Universidade de Aveiro Departamento de Biologia
Ano 2019

Rafael dos Santos Tavares

Pathogenic and antibiotic-resistant strains of *Escherichia coli* in wastewater effluents treated with UV radiation

Estirpes de *Escherichia coli* patogénicas e resistentes a antibióticos em efluentes de águas residuais tratadas com radiação UV

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Doutora Isabel Henriques, Professora Auxiliar do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra, e da Doutora Marta Tacão, Investigadora do Departamento de Biologia da Universidade de Aveiro

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Humanity needs practical men, who get the most out of their work, and, without forgetting the general good, safeguard their own interests. But humanity also needs dreamers, for whom the disinterested development of an enterprise is so captivating that it becomes impossible for them to devote their care to their own material profit.

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o júri

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Por fim, mesmo tendo já utilizado 400 palavras nesta secção, estou certo que o mais provável é ter-me esquecido de mencionar alguém. É o que acontece quando se é afortunado o suficiente para trabalhar num ambiente tão espectacular como este. A todos aqueles que me ajudaram (até na mais ínfima das coisas), peço desculpas pela omissão e deixo aqui os meus mais sinceros agradecimentos.

palavras-chave

ETARs, resistência a antibióticos, virulência, persitência ambiental, risco

resumo

A resistência a antibióticos compromete o tratamento de infecções bacterianas a nível mundial, sendo reconhecido o papel dos reservatórios clínicos e ambientais na sua disseminação. As Estações de Tratamentos de Águas Residuais (ETAR) são fontes de contaminação dos sistemas aquáticos com bactérias resistentes a antibióticos e genes de resistência. Por esta razão são consideradas nodos importantes onde pode ser contida a transferência de resistência no eixo microbiota humano-ambiental. A desinfecção dos efluentes tratados (e.g. por irradiação com UV-C) é uma estratégia promissora. No entanto, algumas bactérias de relevância clínica sobrevivem à desinfeção, desconhecendo-se as suas características fenotípicas e genotípicas. Neste estudo, caracterizámos estirpes de *Escherichia coli* produtoras de beta-lactamases de espectro alargado isoladas de um efluente tratado com radiação UV-C, com o objetivo de identificar potenciais riscos para a saúde humana associados a estes efluentes.

Vinte e cinco estirpes de *E. coli* resistentes a antibióticos e produtoras de CTX-M foram submetidas a genotipagem por rep-PCR, eletroforese em campo pulsado, sequenciação de múltiplos *loci* e determinação de filogrupos. Genes de resistência a antibióticos (GRAs) e genes de virulência (GV) foram detectados por PCR. Os plasmídeos foram analizados por restrição enzimática e a sua transferência avaliada por ensaios de conjugação. A produção de biofilmes, sideróforos e hemolisinas foi determinada fenotipicamente. Foi também avaliada a citoxicidade e invasão celular, usando células Vero, e a infecção de larvas de *Galleria mellonella*. Os genomas de 6 estirpes foram sequenciados e a sua persistência em água doce foi avaliada em microcosmos.

A análise dos perfis de rep-PCR separou as estirpes em 2 grupos: 1) estirpes do filogrupo B2-sgI (n=7 isolados) e 2) estirpes dos filogrupos A (n=16) e C (n=2). As estirpes foram afiliadas a 8 clones conhecidos: 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) e A:ST1284 (n=3). Dos 18 GRAs investigados por PCR, 9 foram detectados (i.e. *sul1*, *sul2*, *sul3*, *tet(A)*, *tet(B)*, $bla_{\text{OXA-1-like}}$, *aacA4*, *aacA4-cr* and *qnrS1*). Nenhum GV foi identificado por PCR. Uma elevada diversidade de plasmídeos foi observada e foram obtidos transconjugantes resistentes à cefotaxima para 8 estirpes, em dois dos casos manifestando um fenótipo de multirresistência. Todas as estirpes foram classificadas como citotóxicas (9 significativamente mais citotóxicas que o controlo positivo), 10 em 21 estirpes eram invasivas (particularmente estirpes do grupo B2:ST131) e 10 estirpes seleccionadas eram patogénicas para larvas. Vinte e quatro e 7 das 25 estirpes produziram sideróforos e hemolisinas, respectivamente. Aproximadamente 65% das estirpes testadas formavam biofilmes, 11 das quais em duas condições experimentais. A análise dos genomas identificou GRAs adicionais (e.g. catB3, strA, strB) e vários GV codificantes de toxinas, sideróforos e factores de colonização, adesão e invasão. Quatro das 6 estirpes foram detectadas por cultivo e/ou qPCR após 28 dias de incubação em microcosmos, sendo que os seus fenótipos de resistência permaneceram inalterados.

Neste estudo verificou-se que o efluente tratado com radiação UV-C é uma fonte de estirpes de *E. coli* multirresistentes e/ou virulentas, algumas das quais poderão persistir no sistema aquático recetor. Como tal, a água residual desinfetada com radiação UV pode ainda constituir um perigo para a saúde pública e para a eficácia geral dos antibióticos, comprometendo a sua reutilização. Uma avaliação mais detalhada de estirpes isoladas de outros efluentes de ETARs é urgente para que possamos desenvolver novos tratamentos, ou combinações sinérgicas destes, capazes de reduzir a libertação destas bactérias para o ambiente.

keywords

WWTP, antibiotic resistance, virulence, environmental persistence, risk

abstract

Antibiotic resistance jeopardizes the treatment of bacterial infections worldwide, with clinical and environmental compartments being recognized in its pandemic dissemination. Wastewater Treatment Plants (WWTPs) are notorious sources of antibiotic resistance into the aquatic systems and are thus considered a key node for containing the antibiotic resistance dissemination across the human-environmental microbiota axis. In this extent, disinfection of effluents before their discharge (e.g. by UV-C irradiation) is a promising strategy. However, some clinically relevant bacteria have been shown to survive such disinfection steps, though a knowledge gap exists in what regards their phenotypic and genotypic features. In this study we characterized a collection of clinically relevant extended-spectrum beta-lactamase-producing *Escherichia coli* isolated from an WWTP's UV-C-irradiated effluent, aiming to identify putative human health risks associated with such effluents.

Twenty-five strains of antibiotic-resistant, CTX-M-producing *E. coli* were genotyped (rep-PCR, Pulsed-Field Gel Electrophoresis, Multilocus Sequence Typing and Clermont phylogrouping), antibiotic-resistant genes (ARGs) and virulence genes (VGs) were PCR-detected, plasmids were analysed by enzymatic restriction and conjugal transfer was evaluated by mating assays. Biofilm, siderophore and haemolysin production, cytotoxicity and invasion into Vero cells and infection of *Galleria mellonella* larvae were phenotypically assessed. Illumina whole-genome sequencing and evaluation of persistence in freshwater microcosms was performed for 6 selected strains.

Analysis of rep-PCR profiles separated strains into 2 major groups, including strains affiliated either with phylogroup B2-sgI (n=7 isolates) or with phylogroups A (n=16) and C (n=2); and further separated into 8 known 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) and A:ST1284 (n=3). Of 18 PCR-screened ARGs, 9 were detected (i.e. *sul1*, *sul2*, *sul3*, *tet(A)*, *tet(B)*, $bla_{\text{OXA-1-like}}$, *aacA4*, *aacA4-cr* and *qnrS1*). No VGs were identified by PCR. Plasmid restriction indicated high diversity of plasmid profiles among strains and mating assays yielded cefotaxime-resistant transconjugants for 8 strains, two of which displaying a multi-drug resistant (MDR) phenotype. All strains were classified as cytotoxic (9 significantly more cytotoxic than the positive control), 10 of 21 strains were invasive (particularly B2:ST131 strains) and 10 selected isolates were pathogenic to larvae. Twenty-four and 7 of the 25 strains produced siderophores and haemolysins, respectively. Approximately 65% of the tested strains formed biofilms, 11 in two distinct experimental conditions. Genome analysis identified additional ARGs (e.g. *catB3*, *strA*, *strB*) and several VGs encoding toxins, siderophores, and colonizing, adhesion and invasion factors. Four of 6 strains were still detected by cultivation and/or qPCR after 28 days of incubation in freshwater microcosms, and resistance phenotypes remained unaltered.

In this study, we confirmed WWTP's UV-C-treated outflow as an input source of MDR and/or virulent *E. coli* strains, some probably capable of persisting in freshwater, and carrying conjugative antibiotic resistance plasmids. Hence, UV-disinfected wastewater may still represent a risk for human health and antibiotic stewardship, which implies a lack of efficiency of this treatment to remove pathogens from wastewater, compromising water reuse. More detailed evaluation of strains isolated from other wastewater effluents is urgent, in order to design new treatments or establish synergistic combinations that can mitigate the release of such bacteria into the environment.

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Chapter I

General Introduction

I. Antibiotics and antibiotic resistance: two halves of the same coin

Historically, infections have been the leading cause of death and the greatest constrain on human life expectancy since record keeping began and until the mid-twentieth century. In fact, in the early 20th century, diseases such as pneumonia, diarrhea and tuberculosis were the leading cause of death, whereas today, cancer and cardiovascular diseases are the major killers (Figure 1; Ritchie & Roser, 2019). This paradigm shift is the result of the introduction of antibiotics in clinical practice along with improved sanitation and vaccination.

Antibiotics are defined as chemical substances used for the treatment of bacterial infections, and can have either a bacteriostatic effect, inhibiting bacterial growth, or can be bactericidal and lead to cell lysis. However, at therapeutic concentrations they shouldn't be toxic to the host's eukaryotic cells, such as antiseptics, nor non-specific like disinfectants (Singleton & Sainsbury, 1996).

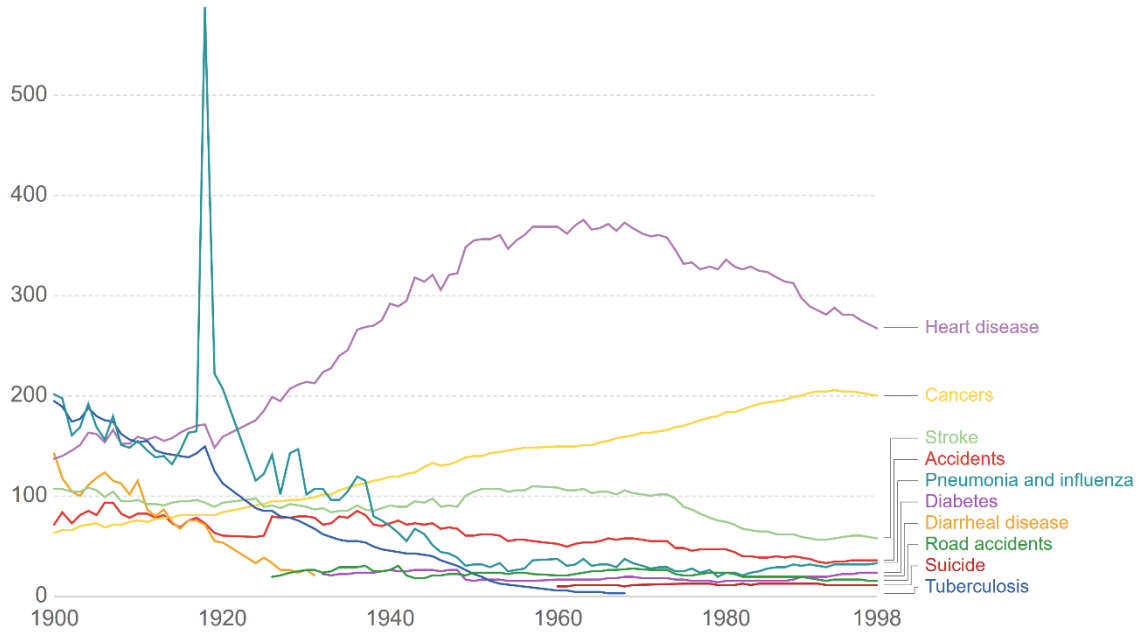


Figure 1. Total mortality rates per cause of death in the USA during the 20th century. Value on YY axis represent the number of deaths per 100,000 population and on XX axis the time frame. The data source is the Center for Disease Control and Prevention (CDC). Adapted from Ritchie & Roser, 2019.

The first antibiotic-like compounds to be discovered were arsphenamines in 1910 and were used to effectively treat syphilis. The strategy used for their discovery became the mould for the screening and development of future antibiotics (Aminov, 2010 and 2017). Sulpha-drugs (or sulphonamides) were later discovered but were the first to be produced in large-scale. However, the discovery of penicillin by Sir Alexander Fleming in 1929 was the true “game changer” for the antibiotic revolution, due to its low toxicity and broad-spectrum. Its introduction in the market was only observed in 1940s, saving thousands of lives both during the World War II as well as after (Aminov, 2010 and 2017; Gaynes, 2017). Many other antibiotics (some of novel classes) were discovered, modified and synthetized in the following decades, with the period between 1950s and 1970s being recognized as the “Golden Age” of antibiotic discovery (Aminov, 2010 and 2017).

Using a chemical structure-based classification, the major groups of antibiotics now available are beta-lactams, fluoroquinolones, tetracyclines, sulphonamides, aminoglycosides and macrolides. Antibiotics can also be classified by their targets/modes of action (Kümmerer *et al.*, 2009). They typically target cellular essential machinery or pathways (Figure 2). Fluoroquinolones inhibit DNA replication by binding to DNA topoisomerases, consequently arresting the replication fork, whereas rifamycins (includes rifampicin) block RNA synthesis by targeting active RNA-polymerases. Protein synthesis is the most generic target of several antibiotics, with the macrolides, amphenicols, lincosamides, streptogramins and oxazolidinones targeting the ribosomal subunit 50S, and tetracyclines and aminoglycosides targeting the 30S subunit. Cell wall synthesis can be disrupted by beta-lactams and glycopeptides (Kohanski *et al.*, 2010; van Bambeke *et al.*, 2017; Figure 2). Sulpha-drugs inhibit the folic acid metabolism, which is essential for DNA synthesis, while polymyxins (lipopeptides), like colistin, destabilize the cellular membrane, leading to cell lysis (van Bambeke *et al.*, 2017; Figure 2).

Although, most antibiotics now in use are synthetic or semi-synthetic formulations, their structure is based on natural compounds produced by members of the environmental microbiota for billions of years, typically to confer an adaptive advantage by inhibition of other microorganisms (Aminov, 2009 and 2017; Waglechner *et al.*, 2019). As such, the existence as well as the development of antibiotic resistance is a natural phenomenon resulting from Darwinian evolution, i.e. exposure of bacteria to natural antibiotics (and later, synthetic ones) selected those who had strategies to survive this stress (acquired by mutagenesis, horizontal genes transfer (HGT) and/or recombination) (Rodríguez-Rojas *et al.*, 2013). Furthermore, antibiotic-producing bacteria need to be intrinsically resistant to the

compound they synthesize or else this feature would result in their death. Interestingly, antibiotic resistance may also represent a signalling strategy in natural bacterial communities (Aminov, 2009). Regardless, the anthropogenic mass-production and use of antibiotics in medicine, aquaculture, agriculture as well as other activities provided an enormous boost in the development and diversification of antibiotic resistance mechanisms (Aminov, 2009; Rodríguez-Rojas *et al.*, 2013). Unfortunately, with the decay of antibiotic discovery pipelines, few antibiotics were introduced into medical practice in the last 50 years (e.g. daptomycin, fidaxomicin and quinupristin-dalfopristin) (Lewis, 2013). In fact, old antibiotics, like colistin and fosfomycin have been re-introduced into medical use, due to lack of option available (Theuretzbacher *et al*., 2015). Quite recently, the U.S. Food and Drug Administration (FDA) approved a new tetracycline (eravacycline) and a new aminoglycoside (plazomicin), which are promising antibiotics for the treatment of complicated intra-abdominal infections and complicated urinary tract infections, respectively (Eljaaly *et al*., 2019; Lee & Burton, 2019). Combinations of antibiotics with inhibitors, like ceftolozane-tazobactam, ceftazidime-avibactam, meropenem-vaborbactam and imipenem-

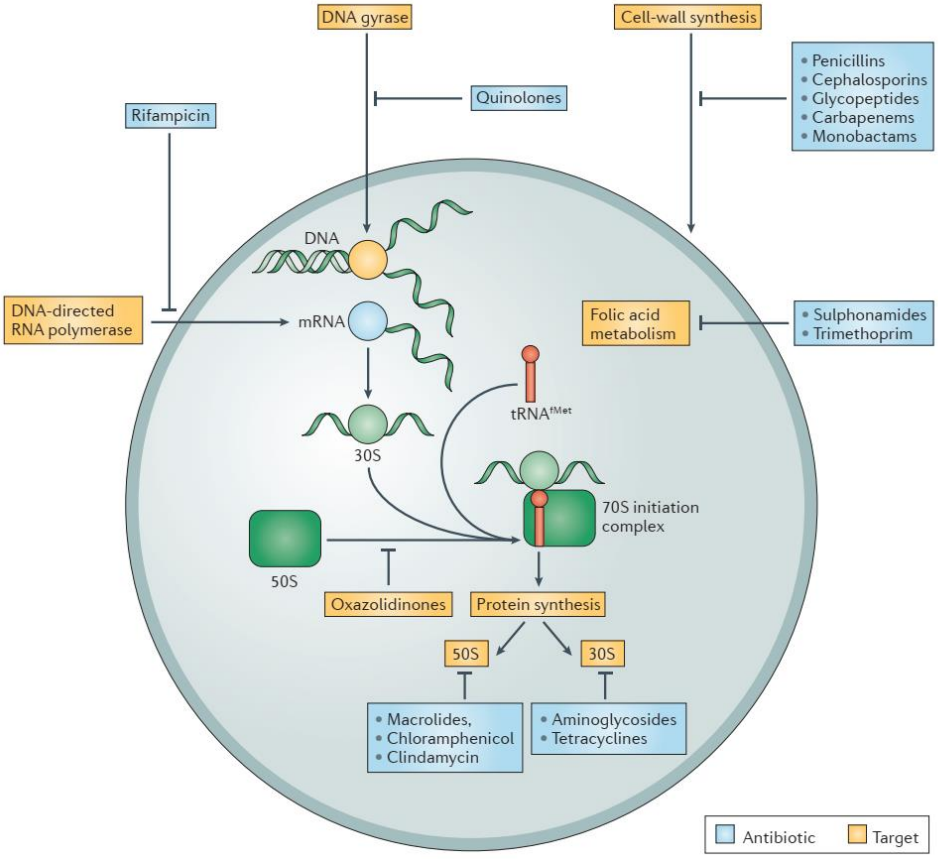


Figure 2. Essential cellular machinery and pathways targeted by the different classes of antibiotics. Schematic adapted from Lewis *et al.*, 2013.

cilastatin-relebactam have also been approved by FDA, displaying relevant activity against key antibiotic resistant pathogens (Leone *et al*., 2019; FDA, www.fda.gov/drugsatfda). However, antibiotic therapeutic options are still limited.

Decreases in the discovery rates of novel antibiotics combined with increasing antibiotic resistance levels presents a serious conundrum. The World Health Organization (WHO) has already coined antibiotic resistance as one of the biggest threats not only to global health but also to food safety and development, and warns about a possible post-antibiotic era – a scenario where simple infections and injuries may result in death (WHO, 2014). In general terms, antibiotic resistance can be defined as the reduction in susceptibility of a bacterial strain to a given antibiotic, which ultimately decreases its effectiveness in the treatment of an infection caused by that same strain. Unfortunately, not only infection treatment is compromised, since several medical procedures, such as surgery, cancer chemotherapy and organ transplants, require the prophylactic use of antibiotics (O'Neill, 2014). By 2014, in Europe and US at least 50,000 deaths occurred as a result of antibiotic resistant infections, and at a global scale more than 700,000 lives were lost per year. But these numbers are small when compared to the 10 million people that are estimated to die from antibiotic-resistant infections by 2050 and the predicted wealth losses in the order of 60-100 trillion US dollars, if no actions are taken (O'Neill, 2014). Currently, there are no doubts of the increasing resistance rates in known pathogens, such as *Acinetobacter*, *Pseudomonas aeruginosa*, staphylococci and enterococci and many members of the *Enterobacteriaceae* family. In fact, the Infectious Diseases Society of America has already alerted for the rise of the ESKAPE pathogens (i.e. *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.), whose features allow them to escape antibiotic therapy (Pendleton *et al.*, 2013). For example, in Slovakia by 2015, resistance level to fluoroquinolones and aminoglycosides in *Klebsiella pneumoniae* was of 70.0% and 66.5%, respectively. In *Acinetobacter* spp., levels of 90.4% and 93.5% have been reported for aminoglycosides (in Lithuania) and carbapenems (in Greece), and vancomycin-resistant *E. faecium* was detected in Ireland with a percentage of 45.8% (ECDC, 2017).

Four main different mechanisms of resistance have been described so far for planktonic bacterial cells: (i) reduced cell permeability, either by having an impermeable barrier to the antibiotic that prevent its entry on the cell (e.g. Gram-negative outer membrane) or by drug active pumping through efflux pumps (that reduce intracellular antibiotic concentrations); (ii) enzymatic antibiotic inactivation by hydrolysis (e.g. beta-

lactamases) or transfer of a chemical group (e.g. aminoglycosides acetyltransferases); (iii) target alteration by mutations in the encoding sequence (e.g. *parC* and *gyrA* point mutations) or by synthesis of enzymes that alter/protect the target (e.g. ribosomal methyltransferases and enzymes of the pentapeptide repeat family encoded by *qnr* genes), thus preventing the binding of the antibiotic; and (v) development of alternate metabolic pathways (Blair *et al.*, 2015; Penesyan *et al.*, 2015; Figure 3). The genes encoding these mechanisms, i.e. antibiotic resistance genes (ARGs), can be located on the bacterial chromosome or in mobile genetic elements (MGE), like transposons and/or plasmids, which can be transferred horizontally between non-related cells (Barbier & Luyt, 2016). Evidently, transferable resistance is the most concerning in terms of antibiotic stewardship. ARGs can easily disseminate since they dictate, in the presence of antibiotic pressure, an increase in bacterial fitness. Major HGT processes are transduction (transfer mediated by bacteriophages), transformation (uptake of naked DNA from the extracellular environment)

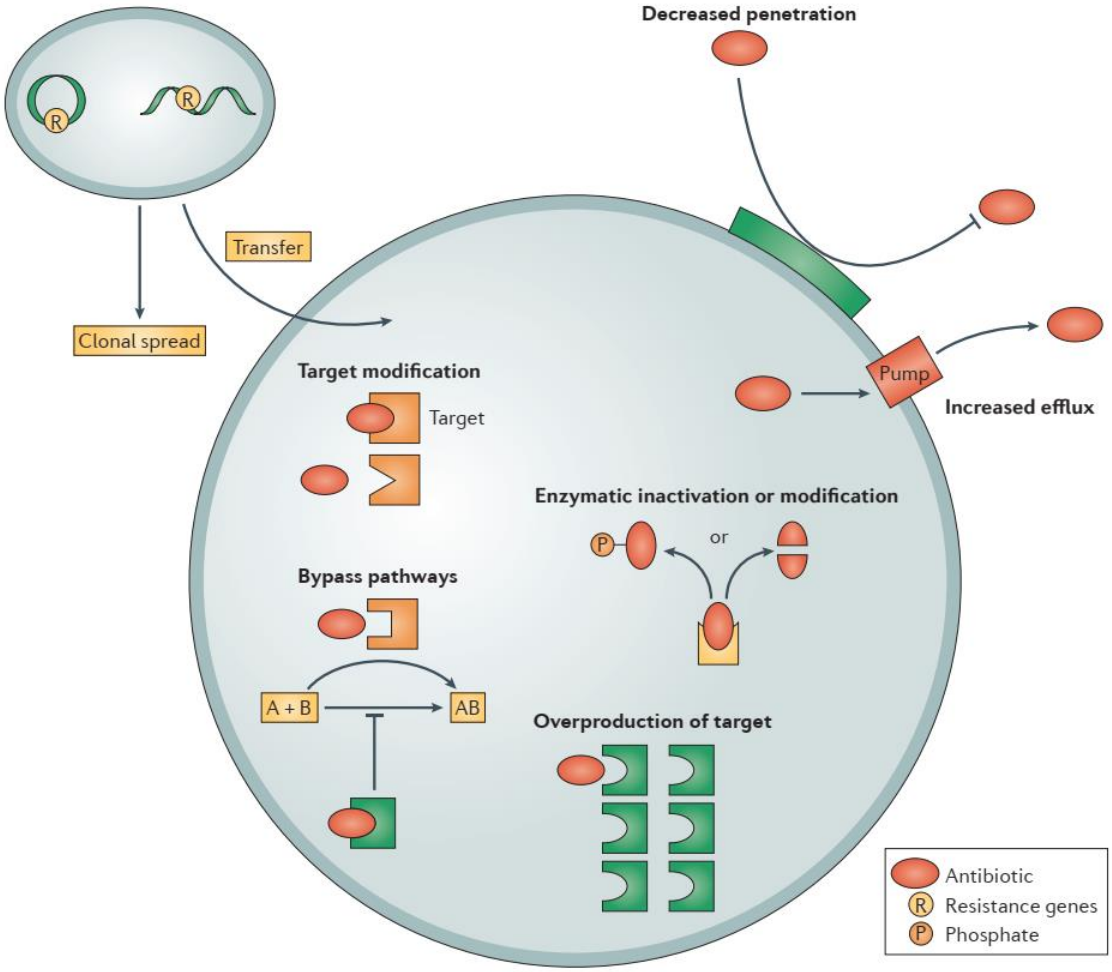


Figure 3. Different antibiotic resistance mechanisms available to the bacterial cell to resist to antibiotic exposure. Schematic adapted from Lewis *et al.*, 2013.

and conjugation (also known as bacterial mating; transfer mediated by plasmids or conjugative transposons) (Darmon & Leach, 2014). Conjugation is of particular relevance, since plasmids are mosaic structures (that assemble other elements such as insertion sequences, integrons and transposons) that often concomitantly carry several ARGs, virulence factors and other genes that enable their maintenance in natural ecosystems (e.g. addiction systems; metal resistance operons) (Bennett, 2008; Carattoli, 2013). Hence, a single transfer event can provide to a bacterial strain resistance to multiple antibiotics and to other compounds, when dealing with a multi-resistance plasmid.

In natural environments, however, bacteria commonly switch from a planktonic to a sessile lifestyle by forming biofilms, which are basically bacterial cells enclosed on a polysaccharide matrix. As part of these structures, bacteria may display resistances to antibiotics 100- to 1000-fold higher than when in a planktonic state (Olsen, 2015). This is related with a multiplicity of factors, such as difficulty of antibiotics in penetrating the biofilm's matrix and slow or altered cell metabolism (Olsen, 2015; Penesyan *et al.*, 2015). Biofilm's higher level of resistance is relevant not only in an environmental framework but also in terms of treating biofilm-forming persistent infections.

Antibiotic resistance dissemination has already made some groups of antibiotics nearly obsolete, however one group that continues to uphold an extreme importance in healthcare treatment and continues to save countless lives are the beta-lactams.

II. Beta-lactams and beta-lactamases: a raging war

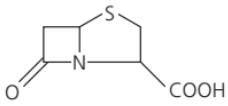
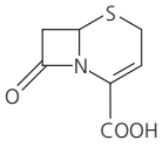
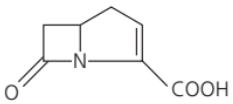
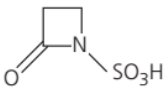
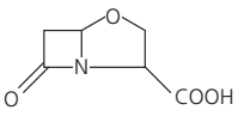
A vital piece of our antibiotic armamentarium is the beta-lactams group of antibiotics, which encompass not only first-line antibiotics but also last-resort drugs. The fact that they have broad-spectrum activity, high therapeutic effectiveness, low toxicity, high diversity of compounds available and usually low prices made them key therapeutic options, reason why they represent in most countries more than 50% of antibiotic consumption (Adler *et al.*, 2016; Courvalin *et al.*, 2010; ECDC, 2015; Resistance map, https://resistancemap.cddep.org/index.php). In fact, consumption of broad-spectrum penicillins and cephalosporins alone represented 55% of the overall antibiotics consumed in 2010 (data from 71 countries; Van Boeckel *et al.*, 2014).

The cellular target of beta-lactams is cell wall synthesis. The cell wall is vital for bacterial survival in structural, defensive and osmotic terms. The major component of this structure is peptidoglycan and its final assembly step (i.e. cross-linking) relies on a transpeptidation reaction catalysed by proteins known as penicillin-binding proteins (PBPs). All beta-lactams have a cyclic backbone known as the beta-lactam ring (Table 1), fundamental to their activity, since it irreversibly binds to the PBPs (neutralizing this enzyme by acylation of a serine in its active site) and thus prevents the cross-linking of the peptidoglycan sidechains. Since there is a homeostatic equilibrium between cell wall degradation and synthesis in an active bacterial cell, blocking of the later by these antibiotics leads to cell lysis (Bonomo, 2017; Bush *et al*., 2018; Kohanski *et al.*, 2010).

This diverse group of antibiotics is divided into penicillins (i.e. penams), cephalosporins (i.e. cephams), carbapenems, monobactams and clavams according with their chemical structure (van Bambeke *et al.*, 2017; Table 1). While penicillins are often used as first-line antibiotics, cephalosporins are more robust antibiotics mostly used for the treatment of more serious infections, though in some cases are also used as first-line antibiotics. Carbapenems are currently last resort drugs used for the treatment of multidrug resistant infections, with their administration restricted to hospital settings in some countries of the European Union. Though most beta-lactams exhibit a broad-spectrum of activity, more recent drugs, such as the later generations of cephalosporins, were designed to retain activity towards more specific bacterial groups, some of which already non-susceptible to key antimicrobial compounds. For example, while 1st generation cephalosporins are

significantly relevant for the treatment of Gram-positive bacteria and 3rd generation cephalosporins have reinforced activity towards Gram-negative bacteria, ceftobiprole (a 5th generation cephalosporin) has been specially developed to tackle methicillin-resistant *Staphylococcus aureus* (Fernandes *et al.*, 2013).

Table 1. Beta-lactam antibiotics' classification and corresponding features with relevant examples. Adapted from van Bambeke *et al*., 2017.

Beta-lactam group	General structure	Brief description	Examples
Penams or penicillins		Include 5 generation of penicillins with narrow- and broad-spectrum of activity, some resistant to beta-lactamase activity.	Penicillin G, methicillin, ampicillin, ticarcillin and piperacillin.
Cephems or cephalosporins		Include 5 generations of cephalosporins. Later generations, such as 3rd generation cephalosporins, have reinforced activity towards gram negative bacteria.	Cephalothin, cefuroxime, cefotaxime, cefepime and ceftolozane.
Carbapenems		Extended spectrum compounds used nowadays as last-resort drugs for the treatment of multi-drug resistant infections.	Meropenem, ertapenem, doripenem and imipenem.
Monobactams		Only active against Gram-negative bacteria, but resistant to beta-lactamases.	Aztreonam and tigemonam.
Clavams		Are considered weak antibiotics and are used in clinical practice as beta-lactamase inhibitors.	Clavulanic acid.

Resistance to beta-lactams was described even before the introduction of penicillin in the market in 1943 (Abraham & Chain, 1940). The most infamous mechanisms of resistance to these compounds are (i) the production of mutant PBPs with reduced binding affinity for the beta-lactams, particularly notorious in *S. aureus*, or (ii) the production of beta-lactamases, enzymes that bind and hydrolyse the beta-lactam ring, inactivating the beta-lactam antibiotics (Fernandes *et al.*, 2013; van Bambeke *et al.*, 2017). Porin loss and/or active efflux systems are also relevant for resistance of Gram-negative bacteria to beta-

lactams, such as is in the case of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Fernandes *et al.*, 2013; Thomson & Bonomo, 2005; van Bambeke *et al.*, 2017).

Beta-lactamases had their origin more than two billion years ago and are currently regarded as the major resistance mechanism to beta-lactams in Gram-negative bacteria (Bush, 2018; Hall & Barlow, 2004). Nowadays, there are 4,453 different entries in the Beta-Lactamase Database (BLDB, http://www.bldb.eu/, accessed in 01/10/2019; Naas *et al.*, 2017), and these enzymes are separated into 4 classes (A, B C and D) according to the Ambler's structural classification scheme (Table 2). Class B includes the metallo-beta-lactamases (require at least one metal ion, frequently zinc, for activity), whereas class A, C and D encompass the serine-beta-lactamases (possess a serine in their active site) (Bush & Jacoby, 2010). A functional classification scheme was proposed by Bush and colleagues and divides beta-lactamases into 4 classes (and 17 subgroups) accordingly with hydrolytic activity and inhibition profiles. In brief, group 1 encompasses the AmpC enzymes, group 2 includes a plethora of enzyme families, such as TEM, SHV, CTX-M, the only serine-beta-lactamases with carbapenemase activity (e.g. KPC) and all the oxacillinases (i.e. OXA-type), while group 3 includes the metallo-enzymes with carbapenemase activity (e.g. IMP and NDM) (Table 2; Bush & Jacoby, 2010).

Cephalosporins, particularly 3rd generation cephalosporins, are among the most potent antibiotics still in play in medical practice, but their efficacy has been largely impaired by the colossal spread of extended-spectrum beta-lactamases (ESBLs). ESBLs are enzymes capable of hydrolysing penicillins, 3rd and 4th generation cephalosporins, and monobactams, but not cephamycins nor carbapenems, while also retaining susceptibility to inhibitors (Fernandes *et al.*, 2013; ur Rahman *et al.*, 2018; Table 2). They are often plasmid-born, which in part explains their enormous dissemination success, particularly in *Enterobacteriaceae*. Nonetheless, association with widespread bacterial clones also plays a pivotal role in their dissemination (ur Rahman *et al.*, 2018). Historically speaking, initial ESBLs were derived from already prevalent broad-spectrum beta-lactamases, i.e. TEM-1 and SHV-1, by point mutation (due to antibiotic selective pressure), thus acquiring a broader hydrolytic profile (ur Rahman *et al.*, 2018). However, TEM and SHV ESBLs were later overshadowed in terms of predominance by enzymes naturally occurring in environmental bacteria (e.g. *Kluyvera* spp.) and that were selected by antibiotic selective pressure – the CTX-M family. All the members of this family are ESBLs, and variants such as CTX-M-15 have disseminated successfully over the globe, mostly due to their association with insertion sequences (e.g. IS*CR1* and IS*Ecp1*), integrons, transposons, promiscuous conjugative

plasmids (often concomitantly carrying other resistance determinants, which can lead to co-selection of *bla*$_{CTX-M}$ genes) and highly successful clones (e.g. *E. coli* ST131) (reviewed by Fernandes *et al.*, 2013; Cantón *et al*., 2012; ur Rahman *et al.*, 2018). The spread of ESBL encoding genes resulted in a reduction in the antibiotic arsenal available for treatment of these infections, which led to increasing reliance on carbapenems (between 2000 and 2010, carbapenem consumption increased by 40% (data from 71 countries; Van Boeckel *et al*., 2014)). This was logically accompanied by increasing carbapenem resistance levels, namely by the spread of genes encoding beta-lactamases with carbapenemase activity (e.g. KPC and NDM) (Bush, 2018). In fact, the WHO, in its priority pathogen list, has already indicated ESBL-producing and carbapenem resistant-*Enterobacteriaceae* as critical bacterial targets in terms of need for research and development of new antibiotics (WHO, https://www.who.int/). The Centers for Disease Control and Prevention (CDC) has also allocated ESBL-producing *Enterobacteriaceae* at a hazard level of serious in the USA and estimates mortality increases by 57% and wealth losses of 40,000 US dollars in bloodstream infections due to ESBL carriage by 2013 (CDC, 2013). Beta-lactams efficiency will in the future likely rely on beta-lactamase inhibitors, such as the new avibactam (can inhibit serine-carbapenemases) or in the development of novel synthetic compounds, though resistance emergence is inevitable (Bush & Macielag, 2010; Bush, 2018).

Henceforth, surveillance of ESBL-producing bacteria in environmental and clinical settings is vital, especially to understand their dissemination pathways and create mitigation strategies.

Table 2. Beta-lactamases classification schemes and characteristics of each group as well as relevant examples. Adapted from Bush & Jacoby, 2010.

Structural classification	Number of entries in BLDB[1]	Functional classification	Brief description	Inhibited by clavulanic acid or tazobactam	Examples
A	1416	2a	Penicillinases inhibited by clavulanic acid.	+	PC1
		2b	Broad-spectrum (both penicillinases and cephalosporinases).	+	TEM-1, SHV-1
		2be	Extended-spectrum cephalosporinases. Activity towards monobactams.	+	TEM-3, SHV-2, all CTX-M
		2br	Penicillinases resistant to beta-lactamase inhibitors.	-	TEM-30, SHV-10
		2ber	Extended-spectrum cephalosporinases with activity towards monobactams and resistant to inhibitors.	-	TEM-50
		2c	Carbenicillinases.	+	PSE-1
		2ce	Carbenicillinases with reinforced activity towards cefepime and cefpirome.	+	RTG-4
		2e	Cephalosporinases inhibited by clavulanic acid.	+	CepA
		2f	Serine-beta-lactamases with carbapenemase activity.	±	KPC-2, IMI-1
B	635	3a	Metallo-beta-lactamases with broad-spectrum activity (including carbapenems but not monobactams).	-	IMP-1, VIM-1
		3b	Metallo-beta-lactamases with preferential carbapenem hydrolysis.	-	CphA
C	1360	1	Cephalosporinases not inhibited by clavulanic acid and capable of cephamycin's hydrolysis.	-	AmpCs
		1e	Cephalosporinases with reinforced activity towards ceftazidime and oxymino-beta-lactams.	-	CMY-37
D	933	2d	Oxacillinases.	±	OXA-1
		2de	Oxacillinases with extended-spectrum activity.	±	OXA-11
		2df	Oxacillinases with carbapenemase activity.	±	OXA-23

[1]Data obtained at 01/10/2019 from BLDB (http://www.bldb.eu/).

III. Wastewater and Wastewater Treatment Plants: the "true" Pandora's box

Water is a basic right to every human being, and non-contaminated drinking water should be accessible to everyone. However, poor management of industrial, agricultural and urban wastewater discharges has compromised the supply of many water sources. Nowadays, at least 2 billion people are forced to use faecal-contaminated drinking water worldwide, which results in diseases like diarrhea, that cause 485,000 deaths per year (WHO, https://www.who.int/news-room/fact-sheets/detail/drinking-water). This situation is only aggravated by population growth and climate change. In fact, in only a matter of 6 years, it is estimated that 50% of the world's population will live in water-stressed regions (WHO, https://www.who.int/news-room/fact-sheets/detail/drinking-water). Therefore, efficient treatment of wastewater is of paramount importance to circumvent water scarcity in the future.

Wastewater Treatment Plants (WWTPs) are facilities designed to remove pollutants (most of anthropogenic provenance) from water. They basically collect wastewater from the sewage systems and apply several treatments that reduce the environmental and public health related hazardous effects of the water, before reuse or discharge into the environment. Most treatments applied (which can be physical, chemical or biological) are based on accelerated natural processes of water purification (EPA, 2004). A wide array of pollutants reach WWTPs, those being: (i) nutrients, such as elevated levels of phosphorus and nitrogen, that if released into the aquatic environment will promote eutrophication and direct toxic effects on wildlife; (ii) oxygen-demanding substances, like ammonia, which reduce water's dissolve oxygen and thus represent a risk for aquatic life (heat also reduces the water's oxygen retention capacity, and so must also be controlled in these facilities); (iii) a plethora of inorganic and synthetic compounds (e.g. heavy metals, detergents, pharmaceutical compounds and pesticides); and biological contaminants, such as antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) (EPA, 2004; Karkman *et al*., 2018; Tran *et al*., 2018).

In simple terms, conventional wastewater processes, which are the minimal requirements for water decontamination, comprise three levels: preliminary, primary and secondary. Primary treatment aims at removing floating debris and solid materials present in wastewater by physical processes, whilst preliminary treatments remove the bulkiest

solids or other material that may damage the subsequent steps in wastewater processing. This is usually accomplished by filtration using bar screens followed by sedimentation of suspended solids in settling tanks. The secondary treatment promotes the removal of nutrients by biological methods, being the most common, activated sludge. In this step, the microbial communities already present in the wastewater are used to remove about 90% of the organic matter present either in aerobic or anaerobic conditions (EPA, 2004; Manaia *et al*., 2018; Michael *et al*., 2013). Lagoons or treatment ponds, land treatment and constructed wetlands are much more natural ways to treat wastewater, though they are mostly used in smaller settings (EPA, 2004). Advanced treatments may be applied in accordance with specific necessities of each WWTP. They can be simple extensions of conventional secondary treatment, to further remove nitrogen and phosphorus content, or can encompass flocculation, membrane filtration, ion exchange, reverse osmosis and/or adsorption steps. A disinfection treatment can also be applied to further remove pathogens (e.g. bacteria and virus) from the wastewater, being the most conventional: chlorination, ozonation and UV-C irradiation (EPA, 2004; Michael *et al*., 2013).

The wastewater compartment and WWTPs have been recognized as a relevant source of ARGs and ARB into the environment (Barancheshme & Munir, 2019; Karkman *et al*., 2018). In fact, these systems not only concentrate high bacterial densities (and thus close proximity between cells) with bacteria of several proveniences (i.e. of animal, human, clinical or environmental sources), but also provide plentiful carbon sources and nutrients, stable pH and temperature that favours bacterial multiplication. Besides, they receive numerous contaminants, such as metals and pharmaceutical compounds, which can exert a selective pressure in the wastewater bacterial community. As such, WWTPs constitute bioreactors that support lateral and *de novo* acquisition of ARGs or selection of resistance mechanisms (Manaia *et al*., 2016 and 2018; Rizzo *et al*., 2013; Figure 4). Considering their central part in the dissemination of resistance in the human-environmental microbiota axis they are potential key points for controlling ARGs and ARB release into the environment (Figure 4).

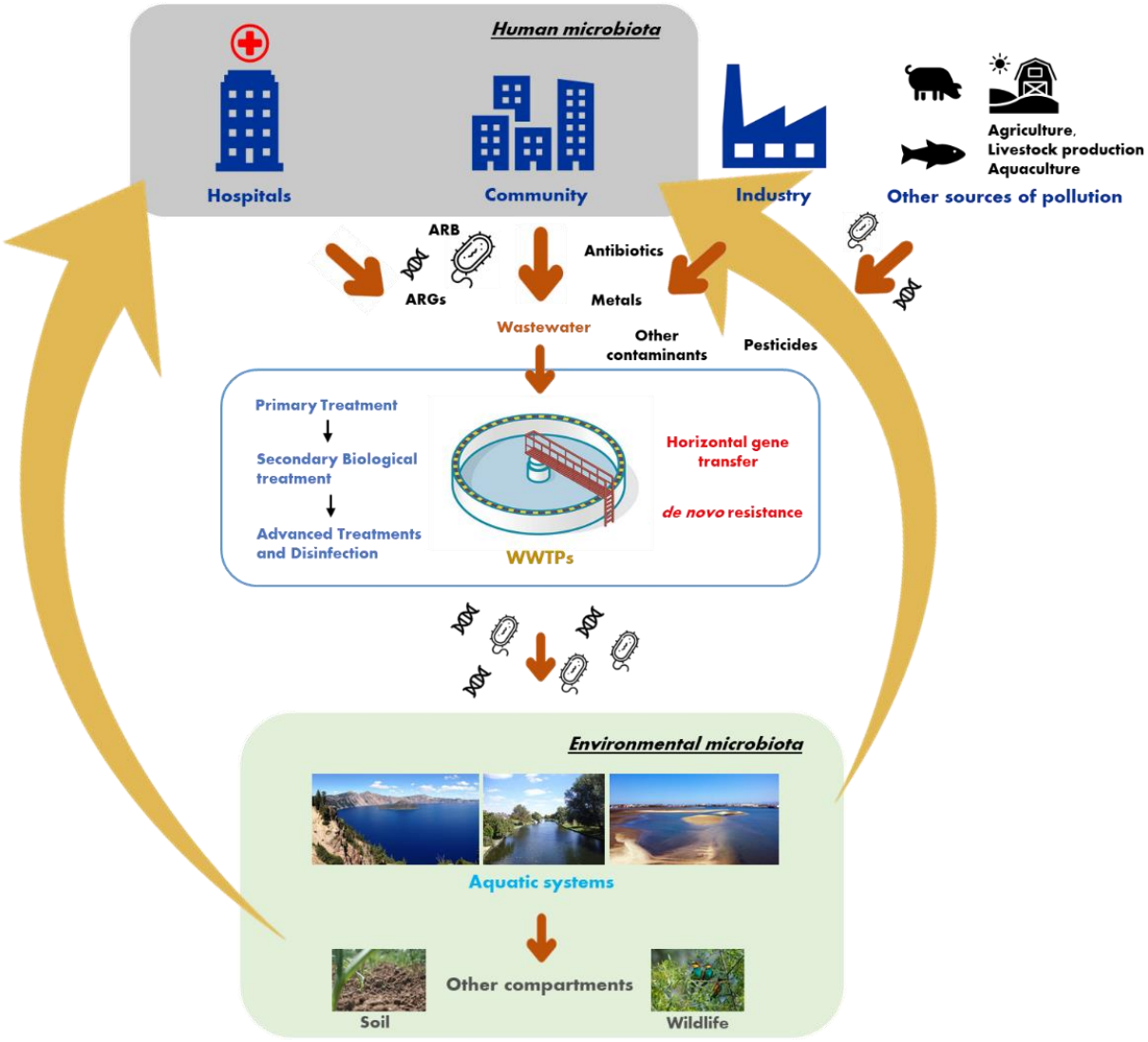


Figure 4. Schematic representation of the antibiotic resistance dissemination from the human microbiota, through domestic, hospital and animal production effluents, into WWTPs and then into the environmental microbiota. WWTPs bring together high bacterial densities, ARGs, ARBs and selective pressures (i.e. contaminants released in industrial, animal production, hospital and community waste) that promote the development and propagation of resistance (i.e. horizontal gene transfer and *de novo* resistance) (Manaia *et al*., 2016 and 2018; Rizzo *et al*., 2013). Aquatic environments provide the ideal distribution system of antibiotic resistance to other compartments, such as soil (e.g. by irrigation with wastewater) and wildlife (e.g. through the food-chain). Through any of these contaminated compartments, antibiotic resistance can re-enter into the human microbiota, either by drinking, consumption of food-products, exposure to aerosols, recreational activities and others (Davies & Davies, 2010; Vaz-Moreira *et al*., 2014). Scheme adapted from Davies & Davies, 2010 and Vaz-Moreira *et al*., 2014.

Considerable amounts of ARGs and ARB have been detected in the final effluent of WWTPs, which indicates that treatments applied cannot remove entirely these contaminants. However, their levels are reduced (Barancheshme & Munir, 2019; Karkman *et al*., 2018; Pärnänen *et al*., 2019) and conventional treatment usually dictates removals of 2-3 log units of the wastewater microbial content (Manaia *et al*., 2016). Secondary treatment alone can significantly reduce bacterial loads due to bacterial aggregation followed by precipitation in secondary settling tanks (Manaia *et al*., 2018), although reduction in nutrient and carbon availability of the wastewater, and competition between member of the microbiota may also explain some of this reduction. Still, the wastewater effluent treated with activated sludge may still represent an input of 10^{12} ARB and 10^{18} ARGs per day into the environment, depending on the size and operational conditions of the WWTPs (Manaia *et al*., 2018). Henceforth, only advanced treatments, such as disinfections steps, will enable to ameliorate the wastewater quality in terms of antibiotic resistance before discharge. Frequently detected ARGs in both WWTPs and their effluents are bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} (resistance to beta-lactams), varied *tet* genes (resistance to tetracyclines), *sul1* and *sul2* (resistance to sulphonamides), *qnrA*, *qnrB* and *qnrS* (resistance to fluoroquinolones), *erm*B and *erm*F (resistance to macrolides) and *str*A and *str*B (resistance to aminoglycosides) (Gatica *et al*., 2015). From large metagenomic surveys, ARGs encoding resistance to tetracyclines, macrolides, aminoglycosides, beta-lactams and sulphonamides were the most abundant in untreated sewage worldwide (Hendriksen *et al*., 2019) and aminoglycosides, multi-drug resistance, sulphonamides and beta-lactams in treated effluents across Europe (Pärnänen *et al*., 2019).

Chlorination is the most common disinfection step applied at WWTPs, however UV-C disinfection and ozonation have gained popularity due to their low hazardous effects to the environment. In fact, broad germicidal activity, short contact times and no residuals release are strong advantages to UV irradiation and the reason why some WWTPs have switched to this advanced treatment, though all treatments have constraints (Bouki *et al*., 2013; Manaia *et al*., 2016). UV kills by inducing DNA damage that leads to loss of reproducibility of the microorganisms (depending on the dose). This also means, that this treatment may impart direct damage on ARGs. Regardless, DNA repair mechanisms, such as photoreactivation or dark repair may neutralize or mitigate these deleterious effects (Rizzo *et al.*, 2013).

Generally speaking, in UV-treated WWTP's outflows the total number of bacteria are significantly decreased, though inconsistencies in ARB removal have been reported, which

is likely related to UV tolerance by these bacteria (Guo *et al*., 2013a,b; Huang *et al*., 2016; Silva *et al*., 2018; Sousa *et al*., 2017; Zhang *et al*., 2009). For ARGs, removal efficiencies may not be as high, in some cases with no significant reductions of the ARG content. At low UV doses, reductions in the order of 52.0-73.5% for five *tet* genes were observed (Zheng *et al*., 2017), a result corroborated by the efficient removal of erythromycin and tetracycline resistance genes observed by Guo and colleagues (Guo *et al*., 2013b). On the other hand, Jäger *et al*. reported removal percentages typically below 50%, with the abundance of bla_{TEM} and $bla_{CTX\text{-}M\text{-}32}$ increasing by 50.1 and 236.3%, respectively (Jäger *et al*., 2018). Other studies showed that the UV disinfection treatments imparted no significant changes (i.e. reduction) on ARG levels (Auerbach *et al*., 2007; Di Cesare *et al*., 2016; Munir *et al*., 2011). These inconsistencies may not only be related to the ARG type and WWTP's operational conditions but also to methodological limitations (e.g. in qPCR). In metagenomic analysis, enteric bacteria seem to be removed by UV-C treatment, though the level of some pathogens (e.g. *Legionella* and *Leptospira*) increase (Numberger *et al*., 2019). Furthermore, significant recovery of the bacterial communities (particularly ARG levels) has been described after 3 days storage at room temperature of UV-disinfected wastewater, which may also undermine treatment efficiencies (Sousa *et al*., 2017).

In fact, many factors do influence wastewater treatment, starting with the composition of the microbial community in the WWTP's influent (reason why studies should provide a more comprehensive analysis of bacterial community shifts and not only of ARGs and ARB abundance; examples of elegant studies are: Narciso-da-Rocha *et al*., 2018 and Numberger *et al*., 2019) and a series of physical and chemical properties of the wastewater (including the contaminants present). To exemplify this variability, studies have shown statistical differences in ARG loads in sewage between high and low-income countries, which correlates with health, environmental and socio-economic factors, and it has also been described a gradient of ARG concentrations in European wastewater (congruent with antibiotic resistance prevalence described in clinical settings), with differences being highlighted between countries with high or low antibiotic consumption (Hendriksen *et al*., 2019; Pärnänen *et al*., 2019). As such, treatments applied should be tailored to each WWTP according with wastewater characteristics, in a case-by-case approach (Manaia *et al*., 2016 and 2018). Furthermore, owing to the variability observed, evaluation of ARGs and ARB removal in each WWTP should be routinely performed, in order to confirm and optimize treatments efficiencies. Although, a significant body of literature has attempted to described the removal efficiency of several wastewater treatments, few efforts were placed in characterizing strains that survived treatment and were released to the environment, which

is vital since it's not likely that any treatment will ever provide a complete removal of these contaminants. Furthermore, considering carriage of ESBLs has been identified in WWTPs with an increasing in prevalence after treatment (Bréchet *et al*., 2014; Silva *et al.*, 2018), it is extremely important to characterize the pathogenicity, environmental persistence and other traits that may potentiate the risk ESBL-producers could pose for human health. Unfortunately, most studies focus on characterizing either antibiotic resistance determinants (phenotype and genotype) or virulence factors (Alouache *et al*., 2014; Diallo *et al*., 2013; Figueira *et al*., 2011; Harnisz & Korzeniewska, 2018; Ojer-Usoz *et al*., 2014; Osińska *et al*., 2017), without providing a comprehensive analysis of the strain's features.

Notwithstanding, it is important to highlight that inefficient wastewater treatment poses risks not only for human health, but also constitutes an environmental problem. Many pharmaceutical compounds (e.g. triclosan, nonsteroidal anti-inflammatory and antiepileptic drugs) are recalcitrant to wastewater treatment and/or suffer biomagnification in downstream ecosystem's food chains, thus possibly exerting toxicity (includes responses such as death or impairment of reproductive function) in wildlife upon chronic exposure (Akpor & Muchie, 2011; Martín *et al*., 2012; Tran *et al*., 2018). Additionally, pharmaceutical drugs (i.e. antibiotics and non-antibiotics; e.g. carbamazepine) and metals released in the final effluent (Karvelas *et al*., 2003; Tran *et a*l., 2018) may also impose a selective pressure towards (or indirectly facilitate the dissemination of) antibiotic resistance in natural bacterial communities (Maier *et al*., 2018; Sandegren *et al*., 2014; Wang *et al*., Zhang *et al*., 2018), compromising antibiotic stewardship.

Assessment of the wastewater quality after WWTP's treatment, particularly in microbiological standards, is directly related with the WHO's 2030 Agenda for Sustainable Development, namely in ensuring availability and sustainable management of water and sanitation worldwide (goal 6). It is also framed in goal 3, since it indirectly safeguards well-being and health of the world's population (WHO, https://sustainabledevelopment.un.org/post2015/transformingourworld). Moreover, the need for surveillance, evaluation of ARB and ARGs removal and characterization of persistent strains is reinforced by the (i) increasing use of treated wastewater for irrigation of agricultural fields; (ii) use of untreated wastewater for animal feeding in aquaculture; and (iii) use of sewage sludge as fertilizer, since these applications provide pathways for ARB to reach and infect humans (i.e. through contamination of soil, water or food-products) or simply a way for ARGs to disseminate (Manaia *et al*., 2016; WHO, https://www.who.int/news-room/fact-sheets/detail/drinking-water). Lastly, with climate

change and more uncertainty regarding water availability, treated wastewater may need to be recycled into drinking water (a practice already established in some countries; Akpor & Muchie, 2011), hence the urgent need to optimize wastewater treatment.

IV. *Escherichia coli* and the golden trio: how antibiotic resistance, virulence and survival capacity can go seriously wrong

The genus *Escherichia* is composed by Gram-negative bacteria that belong to the class Gammaproteobacteria, more specifically to the family *Enterobacteriaceae* (order *Enterobacteriales*), a family which encompasses a plethora of clinically relevant pathogens (e.g. *Salmonella* and *Klebsiella*). It was first described by Theodore von Escherich in 1885, when he discovered *Escherichia coli* (hence the genus name: *Escherichia*), and consist of non-sporing facultative anaerobes whose cells are rod-shaped (Rogers *et al*., 2016; Scheutz & Strockbine, 2015; LPSN, http://www.bacterio.net). There are currently eight validly described species in this genus (*E. coli*, *E. blattae*, *E. adecarboxylata*, *E. albertii*, *E. fergusonii*, *E. hermannii*, *E. marmotae* and *E. vulneris*) (LPSN, http://www.bacterio.net). *E. coli* is by far the most studied bacterial model in microbiology, having been extensively characterized for over 70 years, as indicated by the increasing mentioning in scientific publications over the years, cumulatively referred in 381,627 publications in PubMed (Figure 5; LPSN, http://www.bacterio.net). *E. coli* cells have 1.1-1.5 μm in diameter and 2.0-6.0 μm in length, possess motility through peritrichous flagella and grow optimally at 37ºC. They are naturally found in the gut of warm-blooded animals (reason why it's used as indicator of faecal contamination), where they assist in the breakdown of some substances during digestion, prevent colonization by pathogens and produce vitamin K_2, although they are also present in the environment, such as soil and water (Rogers *et al*., 2016; Scheutz & Strockbine, 2015). In healthy individuals, the average number of *E. coli* cells is in the order of 10^7 CFU/g of faeces (Smati *et al*., 2013), though they represent less than 0.1% of the total intestinal microbiota (Eckburg *et al*., 2005).

Paradoxically, not all *E. coli* are commensal, with pathogenic strains having been implicated in diarrheal and extraintestinal diseases (e.g. urinary tract infection, pneumonia, septicaemia and meningitis). These two distinct lifestyles are enabled by a pool of genes present in MGE (and thus transferable), many of which encoding virulence factors (VFs) - sustain the basis for pathogenicity. In fact, the genome size of pathogenic and commensal strains may be up to 1 Mbp different (Croxen *et al*., 2013). According with the symptomatology of the disease caused, pathogenic strains are divided into 2 major groups:

Intestinal Pathogenic *E. coli* (IPEC) and Extraintestinal Pathogenic *E. coli* (ExPEC) (Robins-Browne *et al*., 2016).

IPEC comprise 7 pathotypes (i.e. groups of strains that provoke the same pathology): Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC), Enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Diffusely-adherent *E. coli* (DAEC) and Adherent-invasive *E. coli* (AIEC) - which are often neatly distinguishable by PCR-based screening of specific virulence genes (Robins-Browne *et al*., 2016; Table 3).

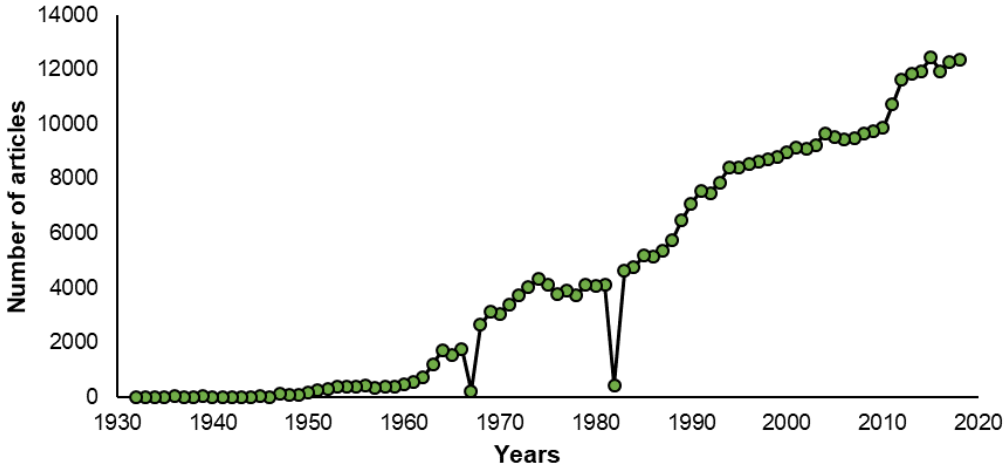


Figure 5. Number of publications available in PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) mentioning the term "*E. coli*" (at any point in the publication) from 1930 to 2018.

EPEC adhere and promote the destruction of the intestinal microvilli provoking distinctive attaching and effacing lesions. The machinery required for inducing such damage is encoded in the locus of enterocyte effacement (LEE; a pathogenicity island), where is found the *eae* gene (encode the adhesion protein intimin). They are further divided according with the presence of a plasmid-born adherence factor, i.e. the bundle-forming pilus (encoded by *bfpA*) – typical EPEC possess both *eae* and *bfpA*, while atypical EPEC only harbour *eae* (Croxen *et al*., 2013; Robins-Browne *et al*., 2016). EIEC are intracellular pathogens (include all strains of the genus *Shigella*), mostly non-motile and whose invasive arsenal is encoded in the invasive plasmid pINV (responsible for penetration, mobility and dispersion). Screening of this pathotype is performed by detection of *ipaH* – present in the pINV (Robins-Browne *et al*., 2016). ETEC synthetize enterotoxins, particularly heat-labile (LT) and/or heat-stable (ST) enterotoxins and are the major cause of diarrhea in 3rd world countries. Colonization factors (CFs) are also present to allow the uptake of toxins into the

intestinal epithelial cells. Standard laboratory detection includes PCR detection of LT (encoded by *elt*), ST (encoded by *est*) and CFs (e.g. *cfa* genes that encode Cfa/I) (Croxen *et al*., 2013). EHEC present phage-located shiga-toxin genes (most common are *stx1* and *stx2*) and provoke haemorrhagic colitis and haemolytic uremic syndrome. EHEC are part of a larger group, the Shiga Toxin-producing *E. coli* (STEC), though strains belonging to other pathotypes may also produce shiga-toxin (and thus also being considered STEC). EHEC typically also harbour the LEE pathogenic island, manifesting similar virulence phenotypes towards the intestinal epithelium to EPEC. PCR-screening of *stx1* and *stx2* is the standard detection method of this pathotype (Croxen *et al*., 2013; Robins-Browne *et al*., 2016). Only STEC strains that can cause haemorrhagic colitis and haemolytic uremic syndrome are termed EHEC (Robins-Browne *et al*., 2016). EAEC strains provoke persistent infection due to the "stacked-brick" aggregation behaviour (in a biofilm) in the intestine wall, where they induce an inflammatory response, and produce enterotoxins (EAST-1 and Pet; encoded in the plasmid pAA). Plasmid-encoded aggregative fimbriae (AAF) and AggR, encoded by *aggR* (both also located on the pAA plasmid), are involved in colonization in typical EAEC. However, little comprehensive information exists for EAEC virulence mechanisms, since they are a very heterogeneous pathotype and the role of some virulence determinants has not yet been elucidated (Robins-Browne *et al*., 2016; Rogers *et al*., 2016; Smith & Fratamico, 2016). Conventionally, screening by PCR of *aggR* is performed. Atypical EAEC exhibit the "stacked-brick" aggregation phenotype but are *aggR* negative (Smith & Fratamico, 2016). DAEC is an heterogenous pathotype distinguishable due to the diffuse adherence pattern observed in cell cultures, for which afimbrial and fimbrial adhesins (Afa-Dr adhesins) have been implicated. Infected cells develop cellular projections that englobe the bacteria. Afa-Dr adhesins are present in 75% of DAEC, whereas other virulence determinants are lacking. The autotransporter toxin encoding gene *sat* has been associated to DAEC (Robins-Browne *et al*., 2016; Rogers *et al*., 2016). However, the presence of ST or LEE in some strains makes classification based on genotype challenging (creates confusion with EPEC and EHEC). Moreover, association of DAEC in diarrheal events has been problematic, since they have been also associated to extraintestinal infections, namely urinary tract infections (UTI) (Robins-Browne *et al*., 2016; Rogers *et al*., 2016). PCR-screening of DAEC usually targets genes present in the operons encoding Afa-Dr adhesins, such as *daaC* (Croxen *et al*., 2013). AIEC, unlike other IPEC, are not associated with diarrhea but with a chronic bowel-related inflammatory disease, known as Crohn's disease. Pathogenesis comprise adherence to epithelial cells and macrophages, followed by invasion into the intracellular environment and replication. Unlike EIEC, AIEC are motile.

Identification of this pathotype is difficult and relies deeply on phenotypical tests, since no specific VF has been correlated with AIEC (Croxen *et al*., 2013; Robins-Browne *et al*., 2016). Likely, more IPEC pathotypes will continue to emerge due to HGT of virulence genes, creating novel combinations (e.g. shiga-toxin producing EAEC) (Robins-Browne *et al*., 2016).

ExPEC are often opportunistic pathogens and are typically divided in Uropathogenic *E. coli* (UPEC), Neonatal Meningitis-associated *E. coli* (NMEC), Sepsis-associated *E. coli* (SEPEC) and Avian Pathogenic *E. coli* (APEC) (Robins-Browne *et al*., 2016; Sarowska *et al*., 2019; Table 3). They possess a multiplicity of virulence genes (regularly found in pathogenicity islands or plasmids) encoding toxins (e.g. *hlyE*, *cnf1*), adhesins (e.g. *afa*/*draBC* and *iha*), protectins and invasins (e.g. *iss*, *ibeA* and *cva*), iron acquisition machinery (e.g. *iroN* and *iutA*) and capsule production (e.g. *kpsM* II or K1 capsule) (Dale & Woodford, 2015; Sarowska *et al*., 2019).

UPEC are one of the principal causes of uncomplicated UTIs and approximately half of the complicated UTIs (often persistent due to the capacity of ExPEC to divide intracellularly) (Sarowska *et al*., 2019). Iron acquisition machinery (e.g. yersiniabactin, aerobactin and salmochelin) is essential in their pathogenesis (as well as for other ExPEC strains). Other VFs include Afa/DraBC adhesins, antigen 43, Sat and Pic toxins (Dale & Woodford, 2015; Sarowska *et al*., 2019). SEPEC enter the blood stream and provoke bacteraemia, while NMEC reach the bloodstream and also invade the meninges of new-borns through the blood-brain barrier, being associated with an elevated death toll (Kaper *et al*., 2004; Sarowska *et al*., 2019). For this reason, NMEC and SEPEC share many virulence genes, such as those encoding invasins (*ibeA*, *ibeB* and *ibeC*), *iss* (increased serum survival), *traT*, *colV*, *cvaC*, *sfa/foc* and *gimB*. Also important is the K1 capsular antigen (*neuA*, *kpsM*) of NMEC (Sarowska *et al*., 2019). APEC provoke colibacillosis in poultry, being responsible for significant economic losses. Typical VFs include Iss, FimC, siderophores IucC and SitA, colicins (CvaC) and haemolysins (HlyE) (Sarowska *et al*., 2019).

However, ExPEC strains exhibit large heterogeneity in terms of virulence armamentarium and pathologies induced, which means that some strains may display, for example, both UPEC and NMEC phenotypes, rendering obsolete ExPEC subdivisions. Some authors screen for ExPEC using multiplex PCRs targeting *afa*/*draBC*, *papA*, *papC*, *sfa*/*foc*, *iutA* and *kpsM II*. If at least 2 genes were present, the test strain was considered ExPEC (Dale & Woodford, 2015; Sarowska *et al*., 2019).

Nonetheless, one of the greatest issues is related to the rise of not only virulent but also antibiotic-resistant strains of *E. coli*. *E. coli* is intrinsically susceptible to most antibiotics with clinical relevance (Poirel *et al*., 2018). However, HGT events enabled the acquisition of a vast arsenal of ARGs, the most concerning being ESBLs, carbapenemases, *mcr* genes (encode colistin resistance), 16S rRNA methylases (confer aminoglycoside resistance) and plasmid-encoded fluoroquinolone resistance (e.g. genes *qnrA* and *aacA4-cr*). In fact, cumulative acquisition of resistance has led multidrug resistant (MDR) *E. coli* to become a worrisome problem (reviewed by Poirel *et al*., 2018). Isolates from European fattening pigs and young calves obtained in 2015 showed broad resistance to tetracycline (54.7 and 45.4%), sulfamethoxazole (44.2 and 36.6%) and ampicillin (39.3 and 31.0%), with 38.1 and 28.6% exhibiting MDR phenotypes, respectively (EFSA & ECDC, 2017). In Europe, 53.7% of the human-related isolates retrieved in 2015 were antibiotic resistant, being ampicillin resistance the most common (57.2% of the isolates) (ECDC, 2017). Some African and European countries reported *E. coli* resistance rates to 3rd generation cephalosporins as high as 80% (in Portugal, 11.3%) (WHO, 2014). Numerous studies endorse the fact that *E. coli* constitute a pertinent reservoir of ARGs (Alves *et al*., 2014; Araújo *et al*., 2017; Moura *et al*., 2014; Varela *et al*., 2015), constituting not only a risk for human health but also for antibiotic resistance dissemination. Noteworthily, *E. coli* ST131 is responsible for the successful dissemination of CTX-M-15 throughout the globe (Cantón *et al*., 2012) and some strains are known UPEC (Robins-Browne *et al*., 2016). In WWTPs, the relative abundance of ARGs has been significantly correlated with the prevalence of phenotypic resistance of clinical isolates of several taxa in Europe, including *E. coli* (Pärnänen *et al*., 2019).

Aggregation of cells in biofilms is not only a major constrain for antibiotic therapy (as previously discussed in Chapter II) but is also considered a relevant virulence determinant, since it prevents effective immunological responses from the host, being often associated with persistent infections (since it also leads to reduced antibiotic susceptibility). In *E. coli*, biofilm formation is a prevalent trait and plays a role in pathologies such as UTIs and Crohn's disease. Cellular structures often associated with this phenotype are curli fimbria (Vila *et al*., 2016).

Table 3. The numerous *E. coli* pathotypes and their corresponding pathologies and symptomatology, virulence determinants and diagnostic strategies. Based on the following revision works: Croxen *et al*., 2013; Dale & Woodford, 2015; Robins-Browne *et al*., 2016; Sarowska *et al*., 2019; Smith & Fratamico, 2016. (Next page)

	Pathotypes	Pathology	Symptomatology	Virulence determinants	PCR-based diagnostic
Intestinal pathogenic *E. coli* (IPEC)	Enteropathogenic *E. coli* (EPEC)	Induce attaching and effacing lesions.	Watery diarrhea, vomiting and fever.	LEE pathogenicity island and EAF plasmid.	*eae* (present in all EPEC) and *bfpA* (only present in typical EPEC)
	Enteroinvasive *E. coli* (EIEC) (Include all *Shigella* spp.)	Bacillary dysentery or shigellosis.	Abundant diarrhea and high fever.	pINV (include type III secretion systems and effectors).	*ipaH*
	Enterotoxigenic *E. coli* (ETEC)	Produce enterotoxins.	Travellers' or watery diarrhea, endemic cholera-like disease in children.	Heat labile (LT) and heat stable (ST) enterotoxins, colonization factors (e.g. CS1, CFA/I).	*est* and *elt*
	Enterohemorrhagic *E. coli* (EHEC) (part of the group: Shiga-toxin-producing or Verotoxigenic *E. coli*)	Haemorrhagic colitis and haemolytic uremic syndrome.	Bloody diarrhea and renal complications.	LEE pathogenicity island and shiga-toxin.	*stx1*, *stx2* and *eae*
	Enteroaggregative *E. coli* (EAEC)	Form mucoid biofilms (aggregation in a stacked-brick manner) in the intestinal walls, provoking inflammation.	Persistent diarrhea in children and HIV infected patients; travellers´ diarrhea; little to no fever and no vomiting.	pAA, EAST-1 and Pet enterotoxin and AAF/I-AAF/V fimbriae.	*aggR* (present in typical EAEC), *aatA*, *aaiC* - difficult to differentiate atypical EAEC from non-diarrheagenic strains (requires observation of phenotype in co-culture with cell lines)
	Diffusely-adherent *E. coli* (DAEC)	Diffusely adherence pattern to epithelial cells.	Watery diarrhea without blood but with vomit; UTI.	Afa-Dr adhesins, AIDA-I, SAT autotransporter toxin.	*daaC*, *daaE*, and *afaB* (in the operons of the Afa/Dr adhesins)
	Adherent-invasive *E. coli* (AIEC)	Crohn's disease.	Inflammatory bowel disease (may lead to severer diarrhea).	Unknown.	Not available
Extraintestinal pathogenic *E. coli* (ExPEC)	Uropathogenic *E. coli* (UPEC)	Cystitis and pyelonephritis.	Dysuria, frequent urination with abnormal colour and abdominal pain.	Afa-Dr adhesins (Afa/DraBC), antigen 43, Iha, PIC and SAT toxins and haemolysin A.	*afa/draBC*, *papA*, *papC*, *sfa/foc*, *iutA* and *kpsM* II (at least 2)
	Neonatal Meningitis-associated *E. coli* (NMEC)	Meningitis in new-born children.	Fever, headache and vomiting.	IbeA, IbeB, IbeC, Iss, TraT, ColV, CvaC, Sfa/Foc, GimB, K1 capsular antigen (NeuA, KpsM), Mat and Irp.	
	Sepsis-associated *E. coli* (SEPEC)	Septicaemia/bacteraemia (i.e. a generalized bloodstream infection).	Dramatic drop on blood pressure and heavy breathing. Can progress into septic shock and death.	IbeA, IbeB, IbeC, Iss, TraT, ColV, CvaC, Sfa/Foc, GimB and Cdt.	
	Avian Pathogenic *E. coli* (APEC)	Colibacillosis in poultry.	Respiratory distress, decrease in feeding and poor growth.	Iss, FimC, IucC and SitA, CvaC and HlyE.	

Though coliform counts are often used as indicator of faecal contamination, several studies have reported the capacity of *E. coli* to persist in environmental settings (e.g. soil and water), becoming part of the natural microbiota (reviewed by Jang *et al*., 2017).

E. coli is abundantly isolated from wastewater. For instance, Bréchet and colleagues described on average 3.54×10^5 and 7.53×10^5 CFU/mL of *E. coli* in hospital and urban wastewater, respectively, and 3.71×10^3 CFU/mL in treated wastewater (Bréchet *et al*., 2014). As such, WWTPs are a source of *E. coli* contamination into the downstream aquatic environments, since wastewater treatment is ineffective in removing in its totality the overall *E. coli* loads (Bréchet *et al*., 2014; Silva *et al*., 2018). Furthermore, numerous studies report the discharge of ESBL-producing *E. coli* into the environment in WWTP's effluents (Amos *et al*., 2014; Bréchet *et al*., 2014; Ojer-Usoz *et al*., 2014; Silva *et al*., 2018) and some ExPEC strains have also been shown to persist wastewater treatment (Bibbal *et al*., 2018; Diallo *et al*., 2013). Survival of the pathogenic *E. coli* O157:H7 in wastewater and activated sludge for at least 20 days has been confirmed, though there is a clear decay in the CFU levels (Czajkowska *et al*., 2008). ETEC strains were shown to persist for up to 3 months in filtered sea and freshwater, maintaining expression of ST and LT (Lothigius *et al*., 2010). But studies to assess the persistence of wastewater-borne strains or pathogenic *E. coli* in water environments are still lacking, particularly those that consider the effect of the indigenous bacterial communities (i.e. do not autoclave or filtrate the microcosm's water). For example, Mauro and co-workers showed that the survival of shiga-toxin producing *E. coli* in freshwater microcosms was deeply affected by the removal of the natural microbiota (Mauro *et al*., 2013).

Hence, *E. coli* strains with persistence traits may reach and disseminate into the community (via environment), becoming a serious health concern, particularly when combined with antibiotic resistance and virulence traits, since it will enable evasion to antibiotic treatment and promote pathogenic behaviour. Monitoring of these characteristics in strains retrieved from wastewater effluents is, therefore, of utmost importance.

V. Scope of this work

As previously discussed, advanced wastewater treatments, such as UV-C irradiation, are one of the few strategies that could prevent the release of large loads of ARGs and ARB into the environment (see chapter III). In a full-scale study, effective removal of *Enterobacteriaceae* and cefotaxime-resistant *Enterobacteriaceae* was observed by application of a UV-C disinfection step (log reductions of 2.1 and 1.8, respectively) (Silva *et al*., 2018). Nonetheless, MDR *E. coli* and *K. pneumoniae* strains were still release in the WWTP's outflow (Silva *et al*., 2018). But knowledge regarding the ARGs they carry is limited, and virulence or environmental persistence is unknown. Besides, UV-C treatment was shown to select for MDR phenotypes (Silva *et al*., 2018). If the same is true for virulence determinants and survival in freshwater environments, despite being effective in reducing ARB loads, this disinfection step may promote the selection of virulent and MDR strains able to persist in the environment ("the golden trio"), thus posing risks for human health and the environment..

This study aimed to characterize the resistome (and lateral transfer), virulence and environmental persistence of MDR *E. coli* strains isolated from a UV-C treated wastewater effluent to infer potential health risks associated with such effluents.

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Chapter II

Genotypic and phenotypic traits of *bla*$_{CTX-M}$-carrying *E. coli* strains that survived an UV-C wastewater treatment

Research Paper

I. Introduction

Wastewater is a relevant source of chemical and biological contamination into the water environments, and despite the improvement of the treatment processes applied in Wastewater Treatment Plants (WWTPs), removal of emerging contaminants such as antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) remains challenging (Bouki *et al.*, 2013; Rizzo *et al.*, 2013; Sharma *et al.*, 2016; Tran *et al.*, 2018). Besides, the inflow of a broad range of contaminants (e.g. antibiotics, metals and faecal coliforms) creates the ideal setting for bacterial growth, lateral gene transfer (LGT) and *de novo* acquisition of antibiotic resistance, probably enriching this compartment in antibiotic resistance determinants before their dispersion into the aquatic systems (Karkman *et al.*, 2018; Manaia *et al.*, 2016; Marti *et al.*, 2014). In fact, 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; Mantilla-Calderon *et al.*, 2016; Miranda *et al.*, 2015; Ojer-Usoz *et al.*, 2014; Piotrowska *et al.*, 2017; Silva *et al.*, 2018; Yang *et al*., 2016), and treated effluents can even be enriched in such xenogenetic contaminants (Bouki *et al.*, 2013; Guo *et al.*, 2013a,b; Miranda *et al.*, 2015; Silva *et al.*, 2018). A promising containment strategy could be the implementation of advanced or disinfection-based tertiary treatments (Rizzo *et al.*, 2013). By 2015, in Europe, more than 70% of the wastewater received tertiary treatment (European Environment Agency, 2017), though in Portugal, only 8.1% of the WWTPs apply such treatments (APA, 2016).

A relatively common tertiary step in WWTPs is UV-C irradiation, since this process possesses a broad range of activity, the water chemical quality is unaffected (since no chemical by-products are generated) and shorter contact times are required (Cutler & Zimmerman, 2011; EPA, 1999). Considering this, over the years many WWTPs have, in fact, switched from chemical disinfection, such as chlorination, to UV irradiation, primarily due to environmental constrains of the previous (Bouki *et al.*, 2013). The underlying bacterial inactivation mechanism is production of DNA by-products (base dimers) that hinders cellular replication, and thus block cellular proliferation (Cutler & Zimmerman, 2011).

In overall, most studies show that the bacterial loads from UV-irradiated effluents are effectively reduced (Silva *et al.*, 2018; Sousa *et al.*, 2017; Zhang *et al.*, 2009), however, data for the removal of ARB can be contradictory, since these bacteria may exhibit higher resistance/tolerance to UV light, and thus be relatively enriched in the WWTPs outflow (Guo *et al.*, 2013a,b; Huang *et al.*, 2016).

Regardless, a total removal of the bacterial content of the wastewater is impossible, and the characteristics of surviving strains remain largely unknown. Hence, understanding the phenotype and genotype of such strains is important to predict their potential biohazard.

Antibiotic-resistant strains of *Escherichia coli*, a known commensal and pathogen, are commonly isolated from wastewater (Bréchet *et al.*, 2014; Conte *et al.*, 2017; Osińska *et al.*, 2017), some displaying virulence factors (Bibbal *et al.*, 2018; Franz *et al.*, 2015; Osińska *et al.*, 2017). Release of ESBL-producing *E. coli* (particularly those carrying $bla_{\text{CTX-M}}$ genes) in the final effluent of WWTPs has been described (Amos *et al.*, 2014; Bréchet *et al.*, 2014; Ojer-Usoz *et al.*, 2014; Silva *et al*., 2018), in one case with a relative enrichment (Bréchet *et al.*, 2014). Despite being considered indicators of faecal contamination, *E. coli* strains have been shown to survive in the environment (Jang *et al.*, 2017), which is concerning since resistance and virulence traits can be unimpacted by wastewater treatments.

UV-C disinfection can lead to increments in the prevalence of ARGs and ARBs, integrases and multi-resistance phenotypes (Guo *et al.*, 2013a,b; Huang et al., 2016; Jäger *et al.*, 2018; Silva *et al.*, 2018; Zhang *et al.*, 2009), further increasing the potential biohazard 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, although the study estimated that 3.0×10^7 cells per m^3 of treated water are released daily in the final effluent, some being $bla_{\text{CTX-M}}$-carrying and multidrug-resistant *E. coli* (Silva *et al.*, 2018). Therefore, in this study we aimed to characterize the diversity, resistome, virulence potential, and persistence and fate in freshwater microcosms of *E. coli* strains that were previously isolated from this final effluent, in order to understand the risk they pose for healthcare.

II. Materials and Methods

II.I. Bacterial strains

The *E. coli* isolates selected for this study were previously obtained from an 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 $bla_{CTX\text{-}M}$ gene (Silva *et al*., 2018). Strains features are presented in Table 1.

II.II. 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 (Wirth et al., 2006; https://enterobase.warwick.ac.uk/warwick_mlst_legacy).

II.III. PCR screening for ARGs and virulence determinants

Total genomic DNA was extracted from all strains, using a Silica DNA Gel extraction kit (ThermoFisher, USA), and used as template in the subsequent screening. PCR-based detection of 19 ARGs (bla_{GES}, $bla_{OXA\text{-}1\text{-}like}$, $bla_{OXA\text{-}2\text{-}like}$, $bla_{OXA\text{-}10\text{-}like}$, *tet(*A*)*, *tet(*B*)*, *tet(*C*)*, *tet(*D*)*, *tet(*E*)*, *tet(*G*)*, *tet(*M*)*, *sul1*, *sul2*, *sul3*, *qnrA*, *qnrB*, *qnrS, aacA4* 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. Amplicons were sequenced to confirm gene identity. For the detection of mutations related to fluoroquinolone resistance, partial amplification by PCR and sequencing of *gyrA* and *parC* amplicons was required.

II.IV. Plasmid characterization 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 PstI and Bst1107I (Thermoscientific, USA). Reaction mixtures of 13 µL consisted of 5 U of each enzyme, 1 µL of 10X Buffer O (Thermoscientific, USA) and 4-6 µg of pDNA. The mixture was incubated for 4 hours at 37ºC and enzymatic digestion was stop 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 (Moura *et al*., 2012). 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 (EUCAST, 2019), except for tetracycline (CLSI, 2018). pDNA was also extracted from transconjugants and enzymatically digested for comparison, as described above.

II.V. 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) and 37ºC as the incubation temperature without shaking, 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 25ºC 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 25 922 was used as positive control (Naves *et al*., 2008) and sterile media as 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.

II.VI. Haemolysin activity and siderophore production

Analysis of haemolytic potential of all strains was assessed by growth on Blood Agar (Biomérriex, France) at 37ºC 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 negative control (MicroLab's strain collection; Alves, unpublished). Test strains were incubated at 37ºC and control strains at 30ºC.

II.VII. Resazurin-based cytotoxicity experiments

Cell-free extracellular extracts were obtained from overnight grown cultures in TSB medium, at 37ºC with 180 rpm of agitation. Cells were removed by centrifugation (20 min; 5,000 rpm) and posterior filtration with 0.2 µm filters. Confluent monolayers of Vero cells (obtained from kidney epithelial cells of African Green monkey, *Cercopithecus aethiops*) 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 (maximum viability) and wells with only resazurin (blank) were included in each 96-well plate. *E. coli* BL21 (non-cytotoxic) and *E. coli* PH20 (shiga-toxin producer; Table S1-A) 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:

$$\text{(A) Cytotoxicity per OD}_{600nm}\ 1.0\ (\%) = \frac{1\ -\ \text{Cell viability}\ (\%)}{\text{OD}_{600\text{nm}}}$$

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

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).

II.VIII. 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 h, 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 MOI (multiplicity of infection) between 1 and 10. Then, after 4 washing steps with PBS the plates were incubated during 1 h 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 during 5 min. 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 are the initial inoculum plate counts (CFU/mL) and LPC are 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 independent assays with varied MOI (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.

II.IX. *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 negative and positive control, respectively. Larvae (n=10 per strain) were injected with 10^5 CFUs of each strain and survival was assessed at 12, 16, 24, 36 and 50 hours. Survival was expressed as the percentage of individuals that were alive (i.e. responded to external stimuli) at each time.

II.X. Whole-genome sequencing

Genomic DNA was extracted from six selected 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 using an Illumina Hiseq 2500 platform (StabVida, Portugal). 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 and Pasteur schemes), *fimH* type, serotype and pathogenic potential relied on MLST 2.0.1, CHTyper 1.0, SeroTypeFinder 2.0.1 and PathogenFinder 1.1, respectively.

II.XI. Microcosms experiments

Strains were grown overnight in M63 minimal media supplemented with 0.8% of glucose and 1 mM of magnesium sulphate at 30ºC. 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 150 mL of freshwater and 1 mL of inoculum (OD_{600nm} of 0.3) in order 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 a period of 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 37ºC. From each microcosm, presumptive *E. coli* colonies were retrieved in the last sampling moment, streaked and typed by BOX-PCR to confirm their identity by comparison with profiles of the original strains. Antibiograms were then 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 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 94ºC for 3 min followed by 40 cycles of denaturation at 95ºC for 10 s and annealing at 60 ºC for 20 s, with fluorescence data acquisition at the end of each cycle. Melting analysis was performed from 55 to 95ºC, with steady 0.1ºC increments at each 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 with the

manufacturer's instructions. DNA standards were prepared for each qPCR experiment by serial dilutions of purified pDNA in ultrapure water.

II.XII. Statistical analysis

Variables were checked for normal distribution by the Shapiro-Wilk test. Analysis 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.

III. Results

III.I. Strains clonal relatedness

The genetic diversity of the *E. coli* strains was evaluated by applying several typing methods (rep-PCR, PFGE and Clermont phylotyping). Rep-PCR analysis (Figure S1) separated strains in two groups that according with Clermont phylogrouping correspond to (i) phylogroup B2-sgI (n=7), and to (ii) phylogroups A (n=16 isolates) and C (n=2) (Table 1). Diversity within each of these groups is evidenced by distinct profiles obtained with rep-PCR and PFGE for the different strains (Figures S1 and S2). MLST analysis identified 8 STs and three isolates belonged to a putative novel ST (Table 1 and Figure 1). Isolates included in phylogroup A were identified with 6 different STs (ST58, ST155, ST453, ST617, ST744 and ST1284). All isolates belonging to phylogroup B2-sgI were affiliated to ST131, and phylogroup C strains were affiliated to ST410.

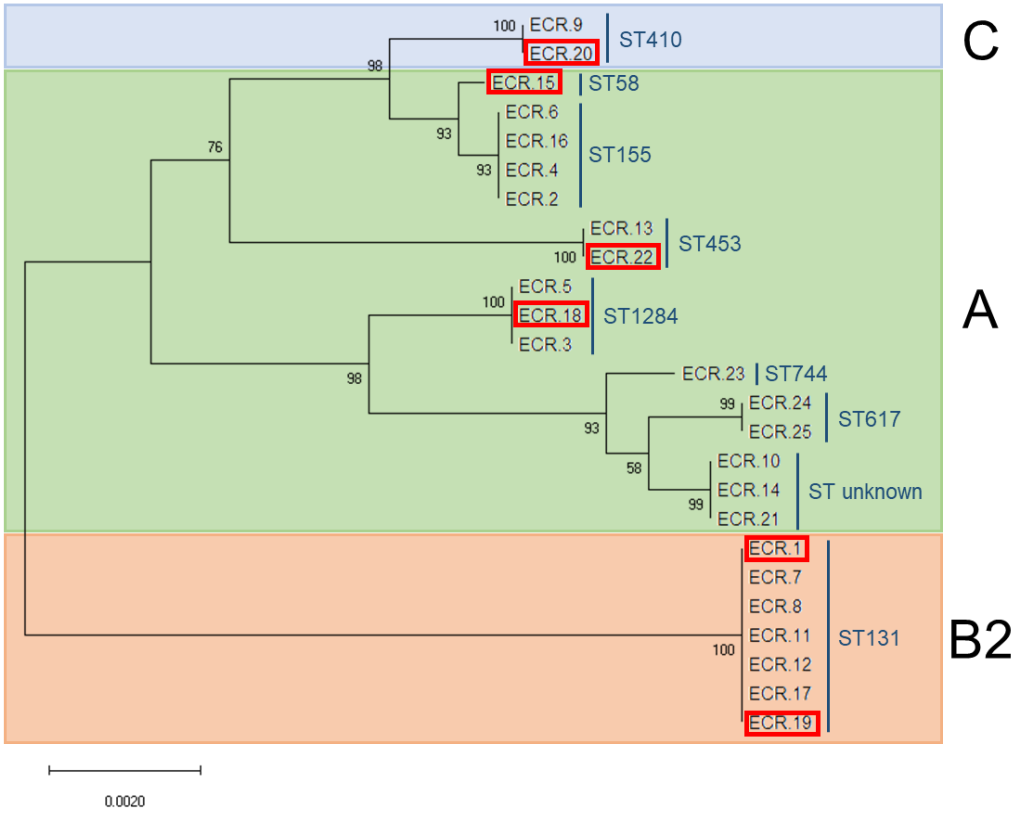

Figure 1. Phylogenetic tree based on the concatenated sequences of the seven housekeeping genes used for MLST and constructed using the Neighbour Joining method (1000 bootstraps). The

evolutionary model that best described the sequence data was kimura 2-parameters gamma distributed with invariant sites. In front of each strain identification is indicated the results from sequence type affiliation. The coloured boxes specify the *E. coli* phylogroup affiliation (orange: B2, green: A and blue: C). Red squares indicate strains whose genome was sequenced. Bootstrap values are showed near each clade.

III.II. Resistome 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). 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%). Conjugal transfer of cefotaxime resistance determinants to rifampicin-resistant *E. coli* CV601 yielded positive results 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*$_{\text{CTX-M-15}}$ (n=3), *bla*$_{\text{CTX-M-32}}$ (n=3) and *bla*$_{\text{CTX-M-1}}$ (n=2) was observed (Table 2). Co-transfer of ARGs (i.e. *bla*$_{\text{TEM}}$, *bla*$_{\text{OXA-1-like}}$, *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-resistance phenotype to the recipient strain (Table 2). In *bla*$_{\text{CTX-M-15}}$-positive transconjugants three replicons of the F family (Inc*FIA*, Inc*FIB* and Inc*F*) were detected, while in *bla*$_{\text{CTX-M-1}}$ transconjugants was detected the Inc*I1* replicon and in *bla*$_{\text{CTX-M-32}}$ transconjugants was detected the Inc*N* replicon (Table 2).

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-sgl	ST131	***bla*CTX-M-15**, **blaTEM**, *blaOXA-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**, *blaOXA-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**, *blaOXA-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**, *blaOXA-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-sgl	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-sgl	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-sgl	ST131	***bla*CTX-M-15**, ***bla*TEM**, *blaOXA-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-sgl	ST131	***bla*CTX-M-15**, ***bla*TEM**, *blaOXA-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. On 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)

III.III. Virulence related features

From the four independent assays performed for biofilm quantification, 14/21 and 17/25 strains formed biofilms at 25ºC with agitation and at 37ºC in static conditions, respectively (Figure 2). 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 37ºC 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).

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 is as follow: (a) red - non-producer, yellow - weak-producer, green - producer; (b) red - negative, yellow – weak-producer; green - abundant producer; (c) red - negative, green - positive; (d) red - non-cytotoxic, yellow - weakly cytotoxic, green - 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:

$Cell\ viability\ (\%) = \frac{ratio\ OD\ 570/600_{extract}}{ratio\ OD\ 570/600_{TSB}}$ and $Cytotoxicity\ per\ OD_{600nm}\ 1.0\ (\%) = \frac{1 - Cell\ viability\ (\%)}{OD_{600nm}}$, respectively. n.d. stands for not determined. (Next Page)

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	-

Only Vero cells monolayers exposed to 50.0% of the raw extracts displayed cell viabilities 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 21 strains were capable of internalization in Vero cells, 7 belonging to the B2:ST131 group (Table 3). To confirm reproducibility of the assays and to provide a quantifiable measurement of invasive potential, 6 strains were selected for 4 independent 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≤0.05) in MOI~5 (Figure 2). All strains displayed invasion indexes significantly lower than the positive control (Figure 3).

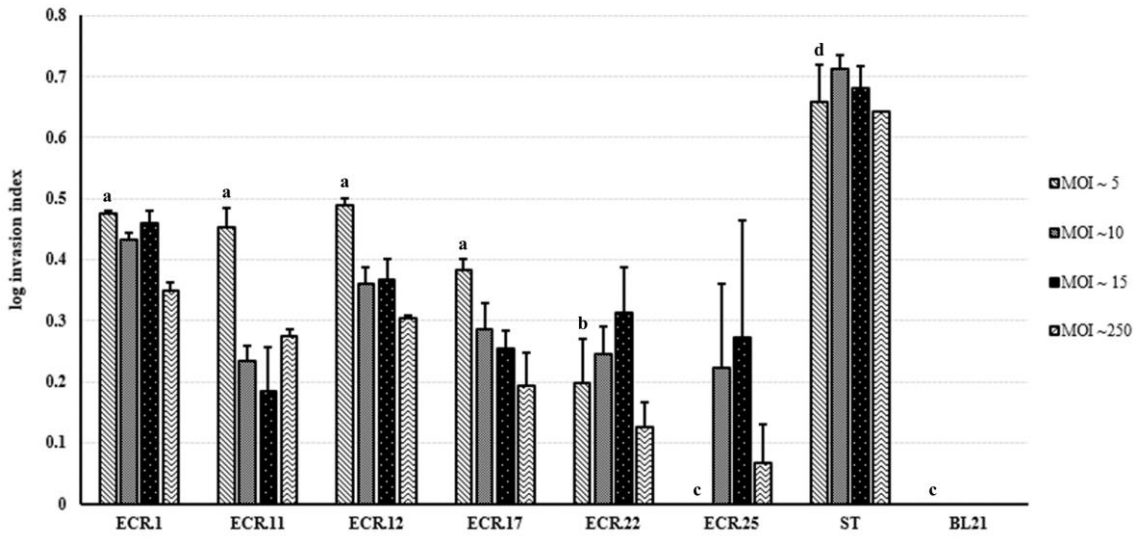

Figure 2. Capacity of selected *E. coli* strains to internalize Vero cells by the gentamicin-protection assay. Four independent assays were conducted with different MOIs (multiplicity of infection). Results are expressed as a logarithmized invasion index. *Salmonella enterica* subsp. *enterica* serovar Typhimurium SC56 (ST) and *E. coli* BL21 were used as positive and negative control, 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).

Assays in *Galleria mellonella* larvae were conducted to evaluate the virulence phenotype of selected strains in live organisms. ECR.18 was the most pathogenic to *G. mellonella*, inducing an identical mortality response to the positive control, followed by ECR.11, ECR.15 and ECR.25 (mortality rates ≥ 70% at 12 h). By the end of the experiment (50 h), 5 of the 10 strains tested killed ≥ 90 % of the larvae. *E. coli* BL21 (used as negative control) induced no mortality to *G. mellonella* (Figure 3 and 4).

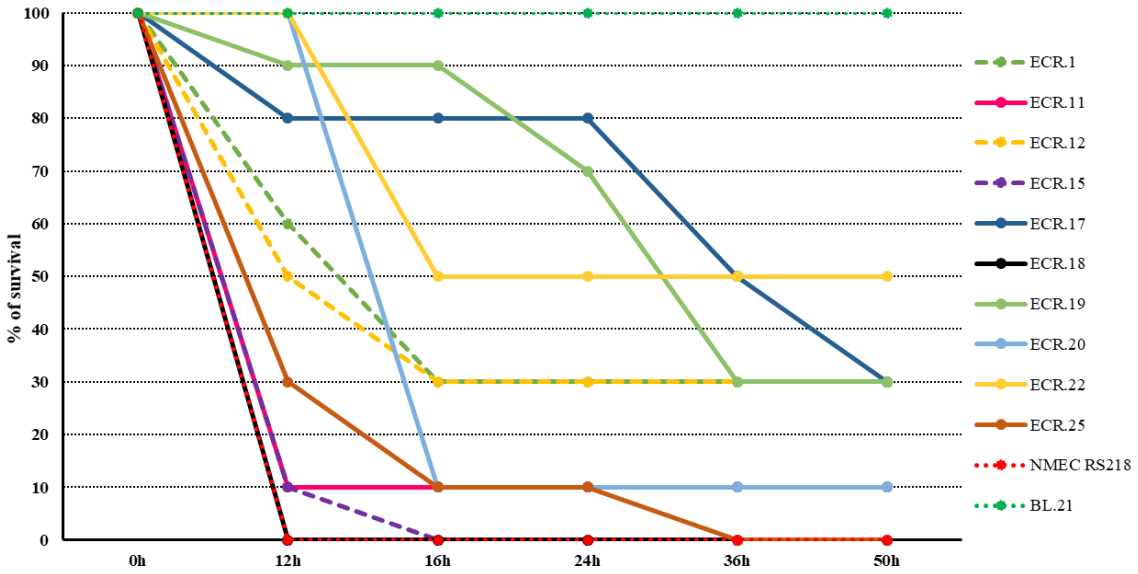

Figure 3. Survival of *Galleria mellonella* larvae (n=10) after inoculation with 10^5 CFUs of different *E. coli* strains over a 50-hour period. *E. coli* BL21 and Neonatal Meningitis-associated *E. coli* (NMEC) strain RS218 were used as negative and positive control, respectively.

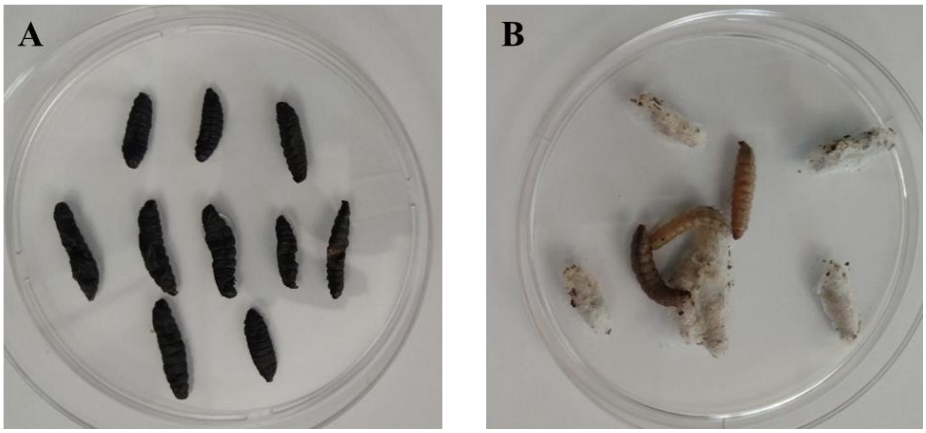

Figure 4. *Galleria mellonella* larvae exposed to the hypervirulent strain ECR.18 (A) and the negative control BL21 (B). The presence of a cocoon is an indicator of survival, which was confirmed by response to external stimuli.

PCR-based screening of IPEC VGs yielded no positive results.

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).

III.IV. Whole-genome analysis

From the 25 *E. coli* strains, 6 were selected for Illumina-based genome sequencing accordingly with clonal relatedness and ARGs content (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). *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 absent. All 6 strains were predicted as human pathogens by PathogenFinder 1.1 (probabilities ≥ 93.0%; Table 4).

In terms of resistome, analysis with ResFinder 3.1.0 confirmed the presence of all previously identified ARGs, and enabled the detection of non-PCR screened 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 multidrug 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). A plethora of genes related with efflux of antibiotics were identified by CARD's RGI (e.g. *mdt*-like and *erm*-like genes; data not presented). Multiple unknown mutations in ribosomal subunits encoding regions, *pmrA*, *pmrB*, *folP* and *ampC* were found by ResFinder 3.1.0 and may aid in expressing resistance phenotypes (data not presented).

Combined used of PlasmidFinder 2.0.1 and pMLST 2.0 confirmed most previously detected replicons and identified Inc*Q1* and Inc*X1* in ECR.22 and *Col*-like replicons in ECR.1 and ECR.18 (Table 4). The pMLST 2.0 tool affiliated Inc*N* replicons to plasmid ST1 (n=2), while the Inc*I1α* belonged to plasmid clonal complex 2 (n=1) or 3 (n=2). Only Inc*P* in ECR.1 and Inc*F* in ECR.18, which were previously detected by PCR and sequencing, were not detected by *in silico* analysis (Table 1 and 3).

In ECR.1, *bla*$_{CTX-M-15}$ 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*$_{CTX-M}$ 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 Inc*N* replicons were identical (100% similarity and 100% query coverage) to regions of *bla*$_{CTX-M-32}$-carrying plasmids (e.g. MF953243.1), supporting the co-transfer of *bla*$_{CTX-M-32}$ and Inc*N* in mating experiments (Table 2). For ECR.18, *bla*$_{CTX-M-15}$, *dfrA17*, *aadA5*, *sul1* and *tet(B)* and for ECR.19, *bla*$_{CTX-M-27}$, *strA*, *strB*, *sul2*, *tet(A)*, *aadA5*, *mph(A)*, *sul1* and *dfrA17* are probably harboured in multi-replicon *FIB-FIA-FII* plasmids, due to high similarity of contigs with plasmid sequences available on the database (CP027130.1 and CP023827.1). In ECR.22, *bla*$_{CTX-M-1}$, *tet(A)* and *sul2* seem to be co-carried in an Inc*I1α* plasmid like MH847571.1, which is corroborated by the conjugation assays results (Table 2).

Using VirulenceFinder 2.0, 11 VGs were detected in the 6 strains, the most prevalent being *iss* (n=5), which enables survival in the serum, followed by *gad* (n=4) and *ipfA* (n=3), which encode a glutamate decarboxylase (enables survival of *E. coli* cells to the stomach's acidity) and an adherence protein, respectively (Table 4). Other virulence determinants detected were *sat* (n=2), *astA* (n=1) and *senB* (n=1) which encode toxins, and *iroN* (n=2; siderophores), *cma* (n=1; colicin M), *iha* (n=1; adherence), *capU* (n=1; hexosyltransferase homolog), and *mchF* (n=1; ABS transporter protein) (Table 4). ECR.22 was the strain harbouring the largest pool of VGs (n=6), followed by ECR.1 and ECR.19 (each n=5) (Table 4). To increase coverage of *E. coli* VGs evaluated, *in silico* screening by VFanalyzer was also performed. The later identified on average approximately 70 genes related to virulence per genome (Table S4). Genes related to adherence (i.e. fimbriae and pilus) and iron uptake (i.e. siderophores) were the most represented, followed by those involved in secretion systems (including non-LEE encoded TTSS effectors and autotransporter mechanisms) (Table S4). *ibeB*, *ibeC*, *eaeH* and haemolysin encoding genes were detected in all strains, and factors related to evasion to the immune system (i.e. capsule) were identified in 4 (Table S4).

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), plasmid replicons (PlasmidFinder 2.0.1 and pMLST 2.0) and virulence genes (VirulenceFinder 2.0).

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

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

III.V. Environmental persistence

To determine the fate and persistence in freshwater of the *E. coli* strains studied in this work, 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 (Figure S5 and S6). 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)] (Figure S4). Significantly elevated *uidA* levels compared with the control in all replicates at this sampling moment corroborates the persistence of this strain (Figure 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 (Figure S4 and S5). ECR.1, ECR.18 and ECR.22 were detected by culture-dependent methods after 28 days in some of the microcosms, but were below the quantification limit, while ECR.15 and ECR.20 were no longer detected (Figure S4). However, for ECR.20 and ECR.22 in 2/3 replicates, *uidA* levels were significantly superior to the control (Dunnet t tests, $p<0.1$; Figure S5). For $bla_{CTX\text{-}M}$ quantification, in most cases this gene levels dropped below the quantification limit at 7 days. $bla_{CTX\text{-}M}$ was not detected in nearly any sample at 28 days (Figure S5). *E. coli* colonies retrieved at 28 days displayed typing profiles and resistance phenotypes and genotypes identical to the inoculated strains (data not presented).

IV. Discussion

Few studies characterized bacterial strains released in the effluents of WWTPs to infer possible health risks to human populations, and those that did had limited scopes (Anastasi *et al*., 2010 and 2013; Dolejska *et al*., 2011; Calhau *et al*., 2015). In this study, we characterized a collection of 25 ESBL-producing *E. coli* strains that survived a UV-C-irradiation step at a full-scale WWTP, and assessed 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 limited therapeutic options are available to treat infections caused by such strains (Rodríguez-Baño & Pascual, 2008).

Strains analyzed were affiliated to phylogroup B2, A, and C, which were consistently clustered by rep-PCR. B2 lineages are often associated with pathogens (Bukh *et al*., 2009). Seven of the analyzed *E. coli* strains were affiliated to ST131, which is a high-risk clone implicated in the successful dissemination of CTX-M-15 (Cantón *et al*., 2012), and thus, its elevated prevalence in cefotaxime-resistant *E. coli* from wastewater was expected based on previous reports (e.g. Dolejska *et al*., 2011). In fact, strains identified as ST131 were all multi-drug resistant (4-5 classes of antibiotics) and displayed relevant phenotypic virulence traits, four of which being predicted as pathogenic (*in silico* and/or *in vivo*) and one being capable of consistently persist in a freshwater microcosm. Moreover, other STs detected in this study had been previously reported in treated wastewater effluents (Bréchet *et al*., 2014; Dolejska *et al*., 2011; Varela *et al*., 2015), and some of them were classified as pathogenic and/or identified as ESBLs-producers, particularly ST131, 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_{KPC-3} (Tacão *et al*., 2017), representing a potential dissemination route of this ST from the hospital to the environment.

Not only different bla_{CTX-M} genes were detected in our isolates, but their putative mobility to new hosts, through mobile platforms that carry other resistance determinants, was also shown. Conjugal transfer of bla_{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 from the F family (Amos *et al*., 2014; Dolejska *et al*., 2011;

Novais *et al*., 2007), *bla*$_{CTX-M-1}$ has been described in conjugative Inc*I1* plasmids from different sources (including WWTPs; Dolejska *et al*., 2013) and *bla*$_{CTX-M-32}$ in Inc*N* plasmids from clinical isolates (Novais *et al*., 2007). However, Dolejska *et al*. (2011) described, as in our study, limited conjugative ability of *bla*$_{CTX-M}$-plasmids isolated from WWTP's effluents (only 4 in 26 isolates conjugated) when compared to other works (Amos *et al*., 2014). Most resistance determinants identified in our study [i.e. *bla*$_{OXA}$, *tet* and *sul* genes, *qnrS1*, *gyrA* and *parC* mutations and *mph*(*A*)] have already been detected in the effluents of WWTPs applying conventional treatment steps (reviewed by Pazda *et al*., 2019).

Several studies report the release of putative virulent strains of *E. coli* in WWTP's 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 difficult pathotype affiliation. Considering that WWTPs act as hotspots for the LGT (Karkman *et al.*, 2018; Manaia *et al.*, 2016), it is possible that barriers between pathotypes have faded due to promiscuous acquisition of VGs encoded in mobile genetic elements during wastewater processing. Still, the presence of increased serum survival factors (encoded by *iss*), P fimbriae (*pap* genes) and abundant genes encoding iron acquisition machinery (e.g. *iutA*, *chuA*, *sitA* and *iroN*) may suggest these strains as extraintestinal pathogenic *E. coli* (ExPEC) (VFanalyser database, http://www.mgc.ac.cn/VFs/main.htm; Sarowska *et al*., 2019). Elevated VG content of these strains may corroborate Anastasi and colleagues' hypothesis that *E. coli* carrying uropathogenic VGs have increasing persistence to WWTPs treatments (Anastasi *et al*., 2010).

Vero cells were used for virulence experiments, since this cell line is often used to detected shiga-toxin production. None of the strains tested possessed shiga-toxin encoding genes, but all displayed relevant cytotoxicity comparatively with *stx2*$^{+}$ *E. coli* PH20, with most presenting cytotoxicity to Vero cells between 20-36%. These levels are in agreement with the results obtained in EHEC 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 also assessed. Variability in the invasiveness of the tested strains according with the MOI can be related with quorum sensing-driven invasion of mammalian cells and experimental limitations (i.e. cell detachment due to production of cytotoxic substance by the strains – confirmed by cytotoxicity assays – thus underestimating internalization capacity; or successive washing

steps that create intra-experimental variations due to discrepant cell detachment). In this sense, MOI of 5 was considered more reliable, since *in vitro* higher cell densities leads to higher cell detachment. B2:ST131 strains were congruently invasive towards kidney epithelial cells, which may suggest these strains as potential uropathogens. 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 comparatively 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). Invasion indexes of an 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), underestimating its pathogenicity. Evidences from invasion and cytotoxicity suggest that at least 10 strains 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 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 50 hours of inoculation at least 50% of the larvae were killed. ECR.18 displayed an identical hypervirulent phenotype to NMEC strain RS218 (positive control), with the survival curve of the latest being concordant with a previous study (Fuentes-Castillo *et al*., 2019). Moreover, five of the tested strains (ECR.15, ECR.18, ECR.20, ECR.22 and ECR.25) were non-invasive in a qualitative invasion assay in Vero cells, but showed pathogenicity to *G. mellonella* larvae. The reliability of *G. mellonella* as a model for bacterial infection lies on the high similarity of their innate immune response to vertebrates. Studies in 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 suggest that most of our isolates can be pathogenic to humans.

Considering that susceptibility to antibiotics and survival on environmental settings may be impacted by biofilm formation capacity, assays were performed to quantify such ability. Such experiments are difficult to standardize. For example, media and the type of quantification protocol used generates 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 terms, biofilm formation was weak to mild in our collection. A credible hypothesis is that previous conventional activated sludge followed by flocculation and precipitation in setting tanks likely removes preferentially bacteria with higher aggregative

behavior, such as strong biofilm producers. Congruently, Čornejová *et al*. (2015) found that most ESBL-producing *E. coli* strains isolated from treated municipal wastewater were weak biofilm producers. Our strongest biofilm producer (ECR.23) carried an Inc*X* 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 differentially survival of *E. coli* K12 depending on temperature. As such, we determined potential persistence of six strains in freshwater microcosms. In general, there was high variability among microcosm 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 at 36 hours) in a freshwater environment 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. However, 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 use of contaminated water, consumption of food products and recreational activities. For example, Leonard and colleagues confirmed the association between surfing, colonization by $bla_{\text{CTX-M}}$ carrying *E. coli* and waters contaminated with these bacteria (Leonard *et al*., 2018). This is especially concerning with the increasing need of recycling treated wastewater into irrigation (already widely implemented) and drinking water due to water scarcity issues (Fatta-Kassinos *et al*., 2011).

V. Conclusion

In the present study we evaluated the antibiotic resistance mechanisms, virulence and environmental persistence of *E. coli* strains present in a UV-C treated effluent. Our data indicates 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 the studied strains, in some cases resulting in multi-resistance phenotypes. In overall, this indicates that the UV-treated effluent analyzed still represents a potential risk for health care and antibiotic stewardship. Detailed evaluation of these traits in strains surviving other wastewater treatments is urgent, since finding an adequate treatment or combination of them that reduce to safe levels antibiotic resistant pathogens from the WWTP's outflow is required to circumvent this environmental and public health risk and enable wastewater reuse.

VI. References

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VII. Supplementary Material

Table S1-A. Primers, conditions and controls used for qualitative PCR.

Target	Primer sequence (5'- 3')	Concentration (µM)	Annealing temperature (ºC)	Thermocycling program	Amplicon size (bp)	Positive control	Reference
bla$_{GES}$	**GES_fwd:** AGT CGG CTA GAC CGG AAA G **GES_rev:** TTT GTC CGT GCT CAG GAT	0.3	57	A	399	*K. pneumoniae* FFUL 22K[1]	Dallenne *et al*., 2010
bla$_{OXA-1-like}$	**OXA-1_fwd:** ACA CAA TAC ATA TCA ACT TCG C **OXA-1_rev:** AGT GTG TTT AGA ATG GTG ATC	0.3	53	B	814	*P. aeruginosa* SOF1[2]	Ouellette *et al*., 1997
bla$_{OXA-2-like}$	**OXA-2_fwd:** CAA GCC AAA GGC ACG ATA GTT G **OXA-2_rev:** CTC AAC CCA TCC TAC CCA CC	0.3	56	B	561	*P. aeruginosa*[2]	Henriques *et al*., 2006a
bla$_{OXA-10-like}$	**OXA-10_fwd:** CGT GCT TTG TAA AAG TAG CAG **OXA-10_rev:** CAT GAT TTT GGT GGG AAT GG	0.3	53	B	652	*P. aeruginosa* ED-1[2]	Huovinen *et al.*, 1988
bla$_{OXA-48}$	**blaOXA48 54I_fwd:** AGC AAG GAT TTA CCA ATA AT **blaOXA48 54I_rev:** GGC ATA TCC ATA TTC ATC	0.3	50	C	571	*Shewanella xiamenensis* C1[3]	Zong, 2012
*tet(*A*)*	**TetA_fwd:** GCT ACA TCC TGC TTG CCT TC **TetA_rev:** GCA TAG ATC GCC GTG AAG AG	0.3	53	D	211	*E. coli* NCTC 50078[4]	Nawaz *et al*., 2006
*tet(*B*)*	**TetB_fwd:** TCA TTG CCG ATA CCA CCT CAG **TetB_rev:** CCA ACC ATC ATG CTA TTC CAT CC	0.3	53	D	391	*E. coli* CSH50[4]	Nawaz *et al*., 2006
*tet(*C*)*	**TetC_fwd:** CTG CTC GCT TCG CTA CTT G **TetC_rev:** GCC TAC AAT CCA TGC CAA CC	0.3	53	D	897	*E. coli* DO7[4]	Nawaz *et al*., 2006
*tet(*D*)*	**TetD_ fwd:** TGT GCT GTG GAT GTT GTA TCT C **TetD_rev:** CAG TGC CGT GCC AAT CAG	0.3	53	D	844	*E. coli* C600[4]	Nawaz *et al*., 2006

*tet(*E*)*	**TetE_fwd:** ATG AAC CGC ACT GTG ATG ATG **TetE_rev:** ACC GAC CAT TAC GCC ATC C	0.3	53	D	744	*E. coli* HB101[4]	Nawaz *et al*., 2006
*tet(*G*)*	**TetG_fwd:** GCG CTN TAT GCG TTG ATG CA **TetG_rev:** ATG CCA ACA CCC CCG GCG	0.3	55	D	803	*Salmonella* sp. P502212[4]	Ng *et al*., 2001
*tet(*M*)*	**TetM_fwd:** GTG GAC AAA GGT ACA ACG AG **TetM_rev:** CGG TAA AGT TCG TCA CAC AC	0.3	55	D	406	Not specified[5]	Ng *et al*., 2001
sul1	**sul1_fwd:** CTG AAC GAT ATC CAA GGA TTY CC **sul1_rev:** AAA AAT CCC ATC CCC GGR TC	0.3	50	D	239	*E. coli* A25[6]	Heuer and Smalla, 2007
sul2	**sul2_fwd:** GCG CTC AAG GCA GAT GGC AT **sul2_rev:** GCG TTT GAT ACC GGC ACC CG	0.3	60	D	293	*E. coli* A7[6]	Kerrn *et al*., 2002
sul3	**sul3_fwd:** AAG AAG CCC ATA CCC GGR TC **sul3_rev:** ATT AAT GAT ATT CAA GGT TTY CC	0.3	50	D	293	*E. coli* A4[6]	Heuer and Smalla, 2007
qnrA	**qnrA_fwd:** TTC TCA CGC CAG GAT TTG **qnrA_rev:** CCA TCC AGA TCG GCA AA	0.3	53	D	521	*Shewanella* B39[3]	Guillard *et al*., 2011
qnrB	**qnrB_fwd:** GGM ATH GAA ATT CGC CAC TG **qnrB_rev:** TTY GCB GYY CGC CAG TCG	0.3	53	D	261	*E. coli* A100[6]	Guillard *et al*., 2011
qnrS	**qnrS_fwd:** GCA AGT TCA TTG AAC AGG GT **qnrS_rev:** TCT AAA CCG TCG AGT TCG GCG	0.3	54	D	428	*E. coli* A152[6]	Cattoir *et al*., 2007
gyrA	**gyrA_fwd:** AAA TCT GCC CGT GTC GTT GGT **gyrA_rev:** GCC ATA CCT ACG GCG ATA CC	0.3	55	B	344	-	Vila *et al*., 1995
parC	**parC_fwd:** CTG AAT GCC AGC GCC AAA TT **parC_rev:** GCG AAC GAT TTC GGA TCG TC	0.3	55	B	168	-	Rodríguez-Martínez *et al*., 2006
aacA4	**aacA4_fwd:** TTG CGA TGC TCT ATG AGT GGC TA **aacA4_rev:** CTC GAA TGC CTG GCG TGT TT	0.3	54	D	482	*Aeromonas* E31[7]	Park *et al.*, 2006
mcr-1	**CLR5_fwd:** CGG TCA GTC CGT TTG TTC **CLR5_rev:** CTT GGT CGG TCT GTA GGG	0.3	58	E	320-350	*E. coli* Ec36[8]	Liu *et al*., 2016
stx1	**STX1_fwd:** ATA AAT CGC CAT TCG TTG ACT AC **STX1_rev:** AGA ACG CCC ACT GAG ATC ATC	0.16 (nM)	52	F	180	*E. coli* PH04[9]	Paton and Paton, 1998

stx2	**STX2_fwd:** GGC ACT GTC TGA AAC TGC TCC **STX2_rev:** TCG CCA GTT ATC TGA CAT TCT G	0.16 (nM)	52	F	255	*E. coli* PH20[9]	Paton and Paton, 1998
eae	**EAE_fwd:** GAC CCG GCA CAA GCA TAA GC **EAE_rev:** CCA CCT GCA GCA ACA AGA GG	0.16 (nM)	54	F	384	*E. coli* PH04[9]	Paton and Paton, 1998
ipaH	**IPAH_fwd:** GTT CCT TGA CCG CCT TTC CGA TAC CGT C **IPAH_rev:** GCC GGT CAG CCA CCC TCT GAG AGT AC	0.15	50	H	600	SB49[10]	Aranda *et al*., 2007
aggR	**AGGR_fwd:** GTA TAC ACA AAA GAA GGA AGC **AGGR_rev:** ACA GAA TCG TCA GCA TCA GC	0.15	50	H	254	17.2[10]	Aranda *et al*., 2007
bfpA	**BFPA_fwd:** AAT GGT GCT TGC GCT TGC TGC **BFPA_rev:** GCC GCT TTA TCC AAC CTG GTA	0.15	50	H	326	E2348/69[10]	Aranda *et al*., 2007
est	**ST_fwd:** ATT TTT MTT TCT GTA TTR TCT T **ST_rev:** CAC CCG GTA CAR GCA GGA TT	0.15	50	H	190	SB9[10]	Aranda *et al*., 2007
elt	**LT_fwd:** GGC GAC AGA TTA TAC CGT GC **LT_rev:** CGG TCT CTA TAT TCC CTG TT	0.15	50	H	450	SB9[10]	Aranda *et al*., 2007
BOX elements	**BOX_A1R:** CTA CGG CAA GGC GAC GCT GAC G	0.4	53	I	variable	-	Versalovic *et al*., 1994
REP elements	**ERIC1:** AAG TAA GTG ACT GGG GTG AGC **ERIC2:** ATG TAA GCT CCT GGG GAT TCA C	0.3	52	I	variable	-	Versalovic *et al*., 1994
adk	**adk_fwd:** ATT CTG CTT GGC GCT CCG GG **adk_rev:** CCG TCA ACT TTC GCG TAT TT	0.3	54	J	583	-	Wirth *et al*., 2006
fumC	**fumC_fwd:** TCA CAG GTC GCC AGC GCT TC **fumC_rev:** GTA CGC AGC GAA AAA GAT TC	0.3	54	J	806	-	Wirth *et al*., 2006
gyrB	**gyrB_fwd:** TCG GCG ACA CGG ATG ACG GC **gyrB_rev:** ATC AGG CCT TCA CGC GCA TC	0.3	60	J	911	-	Wirth *et al*., 2006
icd	**icd_fwd:** ATG GAA AGT AAA GTA GTT GTT CCG GCA CA **icd_rev:** GGA CGC AGC AGG ATC TGT T	0.3	54	J	878	-	Wirth *et al*., 2006

mdh	**mdh_fwd:** ATG AAA GTC GCA GTC CTC GGC GCT GCT GGC GG **mdh_rev:** TTA ACG AAC TCC TGC CCC AGA GCG ATA TCT TTC TT	0.3	60	J	932	-	Wirth *et al*., 2006
purA	**purA_fwd:** CGC GCT GAT GAA AGA GAT GA **purA_rev:** CAT ACG GTA AGC CAC GCA GA	0.3	54	J	816	-	Wirth *et al*., 2006
recA	**recA_fwd:** CGC ATT CGC TTT ACC CTG ACC **recA_rev:** TCG TCG AAA TCT ACG GAC CGG A	0.3	58	J	780	-	Wirth *et al*., 2006
uidA (qPCR)	**uidA_fwd:** CTG CTG CTG TCG GCT TTA **uidA_rev:** CCT TGC GGA CGG GTA T	0.2	60	See methods in main text.	205	-	Kaushik & Balasubramanian, 2012
bla_{CTX-M} (qPCR)	**CTX-M_fwd:** GTG CAG TAC CAG TAA AGT TAT GG **CTX-M_qPCR_rev:** GMA ATC ARY TTR TTC ATS GC	0.2	60	See methods in main text.	215	-	Henriques *et al.*, 2006b This study

[1] Correia *et al*., 2003
[2] provided by P. Nordmann (Centre Hospitalier de Bicêtre, Service de Bactériologie-Virologie-Parasitologie-Hygiène, Le remlin-Bicêtre, France)
[3] Tacão *et al*., 2018
[4] provided by Yvonne Agersø (Danish Institute for Food and Veterinary Research, Copenhagen, Denmark)
[5] provided by Peter Mullany (Eastman Dental Institute, University College London, UK)
[6] Moura *et al*., 2014
[7] Tacão *et al*., 2014.
[8] Tacão *et al*., 2017
[9] provided by Björn Posse (Faculty of Veterinary Medicine, University of Ghent, Belgium)
[10] provided by Josée Harelfrom (Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, QC, Canada)

Table S1-B. Thermocycling conditions for amplification of genes indicated in Table S2-A.

Program	Initial denaturation	Cycles	Final extension
A	10 min at 94 ºC	40 s at 94 ºC, 30 s at annealing temperature, 1 min at 72 ºC for 30 cyles	7 min at 72 ºC
B	5 min at 94 ºC	30 s at 94 ºC, 30 s at annealing temperature, 1 min at 72 ºC for 30 cyles	7 min at 72 ºC
C	2 min at 94 ºC	30 s at 94 ºC, 30 s at annealing temperature, 1 min at 68 ºC for 40 cyles	7 min at 68 ºC
D	5 min at 94 ºC	30 s at 94 ºC, 30 s at annealing temperature, 30 s at 72 ºC for 30 cyles	7 min at 72 ºC
E	15 min at 94 ºC	30 s at 94 ºC, 90 s at annealing temperature, 1 min at 72 ºC for 25 cycles	10 min at 72 ºC
F	3 min at 95 ºC	1 min at 95 ºC, 1 min at annealing temperature, 1 min at 72 ºC for 35 cycles	10 min at 72 ºC
H	5 min at 95 ºC	1 min at 95 ºC, 1 min at annealing temperature, 1 min at 72 ºC for 40 cycles	7 min at 72 ºC
I	7 min at 94 ºC	1 min at 94 ºC, 1 min at annealing temperature, 8 min at 65 ºC for 30 cycles	16 min at 65 ºC
J	2 min at 95 ºC	1 min at 95 ºC, 1 min at annealing temperature, 2 min at 72 ºC for 30 cycles	5 min at 72 ºC

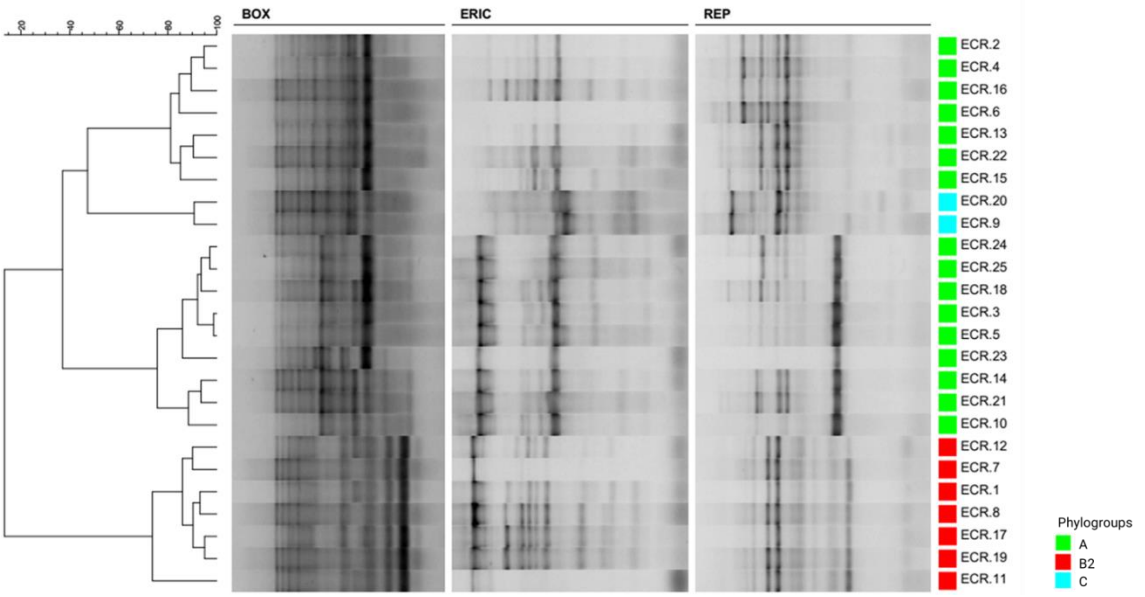

Figure S1. Dendrogram analysis created using Pearson correlation coefficient and the UPMGA clustering method of the combined rep-PCR (BOX, REP and ERIC) fingerprinting patterns of all *E. coli* strains. On red are highlighted the strain affiliated to the B2 phylogroup, on green to the phylogroup A and in blue to the phylogroup C.

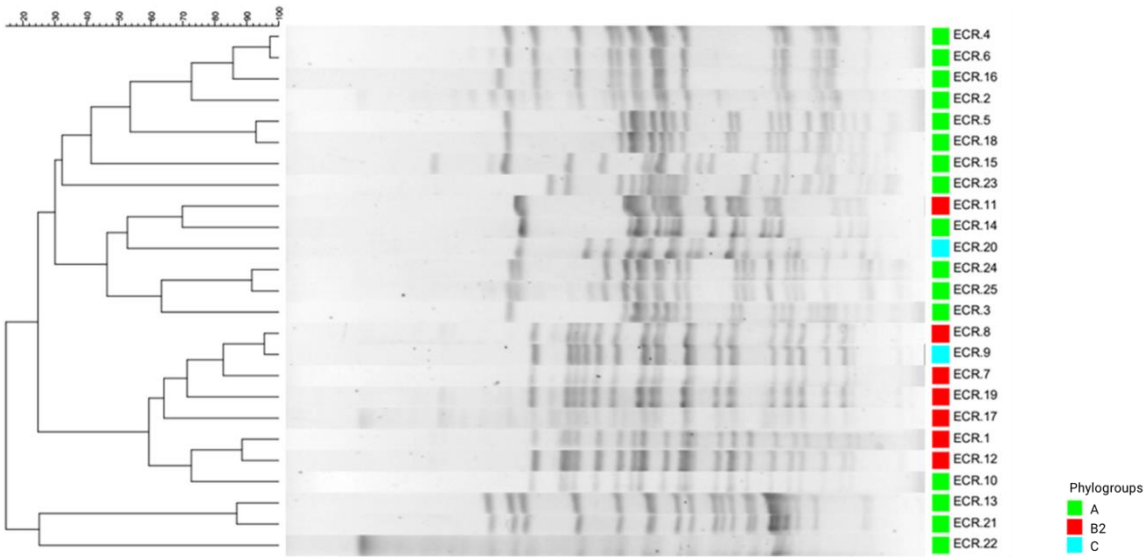

Figure S2. Dendrogram analysis created using Pearson correlation coefficient and the UPMGA clustering method of the Pulse-Field Gel Electrophoresis fingerprinting patterns of all *E. coli* strains. On red are highlighted the strain affiliated to the B2 phylogroup, on green to the phylogroup A and in blue to the phylogroup C.

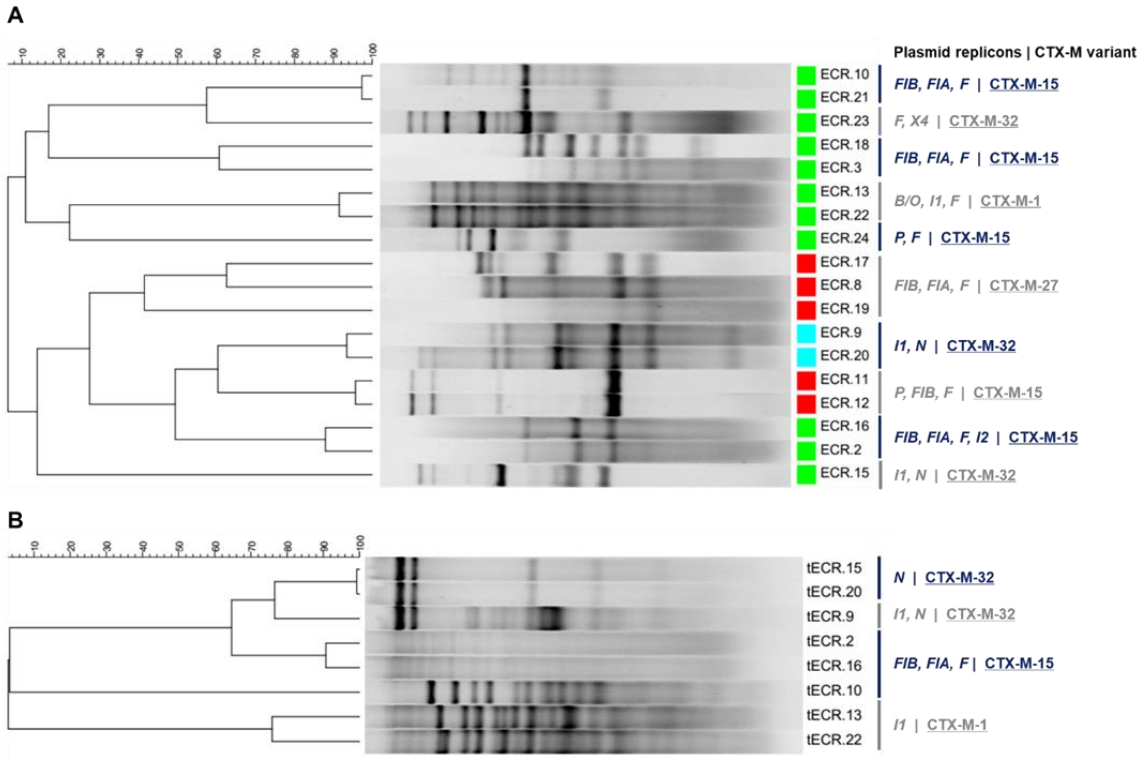

Figure S3. Plasmid restriction analysis using pDNA of all *E. coli* strains (A) or transconjugants obtained during the mating assays (B). Enzymatic restriction was performed with PstI and Bst1107I. The dendrograms were built using the Pearson coefficient and the UPMGA clustering method. Seven profiles were not included in analysis A since they yielded no discernible band patterns. Corresponding PCR-detected replicons and CTX-M variants are also indicated. On A, in red are highlighted the strain affiliated to the B2 phylogroup, on green to the phylogroup A and in blue to the phylogroup C.

Table S2. Genome quality metrics as determined by Quast.

Strains	Number of contigs (>500bp)	Putative genome size (bp)	N50	GC content (%)	Predicted Coding Sequences
ECR.1	93	5,159,736	235,127	50.8	4,874
ECR.15	128	4,917,643	108,986	50.8	4,624
ECR.18	138	4,842,827	88,923	50.8	4,517
ECR.19	95	5,020,022	190,852	50.8	4,710
ECR.20	85	4,816,901	127,460	50.6	4,518
ECR.22	208	5,195,300	107,107	50.6	5,039

Table S3. Culture-dependent weekly monitorization of *E. coli* cells in the freshwater microcosms (0, 7, 14, 21 and 28 days). The 6 tested strains and the control condition are represented. CFU counts were performed in the mFC selective media. Average ± standard deviation log(CFU/mL) values for each microcosm replicate are shown. Average values were used to generate a Green-Yellow-Red gradient. BQL stands for below quantification limit. At the last sampling moment (t-28d), except for ECR.19, qualitative detection of *E. coli* cells was performed by filtering the entire content of each Erlenmeyer into a single filter (i.e. quantification was not possible). The identity of these cells was confirmed by genotyping and comparison with the original inoculated strain. Statistical comparisons with the control were not performed due to obvious differences in colony morphologies between control Erlenmeyers and inoculated Erlenmeyers.

Strain	Replicate	log(CFU/mL)				
		t-0d	t-7d	t-14d	t-21d	t-28d
Control	**R1**	BQL	0.86 ± 0.17	0.10 ± 0.17	BQL	+
	R2	0.10 ± 0.17	BQL	0.20 ± 0.35	BQL	+
	R3	BQL	BQL	BQL	BQL	-
	R4	BQL	BQL	0.76 ± 0.66	0.46 ± 0.14	+
ECR.1	**R1**	4.10 ± 0.02	0.62 ± 0.15	0.11 ± 0.09	BQL	-
	R2	4.10 ± 0.02	0.89 ± 0.19	BQL	BQL	-
	R3	4.05 ± 0.04	1.82 ± 0.04	1.34 ± 0.06	1.07 ± 0.09	+
	R4	4.02 ± 0.03	0.84 ± 0.06	BQL	BQL	-
ECR.15	**R1**	3.83 ± 0.03	BQL	BQL	BQL	-
	R2	3.66 ± 0.05	0.64 ± 0.28	BQL	BQL	-
	R3	3.66 ± 0.10	1.18 ± 0.13	0.22 ± 0.10	BQL	-
	R4	3.45 ± 0.07	0.62 ± 0.15	BQL	BQL	-
ECR.18	**R1**	3.29 ± 0.15	0.72 ± 0.10	0.76 ± 0.02	0.63 ± 0.15	+
	R2	3.33 ± 0.16	BQL	BQL	BQL	-
	R3	3.11 ± 0.10	0.10 ± 0.17	BQL	BQL	-
	R4	3.33 ± 0.05	0.48 ± 0.00	BQL	BQL	-
ECR.19	**R1**	4.49 ± 0.04	2.36 ± 0.07	1.69 ± 0.04	0.69 ± 0.05	0.36 ± 0.03
	R2	4.45 ± 0.13	3.24 ± 0.03	2.81 ± 0.08	1.66 ± 0.14	0.33 ± 0.11
	R3	4.50 ± 0.06	3.18 ± 0.03	2.10 ± 0.05	1.08 ± 0.04	0.28 ± 0.09
	R4	4.55 ± 0.04	3.37 ± 0.03	2.01 ± 0.03	0.96 ± 0.02	1.14 ± 0.00
ECR.20	**R1**	3.47 ± 0.11	0.49 ± 0.20	BQL	BQL	-
	R2	3.42 ± 0.03	0.10 ± 0.17	BQL	BQL	-
	R3	3.51 ± 0.08	1.38 ± 0.11	0.46 ± 0.04	0.36 ± 0.08	-
	R4	3.47 ± 0.08	BQL	BQL	BQL	-
ECR.22	**R1**	4.14 ± 0.04	3.15 ± 0.03	1.62 ± 0.04	BQL	+
	R2	4.10 ± 0.01	1.67 ± 0.08	1.04 ± 0.12	BQL	+
	R3	4.05 ± 0.03	1.82 ± 0.06	1.73 ± 0.05	0.75 ± 0.03	+
	R4	4.11 ± 0.04	0.92 ± 0.14	0.58 ± 0.10	0.18 ± 0.19	-

Table S4. Culture-independent monitorization of the test strains in the freshwater microcosms at 0, 7 and 28 days. The 6 tested strains and the control condition are represented. Average ± standard deviation log(absolute copy number/mL) of *uidA* (*E. coli* genetic marker) and *bla*$_{CTX-M}$ (resistance marker present in all inoculated strains) were determine by qPCR and used to create a Green-Yellow-Red gradient. BQL stands for below quantification limit and indicates that the target genes were below the sensitivity of the qPCR experiment for that specific gene (for *uidA* 10^2 copies and for *bla*$_{CTX-M}$ 10^3 copies). Values on bold indicate statistical differences towards the control in each sampling time (Dunnett t-tests or Mann-Whitney U tests, p>0.1).

Strain	Replicate	log(*uidA* copy number/mL)			log(*bla*$_{CTX-M}$ copy number/mL)		
		t-0d	t-7d	t-28d	t-0d	t-7d	t-28d
Control	**R1**	0.92 ± 0.00	1.11 ± 0.24	1.98 ± 0.03	BQL	BQL	BQL
	R2	0.71 ± 0.00	1.72 ± 0.03	0.98 ± 0.18	BQL	2.36 ± 0.00	BQL
	R3	BQL	1.51 ± 0.05	1.54 ± 0.01	BQL	BQL	BQL
ECR.1	**R1**	**4.12 ± 0.05**	**2.58 ± 0.06**	1.86 ± 0.07	**3.40 ± 0.07**	2.38 ± 0.03	BQL
	R2	**4.03 ± 0.04**	**2.69 ± 0.04**	1.29 ± 0.08	**3.35 ± 0.05**	**2.64 ± 0.06**	BQL
	R3	**3.97 ± 0.02**	**2.64 ± 0.01**	1.50 ± 0.12	**3.37 ± 0.03**	BQL	BQL
ECR.15	**R1**	**3.93 ± 0.04**	1.20 ± 0.16	1.48 ± 0.06	**3.88 ± 0.06**	2.42 ± 0.00	BQL
	R2	**3.52 ± 0.01**	1.15 ± 0.12	**2.14 ± 0.04**	**3.44 ± 0.01**	2.30 ± 0.02	BQL
	R3	**3.87 ± 0.01**	**2.26 ± 0.04**	1.86 ± 0.01	**3.83 ± 0.02**	**2.39 ± 0.00**	BQL
ECR.18	**R1**	**3.95 ± 0.04**	**3.30 ± 0.02**	1.95 ± 0.02	**2.94 ± 0.03**	2.20 ± 0.03	BQL
	R2	**3.63 ± 0.03**	**1.73 ± 0.06**	2.20 ± 0.62	**2.61 ± 0.02**	2.25 ± 0.15	BQL
	R3	**3.72 ± 0.03**	**2.30 ± 0.01**	1.76 ± 0.03	**2.60 ± 0.01**	BQL	BQL
ECR.19	**R1**	**3.72 ± 0.01**	**2.33 ± 0.03**	**2.16 ± 0.13**	**2.89 ± 0.08**	BQL	BQL
	R2	**4.14 ± 0.01**	**1.95 ± 0.08**	**2.13 ± 0.04**	**3.42 ± 0.02**	BQL	BQL
	R3	**4.18 ± 0.01**	**2.42 ± 0.08**	**2.37 ± 0.08**	**3.50 ± 0.04**	BQL	2.11 ± 0.00
ECR.20	**R1**	**4.52 ± 0.00**	**1.28 ± 0.00**	**2.02 ± 0.01**	**4.18 ± 0.02**	BQL	BQL.
	R2	**4.61 ± 0.01**	1.31 ± 0.10	1.31 ± 0.02	**4.42 ± 0.07**	BQL	BQL
	R3	**4.74 ± 0.02**	**1.06 ± 0.49**	**2.01 ± 0.03**	**4.46 ± 0.02**	b.d.l.	BQL
ECR.22	**R1**	**4.51 ± 0.00**	1.37 ± 0.03	**2.02 ± 0.04**	**4.26 ± 0.04**	BQL	BQL
	R2	**4.27 ± 0.03**	1.52 ± 0.09	1.86 ± 0.15	**4.04 ± 0.03**	BQL	BQL
	R3	**4.79 ± 0.03**	**1.80 ± 0.15**	**2.07 ± 0.02**	**4.61 ± 0.02**	BQL	BQL

Table S5. *In silico* virulence genes detected in by VFanalyser, available at VFDB database (http://www.mgc.ac.cn/VFs/main.htm).

VFclass	Virulence factors	Related genes	ECR.1	ECR.15	ECR.18	ECR.19	ECR.20	ECR.22
Adherence	CFA/I fimbriae	*cfaA*	-	+	-	-	+	-
		cfaB	-	+	-	-	+	+
		cfaC	-	+	-	-	+	+
		cfaD/cfaE	-	+	-	-	+	+
	E. coli common pilus (ECP)	*ecpA*	+	+	+	+	+	+
		ecpB	+	+	+	+	+	+
		ecpC	+	+	+	+	+	+
		ecpD	+	+	+	+	+	+
		ecpE	+	+	+	+	+	+
	E. coli laminin-binding fimbriae (ELF)	*elfA*	-	+	+	-	+	+
		elfC	-	+	+	-	+	+
		elfD	-	+	+	-	-	+
		elfG	-	+	+	-	+	+
	EaeH	*eaeH*	+	+	+	+	+	+
	Hemorrhagic *E. coli* pilus (HCP)	*hcpA*	+	+	+	+	+	+
		hcpB	+	+	+	+	+	+
		hcpC	+	+	+	+	+	+
	P fimbriae	*papC*	-	-	-	-	-	+
		papD	-	-	-	-	-	+
		papH	-	-	-	-	-	+
		papI	+	-	-	+	-	-
	Type I fimbriae	*fimA*	+	+	-	-	+	-
		fimC	+	+	-	+	+	+
		fimD	+	+	+	+	+	+
		fimE	+	+	-	-	+	+
		fimF	+	+	+	+	+	+
		fimG	+	+	+	+	+	+
		fimH	+	+	-	+	+	+
		fimI	+	+	-	-	+	+
	Lateral flagella (Aeromonas)	*flgC*	+	-	-	+	-	-
	Type IV pili (*Yersinia*)	*pilQ*	-	+	-	-	+	+
		pilR	-	+	-	-	+	-
		pilS	-	+	-	-	+	+
		pilW	-	+	-	-	+	-
Autotransporter	AatA	*aatA*	-	-	-	-	-	+
	Antigen 43	*agn43*	+	-	-	+	-	-
	Cah	*cah*	+	-	+	+	+	-
	EhaA	*ehaA*	-	+	-	-	-	+
	EhaB	*ehaB*	+	+	+	+	+	+
	EspC	*espC*	+	-	-	+	-	-
	Sat	*sat*	+	-	-	+	-	-

	Temperature-sensitive hemagglutinin	*tsh*	+	-	-	+	-	-
	UpaG adhesin	*upaG/ehaG*	+	+	-	+	+	+
Invasion	Invasion of brain endothelial cells (Ibes)	*ibeB*	+	+	+	+	+	+
		ibeC	+	+	+	+	+	+
Iron uptake	Aerobactin siderophore	*iucA*	+	-	+	+	-	-
		iucB	+	-	+	+	-	-
		iucC	+	-	+	+	-	-
		iucD	+	-	+	+	-	-
		iutA	+	-	+	+	-	-
	Hemin uptake	*chuA*	+	-	-	+	-	-
		chuS	+	-	-	+	-	-
		chuT	+	-	-	+	-	-
		chuU	+	-	-	+	-	-
		chuW	+	-	-	+	-	-
		chuX	+	-	-	+	-	-
		chuY	+	-	-	+	-	-
	Iron/managanease transport	*sitA*	-	-	+	+	-	+
		sitB	-	-	+	+	-	+
		sitC	-	-	+	+	-	+
		sitD	-	-	+	+	-	+
	Salmochelin siderophore	*iroB*	+	-	-	-	-	+
		iroC	+	-	-	-	-	+
		iroD	+	-	-	-	-	+
		iroE	+	-	-	-	-	+
		iroN	+	-	-	-	-	+
	Yersiniabactin siderophore	*fyuA*	+	+	+	+	+	+
		irp1	+	+	+	+	+	+
		irp2	+	+	+	+	+	+
		ybtA	+	+	+	+	+	+
		ybtE	+	+	+	+	+	+
		ybtP	+	+	+	+	+	+
		ybtQ	+	+	+	+	+	+
		ybtS	+	+	+	+	+	+
		ybtT	+	+	+	+	+	+
		ybtU	+	+	+	+	+	+
		ybtX	+	+	+	+	+	+
Non-LEE encoded TTSS effectors	EspL1	*espL1*	-	+	+	-	+	+
	EspL4	*espL4*	-	+	+	-	+	+
	EspR1	*espR1*	-	+	-	-	+	+
	EspR4	*espR4*	-	-	+	-	-	-
	EspX1	*espX1*	-	+	+	-	+	+
	EspX4	*espX4*	-	+	+	-	+	+
	EspX5	*espX5*	-	+	+	-	+	+
	EspY1	*espY1*	-	-	+	-	-	-
Secretion system	ACE T6SS	*Undetermined*	-	+	+	-	-	+
		aec15	-	+	+	-	+	+
		aec16	-	+	+	-	-	+
		aec17	-	+	+	-	-	+

		aec18	-	+	+	-	-	+
		aec19	-	+	+	-	-	+
		aec22	-	+	+	-	-	-
		aec23	-	+	+	-	-	-
		aec24	-	+	+	-	-	-
		aec25	-	+	+	-	-	-
		aec26	-	+	+	-	-	-
		aec27/clpV	-	+	+	-	-	-
		aec28	-	+	+	-	-	-
		aec29	-	+	+	-	-	-
		aec30	-	+	+	-	-	-
		aec31	-	+	+	-	+	+
		aec32	-	+	+	-	+	+
	SCI-I T6SS	Undetermined	+	-	-	+	-	+
		Undetermined	+	-	-	+	-	+
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	-	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
		Undetermined	+	-	-	+	-	-
	Flagella (cluster I) (*Yersinia*)	*fliC*	-	+	-	-	-	-
	icm/dot type IVB locus (*Yersinia*)		-	-	-	-	-	+
Toxin	Colicin-like Usp	*usp*	+	-	-	+	-	-
	Enterotoxin SenB/TieB	*senB*	-	-	-	+	-	-
	Hemolysin/cytolysin A	*hlyE/clyA*	+	+	+	+	+	+
Antiphagocytosis	Capsule (*Klebsiella*)		-	+	+	-	-	-
		wcaI	-	+	-	-	-	-
		wzc	-	+	+	-	-	-
		wzi	-	+	+	-	-	-
		probable *wbaZ*	-	-	+	-	-	-
		wbaP	-	-	+	-	-	-
	Capsular polysaccharide (*Vibrio*)	*wbjD/wecB*	-	-	-	-	+	-
Fimbrial adherence determinants	Stj (*Salmonella*)	*stjC*	-	-	+	-	-	-

Immune evasion	Exopolysaccharide (*Haemophilus*)	*galE*	-	-	+	-	-	+
Serum resistance	LPS rfb locus (*Klebsiella*)		-	+	+	-	+	-
Others	O-antigen (*Yersinia*)		-	-	-	-	+	-

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Chapter III

Final considerations and future work

The study here presented was envisioned within the scope of the project StARE (Stop Antibiotic Resistance Evolution; https://stareeurope.wordpress.com). One of its prime objectives was to gather data regarding the occurrence and prevalence of ARGs and ARB in European wastewater (with standardized protocols), before and after treatment (influent and effluent, respectively), since this kind of information is already available for clinical settings – enabling to study antibiotic resistance evolution and develop tackling measures – but not for the environment (though wastewater has been recognized as major disperser of antibiotics resistance). By doing so, it would be possible to elucidate the effect of wastewater treatments on the resistome and help developing treatments that enable the removal off emerging contaminants, such as ARB and ARGs. In this extent, the effectiveness of UV-C disinfection step was previously evaluated in the removal of the clinically relevant *Enterobacteriaceae* in a Portuguese full-scale WWTP (Silva *et al*., 2018). From this, we learned that, although significant removal took place, some bacterial strains still survived UV-C treatment and were released into the environment. Despite the fact that effectiveness of a given WWTP's treatment is usually measured by its removal capacity, the characteristics of the surviving bacteria are also of relevance when considering treatment efficiencies – often ignored in previous studies. As such, we set to characterize in extent a collection of UV-C-surviving *E. coli* strains, since this species is pathogenic or commensal to humans (Croxen *et al*., 2013), has been shown to persist in environmental settings (Jang *et al*., 2017) and constitutes a reservoir and a vehicle for the dispersion of antibiotic resistance (due to readily acquisition of ARGs) (Poirel *et al*., 2018).

To understand the risk these *E. coli* strains may pose, we employed a three-part analysis, which included antibiotic resistance, virulence and environmental persistence assessment. From this we could conclude that ESBL-producing and MDR *E. coli* survived UV-C irradiation at a WWTP while maintaining virulent attributes (as evidenced by cell line- and larvae-based assays), conjugative plasmids (showed by mating assays) and the potentially ability to survive in natural freshwater environments (at least for 28 days; as implied by microcosm experiments and ST affiliation to successfully disseminated bacterial lineages), where they constitute a risk for antibiotic resistance dissemination and impose health safe concerns.

There are obvious constrains to this study. *E. coli* is not the only potential pathogen found in wastewater, and so, an analysis focused on this species will not provide a

comprehensive picture of the risk to human health imposed by the release of a WWTP's treated effluent. Furthermore, we only characterized a collection of 25 strains (considered low from a sampling effort standpoint), which may shade doubt to the real representativeness of our results. Still, these were necessary compromises for the feasibility of this study. In fact, the selected strains, obtained in the scope of the previous study, were representative (i.e. sharing identical molecular typing profiles) of 93 of the 282 characterized cefotaxime-resistant isolates, which were identified as *E. coli* (n=205), *K. pneumoniae* (n=70), *Citrobacter* sp. (n=6) and *Enterobacter* sp. (n=1) (Silva *et al*., 2018). Another bottleneck of our study was the fact that, although we attempted to extrapolate the potential risk these strains could represent when released in the environment, doses were not evaluated. As such, though we know the amounts of cells released, we are unaware of how much cells actually remain in the environment (and their concentration – dilution factor must be considered) and the concentration of bacteria necessary to cause infection and transfer resistance to non-related taxa. Therefore, we solely indicate a potential qualitative risk.

Further studies in other WWTPs (evaluating other taxa and the same or other wastewater processing steps) are needed since a multiplicity of factors influence the efficiency of treatments. One of the major constrains is actually related with the high variation of the wastewater's bacterial community between WWTPs (related with the physical and chemical properties of the wastewater as well as its origin), with the removal of ARGs conditioned by the persistence of their carriers (Hendriksen *et al*., 2019; Jensen *et al*., 2016; Manaia *et al*., 2018). Besides, the environmental compartment that receives the strains that survived treatment (e.g. water or soil) and its scale (e.g. size of the river or stream) are also major factors for evaluation of risk.

Globally, though UV-C treatment significantly reduced *Enterobacteriaceae* loads to "acceptable" levels (solely considering log reductions, since the Portuguese legislation for wastewater treatment or reuse does not include antibiotic resistance parameters; Decreto-Lei n.º 152/97 and Decreto-Lei n.º 119/2019, respectively), strains with concerning features were not removed. Since biological contaminants, like bacteria, can multiply, even the release of low concentration of these strains cannot be overlooked. As such, more studies are urgently needed to (i) understand the removal efficacy of other wastewater treatments, and the (ii) potential of combining treatments (a promising strategy to circumvent the current bottlenecks of UV-C irradiation), as well as an effort must be undertaken to (iii) characterize collections of strains of different taxa that are found in treated effluents of WWTPs

(subjected to different treatment steps) to infer real effectiveness of a treatment step and understand potential risk, as performed in this study.

Notwithstanding, several questions and future possibilities arise from this work. Below are indicated the most pressing:

I. Virulence phenotypes were explored using Vero cell line (Green monkey kidney epithelial cells) and *G. mellonella* larvae. These approaches suffer from limitations, in the sense of the responses they give, and further experiments including other cell lines (e.g. of intestinal origin) or higher complex models (e.g. zebra fish) could be performed to build a more trustworthy evaluation of *in vitro* and *in vivo* pathogenicity. In fact, the simplicity of cell cultures monolayers (in terms of complexity) and even of the organism *G. mellonella*, compared to the human body, have their limitations, which may under or overestimate the pathogenicity of the tested strains (Barber *et al*., 2016; Tsai *et al*., 2016). Furthermore, since pathogens usually display different virulence to specific tissues, the selection of the cell lines to be used is also essential, when delimiting a cytotoxicity and/or invasion assay. However, when using more complex beings, such zebra fish, ethical limitations exist (Decreto-Lei n.º 113/2013), unlike assays based on *G. mellonella* (Tsai *et al*., 2016). Besides, the later are easily manipulated and their short life cycle is ideal to readily obtain results (Tsai *et al*., 2016).

II. Putative persistence in freshwater was assessed by an easily implementable 28 days long microcosms. A longer incubation period was not performed due to limitations in sample volumes, although considering that ECR.19 consistently survive throughout this experiment, implementing a longer-term microcosm would be ideal. This could unravel the amount of time this strain can persist in river water or if it becomes part of the water's bacterial community (becomes naturalized, and is thus maintained over time) while retaining concerning virulent and antibiotic resistant features – scenario where it would constitute an enormous risk. In fact, while antibiotic resistance was evaluated (and shown to be maintained) in colonies retrieved from the last sampling point of the microcosm, virulence was not determined and could be explored in the future. This could also be executed for transconjugants obtained in the mating assays, to ascertain if virulence features are plasmid-encoded and transferable. Furthermore, persistence of these strains can also be evaluated in river water with higher contamination levels, such as those

found in the river basin to which the WWTP evaluated in this study discharges its effluent.

III. To address limitations in terms of reliability of the experimental setup of the microcosms, we could escalate to a river mesocosm (i.e. an artificial river system; available at DBio, University of Aveiro), where settings will be more complex and present higher similarities to environmental conditions, and thus provide more accurate predictions of persistence.

IV. To further validate persistence of strains in freshwater, the river downstream of the WWTP studied could be sampled, in order to identify any surviving *E. coli* strain that we had previously detected in this study and thus confirm *in situ* survival.

V. Most of the whole-genome analysis conducted in this study were straightforward and as such, a more in-depth bioinformatic analysis should be performed, including: (i) detection of pathogenicity islands and exploring their structure, since these elements are highly related to virulence; (ii) further assembly of contigs belonging to the same plasmids by PCR and Sanger sequencing, to investigate which genes may be co-transferred and what functions are encoded in these elements; and (iii) comparative genomics between all the obtained genomes and genomes available at public databases, to understand if there are genetic features associated with the survival of these strains to UV-C disinfection – may provide an answer as to what optimization can be performed to this treatment to increase its effectiveness and promote the entire removal of the surviving *E. coli* strains here studied.

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