BMC Microbiology

RESEARCH

Open Access

Uropathogenic *Escherichia coli* (UPEC) that hides its identity: features of LC2 and EC73 strains from recurrent urinary tract infections

Linda Maurizi[1], Layla Musleh[1], Francesca Brunetti[1], Antonietta Lucia Conte[1], Anna Riccioli[2], Silvia Sideri[2], Maria Grazia Ammendolia[1*], Daniela Uccelletti[3,4], Emily Schifano[3,4], Marta De Angelis[1], Giusi Ianiro[5], Antonella Niro[5], Antimo Cutone[5], Maria Pia Conte[1] and Catia Longhi[1*]

Abstract

Background Uropathogenic *Escherichia coli* (UPEC) strains are the major causative agents of human urinary tract infections (UTIs). Many patients who develop UTIs will experience a recurrent UTI (RUTI) within 6 months despite antibiotic-mediated clearance of the initial infection. A significant proportion of RUTIs are caused by *E. coli* identical to the original strain. UPEC employs several strategies to adhere, colonize, and persist within the bladder niche. Knowledge about the mechanisms regulating specific host-pathogen interactions that promote bacterial persistence is necessary to develop new approaches to RUTI diagnosis and treatment.

Results LC2 and EC73 UPEC strains were collected from patients with RUTIs. *E. coli* CFT073 and K-12 MG1655 were used as reference strains. UPEC displayed phenotypic profiles like those of the general *E. coli* population. The pan-genome analysis revealed that LC2 harbored many unique genes encoding several different functions such as intracellular trafficking and secretion, and vesicular transport. Contrarily, EC73 was the strain with the lowest number of unique genes involved in replication, recombination, repair and cell wall/membrane/envelope biogenesis. LC2 and EC73 exhibited the capacity to invade bladder monolayers efficiently and to colonize the gut of *Caenorhabditis elegans*, with LC2 being significantly more virulent than EC73. T24 cells infected with EC73 and LC2 strains exhibited significantly increased mRNA levels of IL-6, IL-8, IL-1β and TNF-α. EC73 elicited the strongest cytokine response. Differently, no significant cytokine mRNA induction was detected in T24 cells infected with *E. coli* CFT073. LC2 and EC73 modulated the expression of proteins involved in reactive oxygen species (ROS) balance in infected cells, but to different extents.

Conclusion The acquisition of virulence factors by horizontal transfer of accessory DNA, other than being the cause of transformation to pathogenic strains, is responsible for the genomic plasticity. Our findings suggest that a key role in RUTIs could be played by certain bacterial strains that may benefit from peculiar abilities to adapt and potentially develop reservoirs of persistence across different host environments.

*Correspondence:
Maria Grazia Ammendolia
maria.ammendolia@iss.it
Catia Longhi
catia.longhi@uniroma1.it

Full list of author information is available at the end of the article

© The Author(s) 2025. **Open Access** This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by-nc-nd/4.0/.

Keywords Uropathogenic *Escherichia coli*, Recurrence, Urinary tract infections, Bladder

Introduction

Urinary tract infections (UTIs) rank among the most common bacterial illnesses in women, accounting for approximately one quarter of all reported cases. It is estimated that 50–60% of women will experience at least one episode during their lifetime. UTIs encompass a broad spectrum of clinical presentations, including cystitis, pyelonephritis, and urosepsis [1]. Following an initial UTI, approximately one-fourth of women will experience a second episode within six months and a substantial proportion will go on to develop recurrent UTIs (RUTIs) over the course of their lives [2]. Uropathogenic *Escherichia coli* (UPEC), responsible for about 80% of cases, establishes infection through the expression of a variety of virulence factors (VFs) [3].

The most critical factor contributing to the pathogenicity of UPEC is its ability to adhere to and invade host bladder epithelial cells, a characteristic identified over 20 years ago [4]. The invasion of the bladder wall is facilitated by interactions between the bacterial type I pili and uroplakin proteins expressed on the superficial umbrella cell layer [5]. Once internalized, UPEC may reside freely in the cytosol or within membrane-enclosed vesicles. Free cytosolic UPEC rapidly multiplies and forms intracellular bacterial communities (IBCs) [6, 7]. Simultaneously, it can persist chronically within uroepithelial cells by establishing quiescent intracellular reservoirs (QIRs) [8].

Chronic bladder infections are common and may manifest either latently, as QIRs, or actively, as asymptomatic bacteriuria (ASB/ABU) or chronic cystitis. In mice, as suggested by Hannan et al. (2012), the outcome of a bladder infection – whether QIRs, ASB, or chronic cystitis – is determined within the first few hours of infection [1]. This represents a putative host-pathogen mucosal checkpoint that influences susceptibility to RUTIs [1, 6].

In general, RUTIs can arise via two main mechanisms: reinfection or persistence. Reinfection occurs when a genetically distinct strain, different from the original one, causes a subsequent episode [9]. In contrast, persistence is caused by the original infecting strain, which remains indistinguishable from the initial isolate [10, 11]. For UPEC-related UTIs, more than 60% of recurrences are attributed to the original infecting strain. Same-strain RUTIs have been documented to persist in urine for several years after the initial episode [12]. Moreover, it has been demonstrated that UPEC can persist in the gut even after clearance from the bladder, subsequently recolonizing the urinary tract and triggering new infections [13].

Numerous efforts have sought to determine whether specific UPEC strains possess unique traits that enable bacterial survival, promote adhesion, colonization, and invasion and evasion of host defenses, thus enhancing persistence [14, 15]. However, findings remain inconclusive [16, 17]. It has been suggested that bacteria harboring a higher number of VFs, including adhesins, iron uptake systems, and toxins, are more likely to be associated with same-strain RUTI episodes than those with fewer VFs [18]. Still, no single VF has be found to be sufficient for UPEC to cause UTIs, nor has any specific virulence profile proven predictive of RUTI [12]. The most crucial factor in UPEC persistence appears to be its ability to form biofilm, which increases bladder fitness and antimicrobial resistance [19]. Recent studies have revealed that the low oxygen tension found in the bladder, coupled with the presence of terminal electron receptors in urine, promotes the preferential expression of *E. coli* biofilms [5].

Several studies have demonstrated that RUTIs, especially same-strain recurrences, result from complex interactions between the pathogen and the host [18]. In this context, it has been suggested that UPEC VFs are not only essential for the initial stages of infection but may also modulate host immune responses [20]. The innate immune response triggered by bladder epithelial cells plays a critical role in controlling both acute and persistent infections through several mechanisms. Initially, toll-like receptor 4 (TLR4) on urothelial cells recognizes UPEC and activates a signaling cascade leading to the secretion of pro-inflammatory cytokines, such as interleukin 6 (IL-6) and IL-1β. Upon invasion, urothelial cells eliminate bacteria via exocytosis, a process dependent on the GTPase Rab27B [21]. Furthermore, the host's inflammatory response rapidly recruits neutrophils into the bladder lumen, while infected bladder epithelial cells are exfoliated, effectively reducing bacterial load and lowering reinfection risk [22]. In fact, a large number of exfoliated bladder epithelial cells are often found in the urine UTI patients [23]. Although exfoliation and inflammation are effective in bacterial clearance, they also result in substantial tissue damage that must be repaired to restore the bladder's barrier function [24]. The presence of QIRs suggests that exfoliation can be a double-edged sword: while it reduces the bacterial load initially, it may simultaneously promote long-term persistence and recurrence [25]. UPEC virulence is often assessed in vitro by infecting urinary epithelial cells, where early modulation of inflammatory signaling has been observed [26, 27]. However, these systems fail to fully capture the intricate dynamics of host-pathogen interactions. To address this limitation, *Caenorhabditis elegans* has emerged as a valuable model organism for investigating microbial

pathogenicity, including UPEC-related infections [28–30]. While murine models remain the standard for studying UTI pathogenicity, they have limitations related to costs, labor, and ethical concerns, particularly for large-scale research. In contrast, *C. elegans* offers a low-cost, technically simple, and ethically sound alternative, making it well-suited for high-throughput investigations of bacterial virulence [31]. Studies have revealed that UPEC strains pathogenic to *C. elegans* often share key virulence traits with those infecting mammals, indicating a conserved set of determinants across host species [32]. Given the substantial economic burden of RUTIs and the growing problem of antibiotic resistance, elucidating UPEC virulence mechanisms to identify predictors of recurrence risk is of critical importance.

In this study, we investigated the persistence-related features of two UPEC strains, by conducting a whole genome sequencing and phenotypic analysis. To assess the host response to UPEC infection, we also analyzed the expression of pro-inflammatory cytokines and proteins involved in reactive oxygen species (ROS) balance.

Materials and methods

Bacterial strains and culture conditions

A total of four UPEC strains were analyzed:

i) *E. coli* LC2 was one of two UPEC isolates, collected over 4 years, sharing the same phenotypic profile and belonging to the same clonal group as revealed by random amplified polymorphic DNA (RAPD) analysis, identified in the urine of a woman suffering from RUTIs.
ii) *E. coli* EC73 is one of three UPEC strains (EC71, EC72, and EC73) collected over 2 years from a male suffering from RUTIs [33]. Both strains belonged to the bacterial collection from the Department of Public Health and Infectious Disease, "Sapienza" University of Rome.
iii) *E. coli* CFT073 (ATCC 700928), originally isolated from the blood and urine of a woman with acute pyelonephritis [34], and *E. coli* K-12 MG1655 (ATCC 700926) were used as control strains.

All strains were grown overnight at 37 °C in Brain Heart Infusion (BHI) broth (Oxoid, Rome, Italy) and on Trypticase Soy Agar (TSA) (Oxoid, Rome, Italy) and then stored in BHI with glycerol at – 80 °C.

Identification and susceptibility testing

The identification of strains was performed using both the VITEK 2 (BioMerieux Italia, Firenze, Italy) platform and MALDI-TOF mass spectrometry. The VITEK 2 compact Automated Expert System (AES, BioMérieux Italia, Firenze, Italy) was used for metabolic profiling. Gram-negative bacilli were identified using the VITEK 2 ID-GNB card (BioMérieux Italia, Firenze, Italy), testing the organism's metabolic activity. Results were interpreted by the Advanced Expert System software (AST-N202), using current EUCAST breakpoints (available on the EUCAST website: http://www.eucast.org). The antibiotics tested were: penicillins (amoxicillin, ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam); cephalosporins (cefalotin, cefuroxime, cefuroxime axetil, cefoxitin, cefotaxime, ceftazidime, cefepime); carbapenems (ertapenem, meropenem); monobactams (aztreonam); aminoglycosides (amikacin, gentamicin, tobramycin); fluoroquinolones (ciprofloxacin, ofloxacin); tigecycline and trimethoprim.

Bacterial DNA extraction and sequencing

A single colony of each *E. coli* strain grown on TSA was inoculated into Tryptic Soy Broth (TSB) and incubated overnight at 37 °C. A volume of 1.5 ml from each bacterial culture was centrifuged at 10,000 rpm for 10 min, and DNA extraction was conducted using QIAamp DNA Mini Kit (Qiagen, Germany), as the manufacturer's instruction. The qualitative and quantitative analysis of DNA extraction was assessed with the NanoDropTM spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA).

Genomic samples of EC73 and LC2 were then sequenced using 250-bp paired-end reads on the Illumine MiSeq system by Bio-Fab Research (Rome, Italy), producing about 7 million raw sequence reads in the Fastq format, corresponding to about 300-fold estimated genome coverage. Raw data were imported in Geneious v.7.1.9 (Biomatters, Inc., USA) and trimmed to remove index sequences, adapter sequences, and poor-quality sequenced bases. Genome was de novo assembled by using Velvet v. 1.1 [35] with parameters optimized by using the Velvet optimizer software available at https://github.com/tseemann/VelvetOptimiser.

Pan-genome analysis

The pan-genome of *E. coli* strains EC73, LC2, CFT073 (NCBI accession ID: AE014975.1), UTI89 (NCBI accession ID: NC_007946.1) and MG1655 (NCBI accession ID: ASM584v2) was characterized using the Bacterial Pan Genome Analysis (BPGA) pipeline v. 1.3 [36]. In this pipeline, orthologous gene families were identified based on a protein sequence identity > 80% and their function was predicted using the Clusters of Orthologous Groups (COG) database [37]. Pan-genome analysis, and data visualization were performed using the statistical programming language R v. 4.1.2 [38] along ggplot2 [39], VennDiagram [40] and RColorBrewer [41] packages. Alignments between the protein sequences of traA and fmlC were performed using BlastP [42].

In silico virulence genotyping

The presence of virulence-associated factors of extraintestinal pathogenic *Escherichia coli* (ExPEC) was determined by in silico analysis. Gene sequences were taken from GenBank and tested against the whole genomes of the CFT073, and UTI89 strains using the BLASTn algorithm included in BLAST + v.2.4.0. Hits presenting a query coverage of ≥ 90% and a pairwise identity percentage (percentage of pairwise residues that are identical in the alignment, including gap versus non-gap residues but excluding gap versus gap residues) of ≥ 85% were considered positive. For strains LC2 and EC73, the presence of virulence genes was tested on genomic data produced by next-generation sequencing.

Escherichia coli phylogenetic grouping

Belonging to a specific phylogroup was assessed by a polymerase chain reaction (PCR) assay targeting the genes *chuA* (required for heme transport in enterohemorrhagic O157:H7 *E. coli*), *yjaA* (initially identified in the complete genome sequence of *E. coli* K-12, the function of which is unknown) and a DNA fragment *TspE4. C2*. According to Clermont et al., 2000 [43] and based on the obtained amplification, *E. coli* strains are assigned to phylogroup A, B1, B2, or D. Amplifications were performed in triplicate and their size was determined by comparison with a 100-bp DNA ladder (Biolabs Inc., New England, UK).

Cell line and culture conditions

The T24 human bladder cancer cell line (transitional cell carcinoma) was obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Euroclone, Milan, Italy) and 1% penicillin-streptomycin (Euroclone, Milan, Italy). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

Hemolysis

The hemolysis assay was performed by using plates containing 5% defibrinated sheep blood (Oxoid Ltd., Basingstoke, UK). After bacterial seeding, the plates were examined up to 48 h of incubation at 37 °C for the presence of a hemolysis area around colonies.

Swimming motility assay

A swimming motility assay was performed on soft Luria Bertani (LB) agar plates (Oxoid, Rome, Italy) containing 0.25% agar, after overnight drying at 4 °C. A 2 µL aliquot of bacterial culture was used to seed the plates below the agar surface. Plates were then incubated at 37 °C for 24 h, and the diameters of swimming zones were measured [44].

Biofilm assay

Bacterial biofilm-forming ability was tested according to the method described by Stepanovic et al. [45]. Each well of a 96-well polystyrene microplate filled with 180 µL of TSB was inoculated with 20 µL of each bacterial strain (1–2 × 10^7 colony forming unit (CFU)/mL) and incubated at 37 °C for 24 h, then washed twice with phosphate-buffered saline (PBS) and fixed with methanol (99.8% v/v) for 15 min. Wells were then stained for 20 min with 1% w/v crystal violet (Sigma-Aldrich, USA), rinsed three times with H_2O, and eluted with 95% ethanol. The biofilm was quantified by optical density (OD) at 570 nm in a microplate reader (Tecan Sunrise, X-fluor, Tecan, Männedorf, Switzerland). Based on the cut-off OD, defined as three standard deviations (SDs) above the mean OD of the negative control (ODc), strains were classified as non-biofilm producers (OD ≤ ODc), weak biofilm producers (ODc < OD ≤ 2 × ODc), moderate biofilm producers (2 × ODc < OD ≤ 4 × ODc), strong biofilm producers (4 × ODc < OD).

Adhesion, invasion, and intracellular survival assays

The adhesiveness of *E. coli* strains was assayed on T24 cells seeded in a 24-well plate (0.5 mL/well) at a density of 2 × 10 ^5 cells/mL. After 24 h of incubation at 37 °C in 5% CO2, monolayers were infected with 0.5 mL of bacterial suspension (approximately 2 × 10^6 UFC/mL) at a multiplicity of infection (MOI) of 10 (or 0.1 in the case of *E. coli* CFT073). To synchronize the infection, the plates were centrifuged twice at 500 x g for 2.5 min and incubated at 37 °C in 5% CO2 for 30 min. To determine the number of adherent bacteria, the cells were extensively washed with PBS, lysed adding 0.1% (v/v) Triton X-100, and plated on TSA plates for 24 h at 37 °C. *E. coli* strains were considered adherent when the mean adhesion index (number of adherent bacteria/initial inoculum) was ≥ 0.8%.

For invasion assay, T24 cells, 1 h post-infection, were washed three times with PBS and then incubated for 1 h at 37 °C in 5% CO_2 in a growth medium containing 100 µg/mL gentamicin (GM) (Thermo Fisher Scientific, Waltham, MA, USA), to kill extracellular bacteria, and lysed as above described. For survival assay, after the invasion time, cell monolayers were incubated again with a growth medium containing 50 µg/mL of GM for further 24 h at 37 °C in 5% CO_2 and the number of viable bacteria was determined by plating the dilutions of cell lysates in TSA agar. When the ratio between the number of intracellular bacteria and the initial inoculum was ≥ 0.1%, a strain was considered invasive. Strains were considered able to survive when the number of intracellular bacteria

recovered at 24 h was comparable to that recovered 2 h post-infection (100%). As a negative control, *E. coli* K-12 MG1655 was used in the invasion and survival assays.

Analysis of proinflammatory cytokines induced by UPEC infection

Cytokine expression was evaluated 2 h after infection of human T24 cells with *E. coli* strains. Following the infection time, cells were washed, and total RNA was extracted using TRIzol reagent (Invitrogen Carlsbad, CA) according to the manufacturer's instructions. Total RNA (1 µg/sample) was retro transcribed into cDNA, and Real-Time quantitative PCR (RTqPCR) was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Waltham, Massachusetts, USA). 1 µg of cDNA was used for each Real-Time reaction; analysis was performed in triplicate in 3 independent experiments. The geometric mean of the housekeeping gene β-actin was used to normalize the expression levels of the target genes. Relative mRNA expression was calculated using the ΔΔCT method [46]. Primer sequences are listed in Table 1.

To determine IL-6 production, T24 cells were infected with *E. coli* strains MG1655, LC2 and EC73 strains at a MOI of 10 and with *E. coli* CFT073 at a MOI 0.1 and incubated at 37 °C, as described above. Cell culture supernatants were collected 24 h post-infection and analyzed for human IL-6 levels using an ELISA kit (R&D Systems, Wiesbaden, Germany), according the manufacturer's instructions.

Evaluation of ROS production

ROS production was evaluated in T24 cells 24 h after infection with *E. coli* strains. ROS generation was monitored using the peroxide-sensitive fluorescent compound, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Following infection, monolayers were washed with PBS and incubated with 10 µM H_2DCFDA for 30 min at 37 °C. After three PBS washes, fluorescence intensity was measured by flow cytometer (Perkin-Elmer, Massachusetts, USA) at a wavelength of 488/535 nm. Untreated cells and cells treated with hydrogen peroxide served as negative and positive controls, respectively.

Table 1 Primers used in RT-qPCR for the detection of interleukins

GENE	PRIMER SEQUENCES
IL8-Fw	5'-GTGAAGGTGCAGTTTTGCCA – 3'
IL8-Rev	5'- TCTCCACAACCCTCTGCAC-3'
IL1β-Fw	5'-ACAGATGAAGTGCTCCTTCCA – 3'
IL1β -Rev	5'-GTCGGAGATTCGTAGCTGGAT – 3'
IL6-Fw	5'-GGTACATCCTCGACGGCATCT-3'
IL6-Rev	5'-GTGCCTCTTTGCTGCTTTCAC-3'
TNFα-Fw	5'-AGGCGGTGCTTGTTCCTCA – 3'
TNFα-Rev	5'-GTTCGAGAAGATGATCTGACTGCC – 3'
β Actin -Fw	5'-TGCACCACACCTTCTACAATGA – 3'
β Actin -Rev	5'-CAGCCTGGATAGCAACGTACA – 3'

Western blotting

T24 cells were lysed on ice for 1 h in a buffer containing 25 mM MOPS (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), 2 µM leupeptin, and 2 µM pepstatin. Total protein concentration was assessed using the Bradford method [47]. Protein extracts (40 µg) were separated by Sodium Dodecyl Sulphate – PolyAcrylamid Gel Electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, UK) by electroblotting. After transfer, membranes were incubated for 1 h at room temperature with primary antibodies diluted in PBS containing 5% non-fat dry milk and 0.05% Tween-20 (Blotting-Grade Blocker, PanReac AppliChem, ITW Reagents, Monza, Italy). The primary antibodies used included anti-β-Actin (A2066 Sigma-Aldrich, Milan, Italy) (1:1000), anti-System Xc^- (Ab175186 Abcam, Milan, Italy) (1:1000), anti-SOD-1 (sc-17767) (1:250), anti-SOD-2 (sc-137254) (1:500), anti-GCL (sc-390811, Santa Cruz) (1:1000), anti-Nox2 (sc-130543, Santa Cruz) (1:500), anti-Catalase (SC271803, Santa Cruz) (1:1000), anti-α-Tubulin (sc-23948) (1:1000), and anti-GAPDH (sc-47724) (1:1000). Membranes were then incubated for 1 h at room temperature with the appropriate HRP-conjugated secondary antibody (Biorad) (1:1000) in PBS-T supplemented with 2.5% milk. Protein detection was carried out using the Clarity Western ECL substrate (170–5061, Biorad). Protein expression levels were normalized against β-Actin, α-Tubulin, or GAPDH.

Caenorhabditis elegans infection assays and colonization capability

Exponentially growing *E. coli* cultures were prepared in LB broth at 37 °C. For nematode infection assays, 30 µL of each bacterial culture, standardized to approximately 1 × 10^8 cells, was spread onto Nematode Growth Medium (NGM) agar plates (35 mm). Plates were incubated at 37 °C for 24 h to allow the formation of bacterial lawns, and then seeded with synchronized young adult nematodes grown at 16 °C [48]. Infections were conducted at 25 °C for several days, with nematodes transferred daily to fresh plates. Worm viability was monitored by assessing movement in response to gentle touch. All experiments were performed in triplicate. To estimate the number of viable *E. coli* cells in the intestinal tract of *C. elegans* after 48 h of infection, the method of Schifano and colleagues [49] was adopted.

For persistence assays, synchronized *C. elegans* N2 worms at day 1 of adulthood were exposed to a 1:1

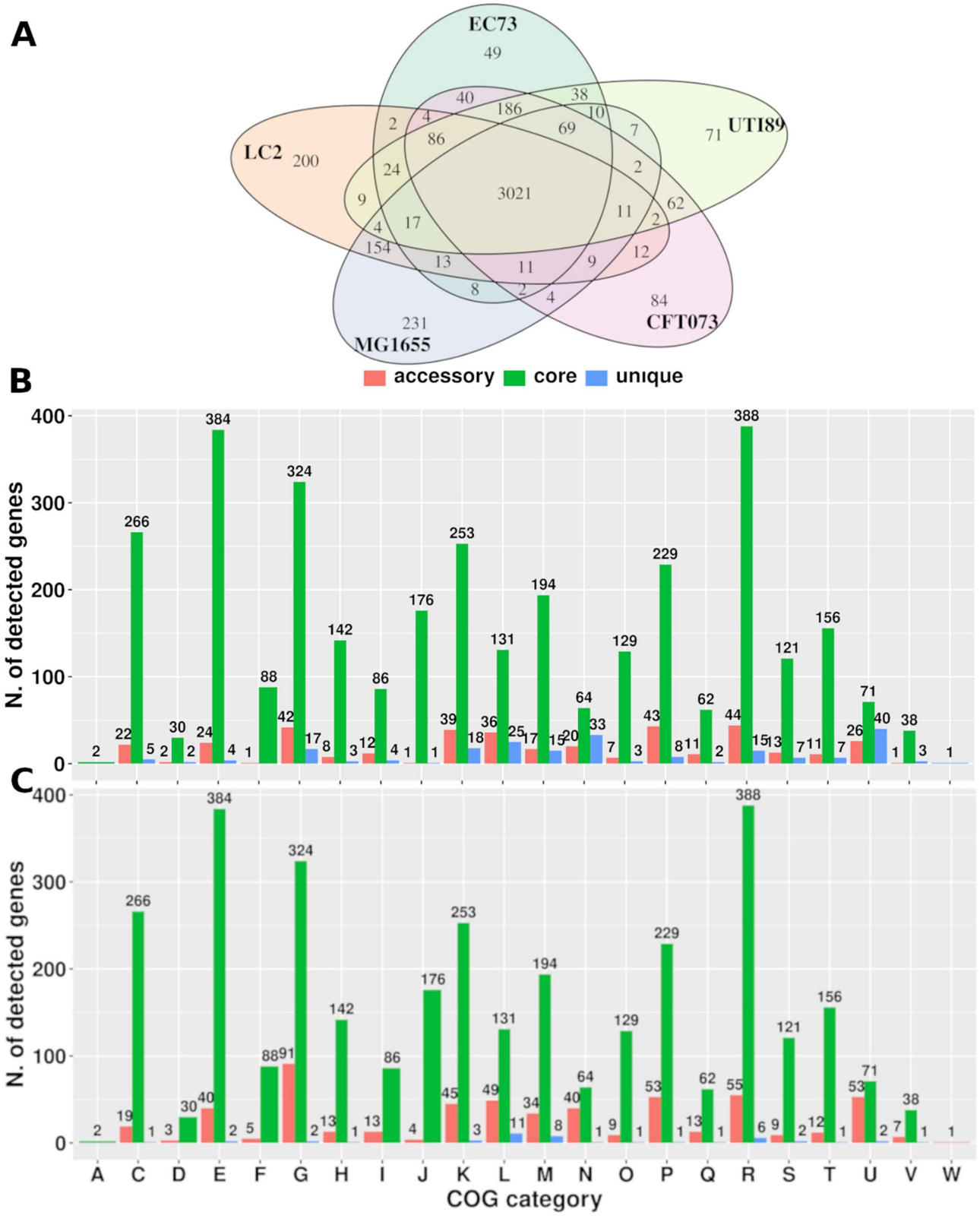

Fig. 1 (See legend on next page.)

(See figure on previous page.)

Fig. 1 Pan-genome analysis of *E. coli* strains LC2, EC73, UTI89, CFT073 and MG1655. **A** Each circle in the Venn diagram represents a single strain (indicated in bold), while the numbers correspond to core, accessory, and unique genes detected in the pan-genome. Overlapping regions reflect the number of genes shared between the corresponding circles (hence, strains). **B**, **C** COG functional analysis of LC2 (**B**) and EC73 (**C**). For each COG functional category, the different bar color indicates the number of associated core-, accessory- and unique-genes detected in the individual strains. COG categories: A = RNA processing and modification; C = Energy production & conversion; D = Cell cycle control, cell division, chromosome partitioning; E = Amino acid transport & metabolism; F = Nucleotide transport & metabolism; G = Carbohydrate transport & metabolism; H = Coenzyme transport & metabolism; I = Lipid transport & metabolism; J = Translation, ribosomal structure & biogenesis; K = Transcription; L = Replication, recombination & repair; M = Cell wall/membrane/envelope biogenesis; N = Cell motility; O = Post-translational modification, protein turnover & chaperones; P = Inorganic ion transport & metabolism; Q = Secondary metabolites biosynthesis, transport & catabolism; R = General function prediction only; S = Function unknown; T = Signal transduction mechanisms; U = Intracellular trafficking, secretion & vesicular transport; V = Defense mechanisms; W = Extracellular structures.

mixture of *E. coli* OP50-GFP and either LC2 or EC73 strains. At 24 and 48 h post-infection, 15 worms per group were anesthetized with sodium azide (20 mmol L^{-1}; Sigma-Aldrich, St. Louis, MO, USA) and examined using a Zeiss Axiovert 25 fluorescence microscope. Each experiment was performed in triplicate. Worms fed exclusively with OP50-GFP were used as a control group. Fluorescence intensity was quantified using ImageJ software.

Results

Pan-genome analysis of UPEC strains

A pan-genome analysis of EC73, LC2, UTI89, CFT073 and MG1655 was performed to assess potential associations with persistent phenotypes. A total of 4,442 gene families were identified in the pan-genome of the five strains (Fig. 1A), where 68% of genes ($n = 3{,}021$) were found in all strains, 17.7% ($n = 786$) were found in multiple strains but not all and 14.3% were found in a single strain ($n = 635$). For clarity, we will refer to these 3 sets of genes as *core*, *accessory*, and *unique*, respectively.

As both LC2 and EC73 show a persistent phenotypic behavior, we first investigated the potential existence of a common pool of genes. Only two genes were found in common between the two strains (Fig. 1A): *tra*A (F-Pilin precursor) and *flm*C (putative F-plasmid maintenance protein C). For both genes, we inferred and compared their protein sequences between the two strains, revealing a sequence similarity of 97.5% and 90% in the case of *tra*A and *flm*C, respectively. Although further studies will be conducted to investigate the features and the role of genes in these strains, these results suggest distinct genetic repertoire for these strains selected for their respective habitat.

To test our hypothesis, we analyzed the set of genes exclusively found in either EC73 or LC2. We observed that LC2 harbored a large number of unique genes ($n = 200$), second only to MG1655 ($n = 231$). These genes account for 5.6% of its genome ($n = 3{,}579$ genes) and encodes for several different functions (Fig. 1B, blue bars), such as intracellular trafficking, secretion and vesicular transport (COG category = *U*;$n = 40$ detected genes), cell motility (COG category = N; $n = 33$), replication, recombination and repair (COG category = *L*;$n = 25$), transcription (COG category = K; $n = 18$), carbohydrate transport and metabolism (COG category = G; $n = 17$), and cell wall/membrane/envelope biogenesis (COG category = *M*;$n = 15$). Contrarily, EC73 was the strain with the lowest number of unique genes ($n = 49$). These genes encoded different functions (Fig. 1C, blue bars) where the majority was involved in replication, recombination and repair (COG category = L; $n = 11$) and cell wall/membrane/envelope biogenesis (COG category = M; $n = 8$).

LC2 and EC73 strains share key virulence factors with UPEC strains

As detailed in Fig. 2, in silico analysis revealed that, unlike *E. coli* CFT073, neither *E. coli* LC2 nor *E. coli* EC73 possessed the *hly*A gene or the *cnf*1 gene, both present in UPEC as *E. coli* UTI 89 and *E. coli* 83,972. The haemolytic activity study demonstrated that only *E. coli* CFT073 exhibited lytic activity on blood agar plates. However, *E. coli* LC2 and EC73 shared several VFs, including the type 1 fimbriae gene cluster (*fim*), a major determinant of adhesion and invasion of both intestinal and urinary tract epithelial cells, and the group II capsule polysaccharide gene cluster (*kps*MTII), which is present in the majority of the ExPEC strains.

LC2 carries virulence factors of diarrhoeagenic E. coli

It has been previously demonstrated that UPEC and enteropathogenic *E. coli* (EPEC) strains such as enteroaggregative *E. coli* (EAEC) share common pathogenic factors [50], including *agg*R, *aap* and *aat*A genes, which are primarily located on the 60-MDa aggregative adherence plasmid (pAA) [51]. In our study, the *aap* and *aat*A genes were present only in *E. coli* LC2 strain. The *aap* gene encodes an anti-aggregation protein that forms a bacterial capsule, preventing bacterial accumulation and facilitating bacteria dispersion [52]. The *aat*A gene encodes part of an outer membrane transport system necessary for the translocation of pathogenic proteins such as aap [50]. Furthermore, *E. coli* LC2 carried the EAEC heat-stable enterotoxin 1-coding gene *ast*A located on plasmid pAA [53], and exhibited the typical EAEC aggregative adherence phenotype on T24 bladder epithelial cells (data not shown). In addition, LC2 had the outer membrane invasion/intimin-like protein gene *eaeX*. *EaeX* encodes for a high molecular weight protein that bears

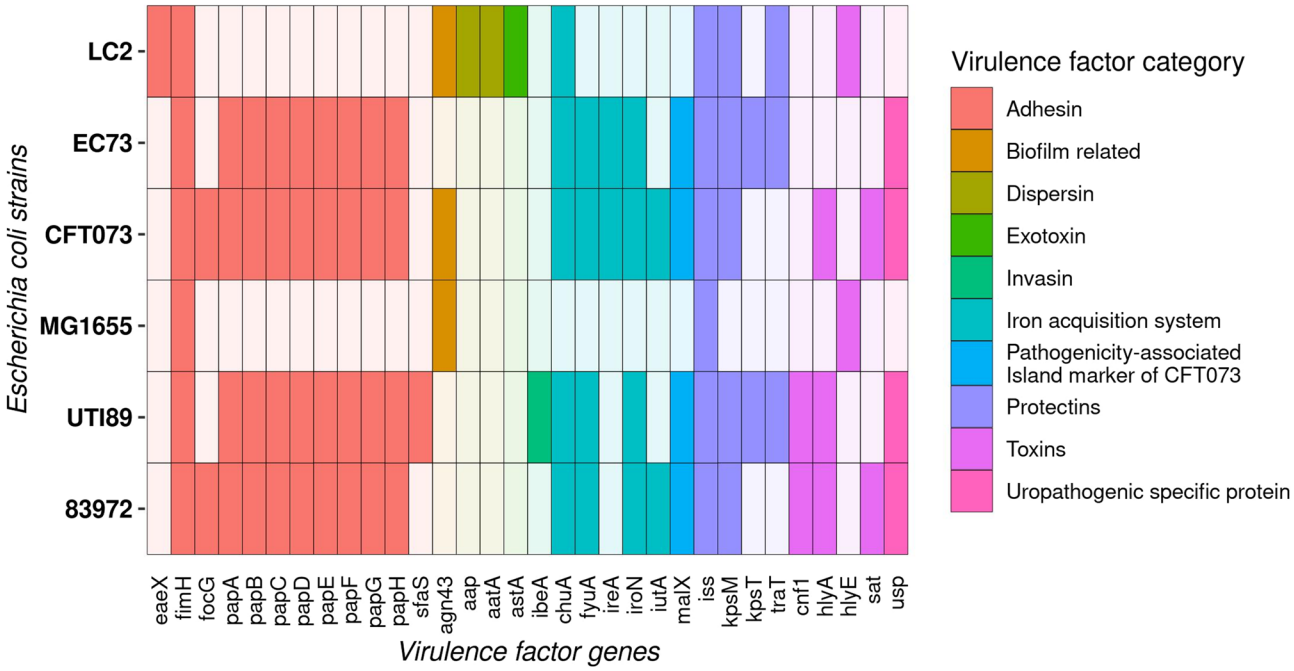

Fig. 2 In silico analysis of VFs typical of UPEC strains. The presence or absence of 30 selected VFs assessed by in silico analysis across *E. coli* strains LC2, EC73, CFT073, MG1655, UTI89, and 83,972. The heatmap displays the distribution of genes according to functional VF categories, each represented by a distinct color

Table 2 UPEC features

	STRAINS			
	***E. coli* LC2**	***E. coli* EC73**	***E. coli* CFT073**	***E. coli* K-12 MG1655**
Phylogenetic group	D	B2	B2	A
Hemolysis	-	-	+	-
Motility	+++	+	+++	+
Biofilm production	MODERATE	STRONG	STRONG	WEAK

significant homology to the outer membrane adhesin invasin of *Yersinia* spp., and intimin of enteropathogenic *E. coli* [54].

Phylogenetic group belonging and UPEC features

E. coli strains were classified into the major phylogenetic classes A, B1, B2, or D, via a rapid and easy phylogenetic grouping technique based on triplex PCR, developed to detect the *chu*A, *yjaA*, and *TspE4* genes. We found that *E. coli* LC2 belonged to the D phylogroup, whereas, as previously shown, *E. coli* EC73 and *E. coli* CFT073 were classified as B2 (Table 2).

The ability of UPEC to orchestrate both adhesion and motility is critical for its successful colonization and ascending infection in the urinary tract [55]. The swimming motility of UPEC was evaluated by measuring the diameters of the swimming zone. As previously demonstrated for *E. coli* CFT073 [56], *E. coli* LC2 also exhibited high motility (Table 2). Since motility enables UPEC to migrate to new areas and facilitate biofilm expansion, biofilm production was quantitatively assessed in our study according to the method described by Stepanović and collaborators [45]. *E. coli* EC73 confirmed its strong ability to form biofilm, while *E. coli* LC2 was a moderate biofilm producer. As expected, *E. coli* CFT073 and *E. coli* MG1655 were confirmed as biofilm producers, albeit to varying extents [57].

Motility was expressed by the mean diameter (centimeters) of the motility zone of each group, based on duplicate measurements obtained from two independent experiments performed on each isolate tested. +: $1 > cm \leq 2$; ++: $2 > cm \leq 3$; +++: >3 cm.

LC2 and EC73 strains exhibit virulence features typically associated with *E. coli* strains responsible for extraintestinal infections

The UPEC isolates displayed biochemical profiles like those of the general *E. coli* population. Metabolic reactions, such as lactate alkalinization, were observed both in *E. coli* LC2 and *E. coli* EC73 strains. Antibiotic resistance screening revealed that *E. coli* LC2 exhibited full susceptibility to fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole, penicillins, and tetracycline. On the other hand, *E. coli* EC73 presented only an intermediate resistance to ciprofloxacin.

LC2 and EC73 strains exhibit the ability to adhere to, invade and survive within T24 cell monolayers

UTIs usually start with UPEC contamination, followed by colonization of the urethra and migration into the bladder lumen. The infection cycle involves a complex sequence of events, including epithelial attachment, invasion of host cells, intracellular proliferation, and eventual rupture of bladder epithelial cells, resulting in the dissemination and reinfection of surrounding epithelial cells. The adhesive, invasive, and intracellular survival capacities of UPEC strains were evaluated by infecting T24 cell monolayers. All tested isolates demonstrated adhesion to cell monolayers (Table 3). The ability of the strains to invade and survive within the infected host cells was evaluated by the gentamicin protection assay. As shown in Table 3, E. *coli* LC2, EC73 and CFT073 exhibited the capacity to invade bladder cells, in contrast to the non-invasive control *E. coli* K-12 strain MG1655. Consistent with previous studies, exposure of bladder cells to the *E. coli* CFT073 strain resulted in significant cytotoxicity [58], thus a sub lytic MOI of 0.1 was used. Following exposure of T24 cell monolayers to *E. coli* LC2 and EC73 strains, the number of intracellular bacteria progressively declined over the next 24 h.

The results were expressed as CFU/mL or as percentage (%) respect to the intracellular bacteria. Data were expressed as mean ± standard deviation (SD).

LC2 and EC73 strains induce inflammatory cytokine expression in bladder epithelial infected cells, but at different extents

To investigate the pro-inflammatory cytokine expression patterns in T24 bladder epithelial cells infected with UTI-associated *E. coli* strains, we performed RT-qPCR on total RNA to evaluate cytokine induction 2 h post-infection. Compared to uninfected controls, T24 cells infected with MG1655, EC73 and LC2 strains exhibited significantly increased mRNA levels of IL-6, IL-8, IL-1β and tumor necrosis factor (TNF)-α. Among these, *E. coli* EC73 elicited the strongest cytokine response, surpassing the levels induced in order by *E. coli* MG1655 and LC2 strains. In contrast, no significant cytokine mRNA induction was detected in T24 cells infected with *E. coli* CFT073 (Fig. 3).

Among the cytokines modulated by UPEC strains, IL-6 plays a key role in limiting pathogen invasion of the uroepithelium and controlling infection. To confirm the transcriptional data, IL-6 protein levels were quantified by ELISA. The results were consistent with those obtained by RT-qPCR analysis: with the exception of CFT073, all tested strains exhibited significantly higher IL-6 levels compared to the control, although with a different extent (Fig. 4).

Table 3 Adhesion, invasion and survival ability of *E. coli* strains, evaluated by gentamicin protection assay

Strains	Adhesion CFU/mL ± SD (%± SD)	Invasion CFU/mL ± SD (%± SD)	Survival CFU/mL ± SD (%± SD)
***E. coli* LC2**	1.40E + 05 ± 228.30 (7.80 ± 0.35)	3.10E + 03 ± 1048.80 (0.34 ± 0.25)	2.35E + 03 ± 120.2 (75.8 ± 35.9)
***E. coli* EC73**	1.00E + 05 ± 675.99 (6.40 ± 2.74)	1.43E + 04 ± 16780.50 (0.90 ± 0.55)	4.33E + 03 ± 369.2 (30.3 ± 29.0)
***E. coli* CFT073**	1.13E + 05 ± 197.65 (10.7 ± 1.54)	2.05E + 02 ± 149.5 (0.51 ± 0.13)	0
***E. coli* K-12 MG1655**	9.10E + 04 ± 229.07 (2.27 ± 0.80)	0	0

LC2 and EC73 strains induce a significant increase in ROS production compared to uninfected bladder cells

The production of ROS species in T24 cells, infected with different UPEC strains, was verified by using the ROS indicator dye H_2DCFA. At 24 h post-infection, all UPEC strains tested induced a significant increase in ROS production compared to uninfected bladder cells (Fig. 5).

LC2 and EC73 strains modulate the expression of proteins involved in ROS balance in bladder epithelial infected cells but at different extents

The expression levels of proteins involved in ROS balance were assessed through Western blot analysis in T24 cells infected with various *E. coli* strains, including the commensal MG1655 and pathogenic LC2, EC73, and CFT073 strains. First, components of the glutathione biosynthesis pathway, specifically System Xc^- and Glutamate-Cysteine Ligase (GCL), were examined. As shown in Fig. 6, T24 cells displayed a significantly diminished expression of System Xc^- upon infection with LC2, EC73, and CFT073 strains compared to uninfected control cells. The MG1655 strain also caused a slight decrease in System Xc^- expression relative to the control, though this change was less pronounced and not statistically significant. When compared to pathogenic strains, MG1655 exhibited a significant difference only with the EC73 strain in influencing the levels of System Xc^-. In line with the downregulation of System Xc^-, a significant reduction in GCL expression was observed in cells infected with all three pathogenic *E. coli* strains compared to uninfected cells or cells infected with the commensal MG1655 strain, which did not induce any change in GCL expression.

Regarding Superoxide Dismutase (SOD) expression, both LC2 and EC73 strains upregulated the expression of SOD-1 and SOD-2 variants in infected cells, indicating stimulation of the antioxidant response, with EC73 inducing the highest increase of both isoforms compared to uninfected cells. Notably, the pathogenic CFT073 strain did not exhibit any ability to enhance the expression of these antioxidant proteins, with levels comparable

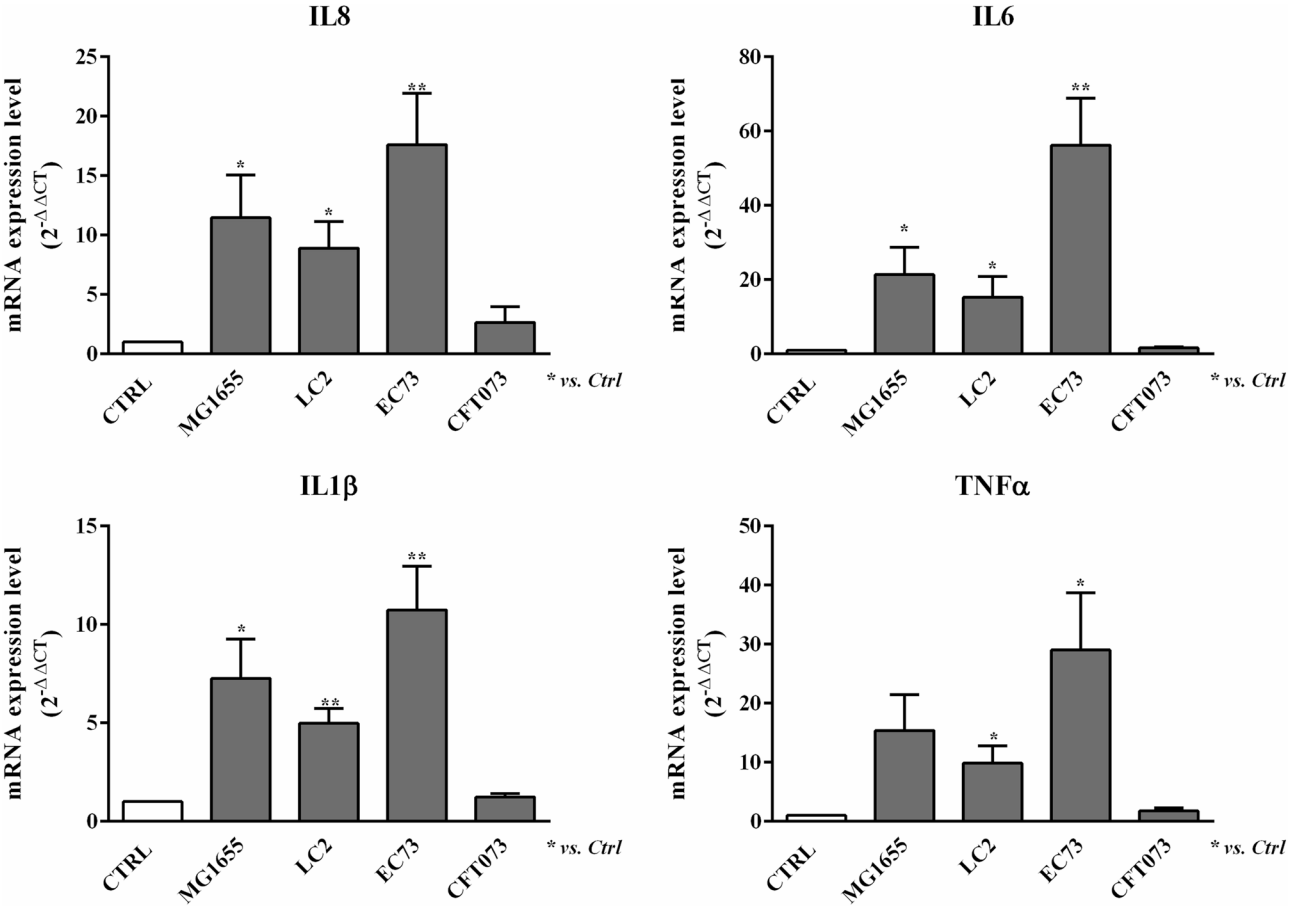

Fig. 3 RT-qPCR analysis of different cytokine mRNA levels in bladder epithelial cells T24 infected with *E. coli* strains. Values were reported as mean ± SD of triplicate samples from three independent experiments. Statistical significance was determined by one-way ANOVA followed by post hoc analysis; asterisks indicate significant differences (** $p < 0.01$; * $p < 0.05$)

to uninfected cells. The commensal MG1655 strain was able to increase, though not significantly, the expression of only the SOD-2 isoform. Interestingly, catalase, the enzyme responsible for decomposing hydrogen peroxide, was overexpressed exclusively upon the infection with the CFT073 strain compared to control or cells infected with other pathogenic strains.

As far as pro-oxidant enzymes, NADPH oxidase 2 (Nox-2) was overexpressed upon infection with both LC2 and EC73 strains compared to uninfected cells, with the highest increase observed upon EC73 infection. In contrast, both the commensal MG1655 and pathogenic CFT073 strains showed no variation in Nox-2 expression, which remained at baseline levels compared to uninfected cells (Fig. 6).

LC2 and EC73 strains exhibit intestinal colonization levels of *C. elegans* but LC2 is significantly more virulent than EC73

To further explore the virulence of the UPEC isolates, an *in vivo C. elegans* infection model was employed. In this assay, the survival of *C. elegans* worms fed on pure cultures of each strain was measured. As shown in Fig. 7A, survival data revealed that approximately half of the LC2-fed worms died by day 5, emphasizing the higher virulence of this strain compared to both EC73 and the control strains, which showed > 50% survival at day 6.

To further assess the extent of infection, bacterial colonization within the *C. elegans* gut was quantified using CFU counts after 3 days of infection. The LC2 and EC73 strains exhibited intestinal colonization levels that were 30% and 40% higher, respectively, than those observed with the CFT073 strain (Fig. 7B). In contrast, colonization by MG1655 was 60% lower than that of the CFT073 strain, demonstrating significantly reduced persistence in the nematode gut. Similar findings were also obtained when *C. elegans* was infected with a reduced load of LC2 or EC73, supplemented together with *E. coli* OP50-GFP (Fig. 8).

Discussion

Many studies provide evidence that RUTIs are mainly driven by the persistence of the original strain in the host despite appropriate antibiotic treatments [59]. Here,

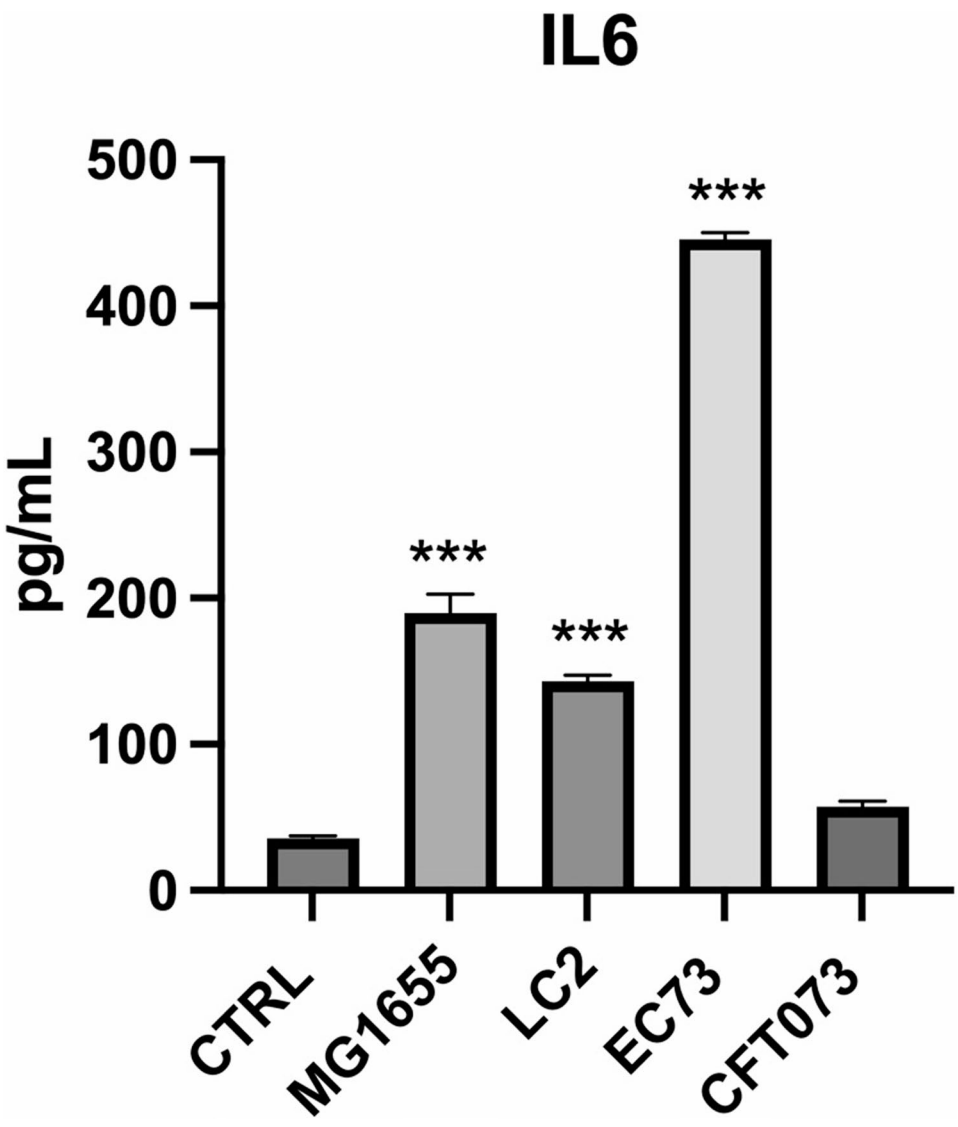

Fig. 4 Quantification of IL-6 protein levels by ELISA assay in bladder epithelial cells T24 infected with *E. coli* strains. Values are expressed as mean ± SD of duplicate samples from two independent experiments. Statistical significance was determined by one-way ANOVA followed by post hoc analysis; asterisks indicate significant differences (***$p < 0.001$).

we conducted a genotypic and phenotypic analysis of UPEC *E. coli* strains aimed at elucidating persistence-promoting factors in two clinical UPEC strains, LC2 and EC73 isolated from patients with recurrent infections. Pan-genome analysis revealed that *E. coli* LC2 harbors a larger number of unique genes compared to EC73 strain, encoding for a larger variety of functions. As previously suggested by Thänert et al., adaptation to specific ecological niches drives the evolution of UPEC genomes [60]. Therefore, we hypothesize that the larger pool of genes uniquely associated with LC2, together with the presence of virulence genes typically found in EPEC strains, may suggest its potential to colonize multiple niches (e.g. bladder and gut). In contrast, the limited set of unique genes identified in EC73 may suggest a high specialization to prostate niche [33]. In our study, in silico analysis of virulence genes revealed that, unlike *E. coli* CFT073, an UPEC strain originally isolated from the blood and urine of a woman with acute pyelonephritis, neither *E. coli* LC2 nor *E. coli* EC73 had *hly*A and *cnf*1 genes (present in UPEC UTI 89 and 83972). Foxman and colleagues reported that the absence of recurrence in UTI patients was associated with a lack of the *cnf*1, *hly*A, and *sfa*/*foc* genes [61]. However, the results of others work are conflicting, suggesting that the VFs presence or absence was not sufficient to explain the different adaptation to the host showed by the different UPEC strains [62–64].

Among our isolates, only LC2 carried the *ast*A gene and exhibited the typical EAEC aggregative adherence

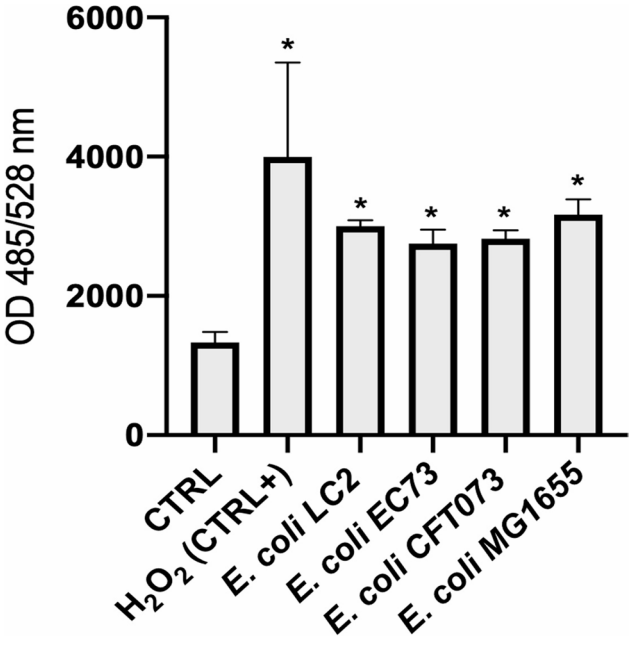

Fig. 5 Evaluation of oxidative stress of T24 cells after 24 h of bacterial infection. Values were reported as mean ± SD. All considered conditions were compared to untreated control. Asterisks indicate significant differences (* $p \leq 0.05$).

phenotype on T24 bladder epithelial cells. Furthermore, LC2 strain carried the *eae* gene, which is characteristic of the EPEC and enterohemorrhagic *E. coli* (EHEC) pathotypes, as well as the *aap* gene, a molecular marker for EAEC detection [65], suggesting a probable persistence of this strain in both the gastrointestinal and urinary tract.

Besides, some *E. coli* strains associated with extraintestinal infections have been found to possess virulence genes correlated to intestinal pathotypes [66]. Mirzarazi et al., 2015 [53] detected EAEC heat-stable enterotoxin 1-coding gene *ast*A in about 10% of UPEC isolates, suggesting that some genes may be transferred from diarrheagenic *E. coli* pathotypes to UPEC.

Recently, the presence of hybrid EAEC strains among *E. coli* isolates from patients with UTIs was investigated [67]. The study highlighted the relationship between *aap* gene and biofilm formation. The authors demonstrated that 88.9% of hybrid strains carrying the *aap* gene were biofilm producers, ranging from weak to strong [67]. In our study, LC2 possessed this gene and was a moderate biofilm producer; on the contrary, EC73, notwithstanding the absence of this gene, was a strong biofilm producer confirming that other genetic elements were involved in this specific bacterial phenotype.

It is well known that *E. coli* isolates causing persistence or relapse are predominantly from phylogenetic groups D and B2 [10, 62, 68]. Our results showed that LC2 belongs to the D phylogroup, while the EC73 strain, like CFT073, belongs to the B2 phylogroup.

Although many *E. coli* sequence types (STs) are involved in extraintestinal infections, the majority of the ExPEC disease burden is attributed to a limited number of lineages, primarily ST131, ST69, ST95, ST73, ST10, ST405, and ST38 [69].

Our in silico analysis showed that LC2 and EC73 strains belong to ST394 and ST95 respectively. ST394 was associated with an in vitro quiescent phenotype [70] and it has been demonstrated that phylogroup D EAEC strains mainly belong to ST131 and ST394 complexes [71]. This evidence suggests that LC2 could be an atypical strain with the potential ability to cause both extraintestinal and intestinal infections in the same patient as previously proposed [72].

The antimicrobial assay showed that both strains exhibited a broad sensitivity to the antibiotics tested. Our results align with those of Luo et al., 2012, who showed that antimicrobial susceptibilities of UPEC isolates had little effect on the development and progression of RUTIs [10].

E. coli has been reported to be enriched in the gut of RUTI patients but extremely rare in healthy controls and non-recurrent individuals and has developed the ability to metabolize a variety of sugars simultaneously [73]. Interestingly, among the UPEC strains used in this study, LC2 showed the ability to metabolize raffinose (data not shown) suggesting a potential metabolic advantage in the gut.

To analyze the urinary colonizing power, we assessed the adhesion, invasion, and survival ability of recurrent LC2 EC73 and CFT073 strains in an in vitro cell model. These strains were able to efficiently adhere and invade T24 bladder cells. According to Naskar et al., 2023 [58], after exposure of the cell monolayers to the LC2 and EC73 strains, the numbers of intracellular bacteria steadily decreased over the next 24 h, although the survival percentage of LC2 was higher than EC73. Regarding *E. coli* EC73, we previously demonstrated that this strain was well adapted to persist in infected prostate cells [33], suggesting a niche-specific adaptation during its persistence [60]. In our study, LC2 and EC73 strains, adapted to different urogenital sites (bladder and prostate respectively) were unable to infect intestinal cell monolayers (Caco-2 cells) [33]. However, both strains were able to colonize the gut in the *in vivo C. elegans* model even if a higher virulence was revealed for LC2 compared to the EC73 and the control strains. This finding was further supported by fluorescence analysis, which highlighted the greater persistence of LC2 in the nematode compared to EC73. However, future studies employing appropriate chronic infection models will be necessary to investigate this aspect in detail.

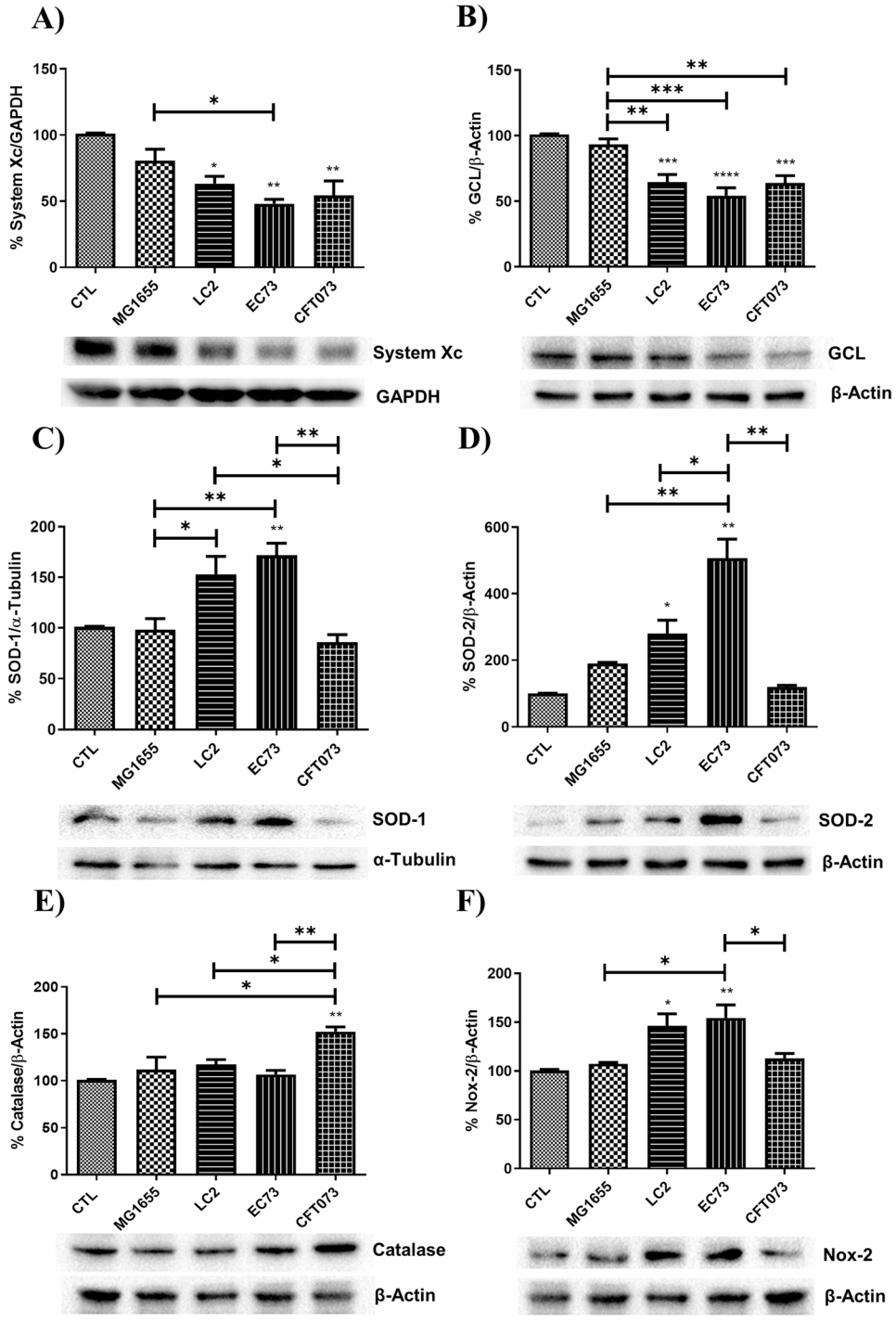

Fig. 6 Western blot and densitometry analysis of System Xc^- (**A**), Glutamine Cysteine Ligase (GCL) (**B**), Superoxide Dismutase-1 (SOD-1) (**C**), Superoxide Dismutase-2 (SOD-2) (**D**), Catalase (**E**) and NADPH oxidase (Nox2) (**F**) in T24 cells infected with *E. coli* strains MG1655, LC2, EC73 and CFT073. Statistical analysis was evaluated by one-way ANOVA with the Tukey post-hoc test; asterisks indicate significant differences (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$).

The association between VFs and pathogenicity in *C. elegans* has been reported for UPEC, although the limitation of the lack of a urinary tract [32, 49]. In particular, Hashimoto et al., 2021 [29], indicated the involvement of UPEC VFs linked to iron acquisition (*chu*A, *fyu*A, and *irp*2) in the high pathogenicity against *C. elegans*. Concurrently, the role of iron capture systems in the development and persistence of RUTIs was debated [10]. Regarding iron uptake systems, our data suggest that although LC2 only has *chu*A gene, it is significantly more virulent in the *C. elegans* model than EC73 which possesses a higher number of iron acquisition genes. This

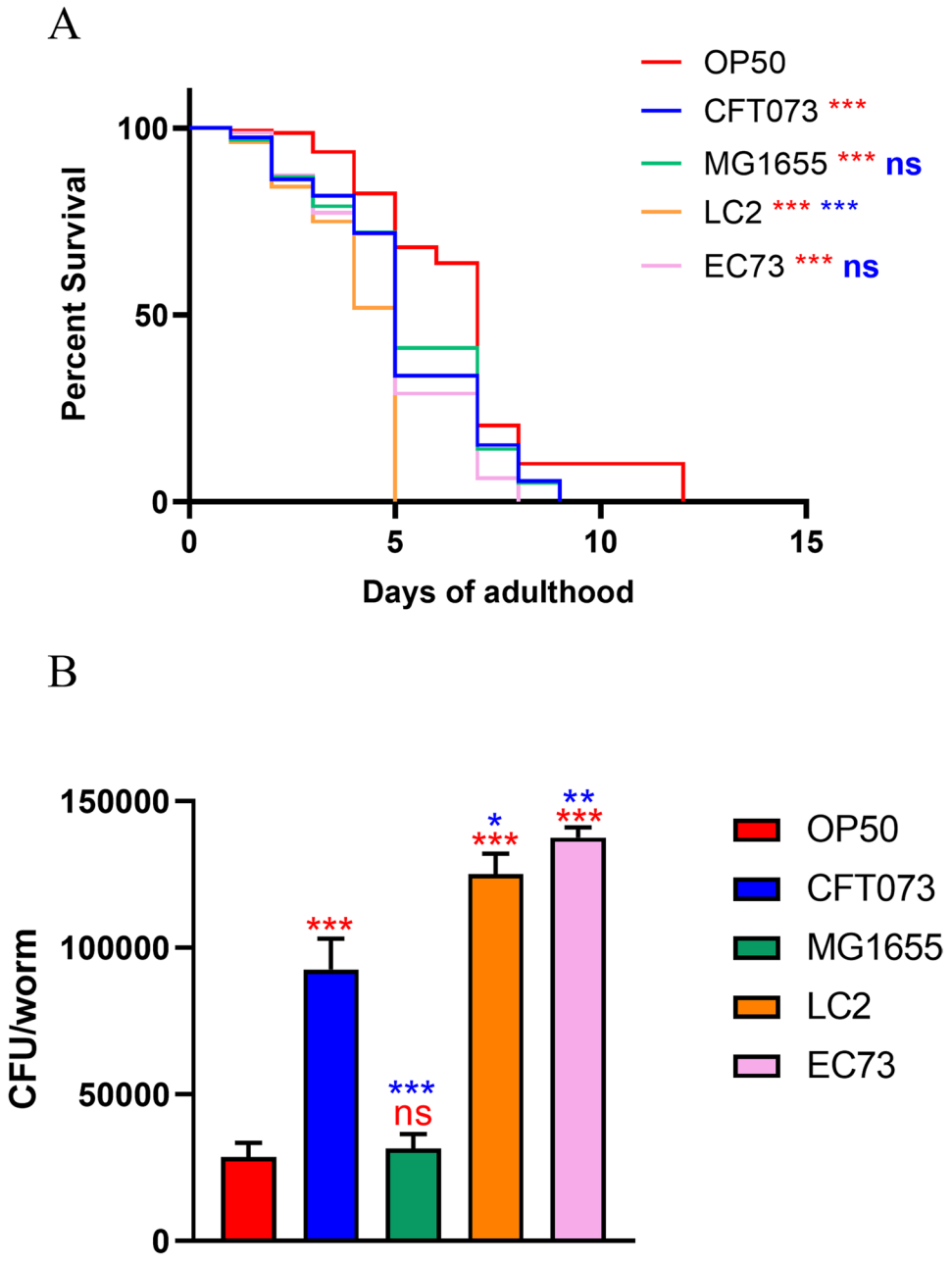

Fig. 7 **A** Kaplan–Meier survival curves of *C. elegans* infected with various *E. coli* strains. **B** Colonization of UPEC strains in the intestinal tract of *C. elegans*. **A** Infections were conducted at 25 °C, and nematode survival was monitored daily. Worms fed with E. coli CFT073 or OP50 served as the control groups ($n = 80$). Statistical significance was assessed using the Log-rank (Mantel-Cox) test; asterisks denote significant differences (***$p < 0.001$; ns: not significant). **B** Differences in colonization levels were evaluated, with asterisks indicating statistically significant differences (*$p < 0.05$; ***$p < 0.001$; ns: not significant). The red asterisks refer to OP50, while the blue ones to CFT073.

leads us to hypothesize that other mechanisms may be involved in the worm's cytotoxicity.

Notably, only LC2 possesses *hly*E producing a cytolytic factor also found in non-pathogenic *E. coli* strains, including the K-12 MG1655 strain [74] suggesting a role also in intestinal colonization [75]. Interestingly, it has been reported that the Cry6A toxin from *Bacillus thuringiensis*, sharing a structural similarity to HlyE, contributed to the intestinal damage of the *C. elegans* [76].

It is well known that in response to UPEC infection, the host produces a rapid pro-inflammatory response, including cytokine release, IL-6 secretion, exfoliation of superficial urothelial cells, and influx of innate immune cells such as neutrophils and macrophages [77]. It has also been reported that the activation of host immunity

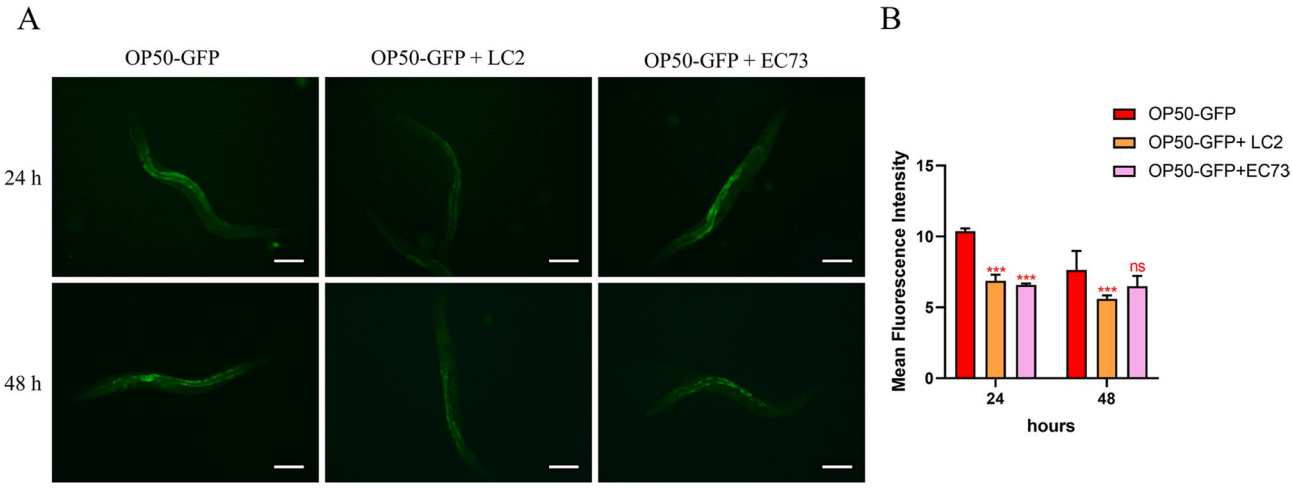

Fig. 8 Persistence of uropathogenic *E. coli* LC2 and EC73. **A** Fluorescence microscopy of wild type N2 worms fed with *E. coli* OP50-GFP and LC2 or EC73 pathogens in a 1:1 ratio and (**B**) related MFI. Scale bar = 100 µm control: worms fed OP50-GFP alone. Statistical analysis was evaluated by one-way ANOVA with the Bonferroni post-test; asterisks indicate significant differences (***$p < 0.001$; ns: not significant). Bars represent the mean of three independent experiments

frequently leads to severe injuries in the urothelium, creating an environment conducive to the recurrence of UTIs in an experienced host [15, 77]. In the present study, T24 bladder epithelial cells infected with both *E. coli* EC73 and LC2 strains upregulated pro-inflammatory cytokine expression, though to varying extents, with EC73 being more efficient than LC2. The reduced IL-6 induction observed in the bladder cell infection model, both at the mRNA and proteins levels, may be linked to the higher virulence of the LC2 strain in the *C. elegans* model. In particular, previous evidence have suggested that IL-6 deficiency, together with increased formation of IBCs, could promote intracellular persistence. Moreover, an inverse correlation has been reported between the level of IL-6 induction in vitro and the clinical severity of UTIs [78].

In contrast, T24 cells infected with *E. coli* CFT073 displayed cytokine expression similar to control cells. This surprising observation is consistent with a previous report from Hilbert et al., 2008 showing that CFT073 dominantly suppresses the innate immune response of T24 bladder epithelial cells via a lipopolysaccharide- and TLR4-independent pathway [79]. As suggested by previous studies, the ability of the cystitis strains to suppress the cytokine response of the epithelium could provide an advantage in invading the tissue and evading early innate immune defenses [27]. Interestingly, KpsMTII and other VFs "protectins", mostly expressed by LC2 and EC73, have been shown to be essential for UTI development and for protecting bacteria against complement-mediated killing.

Moreover, our data revealed that all tested bacterial strains, regardless of their pathogenic profile, induced a significant overproduction of ROS in bladder cells compared to uninfected cells. However, differences were observed in the expression patterns of proteins involved in ROS balance. Both LC2 and EC73 strains significantly upregulated SOD-1 and SOD-2 variants, while downregulating both System $Xc^-$and GCL, compared to uninfected cells or those infected with non-pathogenic strain. Although mitigating excess ROS levels is physiologically important, it has been shown that a regulated elevation of ROS can enhance antibacterial immunity [80]. To our knowledge, only a few studies have explored the effects of intracellular bacteria on antioxidant systems, and their results have been conflicting depending on the cell/animal models and bacterial strains. For instance, SOD-2 upregulation promoted the intracellular survival of *Mycobacterium tuberculosis* in human macrophages [81], while SOD-2 knockdown increased bacterial burden in zebrafish infected with *Pseudomonas aeruginosa* [82]. In this context, the concomitant downregulation of both System Xc^- and GCL observed upon LC2 and EC73 infection could be part of a host defense response to deprive intracellular bacteria of glutathione. This molecule plays a critical role in bacterial pathogenesis, including the activation of virulence gene expression and optimal biofilm formation [83].

Conclusions

There is evidence that niche-adaptation shapes UPEC within-host adaptation. The ability of UPEC to thrive in diverse host environments, such as the gut, urine, bladder, kidneys, and bloodstream is central to its pathogenesis. Both recurrent *E. coli* LC2 and EC73 strains demonstrated the capacity to adhere, invade and survive in bladder cells, suggesting that these strains possess genetic VFs that promote colonization of the urinary

tract. In recent years, the concept that UPEC developed from nonpathogenic strains has been proposed. The acquisition of VFs by horizontal transfer of accessory DNA at the chromosomal or plasmid level, other than being the cause of transformation to pathogenic strains, is responsible for their huge phenotypic and genetic variability. In particular, LC2 strain appeared to exhibit virulence factors intermediate between the diarrheagenic *E. coli* and UPEC strains. The presence of the enteroaggregative genes of *E. coli*, together with the *fim* and *kps*MTII genes typical of ExPEC, suggested that LC2 might be a diarrheagenic strain, probably coming from the gut. On the other hand, both LC2 and EC73 strains possessed mobile genetic factors, such as the *tra*T gene that encodes for a protein exposed on the cell surface involved in interactions between a bacterial cell and its environment. Both strains were also shown to efficiently colonize the gut of *C. elegans*. Probably, this genetic variability allows the UPEC strains to adapt to the different physiological conditions of their host-specific niche which could be the reason behind the lack of common molecular markers responsible for their peculiar phenotypic and genetic characteristics. Notwithstanding, the identification of shared molecular markers between LC2 and EC73, which may contribute to a better understanding of virulence traits of *E. coli* pathotype associated with RUTIs and help predict the course of the infection, the low number of the strains analyzed could represents a limitation of this study.

Abbreviations

UTIs	Urinary tract infections
RUTIs	Recurrent urinary tract infections
UPEC	Uropathogenic Escherichia coli
VFs	Virulence factors
IBCs	Intracellular bacterial communities
QIRs	Quiescent intracellular bacterial reservoirs
ASB/ABU	Asymptomatic bacteriuria
TLR	Tool like receptor
IL	Interleukin
ROS	Reactive oxygen species
RAPD	Random amplified polymorphic DNA
BHI	Brain heart infusion
TSA	Trypticase Soy Agar
AES	Automated expert system
AST-N202	Advanced expert system software
TSB	Tryptic Soy Broth
BPGA	Bacterial Pan genome analysis
COG	Clusters of Orthologous Groups
ExPEC	Extraintestinal pathogenic Escherichia coli
PCR	Polymerase chain reaction
RPMI	Roswell Park Memorial Institute
FBS	Fetal bovine serum
LB	Luria Bertani
CFU	Colony forming unit
PBS	Phosphate-buffered saline
OD	Optical density
SDs	Standard deviations
ODc	Optical density negative control
MOI	Multiplicity of infection
GM	Gentamicin
RTqPCR	Real time quantitative polymerase chain reaction
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
PMSF	Phenylmethanesulfonyl Fluoride
NGM	Nematode Growth Medium
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
EAEC	Enteroaggregative Escherichia coli
EPEC	Enteropathogenic Escherichia coli
TNF	Tumor necrosis factor
EHEC	Enterohemorrhagic Escherichia coli
pAA	Aggregative adherence plasmid
SOD	Superoxide dismutase
GCL	Glutamate-cysteine ligase
STs	Sequence types

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-04287-8.

Supplementary Material 1.

Acknowledgements
Not applicable.

Authors' contributions
A. R., M.G. A., D. U., A. C., M.P. C., C. L., L. M. wrote the main manuscript text; L. M., C.L. contribute to the study conception and design; A.L.C., S. S., E. S., G. I., (A) N., M.D.A. conducted the experiments and analyzed data; C.L., L. M., M.G.A., M.P.C. reviewed and edited the manuscript; F. (B) performed bioinformatic and statistical analysis. All authors reviewed the manuscript.

Funding
This research was funded by Ricerca Scientifica 2023 "Sapienza" University of Rome, to C. Longhi.
Clinical trial number.
Not applicable.

Data availability
The raw sequencing reads of E. coli EC73 and LC2 analyzed in this study have been deposited in the NCBI SRA database under the BioProject PRJNA1234551.

Declarations

Ethics approval and consent to participate
The bacterial strains were collected precisely in 2006 and were present in our Laboratory as collection isolates. At "Sapienza" University of Rome the Ethics Committee for Transdisciplinary Research (CERT in Italian), that ensures that Sapienza University research follows ethical principles defined by international and national regulations and the Sapienza Statute and Code of Ethics, it has been established in 2021 (RD no. 59099/2021) (se https://www.uniroma1.it/en/pagina/ethics-committee-transdisciplinary-research).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Public Health and Infectious Diseases, "Sapienza" University of Rome, Microbiology Section, P. le Aldo Moro 5, Rome 00185, Italy
[2]Department of Anatomy, Histology, Forensic Medicine and Orthopaedics, Section of Histology and Medical Embryology, "Sapienza" University, Rome, Rome 00185, Italy
[3]National Center for Innovative Technologies in Public Health, Italian National Institute of Health, Viale Regina Elena, 299, Rome 00161, Italy
[4]Department of Biology and Biotechnology "C. Darwin", Sapienza University of Rome, Rome 00185, Italy

[5]Department of Biosciences and Territory, University of Molise, Pesche 86090, Italy

Received: 6 March 2025 / Accepted: 29 July 2025
Published online: 25 August 2025

References

1. Hannan TJ, Totsika M, Mansfield KJ, Moore KH, Schembri MA, Hultgren SJ. Host-pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic *Escherichia coli* bladder infection. FEMS Microbiol Rev. 2012;36(3):616–48.
2. McLellan LK, Hunstad DA. Urinary tract infection: pathogenesis and outlook. Trends Mol Med. 2016;22:946–57.
3. Terlizzi ME, Gribaudo G, Maffei ME. Uropathogenic *Escherichia coli* (UPEC) infections: virulence factors, bladder responses, antibiotic, and non-antibiotic antimicrobial strategies. Front Microbiol. 2017;8:1566.
4. Martinez JJ, Mulvey MA, Schilling JD, Pinkner JS, Hultgren SJ. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. EMBO J. 2000;19:2803–12.
5. Klein RD, Hultgren SJ. Urinary tract infections: microbial pathogenesis, host-pathogen interactions and new treatment strategies. Nat Rev Microbiol. 2020;18:211–26.
6. Anderson GG, Dodson KW, Hooton TM, Hultgren SJ. Intracellular bacterial communities of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Trends Microbiol. 2004;12:424–30.
7. Justice SS, Hung C, Theriot JA, Anderson GG, Footer MJ, Hultgren SJ. Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Proc Natl Acad Sci U S A. 2004;101:1333–8.
8. Blango MG, Mulvey MA. Persistence of uropathogenic *Escherichia coli* in the face of multiple antibiotics. Antimicrob Agents Chemother. 2010;54:1855–63.
9. Brauner A, Jacobson SH, Kühn I. Urinary *Escherichia coli* causing recurrent infections–a prospective follow-up of biochemical phenotypes. Clin Nephrol. 1992;38:318–23.
10. Luo Y, Ma Y, Zhao Q, Wang L, Guo L, Ye L, et al. Similarity and divergence of phylogenies, antimicrobial susceptibilities, and virulence factor profiles of *Escherichia coli* isolates causing recurrent urinary tract infections that persist or result from reinfection. J Clin Microbiol. 2012;50:4002–7.
11. Pigrau C, Escolà-Vergé L. Recurrent urinary tract infections: from pathogenesis to prevention. Med Clin (Barc). 2020;155:171–7.
12. Hunstad DA, Justice SS. Intracellular lifestyles and immune evasion strategies of uropathogenic *Escherichia coli*. Annu Rev Microbiol. 2010;64:203–21.
13. Forde BM, Roberts LW, Phan M-D, Peters KM, Fleming BA, Russell CW, et al. Population dynamics of an *Escherichia coli* ST131 lineage during recurrent urinary tract infection. Nat Commun. 2019;10:3643.
14. Ikäheimo R, Siitonen A, Heiskanen T, Kärkkäinen U, Kuosmanen P, Lipponen P, et al. Recurrence of urinary tract infection in a primary care setting: analysis of a 1-year follow-up of 179 women. Clin Infect Dis. 1996;22:91–9.
15. Murray BO, Flores C, Williams C, Flusberg DA, Marr EE, Kwiatkowska KM, et al. Recurrent urinary tract infection: a mystery in search of better model systems. Front Cell Infect Microbiol. 2021;11:691210.
16. Russo TA, Johnson JR. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: expec. J Infect Dis. 2000;181(5):1753–4.
17. Schreiber HL, Spaulding CN, Dodson KW, Livny J, Hultgren SJ. One size doesn't fit all: unraveling the diversity of factors and interactions that drive *E. coli* urovirulence. Ann Transl Med. 2017;5: 28.
18. Silverman JA, Schreiber HL, Hooton TM, Hultgren SJ. From physiology to pharmacy: developments in the pathogenesis and treatment of recurrent urinary tract infections. Curr Urol Rep. 2013;14:448–56.
19. Naziri Z, Kilegolan JA, Moezzi MS, Derakhshandeh A. Biofilm formation by uropathogenic *Escherichia coli*: a complicating factor for treatment and recurrence of urinary tract infections. J Hosp Infect. 2021;117:9–16.
20. Lang T, Hudemann C, Tchatalbachev S, Stammler A, Michel V, Aslani F, et al. Uropathogenic *Escherichia coli* modulates innate immunity to suppress Th1-mediated inflammatory responses during infectious epididymitis. Infect Immun. 2014;82:1104–11.
21. Joshi CS, Mora A, Felder PA, Mysorekar IU. NRF2 promotes urothelial cell response to bacterial infection by regulating reactive oxygen species and RAB27B expression. Cell Rep. 2021;37: 109856.
22. Hayes BW, Abraham SN. Innate immune responses to bladder infection. Microbiol Spectr. 2016. https://doi.org/10.1128/microbiolspec.UTI-0024-2016.
23. Kim M, Ashida H, Ogawa M, Yoshikawa Y, Mimuro H, Sasakawa C. Bacterial interactions with the host epithelium. Cell Host Microbe. 2010;8:20–35.
24. Mysorekar IU, Isaacson-Schmid M, Walker JN, Mills JC, Hultgren SJ. Bone morphogenetic protein 4 signaling regulates epithelial renewal in the urinary tract in response to uropathogenic infection. Cell Host Microbe. 2009;5:463–75.
25. Ting K, Aitken KJ, Penna F, Samiei AN, Sidler M, Jiang J-X, et al. Uropathogenic *E. coli* (UPEC) infection induces proliferation through enhancer of Zeste homologue 2 (EZH2). PLoS One. 2016;11:e0149118.
26. Billips BK, Forrestal SG, Rycyk MT, Johnson JR, Klumpp DJ, Schaeffer AJ. Modulation of host innate immune response in the bladder by uropathogenic *Escherichia coli*. Infect Immun. 2007;75:5353–60.
27. Hunstad DA, Justice SS, Hung CS, Lauer SR, Hultgren SJ. Suppression of bladder epithelial cytokine responses by uropathogenic *Escherichia coli*. Infect Immun. 2005;73:3999–4006.
28. Harbeoui H, Di Martino P, Mayot G. A new killing assay with the *caenorhabditis elegans* PX627 mutant to assess the virulence of uropathogenic *Escherichia coli* strains. J Microbiol Methods. 2023;214: 106823.
29. Hashimoto M, Ma Y-F, Wang S-T, Chen C-S, Teng C-H. Iron acquisition of urinary tract infection Escherichia coli involves pathogenicity in caenorhabditis elegans. Microorganisms. 2021;9:310.
30. Merkx-Jacques A, Coors A, Brousseau R, Masson L, Mazza A, Tien Y-C, et al. Evaluating the pathogenic potential of environmental *Escherichia coli* by using the *caenorhabditis elegans* infection model. Appl Environ Microbiol. 2013;79:2435–45.
31. Wellenberg A, Weides L, Kurzke J, Hennecke T, Bornhorst J, Crone B, et al. Use of C. elegans as a 3R-compliant in vivo model for the chemoprevention of cisplatin-induced neurotoxicity. Exp Neurol. 2021;341: 113705.
32. Lavigne J-P, Blanc-Potard A-B, Bourg G, Moreau J, Chanal C, Bouziges N, et al. Virulence genotype and nematode-killing properties of extra-intestinal *Escherichia coli* producing CTX-M beta-lactamases. Clin Microbiol Infect. 2006;12:1199–206.
33. Conte MP, Aleandri M, Marazzato M, Conte AL, Ambrosi C, Nicoletti M, et al. The adherent/invasive *Escherichia coli* strain LF82 invades and persists in human prostate cell line RWPE-1, activating a strong inflammatory response. Infect Immun. 2016;84:3105–13.
34. Mobley HL, Green DM, Trifillis AL, Johnson DE, Chippendale GR, Lockatell CV, et al. Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. Infect Immun. 1990;58:1281–9.
35. Zerbino DR. Using the Velvet de Novo assembler for short-read sequencing technologies. Curr Protoc Bioinforma Ed Board Andreas Baxevanis Al. 2010;CHAPTER:Unit-11.5.
36. Chaudhari NM, Gupta VK, Dutta C. BPGA- an ultra-fast pan-genome analysis pipeline. Sci Rep. 2016;6: 24373.
37. Galperin MY, Vera Alvarez R, Karamycheva S, Makarova KS, Wolf YI, Landsman D, et al. COG database update 2024. Nucleic Acids Res. 2025;53:D356–63.
38. R: The R Project for Statistical Computing. https://www.r-project.org/. Accessed 18 Feb 2025.
39. Wickham H. ggplot2: elegant graphics for data analysis. New York, NY: Springer; 2009.
40. Chen H, VennDiagram. Generate High-Resolution Venn and Euler Plots. 2022.
41. Neuwirth E. RColorBrewer: ColorBrewer Palettes. 2022.
42. BLAST. Basic Local Alignment Search Tool. https://blast.ncbi.nlm.nih.gov/Blast.cgi. Accessed 18 Feb 2025.
43. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000;66:4555–8.
44. Dusane DH, Hosseinidoust Z, Asadishad B, Tufenkji N. Alkaloids modulate motility, biofilm formation and antibiotic susceptibility of uropathogenic Escherichia coli - PubMed. 2014. https://pubmed.ncbi.nlm.nih.gov/25391152/. Accessed 6 Nov 2024.
45. Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Cirković I, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by Staphylococci. APMIS. 2007;115:891–9.
46. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta delta C(T)) method. Methods San Diego Calif. 2001;25:402–8.

47. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–54.
48. Schifano E, Ficociello G, Vespa S, Ghosh S, Cipollo JF, Talora C, et al. Pmr-1 gene affects susceptibility of *caenorhabditis elegans* to *Staphylococcus aureus* infection through glycosylation and stress response pathways' alterations. Virulence. 2019;10(1):1013–25.
49. Schifano E, Marazzato M, Ammendolia MG, Zanni E, Ricci M, Comanducci A, et al. Virulence behavior of uropathogenic *Escherichia coli* strains in the host model *Caenorhabditis elegans*. Microbiol Open. 2019;8: e00756.
50. Nazemi A, Mirinargasi M, Merikhi N, Sharifi SH. Distribution of pathogenic genes aata, aap, aggr, among uropathogenic *Escherichia coli* (UPEC) and their linkage with StbA gene. Indian J Microbiol. 2011;51:355–8.
51. Nataro JP, Deng Y, Maneval DR, German AL, Martin WC, Levine MM. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. Infect Immun. 1992;60:2297–304.
52. Asadi Karam MR, Rezaei AA, Siadat SD, Habibi M, Bouzari S. Evaluation of prevalence, homology and immunogenicity of dispersin among enteroaggregative *Escherichia coli* isolates from Iran. Iran Biomed J. 2017;21:40–7.
53. Mirzarazi M, Rezatofighi SE, Pourmahdi M, Mohajeri MR. Occurrence of genes encoding enterotoxins in uropathogenic *Escherichia coli* isolates. Braz J Microbiol. 2015;46:155–9.
54. Sheikh J, Dudley EG, Sui B, Tamboura B, Suleman A, Nataro JP. EilA, a HilA-like regulator in enteroaggregative *Escherichia coli*. Mol Microbiol. 2006;61:338–50.
55. Subashchandrabose S, Mobley HLT. Virulence and fitness determinants of uropathogenic *Escherichia coli*. Microbiol Spectr. 2015. https://doi.org/10.1128/microbiolspec.UTI-0015-2012.
56. Yang X, Sha K, Xu G, Tian H, Wang X, Chen S, et al. Subinhibitory concentrations of allicin decrease uropathogenic *Escherichia coli* (UPEC) biofilm formation, adhesion ability, and swimming motility. Int J Mol Sci. 2016. https://doi.org/10.3390/ijms17070979.
57. Sanmukh SG, Admella J, Moya-Andérico L, Fehér T, Arévalo-Jaimes BV, Blanco-Cabra N, et al. Accessing the in vivo efficiency of clinically isolated phages against uropathogenic and invasive Biofilm-Forming Escherichia coli strains for phage therapy. Cells. 2023;12:344.
58. Naskar M, Parekh VP, Abraham MA, Alibasic Z, Kim MJ, Suk G, et al. α-Hemolysin promotes uropathogenic *E. coli* persistence in bladder epithelial cells via abrogating bacteria-harboring lysosome acidification. PLoS Pathog. 2023;19: e1011388.
59. Vitiello A, Ferrara F, Boccellino M, Ponzo A, Sabbatucci M, Zovi A. Antimicrobial resistance in gonorrhea. Microb Drug Resist Larchmt N. 2024;30:297–303.
60. Thänert R, Choi J, Reske KA, Hink T, Thänert A, Wallace MA, et al. Persisting uropathogenic Escherichia coli lineages show signatures of niche-specific within-host adaptation mediated by mobile genetic elements. Cell Host Microbe. 2022;30:1034–e10476.
61. Foxman B, Zhang L, Tallman P, Palin K, Rode C, Bloch C, et al. Virulence characteristics of *Escherichia coli* causing first urinary tract infection predict risk of second infection. J Infect Dis. 1995;172:1536–41.
62. Ejrnæs K, Stegger M, Reisner A, Ferry S, Monsen T, Holm SE, et al. Characteristics of *Escherichia coli* causing persistence or relapse of urinary tract infections: phylogenetic groups, virulence factors and biofilm formation. Virulence. 2011;2:528–37.
63. Flores-Oropeza MA, Ochoa SA, Cruz-Córdova A, Chavez-Tepecano R, Martínez-Peñafiel E, Rembao-Bojórquez D, et al. Comparative genomic analysis of uropathogenic Escherichia coli strains from women with recurrent urinary tract infection. Front Microbiol. 2023;14:1340427.
64. Gao M, Zhao T, Zhang C, Li P, Wang J, Han J, et al. Ferritinophagy-mediated iron competition in rutis: tug-of-war between UPEC and host. Biomed Pharmacother. 2023;163: 114859.
65. Cordeiro F, da Silva Gomes Pereira D, Rocha M, Asensi MD, Elias WP, Campos LC. Evaluation of a multiplex PCR for identification of enteroaggregative *Escherichia coli*. J Clin Microbiol. 2008;46:828–9.
66. Abe CM, Salvador FA, Falsetti IN, Vieira MAM, Blanco J, Blanco JE, et al. Uropathogenic *Escherichia coli* (UPEC) strains may carry virulence properties of diarrhoeagenic *E. coli*. FEMS Immunol Med Microbiol. 2008;52:397–406.
67. Moazeni S, Askari Badouei M, Hashemitabar G, Rezatofighi SE, Mahmoodi F. Detection and characterization of potentially hybrid enteroaggregative *Escherichia coli* (EAEC) strains isolated from urinary tract infection. Braz J Microbiol Publ Braz Soc Microbiol. 2024;55:1–9.
68. Kao C-Y, Zhang Y-Z, Yang D-C, Chen PK, Teng C-H, Lin W-H, et al. Characterization of host and *Escherichia coli* strains causing recurrent urinary tract infections based on molecular typing. BMC Microbiol. 2023;23:90.
69. Manges AR, Geum HM, Guo A, Edens TJ, Fibke CD, Pitout JDD. Global extraintestinal pathogenic *Escherichia coli* (ExPEC) lineages. Clin Microbiol Rev. 2019;32:e00135-18.
70. Sun J, Deering RW, Peng Z, Najia L, Khoo C, Cohen PS, et al. Pectic oligosaccharides from cranberry prevent quiescence and persistence in the uropathogenic *Escherichia coli* CFT073. Sci Rep. 2019;9: 19590.
71. Okeke IN, Wallace-Gadsden F, Simons HR, Matthews N, Labar AS, Hwang J, et al. Multi-locus sequence typing of enteroaggregative *Escherichia coli* isolates from Nigerian children uncovers multiple lineages. PLoS One. 2010;5: e14093.
72. Valiatti TB, Santos FF, Santos ACM, Nascimento JAS, Silva RM, Carvalho E, et al. Genetic and virulence characteristics of a hybrid atypical enteropathogenic and uropathogenic *Escherichia coli* (aEPEC/UPEC) strain. Front Cell Infect Microbiol. 2020;10:492.
73. Maltby R, Leatham-Jensen MP, Gibson T, Cohen PS, Conway T. Nutritional basis for colonization resistance by human commensal *Escherichia coli* strains HS and Nissle 1917 against *E. coli* O157:H7 in the mouse intestine. PLoS One. 2013;8:e53957.
74. Wallace AJ, Stillman TJ, Atkins A, Jamieson SJ, Bullough PA, Green J, et al. *E. coli* hemolysin E (HlyE, clya, SheA): x-ray crystal structure of the toxin and observation of membrane pores by electron microscopy. Cell. 2000;100:265–76.
75. Modgil V, Kaur H, Mahindroo J, Mohan B, Taneja N. Sequence types of enteroaggregative *Escherichia coli* strains recovered from human, animal, and environmental sources: India. Gene Rep. 2024;37: 102017.
76. Dementiev A, Board J, Sitaram A, Hey T, Kelker MS, Xu X, et al. The pesticidal Cry6Aa toxin from *Bacillus thuringiensis* is structurally similar to HlyE-family alpha pore-forming toxins. BMC Biol. 2016;14:71.
77. Bien J, Sokolova O, Bozko P. Role of uropathogenic Escherichia coli virulence factors in development of urinary tract infection and kidney damage. Int J Nephrol. 2012;2012:681473.
78. Horvath DJ, Patel AS, Mohamed A, Storm DW, Singh C, Li B, et al. Association of O-antigen serotype with the magnitude of initial systemic cytokine responses and persistence in the urinary tract. J Bacteriol. 2016;198:964–72.
79. Hilbert DW, Pascal KE, Libby EK, Mordechai E, Adelson ME, Trama JP. Uropathogenic *Escherichia coli* dominantly suppress the innate immune response of bladder epithelial cells by a lipopolysaccharide- and toll-like receptor 4-independent pathway. Microbes Infect. 2008;10:114–21.
80. Mukherjee A, Ghosh KK, Chakrabortty S, Gulyás B, Padmanabhan P, Ball WB. Mitochondrial reactive oxygen species in infection and immunity. Biomolecules. 2024;14:670.
81. Ren X, Dong W, Feng J, Li P, Zheng Y, Wang G, et al. miR-495 regulates cellular reactive oxygen species levels by targeting sod2 to inhibit intracellular survival of Mycobacterium tuberculosis in macrophages. Infect Immun. 2021;89:e0031521.
82. Peterman EM, Sullivan C, Goody MF, Rodriguez-Nunez I, Yoder JA, Kim CH. Neutralization of mitochondrial superoxide by superoxide dismutase 2 promotes bacterial clearance and regulates phagocyte numbers in zebrafish. Infect Immun. 2014;83:430–40.
83. Ku JWK, Gan Y-H. New roles for glutathione: modulators of bacterial virulence and pathogenesis. Redox Biol. 2021;44: 102012.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.