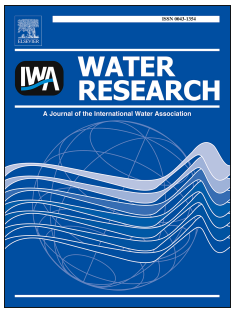


# Genotypic and phenotypic traits of *bla*$_{CTX-M}$-carrying *Escherichia coli* strains from an UV-C-treated wastewater effluent

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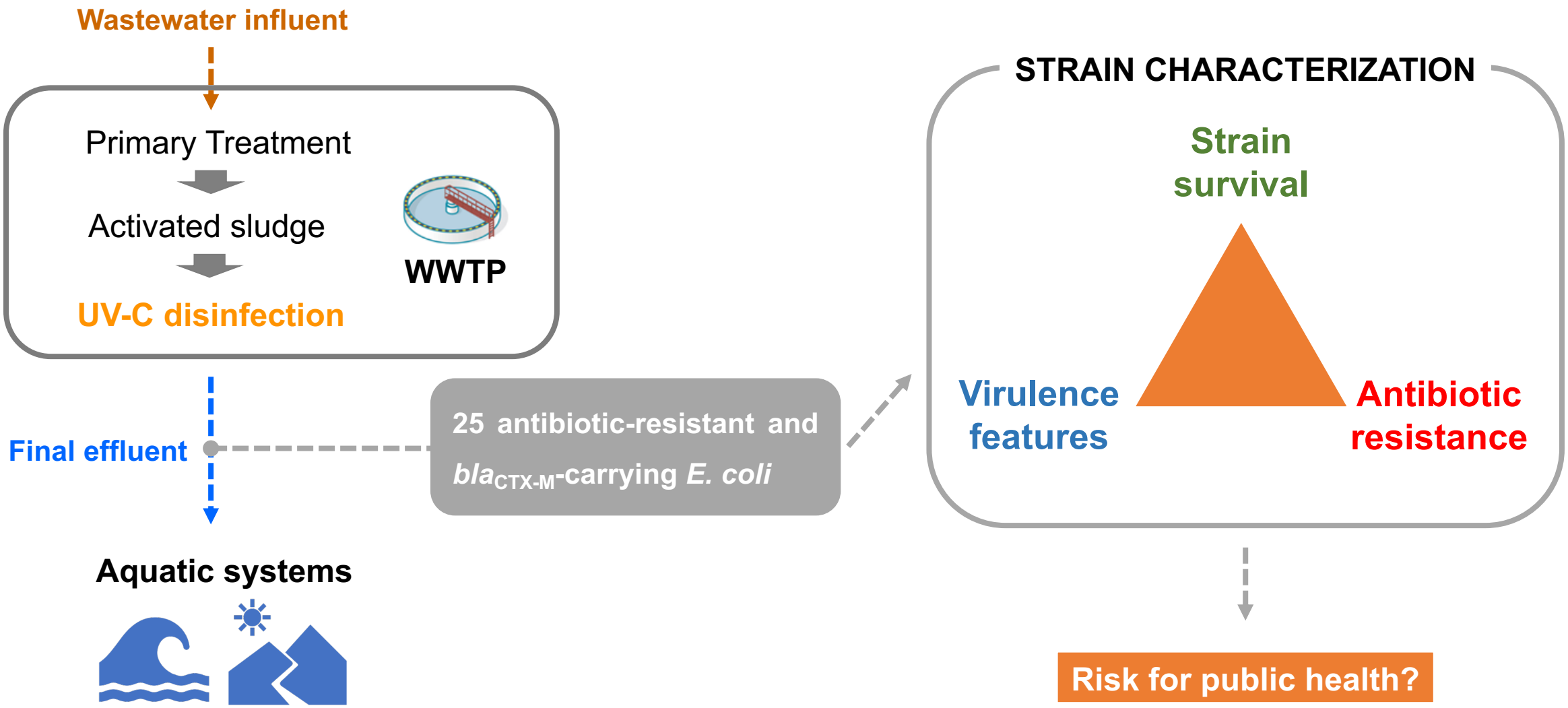
Wastewater influent
Primary Treatment
Activated sludge
UV-C disinfection
WWTP
Final effluent
25 antibiotic-resistant and
bla$_{CTX-M}$-carrying E. coli
Aquatic systems
STRAIN CHARACTERIZATION
Strain
survival
Virulence
features
Antibiotic
resistance
Risk for public health?

**Manuscript**

# Genotypic and phenotypic traits of *bla*$_{\text{CTX-M}}$-carrying *Escherichia coli* strains from an UV-C-treated wastewater effluent

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## ABSTRACT

Wastewater treatment plants (WWTPs) are relevant sources of antibiotic resistance into aquatic environments. Disinfection of WWTPs' effluents (e.g. by UV-C irradiation) may attenuate this problem, though some clinically relevant bacteria have been shown to survive disinfection. In this study we characterized 25 CTX-M-producing *Escherichia coli* strains isolated from a WWTP's UV-C-irradiated effluent, aiming to identify putative human health hazards associated with such effluents. Molecular typing indicated that the strains belong to the phylogroups A, B2 and C and clustered into 9 multilocus sequence types (STs), namely B2:ST131 (n=7), A:ST58 (n=1), A:ST155 (n=4), C:ST410 (n=2), A:ST453 (n=2), A:ST617 (n=2), A:ST744 (n=1), A:ST1284 (n=3) and a putative novel ST (n=3). PCR-screening identified 9 of the 20 antibiotic resistance genes investigated [i.e. *sul1*, *sul2*, *sul3*, *tet*(*A*), *tet*(*B*), $bla_{\text{OXA-1-like}}$, *aacA4*, *aacA4-cr* and *qnrS1*]. The more prevalent were *sul1*, *sul2* (n=15 isolates) and *tet*(*A*) (n=14 isolates). Plasmid restriction analysis indicated diverse plasmid content among strains (14 distinct profiles) and mating assays yielded cefotaxime-resistant transconjugants for 8 strains. Two of the transconjugants displayed a multi-drug resistance (MDR) phenotype. All strains were classified as cytotoxic to Vero cells (9 significantly more cytotoxic than the positive control) and 10 of 21 strains were invasive towards this cell line (including all B2:ST131 strains). The 10 strains tested against *G. mellonella* larvae exhibited a virulent behaviour. Twenty-four and 7 of the 25 strains produced siderophores and haemolysins, respectively. Approximately 66% of the strains formed biofilms. Genome analysis of 6 selected strains identified several virulence genes encoding toxins, siderophores, and colonizing, adhesion and invasion factors. Freshwater microcosms assays showed that after 28 days of incubation 3 out of 6 strains were still detected by cultivation and 4 strains by qPCR. Resistance phenotypes of these strains remained unaltered. Overall, we confirmed WWTP's UV-C-treated outflow as a source of MDR and/or virulent *E. coli* strains, some probably capable of persisting in freshwater, and that carry conjugative antibiotic resistance plasmids. Hence, disinfected wastewater may still represent a

50 risk for human health. More detailed evaluation of strains isolated from wastewater effluents is
51 urgent, to design treatments that can mitigate the release of such bacteria.

52 **Keywords:** WWTP, antibiotic resistance, virulence, environmental persistence, risk.

## 1. INTRODUCTION

Wastewater is a relevant source of chemical and biological contamination into the environment, and despite the improvement of the treatment processes applied in wastewater treatment plants (WWTPs), removal of contaminants such as antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) remains challenging (Bouki *et al.*, 2013; Karkman *et al.*, 2018; Rizzo *et al.*, 2013). Besides, the inflow of a broad range of contaminants (e.g. nutrients, antibiotics, metals and faecal coliforms) may offer adequate conditions for bacterial growth, lateral gene transfer and acquisition of antibiotic resistance, which may jeopardize the capability of wastewater treatment to efficiently remove antibiotic resistance (Karkman *et al.*, 2018; Manaia *et al.*, 2016). A wide variety of ARGs, such as genes encoding extended-spectrum beta-lactamases (ESBLs) and carbapenemases have been reported in WWTPs (Conte *et al.*, 2017; Miranda *et al.*, 2015; Silva *et al.*, 2018). Although such biological contaminants are generally in significantly lower abundance in treated effluents than in pre-treated wastewater, their prevalence (abundance relative to the total number of bacteria) may be identical after treatment or occasionally even higher (Bouki *et al.*, 2013; Guo *et al.*, 2013a,b; Miranda *et al.*, 2015; Silva *et al.*, 2018). Proposed containment strategies include the implementation of advanced or disinfection-based tertiary treatments (Bouki *et al.*, 2013; Rizzo *et al.*, 2013). In 2015, more than 70% of the wastewater of central and northern Europe received tertiary treatment (European Environment Agency, 2017). However, in Portugal, only 8.1% of the WWTPs applied tertiary treatment steps (APA, 2016).

UV-C irradiation is applied in the last stages of wastewater treatment with the intent to disinfect effluents before discharge. Advantages include short contact times and minimal impacts on the water chemical quality since no chemical by-products are generated (Cutler and Zimmerman, 2011; EPA, 1999). Considering this, over the years WWTPs tended to prefer UV irradiation to chemical disinfection, such as chlorination (Bouki *et al.*, 2013). The underlying bacterial inactivation mechanism is the modification of DNA, mainly through the formation of

pyrimidine dimers that hinder cellular replication, and therefore halt cell division (Cutler and Zimmerman, 2011).

Overall, most studies show that bacterial loads from UV-irradiated effluents are effectively reduced (Silva *et al.*, 2018; Sousa *et al.*, 2017). However, data for the removal of ARB can be contradictory, since some studies suggest that some bacteria may survive UV radiation, and thus be relatively enriched in the WWTPs' outflow (Guo *et al.*, 2013a,b).

Antibiotic-resistant strains of *Escherichia coli*, a known species of commensal and pathogenic bacteria, are commonly isolated from wastewater (Bréchet *et al.*, 2014; Conte *et al.*, 2017; Osińska *et al.*, 2017) and some display virulence factors (Franz *et al.*, 2015; Osińska *et al.*, 2017). ESBL-producing *E. coli*, namely those producing CTX-M enzymes (currently the most prevalent ESBLs), are clinically relevant bacteria since they can cause nosocomial and community-acquired infections and are often associated with high mortality rates (Rodríguez-Baño and Pascual, 2008). The release of such strains in the final effluent of WWTPs has been described (Amos *et al.*, 2014; Bréchet *et al.*, 2014; Silva *et al.*, 2018). Despite being considered indicators of faecal contamination, some *E. coli* strains have been shown to survive for long periods and multiply in the environment, potentially establishing themselves in the indigenous microbiota (Jang *et al.*, 2017). This is concerning since resistance and virulence traits can be unimpacted by wastewater treatments.

UV-C disinfection of wastewater can lead to an increase in the prevalence of ARGs and ARB, integrases and multidrug-resistance phenotypes (Guo *et al.*, 2013a,b; Jäger *et al.*, 2018; Silva *et al.*, 2018), further increasing the potential hazard of surviving strains to human health. In a previous study, the efficiency of UV-C irradiation in the removal of cefotaxime-resistant *Enterobacteriaceae* was assessed by culture-dependent methods and it was concluded that the treatment was effective in removing these bacteria. However, this study also estimated that $3.0\times10^7$ cells per $m^3$ of treated water were released daily in the final effluent, some being $bla_{CTX\text{-}M}$-carrying and multidrug-resistant *E. coli* (Silva *et al.*, 2018). Therefore, in this study, we aimed to characterize the diversity, ARG carriage, virulence potential, and persistence and fate in

freshwater microcosms of CTX-M-producing *E. coli* strains that were previously isolated from this final effluent, in order to understand the risk they pose to human health.

## 2. MATERIAL AND METHODS

### *2.1. Bacterial strains*

The *E. coli* isolates selected for this study were previously obtained from a WWTP's final effluent, which applied a final UV-C-irradiation step. These isolates had been previously identified as ESBL-producers and carriers of the $bla_{CTX-M}$ gene (Silva *et al*., 2018). Strains features are presented in Table 1.

### *2.2. Molecular typing*

To determine the clonal relatedness of the selected isolates, rep-PCR and PFGE (Pulsed Field Gel Electrophoresis) were conducted using conditions previously described (Araújo *et al.*, 2017; CDC, 2013, respectively). Determination of *E. coli* phylotypes was performed as described by Clermont and colleagues (Clermont *et al.*, 2013), and members of the B2 group were subtyped by allele-specific PCRs (Clermont *et al*., 2014). Attribution of sequence types (STs) was achieved by PCR amplification and sequencing of seven housekeeping genes (*adk*, *fumC*, *icd*, *purA*, *recA*, *mdh* and *gyrB*) with primers and conditions as described in Warwick´s University MLST database (https://enterobase.warwick.ac.uk/warwick_mlst_legacy).

### *2.3. PCR screening for ARGs and virulence genes*

Total genomic DNA was extracted from all strains, using a Silica DNA Gel extraction kit (ThermoFisher, USA), and used as the template in the subsequent screening. PCR-based detection of 20 ARGs ($bla_{GES}$, $bla_{OXA-1-like}$, $bla_{OXA-2-like}$, $bla_{OXA-10-like}$, *tet(*A*)*, *tet(*B*)*, *tet(*C*)*, *tet(*D*)*, *tet(*E*)*, *tet(*G*)*, *tet(*M*)*, *aacA4*, *aacA4-cr*, *sul1*, *sul2*, *sul3*, *qnrA*, *qnrB*, *qnrS* and *mcr-1*) and 8 virulence genes (VGs) associated with intestinal pathogenic *E. coli* (IPEC) (*stx1*, *stx2*, *eae*, *ipaH*, *aggR*, *bfpA*, *est* and *elt*) was carried out in reactions with a final volume of 25 µL, using 6.25 µL of 5000 U NZYTaq 2x Green Master Mix (NzyTech, Portugal), 0.75 µL of each primer (10 µM), 1 µL of DNA (50-100 ng) and sterile distilled water. Thermocycling conditions and positive controls are listed in Tables S1-A and S1-B. The nucleotide sequences of the amplicons were obtained by Sanger sequencing (GATC Biotech, Germany) and used to confirm the gene

identity. For the detection of mutations related to fluoroquinolone resistance, partial amplification by PCR and sequencing of *gyrA* and *parC* amplicons was required.

### *2.4. Plasmid characterisation and mating assays*

Plasmid DNA (pDNA) was extracted using E.Z.N.A. Plasmid DNA Mini Kit II Spin Protocol (Omega Bio-Tek, USA) or Qiagen Plasmid Mini Kit (Qiagen, Germany) and cut with the restriction enzymes *Pst*I and *Bst*1107I (Thermo Scientific, USA). Reaction mixtures of 13 µL consisted of 5 U of each enzyme, 1 µL of 10X Buffer O (Thermo Scientific, USA) and 4-6 µg of pDNA. The mixture was incubated for 4 hours at 37 ºC and enzymatic digestion stopped by adding 2 µL of a 0.2 M EDTA solution. Fragments were separated in a 0.8% agarose gel.

Conjugation assays were attempted with rifampicin-resistant *E. coli* CV601 as previously described (Araújo *et al.*, 2017). Transconjugants were selected on Plate Count Agar (PCA, Merck, USA) plates supplemented with 8 µg/mL of cefotaxime and 100 µg/mL of rifampicin.

Molecular confirmation of transconjugants was performed with BOX- and ERIC-PCR (Araújo *et al.*, 2017). Genetic determinants previously detected in donor strains were screened on the transconjugants under the same experimental conditions (Silva *et al.*, 2018). Antibiotic susceptibility was assessed by the disc diffusion method. The antibiotics tested were: amoxicillin (AML, 10 µg), amoxicillin with clavulanic acid (AMC, 30 µg), piperacillin (PRL, 30 µg), piperacillin with tazobactam (TZP, 36 µg), ticarcillin (TIC, 75 µg), ticarcillin with clavulanic acid (TIM, 85 µg), cefepime (FEP, 30 µg), cefotaxime (CTX, 5 µg), ceftazidime (CAZ, 10 µg), meropenem (MEM, 10 µg), aztreonam (ATM, 30 µg), ciprofloxacin (CIP, 5 µg), gentamicin (CN, 10 µg), tetracycline (TET, 30 µg), chloramphenicol (C, 30 µg) and trimethoprim/sulfamethoxazole (SXT, 25 µg). For interpretation, EUCAST guidelines were followed for most antibiotics (EUCAST, 2019), except for tetracycline where CLSI guidelines were used (CLSI, 2018). pDNA was also extracted from transconjugants and enzymatically digested for comparison, as described above.

### *2.5. Biofilm production*

Evaluation of biofilm formation capacity was assessed by the microtiter plate assay with a protocol adapted from Stepanovic *et al.* (2000) and Naves *et al.* (2008). Two separated experiments were conducted in duplicate: (1) using the rich medium Tryptic Soy Broth (TSB; Merck, USA) in static conditions and 37 ºC as the incubation temperature, and (2) using the minimal media M63 (2 g/L of ammonium sulphate, 13.6 g/L of monopotassium phosphate and 0.5 mg/L of iron (II) sulphate heptahydrate; pH 7) supplemented with 0.8% of glucose and 1 mM of magnesium sulphate and incubated at 25 ºC with shaking (110 rpm). Bacterial cultures were grown overnight to stationary phase. In flat-bottom 96-well plates, 200 µL of standardized inoculum of approximately $10^3$-$10^4$ CFU/mL (optimal cell densities for biofilm formation of the positive control) was placed in each well (8 replicates for each strain) and incubated during 24 h in the desired conditions. At the end of the incubation period, the $OD_{600nm}$ of each well was measured. After removal of the inoculum and gently washing each well with a saline solution, attached cells were heat-fixed at 50 ºC for 1 hour. Biofilm biomass quantification was achieved by staining with a crystal violet solution at 0.1%, and, after re-washing the wells, the cell-bound stain was solubilized in 30% acetic acid. Then, the $OD_{590nm}$ (corresponds to crystal violet absorption maximum) of each well was measured. *E. coli* ATCC 25922 was used as a positive control (Naves *et al*., 2008) and sterile media as a negative control.

The biofilm formation index (BFI) was calculated from the following equation:

$$\mathrm{BFI} = \frac{\mathrm{OD}_{590\mathrm{nm}}}{\mathrm{OD}_{600\mathrm{nm}}}$$

Strains were classified as non-producers ($BFI \leq BFI_{negative\ control}$), weak-producers ($BFI_{negative\ control} < BFI < 2 \times BFI_{negative\ control}$) or producers ($BFI \geq 2 \times BFI_{negative\ control}$). BFI of the negative control (sterile media) was calculated using the average value of $OD_{590nm}$ of crystal violet stained negative wells and an average $OD_{600nm}$ value of all wells with bacterial growth within the same experiment.

### *2.6. Haemolysin activity and siderophore production*

Analysis of the haemolytic potential of all strains was assessed by growth on Blood Agar (Biomérieux, France) at 37 ºC up until 5 days, with daily observations of the plates. Isolates were considered positive for haemolysin production when a halo was formed around the colonies.

Phenotypic detection of siderophore synthesis was evaluated by growth on Tryptic Soy Agar (TSA, Merck, USA) deferrated with magnesium carbonate (Cox, 1994) followed by overlay with O-CAS media (Pérez-Miranda *et al.*, 2007). *Pseudomonas fluorescens* S3X and *Pseudomonas putida* EAPC8 were used as positive controls (Leite *et al*., 2017) and *Caballeronia* sp. R.N3S1 as a negative control (Lab's strain collection). Test strains were incubated at 37 ºC and control strains at 30 ºC.

### *2.7. Resazurin-based cytotoxicity experiments*

Cell-free extracellular extracts were obtained from overnight cultures grown in TSB medium, at 37 ºC with 180 rpm of agitation. Cells were partly removed from culture broth by centrifugation (20 min; 5,000 rpm) and cell-free extracts were obtained after filtration with 0.2 µm filters. Confluent monolayers of Vero cells (ECACC 88020401, African Green Monkey Kidney cells, GMK clone) were obtained as previously described (Duarte *et al.*, 2015) and exposed in 96-well plates to 50 µL of serial dilutions of the extracts in PBS (Phosphate-Buffered Saline; Gibco, USA), corresponding to 50.0, 25.0, 6.3 and 3.1% of the original extracts (6 replicates per strain). Cell viability, and corresponding cytotoxic potential, was assessed by measuring the metabolization of resazurin into resorufin by the following ratio ($OD_{570nm}/OD_{600nm}$). Wells with cells exposed only to TSB and wells without cells were included in each 96-well plate and used as positive (maximum viability) and negative control (blank), respectively. *E. coli* BL21 (non-cytotoxic) and *E. coli* PH20 (shiga-toxin producer; Table S1-A) extracts were also included. Cell viability was calculated by subtracting the blank and calculating the ratios $OD_{570nm}/OD_{600nm}$ for each well. Cytotoxicity was deduced by cell viability and normalized per $OD_{600nm}$ 1.0 of the culture used for obtaining the cell-free extracts, as indicated in the following formulas:

$$\text{(A) Cell viability (\%)} = \frac{\text{ratio OD } 570/600_{\text{extract}}}{\text{ratio OD } 570/600_{\text{TSB}}} \times 100$$

$$\text{(B) Cytotoxicity per OD}_{600nm}\ 1.0\ (\%) = \frac{100\ - \text{Cell viability (\%)}}{\text{OD}_{600\text{nm}}}$$

Strains were classified by comparison with results obtained for control strains (BL21 and PH20) as non-cytotoxic (≤ BL21), weakly cytotoxic (between BL21 and PH20) and cytotoxic (≥ PH20).

### *2.8. Invasion assays by the gentamicin-protection method*

For assessment of invasion potential of the test strains an adapted gentamicin protection assay was performed (da Silva Santos *et al*., 2015). Briefly, confluent monolayers of Vero Cells were seeded in 12-well plates and, after incubation for 24 hours, were washed thrice with PBS and inoculated with the test strains (in duplicate) in fresh DMEM media (Dulbecco's modified Eagle medium; Gibco, USA) supplemented with 10% FBS (Fetal Bovine Serum; Gibco, USA), in a multiplicity of infection (MOI) between 1 and 10. Then, after 4 washing steps with PBS, the plates were incubated during 1 hour with fresh DMEM supplemented with 100 µg/mL of gentamicin (to remove non-planktonic adherent cells). Afterwards, the mammalian cells were washed again with PBS and lysed by incubation in 1% Triton X-100 for 5 minutes. Lysates were plated in PCA. The percentage of invasiveness of each strain was calculated using the following formula:

$$\%\ \text{of invasion}\ = \frac{\log_{10}(\text{LPC})}{\log_{10}(\text{IPC})} \times 100$$

Where IPC was the initial inoculum plate counts (CFU/mL) and LPC was the lysate plate counts (CFU/mL).

*Salmonella enterica* subsp. *enterica* serovar Typhimurium SC56 (O'Mahony *et al*., 2006) and *E. coli* BL21 were used as positive and negative controls, respectively. Only gentamicin-susceptible strains were included in this assay (n = 21).

Based on the obtained preliminary results, 6 strains were selected for 4 assays with varied MOI (the number of Vero cells were determined by counts in a Neubauer chamber). In these experiments, each strain was inoculated in triplicate on 12-well plates.

### *2.9. In vivo infection experiments in* Galleria mellonella *model*

Infection assays using *Galleria mellonella* larvae were performed for 10 strains (ECR.1, ECR.11, ECR.12, ECR.15, ECR.17, ECR.18, ECR.19, ECR.20, ECR.22 and ECR.25) as previously described (Fuentes-Castillo *et al*., 2019). *E. coli* BL21 and Neonatal Meningitis-associated *E. coli* (NMEC) strain RS218 were used as a negative (non-virulent) and positive control (hypervirulent), respectively. The inoculum was prepared by pelleting an overnight grown culture, washing and resuspending in PBS. Larvae (n=10 per strain) with 250-350 mg of weight were injected with an inoculum corresponding to $10^5$ CFUs of each strain and kept in Petri dishes at 37 ºC during an incubation period of 96 hours. Monitorization of survival was evaluated by the response to physical stimuli and melanisation of the larvae's body. Two independent assays were performed for each strain. Survival curves were plotted using the Kaplan-Meier method.

### *2.10. Whole-genome sequencing*

Genomic DNA was extracted from six isolates (ERC.1, ECR.15, ECR.18, ECR.19, ECR.20 and ECR.22) using Wizard® Genomic DNA purification kit (Promega, USA) and sent for whole-genome sequencing (StabVida, Portugal) using an Illumina Hiseq 2500 platform. Genomic raw reads were assembled with CLC Genomics Workbench 10.0.1. and annotation of the genomes was performed using RAST (http://rast.nmpdr.org/), CARD (https://card.mcmaster.ca/), VFDB database (http://www.mgc.ac.cn/VFs/main.htm) and the tools available at Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). ResFinder 3.1.0 and CARD's Resistance Gene Identifier (RGI) were used for detection of ARGs while VirulenceFinder 2.0 and VFanalyzer were used to identify virulence determinants. Identification of plasmid replicons was performed with PlasmidFinder 2.0.1 and replicons were typed with pMLST 2.0,

whereas *in silico* determination of the sequence type (Warwick´s University scheme), *fimH* type, serotype and pathogenic potential relied on MLST 2.0.1, CHTyper 1.0, SeroTypeFinder 2.0.1 and PathogenFinder 1.1, respectively.

The whole-genome nucleotide sequences have been deposited in GenBank under the BioProject PRJNA612976 with the following accession numbers: JABEXQ000000000 (ECR.1), JABEXR000000000 (ECR.15), JABEXS000000000 (ECR.18), JABEXT000000000 (ECR.19), JABEXU000000000 (ECR.20) and JABEXV000000000 (ECR.22).

### *2.11. Microcosms experiments*

Strains were grown overnight in M63 minimal media supplemented with 0.8% of glucose and 1 mM of magnesium sulphate at 30 ºC. River water was collected from a non-polluted river (Alcofra river; sampling site at 40°37'43.7"N, 8°11'40.9"W; Tacão *et al*., 2012) in sterile flasks and transported to the lab. Microcosms (4 replicates per condition) were prepared by adding to each Erlenmeyer flask 150 mL of freshwater and 1 mL of inoculum ($OD_{600nm}$ of 0.3) to obtain an initial concentration of $10^3$-$10^4$ cells/mL. The experiment included a negative control (non-inoculated river water). Microcosms were sampled weekly for colony counts for 28 days. Colony enumeration was performed by filtering water samples in 0.45 µm grids, which were placed in membrane Faecal Coliform Agar (mFC; Merck, USA) plates and incubated at 37 ºC. From each microcosm, presumptive *E. coli* colonies retrieved in the last sampling moment were streaked and typed by BOX-PCR to confirm their identity by comparison with profiles of the original strains. Also, antibiograms were performed for the original and surviving strains (n=3), as described previously (Silva *et al.*, 2018).

Microcosms were also sampled at 0, 7 and 28 days for whole-community DNA extraction (3 replicates per condition) as previously described (Henriques *et al*., 2004). DNA was used to perform quantitative PCR (qPCR) targeting the *uidA* and $bla_{CTX\text{-}M}$ genes. The 20 µL reaction mixture consisted of 10 µL of NZYSpeedy qPCR Green Master Mix (NzyTech, Portugal), 0.4 µL of each primer, 7.2 µL of ultrapure water and 2 µL of DNA. Primers used are listed in Table

S1-A. The thermocycling program used started with an initial denaturation at 94 ºC for 3 minutes followed by 40 cycles of denaturation at 95 ºC for 10s and annealing at 60 ºC for 20s, with fluorescence data acquisition at the end of each cycle. Melting analysis was performed from 55 to 95 ºC, with steady 0.1 ºC increments at every 5 seconds. To enable an absolute copy number quantification, DNA standards were prepared by inserting the target fragments into the pNZY28 vector and transforming into *E. coli* recipient cells using the NZY-A Speedy PCR cloning kit (NzyTech, Portugal). pDNA was then extracted with NZYMiniprep (NzyTech, Portugal) and residual chromosomal DNA was removed by digestion with Plasmid-Safe- ATP-Dependent DNase (Epicentre, Singapore) according to the manufacturer's instructions. DNA standards were prepared for each qPCR experiment by serial dilutions of purified pDNA in ultrapure water.

### *2.12. Statistical analysis*

Variables were checked for normal distribution by the Shapiro-Wilk test. Analyses of variance were performed with parametric one-way ANOVA followed by Dunnett's or Tukey's post-hoc t-tests or with the non-parametric Kruskal-Wallis test by ranks followed by Mann-Whitney U test, accordingly.

## 3. RESULTS

### *3.1. Strains relatedness*

The group of 25 *E. coli* strains studied in this work (twenty-two of which were representative of phylotypes only detected in the final effluent) resulted from a selection of 76 unique isolates obtained in a previous study (Silva *et al*., 2018). The genetic diversity of these 25 *E. coli* strains was evaluated by several typing methods (rep-PCR, PFGE, MLST and Clermont phylotyping). We identified 9 STs (n=25), 7 belonging to Clermont phylogroup A [ST58 (n=1 isolate), ST155 (n=4), ST453 (n=2), ST617 (n=2), ST744 (n=1), ST1284 (n=3) and a putative novel ST (n=3)], 1 to B2-sgI (ST131, n=7) and another to phylogroup C (ST410, n=2) (Figure 1). The rep-PCR, PFGE fingerprinting and plasmid content analyses (Figure S1, S2 and S3) supported the subsequent characterisation of the 25 strains, although in some cases only slight genomic differences could be found. Nonetheless, these strains could display distinct phenotypes and were, therefore, included in the subsequent characterisation.

### *3.2. ARG content and plasmid transfer capacity*

Besides the ARGs previously reported for these isolates (Silva *et al*., 2018; Table 1), 9 additional genes were detected: *sul1* (n=15 isolates), *sul2* (n=15) and *tet*(*A*) (n=14), followed by $bla_{\text{OXA-1-like}}$ (n=8), *tet*(*B*) (n=8), *aacA4-cr* (n=5), *aacA4* (n=2), *sul3* (n=2) and *qnrS1* (n=1). The presence of carbapenemase encoding genes was not investigated since none of the strains exhibited carbapenem resistance. Mutations that have been described to result in fluoroquinolone resistance were detected in all strains, with the most prevalent being Ser83→Leu and Asp87→Asn in the *gyrA* gene and Ser80→Ile in *parC* (Table 1).

Extraction of pDNA was successful for all 25 strains. Fingerprinting analysis of the restricted plasmid content (Figure S3-A) revealed 14 distinct band patterns (similarities <90%) out of 18 (plasmid restriction yielded no discernible band patterns for 7 strains). Conjugative transfer of cefotaxime resistance determinants to rifampicin-resistant *E. coli* CV601 was detected for 8 out of 25 strains [from phylogroup A (n=6) and C (n=2)], with transconjugant's plasmid content

representing 6 different band patterns (Table 2 and Figure S3-B). Transfer of plasmids harbouring *bla*$_{CTX-M-15}$ (n=3), *bla*$_{CTX-M-32}$ (n=3) and *bla*$_{CTX-M-1}$ (n=2) was observed (Table 2). Co-transfer of ARGs (i.e. *bla*$_{TEM}$, *bla*$_{OXA-1-like}$, *tet*(*A*), *tet*(*B*), *aacA4-cr*, *sul1* and *sul2*) and *intI1* was verified, in 3 cases with the transfer of all ARGs detected in the donor strain. In two cases (i.e. using ECR.2 and ECR.16 as donor strains), plasmid transfer conferred a multi-drug resistance phenotype to the recipient strain (Table 2). In *bla*$_{CTX-M-15}$-positive transconjugants three replicons of the F family (IncFIA, IncFIB and IncF) were detected, while in *bla*$_{CTX-M-1}$ transconjugants was detected the IncI1 replicon and in *bla*$_{CTX-M-32}$ transconjugants was detected the IncN replicon (Table 2).

### *3.3. Virulence related features*

From the four assays performed for biofilm quantification, 14/21 and 17/25 strains formed biofilms at 25 ºC with agitation and at 37 ºC in static conditions, respectively (Table 3). Among the strains that formed biofilms, most were classified as weak biofilm producers, with the exceptions of ECR.3 (A:ST1284) and ECR.24 (A:ST617) at 37 ºC and ECR.23 (A:ST744) at both temperatures. Eleven strains could form biofilms in both experimental models tested and ECR.23 (A:ST744) was the strongest producer (Table 3).

Only Vero cells monolayers exposed to 50.0% of the raw extracts displayed cell viability below 90%. Cytotoxicity of *E. coli* BL21 ranged from 0 to 6.08% and PH20 from 15.32 to 26.81%. Statistical analysis showed that 24/25 strains were significantly more cytotoxic than the negative control (Dunnett t-tests, p=0.000) and 9 were more cytotoxic than PH20 (from which 6 were affiliated to B2:ST131; Dunnett t-tests, p≤0.05). ECR.1 (ST131) was the most cytotoxic strain (Table 3).

Initial screening of the invasion capacity of mammalian cells by *E. coli* strains, indicated that 10 of the 21 strains tested were capable of internalization in Vero cells, 7 belonging to the B2:ST131 group (Table 3). To confirm the reproducibility of the assays and to provide a quantifiable measurement of invasive potential, 6 strains were selected (4 representatives of the

ST131, which were classified as invasive in the qualitative assay, and 2 strains classified as non-invasive) for 4 assays with variable MOIs. Variability of invasion indexes between different MOIs was observed (Figure 2). Multiple comparisons by Tukey's HSD test showed significantly higher invasive ability of B2:ST131 strains (ECR.1, ECR.11 and ECR.12; log invasion index between 0.3-0.5) from the remaining 3 ($p \leq 0.05$) in MOI~5 (Figure 2). All strains displayed invasion indexes significantly lower than the positive control (Figure 2).

Assays in *Galleria mellonella* larvae were conducted to evaluate the virulence of ten selected strains in live organisms. Strains were those selected for whole-genome sequencing analysis and *in vitro* quantitative invasion assays (to evaluate any relationship between *in vitro* and *in vivo* virulence). By the end of the experiment (96 hours), 9 of the 10 strains tested killed > 50% of the larvae. *E. coli* BL21 (used as a negative control) induced no mortality to *G. mellonella* (Figure 3). ECR.25 (ST617) was the most pathogenic to *G. mellonella*, killing all larvae after 50 hours. ECR.15 (ST58) and ECR.18 (ST1284) had identical kill curves.

Growth in Blood Agar indicated that 7 in 25 strains produced haemolysins (Table 3). Siderophore production was positive in nearly all strains (n=24) with most being characterized as abundant producers (n=21) (Table 3). PCR-based screening of IPEC VGs yielded no positive results.

### *3.4. Whole-genome sequence analysis*

From the 25 *E. coli* strains, 6 were selected (representing different lineages with distinct ARG and plasmid content) for Illumina-based genome sequencing (Table 1 and Figure 1).

Quality metrics for sequenced genomes are presented in Table S2. *In silico* sequence type affiliation confirmed the STs obtained by conventional allele amplification and Sanger sequencing (Table 4). Five serotypes were identified: O25:H4 (n=2), O8:H10 (n=1), O23:H16 (n=1), O89/162:H9 (n=1) and H9 (n=1; O antigen-encoding region absent). The *fimH* types detected were 24 (ECR.20), 27 (ECR.15), 30 (ECR.1 and ECR.19) and 31 (ECR.22). For

ECR.18, *fimH* gene was not detected. All 6 strains were predicted as human pathogens by PathogenFinder 1.1 (probability ≥ 93.0%; Table 4).

In terms of antibiotic resistance genes, analysis with ResFinder 3.1.0 confirmed the presence of all ARGs previously detected by PCR, and enabled the detection of additional genes that confer resistance to phenicols (*catB3*), aminoglycosides (*aadA2*, *strA* and *strB*), lincosamides [*lnu*(*F*)] and macrolides [*mph*(*A*)] (Table 4). In all strains, it was also identified a multi-drug resistance gene, *mdf*(*A*), which confers resistance to macrolides, lincosamides and streptogramin B (Table 4). ST131 strains carried the highest number of ARGs (10 each), while ECR.18 (ST1284) and ECR.22 (ST410) carried 9 and 7, respectively (Table 4). Two mutations in *parE* gene conferring resistance to fluoroquinolones (not previously analysed) were detected: Ser458→Ala (n=2) and Ile529→Leu (n=2) (Table 4). Multiple unknown mutations in ribosomal subunits encoding regions, *pmrA*, *pmrB*, *folP* and *ampC* were found by ResFinder 3.1.0 and may contribute to the expression of resistance phenotypes (data not presented). Several genes related with efflux of antibiotics were identified by CARD's RGI (e.g. *mdtA*, *emrA* and *gadW*; data not presented), though further analysis in PATRIC database showed that these genes are often found in *E. coli* genomes (prevalence above 90%) and are thus likely intrinsic.

The combined use of PlasmidFinder 2.0.1 and pMLST 2.0 confirmed most of the previously detected plasmid replicon types and identified IncQ1 and IncX1 in ECR.22 and *Col*-like replicons in ECR.1 and ECR.18 (Table 4). The pMLST 2.0 tool affiliated IncN replicons to plasmid ST1 (n=2), while the IncI1α belonged to the plasmid clonal complex 2 (n=1) or 3 (n=2). Only IncP in ECR.1 and IncF in ECR.18, which were previously detected by PCR and confirmed by amplicon sequencing, were not detected based on the *in silico* analysis (Table 1 and 3).

In ECR.1, $bla_{\text{CTX-M-15}}$ is present in a 96,743 bp contig with 100% similarity to chromosomal assemblies in GenBank. For the remaining strains, the contigs where the $bla_{\text{CTX-M}}$ gene was detected shared 100% similarity (coverages of 100% to ECR.18 and ECR.22, 97% for ECR.19 and 67-68% for ECR.15 and ECR.20) to plasmid sequences available in the database,

suggesting plasmid carriage of these genes. In strains ECR.15 and ECR.20 the contigs that included IncN replicons were identical (100% similarity and 100% query coverage) to regions of $bla_{CTX\text{-}M\text{-}32}$-carrying plasmids (e.g. MF953243.1), supporting the co-transfer of $bla_{CTX\text{-}M\text{-}32}$ and IncN in mating experiments (Table 2). For ECR.18 and ECR.19, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}27}$ are probably harboured in multi-replicon FIB-FIA-FII plasmids harbouring several other ARGs, based on the high similarity of contigs with plasmid sequences available in the database (CP027130.1 and CP023827.1). In ECR.22, $bla_{CTX\text{-}M\text{-}1}$, *tet(A)* and *sul2* seem to be co-carried in an IncI1α plasmid like MH847571.1, which was corroborated by the conjugation assays results (Table 2).

Using VFanalyzer and VirulenceFinder 2.0 we detected 140 different VGs in the genome sequences of the strains analysed (Table S3). The most represented were genes related with adhesion (e.g. haemorrhagic *E. coli* pilus, P fimbriae, long polar fimbriae and Iha), iron acquisition (e.g. yersiniabactin, salmochelin and aerobactin siderophores) and secretion systems (type III and VI), whereas VGs encoding toxins (EAST-1, SenB, haemolysin A and colicins), autotransporters (e.g. Sat, AatA and antigen 43), invasins (IbeB and IbeC), colonizing factors (Gad) and determinants responsible for serum resistance (Iss) and evasion to the immune system (capsular polysaccharides) were also detected (Table S4). Five strains presented approximately 70 VGs each, while ECR.20 only carried 59 VGs (Table S3). The *ibeB*, *ibeC*, *eaeH* and haemolysin encoding genes were identified in all strains. The genes encoding the autotransporter UpaG and factors related with increase serum survival were detected in 5 strains and determinants responsible for evasion to the immune system (i.e. capsule) were found in 4 strains (Table S3). The colicin-like uropathogenic-specific protein-encoding gene (*usp*) was detected in ECR.1 and ECR.19 (Table S4).

### *3.5. Environmental persistence*

To determine the fate and persistence of *E. coli* strains in freshwater, microcosms were established for the 6 genome-sequenced strains, and their presence was monitored by culture-dependent and culture independent-methods. Both methods indicate that after inoculation, the

number of *E. coli* cells tend to decrease over time, although the slope of this decrease is strain-dependent (Table S4 and S5). Of all inoculated strains, only ECR.19 (B2:ST131) was detected in quantifiable levels in the microcosm's water of all replicates after 28 days by cultivation [0.28-1.14 log(CFU/mL)] (Table S4). Significantly elevated *uidA* levels compared with the control in all replicates at this sampling moment corroborates the persistence of this strain (Table S5). In most other cases, CFU levels of the inoculated strains dropped below the quantifiable limit between 7 and 14 days of incubation, with an already strong decay being confirmed by both methods at 7 days (Table S4 and S5). Only ECR.1 and ECR.22 were detected by culture-dependent methods after 28 days in 1/4 of the microcosm's replicates, while ECR.15 (1/3), ECR.20 (1/3) and ECR.22 (2/3) were detected by culture-independent methods at this sampling moment (Table S5). For $bla_{\text{CTX-M}}$ quantification, in most cases, this gene's levels dropped below the quantification limit at 7 days. $bla_{\text{CTX-M}}$ was not detected in any sample at 28 days (Table S5). *E. coli* colonies retrieved at 28 days displayed typing profiles and resistance phenotypes and genotypes identical to the inoculated strains (data not presented).

## 4. DISCUSSION

Few studies characterized bacterial strains released in the effluents of WWTPs to infer possible health risks to human populations (Anastasi *et al*., 2010 and 2013; Dolejska *et al*., 2011; Calhau *et al*., 2015). Henceforth, in this study, we intended to provide a broad description of a collection of ESBL-producing *E. coli* strains that survived a UV-C-irradiation treatment at a full-scale WWTP and assess potential risk based on antibiotic resistance (and its transfer), virulence-related characteristics and environmental persistence. ESBL-producing *E. coli* were evaluated due to their clinical relevance, i.e. because of their increasing prevalence in clinical settings and limited therapeutic options available to treat infections caused by such strains (Rodríguez-Baño and Pascual, 2008; Thaden *et al*., 2016).

We included 25 strains of 9 STs. With the molecular typing results (e.g. MLST, PFGE, rep-PCR and plasmid typing) we observed that strains were closely related within each ST, in some cases suggesting clonality. However, it has been suggested that these methods' resolution may be insufficient to detect dissimilarities between strains (Davis *et al*., 2003; Jonas *et al*., 2003). In fact, from our study, we observed differences between genome sequences from closely related strains such as ECR.1 and ECR.19 (ST131), whose genomes shared 99.85% similarity between orthologous genes (approx. 7.5-7.7 kb differences) and 99.70% in digital DNA-DNA hybridization (approx. 15.0-15.4 kb differences). The strains analysed affiliated to phylogroups B2, A, and C. B2 lineages are often associated with human infections (Bukh *et al*., 2009). Moreover, B2 *E. coli* strains here analysed were identified as ST131, which is considered a high-risk clone implicated in the successful dissemination of $bla_{\text{CTX-M-15}}$ (Cantón *et al*., 2012). The prevalence of ST131 among cefotaxime-resistant *E. coli* from wastewater was expected based on previous reports (e.g. Dolejska *et al*., 2011). All strains identified as ST131 were multi-drug resistant (resistant to 4-5 classes of antibiotics) and displayed relevant phenotypic virulence traits (e.g. cytotoxicity and invasion capacity). All the five tested ST131 strains were predicted as pathogenic by *in vitro* and *in vivo* tests, one (ECR.19, ST131) being capable of consistently persist in a freshwater microcosm. Moreover, six STs detected in this study had

been previously reported in treated wastewater effluents (exceptions were ST617 and ST1284; Bréchet *et al*., 2014; Dolejska *et al*., 2011; Varela *et al*., 2015), and some were classified as pathogenic and/or identified as ESBLs-producers, particularly ST58 and ST155 (Enterobase, https://enterobase.warwick.ac.uk/). ST744 was detected in this study, and, interestingly, has been recently described in a Portuguese hospital, carrying *mcr-1* and $bla_{\text{KPC-3}}$ (Tacão *et al*., 2017).

Not only different $bla_{\text{CTX-M}}$ gene variants were detected, but their putative mobility to new hosts, through mobile platforms that carry other resistance determinants, was also shown. Conjugal transfer of $bla_{\text{CTX-M}}$ was confirmed in nearly 1/3 of our collection (associated with F-like, I1 and N replicons). CTX-M-15 encoding genes are often associated with promiscuous plasmids of the F family (Amos *et al*., 2014; Dolejska *et al*., 2011; Novais *et al*., 2007), $bla_{\text{CTX-M-1}}$ has been described in conjugative IncI1 plasmids from different sources (including WWTPs; Dolejska *et al*., 2013) and $bla_{\text{CTX-M-32}}$ in IncN plasmids from clinical isolates (Novais *et al*., 2007). The conjugative ability of $bla_{\text{CTX-M}}$-plasmids isolated from WWTPs' effluents varies among studies (Amos *et al*., 2014; Dolejska *et al*., 2011), which may represent differences between assay conditions rather than plasmid potential to be laterally transferred.

Several studies report the release of putative virulent *E. coli* strains in WWTPs' effluents (Anastasi *et al*., 2010 and 2013; Calhau *et al*., 2015), with UV irradiation possibly increasing the VG content of surviving strains comparatively with chlorination (Anastasi *et al*., 2013). Whole-genome analysis identified several VGs, though its composition per strains was atypical, making it difficult to define their pathotype. Considering that WWTPs may act as hotspots for lateral gene transfer (Karkman *et al.*, 2018), barriers between pathotypes may have faded due to promiscuous acquisition of VGs encoded in mobile genetic elements during wastewater processing. Still, the presence of adhesion factors (such as P fimbriae and Iha), siderophores (IroN, SitA and IutA), haemolysins (HlyE), invasins (IbeB and IbeC) and increase serum survival (Iss) factors across several of the evaluated strains, indicates them as potential pathogens, which is corroborated by PathogenFinder 1.1 prediction. In particular, the detection

of the genes *iha, agn43, ibeB, ibeC, iroN, sitA, iss, sat* and *hlyA* in these strains suggests extraintestinal pathogenesis since many of these have been linked to extraintestinal pathogenic *E. coli* (ExPEC) strains (Sarowska *et al*., 2019). The VG content of these strains may corroborate Anastasi and colleagues' hypothesis that *E. coli* carrying uropathogenic VGs have a higher ability to survive wastewater treatments (Anastasi *et al*., 2010).

Vero cells were used for virulence experiments since this cell line is often used to detect shiga-toxin production. Though none of the shiga-toxin encoding genes were detected, all strains displayed relevant cytotoxicity comparatively to *stx2*$^{+}$ *E. coli* PH20, with most presenting cytotoxicity to Vero cells between 20-36%. These levels are in agreement with the results obtained for Enterohemorrhagic *E. coli* O26 and O111 carrying *stx1* and/or *stx2* (Lee *et al*., 2008) and for non-*stx* producing *E. coli* from human urine and meat (Roberts *et al*., 2001). Invasion of mammalian cells is also a relevant virulent trait and thus was assessed. Variability in the invasiveness of the tested strains according to the MOI can be related to quorum sensing-driven invasion of mammalian cells and experimental limitations. For instance, cell detachment due to production of cytotoxic substances by the strains (which has been confirmed by cytotoxicity assays) can underestimate internalization capacity and successive washing steps may create additional intra-experimental variations due to discrepant cell detachment. In this sense, a MOI of 5 was considered more reliable, since *in vitro* higher cell densities lead to higher cell detachment. All B2:ST131 strains were invasive (and cytotoxic) towards kidney epithelial cells, which may suggest the uropathogenic potential of these strains. Our results were within the range of invasion indexes reported for isolates retrieved from retail meat and carrying Uropathogenic *E. coli* (UPEC) related VGs (Xia *et al*., 2011), but lower compared with other studies (Barrios-Villa *et al*., 2018; Martinez-Medina *et al*., 2009). Still, large variation in invasion capacity among UPEC of the same serotype, phylogroup and carrying the same VGs has been described (Martinez-Medina *et al*., 2009). The invasion index of a UPEC clinical isolate was shown to be inferior in Vero cells when compared to cell lines from the human urinary tract (Ge *et al*., 2009), indicating that experiments conducted using the Vero cell line

may underestimate these strains *in vitro* pathogenicity. Evidence from invasion and cytotoxicity suggest that at least 10 strains (most belonging to ST131) are pathogenic (since they present both invasiveness and cytotoxicity towards urogenital epithelium cells), which was corroborated by *in silico* pathogenicity prediction and virulence gene screening. To confirm virulent traits determined towards the Vero cell line, we performed infection assays in a living model (*G. mellonella*) and concluded that the 10 selected strains were all pathogenic, since after 96 hours of incubation at least 40% of the larvae were killed. Interestingly, phylogroup A and C strains tested (non-invasive to Vero cells) were shown (in most cases) to lead to higher larvae death rates than B2 strains (invasive to Vero cells). The larvae survival curve corresponding to NMEC strain RS218 (positive control) was concordant with a previous study (Fuentes-Castillo *et al*., 2019), which reflects the assay´s reproducibility. The reliability of *G. mellonella* as a model for bacterial infection lies in the high similarity of their innate immune response to vertebrates. Studies of infection by *E. coli* using this model have been performed, with correlations being established between ExPEC VGs carriage and sequence type (reviewed by Tsai *et al*., 2016). In general, virulence experiments in our study suggest that most of the examined isolates can be pathogenic to humans, either by potentially infecting the urinary tract (B2 strains) or provoking other extraintestinal infections (A and C strains).

Considering that susceptibility to antibiotics and survival in environmental settings may be impacted by biofilm formation capacity, assays were performed to quantify such ability. These experiments are difficult to standardize. For example, different culture media and quantification protocols generate distinct results (Naves *et al*., 2008). We overcame in part the lack of reproducibility by normalizing biofilm biomass with bacterial growth ($OD_{600nm}$). In general, biofilm formation was weak to mild in our collection. A credible hypothesis is that previous conventional activated sludge followed by flocculation and precipitation in settling tanks likely removes preferentially bacteria with higher aggregative behaviour, such as strong biofilm producers. In fact, Čornejová *et al*. (2015) also found that most ESBL-producing *E. coli* strains isolated from treated municipal wastewater were weak biofilm producers, which supports this

hypothesis. Our strongest biofilm producer (ECR.23) carried an IncX plasmid. Plasmids from this incompatibility group have been associated with biofilm formation due to carriage of fimbrial gene cassettes (Burmølle *et al*., 2012).

Pathogenic *E. coli* have been shown to persist in dairy wastewater and activated sludge for more than 20 days (Czajkowska *et al*., 2008) and from 21 to 54 days in lake or river water (Czajkowska *et al*., 2005). In freshwater, Flint (1987) described differential survival of *E. coli* K-12 depending on temperature. As such, we determined the potential persistence of six strains in freshwater microcosms. In general, there was high variability among microcosm's replicates results, either using culture-dependent or culture-independent methods, though data points out to the undeniable persistence of ECR.19 (a multi-drug resistant ST131 strain displaying relevant cytotoxicity, invasiveness and mortality towards *G. mellonella* larvae) in freshwater after 28 days. $bla_{\text{CTX-M}}$ was not detected at days 7 and 28 for ECR.19 (unlike *uidA* and CFU counts), but this is likely associated to the primer binding affinities to different $bla_{\text{CTX-M}}$ variants (in other cases, the discrepancies may also result from the target gene being below the detection limit). Comparison of our findings with other studies is hindered by: (i) effects related to chemical and biological composition of the water; (ii) non-removal of the endogenous microbiota that exerts competition with the inoculated strains (Flint, 1987); and (iii) the inverse proportionality relation between inoculum concentration and persistence in microcosms (Ravva *et al*., 2006). Nonetheless, this time frame might enable these bacteria to reach human populations, through the use of contaminated water, consumption of food products and recreational activities. For example, Leonard and colleagues confirmed the association between surfing in contaminated water and colonisation by $bla_{\text{CTX-M}}$ carrying *E. coli* (Leonard *et al*., 2018). This is especially problematic with the increasing need for recycling treated wastewater for irrigation (already widely implemented) and drinking water due to water scarcity (Fatta-Kassinos *et al*., 2011).

Although the number of isolates here analysed may be seen as a limitation of the study, these strains represented all the distinct BOX-PCR profiles and $bla_{\text{CTX-M}}$ variants detected in the sampled final effluent (Silva *et al*., 2018). Our experimental design did not address seasonal or

geographical variations, since only a single WWTP was sampled over 4 months, though effects are expected. Seasonal differences in antibiotic prescription can influence antibiotic resistance in wastewater, as previously described (Caucci *et al*., 2016), and these fluctuations can have effects on treatment efficiency. For instance, Sui and colleagues showed that ARGs removal in swine WWTPs was higher in winter (Sui *et al*., 2017). The abundance and diversity of ARGs in sewage have also been shown to be distinct in different world regions (Hendriksen *et al*., 2019). From the analysed strains, their relative abundance probably changes across different seasons and other *E. coli* strains with different antibiotic resistance profiles and other virulence and/or persistence behaviours may occur (with consequences for the associated human and environmental health risks). Nonetheless, such differences are expected to be more pronounced across different countries, where the diversity of antibiotic-resistant strains in wastewater probably mirrors the diversity in clinical settings in each country. In fact, this was shown for the WWTPs' resistome (ARGs prevalence and diversity) across Europe (Pärnänen *et al*., 2019). Hence, although our results tackle important knowledge gaps, future studies are needed to elucidate seasonal and geographical variations in terms of the characteristics of strains present in WWTPs' final effluents. Also, future studies should address strains isolated after other advanced treatments and/or belonging to other phylogenetic groups, to better estimate the risk that treated wastewater effluents constitute for human and environmental health.

## 5. CONCLUSION

In the present study, we evaluated the antibiotic resistance mechanisms, virulence and the potential environmental persistence of *E. coli* strains present in a UV-C treated effluent. Our data indicated the presence of successful high-risk clones carrying relevant antibiotic resistance (nearly all multi-drug resistant) and virulence determinants characteristic of ExPEC pathogens, with most strains displaying virulence-related phenotypes, and some, amenable persistence in freshwater microcosms. The transfer of conjugative plasmids carrying numerous ARGs was also confirmed in eight of the studied strains, in some cases resulting in multi-drug resistance phenotypes. In overall, this indicates that the UV-treated effluent analysed still represents a potential risk to environmental and public health. A detailed evaluation of these traits in strains surviving other wastewater treatments is urgent, since finding an adequate treatment that reduces levels of antibiotic-resistant pathogens from WWTPs' effluents is necessary to circumvent adverse environmental and public health impacts and enable wastewater reuse.

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**Tables**

# Genotypic and phenotypic traits of *bla*$_{\text{CTX-M}}$-carrying *Escherichia coli* strains from an UV-C-treated wastewater effluent

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**Table 1.** Phylogroups, sequence type affiliation and phenotypic and genotypic features of the *E. coli* strains.

| Strain[a] | Clermont Phylogroup | Sequence Type | ARGs/Integrons[b] | Mutations responsible for fluoroquinolone resistance | | Phenotypic resistance profile (number of antibiotic classes for which isolates are resistant)[c,d] | Plasmid replicons detected[c] |
|---|---|---|---|---|---|---|---|
| | | | | *parC* | *gyrA* | | |
| **ECR.1** | B2-sgI | ST131 | ***bla*$_{CTX-M-15}$**, **bla$_{TEM}$**, *bla*$_{OXA-1}$, *tet(A)*, *sul1*, *aacA4-cr*/***intI1*\|*dfrA15*\|*aadA1*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TZP-TIC-TIM-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *P*, *FIB*, *F* |
| **ECR.2** | A | ST155 | ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *tet(B)*, *sul1*, *aacA4-cr*/***intI1*\|*dfrA17*\|*aadA5*** | - | Ser83→Ala | AML-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CAZ-ATM-CN-TET-SXT **(4)** | *FIB*, *FIA*, *F*, *I2* |
| **ECR.3** | A | ST1284 | ***bla*$_{CTX-M-15}$**, *tet(B)*, *sul1*, *sul2*/***intI1*\|*dfrA17*\|*aadA5*** | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *FIB*, *FIA*, *F* |
| **ECR.4** | A | ST155 | ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *tet(B)*, *sul1*, *aacA4-cr*/***intI1*\|*dfrA17*\|*aadA5*** | - | Ser83→Ala | AML-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CAZ-ATM-CN-TET-SXT **(4)** | *FIB*, *FIA*, *F*, *I2* |
| **ECR.5** | A | ST1284 | ***bla*$_{CTX-M-15}$**, *tet(B)*, *sul1*, *sul2*/***intI1*\|*dfrA17*\|*aadA5*** | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *FIB*, *FIA*, *F* |
| **ECR.6** | A | ST155 | ***bla*$_{CTX-M-15}$**, *bla*$_{OXA-1}$, *tet(B)*, *sul1*, *aacA4-cr*/***intI1*\|*dfrA17*\|*aadA5*** | - | Ser83→Ala | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CN-TET-SXT **(4)** | *FIB*, *FIA*, *F*, *I2* |
| **ECR.7** | B2-sgI | ST131 | ***bla*$_{CTX-M-27}$**, *tet(A)*, *sul1*, *sul2*/***intI1*\|*dfrA17*\|*aadA5*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-PRL-TIC-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *FIB*, *FIA*, *F* |
| **ECR.8** | B2-sgI | ST131 | ***bla*$_{CTX-M-27}$**, *tet(A)*, *sul1*, *sul2*/***intI1*\|*dfrA17*\|*aadA5*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *FIB*, *FIA*, *F* |
| **ECR.9** | C | ST410 | ***bla*$_{CTX-M-32}$** | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP **(2)** | *I1*, *N* |
| **ECR.10** | A | unknown | ***bla*$_{CTX-M-15}$**, **bla$_{TEM}$**, *tet(A)*, *sul2*/***intI1*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-C-SXT **(5)** | *FIB*, *FIA*, *F* |
| **ECR.11** | B2-sgI | ST131 | ***bla*$_{CTX-M-15}$**, **bla$_{TEM}$**, *bla*$_{OXA-1}$, *tet(A)*, *sul1*, *aacA4*/***intI1*\|*dfrA15*\|*aadA1*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *P*, *FIB*, *F* |
| **ECR.12** | B2-sgI | ST131 | ***bla*$_{CTX-M-15}$**, **bla$_{TEM}$**, *bla*$_{OXA-1}$, *tet(A)*, *sul1*, *aacA4*/***intI1*\|*dfrA15*\|*aadA1*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TZP-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-CN-TET-SXT **(5)** | *P*, *FIB*, *F* |
| **ECR.13** | A | ST453 | ***bla*$_{CTX-M-1}$**, **bla$_{TEM}$**, *tet(A)*, *sul2* | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET **(3)** | *B/O*, *I1*, *F* |
| **ECR.14** | A | unknown | ***bla*$_{CTX-M-15}$**, **bla$_{TEM}$**, *tet(A)*, *sul2*/***intI1*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-PRL-TZP-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-C-SXT **(5)** | *FIB*, *FIA*, *F* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **ECR.15** | A | ST58 | ***bla*$_{\text{CTX-M-32}}$**, *qnrS1*/***intI1*** | - | Ser83→Leu | AML-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP **(2)** | *I1*, *N* |
| **ECR.16** | A | ST155 | ***bla*$_{\text{CTX-M-15}}$**, *bla*$_{\text{OXA-1}}$, *tet(B)*, *sul1*, *aacA4-cr*/***intI1*\|*dfrA17*\|*aadA5*** | - | Ser83→Ala | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CN-TET-SXT **(4)** | *FIB*, *FIA*, *F*, *I2* |
| **ECR.17** | B2-sgI | ST131 | ***bla*$_{\text{CTX-M-27}}$**, *bla*$_{\text{OXA-1}}$, *tet(A)*, *sul1*, *sul2*/***intI1*\|*dfrA17*\|*aadA5*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *FIB*, *FIA*, *F* |
| **ECR.18** | A | ST1284 | ***bla*$_{\text{CTX-M-15}}$**, *tet(B)*, *sul1*, *sul2*/***intI1*\|*dfrA17*\|*aadA5*** | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *FIB*, *FIA*, *F* |
| **ECR.19** | B2-sgI | ST131 | ***bla*$_{\text{CTX-M-27}}$**, *tet(A)*, *sul1*, *sul2*/***intI1*\|*dfrA17*\|*aadA5*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *FIB*, *FIA*, *F* |
| **ECR.20** | C | ST410 | ***bla*$_{\text{CTX-M-32}}$** | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP **(2)** | *I1*, *N* |
| **ECR.21** | A | unknown | ***bla*$_{\text{CTX-M-15}}$**, ***bla*$_{\text{TEM}}$**, *tet(A)*, *sul2*/***intI1*** | Ser80→Ile, Glu84→Val | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-C-SXT **(5)** | *FIB*, *FIA*, *F* |
| **ECR.22** | A | ST453 | ***bla*$_{\text{CTX-M-1}}$**, ***bla*$_{\text{TEM}}$**, *tet(A)*, *sul2* | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET **(3)** | *B/O*, *I1*, *F* |
| **ECR.23** | A | ST744 | ***bla*$_{\text{CTX-M-32}}$**, *tet(B)*, *sul1*, *sul2*/***intI1*\|*dfrA17*\|*aadA5*, *intI2*\|*dfrA*\|*sat*\|*aadA*** | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-SXT **(4)** | *F*, *X4* |
| **ECR.24** | A | ST617 | ***bla*$_{\text{CTX-M-15}}$**, ***bla*$_{\text{TEM}}$**, *tet(A)*, *sul2*, *sul3*/***intI1*** | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-C-SXT **(5)** | *P*, *F* |
| **ECR.25** | A | ST617 | ***bla*$_{\text{CTX-M-15}}$**, ***bla*$_{\text{TEM}}$**, *tet(A)*, *sul2*, *sul3*/***intI1* (empty)** | Ser80→Ile | Ser83→Leu, Asp87→Asn | AML-AMC-PRL-TIC-TIM-FEP-CTX-CAZ-ATM-CIP-TET-C-SXT **(5)** | *P*, *F* |

[a]Underlined are strains whose genome was sequenced;
[b]On bold are indicated ARGs/integrons previously detected by Silva *et al*., 2018.
[c]Features reported by Silva *et al*., 2018.
[d]Antibiotic abreviations: AML - amoxicillin, AMC - amoxicillin/clavulanic acid, PRL - piperacillin, TZP - piperacillin/tazobactam, TIC - ticarcillin, TIM - ticarcillin/clavulanic acid, CTX - cefotaxime, CAZ - ceftazidime, FEP - cefepime, ATM - aztreonam, CIP - ciprofloxacin, GEN - gentamicin, TET - tetracycline, CHL - chloramphenicol, SXT - trimethoprim/sulfamethoxazole.

**Table 2.** Genotypic and phenotypic features of donor strains for which conjugation assays yielded transconjugants. In bold are presented the determinants that were transferred to the recipient *E. coli* CV601.

| Isolate | Genotypic profile | Plasmid replicons | Resistance phenotype (number of antibiotic classes for which the transconjugant/donor strain is resistant) |
|---|---|---|---|
| ECR.2 | ***bla*$_{\text{CTX-M-15}}$**, ***bla*$_{\text{OXA-1-like}}$**, ***tet(B)***, ***intI1***, ***sul1, aacA4-cr*** | ***FIB***, ***FIA***, ***F***, *I2* | **AML**-AMC-**PRL**-TZP-**TIC**-**TIM**-FEP-**CTX**-**CAZ**-**ATM**-**CN**-**TET**-**SXT** (4/4) |
| ECR.9 | ***bla*$_{\text{CTX-M-32}}$** | ***I1***, ***N*** | **AML**-AMC-**PRL**-**TIC**-**TIM**-**FEP**-**CTX**-**CAZ**-**ATM**-CIP (1/2) |
| ECR.10 | ***bla*$_{\text{CTX-M-15}}$**, ***bla*$_{\text{TEM}}$**, *tet(A)*, ***intI1***, *sul2* | ***FIB***, ***FIA***, ***F*** | **AML**-AMC-**PRL**-**TIC**-**TIM**-FEP-**CTX**-**CAZ**-**ATM**-CIP-TET-C-SXT (1/5) |
| ECR.13 | ***bla*$_{\text{CTX-M-1}}$**, *bla*$_{\text{TEM}}$, ***tet(A)***, ***sul2*** | *B/O*, ***I1***, *F* | **AML**-AMC-**PRL**-**TIC**-**TIM**-FEP-**CTX**-CAZ-ATM-CIP-**TET** (2/3) |
| ECR.15 | ***bla*$_{\text{CTX-M-32}}$**, *qnrS1*, *intI1* | *I1*, ***N*** | **AML**-**PRL**-**TIC**-**TIM**-**FEP**-**CTX**-**CAZ**-**ATM**-CIP (1/2) |
| ECR.16 | ***bla*$_{\text{CTX-M-15}}$**, ***bla*$_{\text{OXA-1-like}}$**, ***tet(B)***, ***intI1***, ***sul1, aacA4-cr*** | ***FIB***, ***FIA***, ***F***, *I2* | **AML**-AMC-**PRL**-**TIC**-**TIM**-FEP-**CTX**-**CAZ**-**ATM**-**CN**-**TET**-**SXT** (4/4) |
| ECR.20 | ***bla*$_{\text{CTX-M-32}}$** | *I1*, ***N*** | **AML**-AMC-**PRL**-**TIC**-**TIM**-**FEP**-**CTX**-**CAZ**-**ATM**-CIP (1/2) |
| ECR.22 | ***bla*$_{\text{CTX-M-1}}$**, ***bla*$_{\text{TEM}}$**, ***tet(A)***, ***sul2*** | *B/O*, ***I1***, *F* | **AML**-AMC-**PRL**-**TIC**-**TIM**-FEP-**CTX**-CAZ-ATM-CIP-**TET** (2/3) |

**Table 3.** Phenotypic features of the *E. coli* strains studied. Strains are clustered according with the phylogroup/ST. In quantitative assays, values presented are mean ± standard deviation. The colour code (♦ light grey, ♦ intermediate grey and ♦ dark grey) is as follow: (a) light grey - non-producer, intermediate grey - weak-producer, dark grey - producer; (b) light grey - negative, intermediate grey – weak-producer; dark grey - abundant producer; (c) light grey - negative, dark grey - positive; (d) light grey - non-cytotoxic, intermediate grey - weakly cytotoxic, dark grey - cytotoxic. In siderophore production, (Y) stands for yellow phenotype and (G) for green phenotype in O-CAS media. Data presented for biofilm formation are average values from two independent experiments, in which BFI was the determined from the ratio OD590nm/OD600nm. Cell viability and the percentage of cytotoxicity presented was calculated by the two following formulas: $\text{Cell viability (\%)} = \frac{\text{ratio OD } 570/600_{\text{extract}}}{\text{ratio OD } 570/600_{\text{TSB}}}$ and $\text{Cytotoxicity per OD}_{600nm}\ 1.0\ (\%) = \frac{1 - \text{Cell viability (\%)}}{\text{OD}_{600\text{nm}}}$, respectively. n.d. stands for not determined.

| Phylogroup/ST | Strain | Biofilm production (BFI)[a] | | Siderophore production[b] | | Haemolysin production[c] | Vero cell's assays | |
|---|---|---|---|---|---|---|---|---|
| | | 25ºC with agitation | 37ºC without agitation | | | | Cytotoxicity assays (% of cytotoxicity)[d] | Invasion assays[c] |
| B2:ST131 | ECR.1 | 0.259 ± 0.075 | 0.027 ± 0.000 | ++ | (Y) | - | 53.31 ± 2.70 | + |
| | ECR.7 | 0.182 ± 0.033 | 0.034 ± 0.003 | ++ | (Y) | - | 31.07 ± 2.19 | + |
| | ECR.8 | 0.178 ± 0.027 | 0.037 ± 0.003 | ++ | (Y) | - | 35.87 ± 3.54 | + |
| | ECR.11 | 0.210 ± 0.063 | 0.026 ± 0.005 | ++ | (Y) | + | 35.19 ± 3.76 | + |
| | ECR.12 | 0.197 ± 0.039 | 0.035 ± 0.006 | ++ | (Y) | + | 35.40 ± 3.54 | + |
| | ECR.17 | 0.228 ± 0.093 | 0.042 ± 0.002 | ++ | (Y) | - | 29.46 ± 4.65 | + |
| | ECR.19 | 0.162 ± 0.010 | 0.046 ± 0.002 | ++ | (Y) | - | 27.72 ± 2.03 | + |
| A:ST155 | ECR.2 | 0.087 ± 0.011 | 0.024 ± 0.002 | ++ | (Y) | - | 26.04 ± 4.01 | n.d. |
| | ECR.4 | 0.167 ± 0.039 | 0.022 ± 0.000 | ++ | (Y) | - | 23.90 ± 1.57 | n.d. |
| | ECR.6 | 0.145 ± 0.016 | 0.015 ± 0.001 | ++ | (Y) | - | 17.33 ± 5.17 | n.d. |
| | ECR.16 | 0.139 ± 0.022 | 0.024 ± 0.001 | ++ | (Y) | - | 21.67 ± 6.84 | n.d. |
| C:ST410 | ECR.9 | 0.105 ± 0.007 | 0.020 ± 0.003 | - | | + | 21.49 ± 1.54 | - |
| | ECR.20 | 0.127 ± 0.017 | 0.035 ± 0.006 | ++ | (G) | + | 8.83 ± 7.04 | - |
| A:ST58 | ECR.15 | 0.207 ± 0.047 | 0.037 ± 0.005 | + | (G) | - | 34.67 ± 6.69 | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **A:ST453** | ECR.13 | 0.198 ± 0.106 | 0.046 ± 0.007 | ++ | (G) | - | 20.11 ± 3.51 | - |
| | ECR.22 | 0.222 ± 0.088 | 0.060 ± 0.001 | ++ | (G) | - | 19.53 ± 4.80 | - |
| **A:ST617** | ECR.24 | 0.226 ± 0.078 | 0.065 ± 0.013 | ++ | (G) | + | 25.68 ± 2.62 | + |
| | ECR.25 | 0.277 ± 0.151 | 0.043 ± 0.005 | ++ | (G) | + | 16.07 ± 6.51 | - |
| **A:ST744** | ECR.23 | 0.355 ± 0.251 | 0.095 ± 0.014 | ++ | (G) | + | 30.37 ± 2.13 | + |
| **A:ST1284** | ECR.3 | 0.158 ± 0.024 | 0.074 ± 0.021 | ++ | (Y) | - | 34.00 ± 4.79 | + |
| | ECR.5 | 0.155 ± 0.041 | 0.025 ± 0.002 | ++ | (Y) | - | 26.28 ± 3.16 | - |
| | ECR.18 | 0.194 ± 0.048 | 0.048 ± 0.001 | ++ | (Y) | - | 18.26 ± 2.69 | - |
| **A:ST unknown** | ECR.10 | n.d. | 0.036 ± 0.003 | + | (G) | - | 15.76 ± 5.81 | - |
| | ECR.14 | n.d. | 0.038 ± 0.000 | + | (G) | - | 27.29 ± 1.61 | - |
| | ECR.21 | n.d. | 0.037 ± 0.001 | ++ | (G) | - | 12.70 ± 2.51 | - |

**Table 4.** *In silico* determination of sequence types (Warwick schemes, MLST 2.0.1), serotypes (SeroTypeFinder 2.0.1), *fimH* types (CHTyper 1.0), pathogenicity prediction (PathogenFinder 1.1), ARGs and mutations known to confer antibiotic resistance phenotypes (in this case fluoroquinolone resistance; ResFinder 3.1.0), and plasmid replicons (PlasmidFinder 2.0.1 and pMLST 2.0).

| Strain | MLST | Serotype | *fimH* type | Pathogenecicity prediction | | ARGs[a] | Mutations conferring antibiotic resistance[a] | Plasmid replicons[a] |
|---|---|---|---|---|---|---|---|---|
| | | | | Human pathogen? | Probability | | | |
| **ECR.1** | ST131 | O25:H4 | 30 | yes | 0.930 | ***bla*$_{\text{CTX-M-15}}$**, ***bla*$_{\text{TEM-1B}}$**, ***bla*$_{\text{OXA-1}}$**, ***aacA4-cr***, ***aadA1***, *mdf(A)*, *catB3*, ***sul1***, ***tet(A)***, ***dfrA15*** | <u>*gyrA*</u>: **Ser83→Leu**, **Asp87→Asn**; <u>*parC*</u>: **Ser80→Ile, Glu84→Va**l; <u>*parE*</u>: Ile529→Leu | ***FIB***, ***FII***, *Col*-like |
| **ECR.15** | ST58 | O8:H10 | 27 | yes | 0.935 | ***bla*$_{\text{CTX-M-32}}$**, *aadA2*, ***qnrS1***, *Inu(F)*, *mdf(A)* | <u>*gyrA*</u>: **Ser83→Leu** | ***I1α*** (ST244-like, CC-2), ***N*** (ST1) |
| **ECR.18** | ST1284 | O89/162:H9 | - | yes | 0.935 | ***bla*$_{\text{CTX-M-15}}$**, *strA*, *strB*, ***aadA5***, *mdf(A)*, ***sul1***, ***sul2***, ***tet(B)***, ***dfrA17*** | <u>*gyrA*</u>: **Ser83→Leu**, **Asp87→Asn**; <u>*parC*</u>: **Ser80→Ile**; <u>*parE*</u>: Ser458→Ala | ***FIA***, ***FIB***, *Col*-like |
| **ECR.19** | ST131 | O25:H4 | 30 | yes | 0.936 | ***bla*$_{\text{CTX-M-27}}$**, *strA*, *strB*, ***aadA5***, *mdf(A)*, *mph(A)*, ***sul1***, ***sul2***, ***tet(A)***, ***dfrA17*** | <u>*gyrA*</u>: **Ser83→Leu**, **Asp87→Asn**; <u>*parC*</u>: **Ser80→Ile**, **Glu84→Val**; <u>*parE*</u>: Ile529→Leu | ***FIA***, ***FIB***, ***FII*** |
| **ECR.20** | ST410 | H9 | 24 | yes | 0.937 | ***bla*$_{\text{CTX-M-32}}$**, *mdf(A)* | <u>*gyrA*</u>: **Ser83→Leu**, **Asp87→Asn**; <u>*parC*</u>: **Ser80→Ile**; <u>*parE*</u>: Ser458→Ala | ***I1α*** (ST3, CC-3), ***N*** (ST1) |
| **ECR.22** | ST453 | O23:H16 | 31 | yes | 0.931 | ***bla*$_{\text{CTX-M-1}}$**, ***bla*$_{\text{TEM-1A}}$**, *strA*, *strB*, *mdf(A)*, ***sul2***, ***tet(A)*** | <u>*gyrA*</u>: **Ser83→Leu**, **Asp87→Asn**; <u>*parC*</u>: **Ser80→Ile** | ***B/O/K/Z***, ***FIC(FII)***, ***I1α*** (ST3 or ST214, CC-3), *Q1*, *X1* |

[a]On bold are indicated the genotypic determinants previously detected by Silva et al., 2018 or previously screened in this study (Table 1).

**Figures**

# Genotypic and phenotypic traits of *bla*$_{\text{CTX-M}}$-carrying *Escherichia coli* strains from an UV-C-treated wastewater effluent

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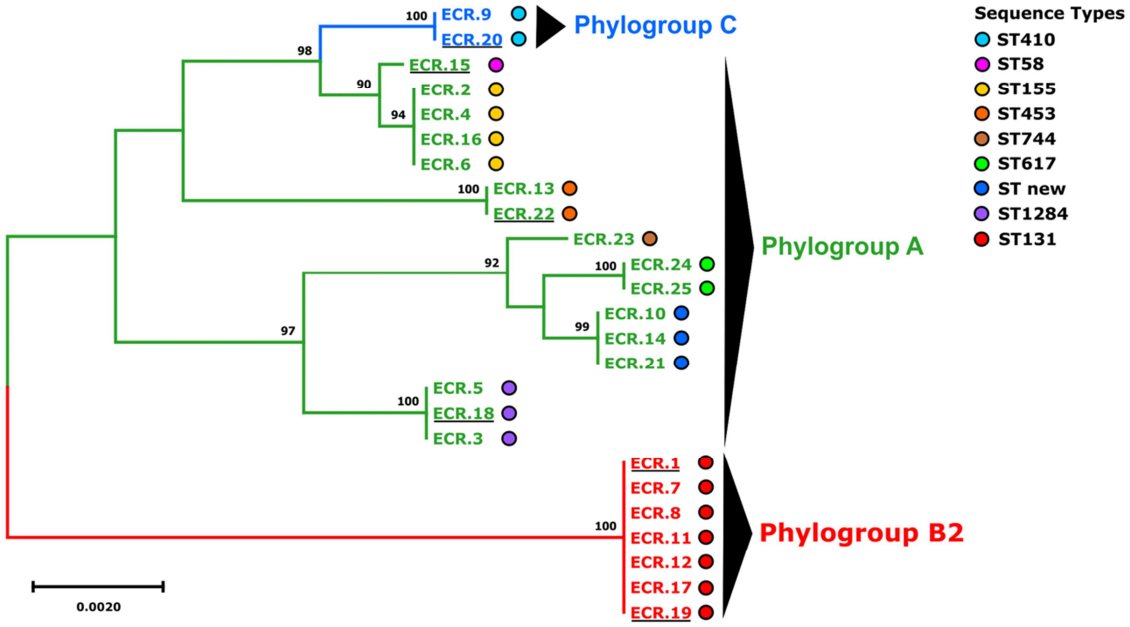



**Figure 1.** Phylogenetic tree based on the concatenated sequences of the seven housekeeping genes used for MLST and constructed using the Neighbour Joining method (1000 bootstraps). The evolutionary model that best described the sequence data was kimura 2-parameters gamma distributed with invariant sites. Strain designation is coloured according with *E. coli* phylogroups (red: B2; green: A; and blue: C). Coloured circles in front of each strain code represents sequence type affiliation. Underlined indicates the strains whose genomes were sequenced. Bootstrap values are showed near each clade.

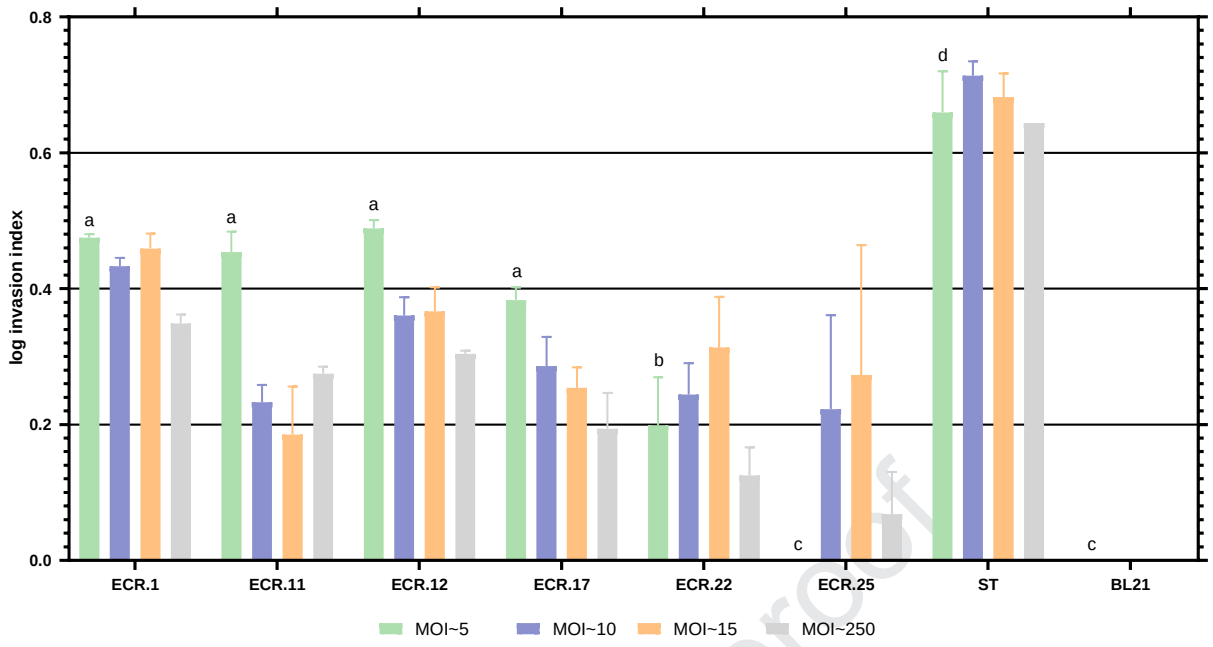
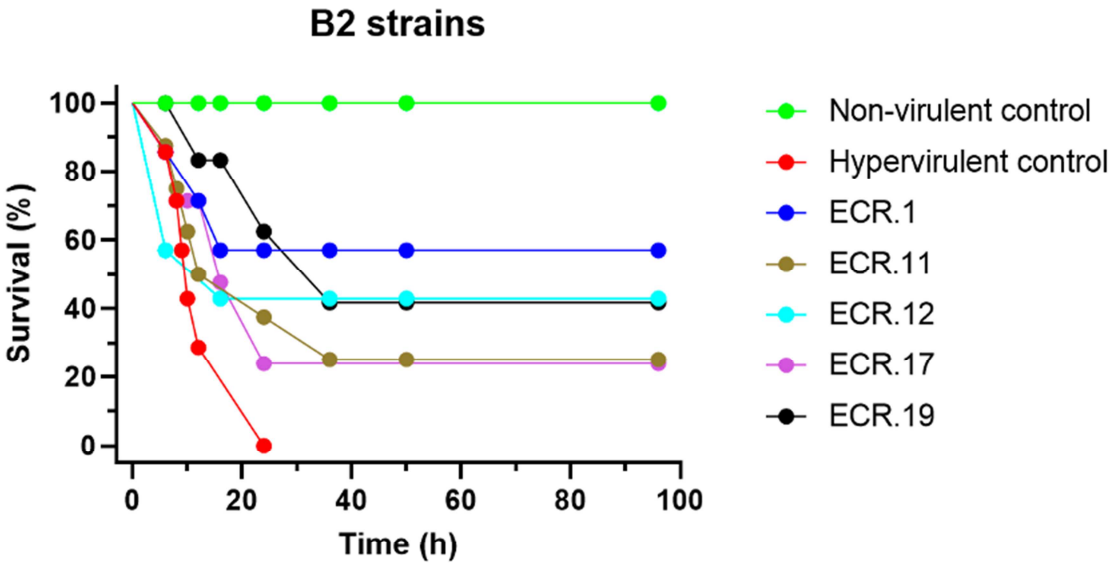
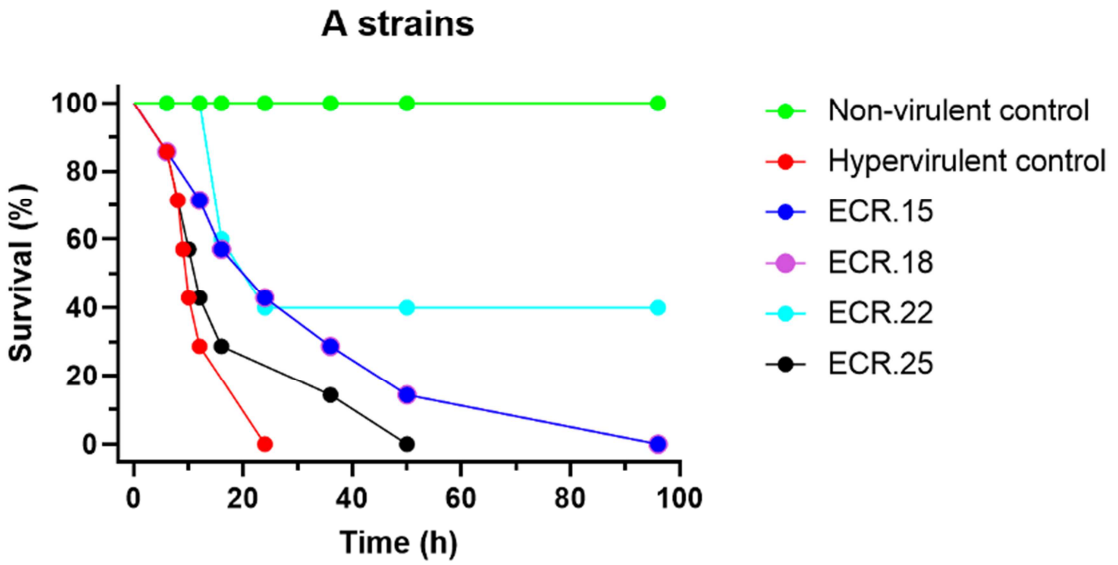



**Figure 2.** Capacity of selected *E. coli* strains to internalize Vero cells by the gentamicin-protection assay. Four assays were conducted with different MOIs (multiplicity of infection). Results are expressed as a logarithmized invasion index. *Salmonella enterica* subsp. *enterica* serovar Typhimurium SC56 (ST) and *E. coli* BL21 were used as positive and negative controls, respectively. Statistical analysis is only presented for MOI~5 (clustered in groups with significant differences – a, b, c, d; Tukey's t-tests, $p<0.05$).


B2 strains
Survival (%)
Time (h)
0
20
40
60
80
100
Non-virulent control
Hypervirulent control
ECR.1
ECR.11
ECR.12
ECR.17
ECR.19


A strains
Survival (%)
Time (h)
0
20
40
60
80
100
Non-virulent control
Hypervirulent control
ECR.15
ECR.18
ECR.22
ECR.25

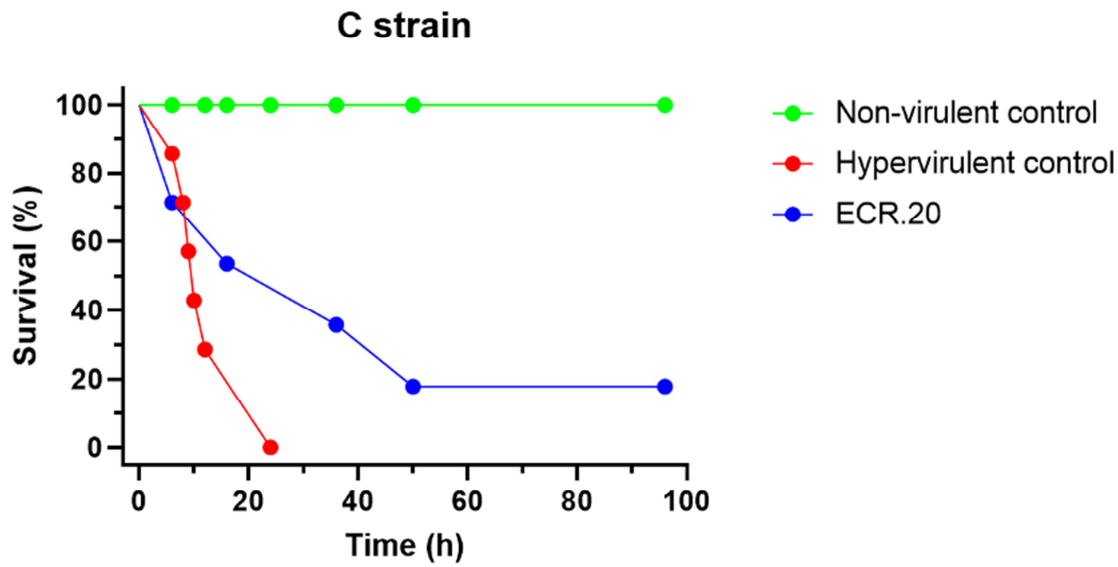

C strain
Survival (%)
Time (h)
0
20
40
60
80
100
Non-virulent control
Hypervirulent control
ECR.20

**Figure 3.** Percentage of survival of *Galleria mellonella* larvae after inoculation with $10^5$ CFUs of different *E. coli* strains over a 96-hour period. *E. coli* BL21 and Neonatal Meningitis-associated *E. coli* (NMEC) strain RS218 were used as negative and positive control, respectively. Test strains were divided according with phylogroup affiliation. For each strain, groups of *G. mellonella* containing ten larvae were evaluated in two independent experiments.

**Highlights**

- CTX-M-encoding E. coli from an UV-treated effluent were studied for human health risk.
- Most strains carried a wide array of antibiotic resistance and virulence genes.
- Transfer of conjugative resistance plasmids was verified in 32% of the strains.
- Tested strains were cytotoxic to Vero cells and pathogenic to *G. mellonella*.
- An *E. coli* ST131 strain persisted in freshwater throughout a microcosms experiment.

**Declaration of interests**

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: