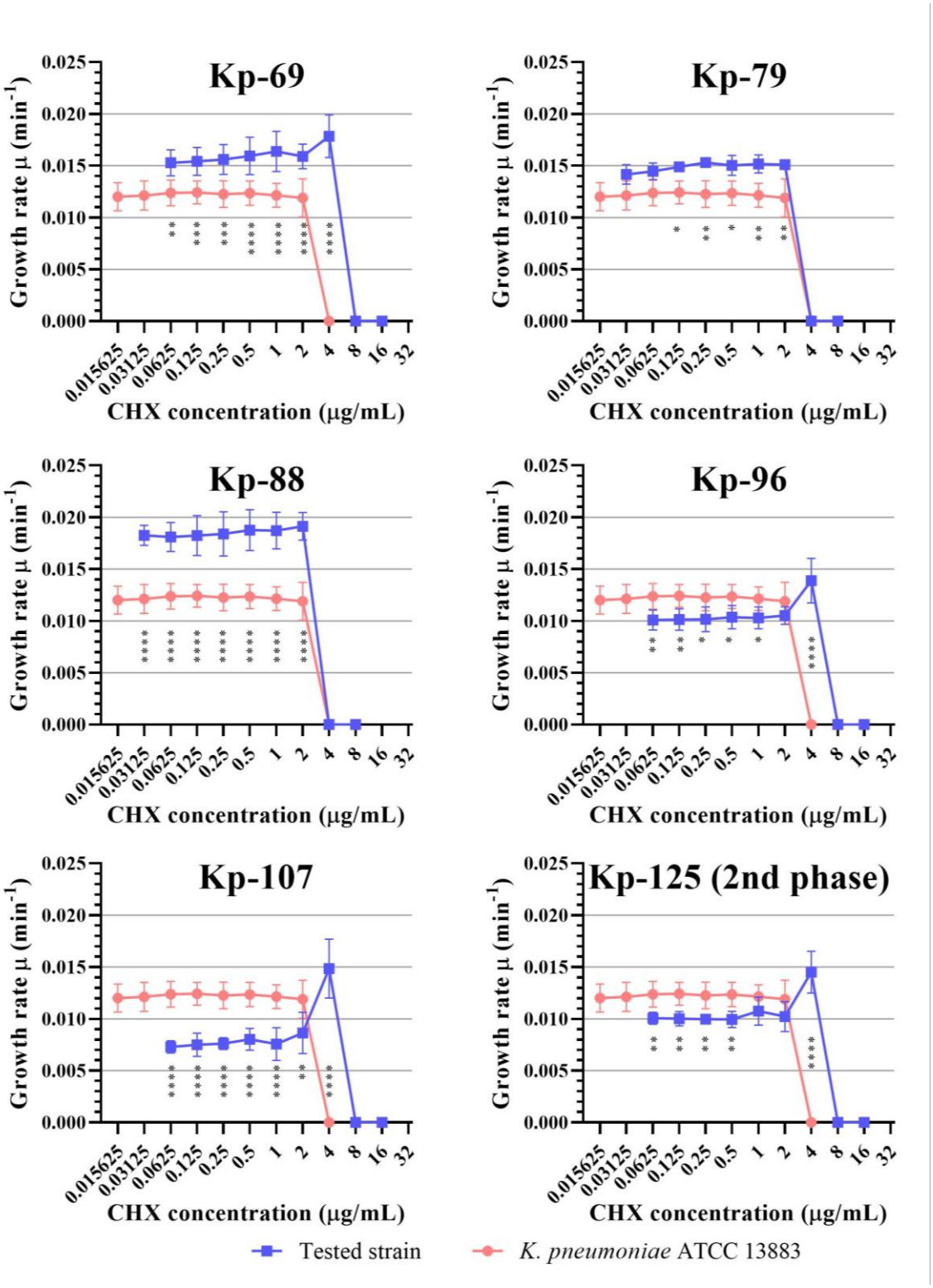

Figure 3-6 – *Continued.*

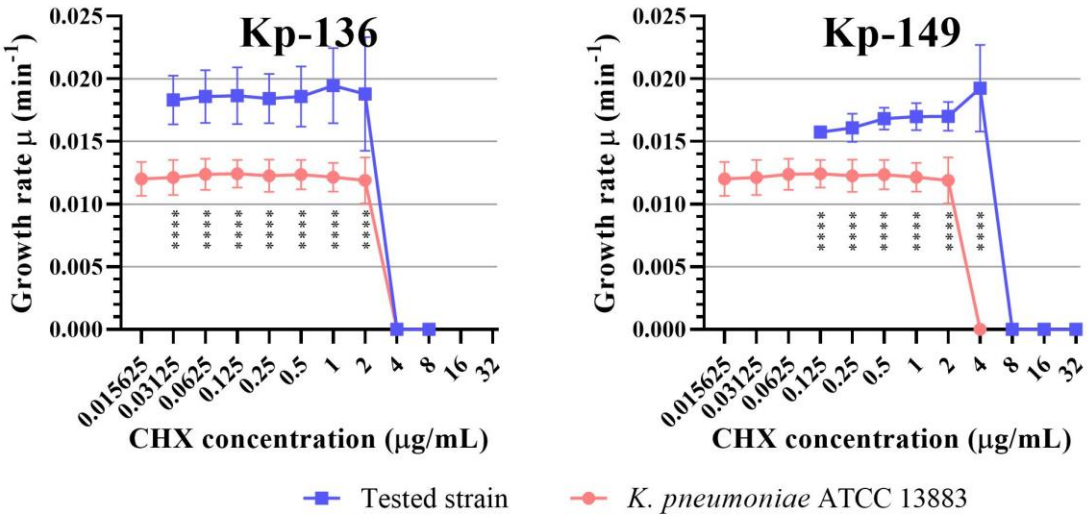

Figure 3-6 – *Continued*.

3.3.2.3 *Escherichia coli* isolates

The detailed results obtained for the *E. coli* reference strain and clinical isolates are presented in appendix X. Results obtained for strains harbouring statistically significant differences for any of the parameters investigated are highlighted in Table 3-11.

Table 3-11. Results obtained from the growth curves study for the *E. coli* isolates.

Experiments were carried at least in triplicate for each condition (n≥3). Values in bold and red corresponded to significant differences (one-way ANOVA followed by Dunnett's post-test; $p<0.05$) observed when compared to the CHX-free R2A growth condition (0 µg/mL CHX). Only isolates and concentrations at which significant differences for at least one of the investigated parameters are displayed, alongside with the control condition. For "variable-growth strains", the concentration at which variability in growth was observed was highlighted in orange. Statistical analyses were not performed on doubling times as they were inferred from the mean growth rate values. SD: standard deviation.

Strain	CHX (µg/mL)	Lag phase duration (min)		Lag phase delay (min)		Growth rate µ (min^{-1})		Doubling time $t_{1/2}$ (min)	OD_{600nm} max	
		Mean	SD	Mean	SD	Mean	SD		Mean	SD
Ec-158	8	**1,436.1**	**0.0**	**1,321.7**	**15.5**	**0.0000**	**0.0000**		**0.030**	**0.027**
	4	**1,436.1**	**0.0**	**1,321.7**	**15.5**	**0.0000**	**0.0000**		**0.027**	**0.025**
	2	147.8	51.7	**33.3**	**38.3**	0.0133	0.0028	52.1	0.222	0.022
	0	114.4	15.5	0.0	0.0	0.0140	0.0027	49.4	0.221	0.004
Ec-165	8	**1,436.1**	**0.0**	**1,321.7**	**9.0**	**0.0000**	**0.0000**		**0.019**	**0.021**
	4	**1,436.1**	**0.0**	**1,321.7**	**9.0**	**0.0000**	**0.0000**		**0.027**	**0.030**
	2	**133.8**	**28.3**	**19.3**	**21.5**	0.0144	0.0047	48.3	0.229	0.018
	0	114.4	9.0	0.0	0.0	0.0147	0.0025	47.0	0.238	0.011
Ec-180	8	**1,436.1**	**0.0**	**1,321.7**	**11.4**	**0.0000**	**0.0000**		**0.020**	**0.021**
	4	**1,436.1**	**0.0**	**1,321.7**	**11.4**	**0.0000**	**0.0000**		**0.028**	**0.028**
	2	**301.1**	**166.9**	**186.7**	**159.5**	0.0143	0.0017	48.4	0.211	0.015
	0	114.4	11.4	0.0	0.0	0.0134	0.0026	51.7	0.229	0.008
Ec-187	8	**1,436.1**	**0.0**	**1,326.3**	**4.0**	**0.0000**	**0.0000**		**0.002**	**0.002**
	4	**1,436.1**	**0.0**	**1,326.3**	**4.0**	**0.0000**	**0.0000**		**0.002**	**0.002**
	2	**1,436.1**	**0.0**	**1,326.3**	**4.0**	**0.0000**	**0.0000**		**0.006**	**0.003**
	1	109.8	4.0	0.0	0.0	**0.0145**	**0.0005**	48.0	0.219	0.004
	0	109.8	4.0	0.0	0.0	0.0129	0.0003	53.5	0.221	0.005
Ec-188	8	**1,436.1**	**0.0**	**1,306.3**	**4.0**	**0.0000**	**0.0000**		**0.004**	**0.002**
	4	**1,436.1**	**0.0**	**1,306.3**	**4.0**	**0.0000**	**0.0000**		**0.006**	**0.004**
	2	**143.1**	**2.3**	**13.3**	**2.3**	0.0130	0.0017	53.2	0.212	0.006
	0.25	129.8	4.0	0.0	0.0	0.0118	0.0017	58.8	**0.236**	**0.003**
	0.125	129.8	4.0	0.0	0.0	0.0115	0.0014	60.5	**0.237**	**0.003**
	0.0625	129.8	4.0	0.0	0.0	0.0116	0.0018	59.5	**0.234**	**0.005**
	0.03125	129.8	4.0	0.0	0.0	0.0121	0.0019	57.3	**0.232**	**0.006**
	0	129.8	4.0	0.0	0.0	0.0124	0.0014	55.9	0.218	0.010

Table 3-11 – *Continued*.

Strain	CHX (µg/mL)	Lag phase duration (min) Mean	SD	Lag phase delay (min) Mean	SD	Growth rate µ (min^{-1}) Mean	SD	Doubling time $t_{1/2}$ (min)	OD_{600nm} max Mean	SD
Ec-191	8	**1,436.1**	**0.0**	**1,315.7**	**6.1**	**0.0000**	**0.0000**		**0.005**	**0.006**
	4	**1,436.1**	**0.0**	**1,315.7**	**6.1**	**0.0000**	**0.0000**		**0.002**	**0.004**
	2	**1,436.1**	**0.0**	**1,315.7**	**6.1**	**0.0000**	**0.0000**		**0.011**	**0.002**
	0.5	120.4	6.1	0.0	0.0	0.0130	0.0016	53.1	**0.240**	**0.004**
	0.25	120.4	6.1	0.0	0.0	0.0129	0.0017	53.9	**0.245**	**0.002**
	0.125	120.4	6.1	0.0	0.0	0.0124	0.0017	56.1	**0.244**	**0.001**
	0.0625	120.4	6.1	0.0	0.0	0.0119	0.0017	58.2	**0.246**	**0.002**
	0.03125	120.4	6.1	0.0	0.0	0.0124	0.0017	56.1	**0.247**	**0.003**
	0	120.4	6.1	0.0	0.0	0.0143	0.0019	48.5	0.228	0.003
Ec-198	4	**1,436.1**	**0.0**	**1,330.6**	**10.3**	**0.0000**	**0.0000**		**0.004**	**0.003**
	2	**1,436.1**	**0.0**	**1,331.1**	**10.2**	**0.0000**	**0.0000**		**0.016**	**0.007**
	1	112.5	10.9	7.0	14.0	0.0123	0.0003	56.2	**0.201**	**0.007**
	0.125	105.5	10.3	0.0	0.0	0.0113	0.0014	61.3	**0.200**	**0.010**
	0	105.5	10.3	0.0	0.0	0.0111	0.0014	62.6	0.184	0.009
Ec-199	4	**1,436.1**	**0.0**	**1,327.3**	**5.5**	**0.0000**	**0.0000**		**0.006**	**0.003**
	2	**1,436.1**	**0.0**	**1,328.1**	**4.8**	**0.0000**	**0.0000**		**0.016**	**0.006**
	0.125	108.8	5.5	0.0	0.0	**0.0113**	**0.0008**	61.5	0.149	0.011
	0.0625	108.8	5.5	0.0	0.0	**0.0114**	**0.0011**	61.0	0.148	0.008
	0	108.8	5.5	0.0	0.0	0.0133	0.0014	52.0	0.157	0.012
Ec-207	16	**1,436.1**	**0.0**	**1,309.9**	**8.7**	**0.0000**	**0.0000**		**0.002**	**0.003**
	8	**1,436.1**	**0.0**	**1,309.9**	**8.7**	**0.0000**	**0.0000**		**0.001**	**0.002**
	4	**797.6**	**699.5**	**671.4**	**703.4**	**0.0077**	**0.0088**	89.8	**0.108**	**0.116**
	0	126.2	8.7	0.0	0.0	0.0140	0.0012	49.5	0.231	0.005

3.3.2.3.1 Differences in MICs between assays

CHX MICs determined with the Bioscreen assay and the previous 96-well plate determination are grouped in Table 3-12. Despite a tendency towards higher values with the Bioscreen assay, no significant differences between the MICs obtained with both methods were detected by Sidak's post-test ($p>0.05$).

Table 3-12. Differences between CHX MICs obtained with the 96-well plate and the Bioscreen assays for the *E. coli* reference strain and clinical isolates.

MICs were determined at least in triplicates (n≥3). No significant differences (two-way ANOVA followed by Sidak's post-test; p>0.05) were observed between both methods. SD: standard deviation.

Strain	96-well plate assay CHX MIC µg/mL		Bioscreen assay CHX MIC µg/mL	
	Mean	SD	Mean	SD
E. coli ATCC 25922	1.67	0.33	2.00	0.00
Ec-158	2.00	0.00	4.00	0.00
Ec-161	1.00	0.00	2.00	0.00
Ec-165	4.00	0.00	4.00	0.00
Ec-172	2.00	0.00	2.00	0.00
Ec-180	4.00	0.00	4.00	0.00
Ec-187	2.00	0.00	2.00	0.00
Ec-188	4.00	0.00	4.00	0.00
Ec-191	1.67	0.58	2.00	0.00
Ec-198	1.67	0.58	2.00	0.00
Ec-199	2.00	0.00	2.00	0.00
Ec-207	4.00	0.00	6.00	2.19

3.3.2.3.2 Impact on the maximum OD_{600nm}

The OD_{600nm} max values obtained for each strain and each concentration tested was compared to the control (0 µg/mL CHX) to see if exposure to CHX had any significant impact on this parameter. The detailed results obtained for each strain are grouped in appendix X; Table 3-11 (page 97) highlights strains for which statistically significant differences were determined. In CHX-free R2A, OD_{600nm} max measured for the *E. coli* isolates ranged from 0.157 (Ec-199) to 0.238 (Ec-165).

Exposure to sub-MIC concentrations of CHX did not have any significant impact (one-way ANOVA followed by Dunnett's post-test; p>0.05) on the OD_{600nm} max for 8/12 *E. coli* strains, including the reference strain *E. coli* ATCC 25922. A significant difference was observed for Ec-207, a "variable-growth strain", when exposed to the concentration at which variability in growth was observed (4 µg/mL). Ec-188, Ec-191 and Ec-198 harboured significantly higher OD_{600nm} max values when compared to the CHX-free control for some of the CHX concentrations tested, but never when exposed to half their MICs.

3.3.2.3.3 Impact on the lag phase duration and delay

The detailed results measured for each isolate are grouped in appendix X; an overview is presented in Figure 3-7. No significant impact on the lag phase duration when isolates were exposed to sub-MIC concentrations of CHX was detected for a majority of isolates (8/12; blue isolates). Regarding Ec-207 (red isolate), a significant increase in the lag phase duration when exposed to the concentration at which variable growth was observed was detected (see Table 3-11 page 97). When exposed to half their MICs, Ec-165, Ec-180 and Ec-188 (black isolates) harboured a significantly extended lag phase duration (respectively 19 min, 187 min and 13 min; Dunnett's test with $p<0.05$).

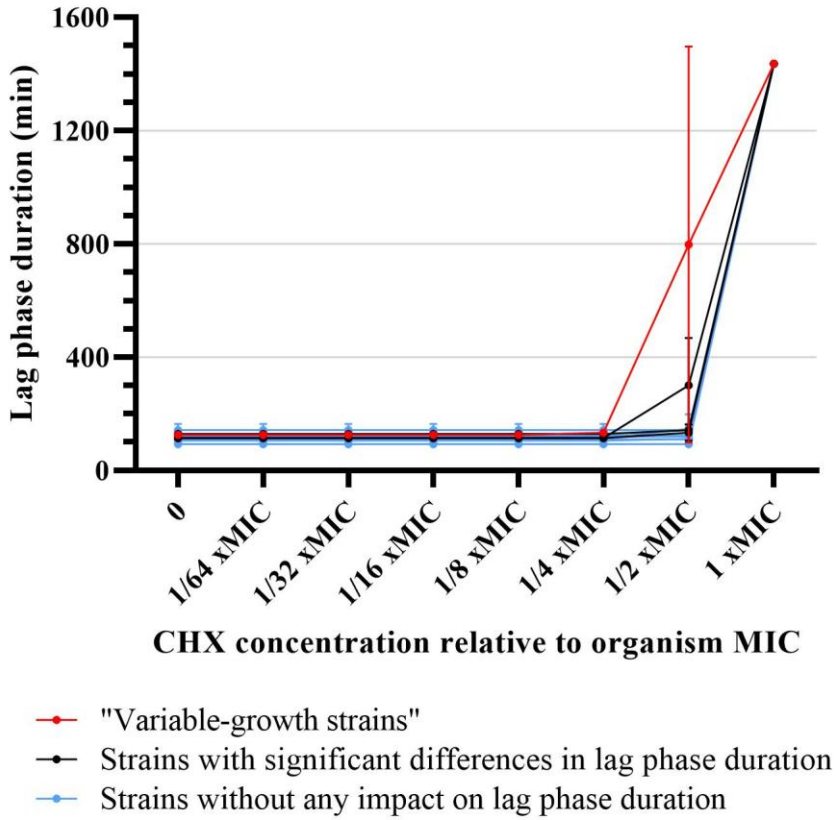

Figure 3-7. Changes in lag phase duration in *E. coli* isolates depending on CHX concentrations expressed as fractions of the organism's MIC.

Experiments were carried at least in triplicate ($n\geq3$) and reported results correspond to mean values and standard deviations. *E. coli* isolates segregated into three different clusters: the "variable-growth strain" (Ec-207, in red), for which variability in growth was observed at concentration corresponding to approximately half of its MIC; the strains for which significant changes in lag phase duration were obtained, mostly when exposed to concentrations corresponding to half their MICs (black); the strains for which no significant

difference in lag phase duration was measured across the different CHX concentrations investigated (blue).

3.3.2.3.4 Impact on the growth rate

The growth rates measured for each strain are presented in appendix X, and significant results highlighted in Table 3-11 (page 97). In biocide-free environment, the growth rates measured for the *E. coli* isolates ranged from 0.0113 min^{-1} (Ec-161; $t_{1/2}$: 61.2 min) to 0.0147 min^{-1} (Ec-165; $t_{1/2}$: 47 min). The growth rates of *E. coli* ATCC 25922, Ec-158, Ec-161, Ec-165, Ec-172, Ec-180, Ec-188, Ec-191 and Ec-198 were not significantly impacted by CHX exposure at any of the tested concentrations, except when exposed to their MICs or above. At 4 µg/mL CHX (concentration at which variability in growth was observed), the growth rate of Ec-207 was significantly decreased when compared to growth in CHX-free R2A. Ec-187's growth rate was significantly increased when exposed to half its MIC. Ec-199 harboured significantly lower growth rates than in R2A only when exposed to 0.0625 and 0.125 µg/mL CHX.

The growth rates of each isolate were compared to the ones obtained with the reference strain *E. coli* ATCC 25922 (appendix XI). When both the tested clinical isolate and the reference strain were able to grow, there was no or almost no significant difference between their growth rates at the different CHX concentrations tested (Sidak's post-test; $p>0.05$).

3.3.3 Cross-resistance mechanisms between biocides and antibiotics

3.3.3.1 Investigation of a hypothetical reduced membrane permeability

The CHX MICs for the 49 selected isolates were determined in the presence of 60 µg/mL EDTA. The results are presented in Table 3-13 for the *K. pneumoniae* isolates and in Table 3-14 for the *E. coli* strains. A significant decrease (Dunnett's post-test; $p<0.05$) in CHX MIC was observed for 22/38 *K. pneumoniae* and for 7/11 *E. coli* isolates in the presence of EDTA; no fold-change ≥ 8.0 was measured (maximum fold-change: 4.8 for Kp-58).

Table 3-13. Comparison between CHX MICs with or without addition of EDTA for *K. pneumoniae* clinical isolates.

CHX MICs were determined at least in triplicates (n≥3). CHX MICs in R2A only were previously measured in chapter 2. CHX MICs with addition of EDTA were measured in the presence of 60 µg/mL EDTA. Statistical significance was determined by two-way ANOVA followed by Dunnett's post-test (significant when $p<0.05$, shown in bold and red). No fold-change higher or equal to 8.0 was detected. SD: standard deviation.

	CHX only		CHX+EDTA		
	CHX MIC (µg/mL)		CHX MIC (µg/mL)		
Strain	Mean	SD	Mean	SD	Fold change
Kp-1	8.00	0.00	3.78	0.39	**2.1**
Kp-7	8.00	0.00	4.00	0.00	**2.0**
Kp-13	6.67	2.31	3.33	0.00	**2.0**
Kp-16	4.00	0.00	3.56	0.77	1.1
Kp-18	4.00	0.00	2.67	0.67	1.5
Kp-19	2.67	1.15	2.00	0.00	1.3
Kp-20	4.00	0.00	2.25	0.50	**1.8**
Kp-22	3.33	1.15	3.11	1.02	1.1
Kp-26	4.00	0.00	3.83	0.34	1.0
Kp-27	8.00	0.00	3.00	0.67	**2.7**
Kp-28	8.00	0.00	3.67	0.39	**2.2**
Kp-30	3.33	1.15	1.56	0.20	**2.1**
Kp-45	6.67	2.31	3.33	1.15	**2.0**
Kp-51	5.33	2.31	2.22	0.39	**2.4**
Kp-58	8.00	0.00	1.67	0.58	**4.8**
Kp-61	2.00	0.00	3.33	0.67	0.6
Kp-63	4.00	0.00	4.00	0.00	1.0
Kp-69	4.00	0.00	3.78	0.39	1.1
Kp-79	2.00	0.00	4.00	0.00	**0.5**
Kp-85	2.00	0.00	2.89	1.02	0.7
Kp-88	2.67	1.15	3.55	0.39	0.8
Kp-94	4.00	0.00	3.78	0.39	1.1
Kp-96	8.00	0.00	3.11	1.02	**2.6**
Kp-99	8.00	0.00	2.22	0.39	**3.6**
Kp-101	4.00	0.00	2.22	0.39	**1.8**
Kp-106	10.67	4.62	2.89	0.38	**3.7**
Kp-107	8.00	0.00	3.56	0.77	**2.2**

Table 3-13 – *Continued*.

Strain	CHX only CHX MIC (µg/mL) Mean	SD	CHX+EDTA CHX MIC (µg/mL) Mean	SD	Fold change
Kp-109	3.33	1.15	3.33	0.00	1.0
Kp-112	6.67	2.31	3.33	0.00	**2.0**
Kp-114	6.67	2.31	3.55	0.39	**1.9**
Kp-115	4.00	0.00	3.78	0.39	1.1
Kp-118	2.00	0.00	0.89	0.19	2.2
Kp-125	8.00	0.00	4.00	0.00	**2.0**
Kp-126	8.00	0.00	3.55	0.39	**2.3**
Kp-136	4.00	0.00	3.33	1.15	1.2
Kp-147	8.00	0.00	3.78	0.39	**2.1**
Kp-149	8.00	0.00	7.11	1.54	1.1
Kp-155	8.00	0.00	2.44	0.77	**3.3**

Table 3-14. Comparison between CHX MICs with or without addition of EDTA for *E. coli* clinical isolates.

CHX MICs were determined at least in triplicates (n≥3). CHX MICs in R2A only were previously measured in chapter 2. CHX MICs with addition of EDTA were measured in the presence of 60 µg/mL EDTA. Statistical significance was determined by two-way ANOVA followed by Dunnett's post-test (significant when $p<0.05$, shown in bold and red). No fold-change higher or equal to 8.0 was detected. SD: standard deviation.

Strain	CHX only CHX MIC (µg/mL) Mean	SD	CHX+EDTA CHX MIC (µg/mL) Mean	SD	Fold change
Ec-158	2.00	0.00	1.50	0.58	1.3
Ec-161	1.00	0.00	0.67	0.24	1.5
Ec-165	4.00	0.00	2.00	0.00	**2.0**
Ec-172	2.00	0.00	0.88	0.25	**2.3**
Ec-180	4.00	0.00	0.92	0.17	**4.4**
Ec-187	2.00	0.00	1.50	0.58	1.3
Ec-188	4.00	0.00	1.61	0.68	**2.5**
Ec-191	1.67	0.58	0.89	0.10	**1.9**
Ec-198	1.67	0.58	0.58	0.38	**2.9**
Ec-199	2.00	0.00	1.56	0.51	1.3
Ec-207	4.00	0.00	1.75	0.50	**2.3**

3.3.3.2 Investigation of a possible enhanced efflux

CHX MICs were determined in the presence of CCCP. These results are summarised in Table 3-15 for *K. pneumoniae* isolates and in Table 3-16 for *E. coli* strains. CHX MICs were found to be significantly decreased (Dunnett's post-test; $p<0.05$) in the presence of CCCP for most strains regardless of the species (37/38 for *K. pneumoniae*, 10/11 for *E. coli*). The fold-change observed was equal to or greater than 8.0 for 21/38 *K. pneumoniae* (up to a 33.8-fold decrease in CHX MIC for Kp-125 in the presence of CCCP) and 1/11 *E. coli* isolates.

Table 3-15. Comparison between CHX MICs with or without addition of CCCP for *K. pneumoniae* clinical isolates.

CHX MICs were determined at least in triplicates ($n\geq3$). CHX MICs in R2A only were previously measured in chapter 2. CHX MICs with addition of CCCP were measured in the presence of 20 µg/mL CCCP, except for Kp-63 where this concentration was decreased to 5 µg/mL. Statistical significance was determined by two-way ANOVA followed by Dunnett's post-test (significant when $p<0.05$, shown in bold and red). Fold-changes equal to or higher than 8.0 were highlighted in green. SD: standard deviation.

	CHX only		CHX+CCCP		
	CHX MIC (µg/mL)		CHX MIC (µg/mL)		
Strain	Mean	SD	Mean	SD	Fold change
Kp-1	8.00	0.00	0.83	0.17	**9.6**
Kp-7	8.00	0.00	0.42	0.14	**19.2**
Kp-13	6.67	2.31	0.42	0.09	**16.0**
Kp-16	4.00	0.00	0.50	0.00	**8.0**
Kp-18	4.00	0.00	0.78	0.25	**5.2**
Kp-19	2.67	1.15	0.39	0.24	**6.8**
Kp-20	4.00	0.00	0.78	0.25	**5.2**
Kp-22	3.33	1.15	0.61	0.33	**5.4**
Kp-26	4.00	0.00	0.42	0.21	**9.6**
Kp-27	8.00	0.00	1.00	0.59	**8.0**
Kp-28	8.00	0.00	0.92	0.17	**8.7**
Kp-30	3.33	1.15	0.61	0.10	**5.4**
Kp-45	6.67	2.31	0.39	0.05	**17.1**
Kp-51	5.33	2.31	0.33	0.09	**16.0**
Kp-58	8.00	0.00	1.16	0.29	**6.9**
Kp-61	2.00	0.00	0.42	0.14	**4.8**

Table 3-15 – *Continued*.

Strain	CHX only CHX MIC (µg/mL) Mean	SD	CHX+CCCP CHX MIC (µg/mL) Mean	SD	Fold change
Kp-63	4.00	0.00	1.00	0.00	**4.0**
Kp-69	4.00	0.00	0.67	0.29	**6.0**
Kp-79	2.00	0.00	0.79	0.25	2.5
Kp-85	2.00	0.00	0.50	0.00	**4.0**
Kp-88	2.67	1.15	0.56	0.23	**4.7**
Kp-94	4.00	0.00	0.61	0.19	**6.6**
Kp-96	8.00	0.00	0.28	0.05	**28.9**
Kp-99	8.00	0.00	0.94	0.10	**8.5**
Kp-101	4.00	0.00	0.83	0.00	**4.8**
Kp-106	10.67	4.62	0.72	0.25	**14.7**
Kp-107	8.00	0.00	1.00	0.00	**8.0**
Kp-109	3.33	1.15	0.39	0.13	**8.5**
Kp-112	6.67	2.31	0.42	0.14	**16.0**
Kp-114	6.67	2.31	1.33	0.34	**5.0**
Kp-115	4.00	0.00	0.72	0.25	**5.5**
Kp-118	2.00	0.00	0.67	0.30	**3.0**
Kp-125	8.00	0.00	0.24	0.02	**33.8**
Kp-126	8.00	0.00	0.72	0.25	**11.1**
Kp-136	4.00	0.00	0.47	0.05	**8.5**
Kp-147	8.00	0.00	0.83	0.17	**9.6**
Kp-149	8.00	0.00	0.94	0.10	**8.5**
Kp-155	8.00	0.00	0.94	0.10	**8.5**

Table 3-16. Comparison between CHX MICs with or without addition of CCCP for *E. coli* clinical isolates.

CHX MICs were determined at least in triplicates (n≥3). CHX MICs in R2A only were previously measured in chapter 2. CHX MICs with addition of CCCP were measured in the presence of 10 µg/mL CCCP. Statistical significance was determined by two-way ANOVA followed by Dunnett's post-test (significant when $p<0.05$, shown in bold and red). Fold-changes equal to or higher than 8.0 were highlighted in green. SD: standard deviation.

	CHX only		CHX+CCCP		
	CHX MIC (µg/mL)		CHX MIC (µg/mL)		
Strain	Mean	SD	Mean	SD	Fold change
Ec-158	2.00	0.00	0.56	0.10	**3.6**
Ec-161	1.00	0.00	0.48	0.17	2.1
Ec-165	4.00	0.00	0.67	0.29	**6.0**
Ec-172	2.00	0.00	0.50	0.00	**4.0**
Ec-180	4.00	0.00	0.56	0.10	**7.2**
Ec-187	2.00	0.00	0.67	0.17	**3.0**
Ec-188	4.00	0.00	0.50	0.00	**8.0**
Ec-191	1.67	0.58	0.78	0.25	**2.1**
Ec-198	1.67	0.58	0.48	0.04	**3.5**
Ec-199	2.00	0.00	0.72	0.25	**2.8**
Ec-207	4.00	0.00	1.78	0.39	**2.3**

3.4 Discussion

Data for antibiotic MICs (performed prior to this work) were not available for all isolates and were, sometimes, expressed in a way not suitable for correlation studies ('> x' or '≤ y'). Discarding these extreme values was not considered, as it would have resulted in the loss of too many data points. Converting the MICs into resistant, intermediate or susceptible status according to the breakpoints provided for Enterobacteriaceae was a possibility but would have had too big an impact on the accuracy and sensitivity of the test. Therefore, as Spearman's test is based on the rank of the values and not the values themselves, MICs expressed as '> x' and '≤ y' were converted into '2x' and 'y', respectively.

There are no established criteria to measure the strength of Spearman's correlations, making difficult the interpretation of results (Kraemer, 2006). When interpretation criteria were detailed in the literature, studies did not seem to agree on thresholds discriminating weak, moderate and strong correlations, e.g. strong correlations corresponding to r scores higher than either 0.3, 0.5, or 0.7 in absolute values (Green, 1978; Chow *et al.*, 1989; Koljalg

et al., 2002; Lambert, 2004; Condell *et al.*, 2012; Mukaka, 2012; Lavilla Lerma *et al.*, 2015; Oggioni *et al.*, 2015; Romero *et al.*, 2017). For this work, the interpretation criteria used was adapted from similar studies where Spearman's and Pearson's (the parametric counterpart of the Spearman's test) tests were used to detect potential links between susceptibility to antibiotics and biocides (Lavilla Lerma *et al.*, 2015; Oggioni *et al.*, 2015; Romero *et al.*, 2017). It is however important to bear in mind that correlations do not involve causality, and do not constitute a proof of cross-resistance for example, only an indication of a potential link (Chow *et al.*, 1989). Regarding the current study, a bias in the establishment of correlations could be that all the investigated microorganisms were multi-drug resistant and carbapenemase producers, therefore harbouring elevated antibiotic MICs for a wide range of compounds. Moreover, for some of the sub-populations investigated (based on the carbapenemase produced), the number of isolates taken into account for the calculation of the Spearman's r scores were low or even insufficient, with the example of the *E. coli* VIM-producer sub-group containing only three isolates, where a minimum of five was necessary for the calculation with Prism GraphPad 5.0 for Windows.

For both species, independently of the carbapenemase produced, positive correlations were found between some biocides, especially between cationic compounds, and to some extent between heavy metals, which could be expected considering their similar chemical properties. However, conflicting reports have been published, some indicating a correlation between similar compounds, e.g. BZC and CHX (Braoudaki & Hilton, 2004; Gadea *et al.*, 2017a; Gadea *et al.*, 2017b; Kampf, 2018; Wand *et al.*, 2019), and others showing no link between them (Romero *et al.*, 2017; Wand *et al.*, 2017; Shepherd *et al.*, 2018). Cross-resistance mechanisms can arise when antimicrobials share a target, an entry route or a mechanism of action (Fernández-Fuentes *et al.*, 2012). For example, BZC and CHX, despite having different mechanisms of action, interact both with the bacterial outer membrane (which harbours a net negative charge) and displace the divalent cations within (Gilbert & Moore, 2005; Ortega Morente *et al.*, 2013). Several mechanisms, including efflux for BZC, CHX (e.g. AcrAB-TolC, Qac proteins, CepA; Poole, 2005; Jaglic & Cervinkova, 2012; Ortega Morente *et al.*, 2013; Anes *et al.*, 2015), CS and SN (e.g. CusCFBA, CopA, Sil proteins; Nies, 2003; Poole, 2005; Ortega Morente *et al.*, 2013; Anes *et al.*, 2015) and permeability alterations (e.g. changes in hydrophobicity, outer membrane protein and fatty acid compositions (Ortega Morente *et al.*, 2013; Wales & Davies, 2015) have been previously reported in *E. coli* and *K. pneumoniae*.

Only few significant correlations between antibiotic and biocide MICs were detected for the overall *E. coli* isolates. When considering sub-groups based on the carbapenemase produced, our data are in accordance with a previous study showing no cross-resistance between CHX and antibiotics when an *E. coli* reference strain was exposed to increasing concentrations of CHX (Thomas *et al.*, 2000). Links between QACs and antibiotics including CIP, (Buffet-Bataillon *et al.*, 2016; Soumet *et al.*, 2016; Henly *et al.*, 2019), IPM, tetracycline, (Braoudaki & Hilton, 2004; Soumet *et al.*, 2012), and cephalosporins, (Soumet *et al.*, 2016; Kampf, 2018) have been reported in the literature. One study claimed no significant differences in antibiotic resistance were observed between households using QACs and the ones who did not (Gerba, 2015). Many potential links were unveiled for the *K. pneumoniae* isolates. This could be surprising as both species belong to the Enterobacteriaceae family. Regarding *K. pneumoniae* isolates, the different correlations observed for the overall population were found in not all but at least 2/4 sub-groups based on the carbapenemase produced. Surprisingly, VIM-producers and NDM-producers did not harbour similar correlation patterns, despite the common features between these carbapenemases; both belong to the Ambler class B (metallo-β-lactamases). Positive correlations were detected between BZC and TIG, MIN and aminoglycosides, except for KPC-producers where the association was inverted. Previous reports showed different correlations between exposure to BZC and changes in MIC for ciprofloxacin and tetracycline (but not for aminoglycosides) in *P. aeruginosa* (Mc Cay *et al.*, 2010; Kim *et al.*, 2018b). CHX was found to be positively associated with carbapenems, cephalosporins, TIG, MIN and to some extent, aminoglycosides; previous studies showed similar associations (Koljalg *et al.*, 2002; Romero *et al.*, 2017). A weak positive correlation was found between CHX and CIP when considering the global population (as previously reported for Gram-negative bacteria; Koljalg *et al.*, 2002), and between CHX and COL only for OXA-48-like-producers. These associations between cationic biocides and antibiotics, especially tetracyclines and derivatives (Ball *et al.*, 1980; McMurry *et al.*, 1980; Srinivasan *et al.*, 2014), and ciprofloxacin (Nikaido & Pages, 2012; Soumet *et al.*, 2012; Slipski *et al.*, 2018), tend to pinpoint towards the expression of efflux-pumps as a potential cross-resistance mechanism. Correlations between CHX and COL were previously shown in *K. pneumoniae* and thought to be linked to efflux or changes in the outer membrane or the LPS (Wand *et al.*, 2017; Hashemi *et al.*, 2019; Zhang *et al.*, 2019). After being left aside for years due to nephrotoxicity side-effects (Gupta, 2016), COL represents now one of the last resort treatments for severe infections. It is therefore paramount to protect this molecule from emerging resistance and possible cross-resistance mechanisms (WHO, 2014; Gu *et al.*, 2016;

Liu *et al.*, 2016). In most cases, CS was found to be negatively correlated with carbapenems and cephalosporins. This was surprising, as copper has been previously reported to be involved in the de-repression of the antibiotic resistance response in *E. coli* (Hao *et al.*, 2014). In the literature, heavy metals and antibiotics could be found to lead to co- or counter-selective consequences, although the latter were seen with mercury and not copper (Wales & Davies, 2015).

CHX was one of the biocides with which most of the correlations with antibiotics were observed. Its use is especially relevant in healthcare settings for a wide range of applications (e.g. dentistry, skin decolonization; Russell, 1986). However, the presence of links between CHX reduced susceptibility and antibiotic resistance, i.e. whether or not cross-resistance mechanisms between these antimicrobials might be involved, remains unclear due to conflicting reports (Oggioni *et al.*, 2013; Kampf, 2016). Here, we chose to focus the scope of this study on CHX, using a limited number of isolates representative of the original population, to pursue further experimental work.

The Bioscreen assay was able to confirm the CHX MICs previously measured in 96-well plates for the investigated isolates. Firstly, determinations for lag phase duration and growth rate were performed by hand, which can be less powerful and accurate than software specifically designed for this task, such as GrowthRate (Hall *et al.*, 2014; Jung *et al.*, 2015). However, due to the very low OD values obtained (due to a combination of both the initial inoculum and the blanking system linked to the decreasing R2A broth OD values previously mentioned), such software could not be used. For some isolates, at specific concentrations of CHX (around their MICs), both growth and no growth were observed across the different replicates for each strain. These isolates were referred to as the "variable growth strains". When exposed to these specific CHX concentrations, statistically significant changes in OD_{600nm} max, lag phase duration and growth rate were sometimes measured, although high standard deviation values were obtained for these parameters. This phenomenon seemed however to be linked to the variability in growth observed under these specific conditions. A minority of isolates (14/39 *K. pneumoniae*, none of the investigated *E. coli*) was not able to grow at the same maximum density (based on OD_{600nm} max values) when exposed to CHX concentrations corresponding to half their MICs, compared to the control conditions. A decrease in the maximum OD was reported in *E. coli* ATCC 10536 when exposed to CHX concentrations corresponding to approximately 2/3 x MIC, but not 1/3 x MIC (Cheung *et al.*, 2012), or to sub-inhibitory concentrations of QACs (Walsh *et al.*, 2003); prolonged exposure to very low concentrations of BZC (0.0002-0.002% the MIC) also resulted in

maximum bacterial density in *E. coli* (Forbes *et al.*, 2019). Exposure to 1/2 x MIC had however a significant impact on the lag phase duration for a majority of isolates (27/39 *K. pneumoniae* and 3/12 *E. coli*), delaying the regrowth of the bacteria for up to 700 min. Extended lag phase duration was previously reported for both *E. coli* and *K. pneumoniae* following exposure to low concentrations of triclosan, and associated with a high fitness cost (Curiao *et al.*, 2015).

When considering the growth rate, no variation was observed for most *K. pneumoniae* (27/39) and *E. coli* (9/12) isolates when exposed to CHX. A similar trend was observed in a previous study where no significant change in growth rate was measured after long-term exposure to CHX in *K. pneumoniae* (Zhang *et al.*, 2019). Several isolates from both species harboured increased growth rate values when exposed to half their MICs, and to some extent to lower concentrations. This observation could be linked to a phenomenon previously observed in *P. aeruginosa* and *S. aureus*, referred to as hormesis, where exposure to a low concentration of biocide could result in a stimulating effect rather than a neutral or deleterious one (Morales-Fernández *et al.*, 2014). Another explanation for these elevated growth rates could be linked to the design of the assay itself. The initial inoculum used (5×10^5 CFU/mL) resulted in low turbidity during the lag phase, which in turn corresponded to very low logarithm values. It has been previously reported that low OD values could lead to abnormal growth rates (Jung *et al.*, 2015), which could be the case here. Regarding Kp-125 (first growth phase only), a significant decrease in growth rate was observed when exposed to half its MIC. It is however of importance to keep in mind that only one replicate was obtained for this condition. Nevertheless, exposure to QACs was shown to negatively impact the growth rates in *P. aeruginosa* and *E. coli* (Walsh *et al.*, 2003). Effects of QACs were thought to be linked to interactions with non-vital targets in this previous study. Long-term exposure to sub-inhibitory concentrations of BZC or octenidine (a biguanide like CHX) also resulted in a decreased growth rate when compared to the original strain in *P. aeruginosa* (Mc Cay *et al.*, 2010; Shepherd *et al.*, 2018). The growth rates of the clinical isolates and the reference strains were compared for both species. No significant differences were observed between clinical and reference strain for *E. coli* (except at concentrations at which *E. coli* ATCC 25922 was not able to grow). Similar observations were made for most *K. pneumoniae* isolates (21/38 clinical isolates). A group of *K. pneumoniae* outgrew the reference strain (13/38), while *K. pneumoniae* ATCC 13883 was able to outcompete five clinical isolates at lower CHX concentrations. For these isolates, the minimum selective concentration (MSC) i.e. the minimal concentration of antimicrobial able to select for resistance, could be estimated (2 μg/mL). The rationale behind the MSC

determination is illustrated in Figure 3-8. The 'traditional' selective window is based on the theory that enrichment for resistant bacteria can only occur within a range of concentrations comprised between the MICs of the susceptible (corresponding to the reference strain in this study) and of the resistant strain. However, Gullberg and colleagues demonstrated that enrichment in resistant bacteria can also happen at lower concentrations, in the 'sub-MIC' selective window. This range of concentrations corresponds to values between the MIC of the susceptible strain and the concentration at which the susceptible strain is able to outcompete the growth of the resistant strain, corresponding to the MSC (Gullberg *et al.*, 2011).

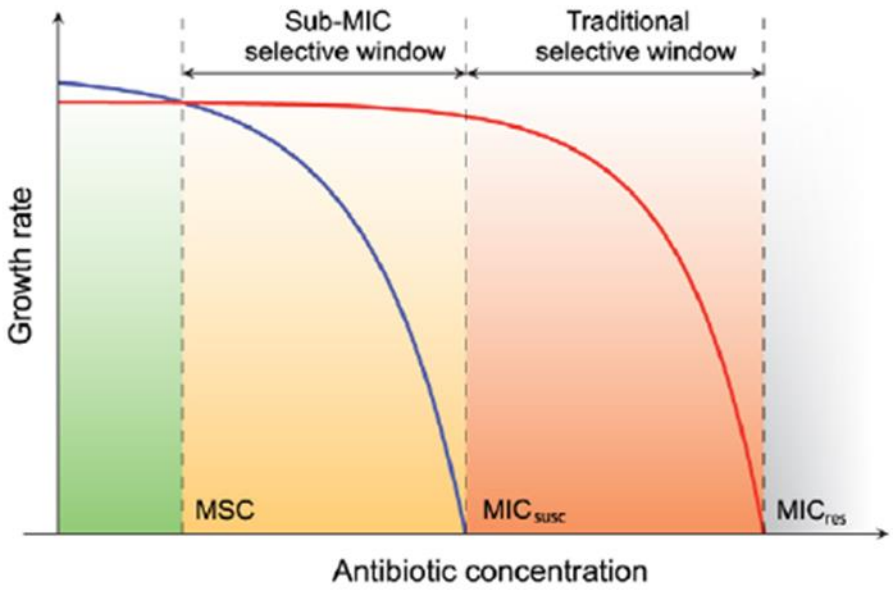

Figure 3-8. Minimum selective concentration determination rationale.
Figure from Gullberg *et al.*, 2011. The 'traditional' selective window (red background) corresponds to the concentrations between the MIC measured for the resistant strain (MIC_{res}) and the one for the susceptible strain (MIC_{susc}). The 'sub-MIC' selective window (orange background) corresponds to concentrations lower than the MIC_{susc} while the growth rate measured for the resistant strain is still higher than the one for the susceptible strain. The concentration corresponding to the point where the reference strain is able to outcompete the resistant strain corresponds to the minimum selective concentration (MSC).

When investigating cross-resistance mechanisms with the addition of CCCP or EDTA for CHX MIC determinations, only differences in MICs equal to or higher than 8-fold between CHX alone and CHX+compound were considered relevant. Indeed, for a given condition, a 2-fold variation in MIC values is common when determining MICs (Osei

Sekyere & Amoako, 2017). As a consequence, if between two distinct conditions MICs differ by 4-folds or less, the value ranges could overlap, and the difference in MICs would not be significant. Therefore, a minimum of 8-fold variation in MIC values enabled the discrimination between significant and non-significant differences.

No potentiation (changes in MICs ≥ 8.0-folds) of the effects of CHX was observed when EDTA was added for *E. coli* or *K. pneumoniae* isolates at the concentration tested, which seemed consistent with previous observations (Russell, 1986). Similar findings were published with QACs in *E. coli* (Walsh *et al.*, 2003). EDTA increases membrane permeability by chelating the divalent cations required for its stability (Pelletier *et al.*, 1994; Sawer *et al.*, 1997; Denyer & Maillard, 2002). This could indicate that changes in the membrane permeability were not involved as a mechanism to counteract the effects of CHX in this study.

In the presence of CCCP, almost all isolates, regardless of the species, harboured a statistically significant decrease in CHX MIC. A fold-change equal to or higher than 8.0 was observed for approximately half of the studied *K. pneumoniae* isolates and only one *E. coli*. CCCP had been previously shown to potentiate CHX (Abuzaid *et al.*, 2012; Wand *et al.*, 2017) but also colistin (Ni *et al.*, 2016; Osei Sekyere & Amoako, 2017) and ciprofloxacin (in *Acinetobacter baumannii*; Ardebili *et al.*, 2014). CCCP disrupts the proton-motive force, which is necessary for efflux pumps belonging to the SMR, MFS, RND and to some extent, the MATE superfamilies, to extrude their substrates (Marquez, 2005; Alav *et al.*, 2018). However, CCCP can interfere with other bacterial functions, including cell division and metabolism, and impact membrane permeability (Anes *et al.*, 2015; Alav *et al.*, 2018). In that regard, potentiation of colistin using CCCP could be linked to the restoration of negative charges on the membrane surface rather than efflux; indeed, colistin, as well as CHX, is a cationic compound, therefore a reduction of the net negative charge (e.g. following a change in the LPS composition) on the membrane would decrease the affinity of these compounds for the structure (Ni *et al.*, 2016; Wand *et al.*, 2017). A cooperation between both increased efflux (through SmvA, a member of the MFS superfamily) and changes in LPS structure has been previously reported to decrease CHX susceptibility in *Proteus mirabilis* as well (Pelling *et al.*, 2019).

When investigating common resistance mechanisms between antibiotics and biocides, the scientific community does not seem to reach a consensus, especially about their clinical relevance (Maillard *et al.*, 2013; Oggioni *et al.*, 2013; Kampf, 2016). When cross-resistance mechanisms between biocides and antibiotics are investigated, one of the most

encountered hypotheses is the role of efflux pumps (Braoudaki & Hilton, 2004; Baker-Austin *et al.*, 2006; Gnanadhas *et al.*, 2013), although this does not exclude the possibility that several distinct mechanisms may be involved at once, such as alterations in the membrane structure as well (Wand *et al.*, 2017; Pelling *et al.*, 2019). Conflicting observations are reported about links between the presence of these efflux pump genes and elevated MICs for biocides. For instance, *qacΔE*, reported to encode an efflux pump widely distributed among Gram-negative bacteria (Jaglic & Cervinkova, 2012), or *cepA* and *smvA* present in *K. pneumoniae*, among others, are thought to be involved in reduced susceptibility to CHX or QACs (Wand *et al.*, 2017; Slipski *et al.*, 2018; Wand *et al.*, 2019). Some studies reported a correlation between the presence of one or more of these genes and reduced susceptibility to biocides (Abuzaid *et al.*, 2012; Guo *et al.*, 2015; Wu *et al.*, 2015; Zhang *et al.*, 2019), while others did not find any link between them (Naparstek *et al.*, 2012; Azadpour *et al.*, 2015; Vijayakumar *et al.*, 2018). Efflux pumps are associated with an important fitness cost and might be unlikely to be selected at very low concentrations (Andersson & Hughes, 2012). In addition, additional information is still required to elucidate the complete mechanism of extrusion linked to several efflux systems, including the MFS superfamily. Indeed, these efflux pumps, like SmvA, are located within the inner membrane and expel their substrates into the periplasmic space, where CHX is likely to cause damage; other transporters, such as outer membrane proteins, would be needed to release CHX in the outside environment (Slipski *et al.*, 2018; Pelling *et al.*, 2019). Another explanation could be that these proteins might not be single components but part of tripartite systems, similarly to the RND superfamily, e.g. the AcrA-AcrB-TolC system (Pasqua *et al.*, 2019; Pelling *et al.*, 2019).

3.5 Conclusion

Overall, the findings in this study (correlations between antibiotic and biocide MICs, extended lag phase when exposure to sub-inhibitory concentrations, impact of CCCP on MIC values) indicate the expression of efflux pumps may account as a resistance mechanism between CHX and some antibiotics, mainly in the *K. pneumoniae* isolates studied. The next chapter will explore the phenotypic and transcriptomic consequences following exposure to sub-inhibitory concentrations of CHX.

Chapter 4. Effects of exposure to sub-inhibitory concentrations of chlorhexidine

4.1 Introduction

4.1.1 Conditions leading to exposure to sub-inhibitory concentrations of antimicrobials

Sub-inhibitory concentrations of antibiotics can be found in various environments, generating gradients of antibiotic concentrations and applying selective pressure on the bacterial communities within, as highlighted in Figure 4-1 (Andersson & Hughes, 2014).

In human medicine, sub-inhibitory concentrations of antibiotics can be found within the body, linked to poor pharmacokinetic properties (poor distribution and/or penetration in the targeted tissues) and poor patient compliance, among other factors (Andersson & Hughes, 2014). In animal production, antibiotics are also used as feed additives to promote growth, and consequently administered at low doses over longer periods of time, exposing the animals' microbiota to sub-inhibitory concentrations (Chattopadhyay, 2014). Residual concentrations of antibiotics were reported in animal-derived products destined for human consumption, including meat and milk (Aalipour *et al.*, 2013; Ramatla *et al.*, 2017).

After antibiotic therapy, up to 80 % of the administered antibiotics can be excreted without undergoing any change, mainly through urine but also faeces and reach wastewater and the global environment without being fully degraded by wastewater treatment plants for example (Diwan *et al.*, 2010; Andersson & Hughes, 2014; Van Epps & Blaney, 2016). Antibiotic can persist over long periods of time in the environment, including soil and water, and enrich their ecosystems in antibiotic resistance determinants and, therefore, antibiotic resistant bacteria (Van Epps & Blaney, 2016; Cycon *et al.*, 2019).

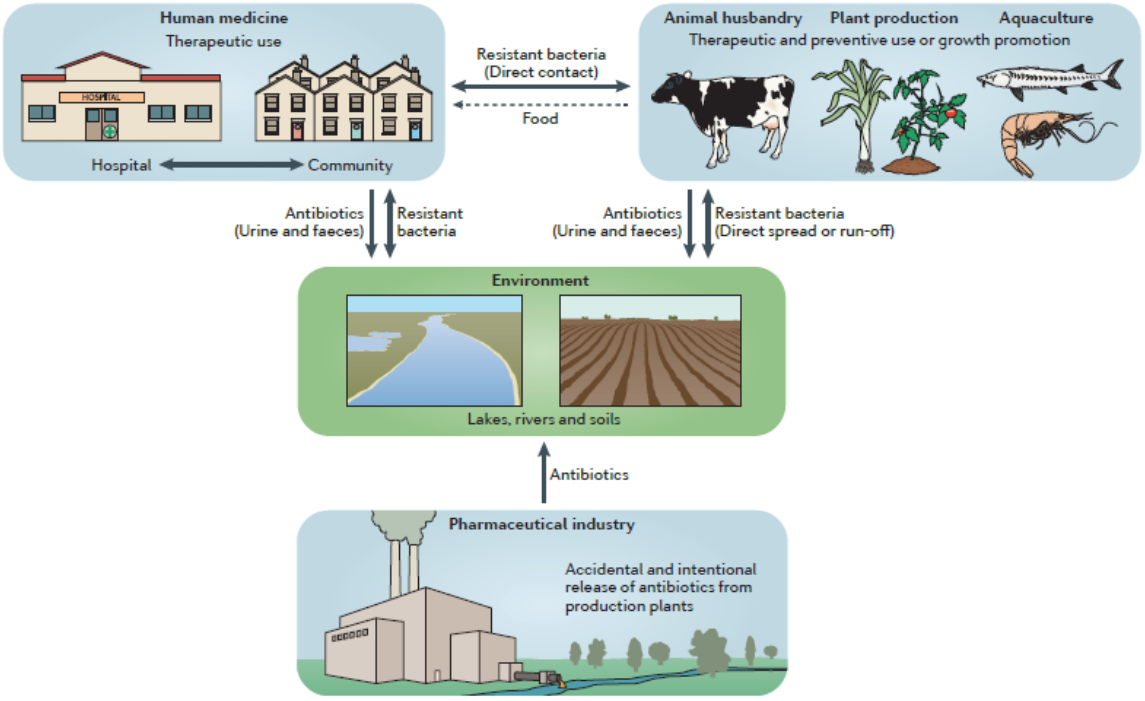

Figure 4-1. Residual antibiotic concentrations and associated resistance are transferred between interconnected environments.

Figure from Andersson & Hughes, 2014. Residual, sub-inhibitory concentrations of antibiotics are found in the environment, from the surroundings of their production to their utilization in human and veterinary medicine or in other agricultural-related settings, resulting in elevated selective pressure enabling the rise and transfer of antibiotic resistance.

Biocides, especially in formulations designed for surface and/or skin decontamination, usually involve a mixture of several active compounds (SCENIHR, 2009). Different parameters have been reported to impact the efficacy of biocides. Some are linked to the biocidal product itself: its chemical properties, its formulation (e.g. possible incompatibility due to interaction with excipients), its stability (especially towards changes in pH and temperature) and its concentration and the subsequent effects of dilution upon activity (Maillard, 2018). Other parameters are related to the surface treated and the use of the biocidal product: extended contact time are correlated with enhanced efficacy, and high organic loads on the surface could quench the activity of the biocide (Maillard, 2005). Finally, some factors are related to the micro-organisms themselves: the type of micro-organisms (bacterial spores being the least susceptible and viruses the most susceptible), the number of micro-organisms (higher number will be more difficult to eradicate), and their physiological state (e.g. presence of biofilm with protecting matrix and reduced metabolism harder to disinfect than planktonic bacteria) (Maillard, 2005; Maillard, 2018). As a

consequence, an improper use, e.g. incorrect dilution, short contact time, dirty surface, and/or a degradation of the biocide (e.g. bacterial degradation of QACs in aerobic conditions; Tezel & Pavlostathis, 2015) could lead to microbial exposure to sub-inhibitory concentrations of biocides.

4.1.2 Impact of exposure to sub-inhibitory concentrations of biocides

As highlighted in the previous section, contact time is an important parameter in biocide efficacy. Indeed, short or prolonged exposure to biocides can have various detrimental effects on bacteria, as illustrated in Table 4-1. Exposition to sub-inhibitory concentrations of biocides could trigger different mechanisms (see below) leading to a further decrease of intracellular concentration of biocides; this decrease could enable bacteria to survive rather than suffering irreversible damage if the exposure was prolonged and/or at higher concentrations of biocides (Maillard, 2018).

Table 4-1. Global impact of short-term or prolonged exposure to biocides on bacterial functions.

Table adapted from Maillard, 2018. A short-term or prolonged exposure to biocides can lead to various cellular effects engendering reversible (highlighted in blue) or irreversible (highlighted in red) consequences on bacterial growth and survival.

	Degree of damage	Consequences	
Short exposure	Disruption of the transmembrane proton-motive force, leading to an uncoupling of oxidative phosphorylation and inhibition of active transport across the membrane		**Reversible**
	Inhibition of respiratory or catabolic/anabolic reactions		
Prolonged exposure	Disruption of metabolic processes		
	Disruption of replication		
	Loss of membrane integrity, resulting in leakage of essential cellular constituents (potassium ions, inorganic phosphate, pentoses, nucleotides and nucleosides, proteins)	Imbalance of pH	**Irreversible**
	Coagulation of intracellular materials	Commitment to cell death (autocidal pathway)	
	Lysis	Cell death	

Exposure to sub-inhibitory concentrations of biocides have been reported to trigger two major mechanisms in bacteria: the induction of the SOS response, and the promotion of mutations, as illustrated in Figure 4-2 (Tezel & Pavlostathis, 2015). The SOS response corresponds to a generalised regulatory response triggered by DNA damage or oxidative stress for example, in order for the bacteria to ensure its survival (Simmons *et al.*, 2008; Baharoglu & Mazel, 2014). The expression of a wide range of genes can subsequently be altered (e.g. involved in efflux, virulence, global metabolism; Kreuzer, 2013), especially in order to promote the uptake of mobile genetic elements (including transposons, integrons, plasmids, via horizontal gene transfer [HGT] and homologous recombination (Tezel & Pavlostathis, 2015). The SOS response has been shown to be induced following exposure and/or adaptation to BZC or CHX in *E. coli* (Bore *et al.*, 2007; Moen *et al.*, 2012; Curiao *et al.*, 2015). Moreover, exposure to sub-inhibitory concentrations of CHX resulted in an increase HGT rate in the same bacterium (Jutkina *et al.*, 2018). On the other hand, increase in mutation rates has also been reported following exposure to sub-inhibitory concentrations of biocides; for example a mutation in *mutS*, involved in mismatch repair, was observed following adaptation to CHX in *K. pneumoniae* (Wand *et al.*, 2017).

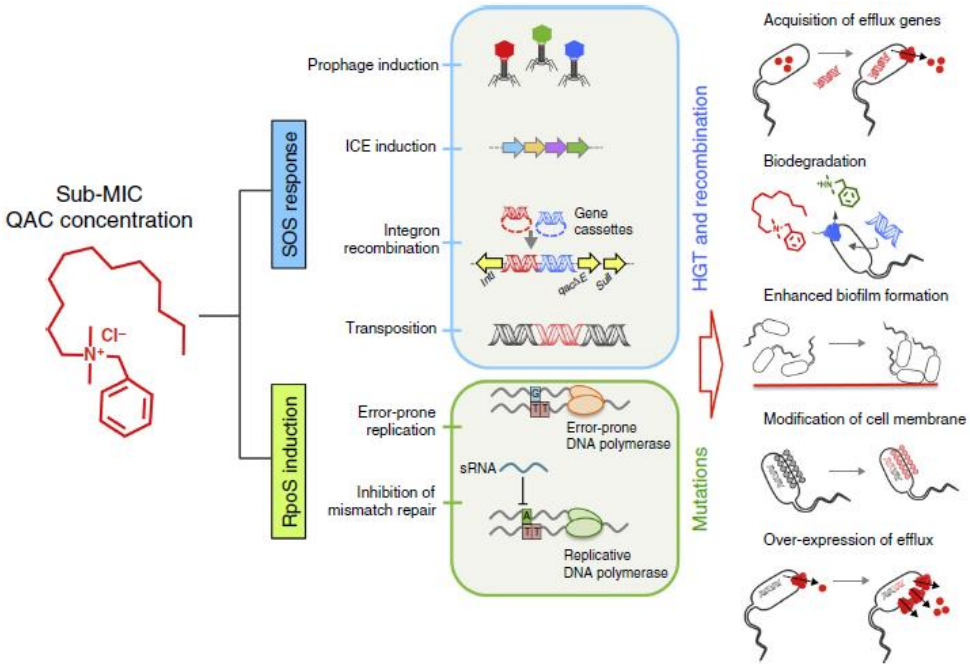

Figure 4-2. Global bacterial response following exposure to sub-inhibitory concentrations of biocides.

Figure from Tezel & Pavlostathis, 2015. Exposure to sub-inhibitory concentrations of biocides (QACs in this example) can induce the SOS response, impacting the global regulation of the expression of a wide range of genes and promoting acquisition of genetic determinants through horizontal gene transfer (HGT). The apparition of mutations can also be promoted, potentially leading to reduced susceptibility or resistance mechanisms.

4.1.3 Aim

In this chapter, the aim is to assess the phenotypical and molecular responses of two selected *K. pneumoniae* isolates following exposure to sub-inhibitory concentrations of CHX.

4.2 Methods

4.2.1 Rationale for the selection of strains and concentrations

Two *K. pneumoniae* isolates were selected to investigate the effects of exposure to sub-inhibitory concentrations of CHX. *E. coli* isolates demonstrated few differences in growth pattern when exposed to CHX, or in their CHX MICs with the addition of either CCCP or EDTA (see chapter 3). *K. pneumoniae* isolates were chosen according to the observed fold-change in CHX MIC when CCCP was added, and to the availability of results

generated by the growth pattern analysis assay at the time of the selection; isolates for which changes in lag phase duration and/or changes in growth rate when exposed to CHX were preferred.

The two selected *K. pneumoniae* were Kp-1 and Kp-125. Kp-125 showed the most important fold-change in CHX MIC when CCCP was added (33.8), and Kp-1 CHX MIC was reduced by 9.6-fold in the same condition. Both isolates presented an extended lag phase when exposed to half their MICs (corresponding to 4 μg/mL CHX) and the growth rate of Kp-125 during its first growth phase was significantly decreased when exposed to the same concentration of CHX and 1/4 x MIC (2 μg/mL CHX). Moreover, Kp-125 belongs to ST258, a clone especially significant in the clinic (Mathers *et al.*, 2015).

4.2.2 Exposure to sub-inhibitory concentrations of chlorhexidine

A schematic view of the CHX sub-inhibitory concentration exposure and following assays is presented in Figure 4-3.

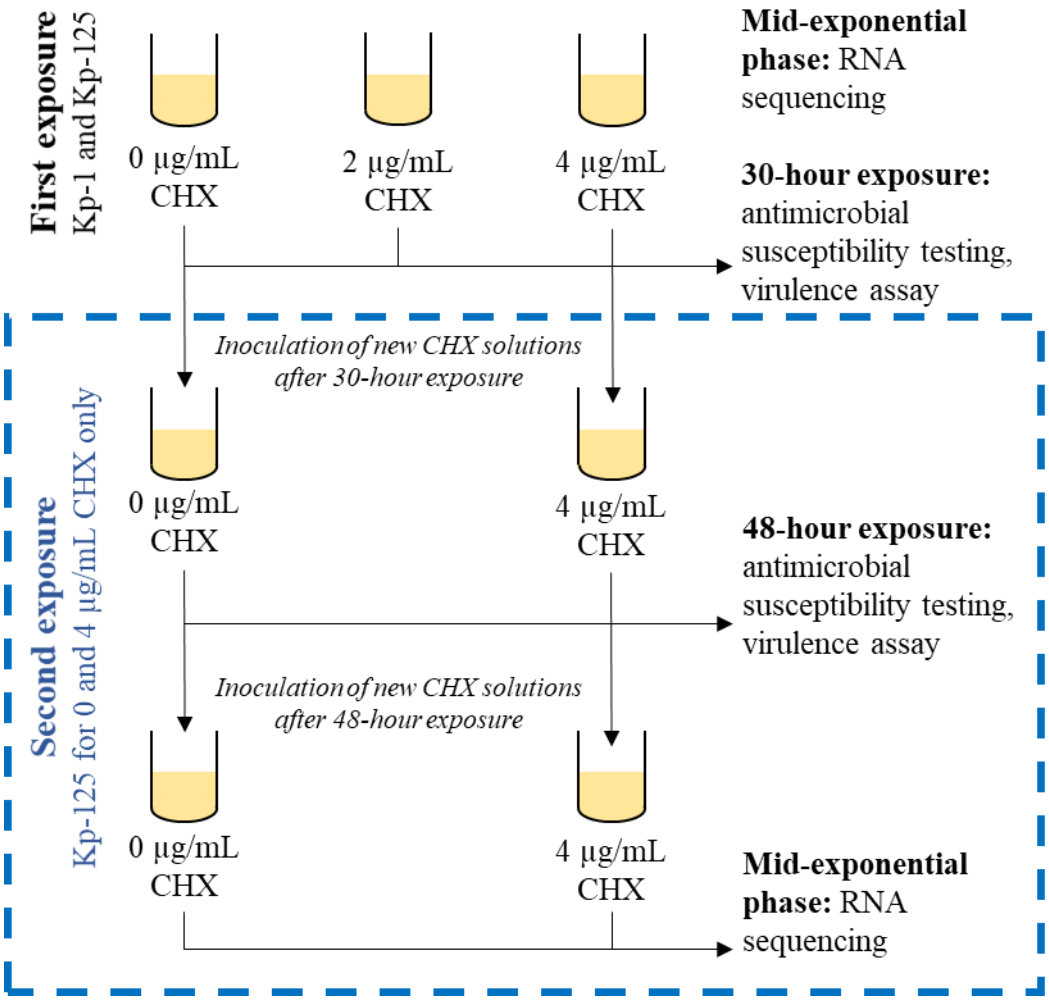

Figure 4-3. Outline of the exposure to sub-inhibitory concentrations of CHX and subsequent assays.

Kp-1 and Kp-125 were firstly exposed to either 0, 2 or 4 µg/mL CHX until they reached mid-exponential phase or for 30 hours depending on the assay. For Kp-125 exposed to 0 and 4 µg/mL only (see dashed blue box), a second exposure to the same concentrations of CHX was performed for 48 hours. Antimicrobial susceptibility testing and virulence assays were performed directly after this second exposure (PRE; post re-exposure). For RNA sequencing, as bacteria needed to be harvested during mid-exponential growth phase, OD_{625nm}-adjusted bacterial suspensions obtained after re exposure were used to inoculate fresh R2A containing CHX concentrations (0 or 4 µg/mL) corresponding to prior exposure. These suspensions were incubated until they reached their mid-exponential growth phase for RNA sequencing.

Isolates were grown overnight in 25 mL TSB at 37 ± 1ºC and suspensions were washed with R2A, OD_{625nm}-adjusted (values ranged from 0.150 to 0.175) and diluted in R2A to reach bacterial density of approximately 1.0 $x10^6$ CFU/mL as detailed in section 2.2.4.1 (page 31). Ten mL from these suspensions was then added to 10 mL of either R2A (0 µg/mL

CHX, control condition) or twice concentrated CHX solutions in R2A corresponding to final CHX concentrations of either 2 (1/4 xMIC) or 4 µg/mL (1/2 xMIC), e.g. 10 mL of bacterial suspension added to 10 mL of a solution containing 4 µg/mL CHX for a final concentration of 2 µg/mL CHX. Suspensions were incubated at 37 ± 1ºC with shaking (150 rpm) until reaching either mid-exponential phase (RNA sequencing) or after 30 hours of culture, enabling bacteria for all tested conditions to reach the plateau phase (antimicrobial susceptibility testing, virulence assay). Based on growth curve studies using the Bioscreen (see chapter 3), both isolates reached mid-exponential phase at OD_{625nm} values of approximately 0.070-0.100. Several studies proposed exposure over 24 hours before performing antimicrobial susceptibility testing (Bore *et al.*, 2007; Henly *et al.*, 2019). It was however not possible in this work as, when growing in 4 µg/mL CHX, both strains could take up to 28 hours to reach the plateau phase.

An additional CHX sub-inhibitory concentration exposure was performed for Kp-125 only at 0 and 4 µg/mL CHX to investigate if an extended exposure could lead to additional or more important responses; several multi-step exposures are described in the literature (Forbes *et al.*, 2019; Henly *et al.*, 2019). After a first 30-hour exposure to either 0 or 4 µg/mL CHX, bacterial suspensions were washed and OD_{625nm}-adjusted as described in the previous paragraph (values ranged from 0.150 to 0.175), before inoculating fresh R2A containing 0 µg/mL CHX if the first exposure was performed in the presence of 0 µg/mL CHX, or 4 µg/mL CHX if the first exposure was performed in the presence of 4 µg/mL CHX. Suspensions were incubated for 48 hours at 37 ºC ± 1ºC. Suspensions were subsequently processed for antimicrobial susceptibility testing and virulence assay (see sections below). As sampling for RNA sequencing had to be performed during mid-exponential growth phase, these suspensions were in parallel used to inoculate CHX-containing R2A, i.e. containing 0 µg/mL CHX if previous exposure was performed in the presence of 0 µg/mL CHX, or containing 4 µg/mL CHX if previous exposure was performed in the presence of 4 µg/mL CHX. These suspensions were incubated at 37 ºC ± 1ºC until mid-exponential growth phase was reached. The control (0 µg/mL) and the highest tested CHX concentration (4 µg/mL) were investigated for the effects post re-exposure (PRE), as most of the observed effects were obtained with 4 µg/mL CHX (see section 3.3.2.2 page 82).

4.2.3 Phenotypic impact of exposure to sub-inhibitory concentrations of chlorhexidine

4.2.3.1 Determination of susceptibility profiles to antimicrobials

After 30 hours of exposure to either 0 (control condition), 2, or 4 μg/mL CHX, the bacterial suspensions were centrifuged at 5,000 g for 10 min at 20ºC. The pellets were resuspended in 10 mL of the corresponding broth (see below) and suspensions were adjusted to OD_{625nm} ranging between 0.150 to 0.175, corresponding to approximately 1.0 x10^8 CFU/mL bacteria. Subsequent steps depended on the protocol followed, as detailed below. Experiments were performed at least in triplicate (n≥3).

4.2.3.1.1 MIC determinations using broth microdilution protocol

Susceptibility testing for BZC and CHX was performed as previously described in section 2.2.4 (page 30) using R2A broth. The OD_{625nm}-adjusted suspensions (range: 0.150-0.175) were diluted 1/100 in R2A (corresponding to approximately 1.0 x10^6 CFU/mL) before inoculation of the microtitre plates. Viable counts were performed as previously detailed (see section 2.2.4.2 page 32). BZC or CHX tested concentrations ranged from 128 to 0.125 μg/mL. The final bacterial concentration corresponded to approximately 5.0 x10^5 CFU/mL. *K. pneumoniae* ATCC 13883 was included as a quality control. Plates were incubated at 37 ± 1ºC and MICs were read after 24 hours and corresponded to the lowest concentrations able to inhibit growth as seen by a naked eye.

MIC determinations for TIG and COL were performed in a similar way, with the use of CAMHB instead of R2A and polystyrene, non-treated, round bottom microtitre plates (Corning, Ewloe, UK) instead of the tissue-culture treated ones following the standard ISO 20776-1:2006 protocol (International Organization for Standardization, 2006). CAMHB was prepared by adding sterile magnesium chloride and calcium chloride solutions to sterile Mueller-Hinton broth (MHB; Oxoid, Basingstoke, UK) in order for it to reach final concentrations of Ca^{2+} and Mg^{2+} of 25 mg/L and 12.5 mg/L, respectively. Stock solutions of 10 mg/mL magnesium chloride (Acros Organics, Geel, Belgium) and 10 mg/mL calcium chloride (Fisher Scientific, Loughborough, UK) were prepared in distilled water and sterilised by filtration through a 0.20 μm cellulose acetate membrane filter. Cation stock solutions and CAMHB were kept at 4 ºC. TIG and COL (both from Sigma-Aldrich, Gillingham, UK) stock solutions were prepared at 640 and 1280 μg/mL, respectively, in distilled water and filtered through a 0.20 μm cellulose acetate membrane filter. The COL

solution was kept at 4 ºC up to 60 days following manufacturer's recommendations. TIG solutions had to be prepared on the day of the testing due to stability issues.

The OD_{625nm}-adjusted suspensions (OD range: 0.150-0.175) were diluted 1/100 in CAMHB (corresponding to approximately 1.0 $x10^6$ CFU/mL) before dispensing to the microtitre plates. Tested concentrations ranged from 32 to 0.03125 µg/mL for both antibiotics. The final bacterial concentration corresponded to approximately 5.0 $x10^5$ CFU/mL. *E. coli* ATCC 25922 (susceptible strain) and *E. coli* NCTC 13846 (COL resistant) were included as quality controls, as recommended by EUCAST (EUCAST, 2019e). MICs were determined after 24 hours incubation at 37 ± 1ºC as mentioned in the previous paragraph. The susceptible, intermediate, or resistant status towards TIG and COL was determined according to EUCAST's breakpoints (EUCAST, 2019b).

4.2.3.1.2 Antibiotic susceptibility testing using disc-diffusion assay

Disc-diffusion assays were performed according to EUCAST's recommendations (EUCAST, 2019a) to assess possible changes in susceptibility profiles following exposure to CHX for other antibiotics, including two aminoglycosides (AK and CN), three carbapenems (IPM, MEM, ETP), three cephalosporins (CAZ, FOX, CPM) and a monobactam (ATM). Disc cartridges were stored at -20ºC before first use, and at 4ºC in the presence of a desiccant when opened. These antibiotics corresponded to the ones for which correlations were observed in the previous chapter (see section 3.3.1.1.2 page 75). Antibiotic-containing and blank (control) discs were purchased from Oxoid (Basingstoke, UK). Mueller-Hinton agar (MHA; Oxoid, Basingstoke, UK) was prepared according to the manufacturer's recommendation, sterilised by autoclave at 121 ± 3ºC for a minimum holding time of 15 min, and 25 mL was poured in 90 mm diameter Petri dishes (corresponding to a 4 mm height of agar as recommended by EUCAST).

After a maximum duration of 15 min after their preparation, each OD_{625nm}-adjusted bacterial suspension (ranging from 0.150 to 0.175, corresponding to approximately 1.0 $x10^8$ CFU/mL) was swabbed onto the surface of the agar in three directions, ensuring no gap was left between the streaks. Antibiotic discs were applied using a disc dispenser (Oxoid, Basingstoke, UK), with a maximum of six discs per agar plate, and within 15 min after swabbing. Discs were gently pressed upon to ensure their close and even contact with the surface of the agar. Plates were incubated for 24 hours at 37 ± 1ºC before measuring the inhibition diameters. *E. coli* ATCC 25922 was included as a quality control (susceptible strain). Inhibition diameters lower than 6 mm could not be measured, as this value

corresponded to the disc diameters. The susceptible, intermediate or resistant status towards the investigated antibiotics was determined according to breakpoints provided by EUCAST (EUCAST, 2019b).

4.2.3.1.3 Statistical analysis

Mean and standard deviation were calculated for each antimicrobial MIC or inhibition diameter, for both strain and under each tested condition. Statistically significant differences were assessed using one-way ANOVA followed by Tukey's multiple comparison test using Prism GraphPad 8 for Windows (significant when $p<0.05$).

4.2.3.2 Virulence assay using the wax moth larvae model *Galleria mellonella*

4.2.3.2.1 Bacterial suspension wash and OD adjustment

After a 30-hour exposure to either 0 (control condition), 2, or 4 µg/mL CHX, the bacterial suspensions were centrifuged at 5,000 g for 10 min at 20ºC. The pellets were resuspended in 10 mL sterile distilled water (sdH_2O; obtained by filtration through a 0.20 µm cellulose acetate membrane filter) and the OD_{625nm} was adjusted to reach a value between 0.150 and 0.175, corresponding approximately to 1.0 $x10^8$ CFU/mL bacteria. Subsequent ten-fold dilutions were prepared in sdH_2O down to 1.0 $x10^6$ CFU/mL. Viable counts were performed as previously described (see section 2.2.4.2 page 32) to control the bacterial concentration.

4.2.3.2.2 Galleria mellonella *maintenance and injection*

The wax moth larvae *Galleria mellonella* is recognised as an alternate infection model to estimate bacterial virulence (Wand *et al.*, 2011). *G. mellonella* larvae (Livefood UK Ltd, Rooks Bridge, UK) were kept in the dark at 4ºC for a maximum of two weeks to avoid the formation of pupae (Leuko & Raivio, 2012). To limit the impact of bacteria naturally occurring on the larvae's skin, they were bathed for few seconds in 70 % (v/v) ethanol prior injection. They were subsequently placed in sterile 90 mm diameter Petri dishes by groups of 10 individuals and allowed to equilibrate to room temperature for an hour before injection (Insua *et al.*, 2013).

G. mellonella larvae were injected with 10 µL of bacterial suspensions containing either 1.0 $x10^8$, 1.0 $x10^7$ or 1.0 $x10^6$ CFU/mL, corresponding to bacterial loads of 10^6, 10^5 and 10^4 CFU, respectively. Injections were performed in the last haemocoel using a

Hamilton syringe with a 22-gauge cemented needle (Hamilton, Birmingham, UK); the syringe was decontaminated with 70 % (v/v) ethanol and rinsed with sterile distilled water (sdH_2O) prior to the first injection and between each injection corresponding to different bacterial test conditions (Leuko & Raivio, 2012; Insua *et al.*, 2013). The injection protocol was validated by including a toxicity control where individuals were injected with 10 µL of 100 % DMSO (Harding *et al.*, 2013; Allegra *et al.*, 2018). Additional controls, including injection of 10 µL sdH_2O, individuals 'stabbed' without injection, and untreated larvae, were also performed to ensure death was not linked to injection trauma or poor health (Insua *et al.*, 2013). A schematic view of the injection protocol is presented in Figure 4-4.

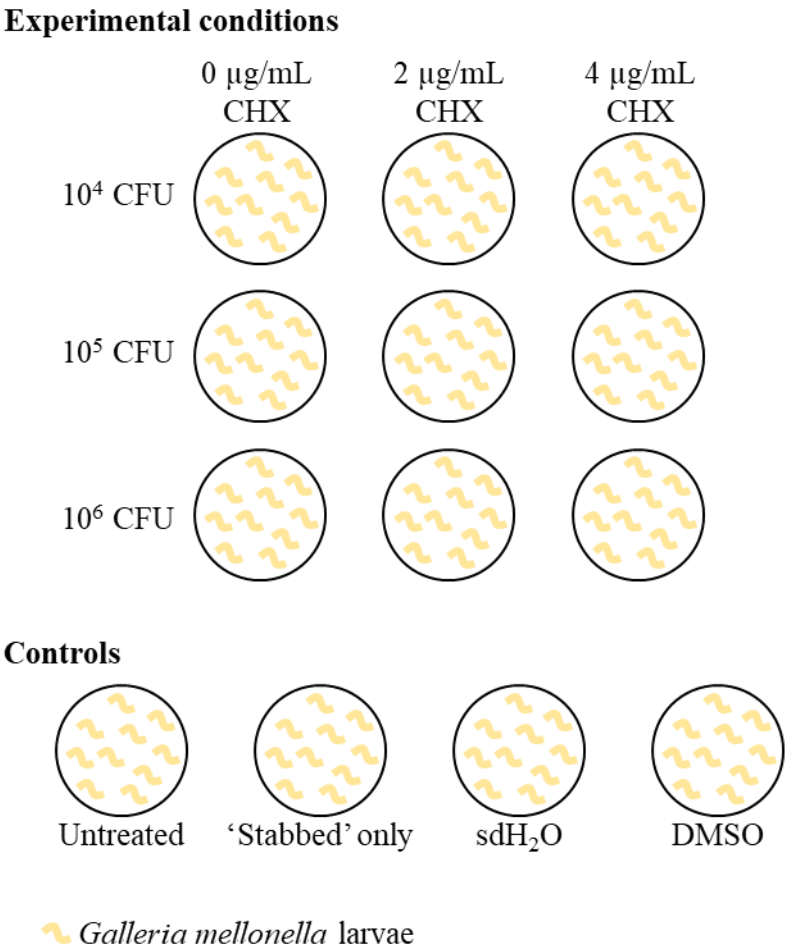

Figure 4-4. Outline of the virulence assay using *G. mellonella* larvae.

G. mellonella larvae were injected with 10 µL of bacterial suspensions containing either 10^4, 10^5 or 10^6 CFU, after exposure to either 0, 2 or 4 µg/mL CHX. Control conditions included untreated larvae, larvae 'stabbed' but without any injection performed, larvae injected with

10 µL sdH_2O and larvae injected with 10 µL 100 % DMSO. Ten larvae were injected for each condition.

After injection, the wax moth larvae were incubated at 37 ± 1ºC and survival was monitored every 24 hours for a period of five days. Larvae were considered dead when no movement was observed after gentle stimulation with a sterile pipette tip (Wand *et al.*, 2011; Insua *et al.*, 2013). Ten larvae were used for each condition, and experiments were performed at least in triplicate (n≥3).

4.2.3.2.3 Statistical analysis

The mean and standard deviation (SD) of *G. mellonella* survival were determined for each tested condition using Prism GraphPad 5 for Windows. The differences observed were assessed by two-way ANOVA followed by Bonferroni's multiple comparison test and were considered significant when $p<0.05$.

4.2.4 Molecular mechanisms involved after exposure to sub-inhibitory concentrations of chlorhexidine

4.2.4.1 Investigation of the presence of plasmids

The presence of plasmids within Kp-1 and Kp-125 was investigated by S1 digestion followed by pulsed field gel electrophoresis (PFGE) with the help of Dr. Refath Farzana (School of Medicine, Cardiff University). The S1 nuclease, purified from *Aspergillus oryzae*, is able to linearize plasmids by targeting supercoiled DNA, enabling the migration of plasmids in agarose gels to their true molecular size (Rittie & Perbal, 2008; Toleman, 2018). The following sections are based on a previously established protocol (Centers for Disease Control and Prevention, 2017; Toleman, 2018).

4.2.4.1.1 Preparation of plugs

Isolates were streaked onto Brilliance UTI (urinary tract infection) Clarity agar (Oxoid, Basingstoke, UK) and grown overnight at 37 ± 1ºC. Two loopfuls of bacteria were then transferred in 2 mL of cell suspension buffer (100 mM Tris, 100 mM EDTA, in ultrapure sterile water, pH 8.0; both from Sigma-Aldrich, Gillingham, UK) and the suspensions OD_{610nm} were adjusted to 0.8-1.0 (approximately 1.0 $x10^9$ CFU/mL bacteria).

Twenty µL of proteinase K (20 mg/mL stock solution, corresponding to 400 µg) was added to 400 µL OD_{610nm}-adjusted suspension (approximately 1.0 $x10^9$ CFU/mL). A subsequent volume of 400 µL of melted agarose (55-60 ºC) was added, and the mixture was poured in plug moulds and enabled to solidify. The agarose was prepared using 1 % SeaKem Gold agarose (Lonza, Slough, UK) in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, in ultrapure sterile water, pH 8.0).

4.2.4.1.2 Bacterial cell lysis in agarose plugs

Each agarose plug (containing bacteria) was transferred into a 5 mL cell lysis solution (50 mM Tris, 50 mM EDTA, 1 % N-Lauroylsarcosine sodium salt [Sigma-Aldrich, Gillingham, UK], pH 8.0) containing 0.1 mg/mL proteinase K. Preparations were incubated for 1.5 to 2 hours at 55ºC with continuous shaking (175 rpm). The lysis buffered was then removed and plugs were washed with 15 mL pre-heated (55ºC) sterile ultrapure water before another 15 min incubation at 55ºC with shaking (step repeated twice). After discarding the water, seven washes were performed with 15 mL pre-heated (55ºC) TE buffer and 15 min incubation at 55ºC with shaking. After the last wash, 10 mL of fresh TE buffer was added to the plugs.

4.2.4.1.3 Digestion with S1 nuclease

Plugs were sliced using a single edge razor blade into a 2.0 to 2.5 mm wide section; one section per isolate was then transferred in 200 µL 1X restriction buffer (Roche Life Science, Welwyn Garden City, UK). The preparation was incubated for 10 min at 37 ± 1ºC and the buffer discarded. A volume of 200 µL of S1 nuclease mix (50 U S1, 20 µg bovine serum albumin, in 1X restriction buffer; Roche Life Science, Welwyn Garden City, UK) was added per plug slice, and preparations were incubated at 37 ± 1ºC for 1.5 to 2 hours.

4.2.4.1.4 Pulsed field gel electrophoresis

The S1 nuclease mix was discarded and 200 µL of 0.5X Tris-borate EDTA (TBE) buffer (5.4 g/L Tris, 2.75 g/L boric acid, 1 mM EDTA, pH 8.0) was added to the plug slices and incubated for 5 minutes at room temperature. Each plug slice was then loaded at the bottom of a well of the PFGE agarose gel (1 % SeaKem gold agarose in 0.5X TBE). The Lambda PFG ladder (New England BioLabs, Ipswich, UK) was also loaded on the gel to enable the determination of plasmid sizes. Wells were sealed with a small volume of warm PFGE agarose gel.

The loaded PFGE agarose gel was then placed into a CHEF DR-II PFGE machine (Bio-Rad, Watford, UK) in the presence of approximately 2 L 0.5X TBE. Ethidium bromide (Sigma-Aldrich, Gillingham, UK) was added to the TBE at a final concentration corresponding to 0.1 μg/mL. The cooling module was set at 10ºC with a pump flow rate of 1 L/min. The electrophoresis parameters corresponded to a voltage of 6 V/cm of gel for a running time of approximately 20 hours. The presence of plasmids was revealed using a UV transilluminator and photographed using a UVP Geldoc II imaging system (UVP, Cambridge, UK).

4.2.4.2 Whole genome sequencing

4.2.4.2.1 Genomic DNA extraction, DNA library preparation and sequencing

The genomic DNA extraction, DNA library preparation and the DNA sequencing were performed by Public Health England prior to this study. DNA was extracted using the Wizard Genomic DNA Purification kit (Promega, Chilworth Southampton, UK) according to the manufacturer's protocol (Greig *et al.*, 2018). DNA libraries were prepared following the manufacturer's recommendations with the Nextera XT DNA library preparation kit and sequenced using a HiSeq 2500 system (both from Illumina, San Diego, USA) with a sequencing depth of approximately 100x per sample (Findlay *et al.*, 2016; Martin *et al.*, 2017).

4.2.4.2.2 Genome assembly and annotation

The WGS data assembly and annotation steps were performed by Nicholas Ellaby (Public Health England). The sequences were assembled into several contigs using SPAdes (http://cab.spbu.ru/software/spades/ accessed on 28/09/2019) with default settings and filtering contigs with a coverage higher or equal to 20x and of length greater than 200 bp (Bankevich *et al.*, 2012), and Prokka (https://github.com/tseemann/prokka accessed on 28/09/2019) was used to annotate the genomes (Seemann, 2014).

4.2.4.3 RNA sequencing

4.2.4.3.1 Total RNA extraction

Bacteria were exposed to either 0 (control), 2 or 4 μg/mL CHX until they reached their exponential growth phase (corresponding to an OD_{600nm} of approximately 0.100). Four replicates were performed for each condition, except stated otherwise (n=4). Suspensions were then snap-frozen by plunging the centrifuge tubes for 1-3 seconds in a mix of absolute ethanol and dry ice (approximately -70ºC) in order to inactivate enzymes and prevent RNA degradation (Salehi & Najafi, 2014). The suspension was vortex-mixed and the operation repeated two additional times before centrifugation at 5,000 g for 10 min at 4ºC. The supernatant was discarded and the pellet frozen at -80ºC until recovered after a maximum of one week for total RNA extraction. The exposure, total RNA extraction and subsequent steps leading to RNA sequencing were performed on four separate replicates, except if stated otherwise (n=4).

The total RNA was extracted using the RiboPure Bacteria kit (Fisher Scientific, Loughborough, UK) according to the manufacturer's instructions. This protocol is based on a phenol extraction followed by glass-fibre filter purification of the total RNA. All centrifugation steps were performed at 16,000 g at 4ºC. The final elution was performed in two steps with an elution of 25 μL each and was followed by DNase I treatment. Samples' quality and concentrations were assessed (see below) and frozen at -80ºC until needed.

The concentration of total RNA recovered was measured using the Qubit RNA Broad-Range assay kit with the Qubit 3.0 reader (Invitrogen-Fisher Scientific, Loughborough, UK) following the manufacturer's instructions. The quality of the samples was assessed using a 2200 TapeStation with an RNA ScreenTape (both from Agilent Technologies, Stockport, UK) according to the manufacturer's instructions.

4.2.4.3.2 Messenger RNA enrichment

The total RNA extracted underwent messenger RNA (mRNA) enrichment using the MICROBExpress kit (Fisher Scientific, Loughborough, UK) according to the manufacturer's instructions. The mRNA enrichment is performed by capturing and removing the 16S and 23S ribosomal RNA (rRNA) from the total RNA population. After measuring their concentrations and evaluating their quality, mRNA-enriched samples were stored at -80ºC until needed.

The concentration of each mRNA-enriched sample was assessed using the Qubit RNA High-Sensitivity assay kit with the Qubit 3.0 reader (Invitrogen-Fisher Scientific, Loughborough, UK). The samples' quality was determined using a 2200 TapeStation with an RNA ScreenTape (both from Agilent Technologies, Stockport, UK) as mentioned above.

4.2.4.3.3 Library preparation and RNA sequencing

The library preparation and sequencing steps were performed by Angela Marchbank (Genome Hub, School of Biosciences, Cardiff University). The mRNA samples were used as templates for the synthesis of complementary DNA enabling the generation of the library preparations using the TruSeq Stranded mRNA sample preparation guide (Illumina, San Diego, USA) with dual index adapters, according to the manufacturer's recommendations. The sequencing was carried using a NextSeq 500 with a high-output cartridge (1x75, single-ended; both from Illumina, San Diego, USA) enabling the generation of approximately 400 million reads in total (corresponding to approximately 13 million reads per sample).

4.2.4.3.4 Data analysis and interpretation

The assembly, annotation and differential expression analysis were performed by Dr. Barbara Szomolay (School of Medicine, Cardiff University) and Thomas Whalley (School of Medicine, Cardiff University). A transcriptome was generated using Trinity with default settings and the '--normalize by read set flag' (Grabherr *et al.*, 2011); reads were trimmed with Trimmomatic (Bolger *et al.*, 2014). For quality control purposes, the raw reads were mapped to the newly created transcriptome using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml accessed on 28/09/2019; Langmead & Salzberg, 2012). As over 80 % of the raw reads mapped to the transcriptome, this suggested the assembly was correct.

The raw transcripts created by Trinity were then annotated using Trinotate (https://github.com/Trinotate/Trinotate.github.io/wiki/Software-installation-and-data-required accessed on 28/09/2019). The reference database was generated following recommendations on the software's website (Haas, 2018). This enabled the creation of a reference database of transcripts from Uniprot (https://www.uniprot.org/ accessed on 28/09/2019), with predicted proteins using Transdecoder (https://github.com/TransDecoder/TransDecoder/releases accessed on 28/09/2019), predicted protein domains using HMMER (http://hmmer.org/ accessed on 28/09/2019, predicted signal peptides using SignalP (http://www.cbs.dtu.dk/cgi-bin/nph-

sw_request?signalp accessed on 28/09/2019), predicted transmembrane regions using TMHMM (http://www.cbs.dtu.dk/cgi-bin/nph-sw_request?tmhmm accessed on 28/09/2019) and rRNA identification using RNAMMER (http://www.cbs.dtu.dk/cgi-bin/sw_request?rnammer accessed on 28/09/2019). These annotations were then merged with the names in the downstream expression matrix with a utility perl script supplied with Trinotate. When several different genes were associated with one sequence, the first one indicated by the software was used for further analysis, as this corresponded to the best hit for its identification. However, no confidence interval or probability was associated to the gene identified by the software.

The raw reads were then mapped onto the transcriptome using Kallisto (https://pachterlab.github.io/kallisto/about accessed on 28/09/2019; Bryant *et al.*, 2017) with default settings and an estimated fragment length of 200 ± 30 bp. Raw reads were also quantified during mapping.

To visualise and detect the presence of outliers, the raw transcript level reads were transformed with the variance stabilizing transform using the DESeq2 R package (https://bioconductor.org/packages/release/bioc/html/DESeq2.html accessed on 28/09/2019; Love *et al.*, 2014). Outliers were identified by using the Mahalanbois distance after performing principal component analysis and removed from the subsequent analyses. Differential expression between the different conditions was performed using the DESeq2 R package: an internal normalization was performed by dividing the counts obtained for a given gene under one specific condition by the mean count measured for the same gene across all exposure conditions, therefore enabling the measurement of $\log_2$ fold-changes in gene expression between two distinct conditions (Love *et al.*, 2014). Genes were considered differentially expressed when they had a Benjamin-Hochberg adjusted p-value lower than or equal to 0.05 and a $\log_2$ fold-change in expression of at least ± 1 (Hassan *et al.*, 2013).

4.3 Results

4.3.1 Phenotypic response after exposure to sub-inhibitory concentrations of chlorhexidine

4.3.1.1 Effects on susceptibility profiles to antimicrobials

The MICs and inhibition diameters obtained for the different investigated antibiotics were within the EUCAST's recommended values (EUCAST, 2019e), except for *E. coli*

ATCC 25922 with COL where the MIC measured (0.11 ± 0.03 µg/mL) was below the range (0.25 to 2 µg/mL). However, the COL MIC obtained for the second reference strain, *E. coli* NCTC 13846 (3.6 ± 1.1 µg/mL) agreed with the EUCAST's target (4 µg/mL, exceptionally 2 or 8 µg/mL).

The mean MIC values measured for TIG, COL, BZC and CHX, before and after exposure to either 0, 2 or 4 µg/mL CHX are grouped in Table 4-2. Overall, no statistically significant differences (one-way ANOVA followed by Tukey's test; $p>0.05$) were obtained for any of the determined MIC when comparing before and after exposure, and between the different exposure conditions, for both isolates. Few statistically significant differences ($p<0.05$) were observed; when comparing the untreated control and all other conditions for BZC MIC in Kp-1, between the untreated control and Kp-1 exposed to 0 µg/mL CHX (R2A only) for CHX, between the untreated control and the PRE conditions for COL in Kp-125 and between the untreated control and the PRE condition (0 µg/mL CHX only) for BZC in Kp-125. However, the fold-changes in MICs were of approximately 2, which correspond to the accepted variability when performing MIC determination (Turnidge & Paterson, 2007). No change in susceptibility (susceptible to resistant or vice-versa) was observed for neither Kp-1 nor Kp-125 under any of the tested conditions.

Table 4-2. TIG, COL, BZC and CHX MICs obtained before and after exposure to 0, 2 or 4 µg/mL CHX.

A), MIC values obtained for Kp-1 before exposure (first column) or after exposure to 0 (Kp-1 + 0), 2 (Kp-1 + 2) or 4 (Kp-1 + 4) µg/mL CHX. B), MIC values obtained for Kp-125 before exposure (first column) or after exposure to 0 (Kp-125 + 0), 2 (Kp-125 + 2) or 4 (Kp-125 + 4) µg/mL CHX. C), MIC values obtained for Kp-125 after a 48-hour re-exposure to 0 (Kp-125 + 0 PRE) or 4 (Kp-125 + 4 PRE) µg/mL CHX. Experiments were performed at least in triplicate ($n\geq3$). A red background indicates a resistant phenotype towards the corresponding antibiotic and a green background indicates susceptibility, according to EUCAST's breakpoints (EUCAST, 2019b). SD: standard deviation.

A	**Kp-1 MIC (µg/mL)**		**Kp-1 + 0 MIC (µg/mL)**		**Kp-1 + 2 MIC (µg/mL)**		**Kp-1 + 4 MIC (µg/mL)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
TIG	8.0	0.0	9.5	4.8	9.9	3.7	6.4	2.2
COL	0.18	0.07	0.13	0.10	0.16	0.07	0.12	0.03
BZC	16.0	0.0	7.6	0.7	7.6	0.7	7.5	1.2
CHX	8.0	0.0	4.7	1.9	5.0	1.7	6.1	1.5

Table 4-2 – *Continued*.

B	Kp-125 MIC (µg/mL)		Kp-125 + 0 MIC (µg/mL)		Kp-125 + 2 MIC (µg/mL)		Kp-125 + 4 MIC (µg/mL)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
TIG	4.0	1.9	3.2	1.0	3.0	1.2	3.3	1.6
COL	0.23	0.15	0.31	0.10	0.35	0.14	0.27	0.10
BZC	16.0	0.0	21.3	6.2	25.3	6.7	26.7	7.5
CHX	8.0	0.0	5.5	2.6	6.0	1.3	7.0	2.0

C	Kp-125 + 0 PRE MIC (µg/mL)		Kp-125 + 4 PRE MIC (µg/mL)	
	Mean	SD	Mean	SD
TIG	5.0	2.0	4.0	0.0
COL	0.50	0.00	0.38	0.05
BZC	32.0	0.0	28.0	5.1
CHX	8.0	0.0	8.0	0.0

The mean inhibition diameters obtained for the different tested antibiotics before and after exposure are presented in Table 4-3. No statistically significant difference (one-way ANOVA followed by Tukey's test; $p>0.05$) in inhibition zone measure was obtained for any antibiotic, for neither Kp-1 nor Kp-125, and under any of the investigated exposure conditions. Similarly, no change in susceptibility towards the different investigated antibiotics was obtained after exposure to the different CHX concentrations investigated.

Table 4-3. Antibiotic inhibition diameters obtained before and after exposure to 0, 2 or 4 µg/mL CHX

A), Inhibition diameters obtained for Kp-1 before exposure (first column) or after exposure to 0 (Kp-1 + 0), 2 (Kp-1 + 2) or 4 (Kp-1 + 4) µg/mL CHX. B), Inhibition diameters obtained for Kp-125 before exposure (first column) or after exposure to 0 (Kp-125 + 0), 2 (Kp-125 + 2) or 4 (Kp-125 + 4) µg/mL CHX. C), Inhibition diameters obtained for Kp-125 after a 48-hour re-exposure to 0 (Kp-125 + 0 PRE) or 4 (Kp-125 + 4 PRE) µg/mL CHX. Experiments were carried at least in triplicate (n≥3). A red background indicates a resistant phenotype towards the corresponding antibiotic and a green background indicates susceptibility, according to EUCAST's breakpoints (EUCAST, 2019b). AK: amikacin; ATM: aztreonam; CAZ: ceftazidime; CIP: ciprofloxacin; CN: gentamycin; CPM: cefepime; ETP: ertapenem; FOX: cefoxitin; IPM: imipenem; MEM: meropenem; SD: standard deviation.

A	**Kp-1 Inhibition (mm)**		**Kp-1 + 0 Inhibition (mm)**		**Kp-1 + 2 Inhibition (mm)**		**Kp-1 + 4 Inhibition (mm)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
IPM	14.3	1.2	15.6	0.9	16.0	0.9	15.8	1.2
MEM	13.0	1.0	14.8	1.0	14.5	1.6	14.8	0.8
ETP	10.3	0.6	11.1	0.6	10.6	2.0	11.2	0.8
ATM	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
CIP	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
CAZ	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
CPM	12.3	0.6	11.9	1.4	12.5	0.5	12.2	1.2
FOX	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
AK	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
CN	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0

B	**Kp-125 Inhibition (mm)**		**Kp-125 + 0 Inhibition (mm)**		**Kp-125 + 2 Inhibition (mm)**		**Kp-125 + 4 Inhibition (mm)**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
IPM	8.0	0.0	9.5	0.6	9.8	0.5	9.0	0.0
MEM	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
ETP	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
ATM	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
CIP	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
CAZ	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
CPM	7.3	0.6	7.3	0.5	7.3	0.5	7.0	0.0
FOX	6.0	0.0	6.0	0.0	6.0	0.0	6.0	0.0
AK	14.0	0.0	14.5	0.6	14.3	0.5	14.5	0.6
CN	18.3	0.6	19.0	0.0	18.8	0.5	19.0	0.0

Table 4-3 – *Continued*.

C	Kp-125 + 0 PRE Inhibition (mm)		Kp-125 + 4 PRE Inhibition (mm)	
	Mean	SD	Mean	SD
IPM	10.0	0.8	9.8	0.5
MEM	6.0	0.0	6.0	0.0
ETP	6.0	0.0	6.0	0.0
ATM	6.0	0.0	6.0	0.0
CIP	6.0	0.0	6.0	0.0
CAZ	6.0	0.0	6.0	0.0
CPM	7.0	0.0	7.3	0.5
FOX	6.0	0.0	6.0	0.0
AK	14.8	0.5	14.0	0.0
CN	18.8	0.5	19.0	0.8

4.3.1.2 Effects on virulence

The detailed results obtained for the virulence assay are presented in appendix XII. The untreated, 'stabbed' (no injection performed) and injected with sdH_2O groups harboured survival rates of 97.5, 96.3 and 100 % after five days of incubation, respectively. All the larvae injected with DMSO had died 24 hours after injection. Important variability in survival rates was measured for all the isolates-CHX exposure-bacterial loads combinations. No statistically significant (two-way ANOVA followed by Bonferroni's post-test; $p>0.05$) difference was observed between the different conditions regardless of the isolate when larvae were injected with 10^4 CFU, with survival rates ranging from 83.3 to 96.0 % after five days of incubation. For both bacterial isolates, and under all the tested conditions, significantly higher (Bonferroni's post-test; $p<0.05$) death rates were obtained with increasing bacterial loads.

The results obtained for Kp-1 are presented in Figure 4-5. For a set bacterial load, no statistically significant differences were observed in virulence when Kp-1 was exposed to the different CHX concentrations.

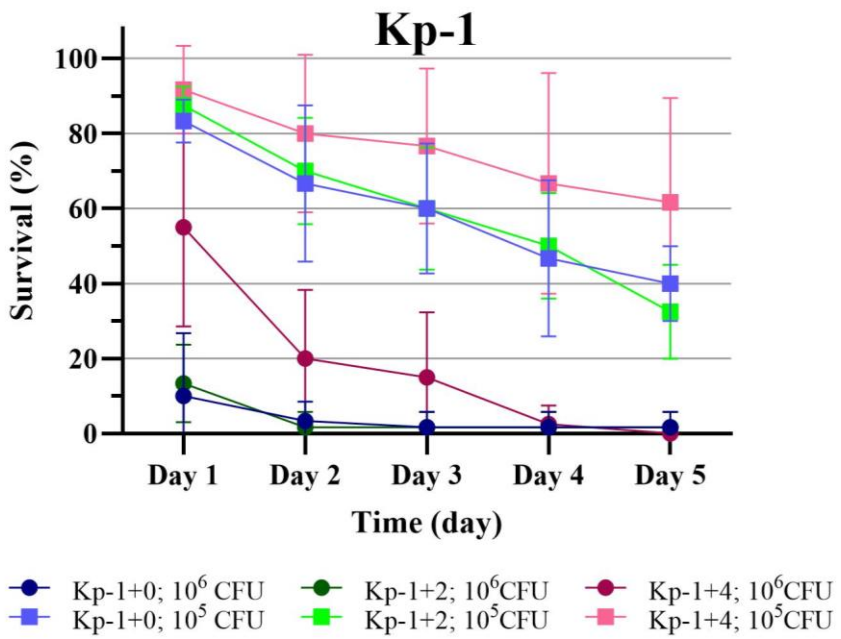

Figure 4-5. *G. mellonella* larvae survival rates after injection of several bacterial loads of Kp-1 previously exposed to different CHX concentrations.

Experiments were carried out at least in triplicate (n≥3). Survival rates (in %) were measured after one to five days incubation following injection of either 10^6, 10^5 or 10^4 CFU of Kp-1 previously exposed to either 0 (Kp-1+0), 2 (Kp-1+2) or 4 (Kp-1+4) µg/mL CHX. Reported values corresponded to mean and standard deviation obtained for each condition.

The results obtained for Kp-125 are presented in Figure 4-6. When 10^6 CFU were injected, a significant (Bonferroni's post-test; $p<0.05$) decrease in survival was observed with increasing concentrations of CHX the isolate was exposed to, for the first exposure only (no difference was obtained in survival rate when comparing secondary exposure to either 0 or 4 µg/mL CHX at this bacterial load). This phenomenon was however not observed when 10^5 CFU were injected. No statistically significant differences were observed in virulence after re-exposure between exposure 0 and 4 µg/mL CHX (Bonferroni's post-test; $p>0.05$). For a set CHX concentration, survival rates were found to be significantly lower after re-exposure for 0 µg/mL (R2A only) when compared to virulence measured after the first exposure to the same concentration (with 10^6 CFU only). The virulence observed in Kp-125 after re-exposure to 4 µg/mL CHX with a bacterial load of 10^5 CFU was significantly less important than the one measured for Kp-125 after the first exposure to the same CHX concentration.

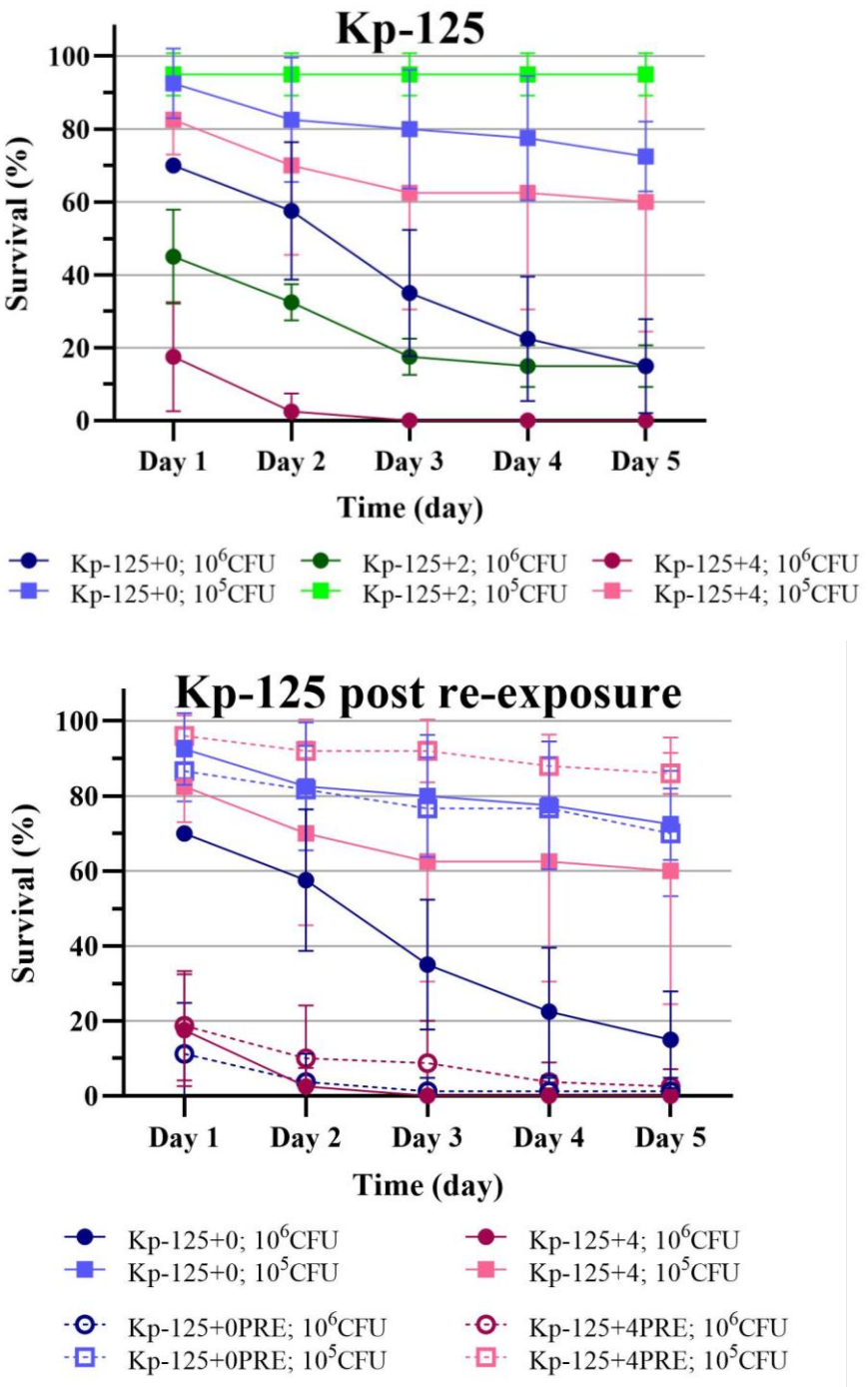

Figure 4-6. *G. mellonella* larvae survival rates after injection of several bacterial loads of Kp-125 previously exposed to different CHX concentrations.

Experiments were carried out at least in triplicate (n≥3). Survival rates (in %) were measured after one to five days incubation following injection of either 10^6, 10^5 or 10^4 CFU of Kp-125 previously exposed to either 0 (Kp-125+0), 2 (Kp-125+2) or 4 (Kp-125+4) µg/mL CHX, or after a second exposure to 0 (Kp-125+0PRE) or 4 (Kp-125+4PRE) µg/mL CHX. Reported values corresponded to mean and standard deviation obtained for each condition.

When comparing both isolates, Kp-1 was found to be significantly (Bonferroni's post-test; $p<0.05$) more virulent than Kp-125. Indeed, the survival rates observed when injecting 10^6 CFU after exposure to any of the CHX concentrations were lower in Kp-1 than in Kp-125 (similar observations were made with a bacterial load of 10^5 CFU).

4.3.2 Molecular changes after exposure to sub-inhibitory concentrations of chlorhexidine

4.3.2.1 Detection of plasmids

Several plasmids were detected by PFGE following S1 digestion for both isolates, as illustrated in Figure 4-7. The top, very bright band observed in both lanes corresponded to the chromosomal DNA. Three bands were observed for Kp-1 (approximately 340 kb, 240 kb and 190 kb) and four bands were obtained for Kp-125 (approximately 194 kb, 150 kb, 97 kb and < 48 kb).

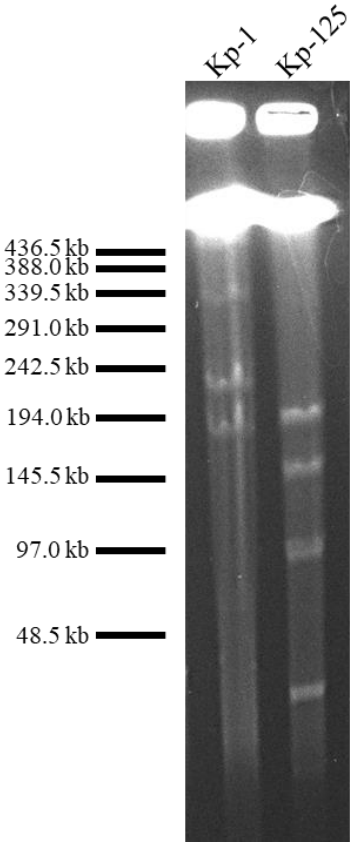

Figure 4-7. Plasmid detection using PFGE following S1 digestion for Kp-1 and Kp-125. S1 digestion linearized the isolates' plasmids, enabling them to migrate to their true molecular size on the electrophoresis gel.

4.3.2.2 Whole genome sequencing

An overall of 495,706 and 247,697 bp were obtained from WGS for Kp-1 and Kp-125, respectively. These numbers went down to 487,543 and 245,509 when considering contigs with a minimum coverage of 20x and a minimum length of 200 bp. This represented however only a small fraction (<10 %) of the expected genome size; indeed, another *K. pneumoniae* ST258 isolate possesses a chromosome of approximately 5,400,000 bp (GenBank access number: LAKK01000012.1). Because of this, a *de novo* transcriptome assembly was preferred for the differential expression analysis instead of mapping the reads to a reference genome.

4.3.2.3 RNA sequencing

The genes for which expression was statistically significant (Benjamin-Hochberg adjusted p-value; $p<0.05$, and $\log_2$ fold-change of at least ± 1.0) between the different exposure conditions for Kp-1 are grouped in Table 4-4. Overall, more genes had their expression altered by exposure to 4 μg/mL than by 2 μg/mL CHX when compared to the control condition (0 μg/mL CHX). When the same gene's expression was altered under both CHX treatments, the fold-change in expression was equivalent or more important at 4 μg/mL CHX. A wide range of cellular activities were impacted by exposure to CHX, including various metabolism pathways, cellular respiration and electron transfer (upregulation up to 2 $\log_2$ fold-change after exposure to both CHX concentrations when compared to the control), stress response (mainly upregulation after CHX exposure) and efflux and transport activities (mainly upregulation after exposure to 4 μg/mL CHX). Regarding efflux, several genes encoding multi-drug efflux pumps were found to be upregulated after exposure to 4 μg/mL CHX, including *acrD* ($\log_2$ fold-change: 1.1), *mdtB* (1.2) and the cationic efflux pump-encoding gene *smvA* (3.5). The expressions of genes encoding a DNA polymerase (*dnaE*) and a DNA gyrase (*gyrA*) involved in cell replication activities were found to be downregulated after CHX treatment. The expression of genes encoding for chaperonins were upregulated, except for *asmA* which expression was downregulated (involved in the prevention of misfolding for outer membrane protein mutants). Genes encoding proteins involved or associated with ribosomes were globally downregulated. The production of fimbriae was decreased after CHX treatment, and degradation of putrescine was increased. No change in the expression profiles of global regulators including *mar* and *sox* were detected, but increased expression of *phnM* (part of the Pho regulon) was observed after exposure to both CHX concentrations.

Table 4-4. Differential expression analysis between exposure to different CHX concentrations for Kp-1.

Exposure of Kp-1 to either 0 or 4 μg/mL CHX was performed in quadruplicate (n=4), and exposure to 2 μg/mL CHX was performed in triplicate (one outlier removed; n=3). Differential expression analysis was performed by comparing Kp-1 exposed to 2 μg/mL CHX (Kp-1 2/0) or exposed to 4 ug/mL CHX (Kp-1 4/0) to the control condition (0 μg/mL CHX). Differential expression was expressed in $\log_2$ fold-change (FC). Only genes for which the differential expression was statistically significant (Benjamin-Hochberg adjusted p value; $p<0.05$) and with at least ± 1 $\log_2$ fold-change are presented in this table. Upregulated genes are highlighted in green, and downregulated genes in red. When numbers are indicated in brackets, it means several different sequences were identified as the same gene in the database. ns: not significant and/or with a $\log_2$ fold-change lower than ± 1; OM: outer membrane; OMP: outer membrane protein; rRNA: ribosomal RNA; tRNA: transfer RNA.

Gene name	Kp-1 2/0 $\log_2$ FC Mean	SD	Kp-1 4/0 $\log_2$ FC Mean	SD	Associated function	Reference
Antibiotic resistance						
ampC	ns		-1.0	0.2	β-lactamase	Jacoby, 2009
Cellular respiration / electron transfer						
fdhF	ns		1.5	0.6	Formate dehydrogenase	Wang & Gunsalus, 2003
fdnG	ns		2.1	0.7	Formate dehydrogenase	Wang & Gunsalus, 2003
fhlA	ns		1.3	0.5	Formate dehydrogenase	Maupin & Shanmugam, 1990
narK	ns		2.0	0.7	Nitrate/nitrite transporter	Bueno *et al.*, 2012
narU	1.8	0.2	2.1	0.1	Nitrate/nitrite transporter	
narZ	ns		1.3	0.4	Nitrate reductase	
nasR	1.9	0.6	1.8	0.5	Nitrate regulation	Goldman *et al.*, 1994
nfsB	ns		1.5	0.1	Nitrate reductase	Rau & Stolz, 2003
Cell wall synthesis and/or maintenance						
dapE	-1.1	0.3	ns		Diaminopimelic acid synthesis	Wu *et al.*, 1992
mltC	1.0	0.3	ns		Murein transglycolase	Artola-Recolons *et al.*, 2014
DNA modification and/or transfer						
dnaE	ns		-1.4	0.4	DNA polymerase	Tomasiewicz & McHenry, 1987
gyrA	ns		-1.6	0.4	DNA gyrase	Pourahmad Jaktaji & Mohiti, 2010
mukB	10.5	1.7	12.1	1.6	Chromosome partition	Niki *et al.*, 1992

Table 4-4 – *Continued*.

Gene name	Kp-1 2/0 log_2 FC Mean	SD	Kp-1 4/0 log_2 FC Mean	SD	Associated function	Reference
Efflux and transport						
acrB	ns		-1.4	0.5	Multi-drug efflux pump, RND family	Alav *et al.*, 2018
acrD	ns		1.1	0.3	Multi-drug efflux pump, RND family	Alav *et al.*, 2018
bepG	ns		1.6	0.2	Multi-drug efflux pump, RND family (*Brucella* spp.)	Martin *et al.*, 2009
mchF	ns		1.1	0.3	Microcin secretion, bacteriocin transport	Poey *et al.*, 2006
mdtB	ns		1.2	0.3	Multi-drug efflux pump, RND family	Kim & Nikaido, 2012
mdtM	ns		1.1	0.2	Multi-drug efflux pump, MFS family	Krizsan *et al.*, 2015
rbtT	ns		1.4	0.3	Ribitol transporter, MFS family	Pao *et al.*, 1998
smvA	3.1	0.4	3.5	0.3	Cationic compounds efflux pump, MFS family	Villagra *et al.*, 2008
Stress response						
acnA	ns		1.1	0.2	RNA regulatory protein linked to oxidative stress	Gruer & Guest, 1994
adhP	ns		1.4	0.2	Alcohol dehydrogenase linked to DNA damage	Khil & Camerini-Otero, 2002
gcvA	-1.1	0.3	ns		Transcriptional activator linked to UV exposure	Sargentini *et al.*, 2016
otsA	ns		1.0	0.2	Involved in trehalose synthesis and osmoprotection	Joseph *et al.*, 2010
Transcription, translation and post-translation modifications						
asmA	ns		-1.3	0.2	Prevents misfolding of mutant OMPs	Prieto *et al.*, 2009
groL (1)	1.2	0.4	1.2	0.3	Chaperonin	Goltermann *et al.*, 2015
groL (2)	1.2	0.3	1.2	0.3		
groL (3)	1.6	0.5	1.5	0.4		
lysS	ns		1.9	0.5	Lysine-tRNA ligase	Leveque *et al.*, 1990
rbbA	ns		-1.6	0.6	Ribosome-associated ATPase	Ganoza & Kiel, 2001
rlhA	-6.0	1.9	ns		Modification of 23S rRNA	Kimura *et al.*, 2017
Virulence-associated						
fimD (1)	ns		-11.1	3.0	Involved in export/assembly of fimbriae	Nishiyama *et al.*, 2008
fimD (2)	ns		-3.1	0.8		
fimD (3)	ns		-1.3	0.3		
puuB	ns		1.5	0.2	Involved in putrescine degradation	Nemoto *et al.*, 2012

Table 4-4 – *Continued*.

Gene name	Kp-1 2/0 log_2 FC Mean	SD	Kp-1 4/0 log_2 FC Mean	SD	Associated function	Reference
Other						
aldA	1.3	0.4	1.6	0.4	Lactaldehyde dehydrogenase	Limon *et al.*, 1997
argI	ns		1.1	0.3	Involved in arginine biosynthesis	Celis, 1977
bisC	-2.1	0.4	-1.3	0.4	Biotin sulfoxide reductase	Pierson & Campbell, 1990
budB	ns		1.7	0.4	Acetolactate synthase	Blomqvist *et al.*, 1993
cbiD	ns		1.1	0.4	Involved in cobalamin synthesis	Moore *et al.*, 2013
cbiF	ns		1.7	0.5	Involved in cobalamin synthesis	
crnA	ns		1.3	0.5	Creatinine amidohydrolase	Beuth *et al.*, 2003
cysI	ns		4.7	1.4	Sulfite reductase	Ostrowski *et al.*, 1989
cysN	ns		-1.2	0.3	Sulfate adenylyl-transferase	Hummerjohann *et al.*, 1998
dcp	ns		2.8	0.9	Carboxypeptidase	Henrich *et al.*, 1993
eutB	1.7	0.5	2.0	0.5	Ethanolamine degradation	Bovell & Warncke, 2013
gapA	ns		1.1	0.2	Involved in glycolysis and glyconeogenesis	Charpentier & Branlant, 1994
gltA	-1.2	0.4	-1.0	0.3	Citrate synthase	Park *et al.*, 1994
mtfA	-1.1	0.3	-1.2	0.3	Glucose-related peptidase, involved in entry into stationary growth phase	Gohler *et al.*, 2012
phnM	1.2	0.4	1.7	0.3	Part of the Pho regulon, global regulation	Jochimsen *et al.*, 2011; Santos-Beneit, 2015
rutG	ns		2.4	0.6	Pyrimidine permease	Kozmin *et al.*, 2013
Unknown / putative						
ybgA	3.0	0.9	ns		Uncharacterized protein	UniProt (YBGA_ECOLI)
ybhD	ns		1.1	0.3	Uncharacterized, putative transcriptional regulator	Flores-Bautista *et al.*, 2018
ydhK	ns		1.7	0.4	Uncharacterized protein	UniProt (YDHK_ECOLI)
ydhS	ns		1.9	0.6	Uncharacterized protein	UniProt (YDHS_ECOLI)
yejA	2.6	0.7	ns		Uncharacterized, might be part of the ABC transporter family	Vondenhoff *et al.*, 2011
ygaD	ns		1.7	0.5	Uncharacterized protein	UniProt (Q1R800_ECOUT)
yhdP	1.0	0.3	ns		Uncharacterized, might be involved in OM permeability	Mitchell *et al.*, 2017; Mitchell *et al.*, 2018

The genes for which expression was statistically significant (Benjamin-Hochberg adjusted p-value; $p<0.05$, and $\log_2$ fold-change of at least ± 1.0) between the different exposure conditions (first exposure only) for Kp-125 are grouped in Table 4-5. Similarly to Kp-1, more genes were differentially expressed after exposure to 4 μg/mL than 2 μg/mL when compared to the control condition (0 μg/mL CHX), and when the expression of a gene was altered in both conditions, the effect was mainly more important after exposure to 4 μg/mL CHX than to 2 μg/mL CHX. The impact of CHX exposure on the different cellular activities seemed more contrasted, with genes linked to the same cellular activity found both upregulated and downregulated. As seen with Kp-1, most genes involved in cellular respiration and several multi-drug efflux pump-encoding genes were found to be upregulated, especially after exposure to 4 μg/mL CHX: *acrD* ($\log_2$ fold-change: 1.2), *emrD* (1.3 after exposure to 2 μg/mL CHX) and *smvA* (1.9). However, genes encoding proteins related to iron transport were downregulated. The expression of several DNA-related genes (DNA polymerase and gyrase, helicase, integrase, transposase, recombinase) was found to be downregulated after CHX exposure, while other were upregulated (genes related to plasmid transfer and conjugation, mismatch repair). An overall decrease of the expression of genes involved in transcriptional, translational and post-translational modifications, and related to virulence, was observed. The expression of *phnM*, as previously seen for Kp-1, was also increased after CHX exposure in Kp-125.

Table 4-5. Differential expression analysis between exposure to different CHX concentrations for Kp-125.

Exposure of Kp-125 to either 0, 2 or 4 μg/mL CHX was performed in quadruplicate (n=4). Differential expression analysis was performed by comparing Kp-125 exposed to 2 μg/mL CHX (Kp-125 2/0) or exposed to 4 ug/mL CHX (Kp-125 4/0) to the control condition (0 μg/mL CHX). Differential expression was expressed in $\log_2$ fold-change (FC). Only genes for which the differential expression was statistically significant (Benjamin-Hochberg adjusted p value; $p<0.05$) and with at least ± 1 $\log_2$ fold-change are presented in this table. Upregulated genes are highlighted in green, and downregulated genes in red. When numbers are indicated in brackets, it means several different sequences were identified as the same gene in the database. ns; not significant and/or with a $\log_2$ fold-change lower than ± 1.

Gene name	Kp-125 2/0 $\log_2$ FC Mean	SD	Kp-125 4/0 $\log_2$ FC Mean	SD	Associated function	Reference
Cellular respiration / electron transfer						
ccmF	ns		-1.1	0.3	Cytochrome c synthesis	Thony-Meyer *et al.*, 1995
hemA	ns		-9.6	2.4	Involved in heme synthesis	Darie & Gunsalus, 1994
narU	1.1	0.2	1.7	0.2	Nitrate/nitrite transporter	Bueno *et al.*, 2012
nfsB	ns		1.2	0.3	Nitrate reductase	Rau & Stolz, 2003
pntB	7.9	1.6	10.3	1.6	NAD(P) transhydrogenase	Clarke *et al.*, 1986
Cell wall synthesis and/or maintenance						
nlpC	ns		1.5	0.4	Endopeptidase involved in peptidoglycan turnover	Xu *et al.*, 2015
rfbD	1.4	0.4	1.6	0.4	Involved in O antigen synthesis	Boels *et al.*, 2004
DNA modification and/or transfer						
dnaE	ns		-1.1	0.2	DNA polymerase	Tomasiewicz & McHenry, 1987
gyrA (1)	ns		-1.8	0.5	DNA gyrase	Pourahmad Jaktaji & Mohiti, 2010
gyrA (2)	ns		-1.4	0.5		
helD	-2.4	0.8	ns		DNA helicase	Mendonca *et al.*, 1993
intB (1)	-1.0	0.2	-1.1	0.2	Integrase	Hochhut *et al.*, 2006
intB (2)	ns		-1.1	0.2		
mobA	1.5	0.5	2.1	0.5	Involved in plasmid transfer	Henderson & Meyer, 1999
mukB	1.4	0.5	2.2	0.5	Chromosome partition	Niki *et al.*, 1992
mutS	4.9	1.2	6.0	1.2	Mismatch repair	Acharya *et al.*, 2003
recC	-1.4	0.4	-1.1	0.4	DNA repair and recombination	Myers & Stahl, 1994
sopA	1.0	0.2	ns		Plasmid partition	Kim & Shim, 1999
tnpA	-2.1	0.5	-1.4	0.4	Transposase	Horak & Kivisaar, 1998
traH	2.2	0.6	2.7	0.6	Involved in F pilus formation (conjugation)	Arutyunov *et al.*, 2010
xerC	ns		-1.1	0.3	Recombinase	Cornet *et al.*, 1997

Table 4-5 – *Continued*.

Gene name	Kp-125 2/0 log_2 FC Mean	SD	Kp-125 4/0 log_2 FC Mean	SD	Associated function	Reference
Efflux / transport						
acrD	ns		1.2	0.3	Multi-drug efflux pump, RND family	Alav *et al.*, 2018
appF	1.9	0.4	2.7	0.4	Oligopeptides ABC transporter (*Bacillus* spp.)	Koide & Hoch, 1994
aroP	2.0	0.6	ns		Permease involved in aromatic amino acid transport	Cosgriff & Pittard, 1997
emrD	1.3	0.4	ns		Multi-drug efflux pump, MFS family	Alav *et al.*, 2018
entE	-1.9	0.5	-1.6	0.5	Enterobactin synthesis (iron transport)	Raymond *et al.*, 2003
entF	-1.8	0.5	ns			
fcuA	ns		-1.0	0.1	Associated with iron transport	Ozenberger *et al.*, 1987; Miethke & Marahiel, 2007; Grim *et al.*, 2012
fepA	-2.5	0.6	ns			
fepC	-2.0	0.4	-1.6	0.4		
fes	-2.1	0.6	ns			
kdpA	ns		1.6	0.4	P-type ATPase, involved in cation transport (potassium)	Sardesai & Gowrishankar, 2001
pgtP	ns		1.2	0.2	Phosphoglycerate transport, MFS family	Varadhachary & Maloney, 1991
rbtT (1)	ns		1.7	0.3	Ribitol transporter, MFS family	Pao *et al.*, 1998
rbtT (2)	1.6	0.4	2.5	0.4		
smvA	1.4	0.3	1.9	0.3	Cationic compounds efflux pump, MFS family	Villagra *et al.*, 2008
ssuA	ns		-1.2	0.4	ABC transporter involved in uptake of sulfonates	Eichhorn *et al.*, 2000
trkA	-2.2	0.6	ns		Involved in potassium uptake	Bossemeyer *et al.*, 1989
Stress response						
bluF	ns		1.0	0.2	Antirepressor linked to blue light exposure	Tschowri *et al.*, 2009
cspE	1.1	0.3	ns		Involved in acclimatation of nutritionally starved bacteria in fresh media	Bae *et al.*, 1999
otsA	ns		1.1	0.1	Involved in trehalose synthesis and osmoprotection	Joseph *et al.*, 2010

Table 4-5 – *Continued*.

Gene name	Kp-125 2/0 log_2 FC Mean	SD	Kp-125 4/0 log_2 FC Mean	SD	Associated function	Reference
Transcription, translation and post-translation modifications						
epmA	ns		-1.6	0.6	Translation elongation factor aminoacetylase	Vivijs *et al.*, 2016
ileS	ns		-3.5	1.3	Isoleucine-tRNA ligase	Chalker *et al.*, 1994
lysS	1.8	0.2	ns		Lysine-tRNA ligase	Leveque *et al.*, 1990
rapA (1)	ns		-1.1	0.2	RNA polymerase-associated protein	Sukhodolets & Jin, 1998
rapA (2)	ns		1.9	0.5		
rlhA	ns		-23.6	2.9	Modification of 23S rRNA	Kimura *et al.*, 2017
rplA	ns		-4.0	1.4	50S ribosomal protein L1 synthesis	Fu *et al.*, 2013
Virulence associated						
fimD	-1.1	0.3	-1.2	0.3	Involved in export/assembly of fimbriae	Nishiyama *et al.*, 2008
gss	ns		-3.1	1.1	Involved in spermidine metabolism	Jelsbak *et al.*, 2012; Chattopadhyay *et al.*, 2013
hcpA	1.3	0.4	ns		Fimbriae synthesis	Hernandes *et al.*, 2011
higA	ns		-2.8	1.0	Antitoxin	Wood & Wood, 2016
yefM	ns		1.1	0.3	Antitoxin	Cherny & Gazit, 2004
Other						
acs	ns		-22.3	3.9	Acetyl-coenzyme A synthetase	Kumari *et al.*, 2000
agP	ns		1.0	0.2	Glucose-1-phosphatase	Pradel & Boquet, 1989
bisC	ns		-4.0	1.3	Biotin sulfoxide reductase	Pierson & Campbell, 1990
can	ns		1.9	0.7	Carbonic anhydrase	Merlin *et al.*, 2003
carB	1.4	0.2	1.2	0.2	Carbamoyl phosphate synthetase	Nyunoya & Lusty, 1983
cysI	ns		1.7	0.6	Sulfite reductase	Ostrowski *et al.*, 1989
dxs	ns		4.0	0.9	Involved in pyruvate degradation	Handa *et al.*, 2013
glnE	2.1	0.4	1.3	0.4	Glutamine synthetase adenylyl-transferase	Rhee *et al.*, 1985
guaA	-1.3	0.4	-1.4	0.4	Guanosine monophosphate synthetase	Tiedeman *et al.*, 1985
idi	7.6	2.0	ns		Isopentenyl disphosphate isomerase	Hahn *et al.*, 1999
livF	1.1	0.2	ns		Involved in aromatic amino acid accumulation	Koyanagi *et al.*, 2004
lsrK	-6.8	1.9	ns		Transcriptional regulator involved in quorum sensing	Xavier & Bassler, 2005
mscK	1.2	0.4	ns		Mechanosensitive channel involved in ion regulation	Li *et al.*, 2002

Table 4-5 – *Continued*.

Gene name	Kp-125 2/0 log_2 FC Mean	SD	Kp-125 4/0 log_2 FC Mean	SD	Associated function	Reference
Other (*continued*)						
phnM	1.1	0.2	1.5	0.2	Part of the Pho regulon, global regulation	Jochimsen *et al.*, 2011; Santos-Beneit, 2015
pta	1.1	0.4	1.1	0.4	Phosphate acetyl-transferase involved in acetate metabolism	Castano-Cerezo *et al.*, 2009
Unknown / putative						
no name	3.7	1.2	ns		Putative transposase for insertion sequence	UniProt
ybgA	-1.1	0.4	ns		Uncharacterized protein	UniProt (YBGA_ECOLI)
ybhD	ns		1.3	0.5	Uncharacterized, putative transcriptional regulator	Flores-Bautista *et al.*, 2018
yejM	4.9	1.3	4.5	1.3	Inner membrane protein (unknown function)	De Lay & Cronan, 2008
yhfK	ns		4.2	1.5	Uncharacterized protein	UniProt (YHFK_ECOLI)
yjiR	1.2	0.4	ns		Uncharacterized, putative transcriptional regulator	Flores-Bautista *et al.*, 2018
yusV	-1.2	0.2	ns		ATPase associated with iron transport (*Bacillus* spp.)	Ollinger *et al.*, 2006

The results obtained for the differential expression analysis after re-exposure to either 0 or 4 µg/mL CHX for Kp-125 are grouped in Table 4-6. Repeated exposure in R2A only (Kp-125 0PRE/0) seemed to have an impact on a wide range of biological functions, including increased expression of genes involved in cellular respiration and electron transfer, in DNA-related functions, and in iron transport, but not in efflux (no change in *acrB* expression, decreased expression for *smvA*). However, only two replicates were obtained for the second exposure to 0 µg/mL CHX (Kp-125 + 0PRE), which could bias the analysis. Few significant changes were observed when comparing the first and second exposure to 4 µg/mL CHX (Kp-125 4PRE/4), with the most pronounced being an increased expression of *rlhA* (log_2 fold-change: 22.7), involved in translation, and *acs* (19.4), involved in the biosynthesis of acetyl coenzyme A. When compared to the first exposure, the second exposure to 4 µg/mL CHX resulted in less differentially expressed genes (based on a first or second 4 µg/mL CHX exposure *versus* 0 µg/mL CHX exposure differential expression analysis) but with similar effects, e.g. increased expression of respiration-associated genes and of *smvA*. The second exposure to 4 µg/mL CHX had an important impact on a wide range of cellular functions when compared to the second exposure to 0 µg/mL CHX (Kp-125 4PRE/0PRE): cellular respiration and electron transfer-related genes were found to be upregulated, as were efflux

and transport-related genes (including *acrD*, *silA* and *smvA*) except for iron transport, and genes encoding proteins involved in transcriptional, translational or post-translational modifications. Similarly to the differences observed after single exposure to both 0 or 4 μg/mL CHX, chaperonin-encoding genes and several DNA-related genes were found to be downregulated (helicase, integrase, replication initiation, recombinase) while genes related to plasmid transfer and mismatch repair were upregulated.

Table 4-6. Differential expression analysis between before and after re-exposure to different CHX concentrations for Kp-125.

Exposure of Kp-125 to either 0, 2 or 4 µg/mL CHX was performed in quadruplicate (n=4); re-exposure to 0 µg/mL was only obtained over two different experiments (n=4), while re-exposure to 4 µg/mL CHX was performed in quadruplicate (n=4). Differential expression analysis was performed by comparing Kp-125 before and after re-exposure to 0 µg/mL CHX (Kp-125 0PRE/0), before and after re-exposure to 4 µg/mL CHX (Kp-125 4PRE/4), and by comparing Kp-125 re-exposed to 4 µg/mL CHX with the control condition (one exposure to 0 µg/mL CHX; Kp-125 4PRE/0) and with re-exposure to 0 µg/mL CHX (Kp-125 4PRE/0PRE). Differential expression was expressed in $\log_2$ fold-change (FC). Only genes for which the differential expression was statistically significant (Benjamin-Hochberg adjusted p value; $p<0.05$) and with at least ± 1 $\log_2$ fold-change are presented in this table. Upregulated genes are highlighted in green, and downregulated genes in red. When numbers are indicated in brackets, it means several different sequences were identified as the same gene in the database. ns: not significant and/or with a $\log_2$ fold-change lower than ± 1.

	Kp-125 0PRE/0 $\log_2$ FC		Kp-125 4PRE/4 $\log_2$ FC		Kp-125 4PRE/0 $\log_2$ FC		Kp-125 4PRE/0PRE $\log_2$ FC			
Gene name	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Associated function**	**Reference**
Cellular respiration / electron transfer										
fdnG	ns		ns		1.2	0.3	1.4	0.4	Formate dehydrogenase	Wang & Gunsalus, 2003
hemA	-1.1	0.3	ns		ns		1.2	0.3	Involved in heme synthesis	Darie & Gunsalus, 1994
narU	ns		ns		ns		1.8	0.2	Nitrate/nitrite transporter	
narV	ns		ns		1.2	0.3	ns		Nitrate reducatse	
narZ	ns		ns		ns		1.9	0.6	Nitrate reductase	Bueno *et al.*, 2012
nfsB	ns		ns		ns		1.3	0.3	Nitrate reductase	Rau & Stolz, 2003
pntB	-6.5	2.1	ns		ns		7.4	2.0	NAD(P) transhydrogenase	Clarke *et al.*, 1986

Table 4-6 – *Continued*.

Gene name	Kp-125 0PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/4 log_2 FC Mean	SD	Kp-125 4PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/0PRE log_2 FC Mean	SD	Associated function	Reference
Cell wall synthesis and/or maintenance										
rfbD (1)	ns		ns		ns		-6.6	2.3	Involved in O antigen synthesis	Boels *et al.*, 2004
rfbD (2)	-2.6	0.5	ns		ns		3.4	0.5		
DNA modification and/or transfer										
helD (1)	ns		ns		ns		-2.5	0.9	DNA helicase	Mendonca *et al.*, 1993
helD (2)	1.2	0.2	ns		ns		-1.4	0.2		
hupB	-22.3	4.6	ns		ns		13.9	4.5	Nucleoid-associated protein (DNA compaction)	Holowka *et al.*, 2017
intB (1)	1.0	0.2	ns		ns		-1.7	0.2	Integrase	Hochhut *et al.*, 2006
intB (2)	1.2	0.2	ns		ns		-1.2	0.2		
mobA	ns		ns		ns		2.6	0.6	Involved in plasmid transfer	Henderson & Meyer, 1999
mukB	ns		ns		ns		1.8	0.6	Chromosome partition	Niki *et al.*, 1992
mutS	-6.5	1.4	ns		ns		7.8	1.4	Mismatch repair	Acharya *et al.*, 2003
repA	ns		ns		ns		-1.2	0.2	Replication initiation protein	Betteridge *et al.*, 2004e
tnpA	1.9	0.5	ns		ns		-2.1	0.5	Transposase	Horak & Kivisaar, 1998
traE	ns		1.5	0.4	1.5	0.4	2.7	0.5	Involved in F pilus formation (conjugation)	Arutyunov *et al.*, 2010
xerC	1.1	0.3	ns		ns		-1.7	0.3	Recombinase	Cornet *et al.*, 1997

Table 4-6 – *Continued*.

Gene name	Kp-125 0PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/4 log_2 FC Mean	SD	Kp-125 4PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/0PRE log_2 FC Mean	SD	Associated function	Reference
Efflux and transport										
acrB	ns		ns		ns		1.2	0.2	Multi-drug efflux pump, RND family	Alav *et al.*, 2018
appF (1)	ns		1.0	0.2	1.1	0.2	1.4	0.2	Oligopeptides ABC transporter (*Bacillus* spp.)	Koide & Hoch, 1994
appF (2)	-2.1	0.5	ns		1.4	0.4	3.5	0.4		
citH	ns		ns		ns		2.4	0.9	Citrate-proton symporter	Pos *et al.*, 1998
entE	-21.4	4.3	ns		ns		20.6	4.1	Enterobactin synthesis (iron transport)	Raymond *et al.*, 2003
entS	4.0	1.2	ns		ns		ns			
fepC	1.8	0.4	ns		ns		-1.2	0.4	Associated with iron transport	Ozenberger *et al.*, 1987; Miethke & Marahiel, 2007; Grim *et al.*, 2012
fes	2.0	0.7	ns		ns		ns			
kdpA	ns		ns		ns		2.2	0.6	P-type ATPase, involved in cation transport (potassium)	Sardesai & Gowrishankar, 2001
pgtP	ns		ns		ns		1.1	0.2	Phosphoglycerate transport, MFS family	Varadhachary & Maloney, 1991
rbtT (1)	ns		ns		ns		1.6	0.3	Ribitol transporter, MFS family	Pao *et al.*, 1998
rbtT (2)	-2.0	0.5	ns		ns		3.1	0.5		
silA	ns		ns		ns		6.2	2.4	Silver efflux pump, RDN family	Ortega Morente *et al.*, 2013rt
smvA	-1.3	0.4	ns		1.1	0.3	2.4	0.4	Cationic compounds efflux pump, MFS family	Villagra *et al.*, 2008
trkA	ns		ns		ns		2.3	0.7	Involved in potassium uptake	Bossemeyer *et al.*, 1989

Table 4-6 – *Continued*.

Gene name	Kp-125 0PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/4 log_2 FC Mean	SD	Kp-125 4PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/0PRE log_2 FC Mean	SD	Associated function	Reference
Stress response										
adhP	ns		ns		1.5	0.3	2.0	0.4	Alcohol dehydrogenase linked to DNA damage	Khil & Camerini-Otero, 2002
otsA	ns		ns		ns		1.2	0.2	Involved in trehalose synthesis and osmoprotection	Joseph *et al.*, 2010
Transcription, translation and post-translation modifications										
cpdB	ns		ns		ns		-1.1	0.3	Involved in RNA degradation	Lopez-Villamizar *et al.*, 2016
cysS	-1.9	0.5	ns		ns		ns		Cysteine-tRNA ligase	Zhang *et al.*, 2003
epmA	ns		ns		ns		-1.3	0.3	Translation elongation factor aminoacetylase	Vivijs *et al.*, 2016
groL (1)	ns		ns		ns		2.0	0.6		
groL (2)	ns		ns		ns		2.3	0.7		Goltermann *et al.*, 2015
groL (3)	ns		ns		ns		2.7	0.7	Chaperonin	
ileS	ns		ns		-4.2	1.2	ns		Isoleucine-tRNA ligase	Chalker *et al.*, 1994
lysS	-1.8	0.2	ns		ns		1.8	0.2	Lysine-tRNA ligase	Leveque *et al.*, 1990
rapA	-1.6	0.5	ns		ns		2.7	0.5	RNA polymerase-associated protein	Sukhodolets & Jin, 1998
rlhA (1)	ns		ns		1.0	0.2	1.0	0.2		
rlhA (2)	ns		22.7	2.9	ns		ns		Modification of 23S rRNA	Kimura *et al.*, 2017
rlmM	ns		ns		ns		-1.2	0.3	Modification of 23S rRNA	Punekar *et al.*, 2012

Table 4-6 – *Continued*.

Gene name	Kp-125 0PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/4 log_2 FC Mean	SD	Kp-125 4PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/0PRE log_2 FC Mean	SD	Associated function	Reference
Transcription, translation and post-translation modifications (*continued*)										
rplA	ns		ns		ns		-5.1	1.6	50S ribosomal protein L1 synthesis	Fu *et al.*, 2013
rpsT	ns		ns		ns		-1.2	0.4	30S ribosomal subunit S20	Celesnik *et al.*, 2007
Virulence-associated										
fimD	1.8	0.4	ns		ns		-1.9	0.4	Involved in export/assembly of fimbriae	Nishiyama *et al.*, 2008
prr	ns		1.0	0.2	ns		ns		Gamma-aminobutyraldehyde dehydrogenase involved in putrescine degradation	Schneider & Reitzer, 2012
virB4	ns		ns		ns		2.3	0.6	Type IV secretion system	Durand *et al.*, 2010
Other										
acs	ns		19.4	3.9	ns		ns		Acetyl-coenzyme A synthetase	Kumari *et al.*, 2000
adhE	-1.4	0.4	ns		ns		1.2	0.3	Aldehyde alcohol dehydrogenase	Membrillo-Hernandez *et al.*, 2000
amn	ns		ns		ns		-1.1	0.1	Adenosine monophosphate nucleosidase	Morrison & Shain, 2008
bcsE	ns		ns				1.0	0.3	Involved in cellulose synthesis	Fang *et al.*, 2014
bisC	ns		ns		ns		-5.7	1.5	Biotin sulfoxide reductase	Pierson & Campbell, 1990

Table 4-6 – *Continued*.

Gene name	Kp-125 0PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/4 log_2 FC Mean	SD	Kp-125 4PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/0PRE log_2 FC Mean	SD	Associated function	Reference
Other (*continued*)										
budB	ns		1.4	0.3	1.4	0.3	1.7	0.3	Acetolactate synthase	Blomqvist *et al.*, 1993
can	ns		ns		ns		3.6	1.1	Carbonic anhydrase	Merlin *et al.*, 2003
carB	-1.3	0.2	ns		ns		1.2	0.2	Carbamoyl phosphate synthetase	Nyunoya & Lusty, 1983
crnA	ns		1.7	0.5	ns		1.4	0.5	Creatinine amidohydrolase	Beuth *et al.*, 2003
cynT	ns		ns		ns		1.2	0.2	Carbonic anhydrase	Merlin *et al.*, 2003
cysI	ns		-2.4	0.6	-2.1	0.6	ns		Sulfite reductase	Ostrowski *et al.*, 1989
dcp	-10.5	2.0	ns		ns		11.3	1.9	Carboxypeptidase	Henrich *et al.*, 1993
gabD	1.1	0.2	ns		ns		-1.1	0.2	Succinic semialdehyde dehydrogenase	Langendorf *et al.*, 2010
livF	-1.1	0.3	ns		ns		ns		Involved in aromatic amino acid accumulation	Koyanagi *et al.*, 2004
lsrK	7.4	2.1	ns		ns		ns		Transcriptional regulator involved in quorum sensing	Xavier & Bassler, 2005
metE	-1.1	0.4	ns		ns		1.6	0.4	Involved in methionine synthesis	Weissbach & Brot, 1991
mscK	-1.6	0.5	ns		ns		1.3	0.5	Mechanosensitive channel involved in ion regulation	Li *et al.*, 2002
mtfA	ns		ns		ns		-1.1	0.2	Glucose-related peptidase, involved in entry into stationary growth phase	Gohler *et al.*, 2012
pdeB	ns		ns		ns		-1.2	0.3	Phosphodiesterase	Chao *et al.*, 2013

Table 4-6 – *Continued*.

Gene name	Kp-125 0PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/4 log_2 FC Mean	SD	Kp-125 4PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/0PRE log_2 FC Mean	SD	Associated function	Reference
Other (*continued*)										
phnC	ns		ns		ns		1.2	0.5	Part of the Pho regulon, global regulation	Jochimsen *et al.*, 2011; Santos-Beneit, 2015
phnM	-1.2	0.3	ns		ns		1.6	0.3		
phnU	ns		ns		ns		-2.0	0.6		
pta	-1.5	0.5	ns		ns		1.2	0.5	Phosphate acetyl-transferase involved in acetate metabolism	Castano-Cerezo *et al.*, 2009
sufB	ns		ns		ns		1.0	0.3	Involved in iron-sulfur cluster synthesis	Yuda *et al.*, 2017
tenA	-1.2	0.4	ns		ns		1.4	0.4	Involved in regulation of protease production (*Bacillus* spp.)	Toms *et al.*, 2005
Unknown / putative										
kpn78578	-19.6	4.9	ns		ns		21.1	4.7	Putative transport protein	UniProt (Y4047_KLEP7)
ybhD	ns		ns		1.5	0.4	1.8	0.5	Uncharacterized, putative transcriptional regulator	Flores-Bautista *et al.*, 2018
ybjX	ns		1.3	0.2	ns		ns		Uncharacterized protein	UniPrit (YBJX_ECOLI)
ydcI	ns		ns		1.1	0.3	1.0	0.3	Uncharacterized, putative transcriptional regulator	Solomon *et al.*, 2014
yddG	ns		1.1	0.2	ns		ns		Uncharacterized protein, putative drug/metabolite transporter	Santiviago *et al.*, 2002
yddH	ns		1.0	0.3	1.2	0.3	1.3	0.3	Uncharacterized protein	UniProt (YDDH_ECOLI)

Table 4-6 – *Continued*.

Gene name	Kp-125 0PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/4 log_2 FC Mean	SD	Kp-125 4PRE/0 log_2 FC Mean	SD	Kp-125 4PRE/0PRE log_2 FC Mean	SD	Associated function	Reference
Unknown / putative (*continued*)										
yejM	-7.4	1.8	ns		ns		6.8	1.8	Inner membrane protein (unknown function)	De Lay & Cronan, 2008
yjiR	ns		ns		ns		-2.3	0.7	Uncharacterized, putative transcriptional regulator	Flores-Bautista *et al.*, 2018
yncG	ns		ns		ns		1.1	0.3	Uncharacterized protein	UniProt (YNCG_ECOLI)
yxjC	ns		1.2	0.3	ns		ns		Uncharacterized, putative transporter (*Bacillus* spp.)	UniProt (YXJC_BACSU)

The comparison between the differential expression profiles obtained for both Kp-1 and Kp-125 following a single exposure to either 2 or 4 µg/mL CHX when compared to the control condition (R2A only, 0 µg/mL CHX) is presented in Figure 4-8. Although a majority of the genes of which the expression was altered is present only in one isolate, some were consistently up- or down-regulated consistently across both isolates. Among them, efflux-related genes, such as *acrD* and *smvA*, were found to be upregulated, as well as *phnM*, a part of the Pho regulon. The expression of two genes associated with DNA modification, *dnaE* (DNA polymerase) and *gyrA* (DNA gyrase), was downregulated following CHX exposure in both isolates, as well as *rlhA* (modification of 23S rRNA) and *fimD* (export and assembly of fimbriae). A gene encoding an uncharacterized protein, *ybgA*, was found to be upregulated following CHX exposure in Kp-1 but downregulated in Kp-125.

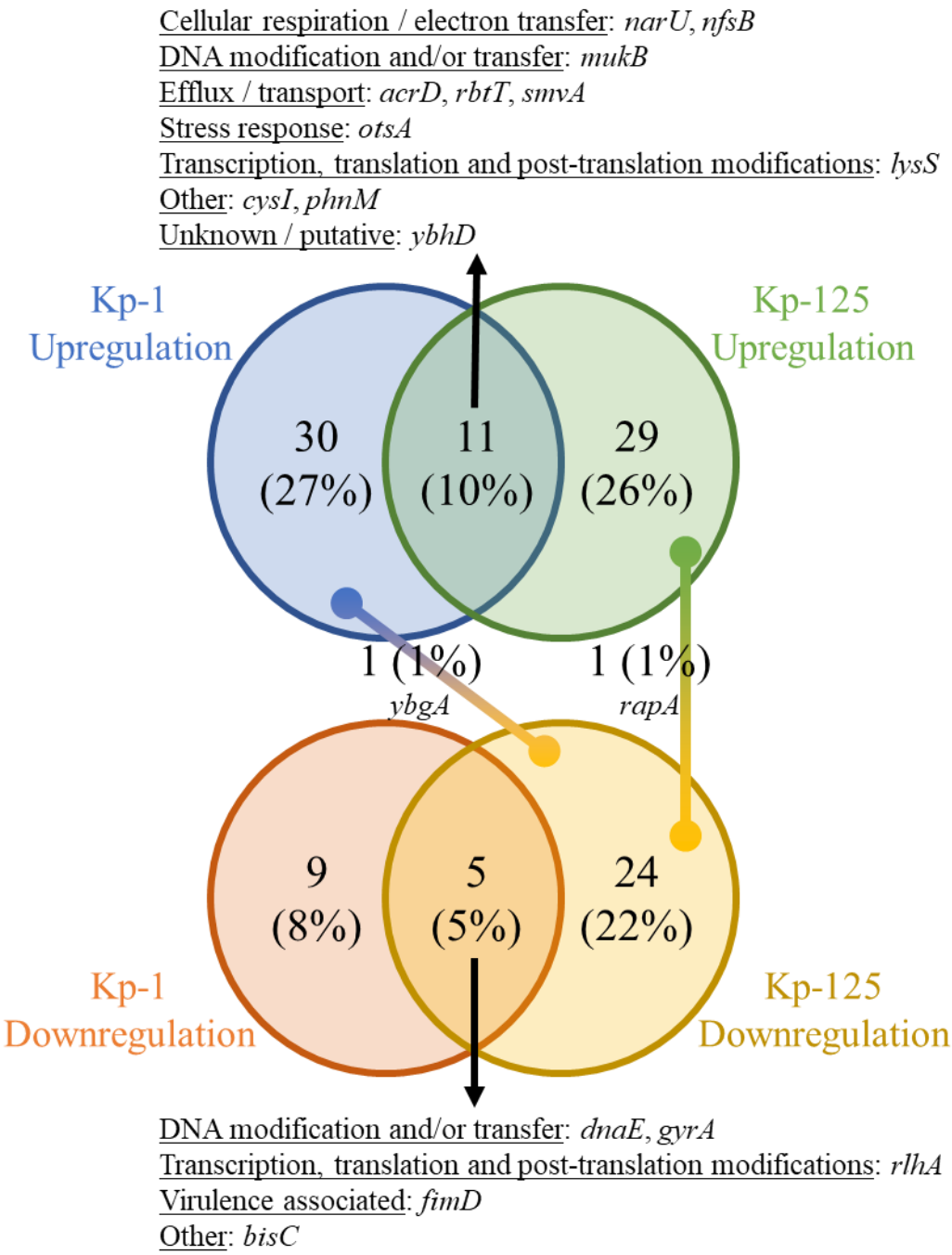

Figure 4-8. Differential expression patterns following a single exposure to either 2 or 4 µg/mL CHX compared to R2A only obtained for both Kp-1 and Kp-125.

Exposure to either 0 (R2A only), 2 or 4 µg/mL CHX was performed at least in triplicate for both isolates. Only genes for which the differential expression was statistically significant (Benjamin-Hochberg adjusted p value; $p<0.05$) and with at least ± 1 $\log_2$ fold-change are considered for this diagram. The number of up- or down-regulated genes for each strain is indicated in the corresponding section, with the associated percentage of the total amount of genes for which the expression was altered indicated in brackets.

4.4 Discussion

Exposure to sub-inhibitory concentrations of CHX did not result in reduced susceptibility to either CHX, BZC or different antibiotics including carbapenems, cephalosporins, ATM, aminoglycosides, TIG and COL. However, for most antibiotics (ATM, CIP, CAZ, FOX, AK and CN for Kp-1; MEM, ETP, ATM, CIP, CAZ and FOX for Kp-125), susceptibility testing was performed using the disc diffusion method, and the inhibition diameters measured before exposure already corresponded to the lowest measurable value (6 mm, corresponding to the disc diameters). As a consequence, it was not always possible to assess a potential reduced susceptibility for these antibiotics after exposure to CHX, which could have biased our observations. Moreover, although the disc diffusion method has advantages, such as a reduced cost and an easy implementation, especially for multiple drug testing as performed in this studies, its results are qualitative (i.e. resistant, intermediate, susceptible), as opposed to quantitative results obtained with broth dilution methods, i.e. MIC values (Dickert *et al.*, 1987; Reller *et al.*, 2009). Indeed, it seems difficult to establish correlations between inhibition zone diameters and MICs (Dickert *et al.*, 1987; Humphries *et al.*, 2018); therefore, changes in inhibition diameters might not indicate a change in MIC, but would be able to show a switch in susceptibility category, e.g. from susceptible to resistant (Reller *et al.*, 2009; Humphries *et al.*, 2018). Regarding colistin, exposure to stepwise increasing CHX concentrations has been reported to lead to reduced susceptibility to COL (which was not observed in this study) but without any impact on BZC or other antibiotic MICs in *K. pneumoniae* isolates, and thought to be linked to an enhanced efflux or mutations in specific genes related to colistin resistance (Wand *et al.*, 2017; Hashemi *et al.*, 2019; Zhang *et al.*, 2019). Such differences in the results obtained could however be linked to the exposure method used, i.e. a single exposure to MIC concentrations corresponding to half the organism's MIC in this study *versus* an adaptation to stepwise increasing CHX concentrations reaching up to 32-fold of the organism' original MIC in the above-mentioned publications. No change in antibiotic susceptibility profile was observed after long-term exposure to BZC in *Klebsiella michiganensis* (Kim *et al.*, 2018b).

Regarding virulence, exposure to CHX did not seem to have an effect on the pathogenesis of Kp-1, but virulence increased with increasing CHX concentrations for Kp-125. However, re-exposure to 4 μg/mL resulted in a decreased virulence when compared to the first exposure. Virulence has previously been reported to be reduced after prolonged exposure to increasing CHX concentrations in *K. pneumoniae* (Wand *et al.*, 2017). In *E. coli*, a prolonged exposure to BZC also led to a decrease in virulence using the *G. mellonella*

model (Henly *et al.*, 2019). Exposure to octenidine, a compound related to CHX, did not result in an increased virulence in *P. aeruginosa* either (Shepherd *et al.*, 2018). Kp-1 was found to be more virulent than Kp-125, despite belonging to a ST less widespread than the 'high-risk' ST258 of which Kp-125 is part of. This seemed however to be in accordance with the literature, where virulence in ST258 *K. pneumoniae* isolates is described as isolate-dependant but not more important than in other STs (Mathers *et al.*, 2015).

Both isolates possessed several large plasmids. *K. pneumoniae* isolates belonging to ST258 appear to possess a pKpQIL-like plasmid of approximatively 113 kb, carrying the bla_{KPC} gene, and the presence of these plasmids seemed to be related to the ability of these isolates to persist and spread in the environment (Mathers *et al.*, 2015). A plasmid of approximately 97 kb was detected for Kp-125 by S1 digestion followed by PFGE, which could be compatible with the presence of a pKpQIL-like plasmid. Unfortunately, the WGS data did not enable the construction of closed sequences corresponding to either the chromosome or the plasmids present within Kp-1 or Kp-125, with a coverage of less than 10 % when compared to the published sequence of the chromosome of a *K. pneumoniae* ST258 isolate (GenBank access number: LAKK01000012.1).

We chose to perform a single exposure to CHX (with one repeated exposure for Kp-125 without increasing the CHX concentration), while most of the studies mentioned in the following paragraphs based their observations following long-term and/or adaptation with increasing concentrations of biocides, which might not reflect real conditions (Soumet *et al.*, 2012). This difference in exposure protocol could explain some of the discrepancies between the literature and the work performed in this study. Repeated exposure in R2A only had a significant impact on various biological functions for Kp-125, including an overall downregulation of genes involved in cellular respiration and electron transfer, which was not consistent with previous findings reporting increased expression of genes related to these functions in *E. coli* (Forbes *et al.*, 2019). No change in the expression of efflux-related (except a decreased expression in *smvA*) was observed after re-exposure to 0 µg/mL CHX. Only two replicates were obtained for this condition (Kp-125+0PRE), which could bias the analysis.

The expression of *groL*, encoding a chaperonin, was found to be upregulated following CHX exposure. Chaperones interact with proteins at a post-translational level to ensure their correct folding. Overexpression of chaperone-encoding genes, especially *groL*, has been previously shown to result in reduced susceptibility to aminoglycosides, as these antibiotics are known to promote translational misreading (Goltermann *et al.*, 2015).

Association between upregulation of *groL* and reduced susceptibility to aminoglycosides was however not observed in this study. Overproduction of chaperones was also found to be related to stress response following exposure to increasing concentrations of CHX in *P. aeruginosa* (Hashemi *et al.*, 2019). Similarly, the expression of *gyrA*, encoding a DNA gyrase, was found to be downregulated following CHX exposure. Quinolones target the DNA gyrase to exert their antimicrobial activity, and mutations in *gyrA* or *gyrB* have been previously reported to impair the effects of these antibiotics (Mc Cay *et al.*, 2010; Pourahmad Jaktaji & Mohiti, 2010; Sanchez-Cespedes *et al.*, 2015). However, changes in CIP susceptibility following CHX exposure could not be assessed as both isolates showed important resistance to this antibiotic already. Regarding resistance to β-lactams, no significant change in expression was observed neither for $bla_{\text{NMD-1}}$ (Kp-1) nor $bla_{\text{KPC-2}}$ (Kp-125); *ampC* was found to be downregulated in Kp-1 only after exposure to 4 μg/mL CHX. No changes in susceptibility profiles to carbapenems, cephalosporins or ATM were observed after CHX exposure, but both isolates already harboured important levels of resistance towards these antibiotics. For polymyxins (including colistin, polymyxin E), increased MICs have been reported following exposure to CHX or BZC, but were mainly linked to mutations in *pmrB*, ultimately resulting in a decrease of the net negative charges of the outer membrane, decreasing the affinity of these antibiotics (Wand *et al.*, 2017; Kim *et al.*, 2018b). Neither increase in colistin MIC nor decrease or change in *pmrB* expression was observed in this study.

For both isolates, exposure to sub-inhibitory concentrations of CHX (especially 4 μg/mL, corresponding to half their MIC) led to an increased expression of efflux systems, including *acrB* or *acrD*, *emrD* (Kp-125 exposed to 2 μg/mL CHX only), *mdtB*, *mdtM*, *smvA* and, to some extent, *silA* (Kp-125 after second exposure to 4 μg/mL CHX only). No statistically significant difference in the expression of *qac* genes (linked to QAC efflux; Abuzaid *et al.*, 2012), or the *cus* efflux system (extruding copper and silver; Ortega Morente *et al.*, 2013) was detected. AcrB, AcrD and MdtB are part of the RDN efflux pump family, with AcrB and MdtB involved in efflux of a wide range of antimicrobial compounds, and AcrD harbouring a narrower spectrum, mainly restricted to aminoglycosides (Anes *et al.*, 2015). EmrD and SmvA are part of the MFS efflux pump family and have been reported to expel amphipathic compounds for EmrD and cationic compounds (including CHX, octenidine, some QACs) among several bacterial species such as *K. pneumoniae* or *P. mirabilis* for SmvA (Yin *et al.*, 2006; Pelling *et al.*, 2019; Wand *et al.*, 2019). MdtM, another member of the MFS family, is thought to be involved in pH regulation and extrusion of bile salts (Holdsworth & Law, 2012; Li *et al.*, 2015). SilA, another member of the RND

family, has been reported to mediate efflux of silver ions in Gram-negative bacteria (Ortega Morente *et al.*, 2013). Similarly to this work, upregulation of the expression of *smvA* linked to the loss of its repressor SmvR (Wand *et al.*, 2017; Wand *et al.*, 2019), or *cepA* (Zhang *et al.*, 2019) following exposure to increasing CHX concentrations in *K. pneumoniae* has been previously reported, and in *P. mirabilis* as well for *smvA* (Pelling *et al.*, 2019). Regarding *smvA*, in the highlighted studies, overexpression of *smvA* was directly linked to increased CHX MICs in the Gram-negative organisms investigated. Exposure to increasing concentrations of BZC has also been reported to increase the expression of *acrB* and *acrF* in triclosan-resistant *K. pneumoniae* (Gadea *et al.*, 2017a), as well as in *E. coli* with increased expression of *acrA, acrB* and *tolC* (Bore *et al.*, 2007; Moen *et al.*, 2012) and *mdtED, mdtNOP* and *mdtM* (Forbes *et al.*, 2019). The expression of *mexA* or *mexB* (but not *smvA*), part of the RND efflux pump family, was upregulated in *P. aeruginosa* following adaptation to CHX or BZC (Mc Cay *et al.*, 2010; Kim *et al.*, 2018a; Hashemi *et al.*, 2019). The equivalent efflux system in *Acinetobacter baumannii, adeABC*, thought to be related to CHX efflux, was also found to be upregulated following CHX exposure in this species (Hassan *et al.*, 2013). Interestingly, increased expression of efflux pump-encoding genes was not linked to a reduced susceptibility to CHX, BZC or any of the investigated antibiotics in this work, as opposed to the above-mentioned studies.

No change in expression of outer membrane protein-encoding genes was detected in this study, while this phenomenon has been reported following exposure to BZC or CHX in several Gram-negative bacteria (Bore *et al.*, 2007; Forbes *et al.*, 2019). However, a decreased expression of *asmA*, of which the corresponding protein is known to be involved in the prevention of misfolding of outer membrane protein mutants (Prieto *et al.*, 2009), was observed.

The expression of global regulators, including the *mar* operon involved in multi-drug resistance or *soxRS* involved in stress response (Duval & Lister, 2013), was not impacted by exposure to sub-inhibitory concentrations of CHX. These findings were similar to another study performed in *E. coli* after long-term exposure to low (0.0002 to 0.002 % of their MIC) BZC concentrations (Forbes *et al.*, 2019). On the contrary, other reports measured an increase in the expression of the global regulators following exposure to increasing concentrations of antimicrobials (Bore *et al.*, 2007; Buffet-Bataillon *et al.*, 2012a; Curiao *et al.*, 2015).

Regarding virulence-related genes, expression of *fimD*, involved in fimbriae assembly and export (Nishiyama *et al.*, 2008), was found to be downregulated following

CHX exposure, while enhanced expression of motility-related genes after long-term exposure to BZC has been previously reported in *E. coli* (Forbes *et al.*, 2019). Fimbriae have been shown to be linked to virulence and pathogenicity especially through enhanced adhesion (Jonson *et al.*, 2005). Putrescine and spermidine, two polyamines involved in the stabilisation of the outer membrane, have been linked to virulence in Enterobacteriaceae (Jelsbak *et al.*, 2012). Genes related to putrescine degradation (*pub* and *prr)* were found to be upregulated, and *gss*, involved in spermidine metabolism, was downregulated following CHX exposure. These findings disagreed with an increase of spermidine biosynthesis observed in *P. aeruginosa* following long-term exposure to BZC (Kim *et al.*, 2018a). After second exposure to 4 μg/mL CHX only, *virB4*, encoding a type IV secretion system, known to be involved in virulence (Voth *et al.*, 2012), was found to be upregulated when compared to its expression following to second exposure to R2A only (0 μg/mL CHX). The overall response following CHX exposure suggested either an unchanged or a decreased virulence for both isolates. This was in accordance with the results observed when performing the virulence assay using *G. mellonella* larvae.

Overall, even though some changes were observed at the molecular level with the RNA-sequencing data, this did not have an impact on the phenotypical level, i.e. no changes in antimicrobial susceptibility or virulence were obtained. It seems important to highlight the fact that increased expression, i.e. increased amount of mRNA obtained for a given gene, does not correlate with an increase in the corresponding protein production (Picard *et al.*, 2012; Pelling *et al.*, 2019). Indeed, several regulation levels are present between both stages, including post-transcriptional regulation with mRNA degradation, small RNAs or RNA-binding proteins, among others (Arraiano, 1993; Van Assche *et al.*, 2015); translational regulation with for example differences in ribosome occupancy and density observed across a global mRNA population (Picard *et al.*, 2012); and post-translational regulation, e.g. protein phosphorylation (Grangeasse *et al.*, 2015; Macek *et al.*, 2019). It is then possible to hypothesize that the alterations in gene expression observed in this study did not result in an increased or decreased production of the corresponding proteins, which could explain the absence of phenotypical changes for both investigated isolates. Complementary assays would have been necessary to understand better the role and involvement of the key genes identified by RNA-sequencing in response to CHX exposure, in order to obtain overlapping information between the different datasets (Bore *et al.*, 2007; Forbes *et al.*, 2019). Among them, quantitative real-time PCR, targeting a smaller amount of genes but less time-consuming and more sensitive than RNA-sequencing, can be used in order to confirm the observations made previously (Bore *et al.*, 2007; Costa *et al.*, 2013); inactivation of some of

these genes by generation of deletion mutants could be of interest in order to assess their direct impact on MIC for example, as performed by Wand and colleagues for *smvA* and *smvR* (Wand *et al.*, 2019); proteomic approaches, where changes in the protein profiles following antimicrobial exposure can be assessed, would be able to provide the missing link between alterations in the transcriptome and the observed phenotype (Bore *et al.*, 2007; Hashemi *et al.*, 2019).

4.5 Conclusion

In conclusion, exposure to sub-inhibitory concentrations of CHX led to an altered expression profile of several genes, including the efflux pump-encoding genes *acrB* and *acrD* involved in antibiotic resistance, and *smvA*, involved in CHX efflux, which were found to be upregulated following a single CHX exposure. However, the effects observed at the molecular level did not engender phenotypical changes, i.e. decreased susceptibility to other antimicrobials or increased virulence.

Chapter 5. General discussion

Susceptibility profiles to biocides and antibiotics have been established for 210 carbapenemase-producing *E. coli* and *K. pneumoniae* clinical isolates in this study. Thanks to standard protocols and available breakpoints for antibiotic susceptibility testing (following BSAC's recommendations in this study: Andrews, 2001; BSAC, 2015), it was possible to categorise these isolates as multi-drug resistant. However, such established guidelines do not exist for biocides making it difficult to compare MICs with the literature. Indeed, MIC values can be impacted by a wide range of factors, including the method (agar dilution, broth macro- or micro-dilution protocol), the media and its composition in nutrients and especially cations (e.g. CAMHB is recommended for antibiotic susceptibility testing using broth microdilution protocol by EUCAST and CLSI, and contains increased concentrations of Ca^{2+} and Mg^{2+}; CLSI, 2012; EUCAST, 2019c), and, for broth microdilution, the material the microtitre plates are made of and the nature (if any) of the coating (Maillard *et al.*, 2013; Wessels & Ingmer, 2013; Bock *et al.*, 2018). The MIC values obtained within this study were within the same order of magnitude as MICs described in the literature for these organisms while being on the lower side of the range (see Table 2-10 page 51 and Table 2-11 page 53), except for CS where MICs measured in this study were found to be up to 1,500 times lower than the ones reported in *E.coli* (lowest CS MIC measured in this study: 7.8 μg/mL; highest CS MIC reported in Table 2-11: 12,500 μg/mL by Biagi *et al.*, 2014). The broth used in our study, R2A, contains less nutrients (including Ca^{2+} and Mg^{2+}) than the CAMHB used by Biagi and colleagues, which could partly explain the differences observed.

For BZC, CHX and CS, *K. pneumoniae* isolates seemed to harbour higher MIC values than *E. coli* isolates, which was consistent with previous reports (Koljalg *et al.*, 2002; Guet-Revillet *et al.*, 2012). It has been previously highlighted that *E. coli* might lack the gene *smvA*, encoding an efflux pump of particular importance in *K. pneumoniae* and involved in extrusion of cationic compounds, including CHX (Wand *et al.*, 2019). In this study, exposure to sub-inhibitory concentrations of CHX for two *K. pneumoniae* also led to an upregulation of *smvA*, which could play a role in the difference observed in CHX susceptibility between *K. pneumoniae* and *E. coli*.

For each biocide, the MIC distribution of both *K. pneumoniae* and *E. coli* isolates was not affected by the type of carbapenemase (NDM, VIM, OXA-48-like or KPC) produced, which disagreed with a previous study where differences in MICs were linked to

the ESBLs produced in *E. coli* (Deus *et al.*, 2017). When Spearman's correlations were determined, different correlations between antibiotic and biocide MICs were obtained according to the type of carbapenemase produced in this study, especially for *K. pneumoniae* where most correlations were obtained for VIM- and OXA-48-like-producers. Following exposure to sub-inhibitory concentrations of CHX, however, no differences in antimicrobial susceptibility profiles were observed for neither Kp-1 (NDM-producer) nor Kp-125 (KPC-producer). Moreover, no significant difference in the expression of $bla_{\text{NDM-1}}$ (Kp-1) or $bla_{\text{KPC-2}}$ (Kp-125) was observed following exposure to CHX. Overall, we did not observe different effects according to the type of carbapenemase produced in the *K. pneumoniae* and *E. coli* populations studied.

The correlation study performed in this work unveiled links between susceptibility to CHX and different antibiotics (amikacin, aztreonam, ciprofloxacin, tigecycline, minocycline) especially when considering the overall *K. pneumoniae* isolates. We formulated the hypothesis of a possible involvement of efflux pumps, a cross-resistance mechanism, especially as efflux has been previously described to be involved in reduced susceptibility to ciprofloxacin and tetracyclines (Ball *et al.*, 1980; Poole, 2007). Another cross-resistance mechanism especially described when cationic compounds like polymyxins, CHX and BZC are involved, is a reduced membrane permeability (Kim *et al.*, 2018b; Hashemi *et al.*, 2019). Addition of CCCP, acting as an efflux pump inhibitor through disruption of the associated proton-motive force (Marquez, 2005; Alav *et al.*, 2018), reduced significantly the CHX MIC for most isolates, which was in accordance with previous reports (Abuzaid *et al.*, 2012; Wand *et al.*, 2017). On the other hand, addition of EDTA, known to disturb the membrane and enhance permeability (Pelletier *et al.*, 1994; Sawer *et al.*, 1997), did not potentiate the activity of CHX at the tested concentration in this collection of isolates, as previously observed (Russell, 1986). Together, these results seemed to favour the hypothesis of the involvement of efflux pumps rather than altered membrane permeability as a common resistance able to counteract the effects of CHX and several unrelated antibiotics in CPE. Growth pattern analysis revealed a significant increase in lag phase duration for *K. pneumoniae* isolates when growing in R2A containing a CHX concentration corresponding to half their MIC. An extended lag phase has been previously reported in both *E. coli* and *K. pneumoniae* following exposure to sub-inhibitory concentrations of triclosan and associated with an unknown mechanism involving a high fitness cost (Curiao *et al.*, 2015). Similar observations have been made in *P. aeruginosa* following long-term exposure to BZC, where changes in growth patterns were associated with a general decrease in global metabolism (Kim *et al.*, 2018a). Efflux pumps have been shown to be associated with an

important fitness cost in bacteria, rendering them unlikely to be selected at very low antimicrobial concentrations (Andersson & Hughes, 2012). Transcriptomic analysis following exposure to sub-inhibitory concentrations of CHX in two *K. pneumoniae* isolates indicated a global upregulation of genes involved in antimicrobial efflux, including *acrB* involved in the extrusion of various antibiotics and biocides and *acrD*, expelling mainly aminoglycosides (Anes *et al.*, 2015), and *smvA*, described as a cationic compounds efflux pump especially relevant for extrusion of CHX in *K. pneumoniae* (Wand *et al.*, 2019). On the other hand, contradicting expression profiles were observed for genes involved in cell wall synthesis and maintenance, sometimes being up- or down-regulated following CHX exposure. While it was not possible to draw conclusions regarding the involvement of altered membrane permeability as a common resistance mechanism between antibiotics and CHX, this work showed that upregulation of efflux pump-encoding genes was part of the response observed following CHX exposure in CPE. However, it seems of importance to highlight the fact that a cooperation between altered permeability and increased influx is possible and act synergistically to confer a reduced susceptibility to some antimicrobials; in *K. pneumoniae*, but also in *P. mirabilis*, an increased expression of *smvA* was observed following CHX exposure, as well as changes in the LPS structure, reducing the negative net charges on the membrane and consequently decreasing the affinity of CHX, a cationic biocide, for it (Pelling *et al.*, 2019; Wand *et al.*, 2019).

We chose to investigate the effects of exposure to CHX concentrations corresponding to half or a quarter of the organism's MIC. Indeed, these were the concentrations at which most effects were observed on growth rates and lag phase durations at the time of the design of the following set of experiments (i.e. evaluation of the phenotypical and molecular effects following exposure to sub-inhibitory concentrations of CHX). These concentrations were within the 'traditional selective window' as defined by Gullberg and colleagues, i.e. between the MIC measured for the isolate harbouring reduced susceptibility (upper limit) and the one measured for the reference, susceptible strain (lower limit), as opposed to the 'sub-MIC selective window' corresponding to lower concentrations comprised between the MIC of the reference strain (upper limit) and the concentration up to which the reference strain is able to outcompete the growth of the less susceptible strain (lower limit, corresponding to the MSC), as shown in Figure 3-8 page 111 (Gullberg *et al.*, 2011). In our study, MSCs could only be defined for a minority of *K. pneumoniae* isolates (and corresponded to 2 µg/mL CHX in these cases), and CHX MICs measured for the clinical isolates and the *K. pneumoniae* ATCC 13883 reference strain were within the same magnitude order (CHX MICs ranged from 2-16 µg/mL for the clinical isolates, and CHX MIC measured for the reference strain

ranged from 1-2 μg/mL). It has however been shown that long-term exposure to concentrations as little as ones corresponding to 0.0002 and 0.002 % of the organism' BZC MIC was enough to alter the transcriptomic profile in *E. coli*, including for the upregulation of efflux-related genes (Forbes *et al.*, 2019). Moreover, we investigated the effects following a single (or once repeated for Kp-125) exposure to CHX. While adaptation protocols involving exposure to increasing concentrations of antimicrobials are widely described (Wand *et al.*, 2017; Henly *et al.*, 2019; Zhang *et al.*, 2019), they might not specifically reflect real conditions of exposure e.g. in the healthcare environment (Soumet *et al.*, 2012; Forbes *et al.*, 2019). However, the increased effects observed in these studies compared to the present work could potentially be linked to the discrepancies in the protocols followed (adaptation to increasing concentrations of antimicrobials over a long period of time *versus* single to dual exposure to constant CHX concentration in this study).

Mechanisms leading to reduced susceptibility to antimicrobials can appear through mutations but also resistance determinant acquisition, including by HGT (Tezel & Pavlostathis, 2015). HGT can occur through three different events: transformation, corresponding to the ability of bacteria to acquire naked DNA from the environment; transduction, corresponding of the transfer of genetic material between two bacterial cells through a bacteriophage; conjugation, corresponding to an exchange of DNA (e.g. plasmids) mediated by cell-to-cell contact *via* a pilus produced by the donor bacteria (Blakely, 2015; Sun, 2018). No measurement of plasmid transfer rates was performed in our study. Indeed, both Kp-1 and Kp-125 possessed several, large plasmids of unknown sequences, and both harboured resistant phenotypes towards a wide range of antibiotics, limiting the possibilities for antibiotic selection following conjugation. The results obtained from the RNA sequencing seemed contradictory regarding horizontal gene transfer. No significant changes were observed in the expression of genes involved in plasmid transfer for Kp-1. However, for Kp-125, after exposure to 4 μg/mL CHX, *traH*, involved in the formation of a conjugative pilus (Arutyunov *et al.*, 2010), was found to be upregulated, as well as *mobA*, involved in plasmid transfer during conjugation (Henderson & Meyer, 1999). On the other hand, *intB*, and *xerC*, two recombinases involved in site-specific recombination, and *recC*, involved in homologous recombination (Myers & Stahl, 1994; Cornet *et al.*, 1997; Hochhut *et al.*, 2006; Blakely, 2015), were downregulated following CHX exposure. These conflicting results did not enable us to identify a trend regarding altered conjugation rates following exposure to sub-inhibitory concentrations of CHX in *K. pneumoniae*; phenotypical assessment of the impact of exposure to sub-inhibitory concentrations of CHX on HGT would have been necessary to conclude. Indeed, Jutkina and colleagues reported an

increase in bacterial conjugation rates following exposure to tetracycline concentrations equivalent to 1/150 of the micro-organism's MIC (Jutkina *et al.*, 2016) and to triclosan (a widely used biocide) and CHX concentrations equivalent to 1/20 and 1/200 of the micro-organism's MIC, respectively (Jutkina *et al.*, 2018), using an *E. coli* recipient strain. These findings highlighted the risk of transmission of resistance determinants between bacteria under selective pressure applied by both antibiotics and biocides, which could enhance the spread of multi-drug resistant bacteria, especially within healthcare settings.

In this study, we investigated the effects of exposure to CHX in planktonic bacteria. Biofilms, corresponding to bacterial communities attached onto a surface and living within an extracellular matrix (containing a wide range of substances including lipids, polysaccharides, proteins) represent however the prevailing lifestyle for bacteria (Lindsay & von Holy, 2006; Hobley *et al.*, 2015). Biofilm formation follows the attachment and multiplication of bacterial cells onto a surface, and is especially relevant in healthcare settings (Donlan, 2001; Lindsay & von Holy, 2006). Indeed, biofilms have been previously associated with medical devices such as endoscopes and protheses (Donlan, 2001; Vickery *et al.*, 2013), but also with hospital surfaces as 'dry surface biofilms' where they may be involved in healthcare-associated infections (Ledwoch *et al.*, 2018). Bacteria living within biofilms have been shown to be less susceptible to antimicrobials through several mechanisms, including protection from the extracellular matrix, acting as a barrier and impacting the penetration and/or stability of the antimicrobial agent, a reduced growth rate, especially for bacteria at the bottom of the biofilm structure (linked to a gradient in oxygen and nutrients from the surface to the bottom of the structure), impairing the effects of antimicrobials acting on active metabolism and/or cell division, and enhanced exchanges of DNA molecules including plasmids containing resistance determinants between the different bacteria present within the biofilm (Donlan, 2001; Lindsay & von Holy, 2006; Bridier *et al.*, 2011; Stewart, 2015). An enhanced biofilm formation has been reported following exposure to CHX in *K. pneumoniae* (Forbes *et al.*, 2014) and after adaptation to BZC in *E. coli* (Henly *et al.*, 2019). The effects of low concentrations on biocides on biofilm formation and bacterial communities living within should consequently not be overlooked.

Overall, biocide MICs measured in this study were much lower than in-use concentrations found in commercial products, as highlighted in Table 2-3 (page 25). Moreover, exposure to sub-inhibitory concentrations of CHX did not result in an increase in MIC for neither CHX nor BZC, both cationic compounds, despite an upregulation of the expression of *smvA* following exposure (8- to 11-fold change when compared to growth in

R2A only). On the contrary, following adaptation from 4 to 128 μg/mL CHX, the expression of *smvA* has been shown to be upregulated at up to 70-fold in *K. pneumoniae*, highlighting its role in bacterial survival in the presence of this biocide (Wand *et al.*, 2019). The difference in the magnitude of the upregulation observed could maybe be linked to the protocol (adaptation *versus* single exposure), as highlighted in a previous paragraph. Exposure to sub-inhibitory concentrations of CHX did not result in reduced susceptibility to antibiotics in this study. Although it was not possible to evaluate changes in susceptibility profiles for some antibiotics including ciprofloxacin due to high level of resistance towards these compounds before exposure; including other isolates harbouring lower antibiotic MICs in the study would have helped identifying a general trend in antibiotic susceptibility profile following CHX exposure. Moreover, for all the tested antibiotics but tigecycline and colistin, the disc diffusion method was used for its easiness to implement when investigating several antibiotics but few isolates; this technique only provides quantitative results which do not correlate with MIC values obtained with broth dilution protocol (Dickert *et al.*, 1987; Reller *et al.*, 2009; Humphries *et al.*, 2018). In several studies however, a reduced susceptibility to colistin especially was observed following CHX exposure in *K. pneumoniae* (Wand *et al.*, 2017; Hashemi *et al.*, 2019; Zhang *et al.*, 2019). The increased expression of efflux-related genes observed at the molecular level, including *acrB* and *acrD* involved in antibiotic efflux, did not translate at the phenotypic level in our study, as similarly observed in *E. coli* exposed to low concentrations of BZC (Forbes *et al.*, 2019). However, it seems important to keep in mind that alterations in gene expression profiles, i.e. changes in the corresponding amount of mRNA, does not always correlate with changes in protein production, as several levels of regulations are involved between mRNA synthesis and the production of the corresponding functional protein (Picard *et al.*, 2012; Pelling *et al.*, 2019). Moreover, pathogenicity, although increased following the first exposure to CHX in Kp-125, was found to be decreased following the second exposure, and virulence in Kp-1 was not affected by exposure to CHX. Taken together, these results would suggest that, despite adaptation to CHX seemed to be ongoing at the transcriptomic level, phenotypical changes, if any, were not clinically relevant, i.e. did not affect susceptibility nor pathogenicity and, consequently, possibilities of treatment of infection due to these organisms. The clinical relevance of common mechanisms between reduced susceptibility to biocides and antibiotics has been widely discussed within the scientific community and does not seem to reach a consensus (Maillard *et al.*, 2013; Oggioni *et al.*, 2013; Harbarth *et al.*, 2014; Kampf, 2016; Kampf, 2019). In the absence of a standardised method to assess susceptibility to biocides and their potential impact on co-selection (co-resistance, cross-resistance, co-regulation) mechanisms

(SCENIHR, 2009; SCENIHR, 2010), precaution principles should be applied to limit the current overuse and misuse of biocides.

Taking this work forward, making a parallel between planktonic-growing and biofilm-growing bacteria might have been of relevance, as biofilms represent the predominant form of bacterial life, as highlighted earlier. The determination of minimum biofilm eradication concentrations (MBEC; Maillard *et al.*, 2013) and the assessment of changes in MBECs following antimicrobial exposure could have brought insight in that regard (Henly *et al.*, 2019). Moreover, after establishing baseline susceptibility data for the 210 isolates, we chose to pursue further experimental work with CHX only; despite its clinical relevance and the fact that most correlations between biocide and antibiotic MICs were obtained with CHX, BZC was also a good candidate. Several studies have reported changes in transcriptomic profiles and/or antibiotic susceptibility profiles following BZC exposure (Kim *et al.*, 2018a; Forbes *et al.*, 2019; Henly *et al.*, 2019); therefore, including BZC in our study might have added additional information to the current knowledge. Regarding our transcriptomic data, confirmation of the changes observed with the RNA-sequencing by quantitative real-time PCR and/or using a proteomic approach might have enabled us to draw more robust conclusions and to explain the discrepancies observed between the molecular and phenotypical levels (Pelling *et al.*, 2019). Furthermore, the addition of experiments assessing the functional consequences of CHX exposure, such as uptake/extrusion studies enabling the visualisation of direct consequences on permeability or efflux (Gnanadhas *et al.*, 2013; Srinivasan *et al.*, 2014) or horizontal gene transfer rate measurements (Jutkina *et al.*, 2016; Jutkina *et al.*, 2018) would also have strengthened our position regarding the impact of exposure to sub-inhibitory concentrations of CHX in CPE. Finally, it would have been interesting to expand this part of our work, i.e. the investigation of transcriptomic and phenotypic changes following CHX exposure, by including other isolates. Indeed, other strains belonging to different STs and/or harbouring lower antibiotic MICs might have highlighted other alterations.

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List of appendices

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