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DOCTORAL DISSERTATION

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Sveučilište u Zagrebu

Prehrambeno-biotehnološki fakultet

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OTPADNE VODE KAO IZVOR ŠIRENJA OTPORNOSTI NA CEFALOSPORINE TREĆE GENERACIJE I KARBAPENEME U VODENI OKOLIŠ

DOKTORSKI RAD

Mentor:
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WASTEWATER AS A SOURCE FOR THE SPREAD OF RESISTANCE TO THIRD-GENERATION CEPHALOSPORINS AND CARBAPENEMS TO THE AQUATIC ENVIRONMENT

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Thesis preformed at Laboratory for Environmental Microbiology and Biotechnology, Division for Marine and Environmental Research, Ruđer Bošković Institute

Supervisor: Nikolina Udiković Kolić, PhD, Scientific Advisor

Short abstract: This dissertation aimed to investigate the removal of enterobacteria resistant to third-generation cephalosporins (3GCs) and carbapenems and the corresponding resistance genes from seven Croatian wastewater treatment plants (WWTPs) and to characterize 3GC- and carbapenem-resistant enterobacterial isolates from hospital and treated wastewater from Zagreb WWTP to reveal their epidemiology and resistance mechanisms. Culture- and quantitative PCR-based methods showed that these WWTPs only partially eliminate these enterobacteria and the resistance genes, which are then released into the environment via the treated wastewater. Phenotypic analysis of the wastewater isolates confirmed their production of extended-spectrum β-lactamases (ESBLs) and/or carbapenemases, and revealed their multidrug-resistant phenotype, while genomic analysis identified ESBL and/or carbapenemase genes as well as resistance genes to other antimicrobial classes. Molecular epidemiology revealed that most *Escherichia coli, Klebsiella spp.,* and *Enterobacter cloacae* complex were human-associated clones, while ST131 and ST361 *E. coli* and ST101 *K. pneumoniae* were recognized as high-risk clones and emerging high-risk clones. These results emphasize the need to use advanced treatment technologies for municipal wastewater treatment and hospital wastewater pre-treatment to control the spread of these critical pathogens, which are resistant to clinically important antibiotics, into the environment.

Key words: Enterobacterales, extended-spectrum β-lactamases, carbapenemases, wastewater treatment plant, treated wastewater, hospital wastewater, antimicrobial resistance

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OTPADNE VODE KAO IZVOR ŠIRENJA OTPORNOSTI NA CEFALOSPORINE TREĆE GENERACIJE I KARBAPENEME U VODENI OKOLIŠ

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Sažetak: Cilj ove disertacije bio je istražiti uklanjanje enterobakterija otpornih na cefalosporine treće generacije (CTG-ove) i karbapeneme te odgovarajućih gena za antimikrobnu otpornost iz sedam hrvatskih uređaja za pročišćavanje otpadnih voda (UPOV-a), kao i karakterizirati odabrane izolate enterobakterija otpornih na CTG-ove i karbapeneme iz bolničkih otpadnih voda te pročišćene otpadne vode zagrebačkog UPOV-a, kako bi se ustanovila njihova epidemiologija i mehanizmi otpornosti. Metodama temeljenim na uzgoju bakterija i kvantitativnom PCR-u analizirani UPOV-i imali su nedovoljnu efikasnost u uklanjanju ovih enterobakterija i gena za otpornost, koji se putem pročišćene otpadne vode ispuštaju u okoliš. Fenotipskom analizom izolata iz otpadnih voda potvrđena je njihova proizvodnja β-laktamaza proširenog spektra (ESBL-a) i/ili karbapenemaza te njihova višestruka otpornost na antibiotike, dok su genomskom analizom identificirani ESBL i/ili karbapenemazni geni i geni za otpornost na druge klase antibiotika. Molekularnom epidemiologijom ustanovljeno je da većina *Escherichia coli*, *Klebsiella* spp. i *Enterobacter cloacae* complex izolata su ljudskog porijekla, dok su ST131 i ST361 *E. coli* i ST101 *K. pnenumoniae,* prepoznati kao visokorizični klonovi i visokorizični klonovi u nastajanju. Ovi rezultati naglašavaju potrebu za primjenom naprednih tehnologija u pročišćavanju komunalne otpadne vode te predtretmana bolničkih otpadnih voda kako bi se smanjilo širenje ovih kritično važnih patogena, otpornih na klinički bitne antibiotike, u okoliš.

Ključne riječi: Enterobacterales, β-laktamaze proširenog spektra, karbapenemaze, uređaji za pročišćavanje otpadnih voda, pročišćena otpadna voda, bolničke otpadne vode, otpornost na antibiotike

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Extended abstract

Antimicrobial resistance (AMR) is one of the most urgent public health problems worldwide and in Croatia. Although measures against AMR require a One Health approach, AMR surveillance programs typically target hospitals and the veterinary sectors, while the environmental sector is often overlooked. Wastewater treatment plants (WWTPs) in urban areas play a crucial role in the spread of AMR, as many of them do not sufficiently remove AMR determinants (bacteria and genes). In Croatia, there is limited evidence on the occurrence of antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) in wastewater after the treatment in WWTPs, especially in relation to clinically important antibiotics such as third-generation cephalosporins (3GCs) and carbapenems. These drugs are crucial for combating infections caused by Enterobacterales pathogens which are difficult to treat due to their resistance to multiple antibiotics. The World Health Organization has classified extended-spectrum β-lactamase (ESBL)-producing (a primary resistance mechanism to 3GCs) and carbapenem-resistant Enterobacterales as critical priority pathogens, underscoring the urgent need for new antibiotics.

The first aim of this dissertation was, therefore, to evaluate the removal efficiency of selected enteric opportunistic pathogens (EOPs), ESBL, and carbapenemase genes, as well as carbapenem- and cefotaxime- (CTX, a representative of the 3GCs) resistant coliform bacteria from seven conventional WWTPs in Croatia. Samples of untreated and treated wastewater were taken from these WWTPs on two occasions (winter and summer). The culture-based and quantitative PCR (qPCR) methods showed that these WWTPs significantly reduced or completely eliminated EOPs such as *Klebsiella pneumoniae* and *Acinetobacter baumannii*. However, CTX-resistant, and carbapenem-resistant coliforms, as well as EOPs such as *Escherichia coli* and *Enterococcus*, could not be completely eliminated. The ESBL genes $bla_{\text{CTX-M-32}}$ and bla_{TEM} were either only slightly reduced or even enriched after the treatment, especially in the summer. The carbapenemase gene $bla_{\text{OXA-48}}$ was removed in almost all WWTPs, while $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-3}}$ were detected sporadically in untreated wastewater and rarely found in the paired treated wastewater. Conversely, the bla_{IMP} and bla_{VIM} genes were frequently enriched during the treatment. In addition, specific WWTP characteristics had a different effect on the CTX and carbapenem-resistant coliforms, EOPs, ESBL, and carbapenemase genes from treated wastewater, indicating that conditions and characteristic of WWTPs may have different impacts on the development of AMR. These findings

underscore the need for advanced wastewater treatment technologies to minimize the risk of environmental contamination with these clinically important pathogens, ARB, and ARGs.

The second aim of the dissertation was to characterize ESBL-producing and carbapenem-resistant Enterobacterales (CRE) isolates from Zagreb's treated municipal wastewater and wastewater of two hospitals, to reveal their epidemiology and underlying antibiotic resistance mechanisms. Of the 200 CTX-resistant isolates from treated wastewater of the Zagreb WWTP, 140 were confirmed as ESBL producers and most of them were identified as *E. coli*. Regarding carbapenem-resistant isolates, 148 of 200 were found to be carbapenemase producers with *Klebsiella, Citrobacter*, and *Enterobacter cloacae* complex (cplx.) being the most frequently identified species. Antimicrobial susceptibility testing showed that most isolates had a multidrug-resistant (MDR) phenotype, with more than half identified as extensively drug-resistant (XDR). Colistin resistance was detected in 40% and 57% of carbapenem-resistant *Klebsiella* and *E. cloacae* cplx. isolates, respectively. PCR and whole-genome sequencing (WGS) analyses revealed that most ESBL-producing isolates, especially *E. coli*, carried $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-116}}$, which are frequently detected together. Among carbapenem-resistant isolates, *Klebsiella pneumoniae* frequently carried $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ together, reflecting the current trend in hospital isolates, while *Klebsiella oxytoca, E. cloacae* cplx. and *Citrobacter* spp. carried $bla_{\text{KPC-2}}$ together with $bla_{\text{NDM-1}}$. Molecular epidemiology by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) showed that most ESBL-producing *E. coli* were associated with high-risk clones (ST131) and emerging high-risk clones (ST361). Most carbapenem-resistant *Klebsiella* spp. and *E. cloacae* cplx. belonged to human-associated clones, and most of them were detected for the first time in Croatia. Based on the dominant clonal types and clinical significance, 17 isolates (ESBL-producing and carbapenem-resistant) were subjected to WGS analysis. In addition to ESBL and/or carbapenemase genes, the sequenced isolates also carried clinically important ARGs for other important antimicrobial classes (e.g., aminoglycosides, fluoroquinolones, trimethoprim, and sulfonamides), virulence genes, and various plasmid replicons, previously associated with the spread of ESBL or carbapenemase genes. Genomic analysis also revealed known and novel resistance mechanisms to colistin in *Klebsiella* spp. and *E. cloacae* cplx. isolates. These mechanisms include known and novel chromosomal point mutations in the *pmrA/B, phoP/Q, mgrB* and/or *crrB* genes, as well as the acquisition of the plasmid-encoded *mcr-4.3* gene. The *mcr-4.3* gene was detected for the first time in *K. pneumoniae* (ST629) and is located on the conjugative IncHI1B plasmid. These results demonstrate the

important role of treated wastewater as a reservoir for the dissemination of clinically relevant enterobacterial strains with clinically important ARGs into the environment.

The subsequent dissertation work covers the phenotypic and genomic characterization of ESBL-producing and carbapenem-resistant isolates from the wastewater of two large hospitals in Zagreb. Out of 200 isolates from hospital wastewater, 69 isolates were confirmed as ESBL producers and 90 isolates as carbapenemase producers. Most isolates were identified as *E. coli*, *Citrobacter* spp., *E. cloacae* cplx. and *K. pneumoniae* species. They were all phenotypically confirmed as MDR. In addition, resistance to colistin was mainly found in carbapenem-resistant isolates. Among the ESBL producers, the $bla_{\text{CTX-M-15}}$ gene was the most prevalent ESBL gene, while the carbapenem-resistant isolates frequently contained the $bla_{\text{KPC-2}}$ and $bla_{\text{NDM-1}}$ genes, followed by $bla_{\text{OXA-48}}$. PFGE and MLST methods revealed that the most frequent clonal types were the high-risk clones ST131 *E. coli*, the emerging high-risk clones ST101 *K. pneumoniae*, and the ST277 *E. cloacae* cplx. clones. Additionally, WGS analysis of selected ESBL-producing and carbapenem-resistant *E. coli*, *K. pneumoniae* and *E. cloacae* cplx. revealed a number of different ARGs conferring resistance to different antimicrobial classes and diverse plasmid replicon types. In addition, the clinically important $bla_{\text{CTX-M-15}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{OXA-48}}$ genes were found to be flanked by mobile genetic elements such as transposons and insertion sequences, indicating their mobilization potential. Finally, colistin resistance in *E. cloacae* cplx. was found to be mediated by chromosomal point mutations in the *pmrA/B* and/or *phoP/Q* genes. These results indicate that hospital wastewater is a potential secondary reservoir for clinically important pathogens and ARGs and must be effectively pretreated prior to discharge into the municipal wastewater system.

Overall, the results of this dissertation show that conventional treatment in Croatian WWTPs is of limited effectiveness in eliminating 3GCs- and carbapenem-resistant enterobacteria as well as ESBL and carbapenemase genes, which are then released into the environment via treated wastewater. Furthermore, our study shows that hospital wastewater is an important source of pathogens and clinically relevant ARGs into the municipal wastewater system. Therefore, pre-treatment of hospital wastewater and advanced technologies for municipal wastewater treatment should be used to reduce the spread of these AMR determinants into the environment.

Keywords: Enterobacterales, extended-spectrum β-lactamases, carbapenemases, wastewater treatment plant, treated wastewater, hospital wastewater, antimicrobial resistance

Proširenisažetak

Antimikrobna otpornost jedna je od najvažnijih javnozdravstvenih problema u svijetu i u Hrvatskoj. Iako mjere protiv antimikrobne otpornosti zahtijevaju pristup "Jedno zdravlje", programi nadzora otpornosti obično su usmjereni na bolničke i veterinarske sektore, dok se okoliš često zanemaruje. Uređaji za pročišćavanje otpadne vode (UPOV-i) u urbanim područjima igraju bitnu ulogu u širenju antimikrobne otpornosti budući da mnogi od njih nedovoljno dobro uklanjanju determinante antimikrobne otpornosti (bakterije i geni). U Hrvatskoj ne postoji dovoljno istraživanja o pojavi bakterija otpornih na antibiotike i gena za otpornost na antibiotike u pročišćenoj otpadnoj vodi iz UPOV-a, a posebice onih bakterija i gena koji su povezani s klinički važnim antibioticima kao što su cefalosporini treće generacije (CTG-ovi) i karbapenemi. Ovi lijekovi su ključni za suzbijanje infekcija uzrokovanih patogenima iz reda Enterobacterales, koje je teško liječiti zbog njihove otpornosti na mnoge antibiotike. Stoga je Svjetska zdravstvena organizacija klasificirala enterobakterije koje proizvode β-laktamaze širokog spektra (primarni mehanizam za otpornost na CTG-ove; engl. *extended-spectrum β-lactamases*, ESBLs) i one koje su otporne na karbapeneme, kao kritički prioritetne patogene, naglašavajući tako hitnu potrebu za novim antibioticima.

Stoga je prvi cilj ove disertacije bio procijeniti učinkovitost uklanjanja odabranih enteričnih oportunističkih patogena (EOP-a), ESBL i karbapenemaznih gena te koliformnih bakterija otpornih na karbapeneme i cefotaksim (CTX, predstavnik CTG-ova) iz sedam konvencionalnih UPOV-a u Hrvatskoj. Uzorci sirove i pročišćene otpadne vode uzeti su iz UPOV-a u dva navrata (u zimi i u ljeti). Metode temeljene na uzgoju bakterija i kvantitativnom PCR-u (qPCR-u) pokazale su da UPOV-i signifikantno reduciraju ili eliminiraju EOP-e, kao što su *Klebsiella pneumoniae* i *Acinetobacter baumannii*. Međutim, koliformi otporni na CTX i karbapeneme te EOP-ovi kao što su *Escherichia coli* i enterokoki, nisu bili u potpunosti eliminirani. ESBL geni, $bla_{\text{CTX-M-32}}$ i bla_{TEM}, bili su ili samo neznatno smanjeni ili obogaćeni, nakon pročišćavanja, pogotovo ljeti. Karbapenemazni gen $bla_{\text{OXA-48}}$ uklonjen je iz gotovo svih UPOV-a, dok su $bla_{\text{NDM-1}}$ i $bla_{\text{KPC-3}}$ bili sporadično detektirani u nepročišćenim otpadnim vodama i rijetko nađeni u istim pročišćenim otpadnim vodama. Tijekom pročišćavanja otpadnih voda često je došlo do obogaćivanja s genima bla_{IMP} i bla_{VIM}. Osim toga, specifične karakteristike UPOV-a imale su različiti učinak na koliforme otporne na antibiotike, EOP-e te ESBL i karbapenemazne gene u pročišćenoj otpadnoj vodi, što ukazuje da uvjeti i karakteristike UPOV-a mogu različito utjecati na razvoj antimikrobne

otpornosti. Ovi rezultati naglašavaju potrebu za naprednim tehnologijama pročišćavanja otpadnih voda kako bi se smanjilo zagađenje okoliša ovim klinički važnim patogenima, bakterijama otpornih na antibiotike i genima za otpornost na antibiotike.

Drugi cilj disertacije bio je karakterizirati izolate iz reda Enterobacterales koje proizvode ESBL enzime te one koje su otporne na karbapeneme, iz zagrebačke pročišćene otpadne vode i otpadne vode dviju zagrebačkih bolnica, kako bi se vidjela njihova epidemiologija i temeljni mehanizam otpornosti na antibiotike. Od 200 izolata otpornih na CTX iz pročišćene otpadne vode zagrebačkog UPOV-a, 140 ih je potvrđeno da proizvode ESBL-ove te ih je većina identificirana kao *E. coli*. Što se tiče izolata otpornih na karbapeneme, za njih 148 od 200, utvrđeno je da proizvode karbapenemaze, a najčešće identificirane vrste bile su *Klebsiella, Citrobacter* i *Enterobacter cloacae* complex (cplx.). Ispitivanjem antimikrobne osjetljivosti pokazalo se da većina izolata ima višestruku otporanost na antibiotike, a više od polovice je identificirano kao ekstenzivno otporni na antibiotike. Otpornost na kolistin je detektirana u 40% *Klebsiella* i 57% *E. cloacae* izolata otpornih na karbapeneme. PCR-om i sekvenciranjem cjelokupnog genoma (eng. *whole-genome sequencing*, WGS) vidjelo se da ESBL-producirajući izolati, a pogotovo *E. coli*, najčešće nose $bla_{\text{CTX-M-15}}$ i $bla_{\text{TEM-116}}$, koji su često detektirani zajedno. Među izolatima otpornima na karbapeneme, *Klebsiella pnumoniae* je često nađena s $bla_{\text{NDM-1}}$ i $bla_{\text{OXA-48}}$, odražavajući tako trenutni trend u bolničkim izolatima, dok su *Klebsiella oxytoca, E. cloacae* cplx. i *Citrobacter* spp. najčešće nosili $bla_{\text{KPC-2}}$, zajedno s $bla_{\text{NDM-1}}$. Molekularnom epidemiologijom, pomoću gel elektroforeze u izmjeničnom polju (engl. *pulsed-field gel electrophoresis*, PFGE) i određivanja nukleotidnih sekvenci na više genskih lokusa (engl. *multilocus sequence typing*, MLST), pokazalo se da su većina ESBL-producirajućih *E. coli* bili viskokorizični klonovi (ST131) i visokorizični klonovi u nastajanju (ST361). Većina karbapenem-otpornih *Klebsiella* i *E. cloacae* cplx. izolata, bili su ljudskog porijekla, a većina ih je bila identificirana po prvi put u Hrvatskoj. Na temelju dominantnih klonskih tipova i kliničkog značaja, 17 izolata (ESBL- i karbapenem otporni) bila su podvrgnuta WGS analizi. Osim ESBL i/ili karbapenemaznih gena, sekvencirani izolati sadržavali su klinički važne gene za otpornost na druge antibiotike (npr. na aminoglikozide, fluorokinolone, trimetoprim i sulfonamide), virulentne gene te razne plazmidne replikone koji su prethodno bili povezani sa širenjem ESBL i karbapenemaznih gena. Genomskom analizom, u *Klebsiella* spp. i *E. cloacae* cplx izolatima nađeni su već poznati i novi mehanizmi otpornosti na kolistin,. Ovi mehanizmi uključivali su poznate i nove kromosomalne točkaste mutacije u *pmrA/B, phoP/Q,*

mgrB i/ili *crrB* genima, kao i stečeni plasmidni *mcr-4.3* gen. Gen *mcr-4.3* po prvi put je nađen u *K. pneumoniae* (ST629) i nalazio se u konjugativnom IncHI1B plazmidu. Ovi rezultati ukazuju na bitnu ulogu pročišćene otpadne vode kao rezervoara za širenje, klinički važnih sojeva enterobakterija koji imaju klinički bitne gene za otpornost na antibiotike, u okoliš.

Slijedom toga ovaj disertacijski rad pokriva i fenotipsku i genomsku karakterizaciju ESBL-producirajućih i karbapenem-otpornih izolata iz otpadnih voda dviju velikih zagrebačkih bolnica. Od 200 izolata iz otpadne vode bolnica, 69 izolata bila su potvrđena kao ESBL proizvođači, a 90 izolata kao proizvođači karbapenemaza. Većina ih je bila identificirana kao *E. coli*, *Citrobacter* spp., *E. cloacae* cplx. i *K. pneumoniae* te su svi bili višestruko otporni na antibiotike. Uz to, otpornost na kolistin je bila uglavnom nađena u izolatima koji su otporni na karbapeneme. Kod ESBL-producirajućih izolata, $bla_{\text{CTX-M-15}}$ bio je najzastupljeniji ESBL gen, dok su karbapenem-otporni izolati često nosili $bla_{\text{KPC-2}}$, $bla_{\text{NDM-1}}$ te $bla_{\text{OXA-48}}$. PFGE i MLST metodama uočeno je da su najčešći klonalni tipovi bili visokorizični klonovi ST131 *E. coli*, visokorizični klonovi u nastajanju ST101 *K. pneumoniae* te ST277 *E. cloacae* cplx. klonovi. Uz to, WGS analizom odabranih ESBL- i karbapenem-otpornih izolata *E. coli*, *K. pneumoniae* i *E. cloacae* cplx. nađen je niz gena za otpornost na različite klase antibiotika te različite vrste plazmidnih replikona. Osim toga, klinički važni geni $bla_{\text{CTX-M-15}}$, $bla_{\text{KPC-2}}$ i $bla_{\text{OXA-48}}$ bili su okruženi mobilnim genetičkim elementima kao što su transpozoni i insercijske sekvence, ukazujući tako na njihov potencijal za mobilizaciju. Na kraju, kolistinska otpornost u *E. cloacae* cplx. bila je posredovana kromosomskim točkastim mutacijama u genima *pmrA/B* i/ili *phoP/Q*. Ovi rezultati pokazuju da je bolnička otpadna voda potencijalni sekundarni rezervoar za klinički važne patogene i gene za otpornost na antibiotike te se mora prethodno učinkovito pročistiti prije ispuštanja u komunalni sustav otpadnih voda.

Sveukupno, rezultati ove disertacije pokazuju da konvencionalno pročišćavanje u hrvatskim UPOV-ima ima ograničenu učinkovitost u eliminaciji enterobakterija otpornih na CTG-ove i karbapeneme, kao i ESBL i karbapenemaznih gena, koji se zatim ispuštaju u okoliš putem pročišćene otpadne vode. Nadalje, bolničke otpadne vode su važan izvor patogena i kliničkih važnih gena za otpornost na antibiotike u komunalnom sustavu otpadne vode. Stoga, implementacijom predtretmana bolničkih otpadnih voda i naprednih tehnologija za pročišćavanje komunalnih otpadnih voda trebalo bi se smanjiti širenje ovih determinanata antimikrobne otpornosti u okoliš.

Ključne riječi: Enterobacterales, β-laktamaze proširenog spektra, karbapenemaze, uređaji za pročišćavanje otpadnih voda, pročišćena otpadna voda, bolničke otpadne vode, otpornost na antibiotike

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Nikolina Udiković Kolić, Ph.D., was born on March 20, 1978, in Ogulin. She holds a Ph.D. in Biotechnology from the Faculty of Food Technology and Biotechnology at the University of Zagreb, where she also completed her bachelor's degree in the same field. Since 2001, she has been working at the Ruđer Bošković Institute in Zagreb, where she currently holds the position of Scientific Advisor and Head of the Laboratory for Environmental Microbiology and Biotechnology. Dr. Udiković Kolić has trained at top institutions abroad, including a year at Yale University in the USA as part of the prestigious Fulbright program. She has led six research projects and contributed to more than ten others. She has published over 45 peer-reviewed articles and a book chapter. Her work has been cited over 3,200 times, with a Google Scholar h-index of 25. She also teaches at doctoral study at the University of Osijek and has successfully supervised or co-supervised 2 PhD students (+1 ongoing) and 5 undergraduate students. She was also the main mentor for 5 postdoctoral students. Dr. Udiković Kolić has participated and given talks at numerous national and international conferences and has received several awards for her scientific contributions. From 2016 to 2021 she was Associate Editor of the Journal of Environmental Quality and now works as Associate Editor for Frontiers in Microbiology. She is also a member of the editorial board of Food Technology and Biotechnology (since 2015). She is a frequent reviewer for many prestigious journals and for national and international science foundations.

Author's publications included in the doctoral dissertation:

Publication No. 1:

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General introduction

Antimicrobial resistance (AMR) is one of the biggest medical problems today. It refers to the ability of microorganisms, including bacteria, viruses, fungi, and parasites, to resist the action of antimicrobial drugs, making infections more difficult to treat and increasing the risk of transmission to others. While natural AMR in bacteria predates antibiotic era, the problem increased over the last two decades. The overuse of antibiotics in both medicine and agriculture has contributed to this trend, leading to the selective pressure on bacterial population and stimulating them to develop and spread AMR. Nowadays, pathogenic bacteria have developed resistance mechanisms to almost all available antibiotics (Ventola, 2015). Due to the difficulties in treating infections caused by such multidrug-resistant (MDR) bacteria and the costs associated with the development of new antibiotics, the World Health Organization (WHO) has identified AMR as one of the most important global threats (WHO, 2014). AMR is not just a problem for humans – it is a One Health issue that involves interactions between the microbiome of humans, animals and the environment (WHO, 2023). Recently, increasing attention has been paid to how the environment, both natural and engineered, contributes to the persistence and spread of AMR (Bengtsson-Palme et al., 2023). This is of particular interest as many clinically relevant antibiotic resistance genes (ARGs) have environmental origins, such as *bla*$_{\text{CTX-M}}$ in *Kluyvera* spp., and *bla*$_{\text{OXA-48}}$ in *Shewanella* spp. (Humeniuk et al., 2002; Poirel et al., 2004). Additionally, bacteria that are clinically important nosocomial pathogens, such as carbapenem-resistant and extended spectrum β-lactamase- (ESBL) producing Enterobacterales, may also be present in the environment (Cherak et al., 2021; Cho et al., 2023). Therefore, it is important to fully understand all potential sources and routes of transmission of antibiotic-resistant bacteria (ARB) and ARGs to enable the development of strategies to reduce their incidence and spread.

In urban areas, wastewater treatment plants (WWTPs) are one of the most important sources for releasing ARB and ARGs into the environment (Marutescu et al., 2023). Urban WWTPs receive wastewater from various sources, including households and hospitals, enabling bacteria from different environments to interact and exchange genes horizontally. This exchange is facilitated by high bacterial densities, biofilms, high nutrient load, and stress caused by pollutant compounds, such as antibiotics and heavy metals (Karkman et al., 2018). Notably, antibiotics and heavy metals also originated from different sources (e.g. pharmaceutical and chemical industry, hospital wastewater, and farms) and are introduced into the treatment process via wastewater (Hama Aziz et al., 2023; Mutuku et al., 2022). In a typical WWTP, ARB and ARGs could be removed by

different processes, although there is also the possibility for their proliferation, if the conditions preferentially select for ARB or promote the potential for horizontal gene transfer (HGT) (Hong et al., 2018; Nguyen et al., 2021). Therefore, WWTPs could discharge relatively large amounts of these dangerous pollutants in the environment (Jäger et al., 2018; Müller et al., 2018) . It has been estimated that approximatively 10^{12} ARB and 10^{18} ARGs per day are continuously released from a conventional WWTPs (i.e. activated sludge used for transformation of dissolved substances into harmless or less harmless products) into the receiving environment (Manaia et al., 2018). Although only certain ARB released from WWTPs pose a direct threat to human or animal health, the risk of enriching the environmental resistome (i.e., the collective pool of ARGs harbored by microorganisms in the environment) whether by selection or HGT, cannot be overlooked. Such enrichment of ARB could eventually lead to increased AMR in pathogenic organisms by HGT (D'Costa et al., 2006; Manaia et al., 2018). This can happen either when the pathogens in the environment encounter commensal ARB or when the environmental ARB come into contact with pathogens in the human body. Resistant pathogens can furthermore be transmitted from person to person, posing a risk of global spread (Ashbolt et al., 2013).

In addition to urban wastewaters, hospital wastewater is essential as a major contributor of clinically relevant ARB and ARGs into municipal WWTPs and, subsequently, into the aquatic environment (Korzeniewska et al., 2013; Laffite et al., 2016; Lamba et al., 2017). The higher proportions of ARB and ARGs in hospital wastewater could be due to their greater prevalence in hospitals then in general population which may be reflected with the higher antibiotic use in hospitals (Kraupner et al., 2021). Therefore, surveillance of hospital wastewater could be a highly resource-efficient way to rapidly generate clinically relevant resistance data. Furthermore, in contrast to many developed countries, hospital wastewater in Croatia does not receive on-site treatment prior to introduction into sewage systems, exacerbating the dissemination potential of resistant pathogens and their ARGs.

Little is known about the occurrence of ARB and ARGs to critically important antibiotics for human medicine, such as third-generation cephalosporins (3GCs) and carbapenems, in wastewater. These types of resistance are increasingly common among Enterobacterales, an order of Gram-negative bacteria, responsible for a wide range of community- and healthcare-associated infections that are often difficult to treat due to their MDR nature (Noster et al., 2021). Carbapenem

antibiotics in particular serve as a last line of defense against such infections. A primary mechanism for resistance to 3GCs and carbapenems is the production of the β-lactamase enzymes ESBLs and carbapenemases (Bonomo, 2017). Typically, the genes encoding these enzymes are located on transferable plasmids together with other ARGs (Tacão et al., 2014). This co-localization favors their rapid co-transmission, leading to the emergence of difficult-to-treat bacteria. In addition, therapeutic options for severe infections, especially caused by carbapenemase-producing MDR strains, remain limited and often rely on colistin, a member of the polymyxin class of antibiotics. Recognizing the urgency of the situation, in 2017 the WHO classified ESBL-producing Enterobacterales (ESBL-E) and carbapenem-resistant Enterobacterales (CRE) as critical priority pathogens that require immediate action to combat their spread and promote the development of new antibiotics (WHO, 2017).

In recent years, there has been increasing interest in investing the presence of ESBL-E and CRE in wastewater (hospital and municipal) and rivers receiving treated wastewater. Studies have shown that ESBL-E and CRE can be detected in both untreated and treated wastewater from municipal WWTPs, as well as downstream waters (Hoelle et al., 2020; Makowska et al., 2020). Increased concentration of 3GCs-resistant *E. coli* has been observed in treated compared to untreated wastewater, in addition to ESBL-producing *E. coli* have been found in rivers downstream of WWTPs (Blaak et al., 2014; Korzeniewska and Harnisz, 2018). In recent studies, CRE were detected in treated wastewater and downstream rivers (Hoffmann et al., 2023) with the potential risk for the transmission into WWTP workers (Rolbiecki et al., 2021). Furthermore, studies have identified both ESBL and carbapenemase genes associated with pathogenic Enterobacterales species in treated wastewater (Hembach et al., 2017; Subirats et al., 2017; Yang et al., 2016). Thus, treated wastewater could serve as a monitoring site for environmental exposure to this dangerous ARB and their mobile ARGs, with the risk of being reintroduced into the community (Larsson et al., 2023). The occurrence of these ARB with ESBL and carbapenemase genes is often associated with inflow of hospital wastewater, although it only comprises of about 2% of total wastewater arriving to the WWTP. Study from Poland showed that the abundance of ESBL and carbapenemase genes were significantly higher in the treated wastewater with higher load of hospital wastewater compared to WWTPs receiving lower percentage of hospital wastewater (Hubeny et al., 2021). In addition, the occurrence of similar carbapenemase genes found in the downstream river were also found in WWTPs which treat wastewater from hospitals (Proia et al., 2018). Further studies

identified the same high-risk *K. pneumoniae* clones carrying ESBL and carbapenemase genes in hospitals, effluents, and downstream rivers (Kehl et al., 2022; Lepuschitz et al., 2019). Therefore, hospital wastewater has been recognized as a critical point for the dissemination of ESBL-E and CRE with significant contribution to their increased levels in treated wastewater (Müller et al., 2018). Consequently, it was suggested that hospital wastewater, as the primary source of these highly resistant bacteria in municipal wastewater, could serve as their early-warning system (Flach et al., 2021). Although in Croatia there has been no systematical studies of hospital wastewater, occurrence of KPC-producing *K. pneumoniae* was found in the downstream river from hospital (Jelić et al., 2019). In addition, high prevalence of resistance to 3GCs and carbapenems among Enterobacterales, especially in *K. pneumoniae* has been reported in Croatian hospital settings (ECDC, 2023a). In 2022, 54.2 % and 24% of clinical *K. pneumoniae* isolates were resistant to 3GCs and carbapenems, respectively (ECDC, 2023b). The possible reason could be the high carbapenem consumption in hospital and community sectors during the COVID-19 pandemic in Croatia (Bedenić et al., 2023; ECDC, 2023c). Therefore, understanding factors that favor the spread of 3GCs-resistant Enterobacterales and CRE, outside hospitals, is crucial to prevent their further dissemination.

However, to date, most of studies on ESBL-E and CRE in wastewater have focused primarily on identifying their phenotypic and molecular mechanisms of resistance. These studies usually utilize culture-based methods in combination with molecular methods such as PCR and/or whole-genome sequencing (WGS). Despite these efforts, the molecular epidemiology of these isolates, characterized by genotyping approaches such as multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE), still remains poorly understood. Furthermore, there has been limited research on the quantification of ESBL and carbapenemase genes by quantitative PCR (qPCR) in both hospital and municipal wastewater. Therefore, this dissertation takes a comprehensive approach using a combination of these methods to investigate the role of hospital and municipal wastewater in Croatia in the spread of these clinically important ARB and ARGs. The aim was to isolate ESBL-E and CRE strains from hospital and treated wastewater and to understand the AMR patterns of these isolates, their phylogenetic relationship to known pandemic high-risk clones, the molecular mechanisms underlying their resistance and their potential virulence. Additionally, efficiency of WWTPs in eliminating selected ESBL and carbapenemase genes from seven Croatian cities was evaluated. The results from this dissertation will provide

important insights on the contribution of hospital and municipal wastewater on the complex dynamics associated with the spread of 3GC and carbapenem resistance in aquatic environment in Croatia.

Chapter 1

Theoretical background

1.1. β-lactam antibiotics

Since the discovery of benzylpenicillin in the 1920s, β-lactam antibiotics have been the most commonly used class of antibiotics for the treatment of infectious diseases. Over the years, various β-lactam classes have been introduced. Their discovery aim to broaden the spectrum of antimicrobial activity against a larger number of bacterial species and to overcome specific resistance mechanisms that occur within the targeted bacterial populations (Bush and Bradford, 2016). In Croatia, in 2022, β-lactam antibiotics accounted for approximatively 58% of all antibiotics prescribed in both hospital and in community (Figure 1A; ECDC, 2023c).

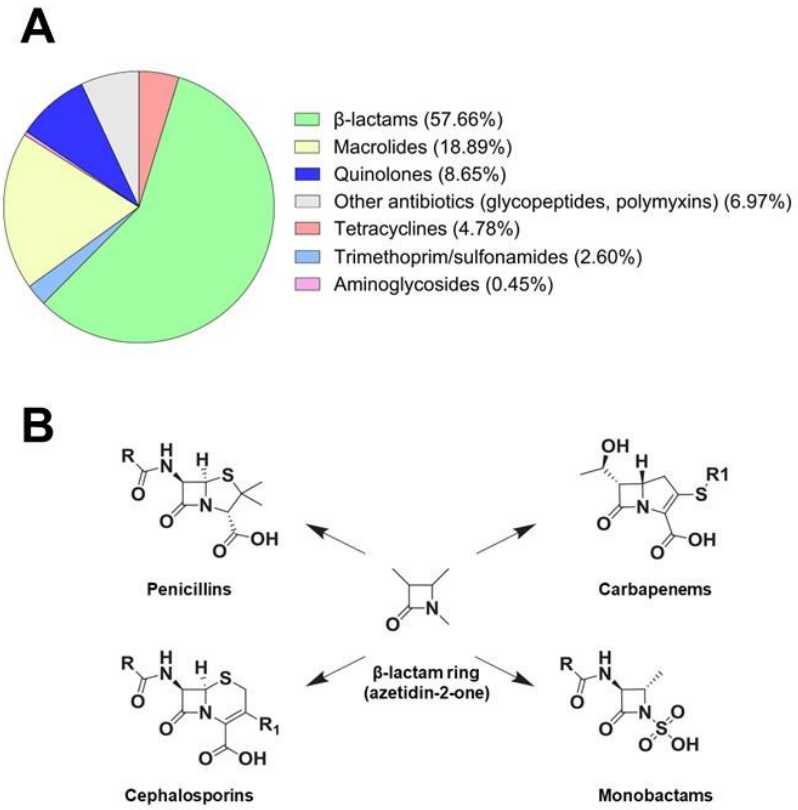

Figure 1. (A) Prescribed antibiotics in hospital and in the community in Croatia for the year 2022. The percentage is expressed as defined daily doses of antibiotics per 1000 inhabitants per day. The percentage is given in brackets (Adapted from ECDC, 2023c https://qap.ecdc.europa.eu/public/extensions/AMC2_Dashboard/AMC2_Dashboard.html, accessed 8th January 2024). (B) Structure of the β-lactam antibiotic groups (Adapted from Vardanyan and Hruby (2016)).

The group of β-lactam antibiotics is divided into two large antibiotic families, the penicillins and the cephalosporins. Additionally, carbapenems and monobactams are newer β-lactam antibiotics, discovered in the 1980s, structurally differing from the conventional β-lactams. Carbapenems have substitution of the sulfur atom in the pyrroline ring with a methylene group, while monobactams have a monocyclic β-lactam structure (Figure 1B). However, all β-lactam antibiotics have a common structural feature – a four-membered β-lactam ring (azetidine-2-one), which is necessary for exhibiting antimicrobial activity (Figure 1B; Vardanyan and Hruby, 2016).

1.1.1. Cephalosporins

Cephalosporins, a key group within the β-lactam antibiotics, are known for their broad spectrum of activity against bacterial infections. Due to their superior resistance to bacterial hydrolytic enzymes, penicillinases, which destroy the β-lactam ring of penicillins, are often used for the treatment of a variety of infections. Therefore, cephalosporins are used to treat infections caused by Gram-negative and Gram-positive bacteria (Das et al., 2019). Their frequent use in clinical pratice is due to their low toxicity, broad spectrum of activity, and ease of administration. Cephalosporins are often prescribed for the treatment of pneumonia, skin and soft-tissue infections, bacteremia and meningitis (Vardanyan and Hruby, 2016).

The cephalosporin compound was first isolated in 1948, from the mold culture *Acremonium strictum* (formerly known as *Cephalosporium acremonium*) in a sewer in Sardinia (Shahbaz, 2017). This mold naturally produces cephalosporin C, which is chemically or enzymatically processed to produce 7-amino cephalosporanic acid, the precursor of all five generations of cephalosporins (Vardanyan and Hruby, 2016). Structurally and functionally similar to penicillins, cephalosporins have the common β-lactam ring, but differ by having a second 6-membered dihydrothiazine ring fused to their 4-membered β-lactam ring via the nitrogen and a tetrahedral carbon atom (Figure 2). This structure is characterized by a carboxyl group on the dihydrothiazine ring at position C-2, which is adjacent next to the nitrogen, and a functionalized amino group at C-7 of the β-lactam ring, which is located opposite to the nitrogen (Figure 2; Roberts, 2001).

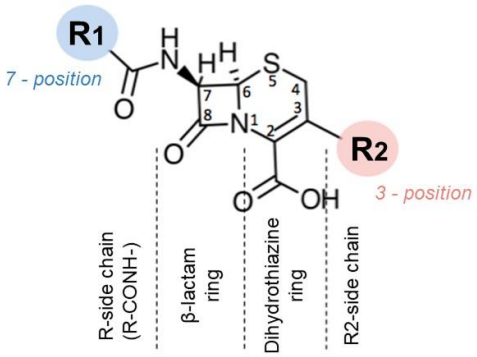

Figure 2. General structure of cephalosporin antibiotics (https://www.ebmconsult.com/articles/penicillin-allergy-cross-reactivity-cephalosporin-antibiotics#jump_ss_101109, accessed 8th January 2024).

Cephalosporins are categorized into five generations, based on their spectrum of activity against Gram-negative and Gram-positive bacteria (Table 1), aligning with the chronological order of their development. Each next generation generally have a broader antimicrobial spectrum (Vardanyan and Hruby, 2016). In particular, the 3GCs such as cefotaxime and ceftazidime, are primarily used against Enterobacterales and other Gram-negative species, as they are very effective against penicillinases.

Table 1 Classification of cephalosporins by generation, including representatives, antimicrobial activity, and medicinal use.

Cephalosporin generations	Representatives	Antimicrobial activity	Use
1st	Cefazolin, Cephalexin	Aerobic Gram-positive cocci (*Streptococcus* spp. and *Staphylococcus* spp. excluding methicillin-resistant *Staphylococcus aureus* (MRSA), *S. epidermidis* and enterococci)), some Gram-negative strains (*E. coli*, *K. pneumoniae*, and *Proteus mirabilis*)	Uncomplicated community acquired skin and soft tissue infections, urinary and respiratory tract infections, and surgical wound prophylaxis
2nd	"True" 2nd gen.-cefuroxime Cephamycins - cefoxitin, cefotetan, and cefmetazole	Gram-positive *Streptococcus* spp. and *Staphylococcus* spp., Expanded Gram-negative bacteria coverage including 1st gen. cephalosporin resistant and indole-positive *Proteus* and *Enterobacter* species (except *Enterobacter cloacae*), *Haemophilus influenza,* and *Bacteroides fragilis*	"True" 2nd gen. cephalosporins are used for community-acquired infections of the respiratory and urinary tract. Cephamycins are used for aerobic-anaerobic skin, soft tissue, intraabdominal, and gynecologic infections, and for surgical prophylaxis.
3rd	Cefotaxime, Ceftazidime	Gram-positive *Streptococcus pneumoniae* and *S. pyogenes* with less activity against staphylococci, Gram-negative Enterobacterales, *Pseudomonas* spp., *Haemophilus influenza*, *Neisseria gonorrhoeae*	Recommended as first line antibiotics for surgical and post-operative prophylaxis, different community-acquired pulmonary, sexually transmitted infections, and meningitis.
4th	Cefepime	Gram-positive methicillin-susceptible *S. aureus*, and streptococci, Gram-negative bacteria such as *E. coli, Klebsiella spp., Enterobacter spp., P. aeruginosa, H. influenzae,* and *N. meningitides*	Treatment of infections caused by drug-resistant microorganism, surgical infections.
5th	Ceftobiprole Ceftaroline	Broad-spectrum against Gram positive bacteria – MRSA, vancomycin-intermediate and vancomycin-resistant *S. aureus*, *Streptococcus pneumoniae*, enterococcus, Gram-negative bacteria except *Pseudomonas* spp.	Treatment of complicated infections of skin and soft tissue.

1.1.2. Carbapenems

Carbapenems are the most powerful subclass of ß-lactam antibiotics, effective against both Gram-positive and Gram-negative bacteria, including anaerobes but are usually reserved for the treatment of the most severe MDR infections caused by Enterobacterales (Doi and Paterson, 2015). However, with the increase of these types of infections, their use in healthcare facilities worldwide is increasing dramatically (Doi and Paterson, 2015).

The first carbapenem, thienamycin, which was isolated from *Streptomyces cattleya* and exhibited high broad-spectrum and β-lactamase inhibitory activity, served as a model for all other carbapenems (Papp-Wallace et al., 2011). Currently, the most commonly used carbapenems include ertapenem, imipenem, meropenem and doripenem as they all have a broad-spectrum of activity against both Gram-positive and Gram-negative bacteria (El-Gamal et al., 2017).

Structurally, carbapenems have a four-membered β-lactam ring fused to a five-membered secondary thiazolidine ring via nitrogen and a neighboring tetrahedral carbon atom (Figure 3A; Papp-Wallace et al., 2011). The stability toward β-lactamases increased with the substitution of a sulfur with a carbon atom at the C-1 position in secondary ring (Figure 3A; Nicolau, 2008). In addition, the hydroxyethyl side chain with *R* configuration at C-8 and *trans* configuration between C-5 and C-6 positions in the β-lactam ring, confers significant antimicrobial properties, stability and resistance to β-lactamase hydrolysis (Figure 3B) (Papp-Wallace et al., 2011). In particular, carbapenems such as ertapenem, meropenem, and doripenem which possess a pyrrolidine ring have an even broader spectrum activity compared to imipenem (Figure 4C). However, due to the instability of the side chains, a considerable number of carbapenems are unsuitable for clinical use, resulting in a limited number of available carbapenem antibiotics (Nicolau, 2008).

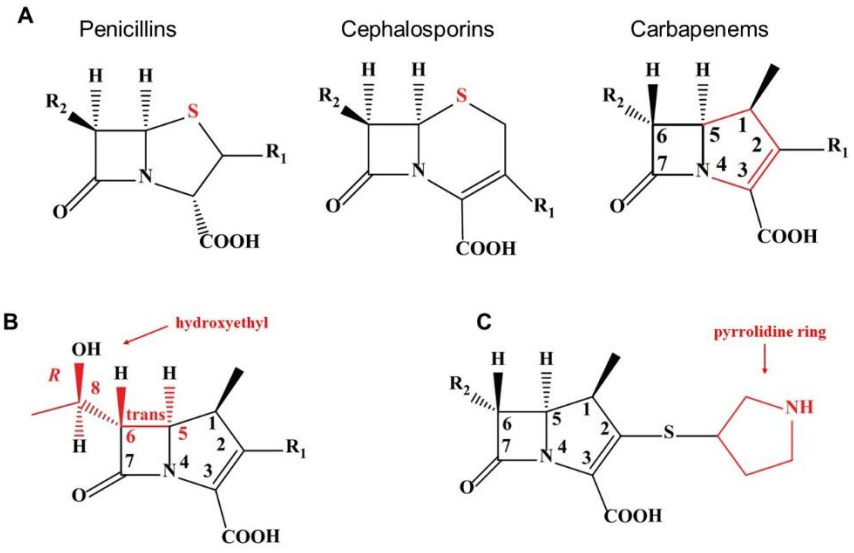

Figure 3. A) Backbone of penicillins, cephalosporins and carbapenems. B) The *R* configuration of hydroxyethyl and *trans* configurations at the C-5 – C-6 bond in β-lactam ring. C) The structure of pyrrolidine ring in carbapenem antibiotics (Adapted from Papp-Wallace et al., 2011).

1.1.3. Mode of action of β-lactam antibiotics

The mechanism by which β-lactams act against bacteria is based on the inhibition of cell wall synthesis. The main component of the bacterial cell wall is the peptidoglycan, which consists of long sugar polymers and the glycan strand cross-linked with peptide chains that extend from the sugars and form cross-links with other peptides (Kapoor et al., 2017). The penicillin-binding proteins (PBPs) facilitate cross-linking of the D-alanyl-alanine part of the peptide chain with the glycine, which strengthens the cell wall (Bhattacharjee, 2016). β-lactams target PBPs because their β-lactam ring, mimics the structure of D-alanyl D-alanine, which is typically bound by PBPs (Kapoor et al., 2017). This interaction between the β-lactam ring and PBPs interferes with normal peptidoglycan synthesis, leading to bacterial cell lysis (Kapoor et al., 2017).

1.2. Mechanisms of AMR and its acquisition

For each class of antibiotics there are several resistance mechanisms that depend on the bacterial species and its genetic structure. The most important mechanisms of resistance include (i) enzymatic modification or inactivation of the antibiotic (e.g. β-lactamases and aminoglycoside-modifying enzymes); (ii) modification of antibiotic target (e.g. resistance to β-lactams via change in PBP2 structure in *S. aureus* by acquisition of the *mecA* gene or chromosomal mutation for overproduction of enzyme dihydrofolate reductase conferring the resistance to trimethoprim); (iii) decreased antibiotic uptake by reduced permeability (e.g. downregulation of porins or mutated porins); (iv) active antibiotic efflux (e.g. overexpression of efflux pumps, encoded by *mefA* and *mefE* genes, that extrude macrolides) (Blair et al., 2015; Munita and Arias, 2016a).

AMR can generally be classified as intrinsic and acquired resistance, which is determined by the way of resistance development (Aslam et al., 2018; Bo et al., 2024). Intrinsic resistance refers to the natural ability of bacteria to resist antibiotics due to inherent structural or functional characteristic (Aslam et al., 2018). This natural defense mechanism, observed in various bacterial strains is independent of the external pressure exerted by antibiotics. The most common intrinsic resistance mechanisms are reduced permeability of the outer membrane, which leads to the inability for the antibiotic to enter the bacterial cell, and natural activity of the efflux pumps, which allows removing toxic substances such as antibiotics from the cell (Reygaert, 2018). For example, in *K. pneumoniae* mutations in *ompK35* gene leads to loss of functions in porins which prevents the

uptake of β-lactam antibiotics (Munita and Arias, 2016). In addition, Enterobacterales species, such as *E. coli* and *K. pneumoniae* have efflux pumps, belonging to AcrAB-TolC system, which are able to extrude a wide array of substrates, conferring resistance to tetracyclines, chloramphenicols, novobiocin, fusidic acid, and fluoroquinolones (Munita and Arias, 2016).

Acquired resistance is usually a significant clinical problem as it has the potential to multiply within bacterial populations. It refers to the development of resistance through the acquisition of a specific resistance mechanism, either through spontaneous mutations (Reygaert, 2018) or through HGT (Nguyen et al., 2021). For example, bacterium that developed point mutations changes parts of its genetic code, which can disrupt the binding site for antibiotic, leading to its ineffectiveness against the bacterial target. Examples of point mutations include resistance to one of the last-resort antibiotics, colistin. In *E. coli*, *Enterobacter, Klebsiella* and *Salmonella* mutations in genes involved in the biosynthesis and modification of lipopolysaccharide (LPS) membranes, lead to the LPS alterations that prevent colistin from binding effectively (Gogry et al., 2021). Although these genetic mutations are still rare, they can become a global health problem due to the clonal spread of the mutants (Gil-Gil et al., 2021). However, it is even more worrying when AMR determinant is acquired through HGT. The HGT allows bacteria susceptible to an antibiotic to developed resistance, which enables its multiplying and spread under its selection pressure (Nguyen et al., 2021). There are three main HGT routes by which bacterial cells can acquire ARGs: transformation (taking extracellular DNA from environment), transduction (transfer of DNA via bacteriophages), and conjugation (transfer of genetic material between two bacterial cells, e.g. via plasmids) (Nguyen et al., 2021). The HGT of ARGs is more likely to occur when ARGs are carried by mobile genetic elements. Insertion sequences (IS), transposons (Tn), and integron/gene cassette system (In) as intracellular mobile genetic elements play important role in capturing and transferring ARGs from chromosome to plasmids in the same cell (Partridge et al., 2018). Plasmids as intercellular mobile genetic elements have a central role in the spread of ARGs through HGT between different bacterial cells, even among genetically unrelated bacteria (Partridge, 2015). These small, circular DNA molecules often carry multiple ARGs and contribute significantly to the diversity of the bacterial resistome. Given their high adaptability and diverse mobile genetic elements, classification of plasmids is often based on replicon typing. Replicons are conservative regions in the plasmid DNA that are important for plasmid replication. Plasmids with the same replicon are incompatible and belong to the same incompatibility group (Inc), which

means that two plasmids with the same replicon cannot stably coexist in the same bacterial cell (Thomas, 2014). There are currently 28 Inc groups found in Enterobacterales (Rozwandowicz et al., 2018). Common plasmids, often belonging to the Inc groups IncA/C, IncF, IncH, IncI, IncL/M, IncN, and IncX, expressing a wide variety of ARGs against different antimicrobial classes, with IncA/C, IncF, and IncX having a global prevalence (Mathers et al., 2015; Rozwandowicz et al., 2018). In addition, IncP, IncR, IncU and IncW are also reported worldwide (Kopotsa et al., 2019). As these plasmids can carry multiple ARGs, a single plasmid conjugation could be sufficient to transfer a MDR phenotype in bacteria (Ruppé et al., 2015).

1.3. Enterobacterales

The Enterobacterales order, belong to the *Gammaproteobacteria* class, and includes a large and diverse group of Gram-negative, facultative anaerobic, non-spore-forming, rod-shaped bacteria (Adeolu et al., 2016). This order comprises of seven genera, each with at least 60% genome-to-genome relatedness, with the *Enterobacteriaceae* family being the most prominent (Adeolu et al., 2016). This family contains well-known genera such as *Escherichia*, *Klebsiella*, *Enterobacter, Salmonella,* and others. Enterobacterales are characterized by being catalase- and lactase-positive, as well as oxidase-negative, which is an important diagnostic feature. All species possess the somatic (O) antigen, and most are motile, possessing the flagellar (H) antigen. Certain genera, including *Klebsiella* and *Enterobacter* are characterized by capsular formation (Kalenić et al., 2013). Enterobacteriales can be found in various environments, including soil, water, and the gastrointestinal tract of humans and animals. Some species are commensals, while other are pathogens that can cause a range of diseases (Octavia and Lan, 2014). The latter includes obligate pathogens such as *Salmonella, Shigella*, and *Yersinia* species. Other genera such as *E. coli*, and specific strains within the *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia, Proteus, Providencia,* and *Morganella* species are referred to as opportunistic pathogens which are often associated with infections in clinical settings (Kalenić et al., 2013). These bacteria are primarily responsible for urinary tract infections, bacteremia, and gastrointestinal tract infections, with an estimated 46% of all hospital-acquired infections are caused by these Enterobacterales species (Dafale and Purohit, 2016). Moreover, *E. coli* and other Enterobacterales, such as *Enterobacter*, *Klebsiella*, and *Citrobacter* are commonly referred to as coliform bacteria. As they are widely distributed in the gastrointestinal tract, they serve as indicators of fecal contamination of various water sources, as

well as in food production (Li and Liu, 2019). Although they do not usually pose a significant health risk, their presence may indicate the potential presence of fecal-derived pathogenic organisms such as obligatory intestinal pathogenic *E. coli* (IPEC) and facultative pathogen extraintestinal pathogenic *E. coli* (ExPEC), which are both found in humans and animals (Sora et al., 2021). Transmission of these pathogenic enterobacteria occurs via the fecal-oral route, through contaminated food or water. This emphasizes the critical importance of maintaining good hygiene practices to prevent their spread.

1.3.1. β-lactam resistance mechanisms in Enterobacterales

As an integral part of the gut microbiome, Enterobacterales are frequently exposed to antibiotics and have evolved mechanisms to overcome their effects. One of these adaptations is the development of the resistance mechanisms against β-lactams, which is mainly enabled by the production of periplasmic enzymes, called β-lactamases. These enzymes, which are usually encoded either in the bacterial chromosome or on plasmids, play the central role in the hydrolysis of the β-lactam ring and preventing antibiotic to reach the PBP (Papp-Wallace et al., 2011).

Currently, over 8000 unique β-lactamase enzymes are listed in the Beta-Lactamase DataBase (BLDB) (Naas et al., 2017). β-lactamases are categorized according to the Ambler molecular classification (classes A to D), which is based on amino acid sequence similarity, and the Bush-Jacoby-Medeiros functional classification (groups 1 to 3), which is based on biochemical function and susceptibility towards β-lactamase inhibitors (Bush and Jacoby, 2010). While the Bush-Jacoby-Medeiros classification is more precise, the Ambler classification is more used due to its simplicity. According to the Ambler classification, classes A, C, and D are serine-based hydrolytic enzymes, while class B, known as metallo-β-lactamases (MBL), contains zinc ions at the active site (Sawa et al., 2020). This zinc ion dependence makes them stabile against inhibition by traditional β-lactamase inhibitors, such as clavulanic acid or sulbactam, which are effective against other types of β-lactamases (Bahr et al., 2022).

The chromosomally induced β-lactam resistance often results from the overexpression of serine-based Ambler class C AmpC β-lactamases which is triggered by the presence of penicillins, clavulanic acid, 1st generation cephalosporins and cephamycins (Ruppé et al., 2015). Under these conditions, spontaneous mutations may occur, leading to overexpression of AmpC and

subsequently conferring resistance to penicillin, aztreonam, 3GCs and even ertapenem. However, it is important to note that these mutations are rare and usually only occur sporadically in bacterial populations (Partridge, 2015; Ruppé et al., 2015).

Moreover, resistance to β-lactams, including 3GCs and carbapenems, among Enterobacterales, is predominantly attributed to plasmid-mediated mechanisms. These mechanisms involve the acquisition of *bla* genes carried on plasmids through HGT process (Partridge, 2015). Enterobacterales strains resistant to both 3GCs and carbapenems often display MDR phenotype, posing a significant public health threat on a global scale. Treating infections caused by these strains often requires the use of last-resort antimicrobials, such as colistin. Therefore, the WHO has identified 3GCs-resistant Enterobacterales and CRE as critical priority pathogens for research and development of new antibiotics (WHO, 2017). In Croatia, there is a notable clinical emergence of these strains, especially *E. coli* and *K. pneumoniae* which have developed resistance to 3GCs and carbapenems. For example, a remarkably high rate of these resistant strains was detected in Croatian hospitals in 2021, with 18% of *E. coli* isolates and a strikingly high 62% of *K. pneumoniae* isolates showed resistance to 3GCs (ECDC, 2023b), where their resistance was primarily attributed to the formation of ESBLs (ISKRA, 2022). In addition, the incidence of carbapenem-resistant *K. pneumoniae* increased by 110% between 2019 and 2022, placing Croatia among the top 10 EU countries with the highest carbapenem resistance in healthcare settings (ECDC, 2023a; ECDC, 2023b).

1.3.2. ESBLs in Enterobacterales

Since the early 1980s, 3GCs have proven to be very successful in the treatment of Gram-negative bacteria (Paterson and Bonomo, 2005). Soon after, ESBL enzymes were discovered. These serine-based β-lactamases, which belong to the Ambler class A and D, are able to hydrolyze most β-lactams antibiotics, including 3GCs such as cefotaxime, ceftriaxone and ceftazidime, as well as monobactams (e.g. aztreonam), but not cephamycins (e.g. cefotetan and cefoxitin), and carbapenems (e.g. meropenem, imipenem, and ertapenem) (Castanheira et al., 2021). ESBL are most commonly found in Enterobacterales, especially in *K. pneumoniae* and *E. coli* (Chao et al., 2023). In addition, many ESBL-E are also capable of acquiring resistance to other classes of antibiotics, such as aminoglycosides, trimethoprim, sulfonamides, quinolones, tetracyclines, and

chloramphenicol (Khadka et al., 2023), therefore, these organisms pose a particular challenge for the treatment (Mancuso et al., 2021).

ESBL enzymes were first detected in clinical isolates resistant to 3GCs in the early 1980s. These enzymes arose from point mutations in the parent enzymes, such as temoniera (TEM) or sulfhydryl reagent variable (SHV) β-lactamase types (Castanheira et al., 2021). These enzymes dominated in the 1980s and 1990s, but after the 2000s, there was a notable change in the genetic profile of ESBLs (Bush and Bradford, 2020). In particular, in *E. coli* and *K. pneumoniae*, there was an increase in the prevalence of a particular enzyme called cefotaxime from Munich (CTX-M) (Peirano and Pitout, 2019). CTX-M enzymes are thought to be derived from an enzyme originally present in the chromosome of environmental *Kluyvera* species (Cantón et al., 2012a). The chromosomal genes (bla_{KLU}) responsible for encoding the enzyme have been mobilized onto conjugative plasmids, which allow easy transfer between pathogens. In addition, the emergence of $bla_{\text{CTX-M}}$ gene appears to have an increased potential to trigger outbreaks (Cantón et al., 2012a). Currently, the CTX-M enzymes are divided into five subfamilies based on its amino acid profiles, namely CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Peirano and Pitout, 2019). The CTX-M-15 enzyme (CTX-M-1 subfamily), which was first detected in *E. coli* from India in 2001, is now the most common ESBL variant worldwide (Bush and Bradford, 2020). CTX-M ESBLs generally hydrolyze cefotaxime and ceftriaxone but are less effective against ceftazidime. However, enzymes from the CTX-M-1 and CTX-M-9 subfamilies showed an increased ability to hydrolyze ceftazidime (Peirano and Pitout, 2019)

Other clinically relevant ESBLs include oxacillinases (OXA), Guiana extended-spectrum β-lactamases (GES), Vietnamese extended-spectrum β-lactamases (VEB) and *Pseudomonas* extended resistant (PER) (Castanheira et al., 2021). OXA-type ESBLs are classified as Ambler class D and to date, 27 OXA enzymes are known to have ESBL activity (Naas et al., 2017; Yoon and Jeong, 2021). However, the global distribution of OXA-types is not as broad as that of CTX-M ESBLs. The less common Ambler A class ESBLs include GES, VEB and PER families. GES-type enzymes are not closely related to any other plasmid-mediated β-lactamases and are more frequently described in *P. aeruginosa* and *Acinetobacter baumannii* than in Enterobacterales (Castanheira et al., 2021). Certain GES-type enzymes, such as GES-2 and GES-5, exhibit carbapenemase activity (Stewart et al., 2015). PER enzymes, originally discovered in *P.*

aeruginosa, have also been detected in various Enterobacterales species, *A. baumannii* and *Aeromonas* spp. (Castanheira et al., 2021). VEB-1 and its variants have been described in various Enterobacterales, *P. aeruginosa*, *A. baumannii*, *Vibrio* spp, and *Achromobacter xylosoyidans* (Castanheira et al., 2021).

1.3.3. Carbapenemases in Enterobacterales

Carbapenems are considered the primary option for infections caused by ESBL-E (Son et al., 2018). However, the global rise in ESBL-E infections has led to a surge in the use of carbapenems, which are often considered as antibiotics of last resort (Aslan and Akova, 2019). This increased use of carbapenems raises concerns about the emergence of CRE. Carbapenem resistance in Enterobacterales is primarily caused by the production of plasmid-mediated carbapenemase enzymes, that hydrolyze carbapenems and other β-lactams (Cantón et al., 2012b). However, in some rare cases, resistance to carbapenem is also attributed to the overproduction of AmpC β-lactamases together with ESBLs and the alternation or loss of outer membrane porins (Hamzaoui et al., 2018). Treatment options for serious CRE infections are extremely limited. Current strategies often involve a combination of different antibiotics, including colistin, tigecycline, fosfomycin, aminogylicosides, and double-carbapenem therapy (Sheu et al., 2019). In addition, carbapenemase-encoding genes, together with resistance genes to other classes of antibiotic, are often carried on conjugative plasmids that can be easily transferred between bacteria, leading to the rapid spread of MDR (Dolejska and Papagiannitsis, 2018). The most clinically relevant carbapenemases are *Klebsiella pneumoniae* carbapenemase (KPC), imipenemase (IMP), Verona integron-encoded metallo-beta-lactamase (VIM), New Delhi metallo-beta-lactamase (NDM) and oxacillinase-48 (OXA-48) (Nordmann et al., 2011).

KPC enzymes belonging to Ambler class A were discovered in *K. pneumoniae* in 1996 (Yigit et al., 2001). Shortly after, the international spread of KPC enzymes began with *K. pneumoniae* sequence type (ST) 258, which first caused outbreaks in the USA and then spread to the rest of the world (Bush and Bradford, 2020). Today, plasmid-mediated KPC is the most commonly detected carbapenemase worldwide, frequently found in *K. pneumoniae, Enterobacter* spp., *Citrobacter* spp., *K. oxytoca*, *E. coli*, and other Enterobacterales (Kazmierczak et al., 2016). The bla_{KPC} gene encoding KPC, is usually located on the composite transposon Tn*4401*, which is found in various plasmids (Chen et al., 2014). In Croatian hospital settings, a KPC-producing *K.*

pneumoniae was detected for the first time in 2011 (Bedenić et al., 2012). Subsequently, the clonal spread of *K. pneumoniae* ST258 was responsible for the dissemination of the KPC-2-type in the northwestern part of the country, where nowadays KPC is detected in all parts of the country (Bedenić et al., 2022; Jelic et al., 2016).

First described plasmid-mediated carbapenemase was IMP-type MBL, found in *P. aeruginosa* and soon after in Enterobacterales species (Ito et al., 1995). Currently, about 100 IMP variants are described in public databases, which are divided into six subgroups based on phylogeny and sequence similarity (Naas et al., 2017; Softley et al., 2020). The IMP-type enzymes are reported all over the world, especially in *P. aeruginosa*, remaining relatively rare among Enterobacterales isolates (Matsumura et al., 2017).

The MBL VIM enzymes were discovered in *P. aeruginosa* in France and Italy in the mid-1990s. By the mid-2000s, *K. pneumoniae* carrying $bla_{\text{VIM-1}}$ was already spreading rapidly throughout southern Europe, with epicenter in Greece (Cantón et al., 2012b). In Croatia, the frequent detection of $bla_{\text{VIM-1}}$ occurred at the beginning of 2010s, mainly in *Enterobacter cloacae* although today is rarely found in clinical isolates (Bedenić et al., 2023; Zujić Atalić et al., 2014).

Last discovered carbapenemase was MBL NDM. It was found in Sweden from an Indian patient, in 2008, and in the less than a decade, it spread rapidly all over the world (Bush and Bradford, 2020). It is often observed in Enterobacterales species, where *E. coli*, *K. pneumoniae*, and *Enterobacter* spp. are accountable for the spread of NDM variants, suggesting heterogeneous acquisition of bla_{NDM} across different species (Wu et al., 2019). In 2009, NDM-1 was detected in *K. pneumoniae* ST25 from a Croatian hospital (Mazzariol et al., 2012). Currently, there is an increase in the occurrence of NDM-1-producing *K. pneumoniae* with additional carbapenemase OXA-48, in Croatia and most of European countries (Bedenić et al., 2023; Ludden et al., 2020).

Only a small number of β-lactamase variants from Ambler class D are classified as carbapenemases. OXA-48-type carbapenemase hydrolyzes imipenem, but shows less efficacy against meropenem (Poirel et al., 2012). In 2007, OXA-48-producing Enterobacterales spread from Turkey to other European countries (Bush and Bradford, 2019). The $bla_{\text{OXA-48}}$ gene is often spread via the transposon Tn*1999* on the conjugative pOXA-48 (IncL/M) plasmids (Poirel et al., 2012). Although OXA-48 is present in various enterobacteria such as *E. coli*, *E. cloacae*, and *C. freundii*,

certain clonal types of *K. pneumoniae* are responsible for its global spread (Pitout et al., 2019). These clonal outbreaks of plasmid-mediated OXA-48 are caused by high-risk and emerging high-risk clones such as *K. pneumoniae* ST101, ST359, ST15, and ST147 (Pitout et al., 2019). In Croatia, outbreaks of OXA-48 are usually associated with *K. pneumoniae* ST15 and ST16 (Bedenić et al., 2018; Jelić et al., 2018). In addition to OXA-48, its variants, including OXA-162, OXA-163, OXA-181, and OXA-232, have also been identified in Enterobacterales (Pitout et al., 2019).

1.4. Wastewater as a source and transmission route of 3GC- and carbapenem-resistant Enterobacterales into the environment

The primary role of urban WWTP is to protect humans and the environment from anthropogenic pollution. Overall, WWTPs have been shown to effectively remove ARB from wastewater prior to discharge into the environment (Karkman et al., 2019). The decreases in ARB abundance in treated wastewater, compared to untreated wastewater is primarily due to the reduction in total bacterial load during treatment processes (Fouz et al., 2020). Despite the observed ARB decrease during the treatment, ARB and ARGs can still be detected in treated wastewater, posing a continued risk of AMR pollution to the environment. For example, studies have found that ESBL and carbapenemase genes are still present in treated wastewater, suggesting that current treatment methods may not be fully effective in eliminating these genes from wastewater (Hembach et al., 2017; Subirats et al., 2017; Yang et al., 2016). It was estimated that conventional WWTP releases up to 10^{12}-10^{18} copies of ARGs daily, and approximately 10^{10}-10^{12} colony-forming units (CFU) per day of *E. coli* resistant to 3GCs (Bréchet et al., 2014; Kwak et al., 2015; Manaia et al., 2016). In addition, studies of treated wastewater have revealed the presence of clinically significant *E. coli* clones that carry various ESBL genes, often associated with conjugative plasmids (Dolejska et al., 2011). Similarly, *K. pneumoniae* isolates carrying different carbapenemases on transferable plasmids were disseminated into receiving river (Hoffmann et al., 2023). This raises the possibility of these plasmids being transferred to commensal or pathogenic bacteria, further exacerbating the spread of resistance to last-resort carbapenems. Moreover, several studies have demonstrated an enrichment of 3GC- or carbapenem-resistant enterobacterial strains during the wastewater treatment. For example, Korzeniewska and Harnisz (2018) observed a higher percentage of *E. coli* resistant to cefotaxime in treated wastewater compare to untreated wastewater. Similarly, Zurfluh et al. (2017) identified clinically important clones of *K. pneumoniae* carrying

bla$_{\text{OXA-48}}$ exclusively in treated wastewater. However, it is assumed that hospital wastewater can contaminate municipal wastewater with these resistant pathogens and clinically important ARGs. In a recent study by Davidova-Gerzova et al. (2023), MDR ESBL-producing *E. coli* strains were detected in both hospitals and treated wastewater from the hospital wastewater-receiving municipal WWTP. The same strains were also found in rivers downstream of the discharge point of this treated wastewater. In addition, clinically significant MDR *K. pneumoniae* producing various carbapenemases was identified in both hospital and treated municipal wastewater (Surleac et al., 2020). The researchers also found clinically significant ESBL- and carbapenemase-producing *K. pneumoniae* isolates downstream of municipal WWTPs receiving wastewater from hospitals which had similar resistance genes to those found in the clinical settings (Kehl et al., 2022; Lepuschitz et al., 2019).

All these mentioned studies point to an important problem that WWTPs, especially those affected by hospital wastewater, release ESBL-E and CRE into the environment. This poses a significant health risk as these bacteria can contaminated downstream aquatic ecosystems used for recreational activities and crop irrigation, and can be ingested by livestock or wildlife, which can promote the spread of resistance to clinically important drugs (Marutescu et al., 2023). Therefore, more efficient technology for municipal wastewater treatment together with on-site hospital wastewater treatment should achieve reduction or even removal of ARB and ARGs.

1.4.1. Current wastewater surveillance

In 2015, the WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) (WHO, 2018), as a crucial step in combating the growing threat of AMR. The GLASS initiative aims to promote comprehensive surveillance of AMR patterns in priority pathogens worldwide while facilitating the early detection of emerging resistance, particularly in healthcare settings. In Croatia, AMR surveillance in healthcare started in 1996 by establishing the Croatian Committee for Antibiotic Resistance Surveillance (Tambic Andrasevic, 2009). Today, the Section for Control of Antibiotic Resistance (ISKRA) tracks AMR at country level and together with the European Surveillance System (TESSy) of the European Center for Disease Prevention and Control (ECDC), interprets and compare AMR data to better update health policy (ISKRA, 2022; Meštrović et al., 2023).

However, all these initiatives focus only on the AMR surveillance of isolates from human clinical samples. Therefore, One Health approach, which links human and animal health with the environment, advocates for AMR surveillance in all settings, including wastewater (WHO, 2023.) However, only a small percentage of research on the WWTPs are focused on One Health approach regarding AMR surveillance (Gholizadeh et al., 2023). In addition, there is currently no legislation for the monitoring of ARB or ARGs in wastewater or surface waters receiving wastewater from WWTPs. Furthermore, there are no established standards for acceptable AMR levels in treated wastewater, so the risk of humans coming into contact with AMR via recipient water needs to be assessed. For this reason, the European Commission recently proposed a revision of the Urban Wastewater Treatment Directive to introduce obligatory monitoring for the presence of AMR in municipal WWTPs (European Commission, 2024). The draft proposes monitoring of emerging pathogens (recommended by the ECDC and WHO) in WWTPs of 100,000 population equivalent (p. e.) or above, at least twice a year in untreated and treated wastewater, which should be adopted by January 1st, 2025. In addition, the WHO proposed Global Tricycle Surveillance guidelines for the global monitoring of ESBL-producing *E. coli* from different environments, including wastewater before and after treatment in WWTPs and in waters downstream of WWTP discharge discharges (WHO, 2021). Although these guidelines provide a framework for the global comparison of ESBL-producing *E. coli*, some experts are concerned that they do not cover all the different resistant Enterobacterales and their ARGs that are spreading into the environment (Milligan et al., 2023).

Monitoring of untreated wastewater may serve as a valuable indicator for tracking the prevalence of ARB and ARGs in the population. Conversely, the monitoring of treated wastewater provides information on the likelihood of exposure to these biological contaminants and their spread in the environment. This information is crucial for assessing the risk of contamination of water sources intended for drinking or agricultural purposes (Larsson et al., 2023). In some countries, monitoring of untreated municipal wastewater has proven to be a valuable method for assessing the prevalence of AMR in certain human pathogens (Huijbers et al., 2020; Hutinel et al., 2019; Rahman et al., 2023). In the Netherlands, wastewater-based surveillance has been an instrument in determining the prevalence of carbapenemase-producing *Enterobacteriaceae* in the national population (Blaak et al., 2021). In addition, the Global Sewage Surveillance Project (GSSP) is pioneering a global initiative that aims to use wastewater to monitor the prevalence of

pathogenic bacteria (Ahrenfeldt et al., 2020). On a smaller scale, in European study, Pärnänen et al. (2019) observed that ARG distribution per country was consistent with the trends observed in clinical isolates. Moreover, a longitudinal study conducted in Ireland on treated wastewater revealed the presence of clinically significant Enterobacterales exhibiting both ESBL and carbapenemase resistance mechanisms, which were discharged from WWTPs and subsequently detected in downstream waters (Hooban et al., 2022). Furthermore, a global survey to monitor cefotaxime-resistant coliforms in treated wastewater has produced worrying results. Many WWTPs have been found to operate under sub-optimal treatment conditions, resulting in the release of more resistant coliforms than required by the new EU regulatory framework for treated wastewater used for irrigation purposes (10 CFU of *E. coli*/100 mL*)* (Marano et al., 2020; EU, 2020). In addition, EU and Croatian legislation regulate that treated wastewater released in surface waters used for bathing and recreation could discharge up to 10^3 CFU of *E. coli*/100 mL (Official Gazette of the Republic of Croatia 26/20). However, Schijven et al. (2015) found out that even though the emission of ESBL-producing *E. coli* from treated wastewater was below regulation limits, those isolates found in recreational waters shared similar genetic and phylogenetic background with *E. coli* from treated wastewater, with an estimated risk of their average ingestion of 13-21%. Therefore, wastewater surveillance should be utilized for preventive purposes and to increase knowledge of the main sources of AMR in the environment.

1.4.2. Methods for monitoring AMR in wastewater

Most research on the monitoring AMR in wastewater uses two main approaches, culture-dependent and culture-independent methods. Culture-dependent methods involve the direct cultivation of ARB on antibiotic-containing media and are generally used for the isolation of indicator organisms, e.g. *E. coli*, as an indicator for fecal contamination. Once the target bacteria have been isolated from a wastewater sample, they are subjected to antibiotic susceptibility testing to accurately determine phenotypic resistance. To complement culture-based methods, Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) is often used for clinical species identification. However, limitations arise as MALDI-TOF MS databases frequently exclude environmental bacteria and are not freely accessible (Ashfaq et al., 2022). Consequently, sequencing of the bacterial 16S rRNA gene may be necessary for accurate species identification. Furthermore, culture-based methods are typically coupled with molecular

techniques to gain a comprehensive understanding of epidemiology of isolates and their resistance mechanisms. This includes employing PFGE and MLST to understand the relatedness of isolates with clinical and environmental origin (Waśko et al., 2022). In addition, PCR is utilized to detected the presence of known AGRs and/or WGS for identification of resistance mechanisms and to determine whether resistance is chromosomal or plasmid mediated (Lepuschitz et al., 2020; Nordmann and Poirel, 2013; Tiwari et al., 2022). However, it is well known that the vast majority of environmental microorganisms (more than 97%) are not culturable under typical laboratory conditions (Tiwari et al., 2022), and therefore are not captured when culture-dependent techniques are employed. This can be partly overcome by the use of culture-independent methods for the study of non-cultured microorganisms, however, these methods also have their limitations.

Quantitative PCR-base approaches (qPCR) are frequently used for the detection and quantification of ARGs in wastewater and other environmental matrices (Liguori et al., 2022). In addition, these methods are used for the direct quantification of pathogens in wastewater samples by targeting their specific taxonomic genes such as *ycc*T for *E. coli*, *glt*A for *K. pneumoniae*, *sec*E for *A. baumannii*, and 23S rRNA for *Enterococcus* species (Hembach et al., 2019, 2017). The qPCR method could be used for systematic wastewater screening due to its high sensitivity, precision in gene targeting, constantly evolving primers, reference conditions, and reproducibility (Manaia et al., 2018). Already, the qPCR results were showed to be comparable at both local and global levels (Cacace et al., 2019; Pärnänen et al., 2019). However, conventional qPCR is not a high-throughput method, which severely limits the potential of information that can be obtained from samples in a timely manner. The high-throughput quantitative PCR-based (HT-qPCR) methods, such as microarrays, offer the invaluable advantage of being able to analyses multiple genes simultaneously (Liguori et al., 2022). However, all qPCR-based methods rely on primers that target known genes, which means that new or unknown genes cannot be detected, and potential future threats cannot be uncovered. These limitations could be circumvented by metagenomics approaches that capture the entire resistome, although ARG annotations still depend on existing gene databases (Karkman et al., 2018; Singh et al., 2022). Another innovative method, emulsion paired isolation and concatenation PCR (epicPCR) links ARGs to their host directly from environmental samples without culturing (Hultman et al., 2018). However, this method is highly technical and time-consuming, making it unsuitable for routine monitoring (Bengtsson-Palme et al., 2023).

1.5. Aims, research objectives and hypotheses

The role of wastewater as a reservoir for resistance to 3GCs and carbapenems is not yet fully understood. In addition, a comprehensive assessment of the prevalence and characteristics of priority WHO pathogens such as ESBL-E and CRE in wastewater systems has not yet been completed. This thesis uses culture-dependent and independent approaches to achieve the following aims:

- To quantify priority enteric opportunistic pathogens (EOPs) and to assess the prevalence of enterobacteria resistant to 3GCs or carbapenems and their associated ARGs in municipal WWTPs;
- To characterize enterobacterial isolates resistant to 3GCs or carbapenems from municipal wastewater, their AMR mechanisms and epidemiology;
- To characterize 3GC- and carbapenem-resistant enterobacterial isolates from hospital wastewater, their epidemiology and the mechanisms underlying their resistance.

Consequently, the following hypotheses were formulated:

(i) Enterobacterales resistant to 3GCs and carbapenems and their associated ARGs are not completely removed from municipal WWTPs;

(ii) Treated municipal wastewater serves as a source for the further spread of MDR enterobacterial strains with acquired resistance to 3GCs and carbapenems into the environment;

(iii) Hospital wastewater is a reservoir for MDR enterobacteria, including high-risk clones that produce ESBLs and/or carbapenemases.

Chapter 2

Publication No. 1: Prevalence of enteric opportunistic pathogens and extended-spectrum cephalosporin- and carbapenem-resistant coliforms and genes in wastewater from municipal wastewater treatment plants in Croatia

Journal of Hazardous Materials

Publication No. 1

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Ana Maravić: Methodology, Writing – review & editing.

Marko Jelić: Formal analysis, Writing – review & editing.

Nikolina Udiković-Kolić: Conceptualization, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

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Research Paper

Prevalence of enteric opportunistic pathogens and extended-spectrum cephalosporin- and carbapenem-resistant coliforms and genes in wastewater from municipal wastewater treatment plants in Croatia

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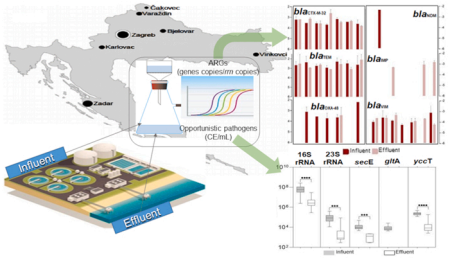
HIGHLIGHTS

- WWTPs reduce but do not eliminate total and resistant *E. coli* and other coliforms.
- Good removal of *A. baumannii* and *K. pneumoniae* was reported in most WWTPs.
- ESBL genes were only slightly reduced or even enriched after treatment.
- CP genes such as bla_{IMP} and bla_{VIM} were frequently enriched during the process.
- Concentrations of bla_{IMP} and bla_{VIM} were affected by specific WWTP characteristics.

GRAPHICAL ABSTRACT



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ABSTRACT

Extended-spectrum β-lactamase (ESBL)- and carbapenemase-producing *Enterobacterales* are a critical global health problem and wastewater treatment plants (WWTPs) can promote their spread into the environment; yet their efficacy is not well characterized. Here, we have used conventional culturing to monitor coliform bacteria and quantitative PCR to monitor 2 ESBL and 5 carbapenemase (CP) genes and 4 enteric opportunistic pathogens (EOPs) in the influent and effluent of 7 Croatian WWTPs in two seasons. In general, levels of total, cefotaxime- and carbapenem-resistant coliforms were significantly reduced but not eliminated by conventional treatment in most WWTPs. Most WWTPs efficiently removed EOPs such as *K. pneumoniae* and *A. baumannii*, while *E. coli* and *Enterococcus* spp. were reduced but still present in relatively high concentrations in the effluent. ESBL genes (bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$) were only slightly reduced or enriched after treatment. CP genes, $bla_{KPC\text{-}3}$, bla_{NDM} and $bla_{OXA\text{-}48}$-like, were sporadically detected, while bla_{IMP} and bla_{VIM} were frequently enriched during treatment and correlated with plant size, number or size of hospitals in the catchment area, and COD effluent concentration. Our results suggest that improvements in wastewater treatment technologies are needed to minimize the risk of environmental contamination with top priority EOPs and ARGs and the resulting public health.

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1. Introduction

One of the greatest threats to human health in the 21st century is the ineffectiveness of antibiotics in treating bacterial infections. Modern living standards have led to uncontrolled, continuous and ubiquitous use of antibiotics for therapeutic purposes in humans and animals, and for growth promotion and prophylaxis in livestock. This has accelerated the emergence and spread of antibiotic-resistant bacteria (ARB) and their antibiotic-resistance genes (ARGs) in both clinical and non-clinical environments (natural and engineered), and threatens global public health (Ashbolt et al., 2013; Bengtsson-Palme et al., 2018; WHO, 2018; Ben et al., 2019).

Of particular concern is the increasing bacterial resistance worldwide to β-lactam antibiotics such as 3rd generation cephalosporins (extended-spectrum cephalosporins, ESC) and carbapenems. ESC are typically used to treat infections caused by Gram-negative bacteria, but with the increase in these types of infections, their use has increased dramatically and contributed to the emergence of resistant enterobacteria. The most common mechanism of resistance to ESC involves the expression of enzymes called extended-spectrum β-lactamases (ESBLs). ESBLs are commonly found in Gram-negative bacteria, and the main types of ESBL variants include TEM, SHV, CTX-M and OXA (Bradford, 2001). However, the increasing prevalence of infections caused by ESBL-producing enterobacteria has led to the increased use of carbapenems as the crucial antibiotics of last resort used to treat these infections. The most common mechanism of carbapenem resistance involves the production of carbapenemase (CP) enzymes (Suay-García, 2019). The most clinically important among them are the KPC, NDM, VIM, IMP and OXA-48 types (Walsh, 2010; Nasri et al., 2017; Makowska et al., 2020). Of even greater concern, genes for ESBLs and CPs are commonly found on plasmids, along with genes for resistance to other classes of antibiotics, and spread readily among different bacterial species (Haller et al., 2018; Sib et al., 2020) Therefore, carbapenem-resistant and ESBL-producing *Enterobacterales* have been identified by World Health Organization (WHO) as the critical antibiotic-resistant "priority pathogens" that pose the greatest threat to human health due to limited therapeutic options (WHO, 2017).

Conventional wastewater treatment plants (WWTPs) consist of a combination of physical and biological processes to remove solids, organic matter and nutrients from wastewater. The most common process for biological treatment of wastewater in a conventional WWTP is activated sludge (Samer, 2015). WWTPs receive wastewater from a variety of sources, including households and hospitals, so environmental bacteria and pathogenic gut bacteria released with the feces (some of which carry acquired ARGs) can interact and exchange genes horizontally. This horizontal gene transfer (HGT) of ARGs is the main cause of the spread of resistance in most Gram-negative bacteria and is facilitated in WWTPs by high bacterial densities, high nutrient loads, and various types of pollutants, including antibiotics and other selectors of antibiotic resistance (Hembach et al., 2017; Karkman et al., 2018). Therefore, WWTPs are considered potential reservoirs for ARGs, but also for enteric opportunistic pathogens (EOPs) and putative hotspots for HGT of ARGs, as well as sources for their dissemination in the environment (Karkman et al., 2018; Pazda et al., 2019; Wang et al., 2020). In general, the abundance of ARB and ARGs is reduced during the wastewater treatment process (Caucci et al., 2016; Wang et al., 2020). However, some ARGs (e.g. bla_{KPC}, bla_{NDM}, bla_{OXA-48}) and ARB (e.g. multidrug resistant *E. coli*, carbapenem-resistant Gram-negative bacteria) have been shown to increase in relative abundance in treated wastewater compared to raw wastewater, and then released into the aquatic environment (Hrenovic et al., 2017; Nasri et al., 2017; Proia et al., 2018; Kumar et al., 2020). Therefore, WWTPs can serve either as a pathway for the spread of antibiotic resistance and EOPs or as a barrier to limit their release into the environment (Nguyen et al., 2021). Further knowledge on the impact of the wastewater treatment process on the abundance and removal of EOPs and ARB/ARGs, especially horizontally transmissible and clinically relevant ARGs, is therefore of great importance.

Resistance to ESC and carbapenems among clinical isolates is increasing and reported in many countries (Khan et al., 2018; Lepuschitz et al., 2019; Krilanović et al., 2020). Croatia is one of the countries with high resistance rates of clinical enterobacteria, especially *K. pneumoniae*, to ESC (53% in 2019; (ECDC, 2020)). In addition, an increase in carbapenem resistance rates from 2% in 2018 to 12% in 2019 was observed in clinical *K. pneumoniae* isolates in Croatia (ECDC, 2020). In addition to characterizing clinical ARB, recent studies have also quantified ESBL and CP genes by quantitative PCR in various European WWTPs. For example, the ESBL genes bla_{TEM} and bla_{CTX-M} were detected in all 16 effluents from WWTPs in 10 countries, while the CP genes bla_{OXA-48} and bla_{KPC} were found in 12 effluents from 10 countries and 9 effluents from 6 countries, respectively (Cacace et al., 2019). On the other hand, CP genes such as bla_{IMP} and bla_{VIM} were found in some influent samples in different EU countries such as Portugal, Spain and Germany (Pärnänen et al., 2019). A recent study looking at ESC-resistant coliforms in WWTPs on 5 continents showed that despite good removal of these coliforms in most WWTPs, significant concentrations ($> 10^3$ CFU/mL) were occasionally found in final effluents (Marano et al., 2020). However, little is known about the prevalence of ESC- and carbapenem-resistant coliforms and the corresponding ARGs and EOPs in Croatian wastewater, which represent a potential dissemination pathway for ESC and carbapenem resistance and EOPs into natural waters. The aim of this study was therefore to quantify and compare the abundance of ARB/ARGs and EOPs in the influent and effluent of WWTPs in seven selected Croatian cities over two seasons (winter and summer). The focus was on culturable cefotaxime (ESC)- and carbapenem-resistant coliforms, selected ESBL (bla_{TEM} and $bla_{CTX-M-32}$) and CP genes (bla_{KPC-3}, bla_{OXA-48}-like, bla_{NDM}, bla_{IMP} and bla_{VIM}) and genetic markers specific for priority EOPs (*E. coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Enterococcus* spp.) to provide information as a basis for risk assessment.

2. Materials and methods

2.1. Sample collection

The 24-hour time-proportional composite samples of untreated wastewater (influent) and treated wastewater (effluent) were collected with automatic samplers installed at the inlet and outlet of the conventional municipal WWTPs of 7 Croatian cities: Zagreb, Zadar, Karlovac, Vinkovci, Bjelovar, Čakovec and Varaždin (Fig. 1, Table 1). The sampling campaigns were performed during three consecutive days (Tuesday, Wednesday and Thursday) on two separate occasions in 2020 – January/February (winter season) and June/July (summer season). The samples were collected in sterile glass bottles (2.5 L), transported in coolers with ice blocks and processed in a laboratory within 2 h.

2.2. Physicochemical analyses

Collected wastewater samples were analyzed for their physicochemical characteristics using internationally validated methods (ISO standards; for more details see Table S1). A number of basic parameters were analyzed, including temperature, pH, conductivity, total suspended solids, chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), nitrogen forms (total N, ammonium-N, nitrate-N) and total phosphorus.

2.3. Enumeration of culturable coliform bacteria

Samples for microbial cultivation were first serially diluted in 0.85% NaCl (tenfold dilutions up to 1:10,000), and then filtered in triplicate through sterile mixed cellulose ester membrane disc filters (47 mm diameter, 0.22-μm pore size, GE Healthcare, Life Science, USA) by using vacuum. The filters were then placed on the Rapid′*E. coli* 2 (Bio-Rad, France) agar plates to enumerate presumptive non-resistant combined

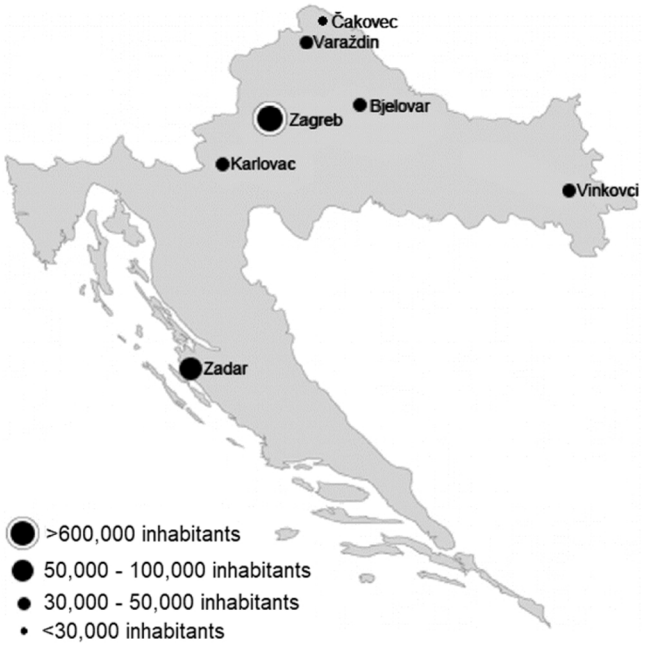

Fig. 1. Map of Croatia with indicated sampling locations.

(*E. coli* + non-*E. coli*) coliform bacteria and supplemented with 4 mg/L cefotaxime (Sigma-Aldrich, USA) to enumerate presumptive cefotaxime-resistant (CTX-R) coliforms (CLSI, 2013). CHROMagar mSuperCARBA (CHROMagar, France) agar plates were used to enumerate presumptive carbapenem-resistant (CR) coliforms. After incubation at 37 °C for 24 h, two types of colonies (based on color) were distinguished and enumerated on Rapid'*E. coli* 2 and CHROMagar mSuperCARBA plates – presumptive *E. coli* and other coliforms (e.g. *Klebsiella, Enterobacter, Citrobacter, Serratia*). Bacterial concentrations for each culture medium and sample were calculated as colony-forming units (CFU) per milliliter of wastewater (CFU/mL).

2.4. DNA extraction and quantitative PCR analyses

For DNA extraction from wastewater, samples (30–100 mL of influent and 100–700 mL of effluent) were filtered in triplicate through the same membrane filters as mentioned above, and the total community DNA was extracted from filters using the DNeasy Powersoil kit (Qiagen, USA). Prior to the extraction procedure, the filters were cut into small pieces with sterile scissors and DNA extraction was performed on the pieces according to the manufacturer's recommendations. DNA quality (260/280 ratio) was assessed using a Nanodrop spectrophotometer (BioSpec Nano, Shimadzu, Japan), and DNA quantity using a Qubit Fluorometer 3.0 (Thermo Fisher Scientific, USA). All extractions were stored at −20 °C until use.

Quantitative PCR (qPCR) was used to quantify two ESBL genes (bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$), five CP genes ($bla_{KPC\text{-}3}$, bla_{NDM}, $bla_{OXA\text{-}48}$-like, bla_{IMP} and bla_{VIM}) and 16S rRNA gene as marker for total bacteria. In addition, marker genes for EOPs were also quantified: *ycc*T (*Escherichia coli*), *glt*A (*Klebsiella pneumoniae*), *sec*E (*Acinetobacter baumannii*) and 23S rRNA (*Enterococcus* spp.). The primers targeting these genes and qPCR conditions are listed in Table S2. All qPCR assays were performed on the ABI 7300 Real-time PCR thermocycler (Applied Biosystems, USA) with Power SYBR® Green PCR Master Mix (10 µL, Applied Biosystems, USA), 1 µM of each primer (Table S2) and 2 ng of DNA template in a total volume of 20 µL.

The qPCR thermal cycling conditions for ARGs and EOPs were as follows: 95 °C for 15 min, 30 cycles (bla_{VIM}), 35 cycles (*ycc*T, *glt*A, *sec*E and bla_{IMP}) or 40 cycles ($bla_{OXA\text{-}48}$-like, bla_{NDM}, $bla_{KPC\text{-}3}$ and 23S rRNA) at 95 °C for 15 s, specific annealing temperature for each gene and primer pair (Table S2) for 30 s, and 72 °C for 30 s, respectively. For quantification of the 16S rRNA gene, thermal cycling conditions were according to López-Gutiérrez et al. (2004).

The plasmids pGEM-T with the corresponding inserts were used as quantification standards for the quantification of the genes bla_{NDM}, $bla_{OXA\text{-}48}$-like, bla_{VIM}, bla_{IMP}, *ycc*T, *sec*E and *glt*A. The pNORM1 plasmid (Rocha et al., 2020) was used to quantify the bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$ genes, while the plasmid pUC19 (Heß et al., 2018) was used to quantify the $bla_{KPC\text{-}3}$ gene. Plasmid DNA was extracted with ExtractNow™ Plasmid Mini kit (Minerva Biolabs GmbH, Germany) and used after linearization to generate a standard curve (10^2-10^8). For enterococci, the 23S rRNA gene from the reference strain (*Enterococcus avium*) was used as the quantification standard. Negative controls (no template controls) were included in each of the assays. Efficiency and accuracy values (Table S2) were determined using six points of serial dilutions of the plasmid carrying ARG. Both samples and standards were analyzed in technical duplicates. Possible qPCR inhibition was assessed by conducting an inhibition test using 10- and 100-fold diluted samples, as previously described (Petric et al., 2011). The detection limit for all target genes was 10^2 gene copies per reaction. ARG abundances were calculated per number of copies of the 16S rRNA gene (*rrn*) (relative abundance), and results were log transformed. Abundances of *ycc*T gene of *E. coli*, *glt*A gene of *K. pneumoniae*, *sec*E of *A. baumannii* and 23S rRNA gene of enterococci and 16S rRNA gene of total bacteria were reported as cell equivalents (CE)/mL (absolute abundance). In the case of *E. coli*, *K. pneumoniae* and *A. baumannii* only one copy of the target gene is present in a cell (Clifford et al., 2012; Gadsby et al., 2015); thus, one copy number corresponds to one cell. However, in the case of enterococci and total bacteria, the average copy number of the 23S rRNA and 16S rRNA genes is five and three, respectively (Stoddard et al., 2015); therefore 23S rRNA and 16S rDNA copies determined by qPCR were divided by 5 and 3, respectively, to convert into CE.

2.5. Data analysis

Bacterial and gene concentration data were first log10-transformed. Before deeper analysis, the data were subjected to a Shapiro-Wilk test to assess their normality. This was performed in R Studio (version 4.0.3.) and confirmed that the data followed a normal distribution. Paired t-tests were performed to compare the average concentrations of culturable bacteria, EOPs or ARGs between the influent and effluent of each WWTP and between seasons. In addition, Welch's t-test was assessed to compare the average concentrations of EOPs from influents and

Table 1
Characteristics of the cities and wastewater treatment plants (WWTPs) included in the study.

	Vinkovci	Bjelovar	Zagreb	Čakovec	Varaždin	Karlovac	Zadar
Population equivalent of WWTPs	43,000	50,000	12,000.000	75,000	140,000	98,500	100,000
Mean wastewater flow (m^3/day)*	16,000(W)	9840(W)	307,556(W)	9548(W)	16,500 (W)	14,990 (W)	9862 (W)
	10,000 (S)	8867 (S)	261,620 (S)	9310 (S)	15,500 (S)	14,605 (S)	11,012 (S)
Hospitals in catchment	1	1	9	1	1	2	1
No. of hospital beds	262	272	6333	346	968	614	483
No. of biological stages	2	2	2	≥ 2	< 2	3	≥ 2

* mean wastewater flow (m^3/day) in two seasons: winter (W) and summer (S)

effluents in all 7 Croatian WWTPs. These analyses were performed using GraphPad Prism version 8.02 for Windows (GraphPad Software, San Diego, California, USA). Log removal values were calculated by taking the logarithm of the ratio of CFU or relative/absolute gene abundance in influent and effluent water of each WWTP.

All further statistical analyses and visualizations were performed in R. Boxplot comparison of relative abundances of ARGs and absolute abundances of gene markers for EOPs from effluent in relation to physicochemical WWTP characteristics was performed using the package 'ggplot2' (Wickham and Chang, 2016). Pearson's rank correlation tests were performed to evaluate the correlations between the physicochemical parameters and the relative abundance of ARGs or the absolute abundance of EOPs. A correlation matrix was constructed using the package 'corrplot' (Wei et al., 2017). A non-metric multidimensional scaling (NMDS) analysis was performed to evaluate the distribution of ARGs in WWTPs' influent and effluent in two seasons based on the Bray-Curtis distance calculated using the package 'vegan' (Oksanen et al., 2018). In addition, the Adonis test was applied with the 'vegan' package to analyze the differences in the relative abundance of ARGs between the influent and effluent of each WWTP and between two seasons. All statistical tests were considered significant at $p < 0.05$.

3. Results

3.1. Abundance of culturable coliforms and their reduction by biological treatment in municipal WWTPs of Croatian cities

We quantified non-resistant presumptive *E. coli* and non-*E. coli* coliforms as well as presumptive CTX-R and CR *E. coli* and other coliforms in influent and effluent water from 7 different Croatian WWTPs by plating on agar media with and without antibiotics.

The average concentrations of presumptive combined coliforms (*E. coli* + non-*E. coli*) in all influent samples ranged from 1.52×10^4 to 1.37×10^5 CFU/mL (Fig. 2) and were generally significantly reduced in the effluents (except in Bjelovar) by about 0.97 – 2.22 log units in winter and by about 1.02 – 2.47 log units in summer (except in Čakovec and Varaždin; Fig. 2).

As shown in Table S3, presumptive *E. coli* concentrations decreased in the effluents to an average of 7.02×10^2 CFU/mL in winter, except in Bjelovar, and to 3.06×10^3 CFU/mL in summer, except in Čakovec and Varaždin. The concentrations of presumptive non-*E. coli* coliforms decreased in the effluents to an average of 1.88×10^3 CFU/mL (Table S3), with no significant variations between seasons ($p > 0.05$, paired t-test).

Average levels of presumptive CTX-R combined coliforms in the influents ranged from 1.01×10^2 to 4.14×10^3 CFU/mL in both seasons (Fig. 2). These levels were significantly lower in all WWTP effluents (average 1.8 log units in both seasons), except in Bjelovar and Varaždin in winter and in Čakovec and Varaždin in summer. The average concentrations of presumptive CTX-R *E. coli* ranged from 1.73×10^1 to 7.83×10^2 CFU/mL in the influents of both seasons, while the concentrations of presumptive CTX-R non-*E. coli* ranged from 8.37×10^1 to 3.36×10^3 CFU/mL (Table S3). However, no significant changes were observed between the influent and effluent of presumptive CTX-R *E. coli* and CTX-R non-*E.coli* coliforms in Bjelovar and Varaždin WWTPs in winter, and in WWTPs from Varaždin (CTX-R *E. coli* and non-*E. coli*), Vinkovci (CTX-R *E. coli*) and Čakovec (CTX-R non-*E.coli*) in summer.

Concentrations of presumptive CR combined coliforms ranged from 4.05×10^1 to 4.81×10^3 CFU/mL in the influents, with no significant seasonal variation for each WWTP ($p > 0.05$, paired t-test; Fig. 2, Table S3). Presumptive CR coliforms were significantly reduced in the effluents of all WWTPs (removal efficiency: 1.39–2.72 log units), except for Varaždin WWTP in both seasons.

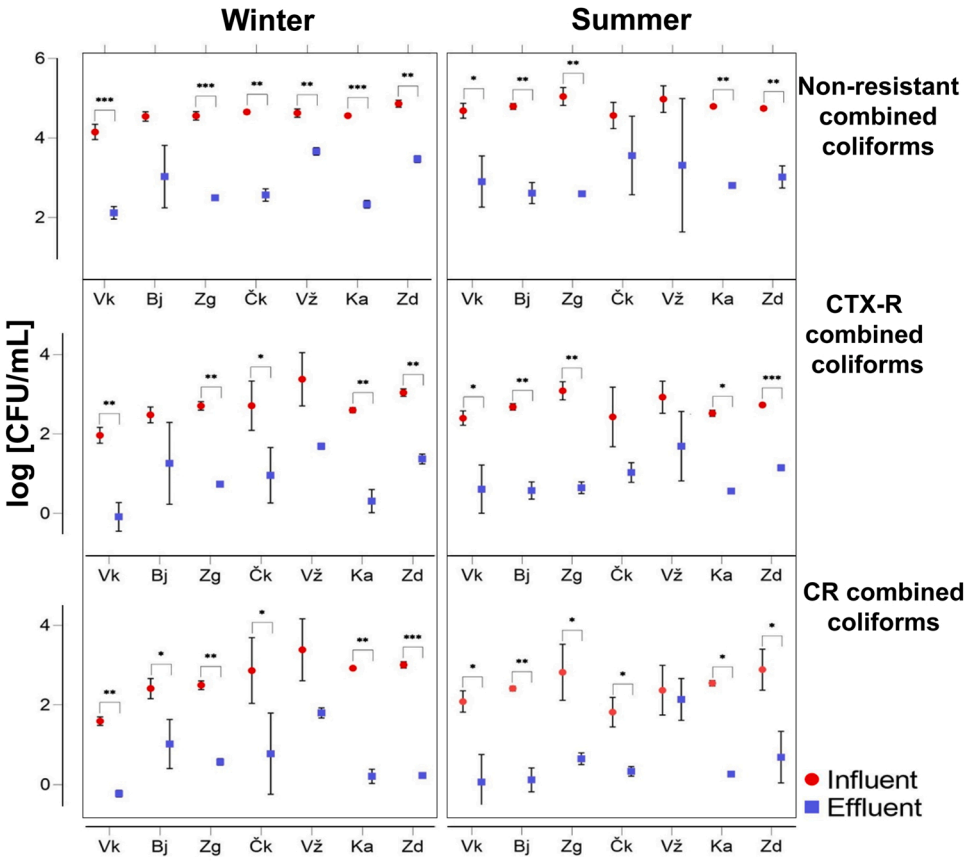

Fig. 2. Quantification of presumptive non-resistant combined coliforms (*E. coli* + non-*E. coli*), cefotaxime-resistant (CTX-R) and carbapenem-resistant (CR) combined coliforms in influent and effluent samples from WWTPs of 7 Croatian cities (Vinkovci, Vk; Bjelovar, Bj; Zagreb, Zg; Čakovec, Čk; Varaždin, Vž; Karlovac, Ka; Zadar, Zd) in winter and summer. Each value is the mean ± SD of three biological replicates. A multiple paired t-test was performed on the log transformed data. Asterisks indicate a statistically significant difference between influent and effluent (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.2. Abundances of EOPs in WWTPs and their removal efficiencies

Apart from the culturable coliforms, the abundance of 16S rRNA gene copies used as a proxy for total bacterial concentration and the abundance of *ycc*T, *glt*A, *sec*E and 23S rRNA genes used as a proxy for total concentration of *E. coli*, *K. pneumoniae, A. baumannii* and *Enterococcus* spp., were determined by qPCR in all influent and effluent samples during winter and summer (Fig. 3 and Table S4).

The qPCR-based analyses showed that the mean concentration of total bacteria in the influent samples was approx. 4×10^8 CE/mL in winter and 7×10^7 CE/mL in summer (Fig. 3). Significant decreases in total bacteria concentrations were achieved at all 7 WWTPs (Fig. 3, Tab. S4), resulting in reductions ranging from 0.13 to 1.92 logs, with no significant seasonal differences ($p > 0.05$, paired t-test). Among the target EOPs, enterococci were most prevalent in the influents (approx. 10^5 CE/mL in both seasons), followed by *E. coli* (approx. 10^4 and 10^5 CE/mL in winter and summer, respectively), *A. baumanni* (approx. 10^4 CE/mL, in both seasons) and *K. pneumoniae* (approx. 10^3 CE/mL in both seasons) (Fig. 3). Concentrations of *Enterococcus* spp. and *E. coli* in effluents decreased by approx. 1.4 log units and 1.7 log units, respectively (two-seasonal average) (Fig. 3 and Table S4). Concentrations of *K. pneumoniae* were below the detection limit in all effluents in both seasons, as were concentrations of *A. baumannii* in winter and in the majority of summer samples (5/7 WWTPs). In only two WWTPs where *A. baummanii* was detected in the effluent, a reduction of 1.50 (Zagreb WWTP) and 0.59 logs (Varaždin WWTP) was observed (Table S4).

A comparison of *E. coli* concentrations in influent and effluent from all studied WWTPs determined by qPCR of the *ycc*T gene with those determined by plating is shown in Fig. S1. The qPCR-based *E. coli* concentrations were higher (0.5–0.99 log units) than the concentrations determined by the culture-based approach in both influent and effluent in both seasons. However, a similar reduction of *E. coli* concentrations due to the conventional treatment was observed for both counting methods (two season average: 1.44 log units – qPCR and 1.83 log units – plating).

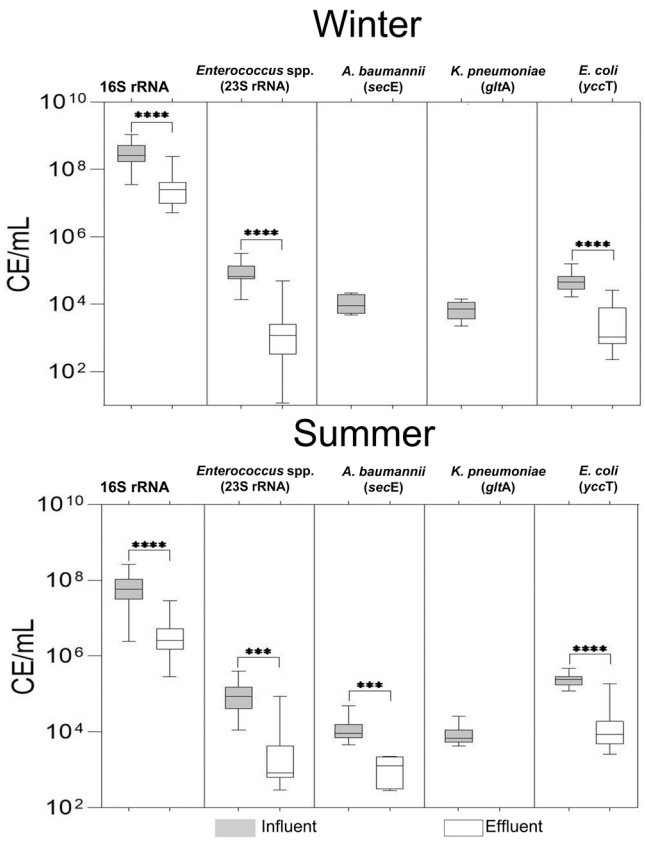

Fig. 3. Absolute abundances of total bacteria (16S rRNA), *Enterococcus* spp. (23S rRNA), *A. baumannii* (*sec*E), *Klebsiella pneumoniae* (*glt*A) and *E. coli* (*ycc*T) determined by qPCR of taxon-specific genes (cell equivalents (CE)/mL) in influents and effluents from all 7 studied WWTPs in two seasons. Boxes indicates the median and quartiles, whiskers represent minimal and maximal values. Significance of reduction is assessed by Welch's t-test and is indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $p < 0.0001$).

3.3. Distribution of ESBL and CP genes in WWTPs

The abundance of two ESBL genes (bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$) and five CP genes ($bla_{KPC\text{-}3}$, bla_{VIM}, bla_{NDM}, bla_{OXA48}-like and bla_{IMP}) in total community DNA from 7 investigated WWTP influents and effluents was analyzed by using qPCR (Fig. 4, Table S5). The NMDS plot in Fig. 4A, based on Bray-Curtis similarity, shows that influent and effluent samples were clustered separately based on the relative abundance of the ARGs analyzed (adonis: R2 = 0.70376, $p < 0.001$). In addition, influent and effluent samples from the different sampling periods (winter/summer) were also grouped separately (adonis: R2 = 0.46506, $p < 0.001$).

Fig. 4B shows the distributions (relative abundance) of two ESBL genes in influent and effluent samples from the studied WWTPs. In general, the relative abundance of the two ESBL genes in the influent and effluent samples of all WWTPs was about one order of magnitude higher in summer than in the corresponding winter samples. The bla_{TEM} gene was detected in measurable concentrations in almost all samples studied, except in the winter effluent of the WWTPs of Karlovac and Čakovec. The average relative abundance of this gene in the winter influent samples was −3.99 log gene copies/*rrn* copies and −2.91 log gene copies/*rrn* copies in the summer influent samples. In most of the studied WWTPs (4/7), the relative abundance of bla_{TEM} in the effluent decreased only slightly compared to the influent in both seasons (Fig. 4B, Table S5). In contrast, during summer, the relative abundance of this gene increased after treatment in some WWTPs (Vinkovci, Zagreb and Zadar). The other ESBL gene, $bla_{CTX\text{-}M\text{-}32}$, was detected in all influents in winter (−5.54 to −4.47 log gene copies/*rrn* copies), but its concentration in effluents from all 7 WWTPs was below the detection limit (Fig. 4B, Table S5). In contrast, this gene was quantified in all analyzed samples in summer, with no statistically significant difference ($p > 0.05$, paired t-test) between influent and effluent levels (average −3.62 log copies/*rrn* copies).

The distribution of CP genes in the WWTP influents and effluents examined is shown in Fig. 4C. $bla_{KPC\text{-}3}$ and bla_{NDM} were detected sporadically in the influent or effluent of some WWTPs only in winter (except bla_{NDM} in influent from Vinkovci WWTP in summer). Gene $bla_{OXA\text{-}48}$-like was mostly quantified in influents of most WWTPs in winter and in summer, and only in effluent from WWTP Bjelovar in winter (0.20 log reduction) and Varaždin in summer (0.19 log increase) (Fig. 4C, Table S5). bla_{IMP} was detected in the effluents of 5 cities (Vinkovci, Bjelovar, Zagreb, Karlovac and Zadar) in winter (average −4.05 log gene copies/*rrn* copies) and in 3 cities (Zagreb, Karlovac and Zadar) in summer (average −3.36 log gene copies/*rrn* copies), but not in the paired influents, except in Vinkovci in winter (Fig. 4C, Table S5). The bla_{VIM} gene was detected in the influents of 4 of the 7 studied cities during winter (average −4.79 log), but only in the paired effluents of Bjelovar and Čakovec it was below the detection limit, while a slight relative increase was observed in the effluents of Varaždin and Zadar (Fig. 4C, Table S5). The bla_{VIM} gene was also quantified in the winter effluents of the Zagreb and Karlovac WWTPs (approx. −4 log copies/*rrn* copies), but not in the paired influents. During the summer, bla_{VIM} was detected in almost all influents (average −3.84 log copies/*rrn* copies), with a slight decrease after treatment in two WWTPs (Bjelovar and Zadar), and an increase in three WWTPs (Vinkovci, Zagreb and Varaždin) (Fig. 4C, Table S5).

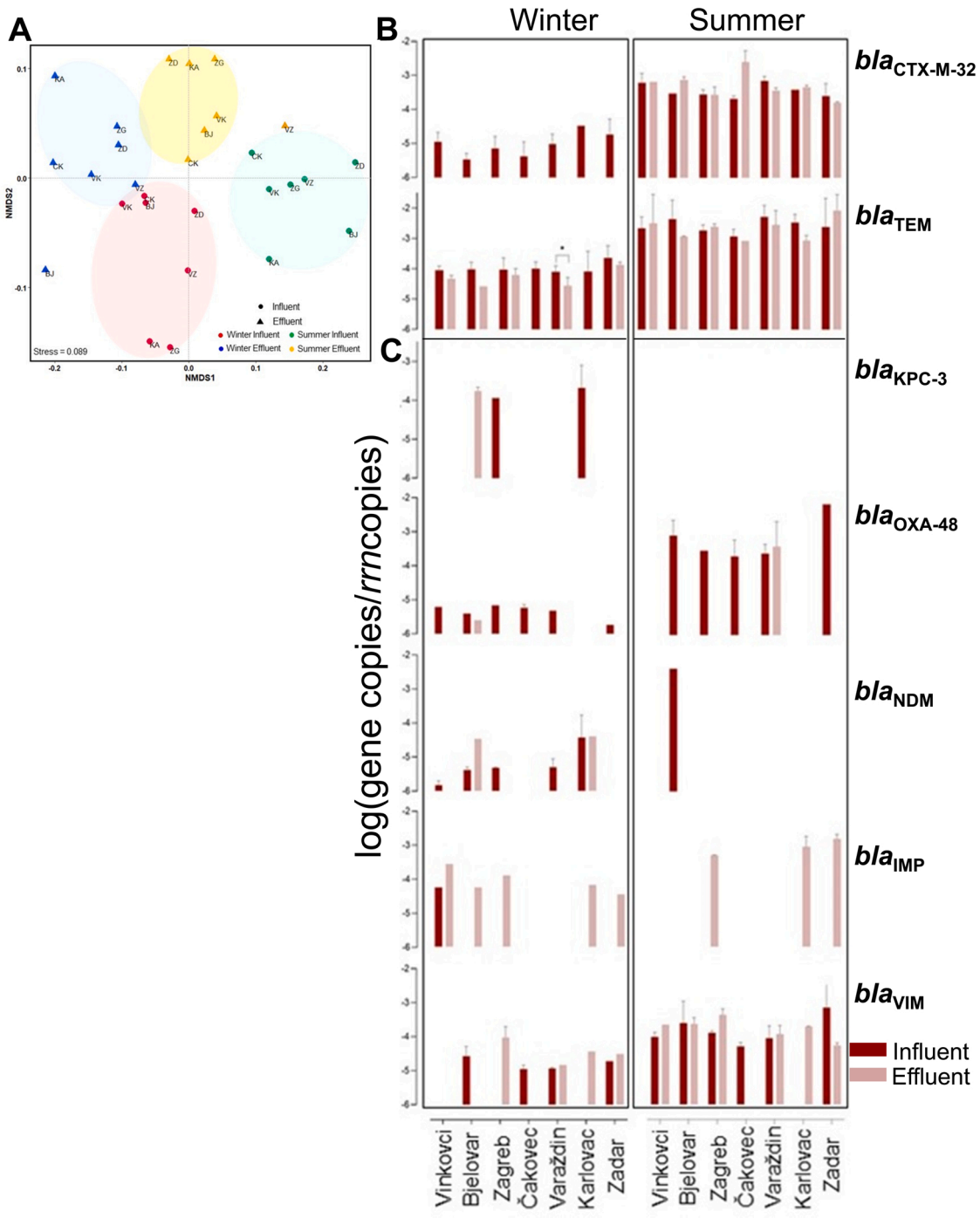

Fig. 4. Analysis of antibiotic-resistance gene distribution in influent and effluent samples from wastewater treatment plants of 7 Croatian cities (Vinkovci, Bjelovar, Zagreb, Čakovec, Varaždin, Karlovac, Zadar). A) Non-metric multidimensional scaling (NDMS) plot of all samples, labelled by sample type (circles and triangles represent influent and effluent samples, respectively) and by sampling time (green and yellow – summer; red and blue - winter). Relative abundance of B) ESBL (bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$) and C) carbapenemase genes ($bla_{OXA\text{-}48}$-like, $bla_{KPC\text{-}3}$, bla_{NDM}, bla_{IMP} and bla_{VIM}) in all samples in winter and summer. Asterisks indicate statistical differences in relative gene abundance between influent and effluent ($p < 0.05$, multiple paired t-test).

3.4. Correlation of ARG/bacterial abundances and physicochemical properties of effluent samples

We performed a Pearson's correlation analysis to investigate the relationship between the target ARGs (relative abundances) and the EOPs (absolute abundances), as well as the presumptive coliforms and the physicochemical parameters of the effluent samples from all 7 WWTPs (Fig. 5). The relative abundances of both ESBL genes, bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$, were strongly and significantly correlated with temperature ($r = 0.86$, $p < 0.001$ and $r = 0.90$, $p < 0.001$, respectively). In addition, bla_{TEM} correlated significantly with *E. coli* ($r = 0.58$, $p < 0.05$) and with $bla_{CTX\text{-}M\text{-}32}$ and bla_{VIM} ($r = 0.79$, $p < 0.001$ and $r = 0.54$, $p < 0.05$, respectively). A significant negative correlation between bla_{TEM} and bla_{NDM} genes was observed ($r = -0.53$, $p < 0.05$). Among the CP genes, $bla_{KPC\text{-}3}$ was significantly correlated with BOD_5 ($r = 0.74$, $p < 0.01$), suspended solids ($r = 0.57$, $p < 0.05$) and bla_{NDM} ($r = 0.66$, $p < 0.01$), and negatively correlated with *E. coli* ($r = -0.62$, $p < 0.05$). The bla_{VIM} showed a significant positive correlation with pH ($r = 0.70$, $p < 0.01$)

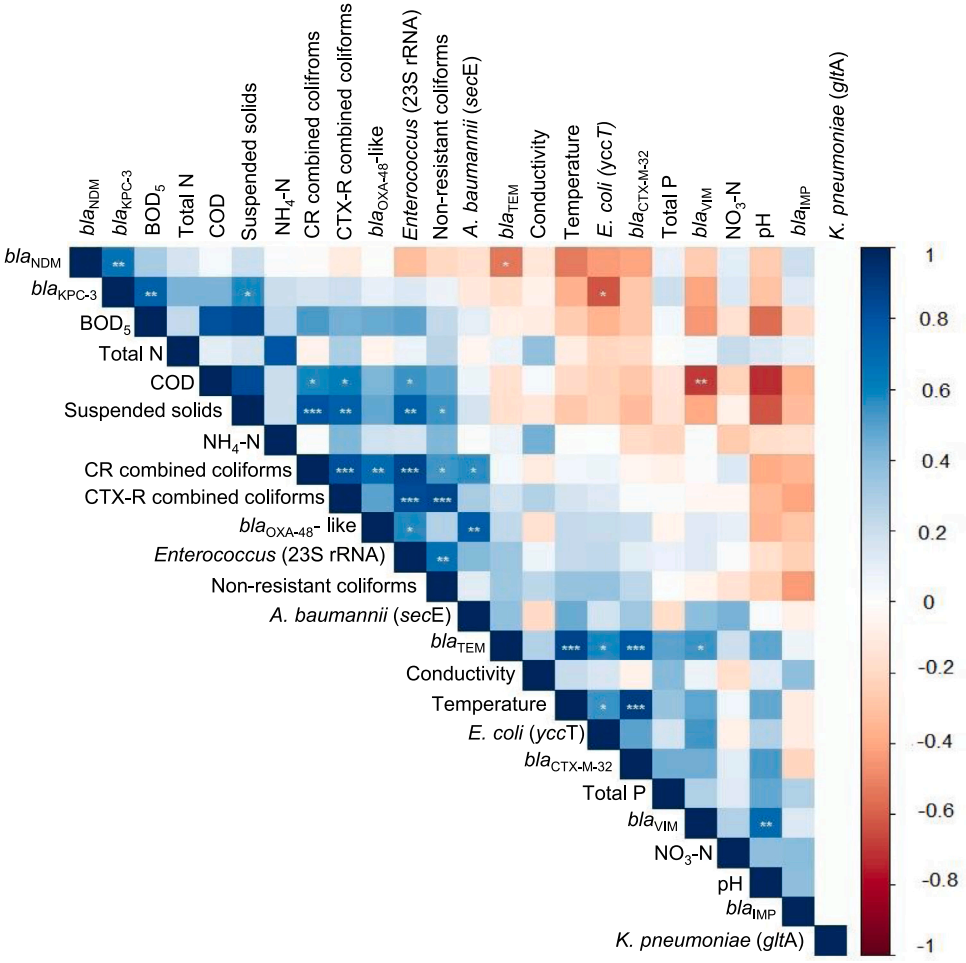

Fig. 5. Pearson's correlation analysis of relative abundance of ARGs (log(gene copies/*rrn* copies)), absolute abundance of EOPs (logCE/mL) and presumptive combined coliform bacteria (CFU/mL) and physicochemical parameters of final effluent samples. Cell colors from blue to red indicate positive and negative Pearson correlations, respectively, white indicates no correlation. Asterisks indicate significant correlations (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

and bla_{TEM} ($r = 0.54$, $p < 0.05$), but a significant negative correlation with COD ($r = -0.69$, $p < 0.01$) (Fig. 5). The bla_{OXA-48}-like correlated significantly with enterococci and *A. baumannii* ($r = 0.57$, $p < 0.05$ and $r = 0.71$, $p < 0.01$, respectively). In addition to correlations with ARGs, *E. coli* was also significantly correlated with temperature ($r = 0.55$, $p < 0.05$), while *Enterococcus* spp. were significantly correlated with COD ($r = 0.55$, $p < 0.05$) and suspended solids ($r = 0.75$, $p < 0.01$) (Fig. 5).

The concentrations of all culturable presumptive coliforms tested (non-resistant, CTX-R and CR combined coliforms) were significantly positively correlated with suspended solids ($r = 0.55$, $p < 0.05$; $r = 0.75$, $p < 0.01$; $r = 0.80$, $p < 0.001$, respectively). In addition, both presumptive CTX-R and CR coliform bacteria had a significant positive correlation with COD ($r = 0.62$ and 0.58, respectively; $p < 0.05$), while presumptive CR coliforms were additionally significantly correlated with bla_{OXA-48}-like and *A. baumannii* (Fig. 5).

3.5. Influence of WWTP characteristics on the abundance of ARGs and EOPs in effluents

The influence of specific WWTP characteristics on the relative abundance of ARGs and the absolute abundance of EOPs (*E. coli*, *K. pneumoniae*, *A. baumannii* and *Enterococcus* spp.) was investigated by dividing the data obtained for each WWTP parameter into two groups and comparing them using the two-sample t-test. Parameters that showed a significant correlation with the tested gene abundances are the WWTP size, the number of hospitals and hospital beds in the catchment, and COD effluent concentration (Fig. 6).

For example, plant size had a significant effect on the bla_{IMP} gene (Fig. 6A). The relative abundance of this gene was significantly increased ($p < 0.05$) in WWTPs with more than 80,000 population equivalents. In addition, both bla_{IMP} ($p < 0.001$) and bla_{VIM} ($p < 0.05$) were significantly increased in plants that had COD levels in effluents below 30 mg O_2/L (Fig. 6B) and two or more hospitals in the catchment area (Fig. 6C). Also, bla_{VIM} was significantly increased ($p < 0.05$) in WWTP catchments with 500 or more hospital beds (Fig. 6D). No significant correlation with resistance or taxonomic gene concentrations was observed for any other parameter examined (Fig. S2).

4. Discussion

The increasing prevalence of resistance to ESC and carbapenems among clinically important pathogens, particularly enterobacteria, is of great concern due to limited antibiotic treatment options worldwide. WWTPs can be an important source for the spread of such ARB and their ARGs as well as EOPs in the environment. Therefore, a better understanding of the fate of ESC- and carbapenem-resistant determinants and various EOPs during wastewater treatment is needed to enable the development of strategies to reduce the risk to public health. Here, we used conventional culturing to monitor presumptive coliform bacteria (non-resistant, CTX-R and CR) and qPCR assays to monitor EOPs (*E. coli*, *A. baumannii*, *K. pneumoniae* and *Enterococcus* spp.) and ARGs of special

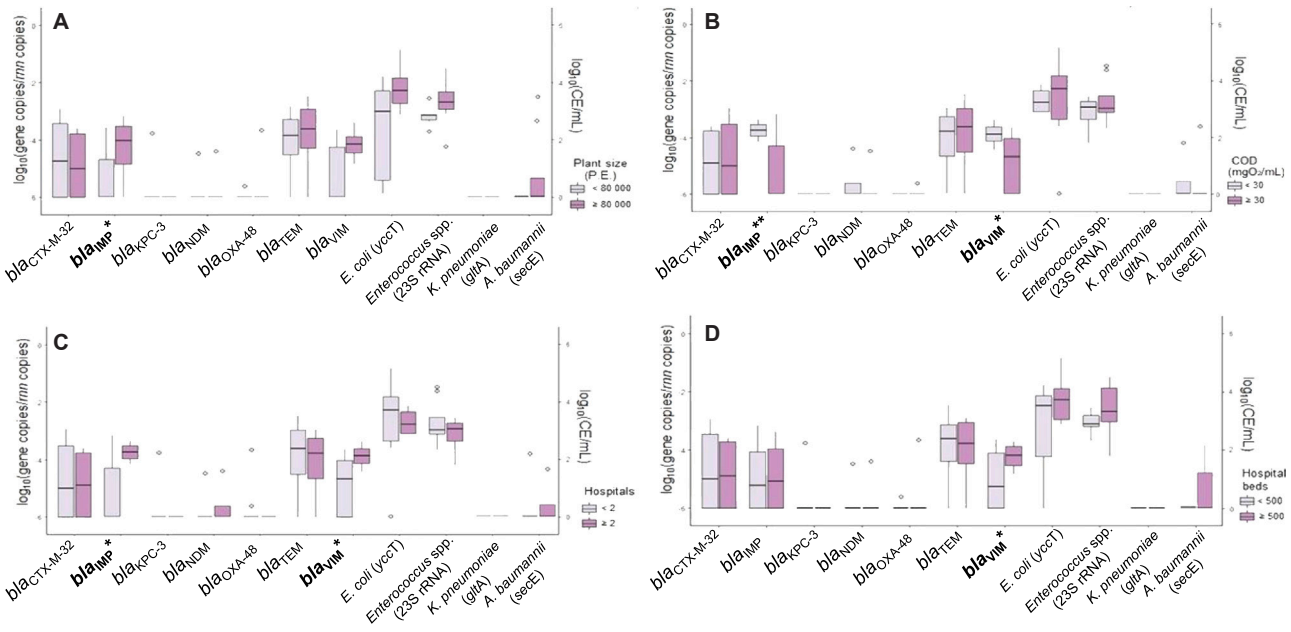

Fig. 6. Boxplot comparison of relative abundance of ARGs or absolute abundance of taxonomic genes as proxies for EOPs (*E. coli*, *Enterococcus* spp., *K. pneumoniae* and *A. baumannii*) between selected WWTP parameters – A, plant size; B, COD concentration; C, number of hospitals; and D, number of hospital beds. Boxes represent quartiles and the median, whiskers are 1.5 x IQR, and circles represent outliers. *indicates significant difference at $p < 0.05$ and ** indicates significant difference at $p < 0.01$ between gene abundances in effluents from two groups of plants.

clinical concern (ESBL and CP genes) in 7 Croatian WWTPs during sampling campaigns in winter and summer. All studied WWTPs operated with conventional activated sludge treatment (CAS), but their design capacities (43,000 – 1,2000,000 p.e.) and the number of hospitals/hospital beds in the catchment areas varied in a rather large range.

Analysis of the levels of presumptive non-resistant, CTX-R and CR coliforms in the influent and effluent of the WWTPs showed a significant decrease in most of the WWTPs in both seasons. Presumptive CTX-R and CR coliforms showed a similar average decrease of approx. 2 log units in both seasons. These results are comparable to those obtained for the removal of CTX-R coliforms in international WWTPs using similar methods (2.1 log reduction for CAS treatment) (Marano et al., 2020). Despite the observed decrease in presumptive CTX-R and CR *E. coli* and other coliforms in effluent samples, the WWTPs of one large (Zagreb) and one small (Varaždin) continental city still discharge up to 10^{11} CTX-R or CR *E. coli* and up to 10^{12} CTX-R or CR other coliforms per day into natural waters. This is consistent with previous studies showing that WWTPs can discharge approx. $10^{10} – 10^{12}$ ESC-resistant *E. coli* daily into the aquatic environment, depending on the size of the plant (Bréchet et al., 2017; Kwak et al., 2015). In addition, the concentrations of presumptive CTX-R and CR coliforms, especially in winter, were usually among the highest in the influents of the Varaždin WWTP, which can be associated with several factors, including contamination from the local poultry industry. On the other hand, the content of presumptive CTX-R and CR coliforms in the influent was not significantly reduced after the treatment, indicating lower effectiveness of the Varaždin WWTP in removing these resistant populations compared to other analyzed plants. In general, the observed differences in the removal efficiency of microbial pollutants between the studied WWTPs could be due to differences in the composition of their influents, the nature of their activated sludge systems, and the operating conditions (Krzeminski et al., 2019). In Croatia, there is a regulation on the presence of *E. coli* in municipal effluent discharged to surface waters for bathing and recreational purposes. The *E. coli* concentration should not exceed 10^3 CFU/100 mL in this effluent (Official Gazette of the Republic of Croatia 26/2020). In the present study, culturable presumptive (non-resistant) *E. coli* were found in concentrations greater than 10^3 CFU/100 mL (up to 10^5 CFU/100 mL) in all final effluents at both seasons, although the receiving waters of the studied WWTPs are not official bathing areas. Moreover, the concentrations of presumptive CTX-R and CR *E. coli* in the effluent of the Varaždin WWTP were above 10^3 CFU/100 mL in both seasons and in Bjelovar in winter. These concentrations were higher than the concentrations of ESC-resistant *E. coli* found in the effluent from Dutch WWTPs (about 10^1 CFU/100 mL; Verburg et al., 2019), but lower than in effluents from Polish WWTPs with different modifications of treatment processes (about 10^4 CFU/100 mL; Osińska et al., 2017). Additionally, positive correlations between parameters such as COD, suspended solids and effluent levels of presumptive CTX-R and CR coliform bacteria were observed in this study. Therefore, our data suggest that wastewater treatment processes in Croatian municipal WWTPs need to be improved and supplemented with disinfection technologies to control the spread of CTX-R and CR *E. coli* and other coliforms via effluents into receiving waters.

Because removal of *E. coli* and other EOPs in WWTPs may be inadequate, discharge of treated wastewater may be a pathway for widespread dissemination of EOPs. We therefore applied qPCR to monitor several EOPs such as *E. coli, A. baumannii, K. pneumoniae* and *Enterococcus* spp. in 7 WWTPs studied. The results show that most WWTPs generally remove *A. baumannii* and *K. pneumoniae* populations efficiently, as they were rarely or not detected in the final effluents. On the contrary, although *E. coli* and enterococci were generally significantly reduced by biological treatment in the majority of the analyzed WWTPs, they were still present in the effluents in high amounts, in the range of 10^4-10^5 CE/100 mL in both seasons. The exception is the WWTP in the city of Varaždin, where the removal of *E. coli* and enterococci was the lowest and therefore, the concentrations in the effluent were the highest (10^6-10^7 CE/100 mL), in agreement with the data for culturable coliforms. The *E. coli* levels observed in the effluents in this study were comparable to those reported by Heß et al. (2016) and Jäger et al. (2018) based on qPCR of the *uid*A and *ycc*T genes, respectively (10^4-10^5 CE/100 mL). However, the enterococci levels found in the effluents in this study (about 10^5 CE/100 mL) were approx. 2 orders of magnitude higher than in two German studies based on qPCR of the 23S rRNA gene (Jäger et al., 2018; Hembach et al., 2019). In addition, higher concentrations of *E. coli* determined by qPCR compared to those determined by culturing are to be expected in this study, as qPCR also detects dead cells

and cells that are in a "viable but not culturable" status. In addition to EOPs, we targeted the 16S rRNA gene as a marker for total bacteria and found that it was present at concentrations of approx. 10^7-10^{10} CE/100 mL after conventional treatment, which is comparable to previous reports on WWTPs with conventional (Czekalski et al., 2012; Nõlvak et al., 2013; Jäger et al., 2018) and advanced treatment (ozonation) (Heß et al., 2016; Hembach et al., 2019).

Further focus on qPCR quantification of β-lactam resistance genes of major importance in clinical setting ($bla_{\text{CTX-M-32}}$, bla_{TEM}, $bla_{\text{KPC-3}}$, $bla_{\text{OXA-48}}$-like, bla_{NDM}, bla_{VIM} and bla_{IMP}) showed that the abundance of these genes in influent and effluent varied greatly depending on the type of gene, the sampling location, and the sampling period. β-lactams are one of the most frequently prescribed classes of antibiotics worldwide, including in Croatia, and the most worrisome types of resistance are ESBLs, which confer resistance to ESC, and carbapenemases which confer resistance to carbapenems and all other β-lactams (Paterson and Bonomo, 2005; Sawa et al., 2020). Here, both ESBL genes, bla_{TEM} and $bla_{\text{CTX-M-32}}$, were present in all influent samples, with relative abundance approx. an order of magnitude lower in winter than in summer. With the exception of $bla_{\text{CTX-M-32}}$ in the winter samples, these two genes were predominantly only slightly reduced or even increased after treatment, indicating variation in relative host abundance or possible HGT to new hosts during treatment. Both bla_{TEM} and $bla_{\text{CTX-M-32}}$ genes were detected in effluent samples from different European WWTPs at concentrations one order of magnitude lower than here (Cacace et al., 2019) and in influent and effluent from Polish WWTP at higher concentrations than here (Zieliński et al., 2021). CTX-M-type β-lactamases are the most common types of ESBLs (Mlynarcik et al., 2021) and TEM type β-lactamases are the most common and widespread plasmid-encoded β-lactamase in the environment (Narciso-Da-Rocha et al., 2014; Proia et al., 2018). Plasmids carrying bla_{TEM} often contain $bla_{\text{CTX-M}}$ genes (Hembach et al., 2017), which is consistent with the strong positive correlation between bla_{TEM} and $bla_{\text{CTX-M-32}}$ observed in this study. The observation that these two genes correlate strongly with temperature suggests that the proliferation and persistance of bacteria carring these genes is stimulated at warmer temperatures. In addition, a significant positive correlation was found between bla_{TEM} and *E. coli,* suggesting that *E. coli* may be the host of this gene, as previously shown in the literature (Adekanmbi et al., 2020).

The most frequent carbapenemases in *Enterobacterales* reported in Europe are KPC, VIM, IMP, NDM and the OXA-48-like enzymes (ECDC, 2013). Of particular concern is the fact that carbapenemases are no longer limited to hospital isolates. In addition to hospitals, they have also been found in long-term care facilities, in the community, in sewage and in receiving waters (Buelow et al., 2018; Proia et al., 2018; Jelić et al., 2019; Sib et al., 2020). Moreover, they continue to spread because their genes, often found in plasmids (Nasri et al., 2017; Freeman et al., 2020), are associated with mobile elements that facilitate their acquisition (e.g., by clonal and horizontal transfer) and their spread from bacterium to bacterium (Subirats et al., 2017; Zhang et al., 2020). Surveillance among clinical enterobacterial isolates in Croatia showed that the most frequent carbapenemases from 2008 to 2012 were VIM and NDM, while from 2015 to 2017, OXA-48 became predominant (Zujić Atalić et al., 2014; Bedenić et al., 2018). In the present study, $bla_{\text{KPC-3}}$, bla_{NDM} and bla_{IMP} were sporadically detected in the influent of some WWTPs, mainly in winter (approx. −3 to −5 log copies/*rrn* copies), which is consistent with reports for some European WWTPs (Subirats et al., 2017; Pärnänen et al., 2019). However, they were rarely detected in effluents, except for bla_{IMP}. Interestingly, in seven cases (4 in winter, 3 in summer), bla_{IMP} was found only in the final effluent but not in the paired influent, as was also observed sporadically for bla_{VIM} and $bla_{\text{KPC-3}}$. The enrichment of these genes could be the consequence of the increase in abundance of their original hosts or of HGT between different hosts during the treatment process. The opposite was observed for $bla_{\text{OXA-48}}$, which was mainly detected in the influent but not in the paired effluents of the different WWTPs (except for two WWTPs), indicating its good removal during conventional wastewater treatment. This could be the result of a good removal of *A. baumannii* as a potential host of this gene in the studied WWTPs (qPCR data), since a positive correlation was found between $bla_{\text{OXA-48}}$ and *A. baumannii.* This pathogen has already been shown to carry $bla_{\text{OXA-48}}$-like genes (Gonçalves et al., 2013; Assem et al., 2017). Finally, the bla_{VIM} gene was detected in the majority of influents and effluents of the studied WWTPs, being enriched in four WWTPs in winter and in summer. This gene was also found in some influent samples in different European countries (Pärnänen et al., 2019) and in effluents from different German WWTPs (Hembach et al., 2019; Sib et al., 2020), but at lower concentrations (10^1 copies/mL) than in the effluents analyzed in this study (10^2–10^3 copies/mL; data not shown).

Finally, our results also showed that specific WWTP characteristics affect the amount of some ARGs released via the final effluent. In contrast to earlier findings (Cacace et al., 2019), we showed that facility size and number of hospitals correlated with concentrations of bla_{IMP} in WWTP effluents, while number of hospitalized patients correlated with concentrations of bla_{VIM} in effluents. However, the concentrations of both bla_{IMP} and bla_{VIM} were found to be inversely correlated with the effluent concentration of COD.

5. Conclusions

This study represents the first comprehensive investigation of the presence and removal of culturable coliforms and a suite of high-priority EOPs and ARGs in municipal WWTPs in several Croatian cities to provide baseline data for emerging antibiotic resistance risks in wastewater. Most previous studies on ARB and ARGs in municipal wastewater have focused on those associated with aminoglycoside, macrolide, tetracycline and sulfonamide resistance. This study highlights the clinical relevance of treated wastewater as it harbours potentially pathogenic bacteria and genes that cause resistance to last-line antibiotics such as carbapenems and ESC. Having demonstrated that some of the top priority EOPs (*E. coli*, enterococci) were not completely eliminated and ARGs of highest clinical concern (bla_{IMP} and bla_{VIM}) were frequently enriched during the process, advanced treatment technologies should be employed to avoid the risk of spreading hazardous ARGs and EOPs and to minimize the impact on public health.

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CRediT authorship contribution statement

Ana Puljko: Investigation, Writing – original draft, Writing – review & editing, Formal analysis, Visualization. **Milena Milaković**: Investigation. **Stela Križanović**: Investigation. **Josipa Kosić-Vukšić**: Investigation, Writing – review & editing. **Ivana Babić**: Formal analysis, Visualisation, Writing – original draft. **Ines Petrić**: Investigation, Writing – review & editing. **Ana Maravić**: Methodology, Writing – review & editing. **Marko Jelić**: Formal analysis, Writing – review & editing. **Nikolina Udiković-Kolić**: Conceptualization, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.128155.

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Chapter 3

Publication No. 2: Treated municipal wastewater as a source of high-risk and emerging multidrug-resistant clones of *E. coli* and other Enterobacterales producing extended-spectrum β-lactamases

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Treated municipal wastewater as a source of high-risk and emerging multidrug-resistant clones of *E. coli* and other Enterobacterales producing extended-spectrum β-lactamases

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High-risk clones

ABSTRACT

Extended-spectrum β-lactamase (ESBL)-producing Enterobacterales are a major public health problem, and wastewater from municipal wastewater treatment plants (WWTPs) is a potential means of spreading them into the environment and community. Our objective was to isolate ESBL-producing *E. coli* and other Enterobacterales from wastewater after treatment at Croatia's largest WWTP and to characterize these isolates by phenotypic and genotypic testing. Of the 200 bacterial isolates, 140 were confirmed as Enterobacterales by MALDI-TOF MS, with *Escherichia coli* and *Klebsiella* spp. predominating (69% and 7%, respectively). All 140 enterobacterial isolates were multidrug-resistant (MDR) and produced ESBLs. The most prevalent ESBL genes among the isolates tested were $bla_{\text{CTX-M-15}}$ (60%), $bla_{\text{TEM-116}}$ (44%), and $bla_{\text{CTX-M-3}}$ (13%). Most isolates (94%) carried more than one ESBL gene in addition to $bla_{\text{CTX-M}}$. Genes encoding plasmid-mediated AmpC, most notably bla_{EBC}, were detected in 22% of isolates, whereas genes encoding carbapenemases ($bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$, $bla_{\text{VIM-1}}$) were less represented (10%). In *E. coli*, 9 different sequence types (ST) were found, with the emerging high-risk clones ST361 (serotype A-O9:H30) and pandemic ST131 (serotype B2–O25:H4) predominating (32% and 15%, respectively). Other high-risk *E. coli* clones included ST405 (3%), ST410 (3%), CC10 (3%), ST10 (3%), and ST38 (2%), and emerging clones included ST1193 (2%) and ST635 (2%). Whole-genome sequencing of three representative *E. coli* from two dominant clone groups (ST361 and ST131) and one extensively drug-resistant *K. oxytoca* revealed the presence of multiple plasmids and resistance genes to several other antibiotic classes, as well as association of the $bla_{\text{CTX-M-15}}$ gene with transposons and insertion sequences. Our findings indicate that treated municipal wastewater contributes to the spread of emerging and pandemic MDR *E. coli* clones and other enterobacterial strains of clinical importance into the aquatic environment, with the risk of reintroduction into humans.

1. Introduction

Antibiotic resistance (AR) is a major health problem worldwide, especially when it is conferred to third-generation cephalosporins (3GCs), which are used to treat infections caused mainly by Gram-negative bacteria (Collignon and McEwen, 2019). The primary mode of resistance to 3GCs is the production of extended-spectrum β-lactamases (ESBLs), and the World Health Organization (WHO, 2019) identifies ESBL-producing Enterobacterales including *Escherichia coli* and *Klebsiella* spp. as the greatest threat to human health. Croatia is one of the nations with significant clinical incidence of these strains conferring resistance to 3GCs. For example, the prevalence of 3GC-resistant *E. coli* and *K. pneumoniae* isolates in clinical settings in Croatia in 2021 was 18% and 62%, respectively (ECDC, 2022), and this resistance is still predominantly caused by the production of ESBLs (ISKRA, 2021).

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Among the various β-lactamases associated with enterobacteria, ESBLs, AmpC, and carbapenemases remain clinically important as they are increasingly common causes of antibiotic-resistant infections worldwide. ESBLs can inactivate penicillins, 1st, 2nd, and 3rd generation cephalosporins, and some 4th generation cephalosporins, but not carbapenems. These enzymes are generally classified into four main types, CTX-M, SHV, TEM and OXA (Castanheira et al., 2021), and their genes ($bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM}, bla_{OXA}) are often located on plasmids so that they can be transferred between different bacteria (Benz et al., 2021). Plasmid-encoded AmpC β-lactamases (pAmpC) are less common than ESBLs, and the most commonly detected enzymes are ECB, FOX, MOX, CMY, DHA, ACT/MIR, and LAT (Coertze and Bezuidenhout, 2019). The genetic background of resistance of enterobacteria to carbapenems is often the production of carbapenemase enzymes that can inactivate all β-lactam antibiotics, and among these enzymes, KPC, NDM, OXA-48, VIM and IMP are of clinical importance (Nordmann et al., 2012a).

Previous research has shown that wastewater treatment plants (WWTPs) contribute to the spread of antibiotic-resistant bacteria (ARB) and their corresponding antibiotic-resistance genes (ARGs) in the environment, which in turn can be transferred to humans through the use of surface waters (Kumar et al., 2020). Studies have shown that *E. coli* is sometimes found in higher concentrations in treated wastewater than in raw wastewater, suggesting that it can better withstand wastewater treatment (Gumede et al., 2021). Notably, extraintestinal pathogenic *E. coli* (ExPEC) lineages such as ST131 or ST10, which often have a multidrug-resistant (MDR) phenotype, have also been found in hospital, municipal wastewater and adjacent rivers (Davidova-Gerzova et al., 2023). Therefore, wastewater testing could be a strategy to monitor high-risk and emerging human-associated ESBL clones.

Our recent study on 3GC-resistant *E. coli* and other coliforms in the same WWTP as the one investigated here (Zagreb WWTP) has shown that despite their significant reduction, a large amount is still discharged into the receiving Sava River (up to 10^{12} 3GC-resistant coliforms per day). In addition, ESBL genes such as $bla_{\text{CTX-M-32}}$ showed only a slight decrease or even an increase after treatment (Puljko et al., 2022). However, there is a lack of information on the diversity and characteristics of 3GC-resistant enterobacteria in treated municipal wastewater in Croatia. The objective of this research was, therefore, to identify and analyze 3GC-resistant and ESBL-producing Enterobacterales in treated wastewater from the WWTP of the Croatian capital (Zagreb). We investigated the AR patterns and the occurrence of ARGs in these isolates utilizing both phenotypic and genotypic methods. Analysis of genetic and epidemiological relationships among isolates was performed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). In addition, whole-genome sequencing (WGS) of MDR *E. coli* from two dominant clone groups and the extensively drug-resistant (XDR) *K. oxytoca* isolate was performed to further characterize their AR profiles.

2. Materials and methods

2.1. Collection of wastewater samples

Automatic samplers were used to collect 24-h composite effluent samples of treated wastewater from Zagreb's conventional WWTP. Sampling was performed on three continuous days in February 2020 (winter). Sterile glass bottles (2.5 L) were used to collect effluents, which were then transported on ice and analyzed as soon as they arrived at the laboratory.

2.2. Isolation and identification of presumptive 3GC-resistant E. coli and other Enterobacterales isolates

The collected effluent samples were subjected to serial tenfold dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) in 0.85% NaCl, and then each dilution was passed through three sterile mixed cellulose ester membrane filters (47 mm diameter, 0.22 μm pore size, Whatman, GE Healthcare, Life Science, USA). Next, the filters were aseptically transferred to Rapid'*E. coli* 2 agar plates (Bio-Rad, France) containing cefotaxime (CTX, 4 mg/L) to isolate 3GC-resistant *E. coli* and other Enterobacterales from the membrane filters. After incubation of the plates at 37 °C for 24 h, suspected colonies of *E. coli* and other Enterobacterales (based on their color) were picked from the membrane filters and subcultured to purity on the fresh Rapid'*E. coli* 2 agar plates. After isolation of 200 colonies, they were stored in 20% glycerol at −80 °C for further analysis.

Frozen isolates were revived by streaking on Rapid'*E. coli* 2 agar plates containing 4 mg/L CTX and incubated overnight (37°C). Subsequently, the isolates were analyzed by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) to confirm their presumptive identity, as previously mentioned (Puljko et al., 2023.). When MALDI-TOF MS could not identify a particular isolate, it was identified by sequencing the 16S rRNA gene (Puljko et al., 2023).

2.3. Screening for ESBL, pAmpC, and carbapenemase production

ESBL production was analyzed using the double disk synergy test (DDST) in accordance with EUCAST guidelines. Detection of pAmpC enzymes was assessed using the phenylboronic acid disk assay as described by Gupta et al. (2014). To determine the production of carbapenemase, we performed the Carba NP assay on isolates that showed resistance to any of the carbapenems (Nordmann et al., 2012b). All of the above protocols were briefly described in a recent paper (Puljko et al., 2023).

2.4. Phenotypic AR screening

All Enterobacterales isolates (n = 140) were subjected to phenotypic screening for resistance to 13 clinically important antibiotics in the disk diffusion assay as previously described (Puljko et al., 2023). In addition, all these isolates were tested for resistance to colistin (COL) using the broth microdilution method as previously described (Puljko et al., 2023). Isolates identified as carbapenem-resistant by the disk diffusion assay were additionally tested for resistance to carbapenems (imipenem (IPM), ertapenem (ETP) and meropenem (MEM)) using the same broth microdilution protocol. Isolates selected for WGS were subjected to repeated phenotypic AR screening after the cultures were revived from −80 °C (see 2.7).

Based on the definitions established by Magiorakos et al. (2012), isolates were sorted into three categories: MDR, XDR and pandrug-resistant (PDR). In addition, isolates were grouped based on the similarity of their AR profiles, and selected representatives from each group were tested for the presence of ARGs by PCR.

2.5. Targeted PCR for β-lactamase genes

A representative subgroup of isolates with various AR patterns (n = 68) was tested for ESBL, pAmpC, and carbapenemase genes by PCR. DNA was isolated from overnight bacterial cultures using the Quick-DNA™ Miniprep Plus Kit (Zymo, USA) and used as a template for PCR. Twenty primer pairs for target ARGs and PCR conditions are listed in Table S1. Primarily, all ESBL-producing isolates were screened by multiplex PCR for β-lactamase genes of the bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{VEB}, bla_{GES}, and bla_{SME} families and by singleplex PCR for the ESBL genes of $bla_{\text{CTX-M}}$ groups 1, 2, and 9. Additionally, screening for pAmpC genes was also performed by multiplex PCR. Finally, all carbapenemase-producing isolates were tested by PCR for the carbapenemase genes bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{\text{OXA-48}}$-like, and bla_{VIM}. Additionally, COL-resistant isolates underwent multiplex PCR for *mcr1-5* genes. All positive amplicons except the pAmpC amplicons were sent to Macrogen (The Netherlands) for Sanger sequencing (forward direction). After editing,

sequences were compared to reference sequences in NCBI using BLASTX.

2.6. Genotyping by PFGE

Genotypic diversity of isolates of *E. coli* (n = 97), *Klebsiella* spp. (n = 10) and *Enterobacter cloacae* complex (cplx.) (n = 9) was assessed by PFGE, as previously described (Jelic et al., 2016). DNA of the bacterial isolates was digested in agarose plugs with *Xba*I (Bio-Rad Laboratories, USA) and separated in a 1% agarose gel using the CHEF-DR III system (Bio-Rad Laboratories, USA) under the following conditions: 6 V/cm; pulse time 6–36 s; final time 19.5 h, at 12 °C. The restriction patterns were analyzed with the software BioNumerics (Applied Maths, Belgium). Dendograms were generated with a positional tolerance of 1.5% using the UPGMA and DICE similarity coefficient. PFGE clusters were established by identifying similarities that were equal to or greater than 85%. For each cluster, one representative isolate was analyzed to determine the sequence type (ST). This was done either by using a genomics service (IDgenomics, Seattle, USA) or by WGS (as explained below).

2.7. WGS and bioinformatic analysis

Three *E. coli* isolates and one *K. oxytoca* isolate were selected for WGS based on their clinical importance and their XDR or MDR phenotype. To revive these isolates, glycerol stocks were used to grow them on LB plates with CTX (4 mg/L). A single colony was selected and cultured overnight in a LB broth supplemented with CTX (4 mg/L). Genomic DNA was isolated from this culture using the same kit as in 2.5. and sequenced on the Ion Torrent PGM instrument as previously described (Puljko et al., 2023). Briefly, sequencing libraries were prepared using the Ion Xpress Plus Fragment Library Kit, which was designed to a fragment size of 400 bp. The assembly *de novo* was done with the software Assembler SPAdes (v.3.1.0.), and the annotation was done with RAST (v.2.0). Genotyping screening of ARGs, STs, and plasmid replicon types in bacterial genomes was performed as previously described (Puljko et al., 2023). *E. coli* phylogroups were determined using the online tool ClermonTyping (Beghain et al., 2018), and SerotypeFinder 2.0 (Joensen et al., 2015) was used for serotype identification under default settings for ST361 and with a minimum length of 40% for ST131. The analysis of virulence factors was conducted using the Virulence Factor Database (VFDB; Chen et al., 2005). Pathogenwatch was used to predict capsule (K) and O serotype in *K. oxytoca* (Argimón et al., 2021).

2.8. Data availability

Sanger sequence data were deposited in GenBank under the accession numbers OR208125 - OR208126 (bla_{OXA-48}), OR208127 (bla_{LEN}), OR208128 – OR208135 (bla_{SHV}), OR208136-OR208137 (bla_{NDM}), OR224481-OR224483 (bla_{VIM}), OR224484 - OR224488 (bla_{GES}), OR224489-OR224538 (bla_{CTX-M}), OR242368-OR242419 (bla_{TEM}). WGS data were deposited at NCBI under the BioProject PRJNA913323 and the following accession numbers: SAMN34152679 (*E. coli* SE_EC_79 isolate), SAMN34152680 (*E. coli* IZ 8–44 isolate), SAMN34152681 (*E. coli* SE_EC_56 isolate) and SAMN34152688 (*K. oxytoca* SE_COL_90 isolate).

3. Results

3.1. Bacterial isolation and identification

Two hundred presumptive CTX-resistant (CTX-R) strains were isolated on Rapid'*E. coli* 2 agar plates amended with CTX (4 mg/L) from effluent samples, including 140 Enterobacterales and 60 non-Enterobacterales isolates (MALDI-TOF MS or 16S rRNA gene sequences). As shown in Table 1, the identified Enterobacterales isolates comprised six genera, with *E. coli* being the most abundant at 69.3% (97/140). The other 5 genera were represented by less than 10%, namely *Klebsiella* spp. (7.2%) *Raoultella* spp. (7.2%), *E. cloacae* cplx. (6.3%), *Kluyvera cryocrescens* (5.7%), and *Citrobacter* spp. (4.3%) (Table 1).

Table 1
Prevalence of enterobacterial genera isolated from wastewater samples.

Name of isolate	Total	
	Number (n)	Percentage (%)
E. coli	97	69.3
Klebsiella spp.	10	7.2
Raoultella spp.	10	7.2
E. cloacae complex	9	6.3
Kluyvera cryocrescens	8	5.7
Citrobacter spp.	6	4.3
Total	**140**	**100**

3.2. Phenotypic detection of β-lactamase enzymes

ESBL production was confirmed in all 140 Enterobacterales isolates presumptive to be CTX-R (DDST test). Of these, 28 isolates (20%) were identified as phenotypic pAmpC producers (combined disk test) and 21 isolates resistant to tested carbapenems (ETP, IMP, and MEM) were confirmed as carbapenemase producers (Carba NP test).

3.3. AR patterns of Enterobacterales isolates

AR patterns of all 140 Enterobacterales isolates were analyzed by agar disc diffusion and broth microdilution methods against clinically relevant antibiotics from 9 different antibiotic classes. Because some enterobacteria have natural AR mechanisms (Magiorakos et al., 2012), the aforementioned resistance results were not considered when reporting the number of resistant isolates.

The distribution of AR profiles for each of the six identified genera is shown in Fig. 1. Most *E. coli* (99%) were MDR, while 51% were XDR. Apart from penicillins and cephalosporins, the antibiotics that *E. coli* were most resistant were ciprofloxacin (CIP, 77%), trimethoprim/sulfamethoxazole (SXT, 65%) and gentamicin (GM, 57%). A similar trend of AR was generally seen in other Enterobacterales isolates tested (*Klebsiella* spp., *Raoultella* spp., and *Citrobacter* spp.), with resistance to GM, SXT, and CIP being lowest in *E. cloacae* cplx. (22%–33%) (Fig. 1). All of these isolates were MDR, with 90% of *Raoultella* spp. and approximately 30% of *Klebsiella* spp. and *Citrobacter* spp. being XDR. Resistance to the three antibiotics within the carbapenem group was variable, with resistance to ETP being more common and most prevalent in *E. cloacae* cplx. (67%), followed by *Citrobacter* spp. (33 %), *Klebsiella* spp. and *Raoultella* spp. (both 30%). *Klebsiella* spp. isolates showed the greatest incidence of resistance to COL (60%), followed by *E. cloacae* cplx. (44%) and *Citrobacter* spp. (17%). *E. coli* was found to have a relatively low resistance to carbapenems and COL, with a range of only 4–7%. In *Kluyvera* spp., only resistance to amoxicillin (AML), cephalexin, and cefuroxime was detected (Fig. 1).

3.4. PCR detection of ARGs

Isolates that produce ESBL were grouped according to their AR profile, and 1–2 representatives from each group (68 isolates in total, Table S2) were selected for further PCR analysis of ESBL, carbapenemase and pAmpC genes.

Of the 68 isolates tested, bla_{CTX-M} (n = 55; 80.9%) and $bla_{TEM-116/135}$ (n = 31; 45.6%) were the most frequently detected groups of ESBL genes (Fig. S1). The gene subtype was determined by amplicon sequencing. Among the isolates with bla_{CTX-M}, four CTX-M subtypes were identified: $bla_{CTX-M-15}$ (n = 41), $bla_{CTX-M-3}$ (n = 9), $bla_{CTX-M-1}$ (n = 4) and $bla_{CTX-M-55}$

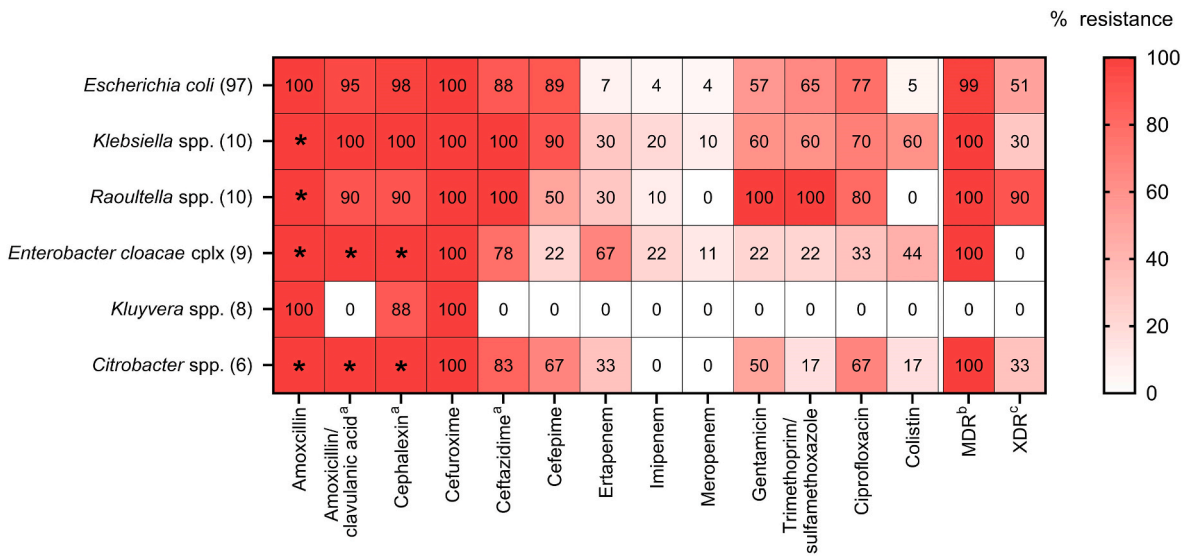

Fig. 1. Percentage of cefotaxime-resistant (CTX-R) Enterobacterales genera from wastewater samples that have AR phenotype. Numbers in brackets indicate the quantity of isolates from each genus. The numbers in the cells represent percentage of resistance and the asterisk represents intrinsic resistance. [a]The percentage of isolates with a resistant phenotype was not 100% because some isolates had an intermediate susceptibility profile. [b] The term MDR refers to resistance to ≥3 antibiotic classes; [c]XDR to susceptibility to ≤2 antibiotic classes.

(n = 1) (Table 2). The most prevalent $bla_{CTX\text{-}M\text{-}15}$ gene was detected mainly in *E. coli* isolates (n = 21), whereas the $bla_{CTX\text{-}M\text{-}1}$ (n = 4) and $bla_{CTX\text{-}M\text{-}55}$ (n = 1) genes were detected only in *E. coli* isolates. Finally, the $bla_{CTX\text{-}M\text{-}3}$ gene was detected mainly in isolates of *K. cryocrescens* (n = 4) (Table 2). Three bla_{TEM} subtypes were identified among the isolates with this gene: $bla_{TEM\text{-}1}$ (n = 35), $bla_{TEM\text{-}116}$ (n = 30) and $bla_{TEM\text{-}135}$ (n = 1) (Table 2). The only non-ESBL variant among them was $bla_{TEM\text{-}1}$, which was detected in *E. coli*, *Klebsiella* spp. (*K. pneumoniae* and *K. oxytoca*), *E. cloacae* cplx. (*E. asburiae*), *Routella* spp. (*R. ornithinolytica* and *R. planticola*), and *Citrobacter* spp. (*C. braakii* and *C. freundii*) (Table 2). The $bla_{TEM\text{-}116}$ subtype was predominantly found in *E. coli* (n = 15) and *Klebsiella* spp. (n = 6), but was also present in *E. cloacae* cplx. (n = 5) and *K. cryocrescens* (n = 4) (Table 2). The $bla_{TEM\text{-}135}$ subtype was detected in only one *E. coli* isolate. Some β-lactamase genes, such as bla_{SHV} (8/64, 12.5%), bla_{GES} (5/64, 7.8%) and bla_{LEN} (n = 1, 1.6%) were detected rather rarely (Table 2). Among the isolates with bla_{SHV}, three SHV subtypes were identified: non-ESBL $bla_{SHV\text{-}1}$ (1 *E. coli*), and ESBL types $bla_{SHV\text{-}12}$ (1 *K. pneumoniae*, *R. planticola* and *C. freundii*) and $bla_{SHV\text{-}28}$ (4 *K. pneumoniae*) (Table 2). The bla_{GES} subtypes included the carbapenemase genes $bla_{GES\text{-}5}$ (2 *E. cloacae* cplx.) and the ESBL genes $bla_{GES\text{-}7}$ (3 *Klebsiella* spp.). The bla_{LEN} gene was found in a single isolate of *K. pneumoniae* (Table 2).

Table 2
Distribution of ESBL, AmpC, or carbapenemase (CP) genes among CTX-R Enterobacterales isolates obtained from effluent samples in this study.

	Species (No of Isolates; Total = 68) / β-lactamase genes		*E. coli* (33)	*Klebsiella* spp. (10)	*E. cloacae* cplx (9)	*Raoultella* spp. (8)	*Kluyvera* spp. (4)	*Citrobacter* spp. (4)
ESBL	blaCTX-M	CTX-M-1	• • •	•				
		CTX-M-3	•	•			•	• • •
		CTX-M-15	• • •	• • • • • • • •	• •	• • •		
		CTX-M-55	•					
	blaTEM	TEM-1*	• • • • • • •	• • • • •	•	• • •		• • • •
		TEM-116	• • • • • •	• • • • • •	• • •		•	
		TEM-135	•					
	blaSHV	SHV-1*	•					
		SHV-12		•		•		•
		SHV-28		• • • •				
	blaLEN	LEN*		•				
	blaGES	GES-7		• • •				
AmpC	ACC							•
	CIT							•
	DHA		•			•		
	EBC		•	• •	• • • • •			
	FOX							•
CP	OXA-48		•	•				
	NDM-1		•	•				
	VIM-1		•		• •			
	GES-5				• •			
	Number of isolates (Σ=68)		1 1 2 1 11 1 2 1 1 10 1 1	1 1 1 1 1 1 2 1 1	2 4 1 1 1	6 1 1	4	1 1 1 1

Black dots – positive gene detection. Cell color represent different number of β-lactamase genes present: white – 1 gene; yellow - 2 genes; green - 3 genes; red - 4 genes; dark red – 5 genes. *non -ESBL variants.

Besides ESBL genes, sixteen ESBL isolates (23.5%) tested positive for pAmpC genes, among which 5 variants were detected: bla_{EBC} (n = 11), bla_{DHA} (n = 2), bla_{ACC} (n = 1), bla_{CIT} (n = 1) and bla_{FOX} (n = 1) (Fig. S1, Table 2). The most dominant AmpC variant, bla_{EBC}, was mostly detected among *E. cloacae* cplx. isolates (Table 2). Genes associated with carbapenem resistance were also found, but less frequently (13.2%), and included bla_{OXA-48} (1 *E. coli* and 1 *K. pneumoniae*), bla_{NDM-1} (1 *E. coli* and 1 *K. pneumoniae*) and bla_{VIM-1} (1 *E. coli* and 2 *E. cloacae*), in addition to the aforementioned bla_{GES-5} (Table 2, Fig. S1).

Isolates with two β-lactamase genes were the most abundant at 66.2% (45/68) and mainly had the combination of two ESBL genes, $bla_{TEM-116}$ and bla_{CTX-M}, found mainly in *E. coli* (n = 13) (Table 2). Nineteen percent of isolates (13/68) were positive for three β-lactamase genes tested, usually the combination of $bla_{TEM-116}$ and $bla_{CTX-M-15}$ with either bla_{SHV} or bla_{GES}, detected mainly in *Klebsiella* spp. (n = 4) (Table 2). Five isolates (4 *Klebsiella* spp. and 1 *Citrobacter* spp.) showed the co-occurrence of 4 β-lactamase genes ($bla_{CTX-M-3}$+$bla_{TEM-116}$+bla_{GES-7}+bla_{NDM-1}, $bla_{CTX-M-15}$+bla_{TEM-1}+bla_{SHV-12}+bla_{EBC}, $bla_{CTX-M-15}$+$bla_{TEM-116}$+bla_{SHV-28}+bla_{EBC}, $bla_{CTX-M-15}$+$bla_{TEM-116}$+bla_{LEN} + bla_{GES-7}, and $bla_{CTX-M-3}$+bla_{TEM-1}+bla_{SHV-12}+bla_{DHA}), and there was one *E. coli* isolate (IZ 8–44) with 5 β-lactamase genes ($bla_{TEM-116}$+bla_{SHV-1}+bla_{DHA} + bla_{OXA-48}+bla_{VIM-1}) (Table 2).

3.5. Molecular typing of isolates

PFGE genotyping of selected isolates of *E. coli* (n = 97), *Klebsiella* spp. (n = 10) and *E. cloacae* cplx. (n = 9) was performed and one representative from each cluster was analyzed to determine the ST. Using the macrorestriction patterns obtained by PFGE, a dendogram was generated and compared with the AR profile, ARG (sub)types and STs (Fig. 2).

Of the 97 *E. coli* isolates, five isolates could not be typed and 27 isolates were not clustered. However, 65 *E. coli* isolates belonged to 12 different clusters (A-L) based on 85% similarity. The largest clusters were H (n = 31) and F (n = 10) (Fig. 2A). Among the clustered *E. coli* isolates, nine STs were found: ST38, ST131, ST361, ST405, ST410, ST635, ST1193, ST3356, ST6329, and a new ST (belonging to ST clonal complex 10, CC10). Two unclustered isolates belonged to ST12193 and ST536, respectively (Fig. 2A). The most abundant STs among *E. coli* were ST361 (31%) and ST131 (14.4%). Some *E. coli* strains showed resistance to different antibiotics even within the same cluster. The largest *E. coli*

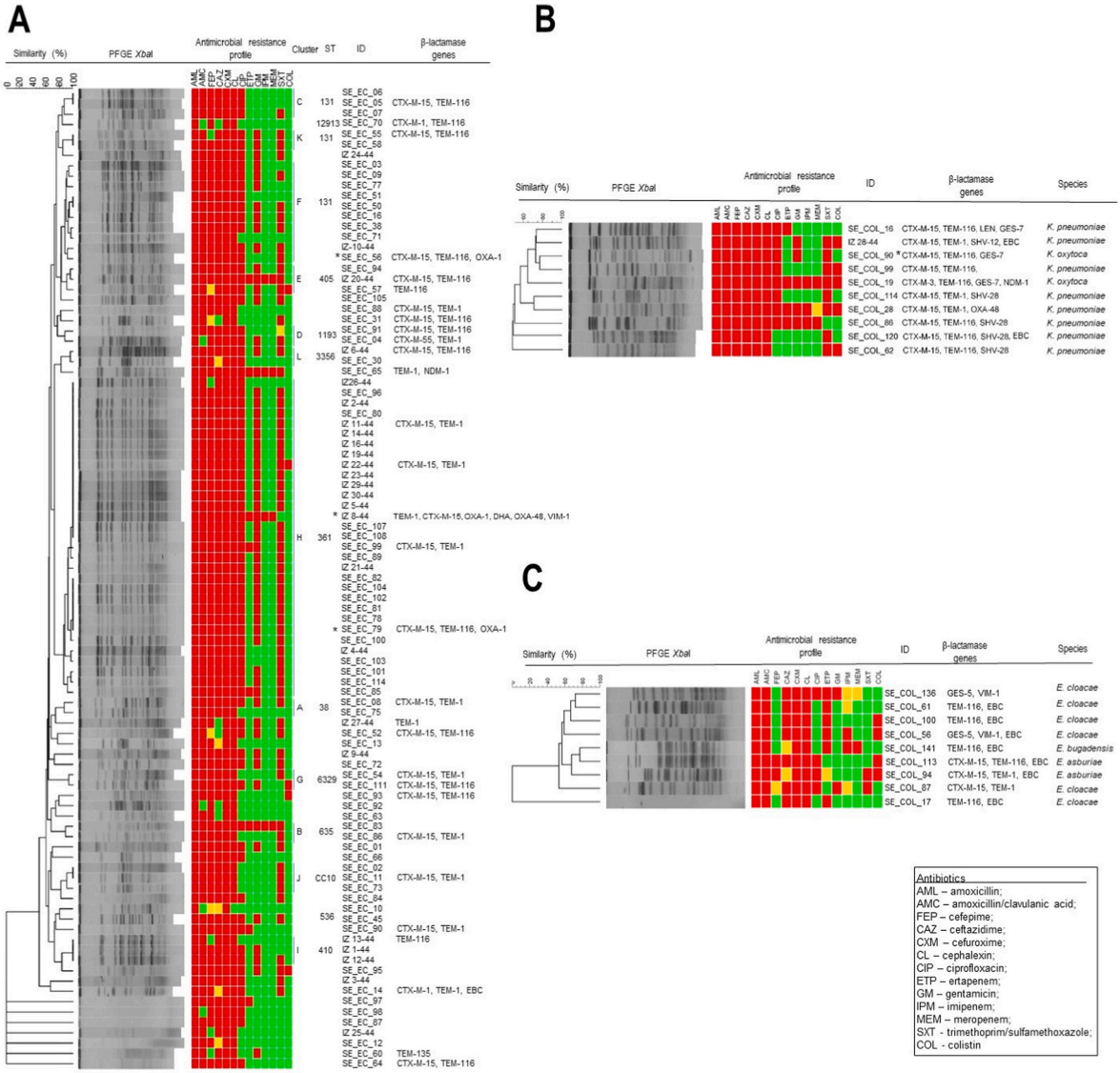

Fig. 2. Dendrogram of PFGE types representing the relatedness between (A) *E. coli*, (B) *Klebsiella* spp., and (C) *E. cloacae* cplx. isolates together with their antibiograms, ARGs present and sequence types (ST). Resistance to antibiotics is indicated by red squares, intermediate resistance by yellow squares, and susceptibility by green squares. ID refers to the name of the isolate, and isolates chosen for WGS analysis were marked with an asterisk (*).

cluster H (n = 31) belonged to ST361, and most isolates from this cluster had the same dominant resistance profile, which included resistance to nine antibiotics. Phenotypic resistance of isolates from this cluster to 3GCs was confirmed by detection of the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}116}$ or $bla_{TEM\text{-}1}$ genes. The *E. coli* in the second largest cluster F (n = 10) were identified as belonging to the epidemiologically important clone ST131, and their ESBL phenotype was linked to two ESBL genes, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}116}$ (Fig. 2A). Isolates from clusters C (n = 3) and K (n = 2) had the same ST (ST131) and ESBL genes ($bla_{CTX\text{-}M\text{-}15}$ + $bla_{TEM\text{-}116}$) as cluster F. In addition to resistance to penicillins and cephalosporins, all but one of the *E. coli* ST131 strains exhibited the phenotype of CIP resistance, and most of them were also resistant to SXT and GM. *E. coli* isolates from clusters A, B, D, G, and J belonging to ST38, ST635, ST1193, ST6329, and CC10 contained $bla_{CTX\text{-}M\text{-}15}$, sometimes together with $bla_{TEM\text{-}116}$ (ST1193 and ST6329). Isolates from cluster I (ST410) contained only $bla_{TEM\text{-}116}$. Isolates from these six clusters were resistant to a smaller number of antibiotics compared to isolates in the largest cluster H. Isolates from the remaining two smaller clusters E and L, belonging to ST405 and ST3356, carried two ESBL genes, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}116}$, and exhibited similar phenotypic AR profile to the second dominant cluster F.

The ten isolates of *Klebsiella* spp. were all singletons (Fig. 2B). Although these isolates exhibited different patterns of AR, all showed an ESBL phenotype and contained $bla_{CTX\text{-}M\text{-}15}$, except for a single isolate which had the $bla_{CTX\text{-}M\text{-}3}$ gene (isolate SE_COL_19). Of these, six isolates possessed an additional two ESBL genes, $bla_{TEM\text{-}116}$ and $bla_{SHV\text{-}28}$ or $bla_{GES\text{-}7}$ (Fig. 2B). Two *Klebsiella* isolates identified as phenotypically resistant to ETP and IPM had $bla_{NDM\text{-}1}$ (isolate SE_COL_19) or $bla_{OXA\text{-}48}$ genes (isolate SE_COL_28). One of these isolates (SE_COL_19) was resistant to all antibiotics tested (PDR), including COL, but no COL resistance genes *mcr1-5* were detected, as was the case with the other four isolates that were phenotypically resistant to COL. In addition to β-lactam resistance, 8/10 *Klebsiella* isolates showed resistance to CIP and SXT. Among these, five were also resistant to GM.

PFGE genotyping was assessed for the *E. cloacae* cplx. isolates per species - two isolates of *E. asburiae*, six isolates of *E. cloacae* and one isolate of *E. bugadensis* (Fig. 2C). All of these isolates were singletons. There were different patterns of AR for each species. Isolates of *E. asburiae* had the genes $bla_{CTX\text{-}M\text{-}15}$ + $bla_{TEM\text{-}1/TEM\text{-}116}$. Of the six *E. cloacae* that had an ESBL phenotype, three contained only $bla_{TEM\text{-}116}$ and one isolate had $bla_{TEM\text{-}1}$ together with $bla_{CTX\text{-}M\text{-}15}$.

The remaining two isolates possessed the carbapenemase genes $bla_{GES\text{-}5}$ and $bla_{VIM\text{-}1}$ and displayed resistance to ETP and/or IPM. In addition, two *E. cloacae* isolates identified as phenotypically COL-resistant had no COL resistance genes *mcr1-5*. Finally, *E. bugadensis* carried $bla_{TEM\text{-}116}$ and tested positive for phenotypic carbapenem resistance (ETP, IPM and MEM) in addition to the ESBL phenotype, but negative for five targeted carbapenemase genes.

3.6. WGS of selected E. coli and K. oxytoca isolates

The complete genomes of three *E. coli* isolates included in dominant clusters F and H and one XDR *K. oxytoca* isolate were sequenced (marked with an asterisk in Fig. 2). Isolates were subjected to analysis of their AR and virulence genes, phylogenetic groups, serotypes, STs and plasmid replicon types (Table 3).

Using the MLST 2.0 tool, the ST of two *E. coli* strains from cluster H was confirmed as ST361 and of *E. coli* from cluster F as ST131, while the *K. oxytoca* isolate could not be typed (ND; Table 3). In addition, ClermonTyping and SerotypeFinder analyses of the *E. coli* isolates showed that isolate ST131 was associated with phylogroup B2 and serotype O25:H4, whereas isolates ST361 was associated with phylogroup A and serotype O9:H30. Moreover, *E. coli* isolate ST131 was identified as presumptive ExPEC strain based on virulence gene composition. VFDB analysis revealed that this isolate contains virulence genes for adherence (*ecp, hcp, pap,* and *fim),* autotransporters (*ehaB, pic,* and *sat*), iron uptake (*iuc*, *iutA, chu, sit* and *ybtA)*, type III secretion system (*espL1),* and toxins (*hly*), of which the *papC* and *iutA* genes were associated with ExPEC (Table S3). Two other *E. coli* ST361 strains also carried a number of virulence genes, although they were not classified as ExPEC (Table S3).

Sequencing revealed that all four isolates showed a genetic match with their corresponding AR phenotype (Table 3). The ESBL phenotype was confirmed by WGS detection of the $bla_{CTX\text{-}M\text{-}15}$ and/or $bla_{OXA\text{-}1}$ genes in these study's isolates. Surprisingly, no $bla_{GES\text{-}7}$ ESBL gene was found in *K. oxytoca* by WGS, although it was found by targeted PCR and Sanger sequencing. In addition, ARGs to other antibiotic classes were detected by WGS (Table 3). In *E. coli* ST131, resistance to CIP was due to carrying the *aac(6′)-Ib-cr* gene. This clone also carried the chloramphenicol (CHL) resistance gene *catB3*, but phenotypic resistance to CHL was not tested. *E. coli* ST361 (IZ 8–44) had ARGs encoding resistance to AML ($bla_{TEM\text{-}1}$), CIP (*aac(6′)-Ib-cr*), GM (*aadA1*, *aph(3′)-Ia, aph(3″)-Ib*), SXT (*dfrA12, dfrA14, sul2, sul3*), CHL (*catB3*), tetracycline (*tetA*) and lincomycin (*lnuF)*. This isolate proved to be phenotypically resistant to carbapenems in the original culture and was identified by targeted PCR as a carrier of $bla_{OXA\text{-}48}$ and $bla_{VIM\text{-}1}$ (Fig. 2A). However, the carbapenem-resistant phenotype changed to a susceptible phenotype after repeated sub-culturing, and no carbapenemase genes were detected in this isolate by WGS analysis (Table 3). Another *E. coli* ST361 isolate, SE_EC_79, had the same resistance profile and carried similar ARGs as isolate IZ 8–44. The *K. oxytoca* isolate (SE_COL_90) carried ARGs encoding resistance to AML ($bla_{TEM\text{-}1}$), CIP (*aac(6′)-Ib-cr*), GM (*aph(3′)-Ia, aac(6′)-Ib*), SXT (*dfrA12*), and CHL (*catB3*).

The genetic context of the β-lactamase genes, $bla_{TEM\text{-}1}$, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}1}$ was further analyzed using RAST annotations (Fig. 3). The $bla_{TEM\text{-}1}$ gene was observed to be flanked by Tn*3* resolvase in *E. coli* ST361 genomes (Fig. 3A) and additionally by IS91 transposase in *K. oxytoca* isolate (Fig. 3B). The $bla_{CTX\text{-}M\text{-}15}$ gene was surrounded by

Table 3
Results of whole-genome sequencing (WGS) analysis for ARGs and plasmid replicon types in sequenced isolates. A positive result is represented by a black square and a negative result is represented by a white square. ST = sequence type.

Isolate No.	Species	Resistance phenotype[1]	MLST	Phylogroup	Serotype	*ExPEC	β-lactamase genes			Other resistance genes										Plasmids				
							bla TEM-1	*bla* CTX-M-15	*bla* OXA-1	*aac(6′)-Ib-cr*	*aadA1*	*aph(3′)-Ia*	*aph(3″)-Ib*	*aph(6)-Id*	*dfrA12*	*dfrA14*	*sul2*	*sul3*	*catB3*	*tet(A)*	*lnu(F)*	Col156	IncFIB	IncHI2	IncHI2A	IncY
SE_EC_56	*E. coli*	AML, AMC, CL, CXM, CAZ, FEP, CIP	ST131	B2	025:H4	YES		■	■	■										■		■				■
SE_EC_79		AML, AMC, CL, CXM, CAZ, FEP, GM, SXT, CIP	ST361	A	09:H30	NO		■	■	■	■		■		■	■		■		■						■
IZ 8-44		AML, AMC, CL, CXM, CAZ, FEP, GM, SXT, CIP	ST361	A	09:H30	NO		■	■	■	■		■		■	■		■		■						■
SE_COL_90	*K. oxytoca*	AML, AMC, CL, CXM, CAZ, FEP, GM, SXT, CIP	ND	KO2	None[2]			■		■				■		■			■					■	

[1]Resistance phenotype: AML – amoxicillin, AMC – amoxicillin/clavulanic acid, CL – cephalexin, CXM – cefuroxime, CAZ – ceftazidime, FEP – cefepime, GM – gentamicin, SXT – trimethoprim/sulfamethoxazole, CIP – ciprofloxacin; [2]does not include serotype K/O; **ExPEC – positive for two or more virulence genes according to Johnson et al. (2005).

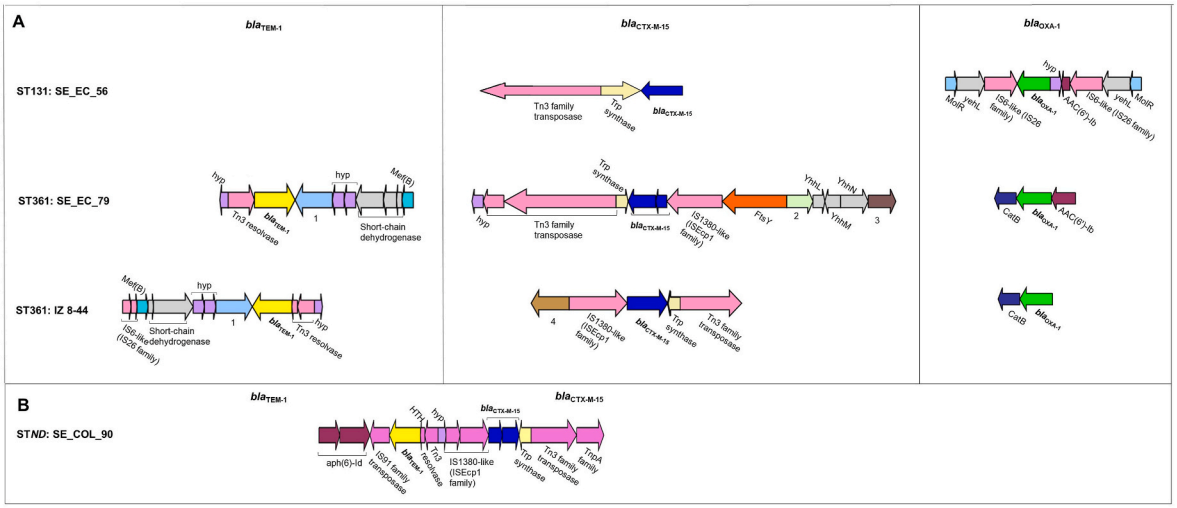

Fig. 3. Schematic representation of the genetic environment of β-lactamase genes (bla_{TEM-1}, $bla_{CTX-M-15}$ and bla_{OXA-1}) in isolates of *E. coli* (**A**) and *K. oxytoca* (**B**). Numbers (1–4) represent predicted regions for dihydropteorate synthase (1), 16S rRNA (guanine(966)-N-(2))-methyltransferase (2), copper-translocating P-type ATPase (3), and phosphatidylglycerol-membrane-oligosaccharide glycerophosphotransferase (4).

insertion sequences (IS*Ecp1* family) and/or transposases (Tn*3* family) in three *E. coli* isolates and *K. oxytoca* (Fig. 3A and B). The bla_{OXA-1} gene was surrounded by insertion sequences (IS6 family) and an aminoglycoside-fluoroquinolone resistance gene (*aac(6′)-Ib*) in the single *E. coli* isolate ST131, whereas this gene was associated with a CHL resistance gene (*catB*) and/or an aminoglycoside-fluoroquinolone gene (*aac(6′)-Ib*) in two ST361 isolates (Fig. 3A).

Finally, WGS analysis revealed that all sequenced isolates had multiple plasmids, with two plasmids in ST361 *E. coli* isolates (IncFIB and IncY) and *K. oxytoca* (IncHI2A and IncHI2) and three plasmids in ST131 *E. coli* (IncFIB, IncY and Col156) were identified (Table 3).

4. Discussion

Current AR surveillance of priority pathogenic enteric bacteria like 3GC-resistant *E. coli* or *K. pneumoniae* in Croatia and other areas focuses primarily on hospitals (ECDC, 2022). However, the role of treated wastewater as a potential pathway for the spread of these and other priority pathogens in the environment and community is much less explored. This study focused on treated wastewater from the Croatian capital to isolate and define the characteristics of ESBL-producing *E. coli* and other Enterobacterales, as they pose a particular challenge for treatment and their prevalence in Croatian hospitals has increased in recent years (ISKRA, 2021). Compared to 29 European countries, Croatia ranked ninth and sixth highest, respectively, in the prevalence of hospital isolates of 3GC-resistant *E. coli* and *K. pneumoniae* in 2021 (ECDC, 2022). Therefore, identifying potential sources of transmission of these resistant bacteria outside of hospitals is critical to support measures to control their further spread. In the present study, 140 ESBL-producing Enterobacterales isolates were successfully isolated from wastewater samples, including opportunistic pathogens such as *E. coli*, *E. cloacae* cplx., *K. pneumoniae* and *K. oxytoca*. Almost all of these isolates were MDR, as they showed resistance to ≥3 unrelated antibiotic classes. In addition, more than 40% of MDR *Enterobacter* and *Klebsiella* isolates were identified as COL-resistant, of which up to 22% were resistant to both carbapenems and COL, which is alarming in the absence of alternative antibiotics. Overall, these results suggest that treated wastewater is a source of opportunistic enteric pathogens that exhibit clinically significant AR phenotypes.

4.1. Genomic traits of ESBL-producing E. coli

By performing additional genomic analyses of *E. coli* wastewater isolates, we were able to determine their relatedness to previously identified *E. coli* clones and identify the genetic origins of their 3GC and carbapenem resistance, as well as assess their pathogenic potential and ability to spread ESBL genes. In our ESBL-producing *E. coli* isolates, β-lactam resistance was mainly mediated by CTX-M- and TEM-type ESBLs, especially CTX-M-15 and TEM-116, which frequently co-occur. Interestingly, CTX-M-15 is the predominant ESBL in *E. coli* isolated from patients in Croatia (Krilanović et al., 2020) and other countries (Ghenea et al., 2022; Paulitsch-Fuchs et al., 2022) and has also been found in *E. coli* isolated from wastewater (Grevskott et al., 2021; Mesquita et al., 2021). Plasmid-mediated AmpC enzymes, including EBC- and DHA-types, and carbapenemases, such as NDM-1 and OXA-48, are poorly represented in our *E. coli* wastewater isolates.

Molecular typing further revealed that majority of *E. coli* producing CTX-M-15 were associated with emerging clinically relevant clones (ST361 and ST1193) and to high-risk pandemic clones (ST131, ST405, ST410, ST38, and CC10), with ST361 and ST131 predominating (together accounting for 47% of all ESBL-positive *E. coli*). Using *in silico* serotyping and phylogroup determination, we demonstrated that the ST361 strains producing CTX-M-15 belong to the clonal group A-O9:H30 previously found in patients from China and Switzerland (Sadek et al., 2022; Huang et al., 2023). In addition to humans, ST361 has already been found in animals and in wastewater from chicken farms (Savin et al., 2020; Tsilipounidaki et al., 2021), but also in Croatian hospital wastewater entering the same WWTP analyzed here (Puljko et al., 2023). This suggests that the ST361 wastewater isolates in Croatia are probably of human origin. Although they carry different virulence markers, neither of the two isolates from the ST361 cluster was classified in the ExPEC group because they did not have enough virulence genes. This indicates that these *E. coli* isolates may not be capable of causing symptomatic infections, so they could be overlooked during hospital surveillance.

In addition, WGS analysis of these two *E. coli* ST361 revealed that they possessed IncFIB- and IncY-type plasmids, and the $bla_{CTX-M-15}$ gene in these isolates was flanked by Tn*3*-family transposase and IS*Ecp1* insertion sequence. This finding and previous studies reporting the spread of $bla_{CTX-M-15}$ through IncFIB and IncY plasmids (Saidani et al., 2019; Rocha-Gracia et al., 2022) suggest that the acquisition of $bla_{CTX-M-15}$ in the ST361 lineage is mediated by plasmids. Our targeted PCR and WGS data also showed that ST361 has more ARGs encoding resistance to β-lactams, including carbapenems (bla_{OXA-1}, bla_{TEM-1}, bla_{OXA-48}, and bla_{VIM-1}), aminoglycosides (*aadA1*, *aph(3′)-Ia, aph(3″)-Ib,* and *aac(6′)-lb-cr*), fluoroquinolones (*aac(6′)-lb-cr*), trimethoprim

(*dfrA12, dfrA14*), sulfonamides (*sul2, sul3)*, chloramphenicol (*catB*), tetracyclines (*tetA*), and lincomycin (*lnuF)*. However, the discrepancy in the identification of carbapenemase genes by PCR and WGS could be due to the loss of plasmid(s) after successive subcultures of ST361 *E. coli* isolate (no carbapenemase genes predicted by WGS) compared to the original culture ($bla_{\text{OXA-48}}$ and $bla_{\text{VIM-1}}$ detected by PCR). This was supported by the change in phenotype of this isolate, which went from carbapenem-resistant to susceptible after subculture. To the best of our knowledge, the carbapenemases OXA-48 and VIM-1 have never been found in *E. coli* ST361, but another carbapenemase, NDM-5, was recently found in *E. coli* ST361 from German hospitals (Hans et al., 2023). The discovery of an XDR clone within the ST361 clone group that produces carbapenemase suggests that this clone has acquired resistance genes to last-line antibiotics, which will worsen treatment options for infections.

The second most abundant clonal group among our *E. coli* isolates is attributed to the high-risk lineage ST131, which is associated with phylogroup B2 and serotype O25:H4 and has been classified as an ExPEC strain due to the presence of genes associated with extraintestinal infections, including *papC* and *iutA* (Johnson et al., 2003). The *E. coli* ST131 is a highly virulent clone widely distributed worldwide (Kocsis et al., 2022). Some studies in Croatia also reported MDR ST131 containing CTX-M-15 in hospitals and nursing homes (Literacka et al., 2009; Krilanović et al., 2020) and hospital wastewater entering the same Zagreb WWTP analyzed here (Puljko et al., 2023), suggesting that the *E. coli* ST131 from our wastewater samples likely originated from human sources. A recent study from the Czech Republic also reported a frequent occurrence of ExPEC MDR ST131 clones containing CTX-M-15 in wastewater from hospitals and urban WWTPs (Davidova-Gerzova et al., 2023), suggesting that these pathogenic clones could spread via wastewater to downstream water bodies. In this work, the mobility potential of the $bla_{\text{CTX-M-15}}$ gene in the isolate of ST131 was supported by the association of this gene with the Tn*3* transposase and with IncFIB- and IncY-type plasmid replicons previously reported to spread this gene (Saidani et al., 2019; Rocha-Gracia et al., 2022). Moreover, the $bla_{\text{OXA-1}}$ and *aac(6')-lb* genes were found in this ST131 surrounded by two IS26, as previously reported for this clone isolated from patients (Livermore et al., 2019). Of note, 2% of *E. coli* isolates from our wastewater were associated with ST1193 (a sister clone of ST131), which has been described as a recent emerging pandemic MDR clone isolated from hospitals in different countries (Kocsis et al., 2022), and less frequently from wastewater/environmental water samples (Biggel et al., 2021; Grevskott et al., 2021).

In addition to ST131, most of the other MDR *E. coli* isolates in the present study were also associated with high-risk clones such as ST405 (3%), ST410 (3%), ST38 (2%) and CC10 (3%) (Kocsis et al., 2022). All these clones are ESBL producers carrying CTX-M-15 type ESBL, except for ST410, which only carries TEM-116. Moreover, these specific STs of *E. coli* have been discovered to cause disease in humans in many countries (Manges et al., 2019). Some of these STs were also previously detected in treated municipal wastewater (ST410, ST38) (Tavares et al., 2020; Grevskott et al., 2021) and hospital wastewater, including the one from Zagreb (ST405) (Puljko et al., 2023). Several STs, including CC10, ST410 and ST405 have been found in animals, food and environmental waters, suggesting a facilitated dissemination across different niches (Raven et al., 2019; Hooban et al., 2022; Zurita et al., 2020).

While most of the *E. coli* found in our wastewater samples were identified as high-risk clones occurring in human infections, some less frequently detected STs such as ST536, ST635, ST3356, ST6329 and ST12913 were not reported as clinically relevant; most of them were only found in the Enterobase database. However, ST635 is considered a highly adapted wastewater-related lineage enriched with resistance and virulence genes, as it is detected in hospital sinks, hospital wastewater and chlorine-treated wastewater and frequently carries ESBL and carbapenemase genes (Zhi et al., 2019; Constantinides et al., 2020; Carlsen et al., 2022).

4.2. Genomic features of ESBL-producing Klebsiella spp.

XDR and MDR *Klebsiella* species, particularly *K. pneumoniae* and *K. oxytoca*, are dangerous bacteria that can cause deadly infections and are often resistant to antibiotic treatment (Martin and Bachman, 2018; Stewart et al., 2022). Analysis of the molecular mechanisms conferring resistance to β-lactams revealed that resistance to cephalosporins in our *Klebsiella* spp. is due to the presence of various CTX-M- and TEM-type ESBLs, mainly CTX-M-15 and TEM-116. In addition, most *Klebsiella* isolates carried both of these genes and some of them in combination with other ESBLs, including the SHV (i.e. SHV-12 and SHV-28) and GES types (i.e. GES-7) as well as the EBC type pAmpC. Other studies also reported a frequent co-occurrence of $bla_{\text{CTX-M-15}}$ with bla_{TEM} and bla_{SHV} genes in *Klebsiella* species isolated from patients in Croatia (Bedenić et al., 2022), but also from hospital wastewater entering the WWTP studied here (Puljko et al., 2023), and on Croatian marine beaches (Maravić et al., 2015). This indicates a probable spread of these species in the environment as well. Furthermore, additional, rather rare β-lactamase genes such as $bla_{\text{GES-7}}$ and bla_{LEN} were found in our isolates, which were not previously found in *Klebsiella* isolates in Croatia. The $bla_{\text{GES-7}}$ gene is usually associated with ESBL activity (Naas et al., 2008) and was previously found in *K. oxytoca* from a hospital in the Czech Republic (Finianos et al., 2022), while the chromosomally encoded bla_{LEN} gene was found as an intrinsic resistance mechanism against narrow-spectrum β-lactams in *K. variicola* (Di et al., 2017).

The *K. oxytoca* which produces three ESBLs, CTX-M-15, TEM-116 and GES-7, was shown by WGS to harbour ARGs for several highest priority antibiotics, including aminoglycosides (*aac(6′)- lb-cr, aph(3″)-la*, and *aph(6)-ld* genes) and fluoroquinolones (*aac(6')- lb-cr*), as well as highly important antibiotics trimethoprim (*dfrA14)* and chloramphenicol (*catB3*). This isolate had the plasmid replicons IncHI2 and IncHI2A, which might be connected to the transfer of ARGs for β-lactams and other antibiotic classes, as previously documented (Pot et al., 2021). Additionally, $bla_{\text{CTX-M-15}}$ in the *K. oxytoca* isolate was surrounded by a Tn*3* transposon and an IS*Ecp1*, which facilitated its mobilization and dissemination, as previously reported (Zhao and Hu, 2013).

Resistance of *K. oxytoca* and *K. pneumoniae* isolates to carbapenems was caused by the carbapenemase genes $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$, respectively, which are also frequently present in *K. pneumoniae* from Croatian hospitals (Bedenić et al., 2022). In addition, the NDM-1-producing *K. oxytoca* isolate (SE_COL_19) proved resistant to all antibiotics tested, including COL, one of the last available antibiotics. However, the isolates resistant to COL did not contain tested *mcr* genes. Therefore, it is likely that they possess a new variation of the *mcr* gene or point mutations in chromosomal genes regulating the lipopolysaccharide in the cytoplasmic membrane known to confer COL resistance (Wang et al., 2021). Given that more than 30% of *K. pneumoniae* from hospitalized patients in Croatia were resistant to carbapenems in 2021 (ECDC, 2022), surveillance of AR *Klebsiella* spp. outside hospitals is essential to monitor and control the rise and spread of resistance rates.

4.3. Genomic characterization of ESBL-producing E. cloacae cplx

Along with *E. coli* and *Klebsiella* spp., Enterobacterales recovered from our treated municipal wastewater samples included ESBL-producing bacteria of the *E. cloacae* cplx. which have become an emerging public health threat. In our ESBL-producing *E. cloacae* cplx. isolates, resistance to 3GCs was primarily caused by various ESBL (i.e., TEM-116, CTX-M-15), pAmpC (i.e., EBC-type), and/or carbapenemase (i.e., GES-5, VIM-1) enzymes. The observed carbapenem resistance in some *Enterobacter* isolates in the present study could be due to the production of pAmpC in combination with ESBLs or loss of porins, as previously reported (Black et al., 2021). In addition, resistance to carbapenems may be caused by the presence of GES-5- and VIM-1-type carbapenemases in two *E. cloacae* isolates (SE_COL_136, and SE_COL_56). However, this resistance, which is phenotypically

manifested mainly against ertapenem, can be explained by the good ertapenem but weaker meropenem hydrolytic activity of GES-5 and VIM-1, as previously documented (Falcone et al., 2009; Ellington et al., 2020). Therefore, the isolation of priority pathogens such as *E. cloacae* cplx. with ARGs against clinically relevant antibiotics from treated municipal wastewater suggests that it may be an additional reservoir in which these bacteria can exist outside medical facilities, leading to their dissemination in the environment and possible reintroduction into society.

5. Conclusion

Clinically relevant MDR and ESBL-producing enteric pathogens are retained in the treated wastewater of Croatia's largest WWTP and discharged into the nearby river. Of particular concern is the transmission risk of ESBL-producing *E. coli*, especially in the context of pandemic high-risk clones and emerging clinically relevant clones, which are mainly associated with the ESBLs CTX-M-15 and TEM-116. Certain *E. coli* isolates are highly virulent and have also been shown to carry transmissible ESBL genes. Genomic analyses also revealed clinically relevant resistance mechanisms to 3GCs and carbapenems in *Klebsiella* and *E. cloacae* cplx. isolates, which could serve as reservoirs for transmissible ARGs of clinical importance. Our results suggest that wastewater treatment needs to be improved to efficiently monitor and control the transmission of priority MDR pathogens in the population.

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CRediT authorship contribution statement

Ana Puljko: Investigation, Visualization, Writing - original draft, Writing - review & editing. **Ivana Babić:** Visualization, Writing - original draft. **Svjetlana Dekić Rozman:** Investigation. **Ivan Barišić:** Conceptualization, Formal analysis, Methodology, Resources, Writing - review & editing. **Marko Jelić:** Formal analysis, Investigation, Visualization, Writing - review & editing. **Ana Maravić:** Investigation, Writing - review & editing. **Marija Parać:** Investigation. **Ines Petrić:** Writing - review & editing. **Nikolina Udiković-Kolić:** Conceptualization, Data curation, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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Chapter 4

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Molecular epidemiology and mechanisms of carbapenem and colistin resistance in *Klebsiella* and other Enterobacterales from treated wastewater in Croatia

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ABSTRACT

Among the most problematic bacteria with clinical relevance are the carbapenem-resistant Enterobacterales (CRE), as there are very limited options for their treatment. Treated wastewater can be a route for the release of these bacteria into the environment and the population. The aim of this study was to isolate CRE from treated wastewater from the Zagreb wastewater treatment plant and to determine their phenotypic and genomic characteristics. A total of 200 suspected CRE were isolated, 148 of which were confirmed as Enterobacterales by MALDI-TOF MS. The predominant species was *Klebsiella* spp. (n = 47), followed by *Citrobacter* spp. (n = 40) and *Enterobacter cloacae* complex (cplx.) (n = 35). All 148 isolates were carbapenemase producers with a multidrug-resistant phenotype. Using multi-locus sequence typing and whole-genome sequencing (WGS), 18 different sequence types were identified among these isolates, 14 of which were associated with human-associated clones. The virulence gene analysis of the sequenced *Klebsiella* isolates (n = 7) revealed their potential pathogenicity. PCR and WGS showed that the most frequent carbapenemase genes in *K. pneumoniae* were $bla_{\text{OXA-48}}$ and $bla_{\text{NDM-1}}$, which frequently occurred together, while $bla_{\text{KPC-2}}$ together with $bla_{\text{NDM-1}}$ was mainly detected in *K. oxytoca*, *E. cloacae* cplx. and *Citrobacter* spp. Colistin resistance was observed in 40% of *Klebsiella* and 57% of *Enterobacter* isolates. Underlying mechanisms identified by WGS include known and potentially novel intrinsic mechanisms (point mutations in the *pmrA/B*, *phoP/Q*, *mgrB* and *crrB* genes) and acquired mechanisms (*mcr-4.3* gene). The *mcr-4.3* gene was identified for the first time in *K. pneumoniae* and is probably located on the conjugative IncHI1B plasmid. In addition, WGS analysis of 13 isolates revealed various virulence genes and resistance genes to other clinically relevant antibiotics as well as different plasmids possibly associated with carbapenemase genes. Our study demonstrates the important role that treated municipal wastewater plays in harboring and spreading enterobacterial pathogens that are resistant to last-resort antibiotics.

1. Introduction

Antimicrobial resistance (AMR) is a multifaceted problem in which the relationships between humans, animals and the environment are closely intertwined. It is therefore essential to fully understand all potential transmission routes of AMR. In addition to surveillance of AMR in clinical settings, wastewater-based surveillance is considered a complementary approach to assess AMR in non-clinical isolates and to assess environmental exposure to potentially hazardous antibiotic-resistant bacteria (ARB) and their resistance genes (Tiwari et al., 2022).

According to the World Health Organization (WHO), Enterobacterales resistant to last-resort antibiotics such as carbapenems, are among the most challenging bacteria to treat in clinics (WHO, 2017). Resistance to carbapenems is often associated with enzymatic

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production of carbapenemases that can hydrolyze most carbapenems and other β-lactam antibiotics (Poirel et al., 2007). This resistance often occurs in combination with resistance to other classes of antibiotics, which limits treatment options and increases mortality (Budhram et al., 2020). In the last decade, the clinically relevant carbapenemases KPC, OXA-48, NDM, VIM and IMP have spread worldwide (Bonomo et al., 2018). In addition, genes encoding carbapenemases are often located on plasmids that can be spread between different species (Ramsamy et al., 2022). In addition to carbapenemases, carbapenem resistance in Enterobacterales is also associated with a reduction in outer membrane permeability in the presence/absence of extended-spectrum ß-lactamase (ESBL) and/or AmpC enzyme production (Barišić et al., 2014; Martínez-Martínez, 2008). The antibiotic colistin is one of the few therapeutic options for severe infections caused by carbapenem-resistant Enterobacterales (CRE), making colistin resistance a serious public health problem (Binsker et al., 2022). Resistance to colistin in Enterobacterales is attributed to mutations in certain chromosomal genes (*pmrA/B*, *phoP/Q*, *mgrB* and/or *crrB*) or to the acquisition of the plasmid-mediated *mcr* genes (Gogry et al., 2021). The detection of isolates carrying carbapenem and colistin resistance genes is therefore of particular importance, as there are only a few therapeutic options left.

In Europe, an increase in the prevalence of CRE, particularly carbapenem-resistant *Klebsiella pneumoniae*, has been observed, with prevalence increasing by almost 50% in the last four years. During the same period, the prevalence of carbapenem resistance in *K. pneumoniae* in Croatia, which causes bloodstream infections, increased by 110% (ECDC, 2023a). Remarkably, Croatia is among the six EU countries with the highest prevalence of carbapenem-resistant *K. pneumoniae* in clinical settings from 2020 to 2022 (ECDC, 2023b). This is in line with the high carbapenem use in the hospital and community sector in Croatia compared to other EU countries in the same three-year period (ECDC, 2023c). Therefore, understanding the factors that favor the spread of CRE, especially *Klebsiella* spp. outside of hospitals, is crucial to prevent their further spread.

Outside of hospitals, wastewater treatment plants (WWTPs) are reported to contribute to the emergence and spread of AMR (Karkman et al., 2018; Marano et al., 2020). Studies have shown the presence of CRE and associated antibiotic-resistance genes (ARGs) in wastewater after treatment in WWTPs (Marutescu et al., 2023). Our recent study in the same WWTP that we analyzed here showed an enrichment of some carbapenemase genes (bla_{VIM} and bla_{IMP}) in the treated wastewater compared to the raw wastewater (Puljko et al., 2022). In addition, the survival of carbapenemase-producing *K. pneumoniae* in the river into which the wastewater is discharged has also been found in Croatia (Jelić et al., 2019) and other countries (Kehl et al., 2022; Lepuschitz et al., 2019). Consequently, treated wastewater could serve as important monitoring sites for environmental exposure to hazardous ARB such as CRE and their mobile carbapenemase genes, with the risk of these being reintroduced into the community (Larsson et al., 2023). However, there is little information on the occurrence and detailed characteristics of CRE in treated municipal wastewater, especially on their epidemiology and genomic characteristics (Gomi et al., 2018; Hoffmann et al., 2023; Zurfluh et al., 2017). In accordance, this work aimed to isolate and identify CRE from treated wastewater and characterize them in terms of their epidemiology, phenotypic AMR and genetic mechanisms underlying resistance to carbapenems. A subset of isolates (n = 13), including isolates with concomitant carbapenem and colistin resistance phenotype (n = 8), was selected for whole-genome sequencing (WGS) to investigate their molecular epidemiology and the presence of ARGs, virulence genes and plasmid replicon types.

2. Materials and methods

2.1. Collection of treated wastewater, isolation and identification of suspected CRE

Treated wastewater samples from the Zagreb WWTP were collected as described in Puljko et al. (2022). Samples were then serially diluted (1:10 to 1:100,000) with 0.85% sterile NaCl, and each diluted sample was filtered through a filter membrane (47 mm diameter, 0.22 µm pore size, Whatman, GE Healthcare, Life Science, USA). For isolation of CRE, filters were aseptically transferred to CHROMAgar mSuperCARBA (CHROMAgar, France) plates and incubated for 24 h at 37 °C. This agar was selected because it can detect OXA-48 producers in addition to other carbapenemase producers (KPC, VIM, NDM and IMP). The suspected CRE colonies (n = 200), which appeared red for *Escherichia coli* and metallic blue for *Klebsiella, Citrobacter* and *Enterobacter*, were subcultured on the same medium to purity and stored at −80 °C in 20% glycerol for further analysis (Puljko et al., 2023).

The isolates were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) at the Ruđer Bošković Institute, as described in Puljko et al. (2023). Three isolates for which MALDI-TOF MS analysis was not successful (two *Enterobacter* and one *Citrobacter*; Table S1) were sent to Macrogen (Amsterdam, Netherlands) for sequencing of the 16S rRNA gene (Puljko et al., 2023).

2.2. Confirmation of carbapenemase production and AMR phenotypes

Isolates identified as Enterobacterales (n = 148) were tested for carbapenemase production using the CarbaNP test (Nordmann et al., 2012). All Enterobacterales isolates were screened for susceptibility to 12 different antibiotics using a Kirby-Bauer disk diffusion test according to the EUCAST (2020) guidelines. Antibiotics screened included amoxicillin (AML) amoxicillin/clavulanic acid (AMC), cephalexin (CL), cefuroxime (CXM), ceftazidime (CAZ), cefepime (FEP), ertapenem (ETP), imipenem (IPM), meropenem (MEM), gentamicin (GM), trimethoprim/sulfamethoxazole (SXT), and ciprofloxacin (CIP). Isolates identified as carbapenem-resistant by the disk diffusion method were additionally tested for their resistance to carbapenems (IPM, ETP and MEM) using the broth microdilution method according to EUCAST (2020) guidelines. In addition, the minimum inhibitory concentration (MIC) of colistin (COL) was determined for all isolates using the same broth microdilution protocol and EUCAST MIC interpretation.

Based on the observed AMR phenotypes and the criteria of Magiorakos et al. (2012), all Enterobacterales isolates were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR).

2.3. Molecular characterization of carbapenemase genes

Enterobacterales isolates were grouped according to their species and phenotypic AMR profiles. Representatives from each group were selected and tested for ARGs by PCR, with the exception of *Klebsiella* isolates, which were all tested by PCR. Briefly, isolates were revived from pure frozen stocks and grown in Luria-Bertani broth (LB) containing IPM (4 mg/L) at 37 °C with agitation (130 rpm). Genomic DNA was extracted from overnight culture using the Quick-DNA™ Miniprep Plus Kit (Zymo, USA). All isolates were screened for carbapenemase genes (bla_{KPC}, bla_{OXA-48}, bla_{NDM}, bla_{VIM} and bla_{IMP}) using the primers and conditions in Table S2. COL-resistant isolates were screened for plasmid-mediated COL resistance genes (*mrc-1-mcr-5*) by multiplex PCR (Table S2). PCR products were Sanger sequenced in forward direction (Macrogen, The Netherlands) and compared with sequences in the NCBI database using BLASTX search after editing.

2.4. Pulsed-field gel electrophoresis (PFGE) analysis and Multi-locus sequence typing (MLST)

E. coli, *Klebsiella* spp. and *Enterobacter cloacae* complex (cplx.) underwent standardized *Xba*I PFGE analysis, as described by Jelic et al. (2016). The dendrogram was constructed using BioNumerics (Applied Maths, Belgium) using the UPGMA and DICE similarity coefficient, and cut-off value of $\geq$ 85%. MLST analysis was performed by IDgenomics service (Seattle, USA) for representative isolates from clusters whose sequence type (ST) was not predicted by WGS (see below).

2.5. WGS, assembly and sequence data analysis

Thirteen isolates (1 *E. coli*, 7 *Klebsiella* spp., 3 *E. cloacae* cplx., and 2 *Citrobacter* spp.) were selected for WGS analysis. The isolates were selected based on their affiliation to the dominant PFGE clusters or the presence or absence of carbapenemase genes. Selected isolates were revived from glycerol stock onto LB plates with IPM (4 mg/L), and a single overnight grown colony was used to isolate genomic DNA using the Quick-DNA^{TM} Miniprep Plus Kit (Zymo, USA) according to the manufacturers recommendations. Sequencing was performed using the Ion Torrent PGM instrument as described in Puljko et al. (2023). SPAdes v.3.15.0 was used to assemble quality-filtered reads with the default parameters, and genomes were annotated with RAST (v.2.0).

2.6. Bioinformatic and phylogenetic analysis

ARGs were found in the assembled genomes and verified using raw reads with ResFinder 4.4.2. (Bortolaia et al., 2020). Plasmid replicon types were identified in the assembled genomes using PlasmidFinder 2.1 (Carattoli et al., 2014). For the *Klebsiella* spp. genomes, prediction of the capsular (K) and lipopolisacharide (LPS) O antigen locus and hypervirulence genes were done by Kleborate (v.2.2.0.) and Kaptive 2.0 tools implemented in Pathogenwatch (Argimón et al., 2021; Lam et al., 2021; 2022). For genomes that were differentially identified by MALDI-TOF MS and WGS, the average nucleotide identity (ANI) values (Goris et al., 2007) were calculated on the basis of a comparison between the genomes tested and the reference genomes using the online service (https://enve-omics.ce.gatech.edu/ani/). The $\geq$ 95% identity criteria were used to identify the species, and four reference genomes were used for comparison: *K. pneumoniae* HS11286 (GenBank accession number: NC_016845.1, *K. quasipneumoniae* subsp. *similipneumoniae* ATCC 700603 (GenBank accession number: NZ_CP014696.2), *K. michiganensis* THO-011 (GenBank accession number: NZ_AP022547.1) and *C. portucalensis* (GenBank accession number: CP044098.1). SerotypeFinder 2.0 and ClermonTyping were used to identify the serotype and phylogroup of *E. coli*, respectively (Beghain et al., 2018; Joensen et al., 2015). The STs of the genomes were identified using the MLST tool 2.0 (Larsen et al., 2012), and the virulence genes were identified using the Virulence Factor Database (VFDB, Chen et al., 2016). The VFanalyzer within the VFDB was used to search for virulence genes in *E. coli* and *Klebsiella* spp. isolates by comparing their nucleotide sequences with the reference sequences (*E. coli* str. K-12 substr. MG1655 and *K. pneumoniae* subsp. *pneumonia* MGH 78578). The virulence genes of *E. cloacae* and *Citrobacter* isolates were predicted based on the alignment of their nucleotide sequences with the nucleotide sequences of VFDB (set B). Nearly identical matches were filtered out based on bitscore values above 90, E-values below $10e^{-5}$, and sequence identity above 90%.

The relationship of the *mcr* gene of *K. pneumoniae* in this study to other *mcr* variants (*mcr-1-mcr-10*) from the NCBI database was analysed by constructing a phylogenetic tree. The tree was inferred using the maximum likelihood method with the LG model and gamma distribution. Bootstrap analysis was performed with 1,000 replicates. The tree was generated using the software MEGA7 (Kumar et al., 2016) and visualised using the online tool iTol (Letunic and Bork, 2016).

The contig containing the *mcr* gene was annotated with RAST and BLASTX. In addition, the entire contig was aligned with the NCBI nucleotide database to find closely related sequences. Visualization was done with Easyfig 2.2.5 (Sullivan et al., 2011).

Mutations in genes conferring COL resistance in sequenced *Klebsiella* and *E. cloacae* cplx. isolates (*pmrA, pmrB, phoP, phoQ, mgrB,* and/or *crrB*) were predicted by alignment of their nucleotide and amino acid sequences with reference sequences in the NCBI database. Reference genomes included wild-type *K. pneumoniae* HS11286 (GenBank accession number: NC_016845.1), *K. oxytoca* KONIH1 (GenBank accession number: CP008788.1), *K. quasipneumoniae* ATCC 700603 (GenBank accession number: NZ_CP014696.2) and *E. cloacae* ATCC13047 (GenBank accession number: CP001918). The effects of the observed mutations (neutral or deleterious) on the functionality of the proteins encoded by COL ARGs were assessed using the PROVEAN tool.

2.7. Data availability

The sequences of the ARGs have been submitted to GenBank under the following accession numbers: bla_{KPC-2} (OR903110-OR903146), bla_{VIM-1} (OR948827-OR948440), bla_{IMP-13} (OR948841), bla_{NDM-1} (PP024889-PP024923), bla_{OXA-48} (PP024935-PP024951) and *mcr-4.3* (OR948842-OR94884). The WGS data have been deposited under the BioProject PRJNA913323 with the BioSample accession numbers: SAMN38849958 (SE_SC_COL_47 *K. pneumoniae*), SAMN34152686 (SE_SC_COL_72 *K. pneumoniae*), SAMN34152684 (SE_SC_COL_96 *K. quasipneumoniae*), SAMN34152685 (SE_SC_COL_102 *K. quasipneumoniae*), SAMN34152690 (SE_SC_COL_103 *K. quasipneumoniae*), SAMN34152683 (SE_SC_COL_148 *K. quasipneumoniae*), SAMN34152689 (SE_SC_COL_140 *K. michiganensis*), SAMN34152691 (SE_SC_COL_159 *E. asburiae*), SAMN34152692 (SE_SC_COL_198 *E. asburiae*), SAMN34152693 (SE_SC_COL_80 *E. cloacae*), SAMN34152682 (SE_SC_COL_21 *E. coli*), SAMN34152695 (SE_SC_COL_83 *C. farmeri*), and SAMN34152694 (SE_SC_COL_124 *C. portucalensis*).

3. Results

3.1. Identification of Enterobacterales isolates

A total of 200 suspected CRE were isolated from treated wastewater samples of the Zagreb WWTP, of which 148 isolates were identified as Enterobacterales species (Fig. 1). The remaining 52 isolates were identified mainly as *Aeromonas* spp. (n = 45) and *Enterococcus* spp. (n = 7) (Fig. 1). Of the 8 Enterobacterales genera identified, the most frequently found genus was *Klebsiella* spp. (n = 47; 32%), consisting of 36 *K. pneumoniae* and 11 *K. oxytoca* isolates. The second and third most common genera were *Citrobacter* spp. (n = 40; 27%) and *Enterobacter cloacae* cplx. (n = 35; 24%). Species such as *Raoultella* spp. (n = 12), *E. coli* (n = 7), *Kluyvera cryocrescens* (n = 5), *Serratia marcescens* (n = 1) and *Leclercia adecarboxylata* (n = 1) together accounted for 18% of the identified CRE species.

3.2. Carbapenemase production and AMR profiles of isolates

The CarbaNP assay confirmed that all 148 CRE isolates produced carbapenemase enzymes. The phenotypic resistance of these isolates to nine clinically relevant antibiotic classes was determined by Kirby-Bauer and broth microdilution methods (Fig. 2, Table S1). The resistance profile to all β-lactam antibiotics tested was similar in each of the 8 identified genera. For instance, all isolates were found to be resistant to AML (penicillins), AMC (penicillin + β-lactamase inhibitor), CL, CXM, and CAZ (1st, 2nd, and 3rd generation cephalosporins, respectively), and ETP and IPM (carbapenems) (Fig. 2). Intermediate resistance profiles to FEP (4th generation cephalosporin) were observed in *Klebsiella*, *Citrobacter, E. cloacae* cplx, *E. coli,* and *Roultella* isolates. In addition, resistance to MEM (carbapenem) was observed in all isolates except a

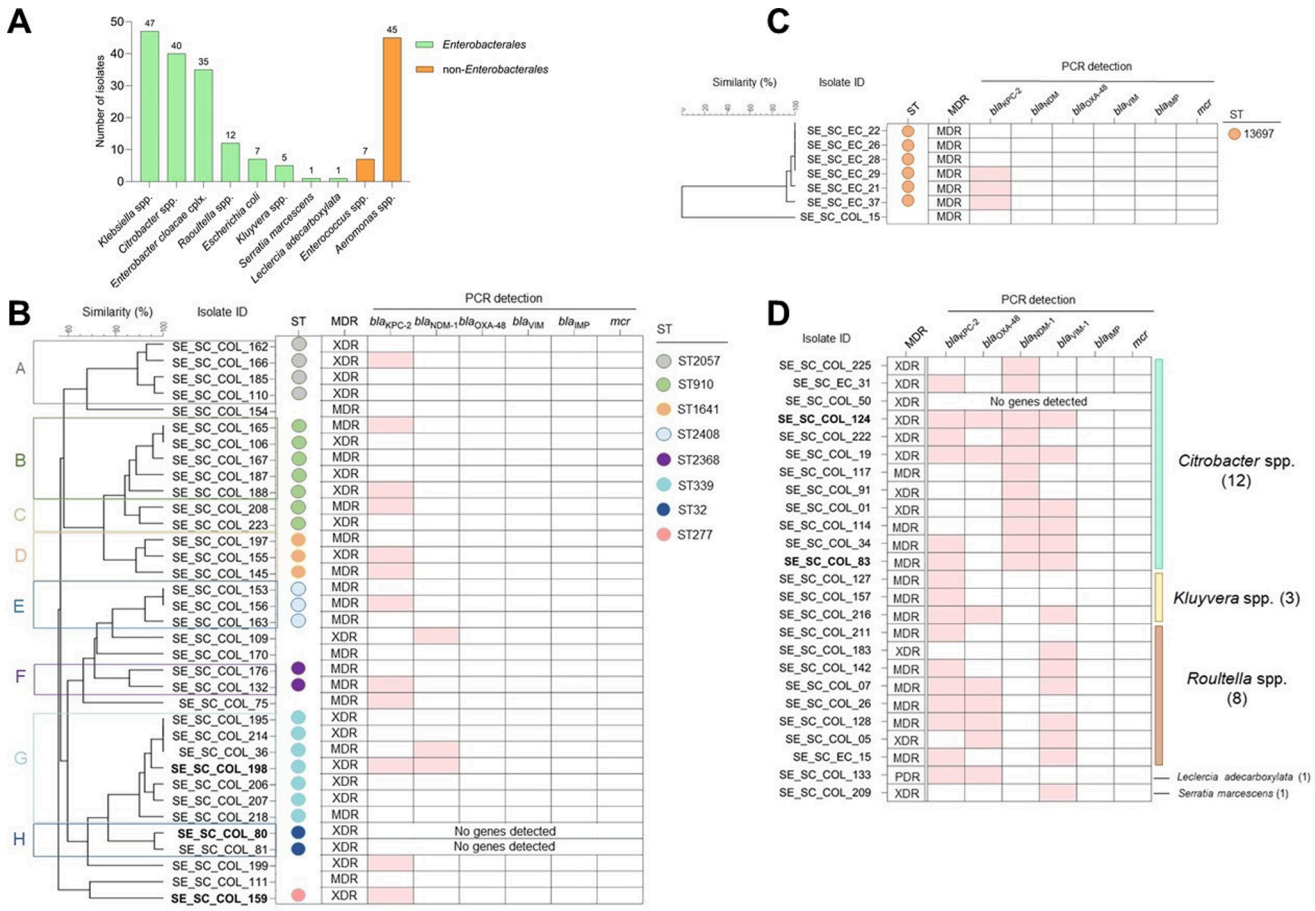

Fig. 1. Identification of 200 suspected CRE species from the treated wastewater samples (A) and cluster analysis based on *Xba*I-PFGE of carbapenem-resistant (B) *E. cloacae* cplx. isolates and (C) *E. coli*. The letters A to H in the dendrogram indicate the identified primary clusters. The coloured circles refer to different sequence types (ST). The light red coloured row in the table indicates the presence of the carbapenemase or colistin genes. (D) Table showing the presence (light red) or absence (white) of genes encoding carbapenemases or colistin resistance in a subset of CRE isolates (*Citrobacter, Kluyvera*, *Roultella, Leclercia,* and *Serratia*). MDR – multidrug-resistant, XDR – extensively drug-resistant, PDR – pandrug-resistant. Isolates subjected to whole-genome sequencing are shown in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

small percentage of *Citrobacter* and *E. cloacae* cplx. (2% and 6%, respectively). Regarding resistance to non-β-lactam antibiotics, most isolates (>65% of each genus) were resistant to CIP (fluoroquinolones). The highest resistance to GM (aminoglycosides) and SXT (trimethoprim/sulfamethoxazole) was observed in *Klebsiella* (51% and 23%, respectively) and *Citrobacter* isolates (53% and 50%, respectively). In addition, the percentage of isolates resistant to COL (i.e. MIC > 2 μg/mL) was generally low, except for *Klebsiella* spp. (40%; MIC = 4–32 μg/mL) and *E. cloacae* cplx. (52%, the majority of which had an MIC > 64 μg/mL) (Fig. 2; Table S1). All 148 CRE were identified as MDR, with more than 50% classified as XDR, and 4 isolates (3 *K. pneumoniae* and one *L. adecarboxylacara*) were PDR (Table S1).

3.3. Genome and phylogenetic analyses of *E. cloacae* cplx. isolates

Phylogenetic typing revealed that 28/35 *E. cloacae* cplx. isolates could be assigned to one of 8 clusters (A-H) belonging to 8 known STs (Fig. 1B). MLST and WGS revealed that ST339 (cluster G) was the most prevalent, followed by ST910, ST2057, ST1641, ST2408, ST2368, and ST32. One nonclustered isolate was identified as ST277 (Fig. 1B). Most isolates associated with these STs carried the carbapenemase gene bla_{KPC-2}, except ST32, in which no tested carbapenemase genes were found, and ST339, which carried bla_{KPC-2} and bla_{NDM-1} simultaneously.

Three *E. cloacae* cplx. isolates (ST32, ST339 and ST277) were subjected to WGS analysis (Fig. 4). The presence of bla_{KPC-2} was confirmed by WGS in ST339 and ST277, as was the absence of known carbapenemase genes in ST32. In addition, other clinically important ARGs were found in sequenced isolates, including ESBLs such as bla_{OXA-1} (in ST277), $bla_{OXA-256}$ (in ST339), and bla_{OXA-10} (in ST32), as well as ARGs against aminoglycosides (*aac(6′)-lb-cr*, *aac(3)-I, aph(6)-Id,* and *aph (3″)-lb*), rifampicin (*aar-3*), chloramphenicol (*catB3*), and antiseptics (*qacE/qacH*) (Fig. 4). Although all three sequenced isolates were phenotypically resistant to COL, no plasmid-mediated *mcr* genes were found in their genomes. However, multiple point mutations were found in the genes corresponding to the PmrA/B, PhoP/Q and/or MgrB proteins, including deleterious (P147A, E218G, and N174S) and neutral mutations (Fig. 4, Table S5). All sequenced *E. cloacae* cplx. isolates contained between 11 and 22 different virulence genes (*fim*, *iutA, fep*, among other genes) (Table S6). PlasmidFinder identified 1 (IncP6), 3 (Col4401, IncFII, and IncR), and 7 plasmid replicons (Col(pHAD28), IncC, IncFIB, IncFII IncHI2, IncP6, and IncX5 types) in *E. cloacae* ST339, *E. asburiae* ST277, and *E. cloacae* ST32, respectively (Fig. 4).

3.4. Genetic characteristics of *E. coli* and other Enterobacterales isolates

Six of the seven MDR *E. coli* isolates formed a cluster belonging to ST13697 and phylogroup A with serotype O154:H4 (Fig. 1C and 4). The carbapenemase type KPC-2 was identified by PCR in isolates from this cluster (Fig. 1C). In addition, WGS identified the ß-lactamase type TEM-1 and the AmpC ß-lactamase type EC as well as the fluoroquinolone resistance gene *qnrS1* in sequenced *E. coli* ST13697. Two plasmid replicon types detected in this sequenced *E. coli* were Col156 and IncP6

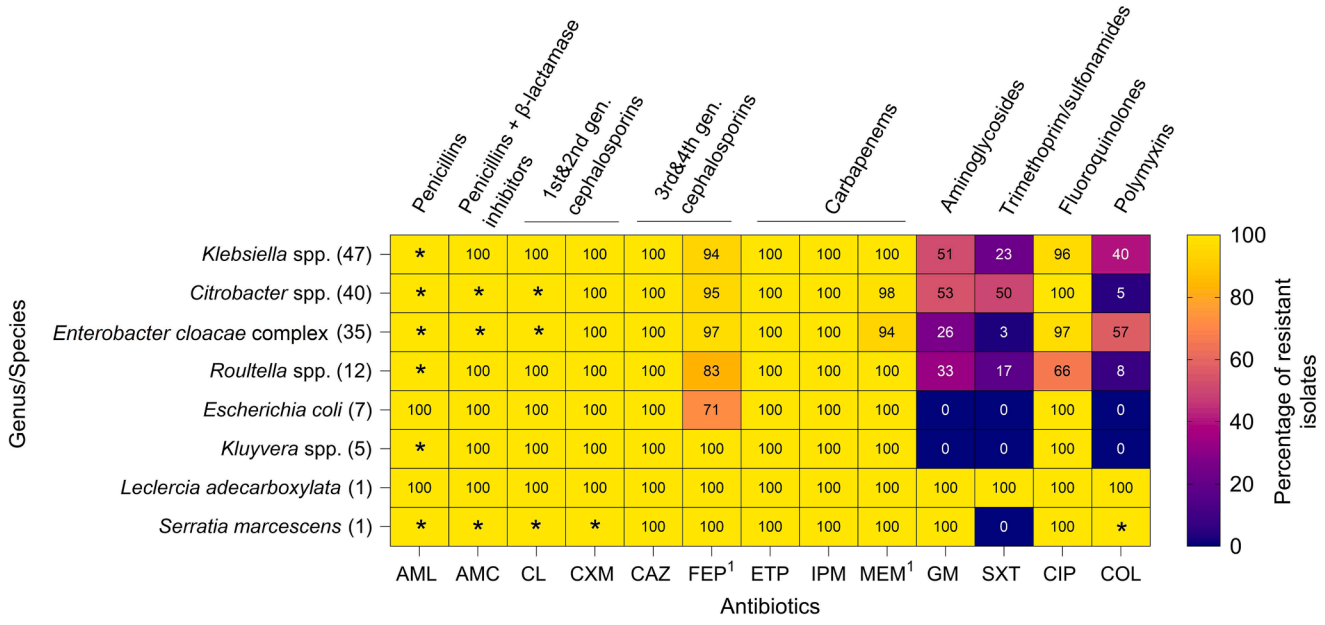

Fig. 2. Antimicrobial resistance patterns of CRE isolated from treated wastewater. Numbers in parentheses indicate the number of isolates from each genus or species. The numbers in the cells indicate the percentage of isolates that were resistant to each antibiotic tested. The asterisk represents intrinsic resistance. [1]The percentage of isolates with a resistant phenotype was not 100% because some isolates were intermediate susceptible. AML: Amoxicillin; AMC: Amoxicillin/Clavulanic acid; CL: Cephalexin; CXM: Cefuroxime; CAZ: Ceftazidime; FEP: Cefepime; ETP: Ertapenem; IPM: Imipenem; MEM: Meropenem; GM: Gentamicin; SXT: Trimethoprim/Sulfamethoxazole; CIP: Ciprofloxacin; COL: Colistin.

(Fig. 4). In addition, this isolate carried 35 different virulence genes related to colonization and biofilm formation, autotransporters, brain endothelial cell invasion etc. (Table S6).

In addition to *E. coli*, a subset of 25 other Enterobacterales isolates with different AMR profiles was selected for further targeted PCR for carbapenemase genes (Fig. 1D, Table S1). In *Citrobacter* isolates, bla_{NDM-1} was the most frequently detected gene (n = 11), followed by bla_{KPC-2} (n = 6), bla_{VIM-1} (n = 6), and bla_{OXA-48} (n = 2). Most *Citrobacter* isolates (n = 8) carried several carbapenemase genes simultaneously, with four carrying two genes (bla_{NDM-1} with bla_{KPC-2} or bla_{VIM-1}), two carrying three genes ($bla_{NDM-1,}$ bla_{KPC-2} bla_{VIM-1}) and two carrying four genes ($bla_{NDM-1,}$ $bla_{KPC-2,}$ $bla_{VIM-1,}$ bla_{OXA-48}). WGS analysis revealed that *C. freundii* was misidentified by MALDI-TOF MS and belonged to *C. portucalensis* (Fig. 4, Table S1). Furthermore, *in silico* MLST analysis associated *C. portucalensis* with ST641 and *C. farmer* with ST600. The bla_{KPC-2} and bla_{VIM-1} were identified by WGS in ST641 *C. portucalensis*, and bla_{KPC-2} was detected in ST600 *C. farmer* (Fig. 4). In addition to the carbapenemases, both sequenced isolates carried genes for TEM-1 ß-lactamase, and OXA-type (OXA-1 or OXA-10) or SHV-12 ESBLs. Genes for resistance to aminoglycosides (*aac(6′)-lb-cr, aph(3)-l* and *aph(6)-ld*), fluoroquinolones (*qnrB9* or *aac(6′)-lb-cr*), sulfonamides (*sul1*), chloramphenicol (*catA1),* trimethoprim (*dfrA1)* and antiseptics (*qacE*) were also found in sequenced isolates (Fig. 4). Both sequenced *Citrobacter* isolates had multiple virulence-associated genes (22 and 26, respectively), including *fepA*, *ompA, nmpC*, *acrB*, among other genes (Table S6). Analysis of the plasmid replicons revealed the presence of IncC, IncX5, IncX6 and IncY plasmids in the sequenced ST600 isolate, while the ST641 isolate had IncY and IncM1 plasmid replicons (Fig. 4).

Raoultella isolates mainly carried bla_{KPC-2} (6 isolates) or bla_{VIM} (6 isolates), followed by bla_{OXA-48} (4 isolates). Two carbapenemase genes in different combinations were found in 4 isolates. All three isolates of *K. cryocrescens* carried bla_{KPC-2}, one of them together with bla_{OXA-48} and bla_{VIM-1}. In *L. adecarboxylata* bla_{KPC-2} and bla_{OXA-48} were detected, and in *S. marscenscens* only bla_{VIM-1} (Fig. 1D).

3.5. Genome analyses of *Klebsiella spp.* isolates

3.5.1. Genetic diversity and carbapenemase gene variants

The PFGE analysis of *K. pneumoniae* showed that 29/36 tested isolates were clustered in 5 clusters (A-E) (Fig. 3A). MLST and WGS further revealed that isolates from the largest cluster A (n = 8 isolates) belonged to ST3590, while isolates from the next largest cluster B (n = 7 isolates) belonged to ST1697. There was also an unassigned ST (STND) that included 6 isolates that belonged to cluster E. Cluster D (n = 4 isolates) included ST1803, while isolates from cluster C (n = 4) belonged to ST629. Various carbapenemase genes were detected in these isolates based on PCR and sequencing analysis. All ST3590 isolates (XDR or MDR) carried only bla_{KPC-2}. In contrast, all XDR and MDR isolates of ST1697 contained bla_{NDM-1}, except one that contained bla_{NDM-1} together with bla_{OXA-48} and one that contained bla_{OXA-48} together with bla_{KPC-2}. All XDR and MDR isolates of STND were carriers of bla_{NDM-1} and bla_{OXA-48} simultaneously. The bla_{NDM-1} gene was common to most XDR ST1803 isolates, sometimes in combination with bla_{OXA-48}, except for one ST1803 isolate that carried only bla_{KPC-2}. The gene bla_{OXA-48} was detected in all isolates of the ST629 cluster, with most isolates (3/4) also carrying the *mcr-4.3* gene for resistance to COL (Fig. 3A).

The PFGE analysis of *K. oxytoca* (n = 11) grouped them to four clusters (A-D), except for two isolates that did not belong to any cluster (Fig. 3B). The MLST analysis assigned the clustered isolates to ST68 (n = 3), ST1697 (n = 2), ST43 (n = 2), and ST17 (n = 2). The presence of carbapenemase genes was variable among these isolates. The bla_{KPC-2} gene was detected in ST68, ST1697 and ST17 isolates, sometimes in combination with bla_{OXA-48} (in ST68) or bla_{NDM-1} and bla_{VIM-1} (in ST1697). One XDR ST43 isolate carried bla_{NDM-1} and bla_{IMP-13}, while the other ST43 isolate carried only bla_{NDM-1} (Fig. 3B).

3.5.2. Genetic determinants related to AMR, plasmid replicon types and virulence in sequenced isolates

To better understand the characteristics of carbapenem-resistant *Klebsiella* isolates from treated wastewater, five *K. pneumoniae* isolates (1 PDR, 3 XDR, and 1 MDR isolate) representing the above clusters and 2 XDR *K. oxytoca* isolates were selected and subjected to WGS. A summary

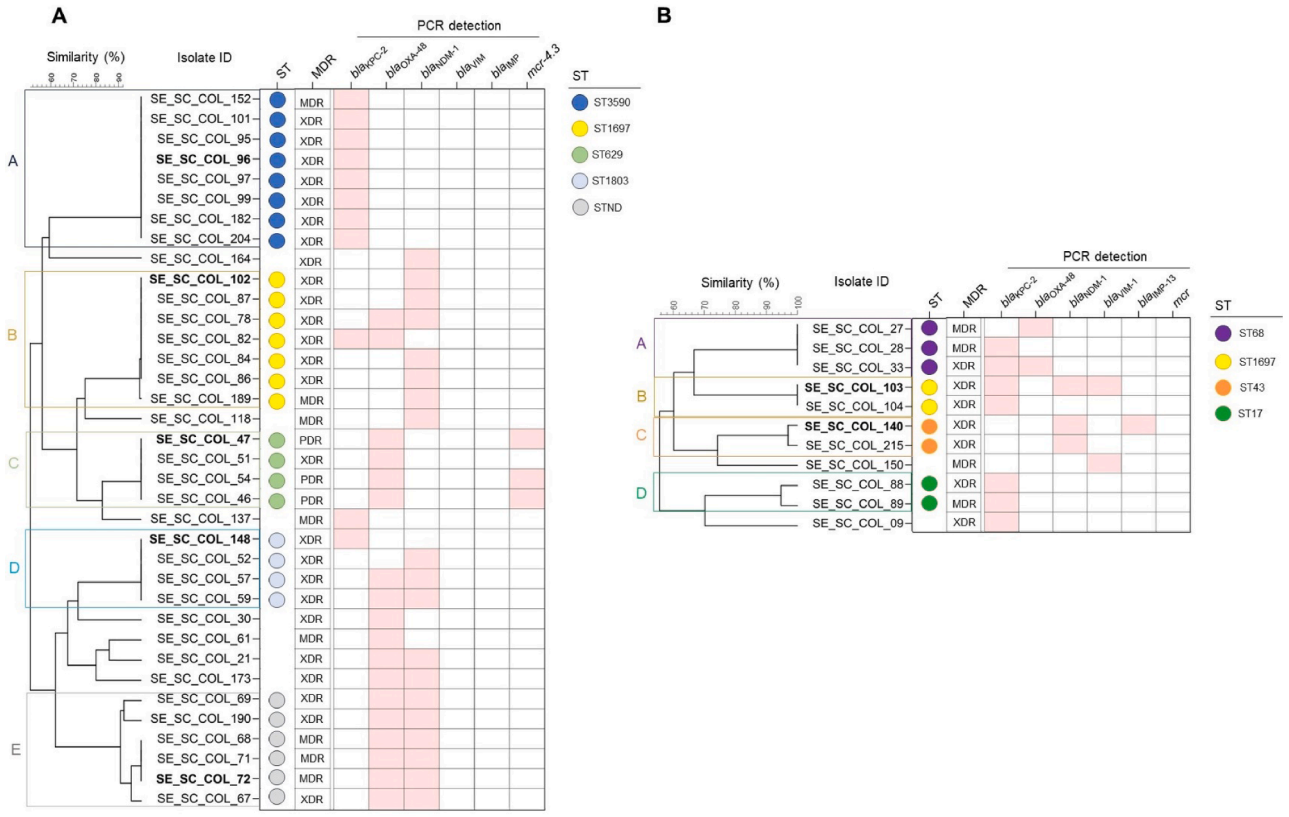

Fig. 3. Cluster analysis based on *Xba*I-PFGE of carbapenem-resistant (A) *K. pneumoniae* and (B) *K. oxytoca* isolates. The letters A through E in the dendrogram denote the primary clusters identified. The coloured circles refer to different sequence types (STs). MDR – multidrug-resistant, XDR – extensively drug-resistant, PDR – pandrug-resistant. The light red row in the table indicates the presence of the carbapenemase gene, and the white row indicates its absence. Isolates that underwent whole-genome sequencing are in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of the WGS data is shown in Fig. 4.

The analysis of 16S rRNA genes in *Klebsiella* genomes revealed that two *K. pneumoniae* isolates (SE_SC_COL_47 and SE_SC_COL_72) identified by MALDI-TOF MS belong to *K. pneumoniae*. In contrast, three *K. pneumoniae* isolates (ST3590, SE_SC_COL_96; ST1697, SE_SC_COL_102; ST1803, SE_SC_COL_148) and one *K. oxytoca* (ST1697, SE_SC_COL_103), previously identified by MALDI-TOF MS, were later identified by WGS as *K. quasipneumoniae* subsp. *similipneumoniae*, while the other *K. oxytoca* (ST43, SE_SC_COL_140) belonged to *K. michiganensis* (Fig. 4). The ANI calculator provided values of $\geq$ 97.7% for *K. quasipneumoniae* subsp. *similipneumoniae* or of 98.8% for *K. michiganensis*. Six sequenced *Klebsiella* isolates harbored various intrinsic ARGs such as $bla_{\text{SHV-1}}$, $bla_{\text{OKP-B}}$, $bla_{\text{OXY-1-7}}$, *fosA* and *oqxA/B*, where $bla_{\text{OKP-B}}$ and *fosA* were only detected in *K. quasipneumoniae* species. Of note, in the *Klebsiella* isolate SE_SC_COL_96 the $bla_{\text{OKP-B}}$ gene was only detected in the raw sequence data (not assembled). Several acquired carbapenemase genes ($bla_{\text{NDM-1}}$, $bla_{\text{KPC-2}}$, and $bla_{\text{OXA-48}}$) and ESBL genes ($bla_{\text{SHV-12}}$, $bla_{\text{SHV-98}}$, $bla_{\text{OXA-1}}$, $bla_{\text{OXA-9}}$, $bla_{\text{C-15}}$ and $bla_{\text{C-3}}$) were identified in various combinations in sequenced *Klebsiella* spp. In addition, ARGs for other antibiotic classes were also found, including ARGs for fluoroquinolones (*qnrB*, *qnrS* and *aac(6′)-Ib-cr*), aminoglycosides (*aac(6′)-Ib-cr*, *aac(3)-I*, *aph(3)-I* and *aadA1*), sulfonamides (*sul1* and *sul2*), trimethoprim (*dfraA14*), macrolides (*mphA, mphE* and *msrE*), tetracyclines (*tetA*), chloramphenicol (*catB3*) and polymyxins (*mcr-4.3*). Two sequenced isolates carried the antiseptic-resistance gene *qacE*.

All sequenced *Klebsiella* isolates carried more than one plasmid

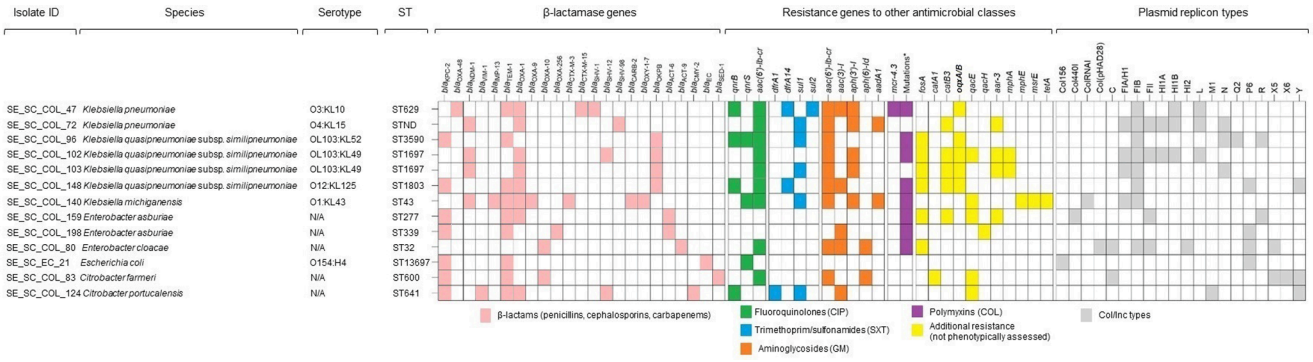

Fig. 4. Summary of whole-genome sequencing data for 13 selected CRE isolates. Colours indicate that the resistance gene or plasmid replicon was present. *The mutations are chromosomal point mutations in the *pmrA/B, phoP/Q, mgrB* and/or *crrB* genes that can confer colistin resistance (see Table S5 for a complete list of point mutations for colistin resistance). N/A – not applicable.

replicon, with IncFIB present in all (Fig. 4). The plasmid replicon type IncFIA/H1 was found in 4 isolates (STND, both ST1697, and ST43). IncHI1B, IncL, and IncN were found in three isolates each.

The VFanalyzer predicted between 16 and 70 different virulence genes in the *Klebsiella* genomes analyzed, with the highest number in ST43 *K. michiganensis* and ST1697 *K. quasipneumoniae* (Fig. 5). Virulence genes included genes encoding biofilm formation (*mrkA-mrkJ)*, adherence (*fimA-fimK)*, siderophores such as aerobactin (*iutA*), enterobactin *(entA-entS* and *fepA-feS)*, yersiniabactin (*fyuA* and *ybt*) and salmochelin (*iroE* and *iroN)*, and secretion systems (T6SS-I, T6SS-II and T6SS-III). Only ST43 *K. michiganesis* possessed yersiniabactin genes, and all isolates possessed genes *acrAB* for efflux pumps associated with AMR and virulence in *Klebsiella* spp. (Fig. 5). Kleborate analysis revealed no hypervirulence-associated genes in these isolates, with the exception of *K. michiganensis*, in which the presence of yersiniabactin-associated genes (*fyuA* and *ybt*) was confirmed. Finally, *K. pneumoniae* isolates had capsules of the KL10- and KL15-type (O3– and O4-type LPS, respectively), *K. quasipneumniae* of the KL52-, KL49- and KL125-type, while *K. michiganensis* had the capsular serotype KL43 (LPS serotype O1) (Fig. 4).

3.5.3. Colistin resistance mechanisms in sequenced Klebsiella spp.

Three ST629 *K. pneumoniae* isolates in this study that were phenotypically identified as COL-resistant contained the *mcr-4.3* gene (Fig. 3A). Phylogenetic analysis revealed that this gene from the sequenced *K. pneumoniae* isolate (SE_SC_COL_47) is closely related (100% nucleotide identity) to *mcr-4.3* from species of 6 genera (*Enterobacter, Acinetobacter*, *Shewanella*, *Salmonella*, *Leclercia*, and *Lelliottia*) (Fig. 6A, Table S3). These isolates were identified on 4 continents in 10 countries (Brazil, China, Czech, Republic, Germany, Italy, Netherlands, Norway, Singapore, Taiwan, and USA). Most of these strains were *E. cloacae* cplx. and *Acinetobacter* species (11/19), mainly isolated from humans. In addition, *mcr-4.3* was also found less frequently in strains from animals (n = 4) and wastewater (n = 1). Furthermore, the phylogenetic tree revealed that *mcr-4.3* and other *mcr-4* variants form a group that is more closely related to the large *mcr-3* gene family (mainly *E. coli* and *Aeromonas* spp.) than to the *mcr-1/2* family (mainly *E. coli* and *Moxarella* spp.) (Fig. 6A).

Sequencing also revealed that the *mcr-4.3* gene was present in the *K. pneumoniae* isolate (SE_SC_COL_47) on a 110,506 bp long contig, which also contains the IncHI1B plasmid replicon (Fig. 6B). In addition, a number of genes involved in conjugation (*tra/trh*) were found downstream of *mcr-4.3* on this contig. The BLASTn search for the entire contig resulted in a significant match (99.95% identity, 75% coverage) with a 230,982 bp plasmid (pC763_1, GenBank accession number: CP067474) from *K. pneumoniae*, which does not contain *mcr-4.3* (Fig. 6B). Nucleotide sequences found upstream of *mcr-4.3* include the *phd* gene of the type II toxin-antitoxin (TA) system and the Tn*3*-family transposon (Fig. 6B). The BLASTn search revealed that this region (Tn*3*-TA-*mcr-4.3*) has 100% identity to the similar region in the genome of *Shewanella frigidimarina* (GenBank accession number: CP000447.1), as well as high similarity (99.97%) to the plasmid of *A. baumannii* (GenBank accession number: NZ_MK360916.1) (Fig. 6B) and other *Acinetobacter* sp. (Table S4).

Finally, WGS showed that four sequenced *Klebsiella* spp. (ST1803, ST3590, ST1697, and ST43) that were phenotypically resistant to COL but lacked plasmid-mediated *mcr* genes had mutations in one or more genes associated with COL resistance: *pmrA/B, phoP/Q, mgrB*, and *crrB* (Fig. 4, Table S5). In ST1803 *K. quasipneumoniae*, 29 point mutations were found that resulted in amino acid changes, 9 of them for the first time (PmrA – S64A, L140Q, E119D; PmrB – T8N, N105S, R256S; PhoP – R165H, Q424L, and PhoQ – Q140K). Of these, R256S and Q424L were predicted to have deleterious effect on PmrB and PhoP function, respectively, based on PROVEAN analysis. In ST3590 *K. quasipneumoniae*, two novel neutral mutations were found in PmrB (M175V) and PhoP (K149E), whereas in ST1697 *K. quasipneumoniae*, a nucleotide deletion of 31 nucleotides (position 601–632) was detected in the *pmrB* gene. In ST43 *K. michiganensis* all investigated genes that can confer resistance to COL were found as wild type. Of note, ST629 *K. pneumoniae,* which contains the *mcr-4.3* gene, also had a neutral mutation in the CrrB protein (Q287K) (Table S5).

4. Discussion

WWTPs are increasingly recognized as a reservoir and pathway for the spread of priority MDR pathogens from the community into natural waters and pose a threat to public health (Lepuschitz et al., 2019). However, there is limited understanding regarding their role in the environmental spread of CRE. We report for the first time the phenotypic and genomic characteristics of CRE remaining in wastewater after treatment in the largest Croatian WWTP, which treats not only municipal wastewater but also wastewater from nine hospitals. A total of 148 carbapenemase-producing Enterobacterales were isolated and characterized, with *Klebsiella* spp. dominating, followed by *Citrobacter* spp. and *E. cloacae* cplx. Some of the isolates of *K. pneumoniae, K. oxytoca* and *C. freundii* were misclassified by MALDI-TOF MS and identified by WGS as *K. quasipneumoniae* subsp. *similipneumoniae, K. michiganensis* and *C. portucalensis*, respectively. This taxonomic reclassification was also supported by ANI analysis (values $\geq$ 97.7%), which has been shown to be a powerful tool for clarifying the distinction between species (Rodríguez-Medina et al., 2019). In addition, the bla_{OKP} gene, normally located on the chromosome of *K. quasipneumoniae*, has been reported to contribute to the reliable differentiation of *K. quasipneumoniae* from other *Klebsiella* spp. (Chew et al., 2021). The detection of $bla_{OKP\text{-}B}$ only in genomes associated with *K. quasipneumoniae* confirmed the correct identification of this species in this study. Of note, the raw data for the detection of $bla_{OKP\text{-}B}$ showed higher sensitivity than the assembled genomes, which could be due to errors in the repeat regions or contamination affecting genome assembly (Cooper et al., 2020). Misidentification of *Klebsiella* spp. and *Citrobacter* spp. by MALDI-TOF

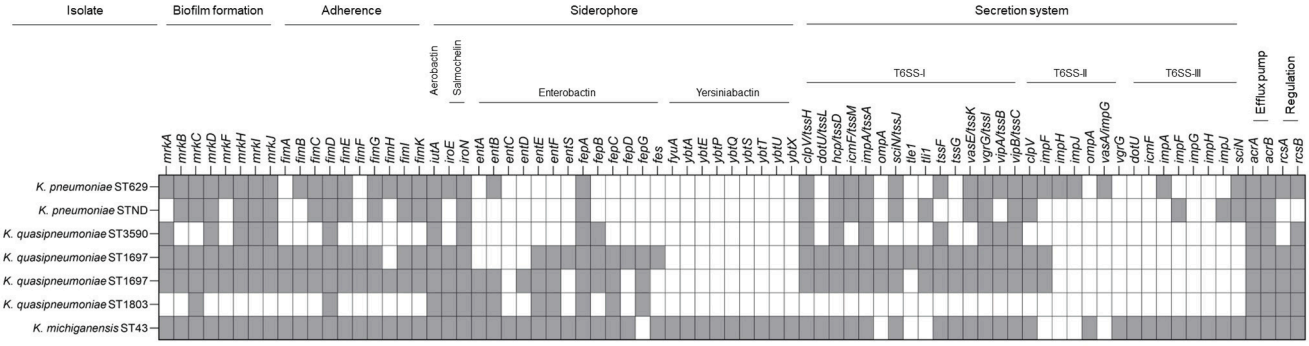

Fig. 5. The content of virulence-encoding genes in relation to their functions in sequenced *Klebsiella* isolates from treated wastewater.

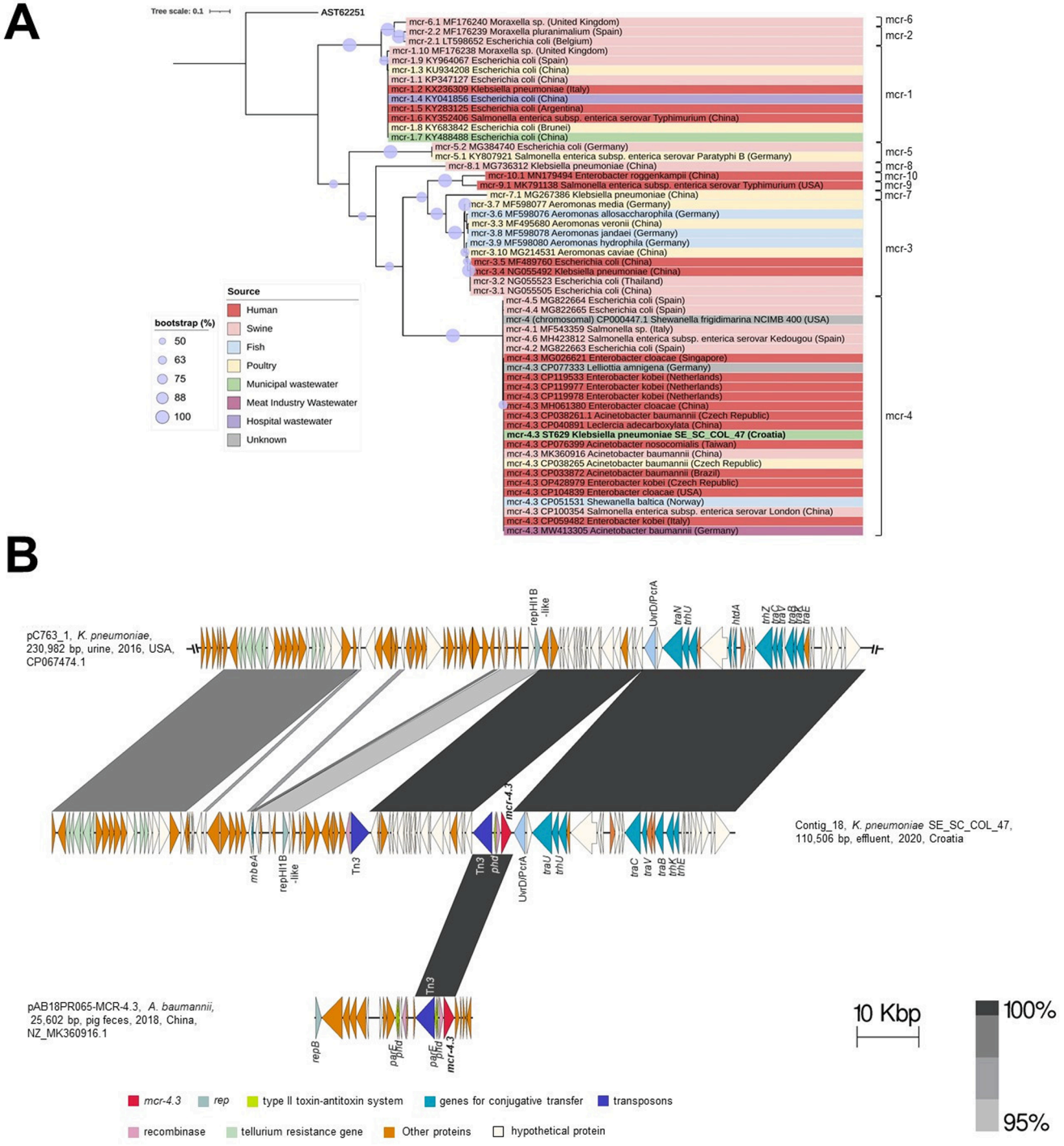

Fig. 6. (A) Phylogenetic tree of *mcr-4* gene sequences. The nucleotide sequence of *mcr-4.3* from *Klebsiella pneumoniae* SE_SC_COL_47 obtained in this study was compared with other known *mcr* variants derived from different isolates and different origins. The tree was rooted with *Escherichia coli* Z1140 with non-functional phosphoethanolamine transferase gene (GenBank accession number: AST66929). The *mcr-4.3* gene from Croatia is indicated in bold letters. The origin of the isolates is noted on the coloured strips according to the indicated key, and the country of isolation is indicated in parentheses. The nucleotide accession number of each member is indicated in the figure. (B) Genetic context of *mcr-4.3* on the contig of *K. pneumoniae* SE_SC_COL_47 together with the linearized partial map of the plasmids of *K. pneumoniae* (GenBank accession number: CP067474.1) and *Acinetobacter baumannii* (GenBank accession number: NZ_MK360916.1). The arrows indicate the direction of transcription of the genes. Grey shaded areas indicate homologous regions with ≥ 95% nucleotide sequence identity with the contig carrying the *mcr-4.3* gene. Different colors indicate the predicted functions of the different genes.

MS has also been observed in other studies (Nobrega et al., 2023; Rodrigues et al., 2018), and it has been hypothesized that *K. quasipneumoniae* is more common in wastewater than *K. pneumoniae* (Liu et al., 2023).

The majority of *Klebsiella*, *Citrobacter* and *E. cloacae* cplx. isolates (≥54% of each genus) were defined as XDR because they were resistant to all but one or two of the antibiotic classes tested. These XDR bacteria, especially *Klebsiella*, pose a significant public health problem as they often cause difficult-to-treat infections and, in some cases, deaths in affected individuals (Navon-Venezia et al., 2017). PCR and WGS

analyses further revealed genetic mechanisms underlying carbapenem resistance in these isolates. The bla_{NDM-1} and the bla_{OXA-48} were the most frequently detected carbapenemase genes, which frequently co-occurred in our *K. pneumoniae* isolates. This carbapenemase combination was also recently detected in *K. pneumoniae* isolates from patients in hospitals and hospital wastewater in Zagreb (Bedenić et al., 2023; Puljko et al., 2023) and in clinical *K. pneumoniae* isolates from 13 European countries, suggesting transboundary dissemination (Ludden et al., 2020). On the other hand, carbapenemase KPC-2 was predominantly detected in our *K. oxytoca* and *E. cloacae* cplx. isolates as well as in *Citrobacter* isolates, but in combination with carbapenemase NDM-1. Interestingly, we frequently observed the presence of two or three different carbapenemases in our Enterobacterales isolates from wastewater. A possible reason for this accumulation of carbapenemase genes in enterobacteria could be the high consumption of carbapenem antibiotics during the COVID-19 pandemic in Croatia (Bedenić et al., 2023). In fact, Croatia is among the three and five EU countries with the highest carbapenem consumption in the hospital and community sectors in 2020 and 2021, respectively (ECDC, 2022). Other carbapenemase genes detected in our isolates were bla_{VIM-1} and bla_{IMP-13}. The bla_{VIM-1} gene was frequently found in *K. oxytoca*, *Citrobacter*, *Kluyvera* and *Roultella* isolates together with two or three other carbapenemase genes. In contrast, bla_{IMP-13} was detected in an XDR isolate of *K. michiganensis* together with bla_{NDM-1}. The identification of the bla_{IMP-13} gene in *K. michiganensis* was an unusual finding, as this gene is normally present in *Pseudomonas aeruginosa* and sporadically in clinical enterobacteria, including *E. coli* and *E. cloacae* cplx, as well as in *Aeromonas media* previously isolated from the same wastewater analyzed here (Cañada-García et al., 2022; Drk et al., 2023).

MLST analysis identified some of the human-associated *K. pneumoniae* (ST3590, ST1803, ST629) and *K. oxytoca* (ST43 and ST17) clones in our wastewater isolates (Aires et al., 2017; Jolley et al.,; Šuto et al., 2022; Takei et al., 2022; Wan et al., 2023). Some of these clones were also found in non-human sources such as animals and soil (ST43 and ST1697) (Jiang et al., 2023; Liao et al., 2019). With the exception of ST629, all these clones were detected for the first time in Croatia. The presence of virulence determinants such as aerobactin, enterobactin and/or yersiniabactin-related genes in *Klebsiella* genomes (ST3590, ST1803, ST629, ST43, STND, and ST1697) has shown that they are likely to be associated with human infections (Shankar et al., 2018). However, due to the absence of other hypervirulence-associated markers, including hypervirulence-associated capsules, these isolates cannot be classified as hypervirulent.

The ST types detected among *E. cloacae* cplx. isolates included mainly human-associated MDR- or XDR-type clones (ST910, ST1641, ST339, ST32, ST277) (Izdebski et al., 2015; Jolley et al., 2018; Zelendova et al., 2023), some of which were also previously detected in wastewater (ST910, ST277, ST32) and waterways (ST910) (Cherak et al., 2021; Cirkovic et al., 2023; Falgenhauer et al., 2019; Puljko et al., 2023). The remaining three (ST2057, ST2408 and ST2368) have already been detected in *Enterobacter* spp. from wastewater, animals and food (Jolley et al., 2018). In addition, STs detected in *Citrobacter* isolates, such as ST641 and ST600 and *E. coli* ST13697, were previously reported in clinical *Citrobacter* spp. (Jolley et al., 2018; Zhou et al., 2020). The profile of virulence genes in *E. cloacae* cplx., *Citrobacter* and *E. coli* isolates suggests that these genes may play an important role in survival in different environments (Amaretti et al., 2020; Mustafa et al., 2020; Yu et al., 2022), although the pathogenic potential of these isolates cannot be determined based on their virulence gene content.

We further determined the plasmid content and potentially associated carbapenem ARGs in draft genomes of *Klebsiella*, *E. cloacae* cplx. and *Citrobacter* isolates to assess their potential for the acquisition and spread of carbapenem resistance. *Klebsiella* isolates carrying the bla_{NDM-1} gene had multiple plasmid replicons of Col-, IncF-, IncH-, IncL-, and IncN-types in various combinations, with IncFIA and IncFIB detected in all four sequenced isolates (STND, both ST1697, and ST43). These two conjugative plasmids and other detected IncH- and IncN-type conjugative plasmids were previously associated with the spread of bla_{NDM-1} in Enterobacterales (Wu et al., 2019). In addition, the two *Klebsiella* isolates ST3590 and ST1803 also had an IncFIB-type plasmid replicon, which may be related to the spread of the bla_{KPC-2} gene, as previously reported for the sewage *K. pneumoniae* isolate in Croatia (Kvesić et al., 2022). Interestingly, the KPC-2-producing *K. quasipneumoniae* ST1803 contained an additional, emerging IncP6 plasmid replicon with a broad-host range. This IncP6 plasmid was reported as a conjugative plasmid (Yao et al., 2017) and was also found in KPC-2-producing *E. cloacae* cplx. ST339 and *E. coli* ST13697 isolates in this study, suggesting that the bla_{KPC-2} gene was likely acquired through IncP6. This is consistent with previous reports of transmission of bla_{KPC-2} by IncP6 in Enterobacterales isolates from wastewater from Croatia and other countries (Ghiglione et al., 2021; Kvesić et al., 2022; Yao et al., 2017). In *E. cloacae* cplx. ST277 and KPC-2-producing *Citrobacter* ST600 and ST641 isolates analyzed in this study, several other plasmid replicons were detected, including IncX5, IncX6, IncM1, IncFII, and IncR types previously reported to be conjugative and associated with transmission of bla_{KPC-2} (Jia et al., 2023; Li et al., 2018; Raro et al., 2020; de Souza et al., 2019; Xie et al., 2023). Seven different plasmid replicon types were found in *E. cloacae* ST32 without the detection of carbapenemase genes. The carbapenem resistance of this isolate may be due to the presence of the AmpC gene bla_{ACT-9} or the ESBL gene bla_{OXA-10} in combination with porin loss, as previously reported (Alonso-García et al., 2023; Lee et al., 2011). In addition, the *C. portucalensis* ST641 carrying bla_{VIM-1} had the plasmid replicon type IncY, which was reported to be conjugative and to harbor bla_{VIM-1} in *E. coli* (Roschanski et al., 2017). Finally, the carbapenemase-producing PDR *K. pneumoniae* ST629 isolate, which carried IncL, IncHI1B and IncFIB plasmid replicons, also had the bla_{OXA-48} gene. The conjugative IncL plasmid (Poirel et al., 2012) may be associated with bla_{OXA-48} transmission, as reported in clinical and environmental isolates in Croatia (Kvesić et al., 2022; Šuto et al., 2022) and other countries (Hamprecht et al., 2019). In addition to carbapenem resistance, this *K. pneumoniae* ST629 isolate exhibits resistance to COL, one of the last available antibiotics, which is mediated by the *mcr-4.3* gene. To the best of our knowledge, this is the first detection of the *mcr-4.3* gene in *K. pneumoniae* and in Croatia in general. Interestingly, the contig with the *mcr-4.3* gene also carried the IncHI1B plasmid replicon, suggesting that the acquisition of *mcr-4.3* in this ST629 isolate may be related to the IncHI1B plasmid. Previous studies have reported the association of the *mcr-2, mcr-3* and *mcr-8* genes with IncHI1B in *K. pneumoniae* (Salloum et al., 2020; Stosic et al., 2021), but an association of the IncHI1B plasmid with *mcr-4.3* has not yet been reported. In addition, the presence of *tra/trh* genes on the contig with the IncHI1B replicon type suggests that IncHI1B may be a conjugative plasmid. Further analysis of this contig revealed that the *mcr-4.3* gene and the upstream neighboring region (Tn*3* transposon and the type II TA system) are identical to the genomic sequence of *S. frigidimarina* and nearly identical to the plasmid sequence of *A. baumannii* and other *Acinetobacter* spp. This supports the conclusion of Zhang et al. (2019) that *mcr-4.3* originated from *S. frigidimarina*, from where it was mobilized onto plasmids in Enterobacterales, potentially including *K. pneumoniae* in this study, but also other bacteria such as *A. baumannii*, as previously reported (Ma et al., 2019). The localization of *mcr-4.3* on the conjugative plasmid may contribute to the rapid spread of COL resistance via wastewater and increase the risk of retransmission to humans.

In addition, novel resistance mechanisms to COL have been observed in some sequenced *Klebsiella* isolates. This is evidenced by novel deleterious mutations in PmrB (R256S) and PhoQ (Q424L) (in *Klebsiella* ST1803) or mutations in *pmrB* by nucleotide deletion (ST1697) or the absence of known resistance mechanisms (ST43 and ST3590). In contrast, known resistance mechanisms to COL were observed in *Enterobacter* isolates. These were based on known deleterious mutations in PmrA, PmrB, PhoP, and/or PhoQ proteins as previously reported for clinical and wastewater *E. cloacae* cplx. isolates (Liao et al., 2022; Puljko

et al., 2023; Uechi et al., 2019).

5. Conclusion

The results of this study show that treated wastewater is a source of hazardous CRE, in particular *Klebsiella* spp. as well as *Citrobacter* spp. and *E. cloacae* cplx. into the aquatic environment. These opportunistic pathogens are phylogenetically related to human-associated MDR-type clones, some of which were detected for the first time in Croatia, and often carry several clinically important carbapenemase genes simultaneously. Five *Klebsiella* spp. were misidentified with MALDI-TOF MS and correctly identified with ANI and by the presence of the species-specific bla_{OKP} gene. WGS analyses also revealed a diverse range of plasmids in the sequenced isolates that are possibly associated with the carbapenemase genes detected. In addition, a significant proportion of *Klebsiella* and *E. cloacae* cplx. isolates demonstrated phenotypic resistance to the last-line antibiotic colistin. The mechanisms underlying its resistance include known and potentially novel intrinsic mechanisms (point mutations in genes for COL resistance) and acquired mechanisms (*mcr-4.3* gene). The colistin resistance gene *mcr-4.3* was identified for the first time in *K. pneumoniae* and in Croatia and is located on the conjugative IncHI1B plasmid. This illustrates the potential of this gene to spread rapidly in the environment and possibly reintroduce into the community, resulting in very limited treatment options. Thus, our monitoring of treated municipal wastewater contributes to the assessment of the risk of spreading resistance to last-line antibiotics from WWTPs into the environment and underlines the need for more effective wastewater treatment.

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CRediT authorship contribution statement

Ana Puljko: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Ivan Barišić:** Writing – review & editing, Writing – original draft, Resources, Formal analysis, Conceptualization. **Svjetlana Dekić Rozman:** Writing – review & editing, Investigation. **Stela Križanović:** Investigation. **Ivana Babić:** Writing – review & editing, Investigation, Data curation. **Marko Jelić:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Ana Maravić:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Nikolina Udiković-Kolić:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108554.

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Chapter 5

Publication No. 4: Resistance to critically important antibiotics in hospital wastewater from the largest Croatian city

Science of the Total Environment

Publication No. 4

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Ines Petrić: Investigation, Writing – review & editing.

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Resistance to critically important antibiotics in hospital wastewater from the largest Croatian city

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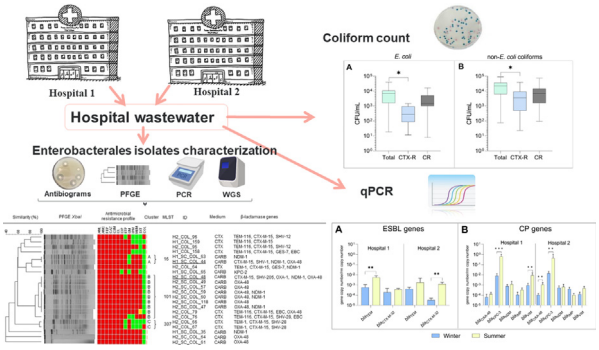
HIGHLIGHTS

- Hospital wastewater contained high concentration of *bla*KPC and *Escherichia coli*.
- The *bla*CTX-M-15 gene dominated among ESBL isolates.
- The *bla*KPC-2 and *bla*NDM-1 genes dominated among carbapenemase-producing isolates.
- Various high-risk enterobacterial clones were detected.
- These clones harboured a diverse plasmidome and exhibited multidrug resistance.

GRAPHICAL ABSTRACT



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ABSTRACT

The emergence of extended-spectrum β-lactamase (ESBL)- and especially carbapenemases in *Enterobacterales* has led to limited therapeutic options. Therefore, it is critical to fully understand all potential routes of transmission, especially in high-risk sources such as hospital wastewater. This study aimed to quantify four enteric opportunistic pathogens (EOPs), total, ESBL- and carbapenem-resistant coliforms and their corresponding resistance genes (two ESBL and five carbapenemase genes) and to characterize enterobacterial isolates from hospital wastewater from two large hospitals in Zagreb over two seasons. Culturing revealed similar average levels of total and carbapenem-resistant coliforms (3.4×10^4 CFU/mL), and 10-fold lower levels of presumptive ESBL coliforms (3×10^3 CFU/mL). Real-time PCR revealed the highest *E. coli* levels among EOPs (10^5 cell equivalents/mL) and the highest levels of the bla_{KPC} gene (up to 10^{-1} gene copies/16S copies) among all resistance genes examined. Of the 69 ESBL- and 90 carbapenemase-producing *Enterobacterales* (CPE) isolates from hospital wastewater, all were multidrug-resistant and most were identified as *Escherichia coli*, *Citrobacter*, *Enterobacter*, and *Klebsiella*. Among ESBL isolates, $bla_{CTX\text{-}M\text{-}15}$ was the most prevalent ESBL gene, whereas in CPE isolates, $bla_{KPC\text{-}2}$ and $bla_{NDM\text{-}1}$ were the most frequently detected CP genes, followed by $bla_{OXA\text{-}48}$. Molecular epidemiology using PFGE, MLST and whole-genome sequencing (WGS) revealed that clinically relevant variants such as *E. coli* ST131 ($bla_{CTX\text{-}M\text{-}15}$/$bla_{TEM\text{-}116}$) and ST541 ($bla_{KPC\text{-}2}$), *K. pneumoniae* ST101 ($bla_{OXA\text{-}48}$/$bla_{NDM\text{-}1}$), and *Enterobacter cloacae* complex ST277 ($bla_{KPC\text{-}2}$/$bla_{NDM\text{-}1}$) were among the most frequently detected clone types. WGS also revealed a diverse range of resistance genes and plasmids in these and other isolates, as well as transposons and insertion sequences in the flanking regions of the $bla_{CTX\text{-}M}$, $bla_{OXA\text{-}48}$, and $bla_{KPC\text{-}2}$ genes, suggesting the potential for

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mobilization. We conclude that hospital wastewater is a potential secondary reservoir of clinically important pathogens and resistance genes and therefore requires effective pretreatment before discharge to the municipal sewer system.

1. Introduction

The threat of increasing antibiotic resistance (AR) of pathogenic bacteria is one of the greatest challenges to global health. Of particular concern is the increasing bacterial resistance to β-lactam antibiotics such as 3rd generation cephalosporins and carbapenems, which are classified as "critically important for human medicine" by the World Health Organization (WHO) (WHO, 2019). However, in 2021, WHO established a new classification in which 3rd generation cephalosporins and carbapenems were placed in the ″Watch″ group of antibiotics (WHO, 2021). It is particularly important to protect the efficacy of these antibiotics and the last-resort antibiotics included in the ″Reserve″ group, as their loss due to AR would result in treatment failures and deaths.

Hospital wastewater is considered a high-risk point source for the spread of antibiotic-resistant bacteria (ARB), antibiotic-resistance genes (ARGs), and enteric opportunistic pathogens (EOPs) in the environment (Hassoun-Kheir et al., 2020). Hospitalized patients are more likely to be treated with antibiotics than the general population, and therefore, higher concentrations of ARB or ARGs are often found in hospital wastewater than in municipal wastewater (Hassoun-Kheir et al., 2020; Paulus et al., 2019). These ARB/ARGs can spread to rivers and lakes through municipal wastewater treatment plants (WWTPs) because they are not always completely removed in WWTPs (Kehl et al., 2022; Puljko et al., 2022). Human-derived bacteria that do not persist in the aquatic environment can transfer ARGs to resident aquatic microorganisms, including pathogens, through horizontal gene transfer (HGT) (González-Plaza et al., 2019; Larsson and Flach, 2022). This could lead to further potential transfer of ARGs from the environment to humans. Therefore, it is important to understand the AR gene pool of hospital wastewater, especially for bacteria of the order *Enterobacterales*, some of which are important nosocomial pathogens that can thrive in both the environment and the human gut, in order to track their spread from the hospital point source to the environment.

In contrast to many developed countries, hospital wastewater in Croatia is discharged directly into municipal WWTPs without any treatment, which may be a cause for concern, even though hospital wastewater represents only a small proportion (<2 %) of the total volume of wastewater treated in WWTP. Therefore, ARB/ARGs, which are typically found in low levels in wastewater, such as CP-producing *Enterobacterales* (CPE) and their mobile ARGs (e.g. bla_{KPC}, bla_{NDM}, bla_{OXA-48}, bla_{VIM} and bla_{IMP}), can spread rapidly and widely, posing a greater risk than other, more common environmental bacteria with intrinsic resistance mechanisms (Manaia et al., 2018). CPE and ESBL (extended spectrum β-lactamase)-producing *Enterobacterales* (ESBL-E) have been listed by the WHO as critical priority pathogens for which research and the development of new antibiotics is urgently needed due to the emergence of multidrug resistance among these pathogens (WHO, 2017). In Europe, these species have been increasingly detected in clinical samples over the past decade (Kazmierczak et al., 2021). Croatia is one of the countries with a high prevalence of these strains, especially *Klebsiella pneumoniae* isolates (62 % resistant to 3rd generation cephalosporins in 2021; ECDC, 2021). In addition, carbapenem resistance rates among clinical *K. pneumoniae* isolates increased from 2 % in 2018 to 32.9 % in 2021 in Croatia (ECDC, 2021). Previous studies have identified hospital wastewater as a high-risk point source for the spread of CPE and ESBL-E (Jelić et al., 2019; Kehl et al., 2022). However, more information on their phylogeny and genomic characteristics is needed to better assess the risk of spreading these clinically important ARB and their ARGs via hospital wastewater.

The most common resistance mechanism to β-lactams in *Enterobacterales* is the production of β-lactamases, and the most important enzymes in this family are the ESBLs, plasmid-mediated AmpC β-lactamases (pAmpC), and carbapenemases (CPs). ESBLs confer resistance to most β-lactam antibiotics, including penicillins, cephalosporins, and monobactam aztreonam, and the most common variants are TEM, SHV, CTX-M, and OXA (Bradford, 2001). pAmpC enzymes are generally less prevalent than ESBLs in *Enterobacterales*, but are still important because they contribute to β-lactam resistance, which can also extend to carbapenems when pAmpC are overproduced in combination with an impermeability defect (Barišić et al., 2014). Carbapenems are considered to be a last-resort treatment for Gram-negative infections, as they retain activity against chromosomal cephalosporinases and ESBLs. The production of CPs can confer resistance to virtually all β-lactams and is the most common mechanism of resistance to carbapenems among Gram-negative bacteria. Acquired CPs of clinical importance include KPC, VIM, NDM, IMP and OXA-48, and their geographic distribution is remarkably diverse (Nasri et al., 2017; Kazmierczak et al., 2021; Neidhöfer et al., 2021).

The role of wastewater from hospitals as a reservoir of potentially pathogenic or ARB of clinical concern, such as CPE and ESBL-E, and the associated risk are not fully understood. To date, studies investigating CPE or ESBL-E in hospital wastewater have mainly used culture-based methods paired with molecular methods such as PCR and sequencing to understand phenotypic and molecular mechanisms of resistance in isolated bacteria (Haller et al., 2018; Lépesová et al., 2020; L. Zhang et al., 2020). However, molecular methods such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) have been used to a much lesser extent to understand the genetic relatedness and epidemiology of isolates (Daoud et al., 2018; Kehl et al., 2022). Similarly, whole-genome sequencing (WGS) and real-time PCR (qPCR) have rarely been used to assess the diversity and abundance of ARGs (Flach et al., 2021; Azuma et al., 2022; Carlsen et al., 2022). Therefore, a combined approach encompassing all of these methods is needed to assess the role of hospital wastewater in the spread of antibiotic-resistant priority pathogens.

In order to comprehensively characterize the wastewater from two hospitals in Zagreb, this study aimed to investigate the prevalence of *Escherichia coli* and other coliforms presumed to be ESBL- or carbapenemase-positive, as well as selected ESBL and CP genes and EOPs using culture-based and molecular methods (real-time PCR). Additionally, a total of 159 enterobacterial isolates (69 ESBL- and 90 CP-producing) was successfully isolated and identified. These isolates were characterized by phenotypic and genotypic assays to determine their AR profiles, molecular epidemiology, and ARGs present in them. In addition, the mechanisms of AR and their potential mobility in the selected isolates of *E. coli*, *Klebsiella* spp., and *Enterobacter cloaceae* complex were characterized using WGS.

2. Materials and methods

2.1. Sample collection

Untreated wastewater samples were collected from two large hospitals (abbreviated as H1 and H2) in Zagreb, Croatia. Both hospitals provide primary health care and emergency services and differ in the number of hospital beds (H1 – 1510 beds, and H2 – 570 beds). Samples were taken at three consecutive days (biological replicates) in winter (from January 28 to 30) and summer (from July 24 to 26) of 2020. Grab wastewater samples (2000 mL) were collected from the sewer system in sterile 2.5 L glass bottles before being discharged into the municipal sewer system. Hospital wastewater is not treated at the hospital before it enters the municipal sewers, as is common for all hospitals in Croatia. The collected samples were transported on ice in cool boxes to the laboratory and processed within 2 h.

2.2. DNA extraction and qPCR assays

For DNA extraction from wastewater, each sample was filtered separately in triplicate (subsamples of 50–90 mL) through mixed cellulose ester membranes (47 mm diameter, 0.22 μm pore size, GE Healthcare, Life Science, USA). Total community DNA was extracted from each filter separately using the DNeasy Powersoil kit (Qiagen, USA) according to the manufacturer's recommendations. Before the extraction procedure, the filters were cut into small pieces with sterile scissors. The three eluates from each sample were pooled. DNA quality (260/280 ratio) was determined using a Nanodrop spectrophotometer (BioSpec Nano, Shimadzu, Japan), and DNA quantity was determined using a Qubit Fluorometer 3.0 (Thermo Fisher Scientific, USA). All extracts were stored at −20 °C until use.

qPCR was used to quantify two ESBL genes (bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$), five CP genes ($bla_{KPC\text{-}3}$, bla_{NDM}, $bla_{OXA\text{-}48}$-like, bla_{IMP}, and bla_{VIM}), colistin resistance gene (*mcr-1*), and the 16S rRNA gene (*rrn*) as a marker for total bacteria. In addition, marker genes for EOPs belonging to the WHO priority pathogens were also quantified: *ycc*T (*E. coli*), *glt*A (*K. pneumoniae*), *sec*E (*Acinetobacter baumanii*), and 23S rDNA (enterococci). Primers, qPCR conditions and generation of standard curves are as described in Puljko et al. (2022). The amplification efficiency values for the latter taxonomic marker genes are listed in Table S2 and ranged from 77 to 115 %. All qPCR assays were performed on the ABI 7300 real-time PCR thermocycler (Applied Biosystems, USA) with Power SYBR® Green PCR Master Mix (10 μL, Applied Biosystems, USA), 1 μM of each primer (Puljko et al., 2022, Tables S1, S2), and 2 ng of DNA template in a total volume of 20 μL. All samples were analysed in triplicate. Gene abundances were calculated per 1 mL sample (absolute abundance) and per number of *rrn* copies (relative abundance). The abundances of the *ycc*T gene of *E. coli*, the *glt*A gene of *K. pneumoniae*, *sec*E gene of *A. baumannii*, the 23S rRNA gene of enterococci, and the *rrn* gene of total bacteria were expressed as cell equivalents (CE)/mL. In the case of *E. coli*, *K. pneumoniae*, and *A. baumannii*, only one copy of the target gene is present in a cell (Clifford et al., 2012; Gadsby et al., 2015); thus, one copy number is equivalent to one cell. However, in enterococci and total bacteria, average copy number of 23S rRNA and 16S rRNA genes is five and three, respectively (Stoddard et al., 2015); therefore, 23S rRNA and 16S rDNA copies determined by qPCR were divided by 5 and 3, respectively, to convert them to CE.

2.3. Coliform counts and isolation of ARB

To enumerate *E. coli* and non-*E. coli* coliforms, a series of dilutions of wastewater samples were prepared in 0.85 % NaCl (tenfold dilutions up to 1:10,000), and then filtered in triplicate through sterile mixed cellulose ester membrane filters (47 mm diameter, 0.22 μm pore size, Whatman, GE Healthcare, Life Science, SAD). Filters were then placed on 1) Rapid'*E. coli* 2 (Bio-Rad, France) for enumeration of total *E. coli* and non-*E. coli* coliforms; 2) Rapid'*E.coli* 2 agar plates supplemented with 4 mg/L cefotaxime (CTX) representing 3rd generation cephalosporins for enumeration of CTX-resistant (CTX-R) *E. coli* and non-*E. coli* coliforms and 3) CHROMagar mSuperCARBA (CHROMagar, France) agar plates for enumeration of carbapenem-resistant (CR) *E. coli* and non-*E. coli* coliforms. Plates were incubated at 37 °C for 24 h, and colonies of total, CTX-R and CR *E. coli* and non-*E. coli* coliforms were enumerated, and their concentrations were calculated as colony-forming units (CFU) per milliliter of wastewater (CFU/mL).

For isolation of ARB, a total of 200 colonies of presumptive *E. coli* and other coliforms were picked from Rapid'*E.coli* 2 with CTX and CHROMagar mSuperCARBA plates and re-streaked on the same medium to purity. The purified colonies were stored in a 20 % glycerol stock at −80 °C.

2.4. Identification of isolates

Bacterial isolates were sent to the Laboratory for Mass Spectrometry and Functional Proteomics at the Ruđer Bošković Institute for identification using Matrix Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) analysis. Isolates were streaked on Mueller-Hinton plates (Oxoid, UK) and incubated overnight for 18–24 h at 37 °C. Colony material of pure cultures was transferred by direct smearing onto spots of the MALDI-TOF MS target with tooth-picks. Bacterial identification was reported to the species level if the score value was above 2.00 or to the genus level if the score was between 1.70 and 1.99. A minority of isolates that could not be successfully identified by MALDI-TOF MS were identified by sequencing of the 16S rRNA gene. For this purpose, a 1465 bp fragment of the 16S rRNA gene was amplified by PCR using primers 27F and 1492R (Weisburg et al., 1991). Thermocycling conditions were as follows: 5 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 1 s at 55 °C and 1:30 min at 72 °C, and a final extension step at 72 °C for 10 min. Amplicons were sent to Macrogen (Amsterdam, Netherlands) for Sanger sequencing in the forward direction. The resulting sequences were characterized using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/). All sequences were identified to species level (≥ 99 % sequence identity).

2.5. Antibiotic susceptibility testing

All isolates were subjected to antibiotic susceptibility testing using the disk diffusion method (EUCAST, 2020). The antibiotics used were amoxicillin (AML, 25 μg), amoxicillin/clavulanic acid (AMC, 30 μg), cephalexin (CL, 30 μg), cefuroxime (CXM, 30 μg), ceftazidime (CAZ, 10 μg), cefepime (FEP, 30 μg), ertapenem (ETP, 10 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), gentamicin (GM, 10 μg), trimethoprim/sulfamethoxazole (SXT, 1.25/23.75 μg) and ciprofloxacin (CIP, 5 μg). The AML, CL and CAZ disks were purchased from Oxoid, and the others from BD (BBL, USA). Isolates that were resistant to any of the carbapenems underwent minimum inhibitory concentration (MIC) determination by serial broth microdilution according to EUCAST (2020) guidelines. In addition, colistin (COL) resistance of all isolates was determined by MIC. Briefly, in a sterile 96-well plate, a starting concentration of 64 mg/L (COL, IPM, and MEM) or 16 mg/L (ETP) was used and serially diluted twofold to a final concentration of 1 mg/L (COL, IPM, and MEM) or 0.25 mg/L (ETP). The remaining two columns were used as a positive control for bacterial growth (no antibiotic) and a negative control (no bacteria added). The wells contained 90 μL of Mueller-Hinton broth (Merck, Germany) or cation adjusted Mueller-Hinton broth 2 (Sigma-Aldrich, Germany; in the case of COL) and serially diluted target antibiotics. Overnight bacterial cultures were diluted to a concentration of 5×10^5 CFU/mL, and each well was inoculated with 10 μL of the culture. The plates were incubated overnight at 37 °C, and the lowest concentration at which no visible growth was observed was determined as the MIC of the sample. Strains *Escherichia coli* ATCC 25922 and *Escherichia coli* NCTC 13846 were used as quality controls. The isolates were classified as multidrug-resistant (MDR), extensively drug-resistant (XDR), or pandrug-resistant (PDR) according to the definitions of Magiorakos et al. (2012). Finally, isolates were clustered into groups according to the similarity of their resistance patterns, and representatives of each group were used for further targeted PCRs.

2.6. Phenotypic identification of ESBLs, pAmpC and carbapenemases

For the detection of ESBL production, CTX-R isolates underwent the double disk synergy test according to EUCAST guidelines (EUCAST, 2017). Briefly, overnight cultures of isolates were diluted in saline to 0.5 McFarland concentration and plated on Mueller-Hinton agar plates with a sterile cotton swab. Paired CAZ (30 μg) and CTX (30 μg) disks were used, which were 20 mm and 30 mm (centre to centre) from the amoxicillin-clavulanate disk (AMC, 20 + 10 μg), respectively. Plates were incubated overnight at 37 °C. An increase in the zone of inhibition (synergy with clavulanate) for one of the 3rd generation cephalosporins was considered a positive result for ESBL production.

To screen for pAmpC production, CTX-R isolates were subjected to a combined disk test using phenylboronic acid (Gupta et al., 2014). Briefly, the cefoxitin disks (30 μg) alone and in combination with phenylboronic acid (300 μg) were placed on the inoculated Mueller-Hinton agar plates.

After overnight incubation at 37 °C, an increase in the zone of inhibition of ≥5 mm indicated pAmpC production.

To detect carbapenemase production, CR isolates were subjected to the in-house Carba NP test (Nordmann et al., 2012). Briefly, a calibrated loop (10 μL) of bacterial colonies was resuspended in Tris-HCL lysis buffer and mixed with 100 μL phenol red solution containing $ZnSO_4 \times 7H_2O$ (0.1 mM) and imipenem-cilastatin (12 mg/mL). After incubation at 37 °C for a maximum of 2 h, the bacterial strains that changed the color of the suspension from red to orange or yellow were considered to be carbapenemase producers.

2.7. Targeted PCRs

Targeted PCRs were performed on a subset of isolates with different AR profiles. Total bacterial DNA was extracted from bacterial overnight cultures using the Quick-DNA™ Miniprep Plus Kit (Zymo, USA) according to the manufacturer's instructions. Isolates with confirmed ESBL production were tested for the presence of ESBL genes by multiplex PCR (bla_{TEM}, bla_{SHV}, bla_{PER}, bla_{VEB}, bla_{GES} and bla_{SME}) and singleplex PCR ($bla_{CTX\text{-}M}$ groups 1, 2, and 9). In addition, these isolates underwent multiplex PCR for pAmpC genes (bla_{MOX}, bla_{CIT}, bla_{DHA}, bla_{ACC}, bla_{EBC}, and bla_{FOX}). All isolates with confirmed CP production underwent PCR for the following CP genes: bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{OXA\text{-}48}$, and bla_{VIM}. Bacterial isolates that were identified as colistin-resistant in broth microdilution assays underwent multiplex PCR for the following genes: *mcr*-1, *mcr*-2, *mcr*-3, *mcr*-4 and *mcr*-5. Primer sequences and thermocycling conditions are listed in Table S3. All positive PCR products were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany) and underwent Sanger sequencing in the forward direction (Macrogen). Resulting sequences were edited and compared with reference sequences in the NCBI database using the online BLASTX search.

2.8. Genotyping by PFGE and MLST

Isolates of *E. coli*, *Klebsiella* spp., and *Enterobacter* spp. were subjected to genetic relatedness determination by PFGE of *Xba*I-digested genomic DNA using the CHEF-DR III system (Bio-Rad Laboratories, USA), as previously described (Jelic et al., 2016). Restriction patterns were analysed with BioNumerics software (Applied Maths, Belgium) using the DICE coefficient (tolerance 1.5 %), and the dendrogram was generated with UPGMA. Isolates that had a similarity cut-off of ≥85 % of their banding patterns were assigned to the same cluster.

One representative of each cluster was analysed for the presence of a sequence type (ST) using the commercial service IDgenomics (Seattle, USA) or by WGS (see below). In the case of the commercial service, the sequences of 7 housekeeping genes of 3 *E. coli* and 1 *K. pneumoniae* were typed using the database https://pubmlst.org/.

2.9. WGS and sequence analysis

Based on the results of antibiotic susceptibility testing and their clinical significance, selected isolates of *E. coli*, *K. pneumoniae*, and *E. cloacae* complex (cplx) were subjected to WGS, resulting in the sequencing of 4 *E. coli*, 2 *K. pneumoniae*, 2 *Enterobacter asburiae*, 3 *Enterobacter cloacae*, 1 *Enterobacter ludwigii* and 1 *Enterobacter kobeii*. DNA was extracted from frozen isolates revived by two consecutive smears on LB agar plates with CTX or IPM (4 mg/L) and subculture overnight in LB broth with the appropriate antibiotic (4 mg/L CTX or IPM). Sequencing was performed on the Ion Torrent PGM platform (Life Technologies, USA) according to the manufacturer's instructions. The Ion Xpress Plus Fragment Library Kit was used to enzymatically shear 100 ng of genomic DNA. The target fragment size was 400 bp. Subsequently, the fragmented DNA was processed using the Ion DNA Barcoding Kit (Life Technologies, USA) and its size was selected using the E-Gel SizeSelect 2 % Agarose Kit (Life Technologies, USA). The size and distribution of DNA fragments were analysed using the High Sensitivity Kit (Agilent, USA). Further sample preparation was performed using the Ion OneTouch Kit (Life Technologies, USA). Finally, the amplified DNA was sequenced using the 318 Chip (Life Technologies, USA). The raw data were assembled de novo using the Assembler SPAdes software, ver. 3.1.0., which is part of the Assembler plugin on the Ion Torrent server. Genomes were annotated using the Rapid Annotation using Subsystem Technology (RAST) database (Aziz et al., 2008; Overbeek et al., 2014). ARGs were found using ResFinder (Bortolaia et al., 2020). STs and plasmid replicon types were identified using tools from the Center for Genomic Epidemology website (Larsen et al., 2012; Carattoli et al., 2014).

Screening for chromosomal mutations in genes associated with colistin resistance was performed using the reference genome of *E. cloacae* ATCC 13047 (NCBI GenBank Accession No. CP001918). *E. cloacae* ATCC 13047 was screened for reference amino acid sequences of PmrA, PmrB, PhoP, PhoQ, and MgrB. Whole genome sequences of 6 *Enterobacter* spp. isolates were used to search for chromosomal mutations causing resistance to colistin in *Enterobacter* spp. by sequence BLASTing (http://blast.ncbi.nlm.nih.gov). The effect of mutation as neutral/detrimental was determined using the freely available PROVEAN (Protein Variation Effect Analyzer) v1.1.3 software (http://provean.jcvi.org/seq_submit.php).

2.10. Data analysis

Bacterial and gene concentration data were first log10-transformed before further analysis. The unpaired Welch's *t*-test was used to compare the average concentration of target organisms (total coliforms, *E. coli*, other coliforms) in hospital wastewater and the absolute concentration of EOPs between seasons. In addition, the relative abundance of ESBL and CP genes between seasons in each hospital wastewater was assessed using a multiple unpaired t-test. All statistical analyses and data visualisations were performed using GraphPad Prism ver. 9.4.0 for Windows (GraphPad Software, San Diego, California, USA).

2.11. Data accessibility

The Sanger sequence data were submitted to GeneBank, and the accession numbers for each gene are as follows: bla_{VIM} (OQ130426-OQ130434), $bla_{OXA\text{-}48}$ (OQ133342-133360), *bla*GES (OQ133361-133362), bla_{SHV} (OQ133363-OQ133367), $bla_{CTX\text{-}M}$ (OQ145335-OQ145369), bla_{NDM} (OQ145370-OQ145389), bla_{KPC} (OQ145390-OQ145414), and bla_{TEM} (OQ215146-OQ215184). Whole genome sequencing data were submitted under BioProject PRJNA913323 with BioSample accession numbers SAMN32292703–SAMN32292715.

3. Results

3.1. Concentrations of total, CTX-R and CR E. coli and other coliforms

Cultivation on non-selective and two different selective plates revealed the presence of total and presumptive CTX-R *E. coli* and non-*E. coli* coliforms (Biorad Rapid'*E.coli* 2 agar plates with CTX) and presumptive CR *E. coli* and non-*E. coli* coliforms (CHROMagar mSuperCARBA plates) in all hospital wastewater samples (Fig. 1). The average concentration of total *E. coli* and non-*E. coli* coliforms in wastewater from the two hospitals was 9×10^3 and 2.5×10^4 CFU/mL, respectively. In comparison, significantly lower concentrations of presumptive CTX-R *E. coli* (4.8×10^2 CFU/mL) and non-*E. coli* (6.8×10^3 CFU/mL) were measured. However, the concentrations of presumptive CR *E. coli* and non-*E. coli* coliforms in the analysed wastewater samples were slightly higher than the corresponding CTX-R concentrations and were consistent with the total concentrations of the corresponding species (Fig. 1, Table S4). No significant seasonal changes were detected in presumptive CTX-R or CR *E. coli* and non-*E. coli* coliforms.

3.2. Abundance of ESBL and CP genes in hospital wastewater

Two ESBL (bla_{TEM}, $bla_{CTX\text{-}M\text{-}32}$) and five CP genes ($bla_{KPC\text{-}3}$, $bla_{OXA\text{-}48\text{-}like}$, bla_{NDM}, bla_{IMP}, bla_{VIM}) were detected in all hospital wastewater samples using qPCR (Fig. 2). The concentrations of bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$ were

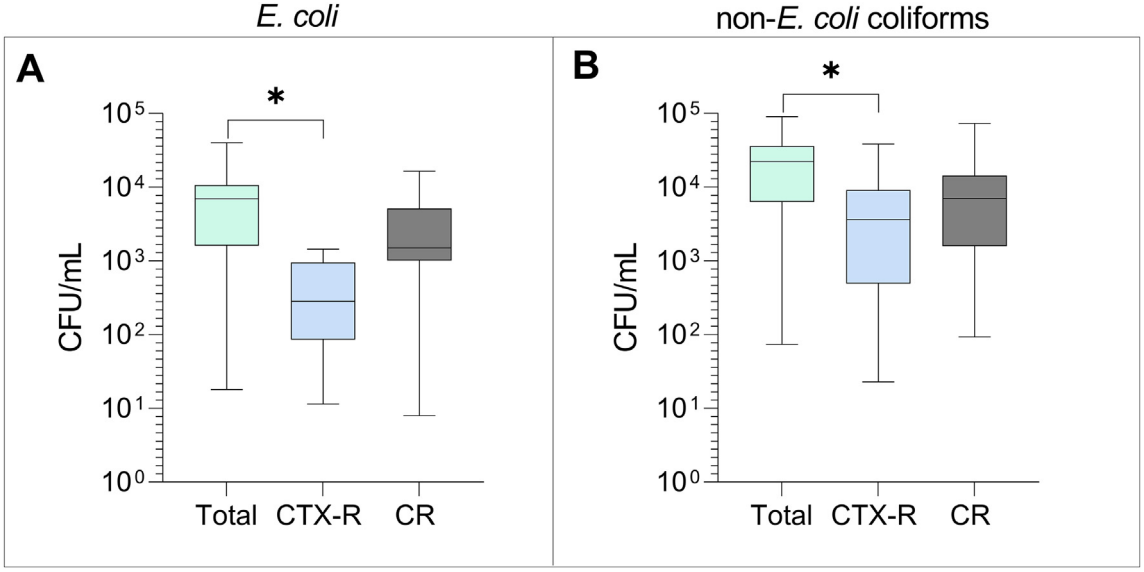

Fig. 1. Boxplot comparison of concentrations (CFU/mL) of total, presumptive CTX-R and CR *E. coli* (A) or non-*E. coli* coliforms (B) in wastewater from both hospitals. Boxes indicate median and quartiles, and whiskers represent minimum and maximum values. Asterisks indicate significant difference between seasons (*$p < 0.05$, multiple Welch's *t*-test).

mostly between approx. 10^{-3} and 10^{-4} gene copies/*rrn* copies (Fig. 2A). Regarding seasonal variations, hospital H1 had significantly higher levels of bla_{TEM} in summer samples, whereas hospital H2 had significantly higher levels of $bla_{CTX\text{-}M\text{-}32}$ in the same season (unpaired *t*-test, $p < 0.01$). Among the CP genes, $bla_{KPC\text{-}3}$ was the most abundant in wastewater of both hospitals, with significantly higher levels in summer (approx. 10^{-1} gene copies/*rrn* copies) compared to winter samples (approx. 10^{-3} gene copies/*rrn* copies) (unpaired *t*-test, $p < 0.01$; $p < 0.001$) (Fig. 2B). The relative abundance of the other CP genes examined mostly ranged from approx. 10^{-3} to 10^{-4} gene copies/*rrn* copies with no significant seasonal differences observed, except for bla_{VIM} in H1 and $bla_{OXA\text{-}48}$ in H2 with significantly higher levels in summer samples. The colistin resistance gene *mcr-1* was not found in any of the hospital wastewater.

3.3. Concentrations of total bacteria and EOPs in hospital wastewater

The qPCR-based analyses of the bacterial *rrn* gene showed that the mean concentration of total bacteria in the winter wastewater samples was approx. 10^8 CE/mL and was significantly lower in the summer samples (10^7 CE/mL) (Welch's t-test, $p < 0.001$) (Fig. 3). In addition, quantification of specific taxonomic markers for EOPs such as *E. coli* (*yccT*), *K. pneumoniae* (*gltA*), *A. baumannii* (*secE*), and *Enterococcus* spp. (23S rRNA) showed that there were no significant differences in gene abundances between seasons, although median levels for *A. baumanii* were considerably higher in summer than in winter samples. In general, *E. coli* was the most abundant species in the hospital wastewater samples (approx. 10^5 CE/mL), whereas the concentrations of the other EOPs were approx. 10^4 CE/mL, with the exception of *A. baumanii* in summer.

3.4. Identification of isolates

A total of 200 presumptive enterobacteria were successfully isolated on selective media supplemented with antibiotics (CTX or carbapenems) from wastewater samples from both hospitals. Of these, we identified 159 members of the order *Enterobacterales* and 41 strains not belonging to this order by MALDI-TOF or 16S rRNA gene sequencing (Fig. S1A). *Enterobacterales* isolates included 69 CTX-R (27 from H1 and 42 from H2) and 90 CR isolates (43 from H1 and 47 from H2) (Fig. S1B). The identified

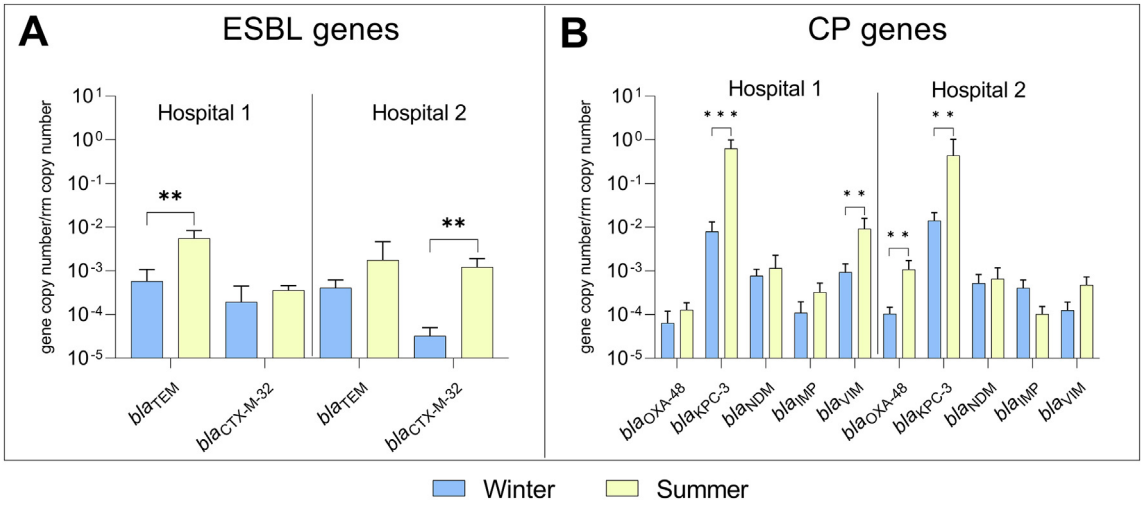

Fig. 2. Relative abundance of ESBL ($bla_{CTX\text{-}M\text{-}32}$ and bla_{TEM}) (A) and carbapenemase (CP) genes ($bla_{OXA\text{-}48}$, $bla_{KPC\text{-}3}$, bla_{NDM}, bla_{IMP}, and bla_{VIM}) (B) in wastewater from two hospitals during winter and summer sampling. A significant difference between gene abundance in samples from different seasons in each hospital was determined using an unpaired t-test and is indicated by asterisks (**$p < 0.01$; ***$p < 0.001$, multiple unpaired t-test).

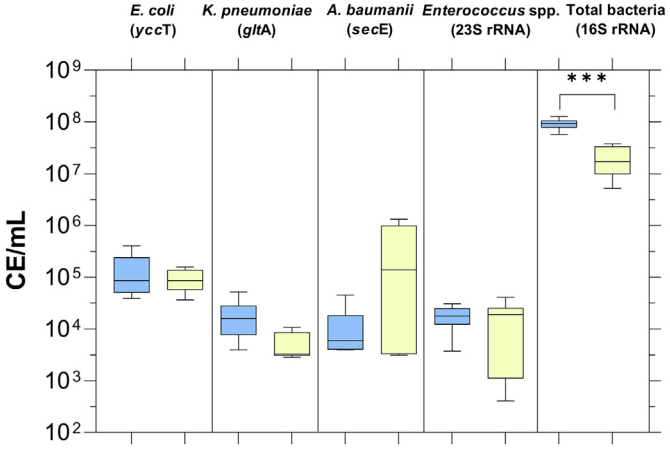

Fig. 3. Quantification of *E. coli* (*ycc*T), *K. pneumoniae* (*glt*A), *A. baumannii* (*sec*E), *Enterococcus* spp. (23S rRNA) and total bacteria (16S rRNA) (cell equivalents (CE)/mL) from winter and summer wastewater samples from two hospitals. The boxes indicate the median and quartiles, and the whiskers represent the minimum and maximum values. Asterisks indicate significant difference between seasons (***$p < 0.001$, multiple Welch's t-test).

Enterobacterales from both hospitals belonged to eight different genera, namely *Escherichia* (n = 58), *Citrobacter* (n = 39), *Enterobacter* (n = 29), *Klebsiella* (n = 23), *Raoultella* (n = 5), *Kluyvera* (n = 3), *Morganella* (n = 1), and *Serratia* (n = 1) (Fig. S1C).

3.5. Phenotypic tests for detection of β-lactamases

All presumptive CTX-R *Enterobacterales* isolates (n = 69) were found to be positive for ESBL production in the double disk synergy test. Of these, 35 % of isolates (n = 24) were also phenotypically positive for pAmpC production in the phenylboronic acid disk test, and 32 % (n = 20) were positive for CP production in the Carba NP test. In addition, all presumptive CR *Enterobacterales* isolates (n = 90) were confirmed as CPE by the Carba NP test.

3.6. Antibiotic susceptibility patterns

All 159 *Enterobacterales* isolates from hospital wastewater (both ESBL-E and CPE) were tested for antibiotic susceptibility by the agar disk diffusion and broth microdilution methods (Fig. 4). Of the β-lactam antibiotics tested, all isolates were resistant to penicillins (AML, AMC), 1st (CL) and 2nd generation cephalosporins (CXM). More than 85 % of ESBL-E and >95 % of CPE isolates were resistant to 3rd (CAZ) and 4th generation cephalosporins (FEP), respectively. Almost all CPE isolates showed resistance to all three carbapenems tested, whereas half of the ESBL-E isolates were found to be carbapenem-resistant (51 %, ETP). Regarding resistance to other classes of antibiotics, high rates of resistance to fluoroquinolones (CIP; ≥87 %) were observed in both groups of isolates. Resistance to aminoglycosides (GM) occurred more frequently in ESBL-E (75 %) than in CPE isolates (57 %). Trimethoprim-sulfonamide resistance (SXT) was confirmed in 36 % of isolates in both groups. Colistin resistance was found at relatively low levels, but in a higher percentage (27 %) in CPE than in ESBL-E isolates (9 %).

Intrinsic resistance was considered in the evaluation of MDR and XDR profiles of *Enterobacterales* isolates (Magiorakos et al., 2012). MDR was found in all isolates (ESBL-E and CPE), and approximately 50 % of ESBL-E and 80 % of CPE isolates were XDR (Fig. 4). In addition, one CPE isolate (*E. kobei*) was classified as PDR, because it was resistant to all antibiotics tested.

3.7. Molecular detection of ARGs

The subset of 42 ESBL-E isolates underwent targeted PCR to detect ESBL and pAmpC genes. Of these isolates, those identified as colistin- or carbapenem-resistant underwent a PCR-based analysis targeting plasmid-mediated colistin resistance genes or CP genes, respectively. Sanger sequencing of the amplicons was used to determine the gene variant (Table 1). The most frequently detected ESBL gene was $bla_{\text{CTX-M-1}}$ group genes, specifically $bla_{\text{CTX-M-15}}$, which was present in 30 isolates, mainly *E. coli* (n = 13) and *K. pneumoniae* (n = 8), while $bla_{\text{CTX-M-3}}$ was detected in 4 isolates (Table 1). This was followed by $bla_{\text{TEM-116}}$, which was detected in 22 ESBL isolates. Other ESBL genes detected were bla_{SHV} ($bla_{\text{SHV-12}}$ and $bla_{\text{SHV-28}}$) and $bla_{\text{GES-7}}$, which were detected only in *Klebsiella* spp. Nineteen ESBL-E isolates possessed two ESBL genes, mainly $bla_{\text{CTX-M-15+TEM-116}}$ (Table 1). Additionally, 12/42 isolates possessed CP genes, mainly $bla_{\text{KPC-2}}$ (n = 6) and $bla_{\text{OXA-48}}$ (n = 5), whereas $bla_{\text{NDM-1}}$ was detected in only one isolate (*K. oxytoca*) (Table 1). The latter *K. oxytoca* was the only isolate in which 5 β-lactamase genes were detected ($bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, $bla_{\text{GES-7}}$, bla_{EBC} and $bla_{\text{NDM-1}}$) (Table 1). In addition, the pAmpC multiplex PCR revealed the presence of bla_{EBC} (6/42 isolates, mainly in *E. cloacae* complex (cplx) and *Klebsiella* spp.), bla_{CIT} (6/42, found only in *Citrobacter* spp.), and bla_{MOX} genes (4/42, found only in *E. coli*).

Of the 43 CPE isolates selected for targeted PCR, CP genes were detected in almost all isolates (41/43). The $bla_{\text{KPC-2}}$ (20/43, 47 %) and $bla_{\text{NDM-1}}$ (19/

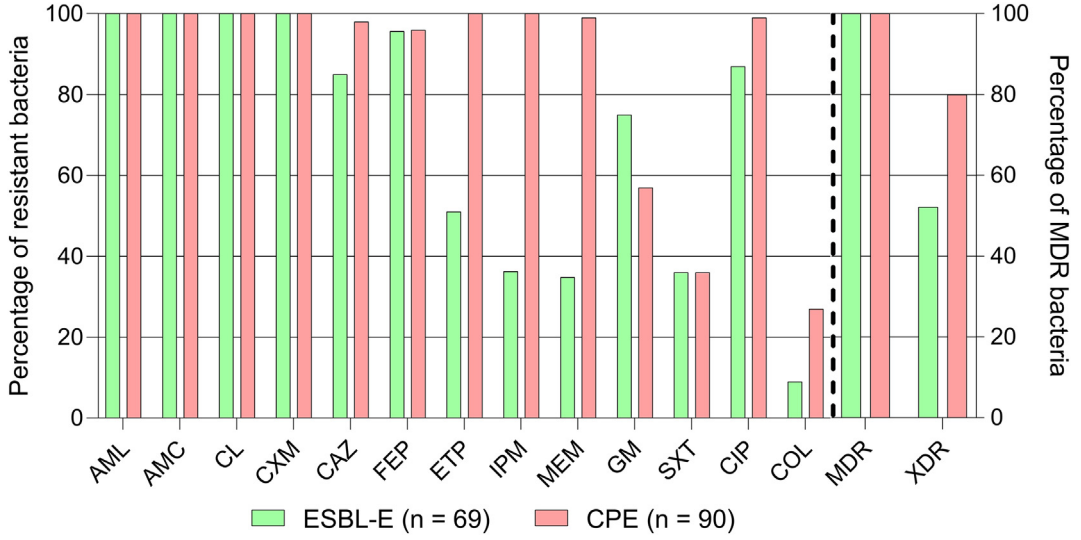

Fig. 4. Percentage of ESBL- and carbapenemase-producing *Enterobacterales* (ESBL-E and CPE) isolates from hospital wastewater samples identified with an antibiotic resistance phenotype. AML: amoxicillin; AMC: amoxicillin/clavulanic acid; CL: cephalexin; CXM: cefuroxime; CAZ: ceftazidime; FEP: cefepime; ETP: ertapenem; IPM: imipenem; MEM: meropenem; GM: gentamicin; SXT: trimethoprim/sulfamethoxazole; CIP: ciprofloxacin; COL - colistin; MDR: multidrug-resistant, XDR: extensively drug-resistant.

43, 44 %) genes were the two most frequently detected ones, especially in *Citrobacter* spp. (Table 2). *E. cloacae* cplx strains (n = 5) and *E. coli* (n = 4) were also frequent carriers of bla_{KPC-2}, whereas *K. pneumoniae* was a frequent carrier of bla_{NDM-1} (n = 7).

Other CP genes detected were bla_{OXA-48} (15/43 isolates, 35 %, mostly in *K. pneumoniae*) and bla_{VIM-1} (9/43, 21 %, mostly in *E. cloacae* cplx and *Citrobacter* spp.) (Table 2), whereas the 17/43 CPE isolates had two or more CP genes. Among these 17 isolates, the most frequent combination of genes was $bla_{OXA-48+NDM-1}$ in *K. pneumoniae* (n = 5) and *Citrobacter* spp. (n = 1) and $bla_{KPC-2+NDM-1}$ in *E. cloacae* cplx (n = 2) and *Citrobacter* spp. (n = 3) (Table 2). The most common combination of three CP genes were $bla_{KPC-2+NDM-1+VIM-1}$, detected in *Citrobacter* spp. (n = 3).

Both ESBL-E and CPE isolates were negative for mobile colistin resistance genes (*mcr*1-*mcr*5).

3.8. Molecular epidemiology of E. coli, K. pneumoniae and E. cloacae cplx isolates

Clonal relatedness of all (ESBL-E and CPE) *E. coli* (n = 58), *K. pneumoniae* (n = 22) and *E. cloacae* cplx isolates (n = 26) was determined by PFGE, and ST was determined for one representative isolate from each cluster.

For *E. coli*, a total of 6 PFGE clusters were found: A (n = 2), B (n = 4), C (n = 7), D (n = 8), E (n = 15) and F (n = 2); 14 isolates could not be assigned to any cluster and 5 could not be typed. Five different STs were found among the clustered isolates: ST216, ST405, ST361, ST541, and ST131. Isolates from clusters D and F had the same ST131, while the ST of the largest cluster (E) could not be determined. The distribution of *E. coli* isolates among clusters showed partitioning between hospitals. The largest cluster (E) included 15 isolates (with bla_{KPC-2} or $bla_{TEM-1/TEM-116}$ + $bla_{CTX-M-15}$ + bla_{MOX} + bla_{KPC-2}), all of which were from H2 hospital wastewater. Most of them showed the same AR profile and were susceptible only to SXT and COL. The *E. coli* strains of another dominant cluster (D) were ST type ST131 (bla_{TEM-1} or bla_{TEM116} + $bla_{CTXM-15}$), and all but one were from H2. The isolates of the third dominant cluster (C) belonged to ST541 (bla_{KPC-2}), were all derived from H1. They were only susceptible to GM, SXT and COL. Isolates from the latter two clusters (C and D) had phenotypic resistance to a lower number of antibiotics tested than those from the largest cluster E.

Table 1
Distribution of ESBL, pAmpC and carbapenemase genes among ESBL-producing *Enterobacterales* from hospital wastewater samples.

Isolates (n = 42)	ESBL, pAmpC and carbapenemase genes
Escherichia coli (n = 18)	TEM-1 (1)
	TEM-1 + CTX-M-15 (6)
	TEM-116 (3)
	TEM-116 + EBC (1)
	TEM-116 + CTX-M-15 (3)
	TEM-116 + CTX-M-15 + MOX + KPC-2 (4)
Klebsiella pneumoniae (n = 8)	CTX-M-15 + SHV-28 (1)
	TEM-1 + CTX-M-15 + SHV-28 (1)
	TEM-1 + CTX-M-15 + SHV-28 + EBC (1)
	TEM-116 + CTX-M-15 (1)
	TEM-116 + CTX-M-15 + SHV-12 (2)
	TEM-116 + CTX-M-15 + GES-7 (1)
	TEM-116 + CTX-M-15 + EBC + OXA-48 (1)
Klebsiella oxytoca (n = 1)	TEM-1 + CTX-M-15 + GES-7 + EBC + NDM-1 (1)
Enterobacter cloacae cplx (n = 5)	TEM-116 (2)
	TEM-116 + EBC (1)
	TEM-116 + CTX-M-15 + EBC (1)
	TEM-1 + CTX-M-15 + EBC + OXA-48 (1)
Citrobacter spp. (n = 6)	TEM −1 + CXT- M-3 + CIT (2)
	TEM-1 + CTX-M-15 + CIT (1)
	TEM-1 + CTX-M-15 + CIT + OXA-48 (1)
	TEM-116 + CTX-M-15 + CIT (2)
Other *Enterobacterales*[a] (n = 4)	TEM-1 + CTX-M-3 + OXA-48 (2)
	TEM-1 + CTX-M-15 + KPC-2 (2)

[a] Other *Enterobacterales* – *Kluyvera cryocrescens* (2), *Raoultella planticola* (1), *Raoultella ornithinolytica* (1)

Table 2
Distribution of carbapenemase genes among CPE isolates from hospital wastewater.

Isolates (n = 43)	Carbapenemase genes
Escherichia coli (n = 4)	KPC-2 (4)
Klebsiella pneumoniae (n = 14)	KPC-2 (1)
	OXA-48 (6)
	NDM-1 (2)
	OXA-48 + NDM-1 (5)
Enterobacter cloacae cplx (n = 9)	KPC-2 (3)
	KPC-2 + NDM-1 (2)
	VIM-1 (2)
	no detected genes (2)
Citrobacter spp. (n = 10)	NDM-1 (1)
	OXA-48 + NDM-1 (1)
	KPC-2 + NDM-1 (3)
	VIM-1 + NDM-1 (1)
	KPC-2 + NDM-1 + VIM −1 (3)
	VIM-1 (1)
Other *Enterobacterales*[a] (n = 6)	KPC-2 (2)
	OXA-48 (1)
	VIM-1 (1)
	KPC-2 + OXA-48 + VIM-1 (1)
	KPC-2 + OXA-48 + NDM-1 (1)

[a] Other *Enterobacterales* – *Kluyvera cryocrescens* (1), *Raoultella planticola* (2), *Raoultella ornithinolytica* (1), *Serratia marcescens* (1), *Morganella morganii* (1).

Among the 22 *K. pneumoniae* isolates, 3 PFGE clusters were found among 13 isolates, whereas the remaining 9 isolates were singletons (Fig. 5B). Three different ST were identified: ST16, ST101, and ST307. The largest cluster B included 9 isolates from H2 wastewater belonging to ST101, and most of them showed resistance to all antibiotics tested, except colistin, and carried bla_{OXA-48} or bla_{OXA-48} + bla_{NDM-1}.

For the *E. cloacae* cplx, PFGE genotyping was assessed per species (Fig. 5C). The *E. cloacae* (n = 16) were grouped into 3 clusters (n = 11), and the remaining 5 isolates were singletons. Cluster C was the largest and included 6 isolates from H2 wastewater that were ST type ST277 and showed phenotypic resistance to carbapenems (bla_{KPC-2}) and colistin but were susceptible to GM and SXT (Fig. 5C). The ST type of the second largest cluster (B) could not be determined (bla_{VIM-1}), whereas 2 isolates of cluster A belonged to ST32. Of the *E. asburiae* isolates (n = 7), only 4 were in a cluster (cluster A), originated from H2 and belonged to ST277 (bla_{KPC-2} + bla_{NDM-1}). The remaining 3 isolates were singletons (Fig. 5C). Three *E. ludwigii* isolates from H2 wastewater had a similarity percentage >94 %, and belonged to ST277. These isolates had the same genotypic (bla_{KPC-2}) and phenotypic resistance profile as *E. cloacae* isolates from cluster C (Fig. 5C). Two *E. bugadensis* isolates were singletons, and one *E. kobei* was not included in this analysis.

3.9. Presence of ARGs, STs and plasmid replicon types in isolates by WGS analysis

A total of 13 isolates were subjected to WGS analysis - four *E. coli*, two *K. pneumoniae*, three *E. cloacae*, two *E. asburiae*, one *E. ludwigii*, and one *E. kobei* (Table S8). This selection mainly included isolates from dominant and distinct clusters with clinically-relevant features, such as resistance to carbapenems and/or colistin.

Of the four different *E. coli* isolates, the STs were identified for three of them, including ST361, ST131, and ST541 while one isolate could not be typed. All three ESBL-producing *E. coli* (ST361, ST131 and ND) contained bla_{CTX-M} genes ($bla_{CTX-M-15}$ and/or $bla_{CTX-M-194}$) and the bla_{OXA-1} gene, whereas one ST361 strain additionally possessed bla_{TEM-1B}. In addition, all *E. coli* isolates contained the pAmpC bla_{EBC} gene. Phenotypic resistance to aminoglycosides was supported by the detection of *acc(6′)-Ib-cr* and/or *aac(3)-IIa* genes, whereas resistance to fluoroquinolones was supported by the presence of *qnr*B1 and/or *acc(6′)-Ib-cr*, to chloramphenicol by the detection of *cat*B3 and to trimethoprim/sulfamethoxazole by *dfr*A12 gene. Other resistance genes found comprised the *lnu*F gene (lincomycin resistance), *tetA* (tetracyline resistance), and *sitABCD* gene (resistance to biocides - hydrogen peroxide). Two ESBL-producing *E. coli* isolates (ST361 and ND) were phenotypically identified as carbapenem resistant, but WGS analysis did not detect carbapenem

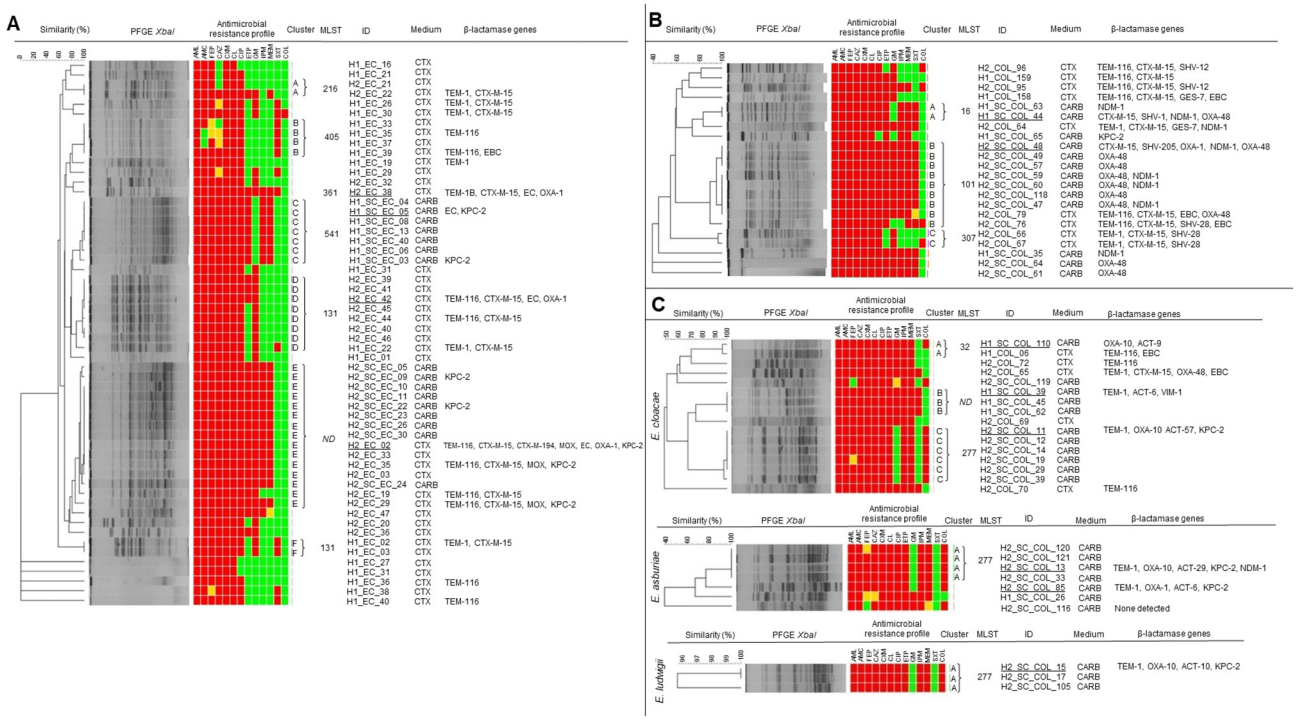

Fig. 5. Dendrogram generated with Bionumerics software showing cluster analysis of *Xba*I-PFGE patterns of (A) *Escherichia coli*, (B) *Klebsiella pneumoniae*, and (C) *Enterobacter* spp. isolates along with their antibiotic resistance phenotypes and genotypes, and multilocus sequence types (MLST). Red squares represent resistance, yellow squares represent intermediate resistance, and green squares represent susceptibility to the indicated antibiotics (AML- ampicillin, AMC – ampicillin/clavulanic acid, FEP – cefepime, CAZ – ceftazidime, CXM – cefuroxime, CL – cefalexin, CIP- ciprofloxacin, ETP – ertapenem, GM – gentamicin, IPM- imipenem, MEM – meropenem, SXT – trimethoprim/sulfamethoxazole, COL –colistin). ID stands for the name of the isolate. ND indicates that a MLST could not be completely identified. Medium indicates the selective antibiotic (CTX – cefotaxime or CARB – carbapenem) contained in the culture medium. Isolates selected for whole genome sequencing are underlined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

resistance genes in them, whereas the presence of bla_{KPC-2} was detected by PCR in one of them. This gene was also detected in CP-producing *E. coli* (ST541), and phenotypic resistance to fluoroquinolones was confirmed by the detection of the *qnr*S1 gene.

Two CP-producing *K. pneumoniae* isolates belonged to ST16 and ST101, respectively (Table S8). According to WGS, these two isolates carried the bla_{OXA-48} gene; however, targeted PCR additionally detected the presence of bla_{NDM-1}. They also possessed ESBL genes such as $bla_{CTXM-15}$, $bla_{SHV-205}$, and bla_{OXA-1}. Additional genes responsible for resistance to trimethroprim/sulfamethoxazole (*dfrA14*), (fluoro)quinolones (*aac(6′)-Ib-cr*, *oqxA*, *oqxB*, *qnrB1*), fosfomycin (*fosA*, *fosA5*), or chloramfenicol (*catB3*) were also detected in these isolates.

E. cloacae cplx included ST277 isolates (2 *E. asburiae*, *E. cloacae*, and *E. ludwigii*), ST32 (*E. cloacae*) and ST501 (*E. kobei*), whereas one isolate could not be assigned into any ST. Carbapenemase production activity was supported by the WGS-based detection of bla_{KPC-2} in ST277 and ST501 isolates or bla_{VIM-1} genes (unknown ST), and targeted PCR additionally detected bla_{NDM-1} in two *Enterobacter* isolates (ST277, ST501). In addition to the bla_{TEM} gene, which was detected in all but one *Enterobacter* spp., other resistance genes for β-lactams were observed, including $bla_{CTX-M-3}$ (in ST501), bla_{OXA-1} (in ST277), bla_{OXA-10} (in ST277, ST32), and bla_{OXA-14} (in ST501). Each isolate contained one bla_{ACT}/bla_{MIR} gene. Three isolates with phenotypic gentamicin resistance contained *aac(6′)-Ib-cr*, *aac(3)-I*, *aph(3″)-Ib*, and *aph(6′)-Id* genes, and four gentamicin-susceptible isolates contained *aac(3′)-1* and *aadA11* or *aac(6′)-Ib-cr*. Of the 7 isolates displaying phenotypic ciprofloxacin resistance, two contained *qnrB1* or *qnrS1* genes and no ciprofloxacin resistance genes were detected in the five isolates. Two *Enterobacter* isolates with phenotypic trimethoprim/sulfamethoxazole resistance contained the *sul1*, *sul2* and *dfrA14* genes. Other genes responsible for resistance to fosfomycin (*fos*A), rifampicin (*arr*-3), chloramphenicol (*catB3*), and the biocides (*qacE*) were also found sporadically. No plasmid-mediated *mcr* colistin resistance genes were detected in ST277, ST32 and ST501 strains that were phenotypically resistant to colistin. These isolates had mutations in three or more genes (*pmrA*, *pmrB*, *phoP*, *phoQ* and *mgrB*) which are associated with colistin resistance. The total number of numerous mutations detected in all six *Enterobacter* spp. isolates was 343 and the analysis in PROVEAN revealed 91 unique mutations (6 deleterious - PmrA – P174A; PmrB – E218G, S308Q, G309R, L310S; PhoP – N174S; and 85 neutral mutations) (Table S9). Multiple amino acid substitutions were noted for all proteins except for MgrB that had one amino acid substitution (V10I) found in 4 *Enterobacter* spp. isolates. The L133I mutation in the *phoQ* gene was reported for the first time in this study, as indicated in Table S9.

The genetic context of the bla_{CTX-M}, bla_{OXA-48}, bla_{KPC-2}, and bla_{VIM-1} genes was analysed using RAST annotations (Fig. 6). The bla_{CTX-M} genes ($bla_{CTX-M-15}$, $bla_{CTX-M-194}$ and $bla_{CTX-M-3}$) were flanked by insertion sequences (*ISEc9* or IS6 family) and/or transposases (Tn*3* family) in all genomes (analysed by WGS) in which these genes were detected (6/13). The bla_{OXA-48} gene was flanked by a *lys*R gene in *K. pneumoniae* ST101 and ST16 and by an IS4 family insertion sequence in *K. pneumoniae* ST101. The bla_{KPC-2} gene was flanked in five *Enterobacter* spp. (ST277 and ST501) by genes belonging to the Tn*3*-based transposon family (*ISKpn6* and *ISKpn27*). Two ST277 *Enterobacter* isolates (*E. cloacae* and *E. ludwigii*) carried bla_{KPC-2} together with the bla_{TEM-1} gene as part of this Tn*3* transposon. Similarly, bla_{KPC-2} was present in ST541 *E. coli* associated with the Tn*3*-based transposon, Tn*4401*. The bla_{VIM-1} was associated in *E. cloacae* (unknown ST) with genes encoding resistance to aminoglycosides (*aac(6′)-Ib-cr*, *aadA1*), biocides (*qacEΔ1*), and sulfonamides (*sul1*).

Finally, plasmids were detected in all 13 sequenced isolates, with eleven isolates containing more than one replicon (Table S8). In total, 19 different

plasmid replicon types were identified in *E. coli*, *K. pneumoniae*, and *Enterobacter* spp. The most frequently detected plasmid replicon types were IncFIB (n = 9) and IncHI2 (n = 6).

4. Discussion

This study focused on the resistance to 3rd generation cephalosporins and carbapenems, especially among *Enterobacterales*, which are now among the most prevalent ARB threatening human health. Monitoring of these bacteria and their ARGs from high-risk point sources such as hospital wastewater is critical to obtain the information needed to track their spread in the environment.

The culture-based enumeration of *E. coli* and other coliforms resistant to CTX (3rd generation cephalosporin) or carbapenems in hospital wastewater analysed here showed that concentrations ranged from 10^3 to 10^4 CFU/mL, which is up to two orders of magnitude higher than in the influent of the receiving WWTP in Zagreb (Puljko et al., 2022). This high prevalence of CTX-R and CR *E. coli* and non-*E. coli* coliforms in wastewater from the two large hospitals in Zagreb is comparable or lower than in several previous studies, including hospital wastewater from neighbouring Austria and Slovenia (Rozman et al., 2020). The lack of on-site treatment of sewage in Zagreb hospitals exacerbates the potential for further spread and resulting health impact of bacterial resistance to last-line antibiotics such as carbapenems.

The further quantification of five CP genes ($bla_{\text{OXA-48}}$, $bla_{\text{KPC-3}}$, bla_{NDM}, bla_{IMP}, and bla_{VIM}) by qPCR in wastewater from both hospitals showed that $bla_{\text{KPC-3}}$ was detected at the highest levels and reached relative levels of up to 10^{-1} gene copies/*rrn* copies. These levels were unusually high compared to previously published concentrations of bla_{KPC} in hospital wastewater samples (around 10^{-5} gene copies/*rrn* copies) (S. Zhang et al., 2020). This high prevalence of bla_{KPC} in the studied hospital wastewater is consistent with the frequent detection of KPC-producing isolates in Croatian hospitals, especially *K. pneumoniae* (Bedenić et al., 2015, 2021; Jelic et al., 2016). The potential risk of this gene would be exacerbated by the possibility of horizontal transmission between strains, as has already been demonstrated in clinical isolates in Croatia (Jelic et al., 2016). Finally, the fact that bla_{KPC} is predominantly associated with hospital wastewater but rarely detected in the environment (Jelić et al., 2019; Hooban et al., 2020) may lead to the prioritisation of monitoring this gene to detect potential leakage from inadequately treated hospital wastewater. The concentrations of other CP genes, $bla_{\text{OXA-48}}$, bla_{NDM}, bla_{VIM}, and bla_{IMP}, as well as two ESBL genes, bla_{TEM} and $bla_{\text{CTX-M-32}}$, were within the range of previous studies ($\sim 10^{-3}$ to 10^{-4} gene copies/*rrn* copies) (Rodriguez-Mozaz et al., 2015; Flach et al., 2021). Moreover, the qPCR-based quantification of the WHO priority pathogens *E. coli*, *K. pneumoniae*, *A. baumanii*, and *Enterococcus* spp. showed that hospital wastewater contained all of these pathogens at concentrations of 10^3 to 10^5 CE/mL (or 10^{-3} to 10^{-5} CE/*rrn* copies), which were comparable to those measured in German hospital wastewater samples (Alexander et al., 2022). All these results confirm that hospital waste is an important reservoir for high-priority pathogens and ARGs and a pathway for their dissemination in water systems.

To place the obtained ARG data in a medical context, culture-based methods paired with molecular methods such as WGS and PCR were used to investigate the phenotypic and molecular mechanisms of resistance in CR and CTX-R enterobacterial isolates. Sixty-nine CTX-R and 90 CR *Enterobacterales* were successfully isolated from our hospital wastewater samples. The mechanisms underlying resistance to CTX and carbapenems in these isolates were the production of ESBL and carbapenemases, respectively. Among the CPE isolates, *Citrobacter* spp. (34 %), *Enterobacter* spp. (26 %), *E. coli* (18 %) and *Klebsiella* spp. (16 %), dominated, whereas *E. coli* (61 %), *Klebsiella* spp. (13 %), and *Citrobacter* spp. (12 %) were predominant among ESBL-E isolates. All isolates tested were found to be MDR, consistent with the ability of enterobacteria to acquire various ARGs via HGT, which is mostly mediated by plasmids (Cantón et al., 2012).

ESBL-E isolates had high rates of resistance to fluoroquinolones (87 %), aminoglycosides (75 %), and even carbapenems (ETP, 51 %), in addition to 3rd and 4th generation cephalosporins. Further genetic characterization of these isolates revealed that the most common ESBL genotype was $bla_{\text{CTX-M-15}}$ (71 %) and $bla_{\text{TEM-116}}$ (52 %), whereas bla_{SHV} (12 %) ($bla_{\text{SHV-12}}$ and $bla_{\text{SHV-28}}$) was rare. Moreover, $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-116/TEM-1}}$ co-occurred in the majority of our *E. coli*, *Klebsiella* spp. and *Citrobacter* spp. isolates, whereas $bla_{\text{CTX-M-15}}$, bla_{TEM}, and bla_{SHV} co-occurred only in *K. pneumoniae* isolates (10 %). This is consistent with previous reports from Zagreb hospitals, including H1 studied here, describing a frequent association

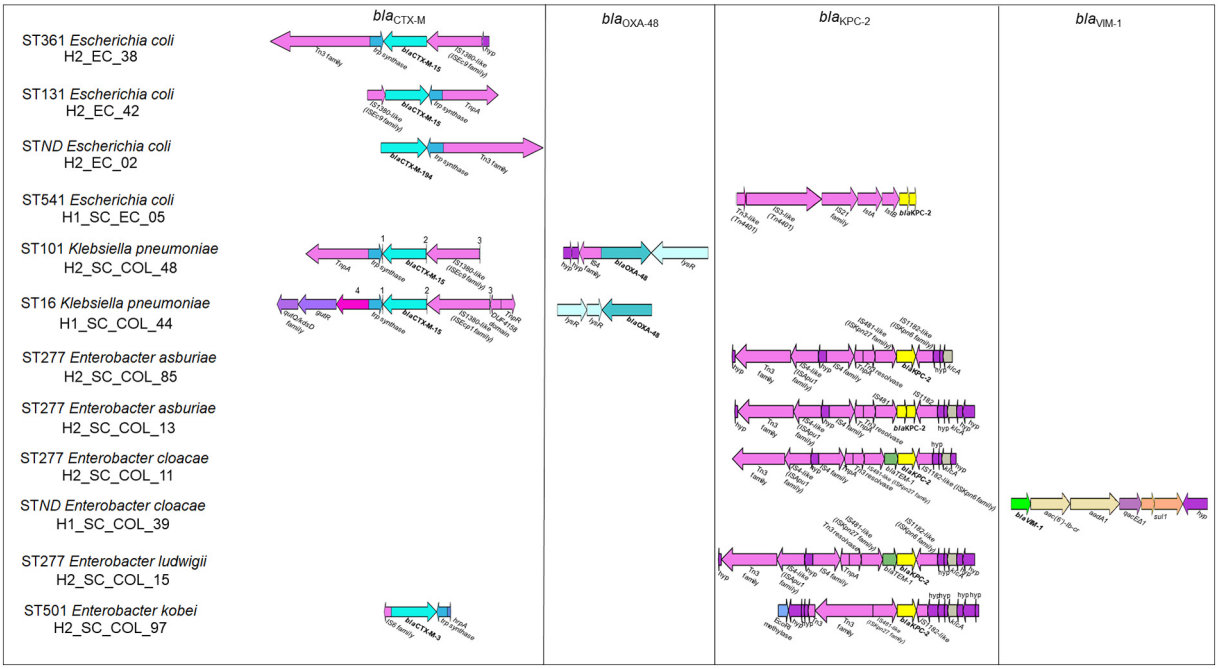

Fig. 6. Schematic presentation of the genetic environment of ESBL ($bla_{\text{CTX-M}}$) and carbapenemase genes ($bla_{\text{OXA-48}}$, $bla_{\text{KPC-2}}$ and $bla_{\text{VIM-1}}$). Numbers (1–3) denote predicted regions for cefepime resistance (1), aztreonam resistance (2), ciprofloxacin resistance (3), and mannose-1-phosphate guanylytransferase (4). ST*ND*- sequence type could not be completely identified. Analyses were performed using RAST annotation and the Gene Graphics web application (https://genegraphics.net).

of these genes in clinical enterobacterial isolates (Bedenić et al., 2016; D'Onofrio et al., 2020). In addition, co-production of ESBL (bla_{CTX-M}, bla_{TEM}, bla_{SHV}), pAmpC (bla_{MOX}, bla_{EBC} or bla_{CIT}), and CP genes (bla_{KPC-2}, bla_{OXA-48} or bla_{NDM-1}) was observed in some of the ESBL-E isolates analysed here, consistent with a worldwide survey of clinical enterobacterial isolates (Kazmierczak et al., 2021).

CPE isolates showed a high rate of resistance to all β-lactam antibiotics tested, including carbapenems, and to fluoroquinolones (99 %), but also to a lesser extent to aminoglycosides (57 %). However, 27 % CPE isolates were resistant to colistin, which may lead to treatment failure if spread further, as carbapenems and colistin are considered as last choice antibiotics for the treatment of MDR bacteria. The bla_{KPC-2} and bla_{NDM-1} were the most frequently detected CP genes in this study, particularly in *Citrobacter* spp. These species are becoming increasingly important in the hospital setting as emerging carriers of CPs, with KPC-2, OXA-48 or VIM predominating depending on the geographic location (Arana et al., 2017; Babiker et al., 2020; Yao et al., 2021).The presence of bla_{VIM-1} and bla_{NDM-1} has also been reported in *Citrobacter freundii* from hospital H1 studied here (Atalić et al., 2014; Bedenić et al., 2016). Our results also show the co-occurrence of two CPs (mostly $bla_{KPC-2+NDM-1}$) in *Citrobacter* spp. which is consistent with some previous studies (Yao et al., 2021), or even three different CPs such as $bla_{KPC-2+NDM-1+VIM-1}$, which to our knowledge has not been reported before. This indicates that these *Citrobacter* spp. could be a relevant reservoir for potentially transmissible carbapenem resistance in hospital wastewater. Moreover, in the present study, carbapenem-resistant *K. pneumoniae*, which are an emerging public health problem in Croatia and other EU countries, were found to contain predominantly bla_{OXA-48} and bla_{NDM-1} genes, which frequently co-occur. This is consistent with previous findings showing that bla_{OXA-48} and bla_{NDM-1} are frequently detected CP genes in clinical *K. pneumoniae* in Zagreb hospitals (Bedenić et al., 2016, 2022) and hospitals from surrounding countries such as Italy (Lorenzin et al., 2022), Slovenia (Benulič et al., 2020) and Serbia (Palmieri et al., 2020). Isolation of this WHO priority pathogen from hospital wastewater provides a secondary reservoir and possible transmission route for these bacteria to natural waters and the community.

Molecular epidemiology using PFGE coupled with WGS identification of ARGs and MLST was further performed on critical priority pathogens (WHO, 2017): ESBL- and CP-producing *E. coli*, *K. pneumoniae*, and *Enterobacter* spp. Among the *E. coli* isolates, the ST of the largest cluster could not be identified, possibly due to their environmental origin. In addition, WGS analysis of a representative of this cluster revealed multiple ARGs for β-lactams, including ESBL ($bla_{CTX-M-15}$, $bla_{CTX-M-194}$, $bla_{TEM-116}$) and CP genes (bla_{KPC-2}), and for other priority antibiotics, as well as the presence of several plasmids, including those already associated with the transmission of $bla_{CTX-M-15}$ (IncFIA and IncFIB) or bla_{KPC-2} (IncR and Col440I) (Chen et al., 2014; Nicolas-Chanoine et al., 2014; Rocha-Gracia et al., 2022). *E. coli* ST131 and ST541 were among the most common sequence types detected in this study. Interestingly, ST131 has also been found in human MDR isolates from hospitals in Croatia (Krilanović et al., 2020) and other countries (Price et al., 2013), and in the environment including municipal wastewater (Hocquet et al., 2016; Lopes et al., 2021). In this study, *E. coli* ST131 had ESBL ($bla_{CTX-M-15+TEM116}$) and pAmpC genes (bla_{EBC}) and was found by WGS to have ARGs to other antibiotics such as gentamycin and chloramfenicol and biocides (peroxides). It also contained the plasmid replicon types IncFIA and IncFIB which have been associated with the spread of the $bla_{CTX-M-15}$ gene (Nicolas-Chanoine et al., 2014; Rocha-Gracia et al., 2022). In another ST, ST541, detected in CP-producing *E. coli* strains, bla_{KPC-2} and ARGs to several other antibiotic classes were detected. This ST is rare and has been detected in livestock in Asia (Chan et al., 2014; Qiu et al., 2019).

Among the *K. pneumoniae* isolates examined in this study, ST101 was the most prevalent multidrug-resistant clone, with phenotypic resistance to all β-lactams including carbapenems. This ST was predominantly associated here with the bla_{OXA-48} gene which was flanked by the IS4 family transposase IS10A, previously found predominantly in pOXA-48 plasmids (Hendrickx et al., 2021). This suggests that ST101 *K. pneumoniae* has the potential to spread carbapenem resistance through horizontal transmission. In agreement with our results, the presence of bla_{OXA-48} and bla_{NDM-1} in ST101 *K. pneumoniae* isolates has recently been reported in Italian and Slovenian hospitals (Nucleo et al., 2020; Benulič et al., 2020). In addition, this ST has also been detected in hospitals and treated hospital wastewater in Serbia and Romania, respectively (Novović et al., 2017; Popa et al., 2021) Other STs detected in *K. pneumoniae* isolates were ST16, associated with CP producers carrying bla_{OXA-48} and/or bla_{NDM-1} and $bla_{CTX-M-15}$ ESBL, and ST307, associated with ESBL producers carrying $bla_{CTX-M-15}$ and bla_{SHV-28}. Previous studies from Croatia have reported the occurrence of bla_{NDM-1} or bla_{OXA-48} in clinical ST16 *K. pneumoniae*, but the co-occurrence of bla_{NDM-1} and bla_{OXA-48} has not yet been reported in this lineage in Croatia (Kocsis et al., 2016; Bedenić et al., 2018; Jelić et al., 2018). In other countries, ST16 is frequently associated with co-occurrence of $bla_{OXA-232}$ and bla_{NDM-1} (Abe et al., 2022; Avolio et al., 2017; Espinal et al., 2019). Furthermore, ST307 has also been described in CTX-M-15-producing *K. pneumoniae*, which caused a nosocomial outbreak in Germany (Haller et al., 2019). In addition, WGS showed that both ST16 and ST101 contained ARGs for several antibiotic classes other than β-lactams and several plasmid replicon types, including IncFIA, IncFIB, and IncR that have previously been associated with the carriage of $bla_{CTX-M-15}$ in *K. pneumoniae* (Wyres et al., 2019; Silva et al., 2022). Apart from the likely plasmid association, $bla_{CTX-M-15}$ was flanked by insertion sequences and Tn*3* type transposon in our *K. pneumoniae* and *E. coli* isolates, highlighting the role of these platforms in its further dissemination (Zhao and Hu, 2013; Grevskott et al., 2020).

The majority of *E. cloacae* cplx isolates analysed in this study were carbapenemase producers belonging to ST277, which to our knowledge has not been previously detected in humans or environmental samples. This ST was MDR with carbapenem and colistin resistance being the most commonly detected resistance phenotypes. WGS showed that these isolates harboured CP genes bla_{KPC-2} or bla_{KPC-2} + bla_{NDM-1} and several other ARGs for β-lactam and other antibiotic classes and biocides, but no mobile colistin resistance genes. However, point mutation analysis of these *E. cloacae* cplx isolates identified mutations in the *pmrA*, *pmrB*, *phoP*, *phoQ* or *mgrB* genes that most likely confer the observed colistin resistance, suggesting chromosomally associated resistance mechanisms. In addition, WGS showed that these isolates contained a diverse plasmidome, including plasmid replicon types associated with the carriage of bla_{KPC-2} (IncFII, IncN, IncP6, IncR, IncX5, and Col440I) (Chen et al., 2014; Souza et al., 2019; Yao et al., 2017) or bla_{NDM-1} (IncFIB, IncN, and IncR) (Wu et al., 2019). This suggests that these CP genes may spread further via HGT. Furthermore, *E. cloacae* isolates whose ST classification could not be successfully identified (cluster B) contained bla_{VIM} and mobilizable plasmids such as IncHI2, IncHI2A, and IncC, which are commonly associated with the transmission of this gene (Arcari et al., 2020; Sadek et al., 2020). The bla_{VIM} gene was genetically linked to several ARGs for other classes of antibiotics or antiseptics in these isolates, suggesting possible common transmission of these genes via HGT. Finally, a ST501 *E. kobei* isolate that had previously successfully colonized and persisted in hospital sinks and plumbing (Aranega-Bou et al., 2021), was phenotypically identified here as PDR. This was supported by the presence of a variety of ARGs, including the carbapenemases KPC-2 and NDM-1, which could be associated with the detected plasmids IncN and IncR, respectively (Chen et al., 2014; Gamal et al., 2016; Wang et al., 2018). Of note, bla_{NDM-1} was not detected in this and several other isolates by WGS, but only by targeted PCR paired with Sanger sequencing, which may be due to loss of plasmid carrying bla_{NDM-1} during repeated subcultures compared to the first subculture used for DNA isolation for targeted PCR. Finally, the analysis of the genomic context of bla_{KPC-2} showed that *Enterobacter* isolates carried this gene as part of a non-Tn*4401* transposon, as also reported in clinical *Enterobacter* isolates from Colombia (De La Cadena et al., 2018) and environmental *Klebsiella* isolates from Brazil (Janssen et al., 2021), suggesting the potential for mobilization.

5. Conclusions

The results of this study, which is the first of its kind in Croatia, show that the wastewater from the two major hospitals in Zagreb contains

relatively high levels of coliform bacteria resistant to 3rd generation cephalosporins and carbapenems, as well as clinically significant ESBL and CP genes. Of concern is the presence of the multidrug-resistant WHO priority pathogens with both intrinsic (point mutations involved in colistin resistance) and acquired resistance mechanisms (ESBL and carbapenemase) previously reported in patients from local hospitals as well. So there is a possibility that these pathogenic strains and antibiotic-resistant strains can be transmitted into the water systems and then back to humans and animals. Effective treatment of hospital wastewater with advanced treatment methods such as UV and ozone treatments must therefore be ensured to reduce or stop the spread of ARB and ARGs of clinical concern in the natural environment.

CRediT authorship contribution statement

Ana Puljko: Investigation, Writing – original draft, Writing – review & editing, Formal analysis, Visualization. **Svjetlana Dekić Rozman:** Investigation. **Ivan Barišić:** Conceptualization, Methodology, Formal analysis, Resources, Writing – review & editing. **Ana Maravić:** Investigation, Writing – review & editing. **Marko Jelić:** Investigation, Formal analysis, Visualization, Writing – review & editing. **Ivana Babić:** Investigation, Visualization, Writing – review & editing. **Milena Milaković:** Investigation. **Ines Petrić:** Investigation, Writing – review & editing. **Nikolina Udiković-Kolić:** Conceptualization, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161805.

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Chapter 6

General discussion

6.1. Efficiency of municipal WWTPs in the removal of EOPs, 3GCs- and carbapenem-resistant coliforms and associated ARGs

In Chapter 2, culture-based and qPCR-based methods were used to investigate the fate of 3GC- and carbapenem-resistant determinants (bacteria and genes) during wastewater treatment. The focus was on culturable 3GC- and carbapenem-resistant coliforms and selected EOPs, ESBL and carbapenemase genes, which were quantified in untreated and treated wastewater from WWTPs of seven Croatian cities (Zagreb, Vinkovci, Bjelovar, Čakovec, Varaždin, Karlovac, and Zadar) during the winter and summer seasons in 2020. All these WWTPs operated with conventional activated sludge treatment and differed in their design capacity (43,000 – 1,200,000 people equivalent) and in the number of hospitals and size (i.e. hospital beds) in their catchment. Given the clinical importance of enterobacteria and their associated genes conferring resistance to 3GCs and carbapenems, it is crucial to assess their removal in WWTPs to provide information as a basis for risk assessment. Such assessments are critical for the development of effective strategies to control the spread of such ARB and their ARGs of high clinical concern into the environment. The results of this part of the research were published in *Publication No. 1*.

The quantification of culturable total coliforms (*E. coli* and other coliforms) as well as cefotaxime-resistant (CTX-R, CTX representing the 3GCs antibiotics) and carbapenem-resistant (CR) coliforms from untreated and treated wastewater, showed a consistent decrease of about 2 log units in most WWTPs in both seasons. This means that the efficiency of the WWTPs in removing of these species from the wastewater was 99%. These results were comparable to those of an European WWTPs where the removal of total and CTX-R coliforms by conventional wastewater treatment was around 2.1-2.3 log units or 99% (Marano et al., 2020). Despite the good efficiency of wastewater treatment, it is concerning that the two largest WWTPs investigated, namely those in Zagreb and Varaždin, still discharged significant quantities of up to 10^{11} CTX-R or CR *E. coli* and up to 10^{12} CTX-R or CR other coliforms per day into the receiving rivers. Previous studies have also shown that WWTPs could discharge around 10^{10}-10^{12} 3GCs-resistant *E. coli* per day (Bréchet et al., 2014; Kwak et al., 2015). Once released, these resistant *E. coli* and other coliforms can serve as a reservoir for cefotaxime and carbapenem ARGs in the environment. These genes are also often located on conjugative plasmids, which facilitates their horizontal transfer to other environmental bacteria or even pathogens. In addition, Croatian legislation only regulates the

presence of *E. coli* in treated municipal wastewater discharged into surface waters used for bathing. According to this regulation, the concentration of *E. coli* in treated wastewater used for bathing should not exceed 10^3 CFU/100 mL (Official Gazette of the Republic of Croatia 26/20). However, the results of this study show that the *E. coli* concentration in all treated wastewater samples exceeded these values in both seasons and reaching up to 10^5 CFU/100 mL. In addition, concentrations of CTX-R and CR *E. coli* in treated wastewater from Bjelovar and Varaždin WWTPs exceeded 10^3 CFU/100 mL in both seasons. Although receiving waters of the studied WWTPs are not official bathing areas, the discharge of these ARB may lead to adverse effects on ecosystem health and aquatic life. It also has potential health hazards resulting from indirect exposure to contaminated water through food consumption (fish) or crop irrigation. Furthermore, the results of the analysis of culturable *E. coli* were consistent with those of the qPCR analysis, in which the *ycc*T gene was specifically selected for the quantification of *E. coli*. This qPCR assessment has shown that the *E. coli* concentration in treated wastewater has decreased significantly compared to untreated wastewater in most of the WWTPs. However, despite this positive trend, the treated wastewater still showed relatively high *E. coli* concentrations, typically between 10^4 and 10^5 cell equivalents (CE) per 100 mL. It is noteworthy that the Varaždin WWTP has the lowest reduction rates for *E. coli*, which is consistent with the minimal decline of these culturable bacteria after the treatment. This observation suggests that the initially high *E. coli* concentration at this WWTP may persist after the treatment, initially influenced by various factors, including contamination from the local poultry industry. The insufficient reduction of *E. coli* in the Varaždin WWTP compared to other Croatian WWTPs could indicate differences in the composition of untreated wastewater, the operating conditions and the characteristics of the activated sludge system (Krzeminski et al., 2019). However, the discharge of such wastewater poses a significant risk for contamination of surface waters with *E. coli*, especially with strains that are resistant to 3GCs and carbapenems and can easily spread in the ecosystem. In contrast, when other EOPs were analyzed using qPCR, a significant reduction was observed. Particularly, the target genes *sec*E for *A. baumannii* in winter, and *glt*A for *K. pneumonia* in both seasons were not detectable in the treated wastewater samples. However, it is important to note that the lack of detection of these pathogens does not definitely indicate their complete removal. Rather, it indicates that their concentrations were below the detection limit, possibly still present and spreading into the receiving rivers. However, similar to *E. coli*, the 23S rRNA gene for enterococci was found in

relatively high concentrations, ranging from 10^6 to 10^7 CE/100 mL, which was particularly noticeable in the Varaždin WWTP. These observations indicate the possibility that various factors within the wastewater treatment processes can selectively influence certain groups of bacteria, while others are effectively eliminated. Therefore, although the overall counts of culturable CTX-R and CR coliforms as well as EOPs has significantly decreased, their continued presence in treated wastewater underscores the urgent need for improved wastewater treatment methods and proactive measures to curb the spread of AMR into the environment.

To assess the impact of wastewater treatment on important β-lactam resistance genes in the clinical settings, a qPCR analysis was performed. This analysis aimed to quantify ESBL genes associated with resistance to 3GCs (*bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$) and carbapenemase genes associated with resistance to cabapenems (*bla*$_{\text{KPC-3}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$, and *bla*$_{\text{IMP}}$) in all seven WWTPs. Gene abundances were normalized to the total bacterial content, which was assessed by quantifying 16S ribosomal RNA genes. The relative abundance of these genes in untreated and treated wastewater varied, depending on the type of gene, the WWTP and the season. For example, the *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$ genes were consistently present in all untreated wastewater samples throughout both seasons, although in winter their amount was about one order of magnitude lower than in summer. This was also reflected in the relative abundance of the two genes in the treated water in summer. It was also consistent with the significant positive correlation observed between *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$ and temperature. The warmer weather may exacerbate bacterial contamination in wastewater as it favors the survival and proliferation of certain bacteria that could carry those genes. Moreover, except for *bla*$_{\text{CTX-M-32}}$ in winter, both *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M-32}}$ genes were only slightly reduced or even increased after the treatment, suggesting possible fluctuations in their host abundance or even HGT to a new host during the treatment. In addition, treated wastewater from Croatian WWTPs investigated here showed an order magnitude higher abundance of these genes, compared to European WWTPs (Cacace et al., 2019). Comparing to clinical settings, *bla*$_{\text{CTX-M}}$ is one of the most dominant β-lactamase gene in enterobacterial isolates from Croatian hospitals (Bedenić et al., 2022). Furthermore, positive correlation between *bla*$_{\text{CTX-M-32}}$ and *bla*$_{\text{TEM}}$ was observed, which is consistent with the occurrence of those two genes on the same plasmids (Hembach et al., 2017). Given their ubiquitous occurrence in both clinical and wastewater system, *bla*$_{\text{TEM}}$ and *bla*$_{\text{CTX-M}}$ has been suggested as indicator genes for monitoring anthropological contamination in environmental settings (Liguori et al., 2022; Narciso-Da-Rocha et al., 2014).

Notably, the detection of *bla*$_{TEM}$ has shown a significant correlation with the amount of qPCR-quantified *E. coli*, suggesting that *E. coli* may be one of the main carriers of *bla*$_{TEM}$.

In addition to ESBL genes, the main focus of this study was on the quantification of carbapenemase genes. These genes are of particular importance in the clinical context as they confer resistance to carbapenems, which are crucial antibiotics used as a last resort to treat infections caused by MDR bacteria. The detection of carbapenemase genes is no longer limited to hospitals, as they have also been found in the community, in wastewater and in receiving waters (Jelić et al., 2019; Proia et al., 2018; Sib et al., 2020). Their dissemination is often associated with mobile genetic elements that may favor their spread across different bacterial species (Subirats et al., 2017; Zhang et al., 2020). In Croatia, the first carbapenemase genes detected in clinical isolates were VIM and NDM, until the prevalence of OXA-48 increased after 2015 (Bedenić et al., 2018; Zujić Atalić et al., 2014). The *bla*$_{OXA-48}$ was successfully eliminated from most Croatian wastewater, indicating its good removal during the wastewater treatment. These results could be due to good removal of *A. baumannii* as a potential host of these genes, since the positive correlation was observed between *bla*$_{OXA-48}$ and *A. baumannii*. Previously studies have shown that *A. baumannii* is a potential carrier of *bla*$_{OXA-48}$ (Assem et al., 2017; Gonçalves et al., 2013). Other targeted carbapenemase genes, such as *bla*$_{KPC-3}$, *bla*$_{NDM}$ and *bla*$_{IMP}$ have been detected sporadically in untreated wastewater from certain WWTPs. Earlier studies have also identified the sporadic presence of these genes in untreated wastewater from various WWTPs in Europe (Pärnänen et al., 2019; Subirats et al., 2017). However, their presence in treated wastewater was remarkably rare, with the exception of *bla*$_{IMP}$. It was observed that the *bla*$_{IMP}$ was exclusively detected in treated wastewater samples (4 in winter and 3 in summer), while it was not present in paired untreated samples, suggesting a possible proliferation of *bla*$_{IMP}$-carrying bacteria or a HGT during the treatment process. Similarly, the *bla*$_{VIM}$ gene showed a pattern of enrichment during treatment. This is worrying because bacteria carrying these genes are spreading into the environment and pose a risk of ARG transfer to other bacteria, including human pathogens, which can lead to infections that are difficult to treat.

To clarify the underlying factors contributing to the observed distribution and variability of CTX-R and CR coliforms, EOPS, as well as ESBL and carbapenemase genes, a comprehensive correlation analysis was performed. The analysis attempted to establish relationship between

culture-based and qPCR data and specific characteristics of WWTPs such as physicochemical parameters of treated wastewater, the size of WWTPs, and the numbers of hospital and hospital beds in the catchment. The results showed that the concentration of CTX-R and CR coliforms, as well as enterococci in treated wastewater was positively influenced by both chemical oxygen demand (COD) and suspended solids. This suggests that certain treatment conditions may actually promote the survival of these ARB and pathogens. However, the concentration of both *bla*$_{VIM}$ and *bla*$_{IMP}$ genes showed an opposite trend, decreasing when the COD concentration increased in the treated wastewater, suggesting that treatment conditions may affect different AMR determinants in different ways. In addition, *bla*$_{TEM}$ and *bla*$_{CTX\text{-}M\text{-}32}$ showed a positive correlation with temperature, as mentioned above, suggesting proliferation and persistence of bacteria with these genes at warmer temperatures (Pärnänen et al., 2019). Moreover, *bla*$_{IMP}$ showed a correlation with the plant sizes and the number of hospitals in its catchment, while *bla*$_{VIM}$ also correlated with the number of hospitals, and the number of beds in hospitals. These results are in contrast to previous findings reported by Cacace et al. (2019), where the number of hospital and hospitals beds had no significant impact on the amount of ARGs released from WWTPs. It is evident that different characteristics of WWTPs may have different impacts on the development of AMR, and predicting an accurate selective effect is challenging due to this complexity. Therefore, the conditions and characteristics of WWTPs should be considered when evaluating AMR monitoring and ultimately maximizing the removal of AMR determinants.

Overall, the findings of Chapter 2 have shown that the conventional treatment processes in seven Croatian WWTPs are insufficient to completely eliminate priority pathogens such as *E. coli* and enterococci, as well as coliform bacteria resistant to 3GCs and carbapenemes and the corresponding resistance genes. Worryingly, the treated wastewater in the majority of these WWTPs often contains elevated relative abundances of ARGs of the high clinical concern such as *bla*$_{VIM}$ and *bla*$_{IMP}$. Based on these results published in *Publication No. 1*, the hypothesis (i) Enterobacterales resistant to 3GCs and carbapenems and corresponding resistance genes are not completely removed from municipal WWTPs is accepted.

6.2. Treated municipal wastewater as a source of MDR Enterobacterales producing ESBLs and carbapenemases

In Chapter 2, culture-based monitoring of treated wastewater was used as a surveillance tool to assess the potential exposure of priority resistant pathogens from several Croatian municipal WWTPs to the environment. The results showed that all WWTPs discharged a significant amount of 3GCs-resistant Enterobacterales and CRE into the environment after the treatment. As a results, Chapters 3 and 4 focused on a detailed investigation of these resistant strains from treated wastewater in the largest Croatian WWTP in Zagreb. Although the cultivation of bacteria allows only about 2% of the environmental microbiome to be grown in the laboratory conditions, it also allows the isolation of pathogens of interest that may be present in the environment (Liguori et al., 2022), and their phenotypic analysis. The aim of this part of the dissertation was to thoroughly characterize these enterobacteria, particularly ESBL and carbapenemase producers, in order to gain insights into their properties. Their AMR patterns, genetic relationship with known high-risk clones, the molecular mechanisms of resistance, and their potential to cause infections were investigated. The results of the ESBL-E strains were presented in *Publication No. 2*, while the results regarding CRE were described in *Publication No. 3*.

6.2.1. Phenotypic and genomic characterization of ESBL-E

In this study, 140 ESBL-E were successfully isolated from treated wastewater of the Zagreb WWTP, including opportunistic pathogens such as *E. coli*, *Klebisella* spp., and *E. cloacae* complex (cplx.) (*Publication No. 2*). A worrying observation was that almost all of these isolates (94%) showed MDR phenotype, i.e. resistance to three or more unrelated classes of antibiotics. Furthermore, over 40% of MDR *Klebsiella* and *E. cloacae* cplx isolates were found to be resistant to colistin, a last-resort antibiotic. Alarmingly, up to 22% of these isolates were resistant to both carbapenems and colistin, highlighting the urgent need for alternative antibiotics in such cases.

PCR screening of ESBL-E isolates for selected ESBL genes showed a remarkable prevalence of the $bla_{\text{CTX-M}}$ gene, especially its variant $bla_{\text{CTX-M-15}}$ in *E. coli* and *Klebsiella* spp. as well as *Roultella* species. Recent Croatian studies have identified $bla_{\text{CTX-M-15}}$ gene as the predominant ESBL gene found in *E. coli* and *K. pneumoniae* from patients (Bedenić et al., 2022; Krilanović et al., 2020). In addition, the same species carrying $bla_{\text{CTX-M-15}}$ have previously been

isolated from wastewater (Davidova-Gerzova et al., 2023; Radisic et al., 2023). These observations indicate a wide distribution of *bla*$_{\text{CTX-M-15}}$ outside the clinical settings, which could be attributed to its association with different mobile genetic elements, including plasmids (Mathers et al., 2015). Furthermore, both *E. coli* and *Klebsiella* isolates carrying *bla*$_{\text{CTX-M-15}}$ often also carried other ESBL genes, particularly *bla*$_{\text{TEM-116}}$, while *K. pneumoniae* also had *bla*$_{\text{SHV}}$ genes, reflecting previous findings in hospitals and marine beaches in Croatia (Bedenić et al., 2016; D'Onofrio et al., 2020; Maravić et al., 2015). The SHV-ESBL variants are often encoded by self-transmissible plasmids that carry resistance genes to other antibiotic classes (Liakopoulos et al., 2016). In addition, *bla*$_{\text{TEM-116}}$ is mostly chromosomally associated gene, however environmental *Acinetobacter* species from Croatia harbored conjugative plasmid carrying *bla*$_{\text{TEM-116}}$ (Maravić et al., 2016), indicating the possibility of HGT between species. Along with *E. coli* and *Klebsiella* species, *E. cloacae* cplx. primarily had *bla*$_{\text{TEM-116}}$ gene for conferring resistance to 3GCs. Interestingly, two *E. cloacae* cplx. isolates did not possess ESBL gene, but carbapenemase genes, *bla*$_{\text{GES-5}}$ and *bla*$_{\text{VIM}}$, which could explain their resistance to both cephalosporins and carbapenems, mainly ertapenem. The *bla*$_{\text{GES-5}}$ gene was previously also observed in *Aeromonas* species in the same treated wastewater (Drk et al., 2023), which could indicate a possible gene transfer from one species to another. In addition, *K. pneumoniae* from treated wastewater in Split WWTP also harbored the *bla*$_{\text{GES-5}}$ (Kvesić et al., 2022). Recently, *bla*$_{\text{GES-5}}$ was found in hospital outbreaks of carbapenemase-producing *E. cloacae* and *K. pneumoniae*, suggesting its clinical relevance (Chudejova et al., 2018; Mendes et al., 2022). In addition to carbapenemase genes, plasmid-mediated AmpC (pAmpC) genes were also detected in *E. cloacae* cplx., where in other species were only detected sporadically. Although carbapenemase genes such as *bla*$_{\text{NDM-1}}$ or *bla*$_{\text{OXA-48}}$ were detected sporadically in ESBL-producing *Klebsiella* spp. from treated wastewater, they are often found in clinical *K. pneumoniae* isolates from Croatia (Bedenić et al., 2023, 2022). In addition, treated wastewater *Klebsiella* spp. isolates were also found to carry the plasmid-mediated ESBL gene *bla*$_{\text{GES-7}}$ and the chromosomally encoded non-ESBL gene *bla*$_{\text{LEN}}$, which are rarely detected and for the first time were found in Croatia. Furthermore, four *Klebsiella* spp., and one *Citrobacter* spp. in this study had four different β-lactamase genes, while one *E. coli* strain carried five β-lactamase genes, including two carbapenemases (*bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$). Unfortunately, the discrepancy in the identification of carbapenemase genes by PCR and WGS in this *E. coli* was observed. This could be due to the loss of plasmids after successive subculturing, as no carbapenemase genes were predicted by WGS. It

was also supported by the change in phenotype of this isolate, which went from carbapenem-resistant to susceptible after the subculture. However, the observation of multiple β-lactamase genes being detected in wastewater isolates, underscores the growing trend observed in clinical isolates (Biez et al., 2022). Moreover, WGS data analysis showed that in addition to β-lactamase genes, sequenced *E. coli* and *K. oxytoca* isolates harbored variety of resistance genes to other classes of antibiotics such as aminoglycosides, fluoroquinolones, trimethoprim/sulfonamides, chloramphenicols, and/or tetracyclines confirming their MDR phenotype. It is noteworthy that *Klebsiella* spp. and *E. cloacae* cplx. resistant to colistin did not test positive for known plasmid-mediated *mcr* genes. However, their colistin resistance could be due to novel variations in the *mcr* gene or, more likely, to point mutations in chromosomal genes responsible for the regulation of lipopolysaccharides in the cytoplasmic membrane (Wang et al., 2021).

To date, there is a significant gap in our understanding of the epidemiology of critically important pathogens in wastewater systems. Therefore, a combination of PFGE and MLST was performed on ESBL-producing *E. coli*, *Klebsiella* spp., and *E. cloacae* cplx. isolates to better understand their genetic relationship and epidemiology. ESBL-producing *Klebsiella* spp. and *E. cloacae* cplx. were all singletons, having a high level of genetic diversity in their populations. In addition, various β-lactamase genes were detected in those isolates, including carbapenemases. Therefore, isolation of these priority pathogens with clinically important ARGs could suggest that treated wastewater is an additional reservoir of these bacteria. On the other hand, the genetic relatedness analysis of *E. coli* showed that the majority of isolates were associated with high-risk pandemic clones, ST131, ST405, ST410, ST38, and clonal complex (CC)10, and emerging clinically relevant clones such as ST361 and ST1193. The MDR ST131 *E. coli* clones carrying *bla*$_{\text{CTX-M-15}}$ have been previously documented in Croatian clinical setting (Krilanović et al., 2020; Literacka et al., 2009) as well as in hospital wastewater entering this WWTP as detailed in *Publication No. 4.* The ST131 is widely recognized as one of the most important international high-risk clones in hospitals and play an important role in the spread of AMR (Kocsis et al., 2022). For example, this lineage is associated with the worldwide spread of *bla*$_{\text{CTX-M-15}}$ in clinical settings (Castanheira et al., 2021). In addition, ST131 carrying the *bla*$_{\text{CTX-M-15}}$ gene has already been found in treated wastewater (Davidova-Gerzova et al., 2023; Mesquita et al., 2021). This occurrence of high-risk ESBL-producing *E. coli* clones in treated wastewater highlights the limitations of current wastewater treatment methods in reliable elimination of these clinically important ARB and allows

their spread into downstream aquatic environments. Phylogroup determination and serotyping using WGS data showed that ST131 *E. coli* was classified within the B2 phylogroup and with O25:H4 serotype. The ST131 B2-O25:H4 is one of the main causes of hospital- and community-acquired urinary tract infections globally (Forde et al., 2019). In addition, the B2 phylogroup is associated with pathogenic ExPEC *E. coli*, which frequently accumulate virulence factors, causing urinary tract infections and bloodstream infections (D'Onofrio et al., 2023). The *in silico* virulence factor analysis of ST131 *E. coli*, confirmed that this isolate is ExPEC due to the virulence genes associated with the extraintestinal infections, mainly *papC* and *iutA*, which are often found in patients with sepsis (D'Onofrio et al., 2023; Johnson et al., 2003). This association with human infections indicates their origins from human sources. However, more alarmingly is that these MDR ExPEC ST131 *E. coli* are disseminating via wastewater to downstream rivers, as recently found in Switzerland (Biggel et al., 2023). This release into natural waterways contributes significantly to environmental contamination, consequently having an impact on human health. Moreover, several other MDR high-risk pandemic *E. coli* clones, including ST405, ST410, ST38, and CC10 carrying mostly $bla_{\text{CTX-M-15}}$ gene have also been previously found in treated wastewater in different countries (Grevskott et al., 2021; Raven et al., 2019; Tavares et al., 2020). The ST405 clone in particular has also been detected in hospital wastewater, as detailed in *Publication No. 4*. In addition, the occurrence of emerging high-risk clone ST1193 *E. coli*, which is considered a sister clone of ST131, has also recently been documented in patients from different geographical regions (Kocsis et al., 2022). However, ST1193 is less common in wastewater and environmental samples than in clinical settings (Biggel et al., 2021; Grevskott et al., 2021). Of note, the predominant clonal *E. coli* group isolated from treated wastewater was associated with emerging high-risk clone ST361, which was frequently found in the clinical settings (Huang et al., 2024). In addition, *in silico* phylogroup and serotype determination of MDR CTX-M-15-producing ST361 *E. coli* clones revealed that they belonged to the commensal phylogroup A with O9:H30 serotype. This clonal group A-O9:H30 was previously found in patients from China and Switzerland (Huang et al., 2023; Sadek et al., 2022). In addition to human sources, the MDR ST361 carrying $bla_{\text{CTX-M-15}}$ genes were also found in treated wastewater from chicken farms (Savin et al., 2020), as well as in hospital wastewater, detailed in *Publicaton No. 4.* Moreover, one ST361 isolate co-harbored two carbapenemase genes $bla_{\text{OXA-48}}$ and $bla_{\text{VIM-1}}$, which have never been found in this strain, although it is responsible for hospital outbreaks of $bla_{\text{NDM-5}}$ all over Europe (Linkevicius et al., 2023). These

results suggest that ST361 acquired resistance genes to the carbapenems and could serve as reservoir for the last-resort resistance. Further WGS data analysis revealed an array of virulent genes. However, the isolates did not have any ExPEC virulence determinants, which confirmed their commensal status. Therefore, this could indicate that these *E. coli* isolates may not be capable of causing symptomatic infections, so they could be overlooked during the hospital surveillance. Although all these *E. coli* clones isolated from treated wastewater have not yet been found in patients from Croatia, with the exception of ST131, they may originate from community. However, this does not exclude their existence in hospital settings in Croatia.

WGS data analysis of plasmid replicon content showed that one ST131 and two ST361 *E. coli* isolates possessed IncFIB and IncY, while *K. oxytoca* had IncHI2 and IncHI2A plasmid types. In addition, genetic context of $bla_{\text{CTX-M-15}}$ in *K. oxytoca* and both *E. coli* showed its association with IS1380 (belonging to IS*Ecp1* family) and Tn*3*-family transposase, which are recognized for the global dissemination of $bla_{\text{CTX-M-15}}$ on various plasmids (Zhao and Hu, 2013). The *ISEcp1* is mostly responsible for translocation of $bla_{\text{CTX-M-15}}$ gene, while IncFIB and IncY plasmids are often associated with its spread between species (Hounmanou et al., 2023; Rocha-Gracia et al., 2022; Saidani et al., 2019). In *K. oxytoca,* the genetic context of $bla_{\text{CTX-M-15}}$ beside insertion sequences and transposons showed *aph(6)-ld* (resistance gene for aminoglycosides) and $bla_{\text{TEM-1}}$ on the same genetic element. Previous studies detected IncHI2 and IncHI2A simultaneous carrying different ARGs, including these β-lactamases and aminoglycoside resistance genes and were responsible for their dissemination across different environmental niches (Berbers et al., 2023; Pot et al., 2021). This could indicate that the spread of $bla_{\text{CTX-M-15}}$ and other ARGs is maintained by mobile genetic elements, which circulate in treated wastewater and could be potentially transferred into commensals or pathogens.

6.2.2. Phenotypic and genomic characterization of CRE

As a part of this dissertation, a comprehensive study of 200 presumptive CRE strains isolated from treated wastewater was conducted (*Publication No. 3*). Of these isolates, 148 were phenotypically confirmed to produce carbapenemases. Based on the MALDI-TOF MS analysis, these isolates were predominantly assigned as *Klebsiella* spp., followed by *Citrobacter* spp., and *E. cloacae* cplx. Interestingly, only a minority of the detected isolates were identified as *E. coli.* Additionally, utilizing the average nucleotide identity (ANI) analysis, a bioinformatics tool for

clarifying the distinction between species using WGS data, revealed misidentification by MALDI-TOF MS. In particular, three *K. pneumoniae* and one *K. oxytoca* submitted to WGS were confirmed to be *K. quasipneumoniae* subsp. *similipneumoniae*, while one *K. oxytoca* was identified as *K. michiganensis*. Misclassification was observed in one *C. freundii* where ANI analysis proved to be *C. portucalensis*. This discrepancy in the MALDI-TOF MS was reported in other studies because commercially available databases often lack reference spectra of diverse species such as *Klebsiella* spp. and *Citrobacter* spp. (Nobrega et al., 2023; Rodrigues et al., 2018). In addition, the species-specific chromosomally encoded bla_{OKP} gene, which was detected in all *K. quasipneumoniae* species analyzed here, could be a reliable marker for differentiating *K. quasipneumoniae* from other *Klebsiella* spp. (Chew et al., 2021). It is interesting to detect *C. portucalensis*, *K. quasipneumoniae* subsp. *similipneumoniae*, and *K. michiganensis* as they are relatively newly identified strains that are often under-represented in the populations. *K. quasipneumoniae* was first identified in patients, and now it is considered to be more common in wastewater settings than *K. pneumoniae* (Liu et al., 2023). *C. portucalensis* was first recognized in the aquatic environment, where nowadays is considered as an emerging MDR pathogen often producing carbapenemases in clinical settings (Luo et al., 2022). Finally, *K. michiganensis* after the first identification was established as an emerging opportunistic pathogen where clinical isolates often carry carbapenemase-encoding genes (Prah et al., 2022).

The phenotypic evaluation included testing of these 148 carbapenemase-producing isolates against 13 antibiotics covering 9 different antibiotic classes. Alarmingly, all isolates exhibited MDR profiles, with over 50% classified as extensively drug resistant (XDR), meaning they were resistant to all but two antibiotic classes as defined by Magiorakos et al. (2012). Furthermore, one *Leclercia adecarboxylata* and three *K. pneumoniae* strains exhibited pandrug-resistant (PDR) phenotype, with the resistance to all tested antibiotics. While colistin resistance among the isolates was generally low, a worrying observation was made in relation to majority of carbapenemase-producing *Klebsiella* spp., and *E. cloacae* cplx. strains, which exhibited resistance to this last-resort antibiotic. This poses a significant public health problem as *Klebisella* species, particularly *K. pneumoniae,* are considered priority pathogens (WHO, 2017). The emergence of resistance to all available antibiotics poses a major challenge to the treatment strategies and often lead to prolonged hospital stays, increased treatment costs and unfavorable patient outcomes (Agyeman et al., 2020).

These findings underscore the urgent need for increased surveillance and innovative approaches to combat AMR in environmental settings.

The use of PCR analysis to determine the genes responsible for carbapenemase production in bacterial isolated led to different results in different species. In particular, a significant prevalence of *bla*$_{\text{NDM-1}}$ was observed in *K. pneumoniae* species, often in association with *bla*$_{\text{OXA-48}}$. This pattern closely matches the co-occurrence of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ in clinical *K. pneumoniae* isolates from Croatian hospitals (Bedenić et al., 2023), as well as in hospital wastewater, as described in *Publication No. 4*. In additional, clinical *K. pneumoniae* with *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ was found in 13 European countries, suggesting transboundary dissemination (Ludden et al., 2020). In contrast, carbapenemase KPC-2 predominated in *K. oxytoca, E. cloacae* cplx., *E. coli* and *Citrobacter* spp., and frequently coexisted with *bla*$_{\text{NDM-1}}$. Interestingly, Enterobacterales isolates, especially *Citrobacter* spp., showed a high incidence of multiple carbapenemase genes. Worryingly, multiple carbapenemase genes have previously been observed in MDR *Citrobacter* strains on conjugative plasmids from patients around the world (Biez et al., 2022; Qiao et al., 2023), suggesting their potential role as reservoirs for the global spread of carbapenemase genes. In addition, *bla*$_{\text{VIM-1}}$ was frequently detected in *K. oxytoca*, *Citrobacter*, *Kluyvera* and *Roultella* species. Remarkably, one *K. michiganensis* isolate contained both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{IMP-13}}$, which is unusual in *Klebsiella* species but has previously been detected in *Pseudomonas aeruginosa* isolates (Cañada-García et al., 2022), and *Aeromonas media* isolate from the same treated wastewater analyzed here (Drk et al., 2023). Additionally, this is the first finding of a *Klebsiella* isolate containing *bla*$_{\text{IMP-13}}$. The accumulation of several carbapenemase genes in Enterobacterales could be at least partly due to the increased consumption of carbapenem antibiotics during the COVID-19 pandemic in Croatia (Bedenić et al., 2023). In fact, Croatia is among the three and five EU countries with the highest carbapenem consumption in hospitals in 2020 and 2021, respectively (ECDC, 2022). Furthermore, the identification of three *K. pneumoniae* strains carrying only *bla*$_{\text{OXA-48}}$ but also harboring the plasmid-mediated *mcr-4.3* gene is of particular concerns, as it confers resistance to two last-line antibiotics and potentially facilitates the spread of these ARGs in downstream environment.

In addition to PCR, WGS analysis was applied for better understanding AMR in *Klebsiella* spp, *E. cloacae* cplx., *Citrobacter* spp., and *E. coli* isolates. In addition to confirmed carbapenemase

genes, all isolates carried other β-lactamase and ESBL genes. Their MDR, XDR or PDR phenotype was also proved by the detection of different ARGs for other antimicrobial classes including fluoroquinolones, aminoglycosides, sulfonamides, trimethoprim, macrolides, tetracyclines, rifampicin, and/or chloramphenicol. Plasmid-mediated colistin resistance *mcr-4.3* gene was confirmed in one PDR *K. pneumoniae*. In other carbapenemase-producing and colistin resistant *Klebsiella* and *E. cloacae* cplx. isolates subjected to WGS, additional genomic analysis revealed known and potentially novel mechanisms underlying colistin resistance. This resistance is caused by point mutation in chromosomal genes, encoding PmrA/B, PhoP/Q, and/or MgrB proteins, responsible for modification of LPS membranes, which leads to alterations of these membranes preventing colistin from binding effectively (Gogry et al., 2021). For example, in one *K. quasipneumoniae,* novel deleterious mutations were found in PmrB and PhoQ proteins, whereas in other *K. quasipneumoniae*, mutation in PmrB was due to nucleotide deletion in corresponding *pmrB* gene. Interestingly, two *Klebsiella* spp. showed no known resistance mechanisms despite their resistance to colistin, underlining the incomplete understanding of this complex mechanism of resistance (Liu et al., 2021). In *E. cloacae* cplx. several point mutations were detected, including deleterious mutations in PmrA, PmrB, PhoP, and/or PhoQ proteins. These findings in *E. cloacae* cplx. mirror previous observation in the same species isolated from hospital wastewater in Zagreb, described in *Publication No. 4*, as well in hospital isolates in other countries (Liao et al., 2022; Uechi et al., 2019).

Furthermore, genomic characterization by PFGE and MLST of *Klebsiella* spp., *E. cloacae* cplx. and *E. coli* was used to analyze their genetic relatedness and epidemiological potential. The genetic relatedness analysis showed that most *K. pneumoniae* strains belonged to human-associated ST3590 clone (Takei et al., 2022), in addition to other smaller clusters ST629 and ST1803 (Aires et al., 2017; Esposito et al., 2018). Second most prevalent clones belonged to ST1697, which was previously only found in animals (Liao et al., 2019). In addition, *K. oxytoca* ST17 and *K. michiganensis* ST43 clones have also been associated with humans (Jolley et al., 2018; Wan et al., 2023), with ST43 also detected in animals (Campos-Madueno et al., 2022). With the exception of ST629, all these *Klebsiella* clones have never been found in Croatia, suggesting either their underrepresentation in hospitals or occurrence in the community and/or animals. Similar to *Klebsiella* spp., MLST analysis of the *E. cloacae* cplx. clusters revealed predominantly human-associated clones such as ST910, ST339, ST1641, and ST32 (Izdebski et al., 2015; Jolley et al.,

2018; Zelendova et al., 2023). Moreover, previous studies have associated ST910, ST2057, ST32 and ST277 with WWTPs and adjacent waterways (Cherak et al., 2021; Cirkovic et al., 2023; Falgenhauer et al., 2019; Jolley et al., 2018; Puljko et al., 2023), indicating a broader ecological presence of *E. claoace* cplx. beyond the clinical settings, extending into environmental niches. In contrast, carbapenemase-producing *E. coli* in this study occurs primarily as clonal type ST13697 that was previously reported in clinical setting (Zhou et al., 2020). *In silico* MLST analysis showed that *C. portucalensis* ST641 and *C. farmeri* ST600 were previously reported in clinical isolates (Jolley et al., 2018).

Furthermore, plasmid replicon types were determined from draft genomes of sequenced *Klebsiella* spp, *E. cloacae* cplx., *Citrobacter* spp., and *E. coli* isolates to assess their potential for acquiring and spreading carbapenemase genes. Four *Klebsiella* isolates (STND, both ST1697, and ST43) with the *bla*$_{\text{NDM-1}}$ gene carried multiple plasmid replicons in various combinations. Particularly, they all possessed IncFIA, and IncFIB replicons, which are known to facilitate transmission of *bla*$_{\text{NDM-1}}$ among Enterobacterales (Wu et al., 2019). The acquisition of *bla*$_{\text{NDM-1}}$ on different and highly promiscuous plasmids only underscores its rapid and worldwide dissemination, which nowadays is no longer reserved for hospital environment. In addition, two *bla*$_{\text{KPC}}$-carrying *K. quasipneumoniae* isolates, ST1803 and ST3590, were identified with IncFIB-type replicons, possibly related to the spread of the *bla*$_{\text{KPC-2}}$ gene, as previously observed in a wastewater *K. pneumoniae* isolate from Croatia (Kvesić et al., 2022). Interestingly, broad-host range IncP6 plasmid, which until recently was rarely associated with *bla*$_{\text{KPC}}$ genes, was detected in *K. quasipneumoniae* ST1803, *E. cloacae* cplx. ST339, and *E. coli* ST13697. This could indicate its role in the increasing spread of *bla*$_{\text{KPC}}$ gene among Enterobacterales, especially in wastewater, as previously reported worldwide, including Croatia (Ghiglione et al., 2021; Kvesić et al., 2022; Ota et al., 2022; Yao et al., 2017). In addition, KPC-2-producing *E. cloacae* cplx. ST277, *Citrobacter* ST600 and ST641 had several other plasmid replicon types, including IncFII, IncM1, IncR, IncX5, and IncX6, all of which have previously been associated with *bla*$_{\text{KPC-2}}$ transmission (Jia et al., 2023; Li et al., 2018; Raro et al., 2020; Souza et al., 2019; Xie et al., 2023). In addition to *bla*$_{\text{KPC-2}}$, *C. portucalensis* ST641 also carried *bla*$_{\text{VIM-1}}$ likely on conjugative IncY plasmid replicon type, consistent with previous report of this plasmid as a carrier of *bla*$_{\text{VIM-1}}$ in *E. coli* (Roschanski et al., 2017). On the other hand, seven different plasmid replicon types were found in *E. cloacae* ST32 without detection of any carbapenemase genes. However, the carbapenemase resistance of this

isolate could be due to the presence of ESBL *bla*$_{\text{OXA-10}}$ and the chromosomally-encoded AmpC *bla*$_{\text{ACT-9}}$ gene, possibly in combination with porin loss, as previously reported (Black et al., 2021). Finally, the *K. pneumoniae* ST629 isolate carried *bla*$_{\text{OXA-48}}$ and an IncL plasmid replicon type, which is consistent with previous reports of the almost constant association of this plasmid with the *bla*$_{\text{OXA-48}}$ in *Klebsiella* isolates from clinical and environmental sources (Hamprecht et al., 2019; Kvesić et al., 2022; Šuto et al., 2022). Remarkably, this wastewater isolate also carried the colistin resistance gene *mcr-4.3*. This is the first detection of the *mcr-4.3* gene variant in *K. pneumoniae*. Analysis of the genomic context of *mcr-4.3* suggest that it was acquired via IncHI1B plasmid, a pattern consistent with previous findings linking different *mcr* variants in *K. pneumoniae* to this plasmid (Salloum et al., 2020; Stosic et al., 2021). The conjugation properties associated with IncHI1B are likely due to the presence of *tra/trh* genes responsible for conjugation, on the same contig. The presence of *mcr-4.3* in *K. pneumoniae* is highly concerning due to its association with IncHI1B plasmid and potential for rapid dissemination via the wastewater system. In particular, analysis of the upstream region of *mcr-4.3* shows sequence homology with *Shewanella frigidimarina,* indication of a plausible origin of this gene and its subsequent mobilization in various bacterial species, including Enterobacterales (Ma et al., 2019; Zhang et al., 2019). This alarming discovery of *K. pneumoniae* associated with humans now carries plasmid-mediated resistance to two last-line antibiotics, increasing the risk of rapid spread via wastewater to downstream settings, and consequently reintroduction to humans.

Finally, the pathogenic potential of the sequenced *Klebsiella* spp., *E. cloacae* cplx., *Citrobacter* spp., and *E. coli* was evaluated based on their virulence gene profile. All sequenced *Klebsiella* isolates were found to harbor virulence determinants such as aerobactin, enterobactin, and/or yersinibactin, which are frequently associated with human infections (Amaretti et al., 2020). However, none of them exhibited hypervirulence-associated markers, such as K1 and K2 capsular types. Analysis of the virulence gene profiles of *E. cloacae* cplx., *Citrobacter* spp., and *E. coli* isolates further suggests that these genes may contribute to their adaptability in different environments (Amaretti et al., 2020; Mustafa et al., 2020; Yu et al., 2022). However, it is important to note that the mere presence of these genes does not directly indicate their pathogenic potential.

Taken together, the findings from Chapters 3 and 4 demonstrated that ESBL-E and CRE isolates from treated wastewater were resistant to multiple critically important antibiotics.

Specifically, their ARGs to 3GCs and carbapenems were associated with mobile genetic elements responsible for their transmission Therefore, based on the results published in *Publications No. 2* and *No. 3*, the hypothesis (ii) treated municipal wastewater serves as a source for the further dissemination of MDR enterobacterial strains with acquired resistance to 3GCs and carbapenems into the environment is accepted.

6.3. Resistance to 3GCs and carbapenems in MDR Enterobacterales from hospital wastewater

Hospital wastewater is considered to be a high-risk point source of clinically significant ARB and ARGs (Hassoun-Kheir et al., 2020; Paulus et al., 2019). In contrast to many developed nations, hospital wastewater in Croatia is discharged directly into the municipal wastewater system without any treatment. Although it accounts for only about 2% of the total volume of wastewater entering the WWTPs, there is concern that increased concentrations of hospital-derived ARB and ARGs entering the municipal WWTPs could potentially be released into the environment. Within the environmental bacterial community, this exposure could increase the likelihood of HGT to commensals and pathogens (Larsson and Flach, 2022). Consequently, it also increases the exposure of humans and animals to ARB, e.g. through irrigation, fishing, recreation, etc., which could eventually lead to life-threatening infections (Stanton et al., 2022). This associated risk is a complex and challenging problem, as it is not yet fully understood, which adds to the concern about the potential impact. Therefore, it is crucial to understand the role of untreated hospital wastewater in the spread of AMR, particularly for the WHO priority pathogens ESBL-E and CRE strains. Therefore, Chapter 5 addresses the phenotypic and genomic characterization of ESBL-E and CRE strains isolated from wastewater of two large hospitals in Zagreb (abbreviated as H1 and H2). The aim was to investigate the molecular epidemiology of these strains using techniques such as PFGE and MLST as well as the molecular mechanisms underlying AMR using molecular methods such as PCR and WGS. The results of this comprehensive study were published in *Publications No. 4.*

Initial culture-based analysis of hospital wastewater in this study found relatively high concentrations of CTX-R and CR coliforms at 10^3 to 10^4 CFU/mL, which was up to two orders of magnitude higher than what is typically found in the untreated wastewater from the receiving WWTP in Zagreb, based on the results from *Publication 1*. This illustrates the potential of the

hospital wastewater to be a significant reservoir for ARB, especially for 3GC-resistant and CR coliform bacteria. In addition, this prevalence of resistant coliforms in the wastewater from Zagreb's major hospitals is comparable or lower than in similar studies from neighboring countries such as Slovenia and Austria (Rozman et al., 2020). To place the obtained data in medical context, isolated strains resistant to 3GCs and carbapenems from hospital wastewater were characterized with regard to the phenotypic and genetic basis of their AMR, and their relationship with strains from human and non-human sources. A total of 200 enterobacterial isolates were successfully isolated from hospital wastewater samples, with 69 isolates phenotypically confirmed as ESBL-producers and 90 isolates as carbapenemase-producers. Based on MALDI-TOF MS analysis, among the ESBL-producers, the most prevalent species included *E. coli*, *Klebsiella* spp., and *Citrobacter* spp., whereas *Citrobacter* spp., *Enterobacter* spp., *E. coli* and *Klebsiella* spp. dominated among the CRE isolates. Phenotypic testing on Enterobacterales isolates against a range of clinically important antibiotics (13 antibiotics in 9 different classes) revealed that all tested isolates were MDR, consistent with the ability of enterobacteria to acquire ARGs through HGT, which is mainly facilitated by plasmids (Nguyen et al., 2021). It is noteworthy that more than 50% of ESBL-E and 80% of the CRE had an XDR profile. This is alarming, as the acquisition of resistance to additional antibiotic classes would further limit therapeutic options. In addition, one *E. kobei* was classified as PDR as it was resistant to all antibiotics tested. Different resistance patterns were observed for ESBL-E and CRE. As expected, almost all CRE showed resistance to carbapenems, while about half of the ESBL-E isolates showed specific resistance to the carbapenem antibiotic ertapenem, in addition to the 3GCs. Both groups were predominantly resistant to fluoroquinolones, while ESBL-E isolates were more frequently resistant to aminoglycosides compared to CRE. This is consistent with previous research findings that aminoglycoside and fluoroquinolone resistance genes are frequently co-localized with ESBL genes on plasmids, especially in clinical *E. coli* and *K. pneumoniae* (Bodendoerfer et al., 2020; Tacão et al., 2014). While only 9% of ESBL-E were resistant to colistin, a remarkable 27% of CRE showed resistance to this antibiotic of last-resort. This poses a significant concern because it could lead to treatment failures if these resistant strains continue to spread. PCR and WGS analyses showed that none of these isolates carried mobile colistin-resistant genes. However, *in silico* analysis, using the PROVEAN tool, on the sequenced *E. cloacae* cplx. isolates, revealed known and novel mutations in the chromosomal genes encoding for PmrA/B, PhoP/Q, and/or MgrB proteins, which are likely

responsible for the observed colistin resistance (Liao et al., 2022; Uechi et al., 2019). These findings strongly suggest the presence of chromosomally associated resistance mechanisms in the *Enterobacter* isolates studied here.

The ESBL-E and CRE isolates underwent PCR analysis for detection of targeted β-lactamase genes. Resistance to β-lactams in ESBL-E isolates was mainly mediated by various ESBL and AmpC β-lactamase enzymes. Resistance to penicillins and cephalosporins in the majority of ESBL-E was determined by the presence of CTX-M-type ESBLs, particularly the $bla_{\text{CTX-M-15}}$ gene, which was found in approximately 70% of isolates. In addition, more than half of the isolates carried a TEM-type ESBL, TEM-116, while SHV types were found exclusively in *K. pneumoniae* isolates. The co-occurrence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{TEM-116/TEM-1}}$ was found in majority of *E. coli*, *Klebsiella* spp., and *Citrobacter* spp., while only 10% of *K. pneumoniae* isolates showed co-occurrence of $bla_{\text{CTX-M-15}}$, bla_{TEM} and bla_{SHV}. All these genes are frequently associated with the clinical ESBL-E isolates identified in Croatian hospitals, especially in hospital H1, underlining their prevalence in the clinical setting (Bedenić et al., 2016; D'Onofrio et al., 2020). Some ESBL-E isolates simultaneously carried ESBL ($bla_{\text{CTX-M}}$, bla_{TEM} or bla_{SHV}), pAmpC (bla_{MOX}, bla_{EBC} or bla_{CIT}) and carbapenemase genes ($bla_{\text{KPC-2}}$, $bla_{\text{OXA-48}}$ or $bla_{\text{NDM-1}}$), which is consistent with the results of a global survey of clinical enterobacterial isolates (Kazmierczak et al., 2021). Interestingly, *K. pneumoniae* and *K. oxytoca* carried the rare $bla_{\text{GES-7}}$ ESBL gene, which was identified in the same species in treated wastewater from *Publication No. 2*, suggesting that this gene is present in both untreated hospital and treated municipal wastewater.

In CRE isolates in this study, resistance to carbapenems was mainly mediated by the $bla_{\text{KPC-2}}$ gene. This carbapenemase gene is also common among clinical Enterobacterales worldwide (Kazmierczak et al., 2021). Additionally, NDM-1 type β-lactamase was the second most frequently identified carbapenemase, observed mainly in *Citrobacter* spp. and frequently co-existing with KPC-2 type carbapenemase. *Citrobacter* spp., which are primarily associated with immunocompromised patients, have been recognized as MDR pathogens responsible for both opportunistic nosocomial and community-acquired infections (Yao et al., 2021). In particular, studies have pointed to the co-occurrence of $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-2}}$ on conjugative plasmids in *Citrobacter* spp. from hospital wastewater, highlighting their role as potential reservoirs for the spread of carbapenem resistance (Li, Y. et al., 2022; Wu et al., 2016). In addition, some *Citrobacter*

isolates from hospital wastewater studied here, carried novel combination of carbapenemase genes such as *bla*$_{\text{NDM-1}}$+*bla*$_{\text{VIM-1}}$+*bla*$_{\text{KPC-2}}$, indicating evolving resistance mechanisms. In contrast, the *K. pneumoniae* isolates in this study frequently had *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{NDM-1}}$ together, which was previously found not only in isolates from Croatian hospitals (Bedenić et al., 2023), but also in *K. pneumoniae* in various European countries (Ludden et al., 2020) and in the treated municipal wastewater from the Zagreb WWTP (*Publication No. 3*). This indicates the widespread distribution of OXA-48- and NDM-1-carrying *K. pneumoniae* in the hospital environment and in the surrounding ecosystems. Therefore, presence of the multiple carbapenemases in the isolates emphasize the role of hospital wastewater as a secondary reservoir and transmission route for these pathogens and their carbapenemase ARGs into wastewaters, potentially allowing them to spread further into the community. Moreover, PCR data paired with WGS identification of resistance genes in selected *E. coli*, *K. pneumoniae* and *E. cloacae* cplx. isolates, confirmed the presence of ESBL and carbapenemase genes. These isolates also harbored ARGs to additionally phenotypic tested antibiotics such as gentamicin, ciprofloxacin and trimethoprim/sulfamethoxazole, proving their MDR phenotype. Moreover, resistance genes to other antimicrobial classes such as lincosamides, phenicols, tetracyclines, fosfomycin, and rifampicin, which were not phenotypic assessed were also detected. Detection of all those ARGs in these isolates poses a particular challenge for the treatment, as the isolates are potentially dangerous if they are reintroduced in the community.

Molecular epidemiologic analysis using PFGE and MLST coupled with WGS analysis was performed for a subset of ESBL-E and CRE isolates, including *E. coli*, *K. pneumoniae*, and *E. cloacae* cplx. isolates. Most *E. coli* isolates were clustered within the high-risk clone ST131 and the rare ST541. The ST131 *E. coli* clones are considered to be the predominant high-risk clones worldwide, which are not only found in hospitals, as they were also detected in hospital wastewater and the corresponding treated wastewater in Czech Republic (Davidova-Gerzova et al., 2023), similar to finding from this study and treated wastewater from Zagreb, detailed in *Publication No. 2*. In addition, the ESBL *bla*$_{\text{CTX-M-15}}$ gene and *aac*(6')-*lb-cr* gene, which confers resistance to fluoroquinolones and aminoglycosides, were also identified in this isolate from hospital wastewater, which are often associated with ST131 lineage from clinical settings (Pitout and Finn, 2020). These results underline the important role of hospital wastewater in the spread of these MDR and highly pathogenic strains via wastewater to natural waters, as found in a recently published

study from Switzerland (Biggel et al., 2023). On the other hand, frequently found KPC-2-producing ST541 in this study was previously detected in the livestock from Asia and it was only recently found in one WWTP from Germany (Carlsen et al., 2024; Qiu et al., 2019). Some *E. coli* strains were also associated with international high-risk clones, such as ST405, which were detected as ESBL producers in treated wastewater from *Publication No. 2*. Interestingly, a rather rare ST216 strain was detected, which was only recently isolated from treated wastewater in Germany and was previously found as KPC-producing *E. coli* in patients and hospital pipes in England (Carlsen et al., 2024; Decraene et al., 2018). In addition, only one *E. coli* ST361 strain associated with the *bla*$_{\text{CTX-M-15}}$ was found in hospital wastewater, previously identified as the most abundant *E. coli* lineage in treated wastewater from Zagreb (*Publication No. 2*). These finding of the different lineage of *E. coli* with various resistance genes in hospital wastewater underline their diversity and could serve as a reservoir for ARGs in wastewater. Further, molecular typing revealed that most *K. pneumoniae* isolates from hospital wastewater were associated with the emerging high-risk opportunistic ST101 clone. PCR and WGS analysis revealed that this clone was associated with the detection of both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$. These findings were previously also observed in *K. pneumoniae* ST101 lineage from H1 hospital in Zagreb (Bedenić et al., 2023), as well as in clinical settings in Slovenia, Serbia, and Italy (Benulič et al., 2020; Novović et al., 2017; Nucleo et al., 2020). Recently, this clone was also found in UV-treated hospital wastewater in Romania (Popa et al., 2021), showing its wide distribution in clinical and related settings. In addition, two smaller *K. pneumoniae* clusters consisting of the high-risk clones ST16 and ST307 were also detected in hospital wastewater. The *K. pneumoniae* ST16 was found to be also associated with the carriage of two carbapenemase *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ genes. Although in Croatian hospitals, co-occurrence of *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ in ST16 lineage did not yet occur, the carriage of either *bla*$_{\text{OXA-48}}$ or *bla*$_{\text{NDM}}$ in this strain was reported before (Bedenić et al., 2018; Jelić et al., 2018; Kocsis et al., 2016). These findings indicate that both *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{OXA-48}}$ co-exist in hospital-associated high-risk *K. pneumoniae* clones, which could be their reservoirs in municipal wastewater system. Furthermore, the *K. pneumoniae* ST307 was previously not been reported in Croatia. However, this clone is often associated with MDR *K. pneumoniae* isolates causing the worldwide nosocomial outbreaks (Peirano et al., 2020), and in recent years it was also detected in hospital and municipal wastewater (Carlsen et al., 2022; Radisic et al., 2023). These findings of high-risk *K. pneumoniae* clones only emphasize the role of hospital wastewater as their reservoir, which could easily be transmitted into

downstream settings. Using molecular typing, most *E. cloacae* cplx. were identified as the clonal type ST277, which was previously only recorded in the PubMLST database (Jolley et al., 2018). This MDR clones had carbapenem and colistin resistance phenotype, where PCR and WGS analysis showed that these isolates harbored carbapenemase genes $bla_{\text{KPC-2}}$ or $bla_{\text{KPC-2}}$+$bla_{\text{NDM-1}}$ but no mobile colistin resistance genes. This resistance is most likely conferred by the known or new deleterious mutations in chromosomal *pmrA*, *pmrB* and *phoP* genes (Liao et al., 2022; Uechi et al., 2019). Furthermore, two clonal *E. cloacae* cplx. types were found, namely ST32 and ST501. The *E. cloacae* cplx. ST32 although resistant to carbapenems and colistin, did not harbor any carbapenemase or colistin resistance genes. Their carbapenemase resistance could come from the presence of ESBL $bla_{\text{OXA-10}}$ and the chromosomally-encoded AmpC $bla_{\text{ACT-9}}$ gene, with porin loss, as observed in the same lineage from treated wastewater, described in *Publication No. 3*. The resistance to colistin could have occurred due to the deleterious mutations found in chromosomal genes responsible for colistin resistance. This clone was previously also associated with carbapenem and colistin resistance from patients (Han et al., 2023; Zelendova et al., 2023). Moreover, the *E. cloacae* cplx. ST501 was found to be mostly associated with hospital sinks and plumbing (Aranega-Bou et al., 2021). Interestingly, combined PCR and WGS analysis confirmed that the PDR *E. cloacae* cplx. ST501 harbors a range of ESBL and carbapenemase genes, including $bla_{\text{CTX-M-3}}$, $bla_{\text{OXA-14}}$, and $bla_{\text{KPC-2}}$, ARGs for other antimicrobial classes and deleterious mutation in chromosomal genes conferring colistin resistance, verifying its PDR phenotype.

Additionally, WGS results revealed the diverse range of plasmids found in sequenced *E. coli*, *K. pneumoniae* and *E. cloacae* cplx. isolates. Clones ST131 and ST361 *E. coli* and ST101 and ST16 *K. pneumoniae* harbored IncFIA, IncFIB and/or IncR plasmid replicon types, which are commonly associated with the spread of the $bla_{\text{CTX-M-15}}$ gene (Nicolas-Chanoine et al., 2014; Rocha-Gracia et al., 2022; Silva et al., 2022). In addition, genomic context based on WGS data in all four isolates revealed that $bla_{\text{CTX-M-15}}$ gene was flanked by IS1380 insertion sequence and Tn*3* family transposase, previously reported to be involved in the dissemination of $bla_{\text{CTX-M-15}}$ (Smet et al., 2010). This could indicate that $bla_{\text{CTX-M-15}}$ gene is acquired via mobile genetic elements in different species, which could disseminate the gene through municipal wastewater system, consequently reaching the environment. In addition, sequenced *K. pneumoniae* harbored $bla_{\text{OXA-48}}$, where genetic context of the gene in *K. pneumoniae* ST101 was associated with the IS10A insertion sequence, belonging to IS4 transposase family, previously found predominantly in pOXA-48

plasmid (Hendrickx et al., 2021). Furthermore, carbapenemase-producing *E. coli* ST541 with *bla*$_{KPC-2}$ had eight different plasmid replicon types, some of which were previously associated with the spread of *bla*$_{KPC-2}$, such as IncM1 and IncR (Chen et al., 2014; Knecht et al., 2022). In addition, the genetic context of *bla*$_{KPC-2}$ showed its embedment within *Tn4401* transposon, which is often responsible for its mobilization. In addition, this mobile genetic element with *bla*$_{KPC-2}$ often is found on the conjugative plasmids, also described here, meaning that it can be disseminated into different species (Chen et al., 2014). Interestingly, sequence *E. cloacae* cplx. ST277 and ST501 harbored *bla*$_{KPC-2}$ with different genetic context then *E. coli*, suggesting its carriage on a non-*Tn4401* transposons, which are in recent years associated with the mobilization of *bla*$_{KPC}$ genes in various Enterobacterales species, often found in both clinical and environmental settings (Yao et al., 2024). Moreover, these isolates presented a diverse plasmidome, including plasmid replicons types such as Col440I, IncFII, IncN, IncP6, IncR, and/or IncX5, which were previously associated with the *bla*$_{KPC-2}$ transfer (Chen et al., 2014; Souza et al., 2019; Yao et al., 2017).

Taken together, the results from Chapter 5 have shown that hospital wastewater is a reservoir for multidrug-resistant WHO priority pathogens such as 3GC- and carbapenem-resistant Enterobacterales, which may have acquired resistance mechanisms to ESBL and carbapenemases, together with chromosomal mutations involved in colistin resistance. In addition, considerable number of *E. coli* and *K. pneumoniae* were phylogenetically related to the globally disseminated high-risk and emerging human-associated clones, some of which had previously been identified in patients from local hospitals and treated municipal wastewater. The potential for these pathogens to be released into the natural environment and reintroduced into humans and animals raises serious public health concerns. To mitigate the risk of spreading such ARB and ARGs of clinical importance, effective pre-treatment of hospital wastewater is critical. All in all, based on the results published in *Publication No. 4*, the hypothesis (iii) that hospital wastewater is a reservoir of multidrug-resistant enterobacteria, including high-risk clones that produce ESBLs and/or carbapenemases is accepted.

Chapter 7

Conclusions

- Conventional treatment processes in seven Croatian WWTPs do not completely eliminate CTX-R and CR *E. coli* and other coliforms, EOPs, ESBL and carbapenemase genes from the treated wastewater, especially the carbapenemase genes *bla*$_{VIM}$ and *bla*$_{IMP,}$ which are often enriched during the treatment process. These results emphasize the need to use advanced treatment technologies to reduce the release of clinically important ARB and ARGs into the environment.
- Specific WWTP characteristics had an effect on the amount of ARB and ARGs remaining in the treated wastewater. In particular, COD and suspended solids positively correlated with CTX-R and CR coliforms and enterococci, while COD showed a negative correlation with *bla*$_{VIM}$ and *bla*$_{IMP}$. The number of hospitals in the WWTP catchment correlated positively with *bla*$_{VIM}$ and *bla*$_{IMP}$, while the *bla*$_{VIM}$ was also influenced with the number of hospital beds, and the *bla*$_{IMP}$ with the size of WWTP. The *bla*$_{TEM}$ and *bla*$_{CTX-M-32}$ correlated positively with temperature. Therefore, WWTP characteristics should be considered in management strategies to maximize the removal of AMR determinants from wastewater.
- Municipal treated wastewater from the Zagreb WWTP and wastewater from two Zagreb hospitals are important sources of MDR and XDR Enterobacterales producing ESBLs and/or carbapenemases. The ESBL phenotype of most ESBL-E isolates was mediated by the production of β-lactamases CTX-M-15 and TEM-116, while carbapenem resistance in most CRE was mainly mediated by NDM-1, KPC-2 and/or OXA-48 carbapenemases, which frequently co-occurred, especially in *K. pneumoniae* isolates. Genes encoding these ESBLs and carbapenemases were frequently associated with different mobile genetic elements, which may contribute to their mobilization within and between bacterial hosts.
- Resistance genes to other clinically important antibiotic classes such as aminoglycosides, fluoroquinolones, trimethoprim, and sulfonamides were identified in sequenced ESBL-E and CRE isolates of *E. coli*, *Klebsiella* spp., *E. cloacae* cplx., and *Citrobacter* spp. Furthermore, a variety of plasmids were observed in these isolates, indicating the potential for HGT. In addition, virulence gene content of some sequenced *E. coli* isolates indicated that they were likely associated with extraintestinal human infections.
- Chromosomal mechanisms of resistance to colistin were found in some CRE *Klebsiella* spp. and *E. cloacae* cplx. from hospital and municipal treated wastewater, indicated by known and novel mutations in the *pmrA/B, phoP/Q, mgrB,* and/or *crrB* genes. In addition, the colistin resistance gene *mcr-4.3* was identified for the first time in *K. pneumoniae* and in Croatia and it is

located on the conjugative IncHI1B plasmid. These findings highlight that this gene can spread into the environment and potentially be re-transmitted to humans via exposure routes, leading to very limited treatment options.

- ESBL-E isolates, especially most *E. coli* isolates from the hospital and municipal wastewater, were associated with the pathogenic high-risk clones (ST131, ST405, ST410, ST10, ST38, and CC10), and the emerging high-risk clones (ST361, ST1193, and ST635), some of which were also detected in Croatian hospitals (ST131).
- CRE isolates from hospital and municipal wastewater were phylogenetically related to human-associated MDR clonal types. In particularly, *K. pneumoniae* isolates from hospital wastewater were related to the high-risk clones ST16 and ST307 and emerging ST101 clones, some of which were detected for the first time in Croatia.
- All these findings underline the need not only to implement advanced wastewater treatment methods but also to integrate on-site pre-treatment of hospital wastewater before it is released into municipal wastewater. Furthermore, our research underlines the importance of looking at AMR from a One Health perspective. It argues for the introduction of environmental surveillance measures for AMR not only in Croatia, but also in other regions worldwide.

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Biography

Biography

Ana Puljko was born on August 21, 1992, in Zagreb. After completing her bachelor's degree in Agroecology, in 2015 she enrolled in the master's study Agroecology, a course Microbial Biotechnology in Agriculture at the Faculty of Agriculture, University of Zagreb. She graduated in 2018, and in 2019 she was employed in a publishing company. In the same year, she was employed as an associate in the Laboratory for Environmental Microbiology and Biotechnology, Division for Marine and Environmental Research, Ruđer Bošković Institute. In the same laboratory, from 2020 until today, she has been employed as an assistant, on the project “Antibiotic resistance in wastewater treatment plants in Croatia: focus on extended-spectrum ß-lactamases and carbapenemases“ of the Croatian Science Foundation. In the academic year 2020/2021, she enrolled in a postgraduate university doctoral study in Biotechnology and Bioprocess Engineering, Food Technology, and Nutrition at the Faculty of Food and Biotechnology, University of Zagreb. During 2022 and 2023, she participated in the Austrian-Croatian bilateral project, where she did sequencing and analysis of bacterial genomes at the Austrian Institute of Technology in Vienna. Her main scientific work of interest includes antibiotic-resistant bacteria from wastewater and their mechanisms of antibiotic resistance. She is the first author of four scientific papers in one of the most prestigious journals in the field of environmental sciences, cited by the Web of Science database, and participated as a co-author on three more scientific papers. She is the winner of the annual award of the Ruđer Bošković Institute for the best published scientific paper for 2022. She participated in several domestic and international scientific congresses. She is a member of the Croatian Microbiological Society and the Federation of European Microbiological Societies.

List of author's publications

- **Puljko, A.**, Barišić, I., Dekić Rozman, S., Križanović, S., Babić, I., Jelić, M., Maravić, A., Udiković-Kolić, N. (2024) Molecular epidemiology and mechanisms of carbapenem and colistin resistance in *Klebsiella* and other Enterobacterales from treated wastewater in Croatia. *Environment International*, **185**, 108554, 1-12 https://doi.org/10.1016/j.envint.2024.108554
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- **Puljko, A.**, Milaković, M., Križanović, S., Kosić-Vukšić, J., Babić, I., Petrić, I., Maravić, A., Jelić, M., Udiković-Kolić, N. (2022) Prevalence of enteric opportunistic pathogens and extended-spectrum cephalosporin- and carbapenem-resistant coliforms and genes in wastewater from municipal wastewater treatment plants in Croatia. *Journal of Hazardous Materials*, **427** 128155, 1-11 https://doi.org/10.1016/j.jhazmat.2021.128155
- Kajić, S., **Puljko, A.**, Rajnović, I., Sikora, S. (2020) Resistance of indigenous *Bradyrhizobium japonicum* strains on moisture deficiency stress. *Journal of Central European agriculture*, **21**, 2; 285-291. https://doi.org/10.5513/JCEA01/21.2.2580

Supplementary materials

Supplementary materials for the Publication No. 1:

Puljko, A., Milaković, M., Križanović, S., Kosić-Vukšić, J., Babić, I., Petrić, I., Maravić, A., Jelić, M., Udiković-Kolić, N. (2022) Prevalence of enteric opportunistic pathogens and extended-spectrum cephalosporin- and carbapenem-resistant coliforms and genes in wastewater from municipal wastewater treatment plants in Croatia. *Journal of Hazardous Materials*, **427** 128155, 1-11 https://doi.org/10.1016/j.jhazmat.2021.128155

Table S1. Physicochemical parameters of WWTP influents and effluents during the summer and winter sampling campaigns

Parameter	Sampling campaign	Sample type	WWTP locations						
			Vinkovci	Bjelovar	Zagreb	Čakovec	Varaždin	Karlovac	Zadar
Air temperature (°C)	Winter		6.4	0.6	-0.9	3.1	7.6	0.9	12.3
	Summer		19.3	20.4	26.2	23.0	23.1	18.4	24.9
pH	Winter	Influent	7.8	7.9	7.7	7.5	7.2	7.8	7.6
		Effluent	7.6	7.6	7.8	7.6	7.6	7.7	7.7
	Summer	Influent	7.9	7.9	7.8	7.7	7.2	7.9	7.3
		Effluent	7.7	7.9	7.9	7.7	7.6	7.9	7.8
Conductivity (µS/cm)	Winter	Influent	979.33	1323.00	1147.67	1331.33	1060.00	1027.67	6696.67
		Effluent	883.33	1256.67	1038.00	1052.67	565.33	913.33	7383.33
	Summer	Influent	828.67	1073.00	933.33	785.00	980.33	977.00	8433.33
		Effluent	776.00	1009.33	781.33	682.67	586.00	721.50	8996.67
COD (mg O_2/L)[a]	Winter	Influent	284.33	333.33	361.67	772.33	1034.00	298.00	718.00
		Effluent	46.00	64.33	29.00	50.33	57.00	<15.00	38.00
	Summer	Influent	416.00	259.67	220.67	355.67	957.33	281.00	956.67
		Effluent	16.33	29.67	15.67	64.67	59.00	15.55	40.07
BOD_5 (mg O_2/L)[b]	Winter	Influent	87.33	148.67	130.33	280.00	411.33	102.67	261.67
		Effluent	19.73	43.00	8.03	13.00	22.33	2.10	8.90
	Summer	Influent	205.00	127.67	82.00	132.00	438.33	113.50	243.00
		Effluent	5.57	17.33	3.13	7.80	27.00	3.20	12.33
Total N (mg/L)	Winter	Influent	30.03	45.77	32.60	61.47	41.30	41.23	65.17
		Effluent	2.11	33.03	21.57	6.44	6.43	6.44	44.10
	Summer	Influent	33.53	30.90	23.17	50.47	43.07	37.15	55.33
		Effluent	6.85	23.03	19.17	12.60	9.21	6.36	8.92
NH_4-N (mg/L)	Winter	Influent	9.26	20.58	18.85	33.45	8.47	18.65	36.43
		Effluent	0.41	10.88	0.56	1.84	2.01	0.22	30.01
	Summer	Influent	15.22	16.80	13.48	20.29	7.64	19.45	34.10
		Effluent	5.47	8.23	0.12	1.21	9.17	0.14	0.19
Total P (mg/L)	Winter	Influent	5.44	5.63	3.87	9.07	8.46	5.80	8.29
		Effluent	0.93	2.90	2.38	1.06	1.03	1.80	0.89
	Summer	Influent	8.61	6.13	3.88	6.84	7.50	5.84	9.94
		Effluent	2.43	3.17	1.51	2.11	1.76	2.77	5.01
NO_3^-N (mg/L)	Winter	Influent	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
		Effluent	0.61	7.93	16.33	0.40	0.38	5.43	1.08
	Summer	Influent	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
		Effluent	1.54	0.79	16.88	7.50	6.70	4.32	4.70
Suspended solids (mg/L)	Winter	Influent	-	-	-	-	-	-	-
		Effluent	8.07	36.33	10.87	15.67	35.33	2.40	11.67
	Summer	Influent	-	-	-	-	-	-	-
		Effluent	3.47	6.67	4.40	11.83	28.67	6.40	8.93

[a]Chemical oxygen demand. [b]Biological oxygen demand

Table S2. Primers and conditions used to quantify antibiotic-resistance genes and taxonomic genes specific for opportunistic pathogens targeted in this study.

	Target gene	Resistance phenotype/enzyme	Primer sequence 5' → 3'	Amplicon size (bp)	Ta* (°C)	Amplification accuracy/efficiency (%)		Reference
						Assay 1	Assay 2	
ANTIBIOTIC-RESISTANCE GENES	***bla*TEM**	β-lactam resistance/ ESBL	TTCCTGTTTTTGCTCACCCAG CTCAAGGATCTTACCGCTGTTG	113	63	0.999/96.44	0.999/98.62	Rocha et al., 2020
	***bla*CTX-M-32**	β-lactam resistance/ESBL	CGTCACGCTGTTGTTAGGAA CGCTCATCAGCACGATAAAG	156	63	0.999/99.04	0.996/83.24	Rocha et al., 2020
	***bla*VIM**	β-lactam resistance/ carbapenemase	AGTGGTGAGTATCCGACAG TCAATCTCCGCGAGAAG	212	60	0.996/105.71	0.998/96.74	This study
	***bla*IMP**	β-lactam resistance/ carbapenemase	GGAATAGRRTGGCTTAAYTCTC GGTTTAAYAAARCAMCCACC	233	60	0.998/94.20	0.999/83.43	This study
	***bla*NDM**	β-lactam resistance/ carbapenemase	GATTGCGACTTATGCCAATG TCGATCCCAACGGTGATATT	189	60	0.987/103.13	0.999/91.59	Subirats et al., 2017
	***bla*KPC-3**	β-lactam resistance/ carbapenemase	CAGCTCATTCAAGGGCTTTC GGCGGCGTTATCACTGTATT	196	59	0.985/112.99	0.997/77.55	Szczepanowski et al., 2009
	***bla*OXA-48**	β-lactam resistance/ carbapenemase	AGGCACGTATGAGCAAGATG TGGCTTGTTTGACAATACGC	189	60	0.999/99.07	0.998/93.75	Subirats et al., 2017
	Target gene	**Organism**	**Primer sequence 5' → 3'**	**Amplicon size (bp)**	**Ta* (°C)**	**Amplification accuracy/efficiency (%)**		**Reference**
						Assay 1	**Assay 2**	
TAXONOMY GENES	***ycc*T**	*Escherichia coli*	GCATCGTGACCACCTTGA CAGCGTGGTGGCAAAA	59	60	0.993/115.08	0.997/107.55	Hembach et al., 2017
	***glt*A**	*Klebsiella pneumoniae*	ACGGCCGAATATGACGAATTC AGAGTGATCTGCTCATGAA	68	60	0.999/105.57	0.999/104.12	Hembach et al., 2017
	***sec*E**	*Acinetobacter baumannii*	GTTGTGGCTTTAGGTTTATTATACG AAGTTACTCGACGCAATTCG	94	60	0.992/114.51	0.999/96.82	Hembach et al., 2017
	23S rRNA	*Enterococcus* sp.	AGAAATTCCAAACGAACTTG CAGTGCTCTACCTCCATCATT	-	60	0.994/114.72	0.999/77.11	Frahm and Obst, 2003
	16S rRNA	Total bacteria	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGCA	196	60	0.996/86.19	0.998/103.62	López-Gutiérrez et al., 2004

*Ta –annealing temperature

Table S3. Quantification of combined coliforms, *E. coli* and non-*E. coli* (CFU/mL) in influent and effluent samples and log reduction from 7 WWTPs in the winter and summer campaign.

WWTP	Season	Sample	Combined coliforms			*E. coli*			Non *E. coli*		
			Non-resistant	CTX-R	CR	Non-resistant	CTX-R	CR	Non-resistant	CTX-R	CR
Vinkovci	Winter	Influent	$1.52x10^4$	$1.01x10^2$	$4.05x10^1$	$7.48x10^3$	$1.73x10^1$	$1.62x10^1$	$7.75x10^3$	$8.37x10^1$	$2.43x10^1$
		Effluent	$1.38x10^2$	1.05	0.61	$6.60x10^1$	0.16	0.19	$7.22x10^1$	0.89	0.42
	log reduction		2.04±0.1	2.05±0.19	1.82±0.19	2.06±0.31	2.03±0.14	1.95±0.22	2.02±0.09	2.06±0.21	1.79±0.22
	Summer	Influent	$5.75x10^4$	$3.00x10^2$	$1.55x10^2$	$3.48x10^4$	$5.33x10^1$	$6.18x10^1$	$2.27x10^4$	$2.47x10^2$	$9.35x10^1$
		Effluent	$1.83x10^3$	8.80	2.92	$1.05x10^3$	2.00	0.56	$7.80x10^2$	6.80	2.36
	log reduction		1.83±0.47	1.81±0.48	2.05±0.76	1.83±0.44	1.64±0.91	2.22±0.86	1.76±0.51	1.81±0.41	1.96±0.72
Bjelovar	Winter	Influent	$3.60x10^4$	$3.28x10^2$	$2.50x10^2$	$2.35x10^4$	$6.17x10^1$	$1.40x10^2$	$1.24x10^4$	$2.67x10^2$	$1.10x10^2$
		Effluent	$3.08x10^3$	$9.35x10^1$	$2.06x10^1$	$1.95x10^3$	$1.10x10^1$	4.22	$1.13x10^3$	$8.22x10^1$	$1.64x10^1$
	log reduction		1.51±0.73	1.22±0.84	1.39±0.45	1.58±0.77	1.17±0.60	1.47±0.15	1.40±0.73	1.25±0.88	1.16±0.53
	Summer	Influent	$7.00x10^4$	$5.57x10^2$	$2.93x10^2$	$3.73x10^4$	$1.02x10^2$	$5.00x10^1$	$3.27x10^4$	$4.55x10^2$	$2.43x10^2$
		Effluent	$5.02x10^2$	4.45	1.63	$2.37x10^2$	0.98	0.078	$2.65x10^2$	3.47	1.55
	log reduction		2.20±0.2	2.10±0.14	2.32±0.24	2.25±0.20	2.00±0.07	2.85±0.34	2.13±0.23	2.15±0.18	2.27±0.22
Zagreb	Winter	Influent	$3.75x10^4$	$5.22x10^2$	$3.23x10^2$	$1.90x10^4$	$1.48x10^2$	$8.00x10^1$	$1.84x10^4$	$3.73x10^2$	$2.43x10^2$
		Effluent	$3.17x10^2$	5.57	3.83	$1.27x10^2$	1.15	0.91	$1.90x10^2$	4.24	2.92
	log reduction		2.07±0.11	1.97±0.15	1.93±0.12	2.18±0.09	2.15±0.32	1.97±0.16	1.96±0.24	1.92±0.17	1.91±0.12
	Summer	Influent	$1.37x10^5$	$1.56x10^3$	$1.67x10^3$	$6.05x10^4$	$1.90x10^2$	$1.13x10^2$	$7.58x10^4$	$1.37x10^2$	$1.56x10^3$
		Effluent	$4.25x10^2$	4.95	4.55	$1.33x10^2$	0.50	0.40	$2.92x10^2$	4.42	4.15
	log reduction		2.47±0.2	2.50±0.19	2.29±0.65	2.65±0.13	2.56±0.21	2.40±0.14	2.34±0.27	2.46±0.2	2.28±0.81
Čakovec	Winter	Influent	$4.60x10^4$	$9.42x10^2$	$2.20x10^3$	$2.12x10^4$	$5.83x10^1$	$5.55x10^2$	$2.48x10^4$	$8.83x10^2$	$1.64x10^3$
		Effluent	$3.87x10^2$	$2.21x10^1$	$3.11x10^1$	$4.83x10^1$	0.15	$1.00x10^1$	$3.38x10^2$	$2.20x10^1$	$2.11x10^1$
	log reduction		2.08±0.16	1.75±0.36	2.09±0.47	2.64±0.03	2.30±0.26	1.81±0.37	1.89±0.14	1.72±0.36	2.29±0.59
	Summer	Influent	$5.08x10^4$	$5.96x10^2$	$8.97x10^1$	$2.37x10^4$	$8.37x10^1$	$3.50x10^1$	$2.72x10^4$	$5.12x10^2$	$5.47x10^1$
		Effluent	$1.75x10^4$	$1.28x10^1$	2.33	$1.17x10^4$	1.15	0.62	$5.77x10^3$	$1.17x10^1$	1.72
	log reduction		1.02±1.23	1.42±0.88	1.51±0.46	0.96±1.42	2.08±0.00	1.65±0.42	1.08±1.03	1.39±0.00	1.43±0.40
Varaždin	Winter	Influent	$4.35x10^4$	$4.14x10^3$	$4.81x10^3$	$1.27x10^4$	$7.83x10^2$	$1.13x10^3$	$3.08x10^4$	$3.36x10^3$	$3.70x10^3$
		Effluent	$4.68x10^3$	$5.03x10^1$	$6.58x10^1$	$1.30x10^3$	$1.02x10^1$	$1.32x10^1$	$3.38x10^3$	$4.02x10^1$	$5.27x10^1$
	log reduction		0.97±0.06	1.68±0.74	1.59±0.87	0.99±0.03	1.60±0.97	1.74±0.81	0.96±0.09	1.70±0.7	1.53±0.92
	Summer	Influent	$1.30x10^5$	$1.23x10^3$	$4.99x10^2$	$2.35x10^4$	$2.18x10^2$	$7.90x10^1$	$1.07x10^5$	$1.01x10^3$	$4.20x10^2$
		Effluent	$2.08x10^4$	$1.52x10^2$	$2.23x10^2$	$7.55x10^3$	$3.33x10^1$	$2.38x10^1$	$1.33x10^4$	$1.18x10^2$	$1.99x10^2$
	log reduction		1.68±1.38	1.25±0.51	0.23±0.44	1.33±1.27	1.20±0.62	0.36±0.72	1.79±1.44	1.25±0.49	0.22±0.40
Karlovac	Winter	Influent	$3.68x10^4$	$4.05x10^2$	$8.52x10^2$	$2.05x10^4$	$3.83x10^1$	$4.08x10^2$	$1.63x10^4$	$3.67x10^2$	$4.43x10^2$
		Effluent	$2.20x10^2$	2.45	1.73	$1.45x10^2$	0.53	0.75	$7.50x10^1$	1.92	0.98
	log reduction		2.22±0.12	2.28±0.35	2.72±0.18	2.16±0.14	1.85±0.06	2.79±0.71	2.35±0.16	2.23±0.46	2.66±0.15
	Summer	Influent	$6.95x10^4$	$3.80x10^2$	$4.00x10^2$	$4.00x10^4$	$1.20x10^2$	$9.50x10^1$	$2.95x10^4$	$2.60x10^2$	$3.05x10^2$
		Effluent	$6.80x10^2$	3.95	1.95	$3.33x10^2$	1.31	0.60	$3.48x10^2$	2.65	1.35
	log reduction		2.01±0.03	1.98±0.09	2.31±0.10	1.39±1.21	1.98±1.14	2.22±0.15	1.92±0.08	1.99±0.15	1.57±0.07
Zadar	Winter	Influent	$7.48x10^4$	$1.12x10^3$	$1.53x10^2$	$3.82x10^4$	$9.33x10^1$	$2.17x10^1$	$3.67x10^4$	$1.03x10^3$	$1.32x10^2$
		Effluent	$3.03x10^3$	$2.45x10^1$	1.78	$1.28x10^3$	1.50	0.067	$1.75x10^3$	$2.30x10^1$	1.72
	log reduction		1.39±0.08	1.66±0.17	1.93±0.06	1.46±0.11	1.78±0.57	2.36±0.26	1.33±0.06	1.66±0.16	1.88±0.10
	Summer	Influent	$6.22x10^4$	$6.18x10^2$	$1.26x10^3$	$3.15x10^4$	$4.83x10^1$	$1.67x10^1$	$3.07x10^4$	$5.70x10^2$	$1.24x10^3$
		Effluent	$1.26x10^3$	$1.55x10^1$	$1.13x10^1$	$4.38x10^2$	2.67	0.43	$8.25x10^2$	$1.28x10^1$	$1.09x10^1$
	log reduction		1.69±0.3	1.60±0.03	2.23±0.57	2.03±0.59	1.38±0.28	0.74±0.00	1.60±0.21	1.64±0.04	2.22±0.58

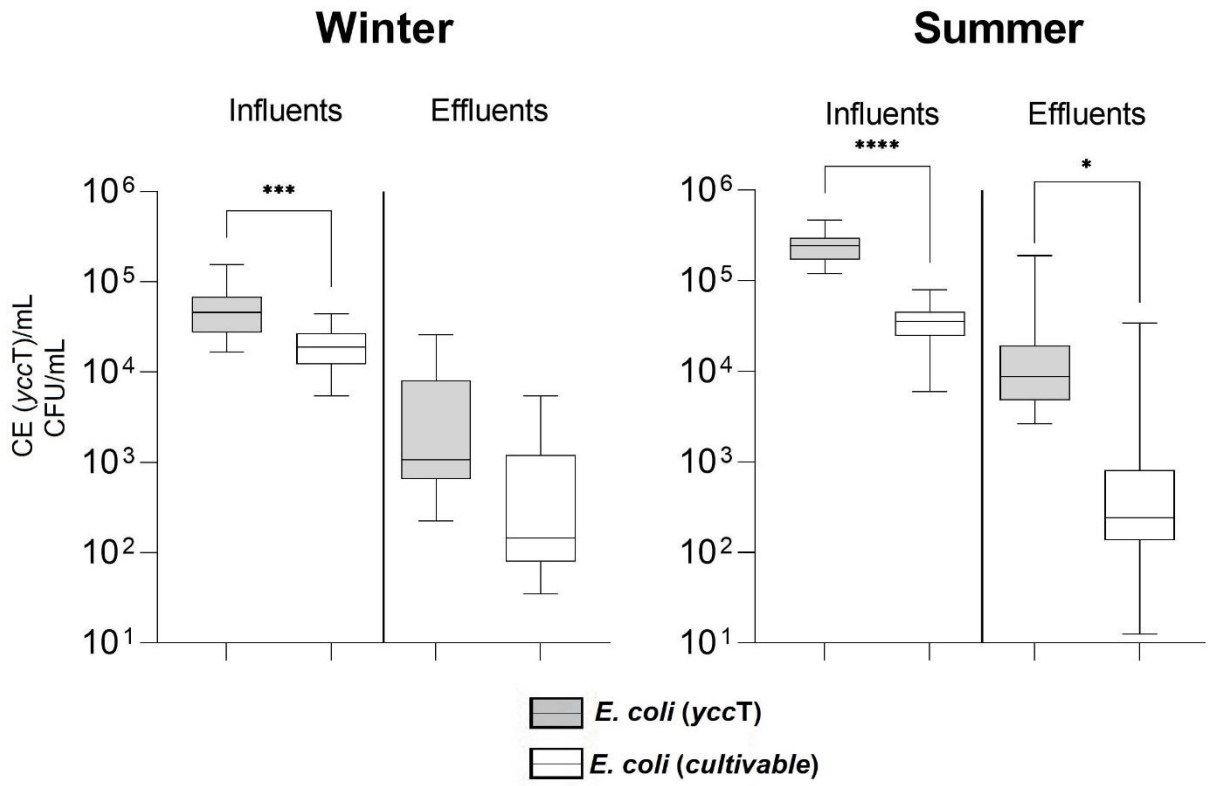
Table S4. Quantification of total bacteria and opportunistic pathogens (CE/mL) with log reduction (± SD) between influent and effluent of 7 Croatian WWTPs from winter and summer campaign.

	Season	Sample	Vinkovci	Bjelovar	Zagreb	Čakovec	Varaždin	Karlovac	Zadar
Total bacteria (16S rRNA)	Winter	Influent	$2.91x10^8$	$3.08x10^8$	$3.83x10^8$	$6.68x10^8$	$3.06x10^8$	$3.62x10^8$	$1.73x10^8$
		Effluent	$9.25x10^6$	$9.82x10^7$	$1.88x10^7$	$3.08x10^7$	$1.64x10^8$	$8.67x10^6$	$3.24x10^7$
		log reduction	1.50±0.04	0.50±0.05	1.31±0.04	1.34±0.12	0.27±0.05	1.62±0.12	0.73±0.03
	Summer	Influent	$5.54x10^7$	$4.01x10^7$	$9.74x10^7$	$8.22x10^7$	$7.05x10^7$	$6.96x10^7$	$1.08x10^8$
		Effluent	$6.70x10^5$	$1.76x10^6$	$2.83x10^6$	$2.88x10^6$	$1.36x10^7$	$2.40x10^6$	$7.99x10^6$
		log reduction	1.92±0.08	1.36±0.09	1.54±0.06	1.46±0.07	0.71±0.1	1.46±0.1	0.13±0.12
***Enterococcus* spp. (23S rRNA)**	Winter	Influent	$6.82x10^4$	$4.58x10^4$	$9.98x10^4$	$8.06x10^4$	$1.99x10^5$	$5.37x10^4$	$2.31x10^5$
		Effluent	$2.46x10^2$	$2.88x10^3$	$1.57x10^3$	$8.43x10^2$	$2.69x10^4$	$9.14x10^1$	$1.32x10^3$
		log reduction	2.44±0.42	1.20±0.14	1.80±0.19	1.98±0.19	0.87±0.13	2.77±1.33	2.24±0.11
	Summer	Influent	$1.15x10^5$	$3.31x10^4$	$1.13x10^5$	$5.31x10^4$	$3.37x10^5$	$1.05x10^5$	$9.14x10^4$
		Effluent	$3.16x10^2$	$8.88x10^2$	$8.98x10^2$	$7.90x10^2$	$6.50x10^4$	$6.32x10^3$	$4.14x10^3$
		log reduction	2.56±0.14	1.57±0.17	2.10±0.11	1.83±0.16	0.71±0.1	1.22±0.28	1.34±0.31
***A. baumanii* (*sec*E)**	Winter	Influent	< LOD	< LOD	$1.41x10^4$	< LOD	$1.19x10^4$	$4.90x10^3$	< LOD
		Effluent	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		log reduction	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	Summer	Influent	$4.64x10^3$	$1.70x10^4$	$1.05x10^4$	$1.04x10^4$	$8.63x10^3$	$4.14x10^4$	$1.29x10^4$
		Effluent	< LOD	< LOD	$3.34x10^2$	< LOD	$2.23x10^3$	< LOD	< LOD
		log reduction	*NA*	*NA*	1.50±0.12	*NA*	0.59±0.01	*NA*	*NA*
***K. pneumoniae* (*glt*A)**	Winter	Influent	< LOD	< LOD	$1.12x10^4$	< LOD	$2.92x10^3$	< LOD	$8.40x10^3$
		Effluent	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		log reduction	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	Summer	Influent	$6.15x10^3$	$7.88x10^3$	$1.61x10^4$	< LOD	$5.93x10^3$	$1.23x10^4$	$5.46x10^3$
		Effluent	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		log reduction	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
***E. coli* (*ycc*T)**	Winter	Influent	$2.75x10^4$	$3.47x10^4$	$8.32x10^4$	$4.12x10^4$	$3.96x10^4$	$4.44x10^4$	$1.10x10^5$
		Effluent	$2.26x10^2$	< LOD	$9.21x10^2$	< LOD	$1.61x10^4$	$7.39x10^2$	$2.60x10^3$
		log reduction	2.09±0.00	*NA*	1.96±0.22	*NA*	0.39±0.22	1.78±0.02	1.63±0.13
	Summer	Influent	$2.08x10^5$	$2.48x10^5$	$2.16x10^5$	$2.19x10^5$	$2.29x10^5$	$3.94x10^5$	$2.06x10^5$
		Effluent	$7.22x10^3$	$4.25x10^3$	$3.72x10^3$	$1.73x10^4$	$1.47x10^5$	$7.33x10^3$	$1.51x10^4$
		log reduction	1.46±0.1	1.77±0.1	1.76±0.12	1.10±0.08	0.19±0.07	1.73±0.07	1.13±0.07

Table S5. Relative abundance (log gene copy number/*rrn* copy ± SD) of targeted ESBL and carbapenemase genes and their log reduction values (±SD) in influent and effluent of 7 Croatian WWTPs over winter and summer sampling campaign.

ESBL and CP genes	Sampling season	Sampling type	WWTP location						
			Vinkovci	**Bjelovar**	**Zagreb**	**Čakovec**	**Varaždin**	**Karlovac**	**Zadar**
***bla*TEM**	Winter	Influent	-4.05±0.14	-4.02±0.24	-4.03±0.39	-4.00±0.23	-4.10±0.19	-4.09±0.67	-3.65±0.41
		Effluent	-4.33±0.11	-4.58±0.00	-4.20±0.20	< LOD	-4.56±0.27	< LOD	-3.87±0.09
		log reduction	0.28±0.06	0.56±0.14	0.17±0.01	*NA*	0.46±0.01	*NA*	0.22±0.12
	Summer	Influent	-2.99±0.34	-2.71±0.57	-3.05±0.17	-3.23±0.21	-2.64±0.34	-2.82±0.25	-2.94±0.85
		Effluent	-2.84±0.86	-3.23±0.01	-2.94±0.09	-3.36±0.00	-2.88±0.43	-3.35±0.16	-2.45±0.47
		log reduction	**-0.15**±0.36	0.52±0.00	**-0.11**±0.05	0.13±0.00	0.24±0.14	0.53±0.00	**-0.49**±0.28
***bla*CTX-M-32**	Winter	Influent	-4.95±0.27	-5.54±0.18	-5.14±0.35	-5.38±0.42	-5.02±0.29	-4.48±0.00	-4.47±0.46
		Effluent	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		log reduction	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	Summer	Influent	-3.48±0.25	-3.77±0.00	-3.79±0.13	-3.90±0.08	-3.43±0.12	-3.67±0.00	-3.84±0.33
		Effluent	-3.34±0.00	-3.41±0.08	-3.80±0.21	-2.93±0.30	-3.70±0.08	-3.60±0.06	-4.01±0.02
		log reduction	**-0.14**±0.00	**-0.36**±0.00	0.01±0.02	**-0.97**±0.16	0.26±0.01	**-0.07**±0.00	0.17±0.09
***bla*KPC-3**	Winter	Influent	< LOD	< LOD	-3.94±0.00	< LOD	< LOD	-3.68±0.58	< LOD
		Effluent	< LOD	-3.7±0.10	< LOD	< LOD	< LOD	< LOD	< LOD
		log reduction	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	Summer	Influent	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		Effluent	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		log reduction	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
***bla*OXA-48**	Winter	Influent	-5.21±0.00	-5.40±0.00	-5.16±0.00	-5.24±0.09	-5.32±0.00	< LOD	-5.74±0.00
		Effluent	< LOD	-5.60±0.00	< LOD	< LOD	< LOD	< LOD	< LOD
		log reduction	*NA*	0.20±0.00	*NA*	*NA*	*NA*	*NA*	*NA*
	Summer	Influent	< LOD	-3.37±0.40	-3.77±0.00	-3.92±0.44	-3.85±0.25	< LOD	-2.54±0.00
		Effluent	< LOD	< LOD	< LOD	< LOD	-3.66±0.66	< LOD	< LOD
		log reduction	*NA*	*NA*	*NA*	*NA*	**-0.19**±0.09	*NA*	*NA*
***bla*NDM**	Winter	Influent	-5.83±0.13	-5.39±0.09	-5.32±0.01	< LOD	-5.30±0.25	-4.43±0.66	< LOD
		Effluent	< LOD	-4.46±0.00	< LOD	< LOD	< LOD	-4.36±0.00	< LOD
		log reduction	*NA*	**-0.93**±0.00	*NA*	*NA*	*NA*	**-0.04**±0.00	*NA*
	Summer	Influent	< LOD	-3.97±0.00	< LOD	< LOD	< LOD	< LOD	< LOD
		Effluent	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		log reduction	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
***bla*IMP**	Winter	Influent	-4.24±0.50	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		Effluent	-3.55±0.20	-4.24±0.00	-3.88±0.31	< LOD	< LOD	-4.16±0.15	-4.44±0.18
		log reduction	**-0.68**±0.11	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
	Summer	Influent	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
		Effluent	< LOD	< LOD	-3.57±0.01	< LOD	< LOD	-3.36±0.28	-3.14±0.12
		log reduction	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*	*NA*
***bla*VIM**	Winter	Influent	< LOD	-4.57±0.28	< LOD	-4.95±0.11	-4.93±0.03	< LOD	-4.72±0.00
		Effluent	< LOD	< LOD	-4.03±0.33	< LOD	-4.83±0.00	-4.43±0.00	-4.51±0.00
		log reduction	*NA*	*NA*	*NA*	*NA*	**-0.10**±0.00	*NA*	**-0.21**±0.00
	Summer	Influent	-4.02±0.13	-3.61±0.65	-3.89±0.06	-4.29±0.11	-4.05±0.37	< LOD	-3.15±0.82
		Effluent	-3.65±0.00	-3.63±0.18	-3.37±0.18	< LOD	-3.93±0.25	-3.71±0.01	-4.26±0.07
		log reduction	**-0.37**±0.22	0.02±0.23	**-0.53**±0.24	*NA*	**-0.12**±0.2	*NA*	1.11±0.2

negative log reduction value indicates an increase in effluent; *NA* - data not available; LOD – limit of detection



Season	Sample type	*E. coli* (*ycc*T)	*E. coli* (cultivable)	Concentration differences (log units)
Winter	Influent	5.44×10^4	1.74×10^4	0.50
	Effluent	4.88×10^3	6.05×10^2	0.91
Summer	Influent	2.38×10^5	3.57×10^4	0.82
	Effluent	3.13×10^4	3.20×10^3	0.99

Figure S1. Difference in abundance of qPCR-based *E. coli* (*ycc*T) (CE/mL) and cultivable *E. coli* (CFU/mL) between influents or effluents from all 7 Croatian WWTPs in two seasons. Significant difference is assessed by Welch's t-test and is indicated by asterisks (*$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$).

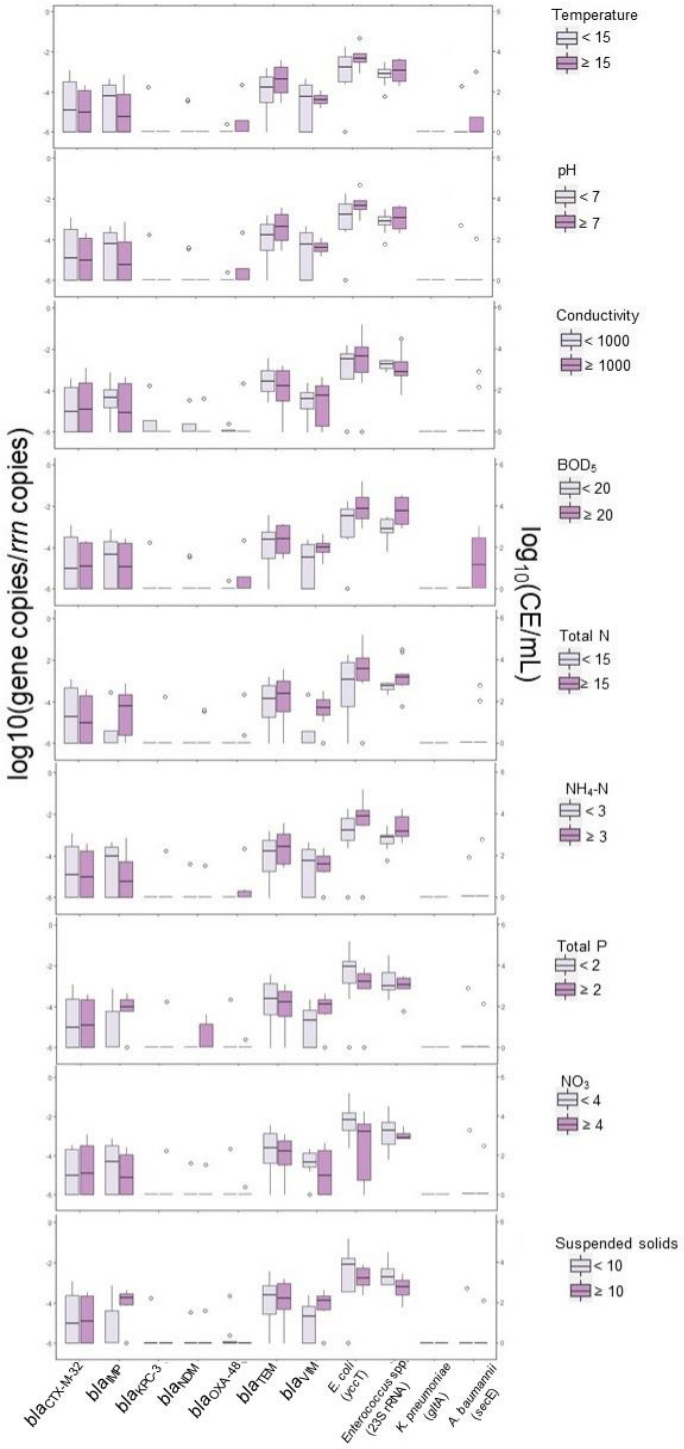


Figure S2. Box plot comparison of relative abudance of ARGs or absolute abudance of taxonomic genes as the proxies for total *E. coli*, *Enterococcus* spp., *K. pneumoniae* and *A. baumannii* between selected WWTP parameters from both sampling seasons. Boxes represent quartiles and the median, whiskers are 1.5 x IQR and circles depict outliers.

Supplementary materials for the Publication No. 2:

Puljko, A., Babić, I., Dekić Rozman, S., Barišić, I., Jelić, M., Maravić, A., Parać, M., Petrić, I., Udiković-Kolić, N. (2024) Treated municipal wastewater as a source of high-risk and emerging multidrug-resistant clones of *E. coli* and other Enterobacterales producing extended-spectrum β-lactamases. *Environmental Research*, **243**, 117792, 1-11 https://doi.org/10.1016/j.envres.2023.117792

Table S1. Primers and thermocycling conditions for PCR detection of β-lactamase and colistin resistance genes.

Target gene		Primer sequence 5' → 3'	Amplicon size (bp)	Amplification conditions	Reference
***bla*$_{CTX-M}$ groups**	***bla*$_{CTX-M-1}$**	GGTTAAAAAATCACTGCGTC TTGGTGACGATTTTAGCCGC	864	Initial denaturation step for 2:30 min at 94°C, followed by 30 cycles of 20 sec at 94°C 25 sec at 55°C 45 sec at 72°C final extension for 2 min at 72°C	Saladin et al., 2002
	***bla*$_{CTX-M-2}$**	ATGATGACTCAGAGCATTCG TGGGTTACGATTTTCGCCGC	866		
	***bla*$_{CTX-M-9}$**	ATGGTGACAAAGAGAGTGCA CCCTTCGGCGATGATTCTC	870		
ESBL-multiplex 1	***bla*$_{TEM}$**	GCGGTAAGATCCTTGAGAGT TACGATACGGGAGGGCTTA	620	Initial denaturation step for 2:30 min at 94°C, followed by 35 cycles of 30 sec at 94°C 30 sec at 55°C 45 sec at 72°C final extension for 2 min at 72°C	Jelić, 2018
	***bla*$_{SHV}$**	TTCGCCTGTGTATTATCTCC CGCCTCATTCAGTTCCG	494		
	***bla*$_{VEB}$**	ATGCCAGAATAGGAGTAGC AATTGTCCATTCGGTAAAGTAAT	673		
ESBL-multiplex 2	***bla*$_{GES}$**	CTAGCATCGGGACACAT GACAGAGGCAACTAATTCG	504		
	***bla*$_{PER}$**	CTGGGCTCCGATAATGA CTGGTCGCCWATGATGA	349		
	***bla*$_{SME}$**	GCTCAGGTATGACATTAGGT CCAATCAGCAGGAACACTA	350		
AmpC-multiplex	***bla*$_{MOX}$**	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTGC	520	Initial denaturation step for 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C 30 sec at 64°C 1 min at 72°C final extension for 7 min at 72°C	Perez-Perez and Hanson, 2002
	***bla*$_{CIT}$**	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462		
	***bla*$_{DHA}$**	AACTTTCACAGGTGTGCTGGGT CCGTACGCATACTGGCTTTGC	405		
	***bla*$_{ACC}$**	AACAGCCTCAGCAGCCGGTTA TTCGCCGCAATCATCCCTAGC	346		
	***bla*$_{EBC}$**	TCGGTAAAGCCGATGTTGCGG CTTCCACTGCGGCTGCCAGTT	302		
	***bla*$_{FOX}$**	AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG	190		
***bla*$_{OXA-48}$-like**	***bla*$_{OXA-48}$**	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	744	Initial denaturation step for 2: 30min at 94°C, followed by 30 cycles of 20 sec at 94°C, annealing temperature: 25 sec at 57°C for *bla*$_{VIM}$, *bla*$_{OXA-48}$, *bla*$_{KPC}$; 55°C for *bla*$_{IMP}$; 58°C for *bla*$_{NDM}$ 1 min at 72°C final extension for 7 min at 72°C	Poirel et al., 2004
***bla*$_{NDM}$**	***bla*$_{NDM}$**	TGGCAGCACACTTCCTATC AGATTGCCGAGCGACTTG	488		Revathi et al., 2013
***bla*$_{KPC}$**	***bla*$_{KPC}$**	AGTTCTGCTGTCTTGTCT CTTGTCATCCTTGTTAGGC	793		Jelić, 2018
***bla*$_{VIM}$**	***bla*$_{VIM}$**	GGTGAGTATCCGACAGTC CAGCACCRGGATAGAAGAG	442		
***bla*$_{IMP}$**	***bla*$_{IMP}$**	GGYGTTTATGTTCATACWTC GGATYGAGAATTAAGCCACTC	235		
***mcr*-multiplex**	***mcr-1***	AGTCCGTTTGTTCTTGTGGC AGATCCTTGGTCTCGGCTTG	320	Initial denaturation step for 15 min at 94°C, followed by 25 cycles of 30 sec at 94°C 90 sec at 58°C 1 min at 72°C final extension for 10 min at 72°C	Rebelo et al., 2018
	mcr-2	CAAGTGTGTTGGTCGCAGTT TCTAGCCCGACAAGCATACC	715		
	mcr-3	AAATAAAAATTGTTCCGCTTATG AATGGAGATCCCCGTTTTT	929		
	mcr-4	TCACTTTCATCACTGCGTTG TTGGTCCATGACTACCAATG	1116		
	mcr-5	ATGCGGTTGTCTGCATTTATC TCATTGTGGTTGTCCTTTTCTG	1664		

Table S2. List of of all isolates for PCR according to 6 Enterobacterales genera and species.

6 Enterobacterales genera	Isolate No.	Species
Escherichia coli **(n = 33)**	SE_EC_65	
	IZ 20-44	
	IZ 8-44	
	SE_EC_99	
	SE_EC_90	
	IZ 22-44	
	SE_EC_57	
	SE_EC_111	
	SE_EC_93	
	IZ 6-44	
	SE_EC_79	
	SE_EC_08	
	IZ 11-44	
	SE_EC_04	
	SE_EC_55	
	SE_EC_52	
	SE_EC_56	*E. coli*
	SE_EC_14	
	IZ 27-44	
	IZ 1-44	
	SE_EC_91	
	SE_EC_110	
	SE_EC_54	
	SE_EC_11	
	SE_EC_31	
	SE_EC_64	
	SE_EC_88	
	SE_EC_13	
	SE_EC_60	
	SE_EC_70	
	SE_EC_05	
	SE_EC_86	
	IZ 13-44	
Klebsiella **spp. (n = 10)**	SE_COL_19	*K. oxytoca*
	SE_COL_90	
	SE_COL_28	
	IZ 28-44	
	SE_COL_62	
	SE_COL_99	*K. pneumoniae*
	SE_COL_86	
	SE_COL_114	
	SE_COL_120	
	SE_COL_16	
Enterobacter cloacae **cplx. (n = 9)**	SE_COL_141	*E. bugandensis*
	SE_Col_113	*E. asburiae*
	SE_COL_94	
	SE_COL_17	
	SE_COL_61	
	SE_COL_100	*E. cloacae*
	SE_COL_136	
	SE_COL_56	
	SE_COL_87	
Raoultella **spp. (n = 8)**	SE_COL_14	
	SE-COL-09	*R. planticola*
	SE-COL-30	
	SE-COL-85	
	SE_col_80	
	SE_COL_83	*R. ornithinolytica*
	SE_COL_79	
	SE-COl_84	
Kluyvera cryocrescens **(n = 4)**	IZ 23-37	
	IZ 17-37	*K. cryocrescens*
	SE_COL_98	
	SE_COL_89	
Citrobacter **spp. (n = 4)**	IZ-29-37	*C. freundii*
	IZ 11-37	
	SE_COL_18	*C. braakii*
	SE_COL_70	

Table S3. Virulence factors found in *Escherichia coli*

ID	ST	ExPEC	UPEC	Adherence	Autotransporters	Iron uptake	Non-LEE encoded TTSS effectors	Toxins	Invasion
SE_EC_56	131	YES: *papC, iutA*	NO: *chuA*	ECP: *ecpBCDER*; HCP: *hcpAC;* P fimbriae: *papC*IX; Type I fimbriae: *fimDFGHI*	*ehaB*; *pic; sat*	Aerobactin: *iucABCD, iutA;* Heme uptake: *chuAY*; Fe/Mg transport: *sitBC*; Yersiniabactin: *ybtA*	*espL1*	Alpha -Hemolysin: *hlyACD*; Hemolysin/cytolysin: *hlyE/clyA*	-
SE_EC_79	361	NO	NO	CFA/I fimbriae: *cfBCD/E*; ECP: *ecpABCD*; ELF: *elfACG*; HCP: *hcpAC*; Type I fimbriae: *fimACDEFGHI*	*cah; ehaB*	Fe/Mg transport: *sitABCD*	*Espl1, espL4, espR1, espX1, espX4, espX5*	Hemolysin/cytolysin: *hlyE/clyA*	Ibes: *ibeBC*
IZ 8-44	361	NO	NO	CFA/I fimbriae: *cfBCD/E;* ECP: *ecpABCDE*; ELF: *elfACG*; eaeH; HCP: *hcpABC*; Type I fimbriae: *fimACDEFGH*	*ehaB*	Fe/Mg transport: *sitABCD*	*Espl1, espL4, espR1, espX1, espX4, espX5*	Hemolysin/cytolysin: *hlyE/clyA*	Ibes: *ibeBC*

ECP: *E. coli* common pilus; HPC: hemorrhagic *E. coli* pilus; ELF: *E. coli* laminin-binding fimbriae; Ibes: Invasion of brain endothelial cell

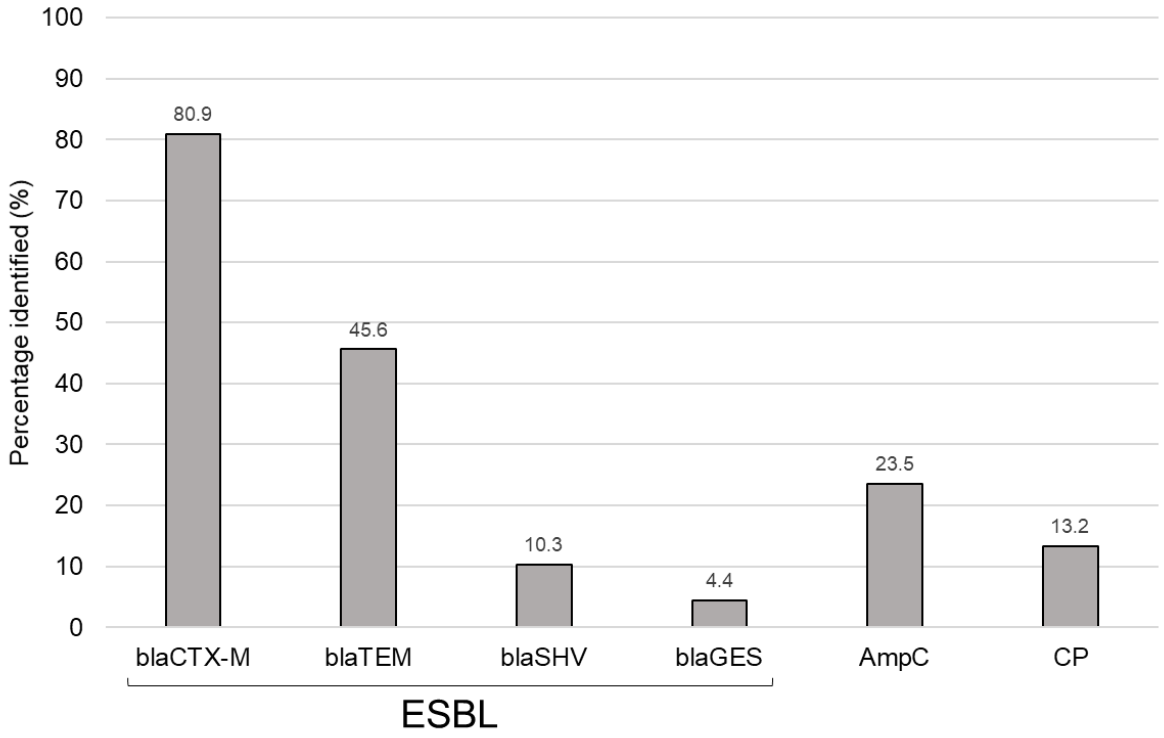


Figure S1. Percentages of main groups of β-lactamase genes: ESBL (*bla*$_{\text{TEM}}$ group*, *bla*$_{\text{CTX-M}}$ group, *bla*$_{\text{SHV}}$ group**, and *bla*$_{\text{GES-7}}$ group), carbapenemase genes (CP), and AmpC genes detected in total of 64 ESBL-producing isolates. **bla*$_{\text{TEM}}$ group - includes only *bla*$_{\text{TEM-116}}$ and *bla*$_{\text{TEM-135}}$; ***bla*$_{\text{SHV}}$ group includes only *bla*$_{\text{SHV-12}}$, and *bla*$_{\text{SHV-28}}$.

Supplementary materials for the Publication No. 3:

Puljko, A., Barišić, I., Dekić Rozman, S., Križanović, S., Babić, I., Jelić, M., Maravić, A., Udiković-Kolić, N. (2024) Molecular epidemiology and mechanisms of carbapenem and colistin resistance in *Klebsiella* and other Enterobacterales from treated wastewater in Croatia. *Environment International*, **185**, 108554, 1-12 https://doi.org/10.1016/j.envint.2024.108554

Table S1. Kirby-Bauer Assay and minimum inhibitory concentration (MIC) assays for carbapenem-resistant Enterobacterales

Species (MALDI-TOF MS)	Isolate ID	Kirby- Bauer Assay (diameter/mm)												MIC assay (mg/L)				Multidrug resistance
		AML25*	AMC30	CL30	CXM30	CAZ10	FEP30	ETP10	IPM10	MEM10	GM10	SXT (1.25/	CIP5	ETP	IPM	MEM	COL	
Klebsiella pneumoniae	SE_SC_COL_54	0	0	0	0	0	0	0	0	0	0	0	0	>16	>64	>64	32	PDR
Klebsiella pneumoniae	SE_SC_COL_47	0	0	0	0	0	0	0	0	0	0	0	0	>16	>64	>64	32	PDR
Klebsiella pneumoniae	SE_SC_COL_46	0	0	0	0	0	0	0	0	0	8	0	0	>16	>64	>64	32	PDR
Klebsiella pneumoniae	SE_SC_COL_96	0	0	0	0	16	17	0	0	0	21	24	13	>16	>64	>64	32	XDR
Klebsiella pneumoniae	SE_SC_COL_95	0	0	0	0	9	15	0	0	0	23	25	13	>16	>64	>64	16	XDR
Klebsiella pneumoniae	SE_SC_COL_97	0	0	0	0	12	16	0	0	0	23	24	13	>16	>64	>64	16	XDR
Klebsiella pneumoniae	SE_SC_COL_99	0	0	0	0	14	15	0	0	0	23	24	12	>16	>64	>64	16	XDR
Klebsiella pneumoniae	SE_SC_COL_182	0	0	0	0	13	15	0	0	0	19	23	12	>16	>64	>64	8	XDR
Klebsiella pneumoniae	SE_SC_COL_204	0	0	0	0	11	14	0	0	0	24	23	13	>16	>64	>64	8	XDR
Klebsiella pneumoniae	SE_SC_COL_101	0	0	0	0	12	15	0	0	0	19	26	14	>16	>64	>64	8	XDR
Klebsiella pneumoniae	SE_SC_COL_67	0	0	0	0	0	15	0	0	0	21	27	21	>16	>64	>64	4	XDR
Klebsiella pneumoniae	SE_SC_COL_84	0	0	0	0	0	8	8	9	8	23	27	15	>16	>64	>64	4	XDR
Klebsiella pneumoniae	SE_SC_COL_102	0	0	0	0	0	8	12	15	12	22	19	15	>16	>64	>64	4	XDR
Klebsiella pneumoniae	SE_SC_COL_82	0	0	0	0	10	19	15	15	17	0	14	15	>16	>64	64	4	XDR
Klebsiella pneumoniae	SE_SC_COL_86	0	0	0	0	0	16	14	19	16	19	20	16	>16	>64	>64	16	XDR
Klebsiella pneumoniae	SE_SC_COL_87	0	0	0	0	0	12	13	19	16	20	20	16	>16	64	>64	4	XDR
Klebsiella pneumoniae	SE_SC_COL_51	0	0	0	0	0	0	0	0	0	0	0	0	>16	>64	>64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_59	0	0	0	0	0	0	0	0	0	0	0	0	>16	>64	64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_57	0	0	0	0	0	0	8	20	14	8	0	0	>16	32	32	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_21	0	0	0	0	0	14	0	0	8	8	28	21	>16	>64	>64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_52	0	0	0	0	0	0	0	0	10	0	21	0	>16	>64	>64	2	XDR
Klebsiella pneumoniae	SE_SC_COL_69	0	0	0	0	0	16	9	15	10	16	21	15	>16	>64	>64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_190	0	0	0	0	0	15	11	21	13	15	25	17	>16	>64	>64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_173	0	0	0	0	10	20	15	16	15	0	22	15	>16	>64	64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_164	0	0	0	0	13	21	15	20	18	0	21	15	>16	>64	64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_148	0	0	0	0	13	21	14	10	18	0	10	16	>16	>64	64	32	XDR
Klebsiella pneumoniae	SE_SC_COL_30	0	0	0	0	0	15	16	17	16	13	28	18	>16	>64	64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_61	0	0	0	0	0	14	0	8	8	9	29	20	>16	>64	>64	<1	MDR
Klebsiella pneumoniae	SE_SC_COL_78	0	0	0	0	0	15	0	12	10	22	0	0	>16	>64	>64	<1	XDR
Klebsiella pneumoniae	SE_SC_COL_72	0	0	0	0	0	16	9	16	12	20	22	17	>16	>64	>64	2	MDR
Klebsiella pneumoniae	SE_SC_COL_152	0	0	0	0	12	15	0	0	0	24	23	13	>16	>64	>64	2	MDR
Klebsiella pneumoniae	SE_SC_COL_189	0	0	0	0	0	18	13	19	18	20	22	16	>16	>64	>64	2	MDR
Klebsiella pneumoniae	SE_SC_COL_137	0	0	0	9	16	24	17	19	18	25	26	14	>16	64	32	2	MDR
Klebsiella pneumoniae	SE_SC_COL_71	0	0	0	0	0	18	10	17	11	25	23	17	>16	>64	>64	<1	MDR
Klebsiella pneumoniae	SE_SC_COL_68	0	0	0	0	0	17	10	15	10	27	22	17	>16	>64	>64	<1	MDR
Klebsiella pneumoniae	SE_SC_COL_118	0	0	0	0	12	26	0	0	0	22	22	13	>16	>64	>64	<1	MDR
Klebsiella oxytoca	SE_SC_COL_215	0	0	0	0	0	12	0	20	19	15	31	10	>16	>64	>64	4	XDR
Klebsiella oxytoca	SE_SC_COL_140	0	0	0	0	0	10	11	18	14	13	26	9	>16	>64	>64	8	XDR
Klebsiella oxytoca	SE_SC_COL_33	0	0	0	0	0	21	16	17	19	0	0	11	>16	>64	64	<1	XDR
Klebsiella oxytoca	SE_SC_COL_88	0	0	0	0	12	27	18	18	20	14	0	19	>16	64	32	<1	XDR
Klebsiella oxytoca	SE_SC_COL_89	0	0	0	0	11	26	16	20	16	26	0	15	>16	>64	64	<1	MDR
Klebsiella oxytoca	SE_SC_COL_150	0	0	0	0	0	12	8	17	16	18	21	17	16	>64	64	<1	MDR
Klebsiella oxytoca	SE_SC_COL_28	0	0	0	0	13	16	0	0	0	22	30	15	>16	>64	>64	<1	MDR
Klebsiella oxytoca	SE_SC_COL_27	0	0	0	0	0	9	0	0	8	0	27	27	>16	>64	>64	<1	MDR
Klebsiella oxytoca	SE_SC_COL_103	0	0	0	0	9	14	0	11	0	10	29	18	>16	>64	>64	<1	XDR
Klebsiella oxytoca	SE_SC_COL_104	0	0	0	0	9	13	0	12	0	9	29	17	>16	>64	>64	<1	XDR
Klebsiella oxytoca	SE_SC_COL_09	0	0	0	10	14	21	20	21	21	16	33	19	>16	>64	64	<1	XDR

Table S1. Continued

Species (MALDI-TOF MS/16S rRNA)	Isolate ID	Kirby- Bauer Assay (diameter/mm)												MIC assay (mg/L)				Multidrug resistance
		AML 25*	AMC 30*	CL 30*	CXM 30	CAZ 10	FEP 30	ETP 10	IPM 10	MEM 10	GM 10	SXT (1.25/	CIP 5	ETP	IPM	MEM	COL	
Citrobacter freundii	SE_SC_COL_225	0	0	0	0	0	0	9	13	10	16	24	19	>16	>64	>64	4	XDR
Citrobacter freundii	SE_SC_EC_31	0	0	0	0	0	15	12	14	13	21	26	16	>16	>64	>64	64	XDR
Citrobacter freundii	SE_SC_COL_23	0	0	0	0	0	14	12	16	13	16	0	9	>16	>64	>64	2	XDR
Citrobacter freundii[1]	SE_SC_COL_22	0	0	0	0	0	9	9	16	10	16	0	14	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_COL_194	0	0	0	0	0	11	11	14	12	0	0	8	>16	>64	>64	2	XDR
Citrobacter freundii	SE_SC_COL_76	0	0	0	0	0	13	16	17	14	16	0	17	>16	>64	>64	2	XDR
Citrobacter freundii	SE_SC_COL_94	0	0	0	0	0	12	13	15	19	9	0	0	>16	>64	>64	2	XDR
Citrobacter freundii	SE_SC_COL_124	0	0	0	0	0	10	0	13	10	0	0	10	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_COL_100	0	0	0	0	0	13	9	15	10	16	0	8	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_COL_24	0	0	0	0	0	12	10	16	11	16	0	14	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_COL_113	0	0	0	0	0	19	14	16	17	9	0	9	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_COL_168	0	9	0	0	0	12	0	19	18	0	0	10	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_COL_50	0	0	0	0	9	0	0	16	11	12	0	0	>16	>64	64	<1	XDR
Citrobacter freundii	SE_SC_COL_25	0	0	0	0	0	14	14	18	13	16	0	9	>16	>64	64	<1	XDR
Citrobacter freundii	SE_SC_COL_31	0	0	0	0	0	0	0	14	10	12	0	0	>16	64	64	<1	XDR
Citrobacter freundii	SE_SC_COL_105	0	0	8	0	0	20	18	21	22	16	0	13	>16	>64	32	<1	XDR
Citrobacter freundii	SE_SC_COL_222	0	0	0	0	0	14	9	13	9	14	32	21	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_COL_93	0	8	0	0	0	19	15	18	19	15	24	21	>16	>64	64	2	XDR
Citrobacter freundii	SE_SC_COL_169	0	12	0	0	7	14	0	20	19	22	0	14	>16	64	64	<1	XDR
Citrobacter freundii	SE_SC_COL_19	0	0	0	0	0	8	7	10	8	24	0	11	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_COL_02	0	0	0	0	0	14	18	19	15	17	0	14	>16	>64	>64	<1	XDR
Citrobacter freundii	SE_SC_EC_05	0	0	0	0	0	17	12	18	15	18	34	21	>16	>64	>64	2	MDR
Citrobacter freundii	SE_SC_EC_04	0	0	0	0	0	12	15	17	14	23	21	19	>16	>64	>64	2	MDR
Citrobacter freundii	SE_SC_COL_117	0	10	0	0	0	15	11	16	13	20	19	16	>16	>64	>64	<1	MDR
Citrobacter freundii	SE_SC_EC_33	0	0	0	0	12	19	14	19	18	22	33	19	>16	>64	64	<1	MDR
Citrobacter freundii	SE_SC_COL_64	0	0	0	0	0	20	15	19	19	17	21	17	>16	>64	32	<1	MDR
Citrobacter freundii	SE_SC_COL_63	0	8	0	0	9	20	11	18	16	18	25	17	>16	64	32	<1	MDR
Citrobacter brakii	SE_SC_COL_91	0	0	0	0	0	9	0	13	9	8	0	12	>16	64	>64	<1	XDR
Citrobacter brakii	SE_SC_COL_92	0	0	0	0	0	14	8	14	9	10	0	0	>16	64	64	<1	XDR
Citrobacter brakii	SE_SC_COL_77	0	0	0	0	0	15	18	18	16	15	0	13	>16	64	64	<1	XDR
Citrobacter brakii	SE_SC_COL_01	0	0	0	0	0	11	0	0	0	16	31	13	>16	>64	>64	<1	XDR
Citrobacter brakii	SE_SC_COL_107	0	0	0	0	11	16	0	0	0	22	29	21	>16	>64	>64	<1	MDR
Citrobacter brakii	SE_SC_COL_90	0	0	0	0	0	14	0	0	0	24	32	12	>16	>64	>64	<1	MDR
Citrobacter brakii	SE_SC_COL_114	0	0	0	0	0	14	0	0	0	22	30	11	>16	>64	>64	<1	MDR
Citrobacter brakii	SE_SC_COL_158	0	0	0	0	9	17	0	19	16	18	29	19	>16	>64	64	<1	MDR
Citrobacter brakii	SE_SC_COL_186	0	0	9	0	0	12	19	19	18	18	14	15	16	>64	32	<1	MDR
Citrobacter brakii	SE_SC_COL_203	0	0	0	0	10	23	13	21	22	26	37	21	>16	64	8	2	MDR
Citrobacter farmeri	SE_SC_COL_83	0	10	0	0	19	24	18	19	18	17	27	21	>16	64	32	2	MDR
Citrobacter farmeri	SE_SC_COL_17	0	9	8	0	0	24	19	20	19	18	29	21	>16	64	16	2	MDR
Citrobacter farmeri	SE_SC_COL_34	0	11	0	0	15	22	19	22	19	17	29	21	>16	>64	32	<1	MDR

Table S1. Continued

Species (MALDI-TOF MS/16S rRNA)	Isolate ID	Kirby- Bauer Assay (diameter/mm)												MIC assay (mg/L)				Multidrug resistance
		AML 25*	AMC 30*	CL 30*	CXM 30	CAZ 10	FEP 30	ETP 10	IPM 10	MEM 10	GM 10	SXT (1.25/	CIP 5	ETP	IPM	MEM	KOL	
Enterobacter cloacae	SE_SC_COL_214	0	8	0	0	16	23	13	17	15	19	35	21	>16	>64	64	>64	XDR
Enterobacter cloacae	SE_SC_COL_199	0	11	0	0	14	23	0	0	0	21	24	20	>16	64	32	>64	XDR
Enterobacter cloacae	SE_SC_COL_223	0	0	0	0	10	16	16	19	17	25	29	12	>16	>64	64	4	XDR
Enterobacter cloacae	SE_SC_COL_198	0	0	0	0	13	19	18	16	18	15	29	30	>16	>64	64	>64	XDR
Enterobacter cloacae	SE_SC_COL_218	0	0	0	0	19	21	8	20	19	18	31	20	>16	>64	64	>64	MDR
Enterobacter cloacae	SE_SC_COL_80	0	0	0	0	0	22	0	9	9	8	29	16	>16	32	32	>64	XDR
Enterobacter cloacae	SE_SC_COL_36	0	0	0	0	18	26	19	24	24	14	28	15	2	8	8	>64	MDR
Enterobacter cloacae [1]	SE_SC_COL_197	0	0	0	0	13	15	0	0	0	23	28	9	>16	>64	>64	<1	MDR
Enterobacter cloacae	SE_SC_COL_75	0	9	0	0	15	21	16	19	17	21	31	15	>16	>64	64	2	MDR
Enterobacter cloacae	SE_SC_COL_111	0	0	0	0	0	15	18	16	18	19	19	21	>16	>64	>64	2	MDR
Enterobacter cloacae	SE_SC_COL_208	0	0	0	0	16	23	8	20	19	26	34	15	>16	>64	64	<1	MDR
Enterobacter asburiae	SE_SC_COL_159	0	0	0	0	13	20	0	13	11	20	29	17	>16	>64	>64	>64	XDR
Enterobacter asburiae	SE_SC_COL_206	0	0	0	0	12	17	13	15	16	16	28	17	>16	>64	>64	>64	XDR
Enterobacter asburiae	SE_SC_COL_207	0	8	0	0	12	18	13	15	16	15	28	16	>16	>64	64	>64	XDR
Enterobacter asburiae	SE_SC_COL_110	0	0	0	0	14	22	16	19	17	22	28	16	>16	>64	64	16	XDR
Enterobacter asburiae	SE_SC_COL_166	0	0	0	0	10	18	0	17	13	23	32	19	>16	>64	>64	8	XDR
Enterobacter asburiae	SE_SC_COL_185	0	0	0	0	15	21	13	20	11	24	31	19	>16	>64	>64	8	XDR
Enterobacter asburiae	SE_SC_COL_162	0	0	0	0	14	21	0	19	16	20	31	18	>16	>64	>64	4	XDR
Enterobacter asburiae	SE_SC_COL_106	0	0	0	0	13	20	15	21	16	27	30	14	>16	>64	>64	4	XDR
Enterobacter asburiae	SE_SC_COL_188	0	8	7	0	16	22	16	20	19	15	32	15	>16	>64	64	4	XDR
Enterobacter asburiae	SE_SC_COL_167	0	0	0	0	10	16	0	16	18	27	31	21	>16	64	64	2	MDR
Enterobacter asburiae	SE_SC_COL_163	0	0	0	0	11	21	0	18	18	21	31	19	>16	>64	>64	2	MDR
Enterobacter asburiae	SE_SC_COL_145	0	0	0	0	14	18	13	18	19	22	29	17	>16	>64	64	2	MDR
Enterobacter asburiae	SE_SC_COL_153	0	0	0	0	15	19	0	16	11	23	25	17	>16	>64	>64	<1	MDR
Enterobacter asburiae	SE_SC_COL_156	0	0	0	0	13	18	0	16	11	21	26	16	>16	>64	>64	<1	MDR
Enterobacter asburiae	SE_SC_COL_170	0	0	0	0	12	16	10	12	8	22	25	15	>16	>64	>64	<1	MDR
Enterobacter asburiae	SE_SC_COL_109	0	0	0	0	0	16	11	19	16	25	0	21	>16	>64	>64	2	XDR
Enterobacter asburiae	SE_SC_COL_154	0	0	0	12	0	22	0	15	14	23	25	18	16	8	4	<1	MDR
Enterobacter kobei	SE_SC_COL_155	0	0	0	0	11	17	0	14	13	23	31	9	>16	>64	>64	4	XDR
Enterobacter kobei	SE_SC_COL_187	0	0	0	0	14	20	16	20	18	15	31	15	>16	>64	64	4	XDR
Enterobacter kobei	SE_SC_COL_81	0	0	0	0	0	21	0	11	9	7	29	18	>16	64	>64	64	XDR
Enterobacter kobei	SE_SC_COL_165	0	0	0	0	9	21	8	19	18	22	30	15	>16	>64	32	2	MDR
Enterobacter ludwigii	SE_SC_COL_132	0	0	0	0	12	21	15	17	14	25	27	15	>16	>64	>64	2	MDR
Enterobacter ludwigii	SE_SC_COL_176	0	0	0	0	11	21	14	16	13	21	29	16	>16	>64	32	2	MDR
Enterobacter ludwigii [1]	SE_SC_COL_195	0	0	0	0	14	20	15	20	18	16	30	18	>16	>64	>64	>64	XDR

Table S1. Continued

Species (MALDI-TOF MS)	Isolate ID	Kirby- Bauer Assay (diameter/mm)												MIC assay (mg/L)				Multidrug resistance
		AML 25	AMC 30*	CL 30	CXM 30	CAZ 10	FEP 30	ETP10	IPM10	MEM10	GM 10	SXT (1.25/	CIP 5	ETP	IPM	MEM	COL	
Raoultella ornithinolytica	SE_SC_COL_211	0	0	0	0	9	21	0	15	16	22	35	25	>16	>64	>64	>64	MDR
Raoultella ornithinolytica	SE_SC_COL_160	0	0	0	0	0	19	0	21	17	9	32	18	>16	>64	64	<1	XDR
Raoultella ornithinolytica	SE_SC_Col_183	0	0	0	0	0	14	16	15	13	14	16	10	>16	64	>64	<1	XDR
Raoultella ornithinolytica	SE_SC_COL_08	0	0	0	0	10	21	14	19	18	19	22	21	>16	>64	>64	<1	MDR
Raoultella ornithinolytica	SE_SC_COL_07	0	8	8	0	9	21	14	19	16	20	23	20	>16	>64	>64	<1	MDR
Raoultella ornithinolytica	SE_SC_COL_142	0	0	0	0	10	19	12	16	12	19	26	21	>16	>64	>64	<1	MDR
Raoultella ornithinolytica	SE_SC_COL_39	0	0	0	8	13	23	18	21	19	20	33	21	>16	>64	64	<1	MDR
Raoultella ornithinolytica	SE_SC_COL_26	0	9	0	8	16	24	17	20	20	23	33	25	>16	>64	32	<1	MDR
Raoultella ornithinolytica	SE_SC_COL_128	0	0	0	18	19	24	0	0	0	24	32	25	>16	>64	64	<1	MDR
Raoultella planticola	SE_SC_COL_03	0	0	0	0	0	12	0	9	9	9	0	16	>16	64	32	2	XDR
Raoultella planticola	SE_SC_COL_05	0	0	0	0	7	11	0	11	10	8	0	16	>16	32	64	2	XDR
Raoultella planticola	SE_SC_EC_15	0	0	0	0	10	15	0	0	0	24	27	25	>16	>64	>64	<1	MDR

Species (MALDI-TOF MS)	Species ID	Kirby- Bauer Assay (diameter/mm)												MIC assay (mg/L)				Multidrug resistance
		AML 25	AMC 30	CL 30	CXM 30	CAZ 10	FEP 30	ETP 10	IPM 10	MEM 10	GM 10	SXT (1.25/	CIP 5	ETP	IPM	MEM	COL	
Escherichia coli	SE_SC_EC_26	0	10	0	0	14	21	18	21	20	23	33	20	>16	>64	>64	2	MDR
Escherichia coli	SE_SC_EC_28	0	12	0	0	16	23	13	21	18	25	32	20	>16	>64	>64	2	MDR
Escherichia coli	SE_SC_COL_15	0	11	0	0	14	22	19	21	21	24	31	21	>16	>64	64	2	MDR
Escherichia coli	SE_SC_EC_22	0	0	0	0	13	16	0	0	0	22	31	19	>16	>64	>64	<1	MDR
Escherichia coli	SE_SC_EC_21	0	0	0	0	11	16	0	0	0	23	28	18	>16	>64	>64	2	MDR
Escherichia coli	SE_SC_EC_29	0	10	0	0	17	25	12	21	22	25	31	18	>16	>64	64	2	MDR
Escherichia coli	SE_SC_EC_37	0	11	0	0	18	25	10	25	25	30	35	21	>16	64	32	2	MDR

Species (MALDI-TOF MS)	Isolate ID	Kirby- Bauer Assay (diameter/mm)											
		AML 25	AMC30*	CL 30	CXM 30	CAZ 10	FEP 30	ETP 10	IPM 10	MEM 10	GM 10	SXT (1.25/	CIP 5
Kluyvera cryocrescens	SE_SC_COL_157	0	2	0	9	19	22	0	18	17	24	27	20
Kluyvera cryocrescens	SE_SC_COL_216	0	0	0	10	19	22	0	10	8	25	27	18
Kluyvera cryocrescens	SE_SC_COL_126	0	0	0	0	16	21	15	20	17	24	21	13
Kluyvera cryocrescens	SE_SC_COL_127	0	0	0	0	14	19	14	18	15	21	25	18
Kluyvera cryocrescens	SE_SC_COL_16	0	0	0	0	16	21	15	19	16	21	25	18

Table S1. Continued

Species (MALDI-TOF MS)	Isolate ID	Kirby- Bauer Assay (diameter/mm)												MIC assay (mg/L)				Multidrug resistance
		AML 25	AMC30	CL 30	CXM 30	CAZ 10	FEP 30	ETP10	IPM10	MEM10	GM 10	SXT (1.25/	CIP 5	ETP	IPM	MEM	COL	
Leclercia adecarboxylata	SE_SC_COL_133	0	0	0	0	0	10	0	13	10	9	0	17	>16	>64	>64	8	PDR

Species (MALDI-TOF MS)	Isolate ID	Kirby- Bauer Assay (diameter/mm)												MIC assay (mg/L)				Multidrug resistance
		AML 25*	AMC 30*	CL 30*	CXM 30*	CAZ 10	FEP 30	ETP10	IPM10	MEM10	GM 10	SXT (1.25/	CIP 5	ETP	IPM	MEM	COL*	
Serratia marcescens	SE_SC_COL_209	0	0	0	0	0	0	0	0	0	8	19	10	>16	>64	>64	>64	XDR

[1] Species confirmed with 16S rRNA Sanger sequencing. *Intrinsic resistance: amoxicillin, amoxicillin/clavulanic acid, cephalexin, cefuroxime, and/or colistin. AML25 - Amoxicillin (25 µg), AMC30 - Amoxicillin/clavulanic (30 µg), CL30 - Cephalexin (30 µg), CXM30 - Cefuroxime (30 µg), CAZ10 - Ceftazidime (10 µg), FEP30 - cefepime (30 µg), ETP10 - Ertapenem (10 µg), IPM10 - Imipenem (10 µg), MEM - Meropenem (10 µg), GM10 - Gentamicin (10 µg), SXT1.25/23.75 - Trimethoprim/Sulfamethoxazole (1.25/23.75 µg), CIP5 - Ciprofloxacin (5 µg), COL - Colistin. MDR - Multidrug resistant, XDR - Extensively drug resistant, PDR – Pandrug-resistant.

Table S2. Singleplex and multiplex PCR primers and thermocycling conditions for detection of carbapenemase and colistin resistance genes.

Target gene		Primer sequence 5' → 3'	Amplicon size (bp)	Amplification conditions	Reference
***bla*OXA-48-like**	***bla*OXA-48**	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	744	Initial denaturation step for 2: 30min at 94°C, followed by 30 cycles of 20 sec at 94°C, annealing temperature: 25 sec at 57°C for *bla*VIM, *bla*OXA-48, *bla*KPC; 55°C for *bla*IMP; 58°C for *bla*NDM 1 min at 72°C final extension for 7 min at 72°C	Poirel et al., 2004
***bla*NDM**	***bla*NDM**	TGGCAGCACACTTCCTATC AGATTGCCGAGCGACTTG	488		Revathi et al., 2013
***bla*KPC**	***bla*KPC**	AGTTCTGCTGTCTTGTCT CTTGTCATCCTTGTTAGGC	793		Jelić, 2018
***bla*VIM**	***bla*VIM**	GGTGAGTATCCGACAGTC CAGCACCRGGATAGAAGAG	442		
***bla*IMP**	***bla*IMP**	GGYGTTTATGTTCATACWTC GGATYGAGAATTAAGCCACTC	235		
***mcr*-multiplex**	***mcr-1***	AGTCCGTTTGTTCTTGTGGC AGATCCTTGGTCTCGGCTTG	320	Initial denaturation step for 15 min at 94°C, followed by 25 cycles of 30 sec at 94°C 90 sec at 58°C 1 min at 72°C final extension for 10 min at 72°C	Rebelo et al., 2018
	***mcr*-2**	CAAGTGTGTTGGTCGCAGTT TCTAGCCCGACAAGCATACC	715		
	***mcr*-3**	AAATAAAAATTGTTCCGCTTATG AATGGAGATCCCCGTTTTT	929		
	***mcr*-4**	TCACTTTCATCACTGCGTTG TTGGTCCATGACTACCAATG	1116		
	***mcr*-5**	ATGCGGTTGTCTGCATTTATC TCATTGTGGTTGTCCTTTTCTG	1664		

Table S3. Sequences producing significant alignments with *mcr-4.3*.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession	Source	Country
Leclercia adecarboxylata strain Z96-1 plasmid pMCR-Z96-1	Leclercia adecarboxylata	3003	3003	100%	0	100	9782	CP040891.1	Human	China
Acinetobacter baumannii strain EH plasmid pEH_mcr4.3	Acinetobacter baumannii	3003	3003	100%	0	100	18786	CP038261.1	Human	Czech Republic
Acinetobacter baumannii strain EC plasmid pEC_mcr4.3	Acinetobacter baumannii	3003	3003	100%	0	100	43093	CP038265.1	Poultry	Czech Republic
Acinetobacter baumannii plasmid pAB18PR065-MCR-4.3	Acinetobacter baumannii	3003	3003	100%	0	100	25602	MK360916.1	Swine	China
Acinetobacter baumannii strain MRSN15313 plasmid pAb-MCR4.3	Acinetobacter baumannii	3003	3003	100%	0	100	35502	CP033872.1	Human	Brazil
Enterobacter cloacae strain En_MCR4 plasmid pEn_MCR4	Enterobacter cloacae	3003	3003	100%	0	100	8639	MH061380.1	Human	China
Enterobacter cloacae strain ENT1164 phosphoethanolamine transferase MCR-4.3 (mcr-4) gene	Enterobacter cloacae	3003	3003	100%	0	100	1626	MG026621.1	Human	Singapore
Enterobacter kobei strain MUMC-1 plasmid pMUMC-1_5	Enterobacter kobei	3003	3003	100%	0	100	12808	CP119978.1	Human	Netherlands
Enterobacter kobei strain MUMC-2 plasmid pMUMC-2_4	Enterobacter kobei	3003	3003	100%	0	100	12808	CP119977.1	Human	Netherlands
Enterobacter kobei strain RIVM_C014549 plasmid pRIVM_C014549_3	Enterobacter kobei	3003	3003	100%	0	100	12786	CP119533.1	Human	Netherlands
Enterobacter kobei strain RIVM_C009363 plasmid pRIVM_C009363_4	Enterobacter kobei	3003	3003	100%	0	100	12808	CP119532.1	Human	Netherlands
Acinetobacter nosocomialis strain 2S8-227 plasmid p2S8-227-mcr4.3	Acinetobacter nosocomialis	3003	3003	100%	0	100	24013	CP076399.1	Human	Taiwan
Enterobacter kobei strain 26153 plasmid pMCR4-26153	Enterobacter kobei	3003	3003	100%	0	100	12808	OP428979.1	Human	Czech Republic
Enterobacter cloacae strain 2022CK-00409 plasmid unnamed3	Enterobacter cloacae	3003	3003	100%	0	100	10802	CP104839.1	Human	USA
Shewanella baltica strain 11FHM2 plasmid pSBP2_mcr4	Shewanella baltica	3003	3003	100%	0	100	86727	CP051531.1	Atlantic Mackerel	Norway
Salmonella enterica subsp. enterica serovar London strain LZ19MPS10 plasmid pYULZMPS10	Salmonella enterica subsp. enterica serovar Londo	3003	3003	100%	0	100	158615	CP100354.1	Swine	China
Acinetobacter baumannii strain LWGS-04-08-26 plasmid pLWGS0408-26	Acinetobacter baumannii	3003	3003	100%	0	100	26839	MW413305.1	Slaugtherhouse processed wastewater	Germany
Lelliottia amnigena strain FDAARGOS 1446 plasmid unnamed2	Lelliottia amnigena	3003	3003	100%	0	100	123770	CP077333.1	Unknown	Germany
Enterobacter kobei strain IB2020 plasmid pIB2020_ColE_MCR	Enterobacter kobei	3003	3003	100%	0	100	12808	CP059482.1	Human	Italy
Shewanella frigidimarina NCIMB 400, chromosome	Shewanella frigidimarina NCIMB 400	3003	3003	100%	0	100	4845257	CP000447.1	Unknown	USA

Table S4. Genetic context of *mcr-4.3* gene from different *Acinetobacter* spp. isolates related to *mcr-4.3* gene from *K. pneumoniae* ST629 SE_SC_COL_47.

Species	Source	Plasmid name/size (bp)	*mcr-4.3*	Query cover (%)	Identity (%)	GenBank accession number	Reference
Acinetobacter baumannii strain EH	Clinical sample, Czech Republic	pEH_mcr4.3 43,093	***mcr-4.3****/recombinase/phd/parE/Tn*3	100	99.96	CP038261	Bitar et al., 2019
Acinetobacter baumannii strain MRSN15315	Clinical sample, Brazil	pAb-MCR4.3 35,502	*Tn3/parE/phd/recombinase/****mcr-4.3***	100	99.96	CP033872	Unpublished
Acinetobacter baumannii strain LWGS-04-08-26	Slaughterhouse wastewater, Germany	pLWGS0408-26 26,839	*Tn3/toxin/antitoxin/resolvase/resolvase/mcr-4.3*	100	99.94	MW413305	Unpublished
Acinetobacter baumannii strain EC	Poultry, Czech Republic	pEC_mcr4.3 43,093	*Tn3/parE/phd/recombinase/****mcr-4.3***	100	99.94	CP038265	Bitar et al., 2019
Acinetobacter nosocomialis strain 2S8-227	Clinical samples, Taiwan	P2S8-227-mcr4.3 24,013	***mcr-4.3****/recombinase/phd/parE/Tn*3	100	99.91	CP076399	Unpublished

Table S5. Summary of amino acid changes in proteins associated with colistin resistance in Enterobacterales identified by whole genome sequencing (WGS).

ST/Isolate ID/Species	PmrA	PmrB	PhoP	PhoQ	MgrB	CrrB*
ST629 SE_SC_COL_47 *K. pneumoniae*	WT	WT	WT	WT	WT	Q287K
ST1803 SE_SC_COL_148 *K. quasipneumoniae*	**S64A,** N131D, **L140Q, E199D,** N219H	**T8N, N105S,** A228T, Q232E, I242V, N224S, T246D, **R256S(*),** E272Q, Q356R	R34K, **R165H**	R64K, Q92K, A106T, E112D, I139V, **Q140K**, L163F, T372S, H410Y, **Q424L(*),** Q482L, Q487E	/	/
ST3590 SE_SC_COL_96 *K. quasipneumoniae*	/	**M175V**	**K149E**	WT	/	/
ST1697 SE_SC_COL_102 *K. quasipneumoniae*	WT	Nucleotide deletion (601-632 nk)	WT	WT	/	/
ST43 SE_SC_COL_140 *K. michigenansis*	WT	WT	WT	WT	WT	WT
ST277 SE_SC_COL_159 *E. asburiae*	A19G, S21A, A31G, V71I, D72A, N89T, S93A, C143R, Q144R, S145D, L146Q, P147A(*), R218S	L76F, Q91K, A94D, H132N, S167A, S171A, A172T, I184V, N206S, D209E, T213E, E218G(*), P233A, A270G, Q271V, R276Q, V277I, K325R, A331G, S344A	L129I, F141L, S142A, N145E, H207Q	E237Q, S244T, R297K, M298L, D407E, N408T, S448A, G464S, S484L	V10I	
ST339 SE_SC_COL_198 *E. cloacae*	A19G, S21A, A31G, E35D, V71I, D72A, N89T, S93A, C143R, Q144R, S145D, L146Q, P147A(*), R218S	L76F, Q91K, A94D, H132N, S167A, S171A, A172T, I184V, N206S, D209E, T213E, E218G(*), P233A, A270G, Q271V, R276Q, V277I, E288D, I295T, K325R, A331G, S344A	L129I, F141L, S142A, N145D, H207Q	K2R, R3G, L4I, M5L, M9L, Q69R, S71T, V102I, E112D, L133I, K141Q, D150N, P168L, D169N, V178I, R190M, G193S, V196I, S244T, R297K, M298L	WT	
ST32 SE_SC_COL_80 *E. cloacae*	/	G72S, Q91K, A94D, H132N, S171T, A172T, I184V, V205I, N210T, P233N, A260P, T267A, A270E, Q271V, D272A, R276L, I280S, I295T, V319L, H323R, T327A, A331G	L129I, F141L, N174S(*), H207Q	K2R, R3G, L4I, M5L, M9L, Q69R, V102I, L133I, L139M, K141Q, A148E, L163I, P168M, D169N, V178I, G193S, S448A, G464S	WT	

*CrrB – only in *Klebsiella* spp.; WT –wild type; bold – first found and reported; (*) – deleterious mutation; / - not detected

Table S6. Virulence genes in Enterobacterales isolates identified with VFDB from whole-genome sequencing (WGS) data.

Isolate ID	Species	ST	Virulence factors
SE_SC_COL_159	*E. asburiae*	ST277	*flhB, iutA, hcp/tssD, rpos, cheAW, flhC, fimZ, ompA, flgK, LPS, rcsB, fepACG, cpsG, rfbK1, entEF, fliL,* T6SS-III
SE_SC_COL_198	*E. cloacae*	ST339	*impG, ompA, rfbKI, icmF1/tssM1, cheM, iutA, narH, fepAC, fimZ, flgK, motA, entA, nmpC, LPS, entBEF,* T6SS-III, capsule formation *(K. pneumonia), gndA*
SE_SC_COL_80	*E. cloacae*	ST32	*nmpC, ipah, fepA, ompA, iutA, acrB, hcp/tssD, flhC, cheA, fliC/fljB,* T6SS-III
SE_SC_EC_21	*E. coli*	ST13697	*cfaBCD/E, cgsAFG, elfACDG, hcpABC, papC, fimACDEFGHI, ehaB, ibeBC, iroN, espL4, espR1, espX1X4X5, aec15, escV, hlyE/clyA, wbjD/wecB*
SE_SC_COL_83	*C. farmeri*	ST600	*rpoS, ompA, acrB, nmpC, fepA, ehaB, fliF, ibeB, iroN, vgr* (T6SS), *hcp/tssD* (T6SSIII), *katG, mrkABFCH, cheA*
SE_SC_COL_124	*C. portucalensis*	ST614	*rpoS, ompA, acrB, nmpC, fepA, sux, stcAC, allC, fliG, fimACDFIW, ipah, fur, chuW, csgB, flhA, pegA, fliI*

Supplementary materials for the Publication No. 4:

Puljko, A., Dekić Rozman, S., Barišić, I., Maravić, A., Jelić, M., Babić, I., Milaković, M., Petrić, I., Udiković-Kolić, N. (2023) Resistance to critically important antibiotics in hospital wastewater from the largest Croatian city. *Science of the Total Environment*, **870**, 161805, 1-13 http://dx.doi.org/10.1016/j.scitotenv.2023.161805

Table S1. Primers and conditions used to quantify antibiotic-resistance genes in this study.

Target gene	Resistance phenotype/enzyme	Primer sequence 5' → 3'	Amplicon size (bp)	Ta* (°C)	Amplification accuracy/efficiency (%)		Reference
					Assay 1	Assay 2	
*bla*CTX-M-32	β-lactam resistance/ ESBL	CGTCACGCTGTTGTTAGGAA CGCTCATCAGCACGATAAAG	156	63	0.999/99.04	0.996/83.24	Rocha et al., 2020
*bla*TEM		TTCCTGTTTTTGCTCACCCAG CTCAAGGATCTTACCGCTGTTG	113		0.999/96.44	0.999/98.62	
*bla*OXA-48	β-lactam resistance/ carbapenemase	AGGCACGTATGAGCAAGATG TGGCTTGTTTGACAATACGC	189	60	0.999/99.07	0.998/93.75	Subirats et al., 2017
*bla*KPC-3		CAGCTCATTCAAGGGCTTTC GGCGGCGTTATCACTGTATT	196		0.985/112.99	0.997/77.55	Szczepanowski et al., 2009
*bla*NDM		GATTGCGACTTATGCCAATG TCGATCCCAACGGTGATATT	189		0.987/103.13	0.999/91.59	Subirats et al., 2017
*bla*VIM		AGTGGTGAGTATCCGACAG TCAATCTCCGCGAGAAG	212		0.996/105.71	0.998/96.74	Puljko et al., 2022
*bla*IMP		GGAATAGRRTGGCTTAAYTCTC GGTTTAAYAAARCAMCCACC	233		0.998/94.20	0.999/83.43	
mcr-1	Colistin resistance	GGGCCTGCGTATTTTAAGCG CATAGGCATTGCTGTGCGTC	183		0.998/92.86	0.999/75.50	Hembach et al., 2017

Table S2. Primers and conditions used to quantify taxonomic genes for enteric opportunistic pathogens and total bacteria in this study.

Target gene	Organism	Primer sequence 5' → 3'	Amplicon size (bp)	Ta* (°C)	Amplification accuracy/efficiency (%)		Reference
					Assay 1	Assay 2	
16S rRNA	Total bacteria	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGGCA	196	60	0.996/86.19	0.998/103.62	López-Gutiérrez et al., 2004
*ycc*T	*Escherichia coli*	GCATCGTGACCACCTTGA CAGCGTGGTGGCAAAA	59		0.993/115.08	0.997/107.55	
*glt*A	*Klebsiella pneumoniae*	ACGGCCGAATATGACGAATTC AGAGTGATCTGCTCATGAA	68		0.999/105.57	0.999/104.12	Hembach et al., 2017
secE	*Acinetobacter baumannii*	GTTGTGGCTTTAGGTTTATTATACG AAGTTACTCGACGCAATTCG	94		0.992/114.51	0.999/96.82	
23S rRNA	*Enterococcus* sp.	AGAAATTCCAAACGAACTTG CAGTGCTCTACCTCCATCATT	-		0.994/114.72	0.999/77.11	Frahm and Obst, 2003

Table S3. Primers and thermocycling conditions for PCR detection of ESBL, pAmpC, carbapenemase, and *mcr* genes.

Target gene		Primer sequence 5' → 3'	Amplicon size (bp)	Amplification conditions	Reference
***bla*$_{\text{CTX-M}}$ groups**	***bla*$_{\text{CTX-M-1}}$**	GGTTAAAAAATCACTGCGTC TTGGTGACGATTTTAGCCGC	864	Initial denaturation step for 2:30 min at 94°C, followed by 30 cycles of 20 sec at 94°C 25 sec at 55°C 45 sec at 72°C final extension for 2 min at 72°C	Saladin et al., 2002
	***bla*$_{\text{CTX-M-2}}$**	ATGATGACTCAGAGCATTCG TGGGTTACGATTTTCGCCGC	866		
	***bla*$_{\text{CTX-M-9}}$**	ATGGTGACAAAGAGAGTGCA CCCTTCGGCGATGATTCTC	870		
ESBL-multiplex 1	***bla*$_{\text{TEM}}$**	GCGGTAAGATCCTTGAGAGT TACGATACGGGAGGGCTTA	620	Initial denaturation step for 2:30 min at 94°C, followed by 35 cycles of 30 sec at 94°C 30 sec at 55°C 45 sec at 72°C final extension for 2 min at 72°C	Jelić, 2018
	***bla*$_{\text{SHV}}$**	TTCGCCTGTGTATTATCTCC CGCCTCATTCAGTTCCG	494		
	***bla*$_{\text{VEB}}$**	ATGCCAGAATAGGAGTAGC AATTGTCCATTCGGTAAAGTAAT	673		
ESBL-multiplex 2	***bla*$_{\text{GES}}$**	CTAGCATCGGGACACAT GACAGAGGCAACTAATTCG	504		
	***bla*$_{\text{PER}}$**	CTGGGCTCCGATAATGA CTGGTCGCCWATGATGA	349		
	***bla*$_{\text{SME}}$**	GCTCAGGTATGACATTAGGT CCAATCAGCAGGAACACTA	350		
AmpC-multiplex	***bla*$_{\text{MOX}}$**	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTGC	520	Initial denaturation step for 3 min at 94°C, followed by 35 cycles of 30 sec at 94°C 30 sec at 64°C 1 min at 72°C final extension for 7 min at 72°C	Perez-Perez and Hanson, 2002
	***bla*$_{\text{CIT}}$**	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462		
	***bla*$_{\text{DHA}}$**	AACTTTCACAGGTGTGCTGGGT CCGTACGCATACTGGCTTTGC	405		
	***bla*$_{\text{ACC}}$**	AACAGCCTCAGCAGCCGGTTA TTCGCCGCAATCATCCCTAGC	346		
	***bla*$_{\text{EBC}}$**	TCGGTAAAGCCGATGTTGCGG CTTCCACTGCGGCTGCCAGTT	302		
	***bla*$_{\text{FOX}}$**	AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG	190		
***bla*$_{\text{OXA-48}}$-like**	***bla*$_{\text{OXA-48}}$**	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	744	Initial denaturation step for 2: 30min at 94°C, followed by 30 cycles of 20 sec at 94°C, annealing temperature: 25 sec at 57°C for *bla*$_{\text{VIM}}$, *bla*$_{\text{OXA-48}}$, *bla*$_{\text{KPC}}$; 55°C for *bla*$_{\text{IMP}}$; 58°C for *bla*$_{\text{NDM}}$ 1 min at 72°C final extension for 7 min at 72°C	Poirel et al., 2004
***bla*$_{\text{NDM}}$**	***bla*$_{\text{NDM}}$**	TGGCAGCACACTTCCTATC AGATTGCCGAGCGACTTG	488		Revathi et al., 2013
***bla*$_{\text{KPC}}$**	***bla*$_{\text{KPC}}$**	AGTTCTGCTGTCTTGTCT CTTGTCATCCTTGTTAGGC	793		Jelić, 2018
***bla*$_{\text{VIM}}$**	***bla*$_{\text{VIM}}$**	GGTGAGTATCCGACAGTC CAGCACCRGGATAGAAGAG	442		
***bla*$_{\text{IMP}}$**	***bla*$_{\text{IMP}}$**	GGYGTTTATGTTCATACWTC GGATYGAGAATTAAGCCACTC	235		
***mcr*-multiplex**	***mcr*-1**	AGTCCGTTTGTTCTTGTGGC AGATCCTTGGTCTCGGCTTG	320	Initial denaturation step for 15 min at 94°C, followed by 25 cycles of 30 sec at 94°C 90 sec at 58°C 1 min at 72°C final extension for 10 min at 72°C	Rebelo et al., 2018
	***mcr*-2**	CAAGTGTGTTGGTCGCAGTT TCTAGCCCGACAAGCATACC	715		
	***mcr*-3**	AAATAAAAATTGTTCCGCTTATG AATGGAGATCCCCGTTTTT	929		
	***mcr*-4**	TCACTTTCATCACTGCGTTG TTGGTCCATGACTACCAATG	1116		
	***mcr*-5**	ATGCGGTTGTCTGCATTTATC TCATTGTGGTTGTCCTTTTCTG	1664		

Table S4. Concentration of total, CTX-R and CR *E. coli* and non-*E. coli* coliforms (CFU/mL±SD)

on selective media from two hospitals in the winter and summer sampling campaigns.

Sample type		Season	Hospital 1	Hospital 2
E. coli	Total	Winter	$2.38x10^4±2.00x10^4$	$1.87x10^3±6.83x10^2$
		Summer	$5.45x10^3±3.21x10^3$	$5.17x10^3±4.47x10^3$
	CTX-R	Winter	$2.75x10^2±2.47x10^2$	$6.72x10^2±6.78x10^2$
		Summer	$3.65x10^2±4.66x10^2$	$5.56x10^2±7.70x10^2$
	CR	Winter	$6.68x10^3±8.53x10^3$	$1.53x10^3±1.15x10^2$
		Summer	$3.22x10^3±4.16x10^3$	$2.34x10^3±3.21x10^3$
Non-*E. coli* coliforms	Total	Winter	$4.97x10^4±3.50x10^4$	$7.37x10^3±1.49x10^3$
		Summer	$2.28x10^4±1.79x10^4$	$2.04x10^4x1.89x10^4$
	CTX-R	Winter	$1.48x10^4±2.06x10^4$	$1.49x10^3±1.70x10^3$
		Summer	$4.12x10^3±3.80x10^3$	$6.82x10^3±5.94x10^3$
	CR	Winter	$2.93x10^4±3.80x10^4$	$5.35x10^3±2.44x10^3$
		Summer	$1.38x10^4±1.46x10^4$	$9.20x10^3±7.98x10^3$

Table S5. Relative abundance (gene copies/*rrn* copies±SD) of ESBL and carbapenemase genes in both hospitals in two seasons. Values in parentheses are $\log_{10}$ (gene copies/*rrn* copies±SD).

β-lactamase gene	Hospital 1		Hospital 2	
	Winter	Summer	Winter	Summer
bla_{TEM}	$5.84x10^{-4}±4.93x10^{-4}$ (-3.33±0.34)	$5.60x10^{-3}±2.79x10^{-3}$ (-2.28±0.20)	$4.11x10^{-4}±2.09x10^{-4}$ (-3.42±0.20)	$1.77x10^{-3}±2.90x10^{-3}$ (-3.45±1.01)
$bla_{CTX-M-32}$	$1.95x10^{-4}±2.55x10^{-4}$ (-4.05±0.72)	$3.60x10^{-4}±9.94x10^{-5}$ (-3.46±0.12)	$3.27x10^{-5}±1.78x10^{-5}$ (-4.53±0.23)	$1.22x10^{-3}±6.92x10^{-4}$ (-2.96±0.23)
bla_{KPC-3}	$7.95x10^{-3}±5.26x10^{-3}$ (-2.16±0.27)	$6.37x10^{-1}±3.53x10^{-1}$ (-0.26±0.33)	$1.43x10^{-2}±7.23x10^{-3}$ (-1.90±0.28)	$4.39x10^{-1}±5.96x10^{-1}$ (-0.80±0.88)
bla_{OXA-48}	$6.46x10^{-5}±5.45x10^{-5}$ (-4.32±0.43)	$1.27x10^{-4}±6.00x10^{-5}$ (-3.92±0.19)	$1.04x10^{-4}±4.27x10^{-5}$ (-4.01±0.21)	$1.08x10^{-3}±6.46x10^{-4}$ (-3.01±0.24)
bla_{NDM}	$7.74x10^{-4}±3.10x10^{-4}$ (-3.14±0.20)	$1.15x10^{-3}±1.13x10^{-3}$ (-3.07±0.41)	$5.19x10^{-4}±3.10x10^{-4}$ (-3.35±0.30)	$6.57x10^{-4}±5.24x10^{-4}$ (-3.28±0.35)
bla_{IMP}	$1.10x10^{-4}±8.66x10^{-5}$ (-4.04±0.32)	$3.24x10^{-4}±2.03x10^{-4}$ (-3.54±0.25)	$4.11x10^{-4}±2.09x10^{-4}$ (-3.42±0.20)	$1.02x10^{-4}±5.19x10^{-5}$ (-4.02±0.23)
bla_{VIM}	$9.36x10^{-4}±5.18x10^{-4}$ (-3.07±0.22)	$9.19x10^{-3}±6.68x10^{-3}$ (-2.11±0.30)	$1.26x10^{-4}±6.74x10^{-5}$ (-3.95±0.27)	$4.74x10^{-4}±2.52x10^{-4}$ (-3.36±0.24)

Table S6. Absolute quantification of EOPs (CE/ml±SD) from H1 and H2 hospitals in two seasons. Values in parentheses represent $\log_{10}$ (CE/mL±SD).

Taxonomic genes for EOPs	H1 hospital		H2 hospital	
	Winter	**Summer**	**Winter**	**Summer**
***E. coli* (*ycc*T)**	$2.36x10^5±1.52x10^5$ ($5.31±0.28$)	$1.18x10^5±4.62x10^4$ ($5.05±0.19$)	$5.05x10^4±1.05x10^4$ ($4.70±0.10$)	$6.81x10^4±3.40±10^4$ ($4.79±0.23$)
***K. pneumoniae* (*glt*A)**	$1.48x10^4±5.73x10^3$ ($4.15±0.18$)	$4.37x10^3±1.84x10^3$ ($3.62±0.17$)	$2.42x10^4±2.48x10^4$ ($4.18±0.56$)	$6.80x10^3±5.61x10^3$ ($3.74±0.41$)
***A. baumaniii* (*sec*E)**	$4.66x10^3±1.16x10^3$ ($3.66±0.10$)	$8.19x10^5±5.34x10^5$ ($5.83±0.37$)	$2.01x10^4±2.17x10^4$ ($4.13±0.46$)	$2.36x10^5±1.52±10^5$ ($5.31±0.28$)
***Enterococcus* sp. (23S rRNA)**	$1.32x10^4±8.53x10^3$ ($4.02±0.39$)	$1.96x10^4±1.02x10^3$ ($4.29±0.02$)	$2.24x10^4±7.78x10^3$ ($4.36±0.16$)	$1.42x10^4±2.31x10^4$ ($3.45±1.04$)
Total bacteria (16S rRNA)	$1.01x10^8±2.38x10^7$ ($7.99±0.10$)	$1.20x10^7±7.07x10^6$ ($7.02±0.28$)	$8.44x10^7±2.38x10^7$ ($7.91±0.14$)	$2.81x10^7±1.21x10^7$ ($7.42±0.22$)

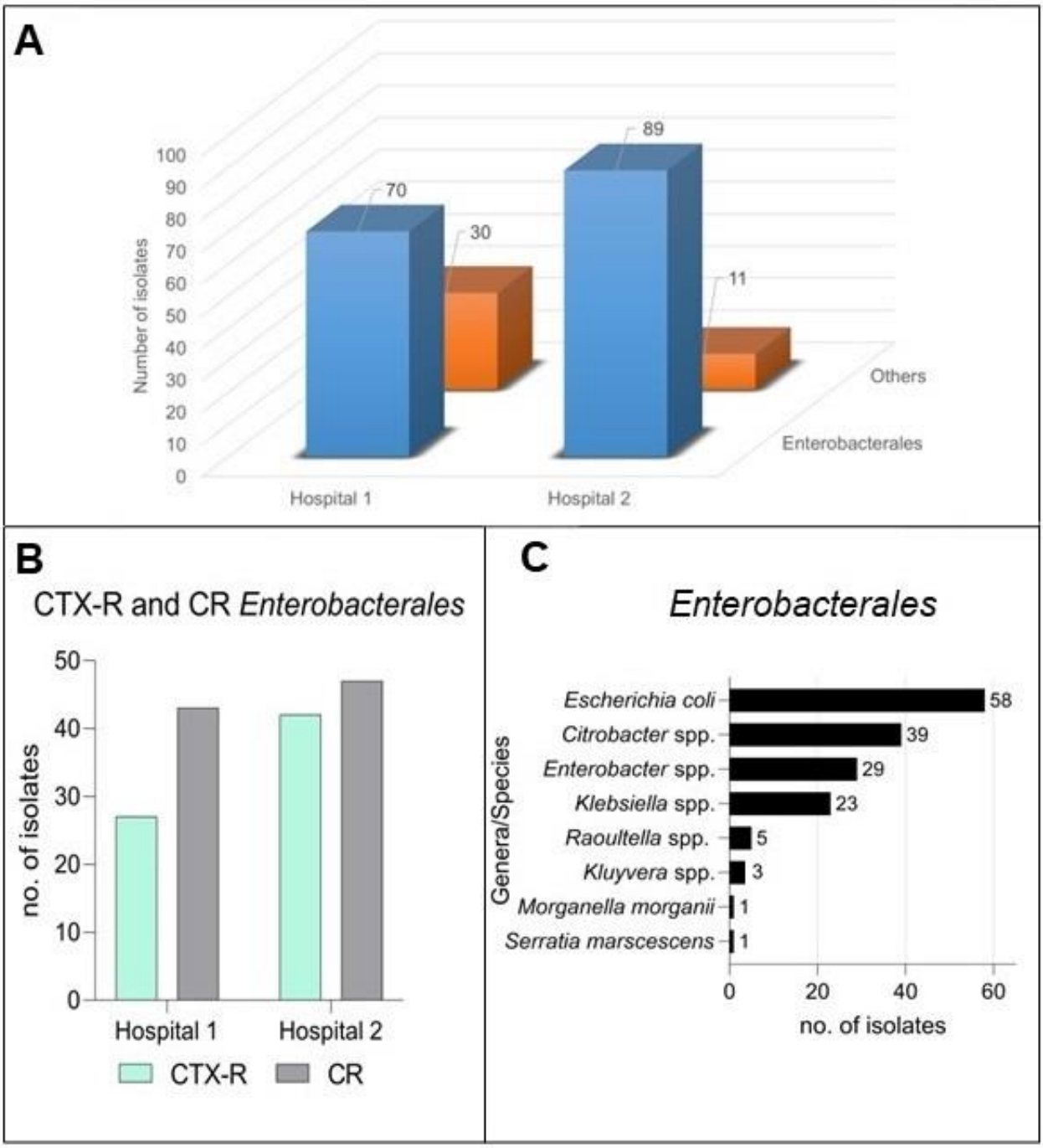


Figure S1. Isolation and identification of bacteria from hospital wastewater samples. A) Number of *Enterobacterales* and non-*Enterobacterales* isolates in each sample type. B) Number of presumptive CTX-R and CR *Enterobacterales* isolates in each hospital wastewater C) Number of each enterobacterial genus identified in both hospital wastewater.

Table S8. A summary of whole genome sequencing (WGS) data (antibiotic-resistance genes, sequence types, plasmid replicons and point mutations in colistin resistance genes) together with the results of targeted PCRs and resistance phenotype of selected isolates from both hospital wastewater.

Species/ Isolate ID	Source/β-lactamase production	Resistance phenotype	Targeted PCR	Whole Genome Sequencing data					
				ST	β-lactamase (*bla*) genes	Other ARGs	Biocide resistance genes	Plasmids	Point mutations in colistin-resistance genes[1]
***Escherichia coli* H2_EC_38**	H2 ESBL	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, GM, SXT, CIP	CTX-M-15, TEM-1	361	TEM-1B, CTX-M-15, OXA-1, EC	*aac(6')-Ib-cr-, aac(3)-IIa, dfrA12, tetA lnuF, catB3*	*sitABCD*	IncFIB	-
***Escherichia coli* H2_EC_42**	H2 ESBL	AML, AMC, CL, CXM, CAZ, FEP, GM, ETP, CIP	CTX-M-15, TEM-116	131	CTX-M-15, OXA-1, EC	*aac(6')-Ib-cr, aac(3)-IIa, catB3*	*sitABCD*	CoIRNAI, IncFIA, Col156, IncFIB(AP001918)	-
***Escherichia coli* H2_EC_02**	H2 ESBL	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, GM, CIP	CTX-M-15, TEM-116, KPC-2	*ND*	CTX-M-194, OXA-1, EC	*aac(6')-Ib-cr, dfrA14 qnrB1, tetA, catB3*	-	IncA, IncHI2, IncM1, IncHI2A	-
***Escherichia coli* H1_SC_EC_05**	H1 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, CIP	KPC-2	541	KPC-2, EC	*qnrS1*	-	IncHI1A(NDM-CIT), IncR, IncM1, IncHI1B(pNDM-CIT), IncFIB(K), IncQ2, IncY, Col440I	-
***Klebsiella pneumoniae* H2_SC_COL_48**	H2 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, GM, SXT, CIP	OXA-48, NDM-1	101	CTX-M-15, SHV-205, OXA-1, OXA-48	*aac(6')-Ib-cr, dfrA14, OqxB catB3, fosA*	-	IncR , Col440II, IncFIA(HI1)	-
***Klebsiella pneumoniae* H1_SC_COL_44**	H1 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, SXT, CIP	OXA-48, NDM-1	16	CTX-M-15, SHV-1 , OXA-48	*aph(3'')-Ib, dfrA14, , qnrB1, OqxA, OqxB, fosA5*	-	IncFIB(K)	-
***Enterobacter asburiae* H2_SC_COL_85**	H2 CARB	AML, AMC. CL. CXM, CAZ, FEP, ETP, IPM, MEM, CIP, COL	KPC-2	277	TEM-1, OXA-1, ACT-6, KPC-2	*aac(6')-Ib-cr, sul1, fosA, catB3, arr-3*	*qacE*	IncFIB(pECLA), IncP6, IncR, IncFII(Yp), Col440I	pmrA (n = 13) pmrB (n = 20) phoP (n = 5) phoQ (n = 25) mgrB (n = 1)

Table S8. Continued

Species/ Isolate ID	Source/β-lactamase production	Resistance phenotype	Targeted PCR	Whole Genome Sequencing data					
				ST	β-lactamase genes	Other ARG	Other resistance genes	Detected Plasmids (Inc groups)	Point mutations in colistin-resistance genes[1]
***Enterobacter asburiae* H2_SC_COL_13**	H2 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, CIP, COL	KPC-2, NDM-1	277	TEM-1, OXA-10, ACT-29 KPC-2	*aadA11, aac(3)-I, fosA*	*qacE*	IncFIB(pECLA), IncX5	pmrA (n = 13) pmrB (n = 20) phoP (n = 5) phoQ (n = 25) mgrB (n = 1)
***Enterobacter cloacae* H2_SC_COL_11**	H2 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, CIP, COL	KPC-2	277	TEM-1, OXA-10, ACT-57, KPC-2	*aadA11, aac(3)-I, fosA*	*qacE*	IncFIB(pECLA), IncP6	pmrA (n=13) pmrB (n = 22) phoP (n = 5) phoQ (n = 23; novel mutation L133I)
***Enterobacter cloacae* H1_SC_COL_39**	H1 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, GM, SXT, CIP	VIM-1	*ND*	TEM-1, ACT-16, VIM-1	*aac(6')-Ib-cr, aph(3'')-Ib, dfrA14, qnrB1, sul1, sul2, catB3*	*qacE*	IncC, IncHI2A, IncHI2, Col(pHAD28)	-
***Enterobacter cloacae* H1_SC_COL_110**	H1 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, GM, CIP, COL	Non detected	32	OXA-10, ACT-9	*aac(6')-Ib-cr, aph(3'')-Ib, aph(6)-Id, fosA*	-	IncC, IncX5, Col(pHAD28), IncP6, IncHI2, IncHI2A, IncFIB(K)	pmrB (n = 22) phoP (n = 4) phoQ (n = 18; novel mutation L133I)
***Enterobacter ludwigii* H2_SC_COL_15**	H2 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, CIP, COL	KPC-2	277	TEM-1, OXA-10, ACT-10 KPC-2	*aadA11, aac(3)-I, fosA*	*qacE*	IncP6, IncFIB(pECLA), IncX5	pmrA (n = 13) pmrB (n = 22) phoP (n = 5) phoQ (n = 26; novel mutation L133I) mgrB (n = 1)
***Enterobacter kobei* H2_SC_COL_97**	H2 CARB	AML, AMC, CL, CXM, CAZ, FEP, ETP, IPM, MEM, GM, SXT, CIP, COL	KPC-2, NDM-1	501	TEM-1, CTX-M-3, OXA-14, MIR-3, KPC-2	*aac(6')-Ib-cr, aac(3)-I, dfrA14, qnrS1, sul1, fosA, arr3*	*qacE*	IncR, IncN	pmrA (n = 12) pmrB (n = 24) phoP (n =2) phoQ (n = 2) mgrB (n = 1)

AML – ampicillin, AMC – ampicillin/clavulanic acid, CL – cefalexin, CXM – cefuroxime, CAZ – ceftazidim, FEP – cefepime, ETP – ertapenem, IPM – imipenem, MEM – meropenem, GM – gentamycin, SXT - trimethoprim/sulfamethoxazole, CIP – ciprofloxacin, COL – colistin.

ND – not detected. ARGs - antibiotic-resistance genes. [1]For a detailed table for colistin point mutations, see Supplementary **Table S9.** The numbers in parentheses represent the determined amino acid changes per protein.

Table S9. Amino acid changes in proteins associated with colistin resistance in *Enterobacter* spp. identified by sequence analysis. Mutations were very similar among the isolates studied, yielding a total of 14, 42, 5, 29 and 1 unique mutations for *pmrA*, *pmrB*, *phoP*, *phoQ* and *mrgB*, respectively.

Isolate	Species	Isolate	Protein				
			PmrA	PmrB	PhoP	PhoQ	MgrB
H2_SC_COL_85	***E. asburiae***	**H2_SC_COL_85**	A19G, S21A, A31G, V71I, D72A, N89T, S93A, C143R, Q144R, S145D, L146Q, P147A (*), R218S	L76F, Q91K, A94D, H132N, S167A, S171A, A172T, I184V, N206S, D209E, T213E, E218G (*), P233A, A270G, Q271V, R276Q, V277I, K325R, A331G, S344A	L129I, F141L, S142A, N145E, H207Q	**K2R, L4I, M5L, M9L, Q69R, S71T, V102I, E112D, L132I, K141Q, D150N, P168L, D169N, V178I, R190M, G193S, V196I, S244T, R297K, M298L, D407E, N408T, S448A, G464S, S484L**	V10I
H2_SC_COL_13	***E. asburiae***	**H2_SC_COL_13**	A19G, S21A, A31G, V71I, D72A, N89T, S93A, C143R, Q144R, S145D, L146Q, P147A (*), R218S	L76F, Q91K, A94D, H132N, S167A, S171A, A172T, I184V, N206S, D209E, T213E, E218G (*), P233A, A270G, Q271V, R276Q, V277I, K325R, A331G, S344A	L129I, F141L, S142A, N145E, H207Q	**K2R, L4I, M5L, M9L, Q69R, S71T, V102I, E112D, L132I, K141Q, D150N, P168L, D169N, V178I, R190M, G193S, V196I, S244T, R297K, M298L, D407E, N408T, S448A, G464S, S484L**	V10I
H2_SC_COL_11	***E. cloacae***	**H2_SC_COL_11**	A19G, S21A, A31G, V71I, D72A, N89T, S93A, C143R, Q144R, S145D, L146Q, P147A (*), R218S	**L76F**, Q91K, **A94D**, **H132N**, **S167A**, **S171A**, A172T, **I184V**, **N206S**, **D209E**, **T213E**, **E218G (*)**, **P233A**, A270G, **Q271V**, **R276Q**, **V277I**, **E288D**, I295T, **S308Q(*)**, **G309R(*)**, **L310S(*)**	L129I, **F141L**, **S142A**, **N145D**, H207Q	K2R, R3G, L4I, M5L, M9L, **Q69R**, S71T, V102I, **E112D**, ***L133I***, K141Q, D150N, P168L, D169N, V178I, R190M, G193S, V196I, S244T, R297K, M298L, **D407E, N408T**	WT
H1_SC_COL_110	***E. cloacae***	**H1_SC_COL_110**	–	**G72S**, Q91K, **A94D**, **H132N**, **S171T**, A172T, **I184V**, **V205I**, **N210T**, **P233N**, **A260P**, **T267A**, **A270E**, **Q271V, D272A, R276L**, **I280S**, I295T, **V319L**, **H323R**, **T327A**, A331G	L129I, **F141L**, **N174S (*)**, H207Q	K2R, R3G, L4I, M5L, M9L, **Q69R**, V102I, ***L133I*****, L139M,** K141Q, **A148E, L163I, P168M,** D169N, V178I, G193S, S448A, G464S	–
H2_SC_COL_15	***E. ludwigii***	**H2_SC_COL_15**	A19G, S21A, A31G, V71I, D72A, N89T, S93A, C143R, **Q144R**, **S145D**, L146Q, P147A (*), R218S	L76F, Q91K, H132N, **S167A**, S171A, A172T, I184V, **N206S**, **D209E**, **T213E**, **E218G (*)**, P233A, **A270G**, Q271V, R276Q, **V277I**, **S278G**, **E288D**, I295T, **K325R**, A331G, **S344A**	L129I, F141L, S142A, **N145E**, H207Q	K2R, R3G, L4I, M5L, M9L, Q69R, S71T, V102I, E112D, ***L133I***, K141Q, D150N P168L, D169N, V178I, R190M, G193S, V196I, S244T, R297K, M298L, D407E, N408T, S448A, G464S, S464L	V10I
H2_SC_COL_97	***E. kobei***	**H2_SC_COL_97**	A19G, S21A, A31G, V71I, D72N, N89T, S93A, **C143Q**, **S145D**, L146Q, **P147A (*)**, R218S	Q91K, H132S, Q135K, A163S, S167A, S171T, A172T, I184V, D209E, T213E, E218G (*), A221T, P233A, A260S, A270E, Q271V, D272E, R276Q, V277I, I295T, V319L, K325R, A331G, S244A	L129I, F141L	R297K, M298L	V10I

WT wild-type; − not determined. The asterisk (*) represents Deleterious mutation. All others were determined as Neutral mutations. Deleterious or Neutral effect of mutations has been checked using PROVEAN software. The bold mutations were this article first found and reported for certain *Enterobacter* spp. *Italic* = this article Novel Neutral mutation *Pho*Q (L133I) for *E. cloacae.* References: Uechi et al., 2018., Liao et al., 2022.