Interplay between uropathogenic *Escherichia coli* and bladder cells and new strategies to counteract bacterial persistence

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1. INTRODUCTION

1.1 *Escherichia coli*

Theodor Escherich, in 1885, identified a bacterium that he believed was associated with infantile diarrhoea. This bacterium was initially referred to as "*Bacterium coli commune*" or "*B. coli commune*" in his research. A few decades later, in recognition of his work, this bacterium was officially named "*Escherichia coli*" in his honour [*Geurtsen et al., 2022*].

E. coli is a Gram-negative bacterium, facultative anaerobe, not sporigenous, belonging to the *Enterobacterales* order. It has a rod shape of roughly 0.4 μm in diameter and 2–3 μm in length. It can be provided or not with a capsule and is generally mobile due to the peritric flagella. *E. coli* can grow in a wide range of temperatures, typically between 15°C and 45°C. This adaptability to temperature is one of the reasons for its prevalence in various environments. *E. coli* can survive in the environment for extended periods, especially in soil and water. This environmental resilience can lead to contamination and the potential for *E. coli*-related infections if ingested or otherwise introduced into the human body *[Sora et al., 2021]. E. coli* is a commensal member of the vertebrate gut microbiota. The concentration of *E. coli* in faeces can range from 10^7 to 10^9 colony-forming units per gram. However, there are several *E. coli* that, thanks to the presence of several virulence factors, have the ability to cause several infectious diseases in humans and animals [*Kaper et al., 2004*].

1.1.1 *E. coli* pathotypes

E. coli can be categorized into various pathotypes based on the presence of specific virulence factors, mechanisms of infection, tissue tropism, interactions with host cells, and the clinical symptoms they cause.

In general, *E. coli* pathotypes can be broadly categorized into two groups:

- Intestinal strains (diarrheagenic, DEC): that can lead to various forms of diarrhoea, including haemolytic and Hemolytic–Uremic Syndrome (HUS).

- Extra-intestinal strains (ExPEC): that can cause urinary tract infections (UTIs), diverse intra-abdominal, pulmonary, skin, and soft tissue infections, newborn meningitis (NBM) and bacteraemia [*Denamur et al., 2021*].

The following pathotypes can be included among the intestinal strains: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), diffusely adherent *E. coli* (DAEC) [*Pokharel et al., 2023*].

The ExPEC group includes the following pathotypes:

- uropathogenic *E. coli* (UPEC)
- *E. coli* associated with neonatal meningitis (NMEC)
- sepsis-associated *E. coli* (SEPEC)
- avian pathogenic *E. coli* (APEC)
- endometrial pathogenic *E. coli* (EnMEC)
- mammalian pathogenic *E. coli* (MPEC)

ExPEC strains often reside in the intestinal microbiota of humans and other animals, and they have the capacity to emerge from their intestinal reservoir to cause extraintestinal infections. This transition can be facilitated by factors such as virulence determinants, and by the ability to adhere to and invade host tissues. This passage may also be associated with factors like host immunosuppression, catheterization, or other predisposing conditions [*Manges et al., 2019*]. ExPEC strains are known for possessing a variety of virulence factors; these factors are often encoded on various genetic elements, such as pathogenicity islands (PAIs), plasmids, and other mobile genetic elements [*Sarowska et al., 2019*]. Given the clinical importance of ExPEC, surveillance systems are crucial for monitoring and preventing the spread of pandemic serotypes of these strains. Phylogenetic analysis is a valuable and versatile method used in the classification and study of *E. coli* strains. It involves constructing phylogenetic trees to understand the evolutionary relationships among different *E. coli* isolates. Phylogenetic analysis classifies *E. coli* strains into different

phylogroups based on genetic relatedness. These phylogroups are labeled A, B1, B2, C, D, E, F, G, and clade I [*Clermont et al., 2019*]. Commensal *E. coli* strains are primarily found in phylogroups A or B1, while ExPEC strains are often associated with phylogroup B2. Strains of group D are also found among ExPEC strains, and group E is related to group D. Group F is associated to the main group B2. Some *E. coli* strains that are genetically diverse but phenotypically indistinguishable are assigned to cryptic clades. Today, multilocus sequence type (MLST) analysis is the *"gold standard"*, used to identify ExPEC strains as well as to evaluate their evolutionary relationships among different lineages. Despite the huge diversity of ExPEC sequence types (STs), it has been recently reported that ExPEC ST12, ST69, ST73, ST95, ST127 and ST131 are the predominant lineages causing extraintestinal infections worldwide [*Longhi et al., 2022*].

This high level of genetic heterogeneity within the species *E. coli* mirrors the genomic plasticity of these organisms which results from frequent acquisition and loss of genomic information as well as high recombination rates. Understanding the genetic diversity and genomic plasticity of *E. coli* is crucial, particularly in the context of Public Health; in fact, genetic plasticity and selection pressures can contribute to the emergence of antibiotic-resistant strains [*Manges et al., 2019*]. This flexibility of the *E. coli* genome also allows for significant intra-species variability. This means that different strains of *E. coli* can exhibit diverse genetic characteristics, contributing to their adaptability to various environments and conditions. All of this led to the spread of hybrid-pathogenic *E. coli*. The term is coined to describe strains that carry new combinations of virulence factors associated with both DEC and ExPEC [*Nascimento et al., 2021]*. These hybrid pathogenic strains often appear more virulent than their ancestral lineages, as exemplified by a hybrid (EHEC/EAEC) strain that caused a large diarrhoea outbreak in Germany in 2011 [*Brzuszkiewicz et al., 2011*]. In UPEC some DEC molecular markers, such as the locus of enterocyte effacement (Locus of Enterocyte Effacement (LEE) region), a chromosomal PAI from EPEC, and the aggregative adherence plasmid (pAA), from EAEC, have been identified [*Toval et al., 2014]*. In addition, some UPEC strains harbour some molecular markers of STEC (*stx1* and/or *stx2* genes) that encode potent cytotoxins that inhibit protein synthesis in eukaryotic cells [*Toval et al., 2014*]. In particular, the LEE region encodes proteins that play a crucial role in inducing a

histopathological lesion known as attaching and effacement (AE) in infected epithelial cells. The AE lesion is characterized by several features, including the intimate adherence of bacteria to the host cell [*McDaniel et al., 1995*]. For example, the *E. coli* isolate serotype O71:H40 is identified as a hybrid EPEC/UPEC, that harbours 41 genes from the LEE region. This isolate produces the adhesin intimin, adheres to both urinary and intestinal cell lineages, and induces AE lesions in HeLa cells [*Valiatti et al., 2020*]. The presence of hybrid virulence factors may contribute to altered disease manifestations in infected individuals, potentially impacting the severity and clinical presentation of infections. In addition, these strains may exhibit a broader range of antibiotic resistance patterns and pathogenic capabilities [*Tanabe et al., 2022*].

1.2 Urinary Tract Infections (UTIs)

Urinary Tract Infections (UTIs) are one of the most common bacterial infections in humans worldwide. Women are more susceptible to UTIs than men, with at least 40% of women experiencing at least one episode of UTI in their lifetime. UTIs still affect around 12% of men and 5% of children [*Serretiello et al., 2021*]. UTIs can be acquired in community and hospital settings and are classified based on various characteristics such as the site of infection, risk factors and onset of infection. UTIs may be classified as uncomplicated or complicated [*Flores-Mireles et al., 2015*]. Uncomplicated UTIs, such as cystitis, are more common in outpatient settings. They typically occur in otherwise healthy individuals without structural or neurologic abnormalities of the urinary tract. Symptoms of uncomplicated UTIs may include dysuria, urgency, frequency, and lower abdominal discomfort. Complicated UTIs, instead, are linked to patient-level factors that compromise urodynamics, such as urinary obstruction or catheterization, or impairment of host defences. Individuals with complicated UTIs are often at an increased risk of developing more severe infections, which may involve the upper urinary tract (pyelonephritis) [*Flores-Mireles et al., 2019*].

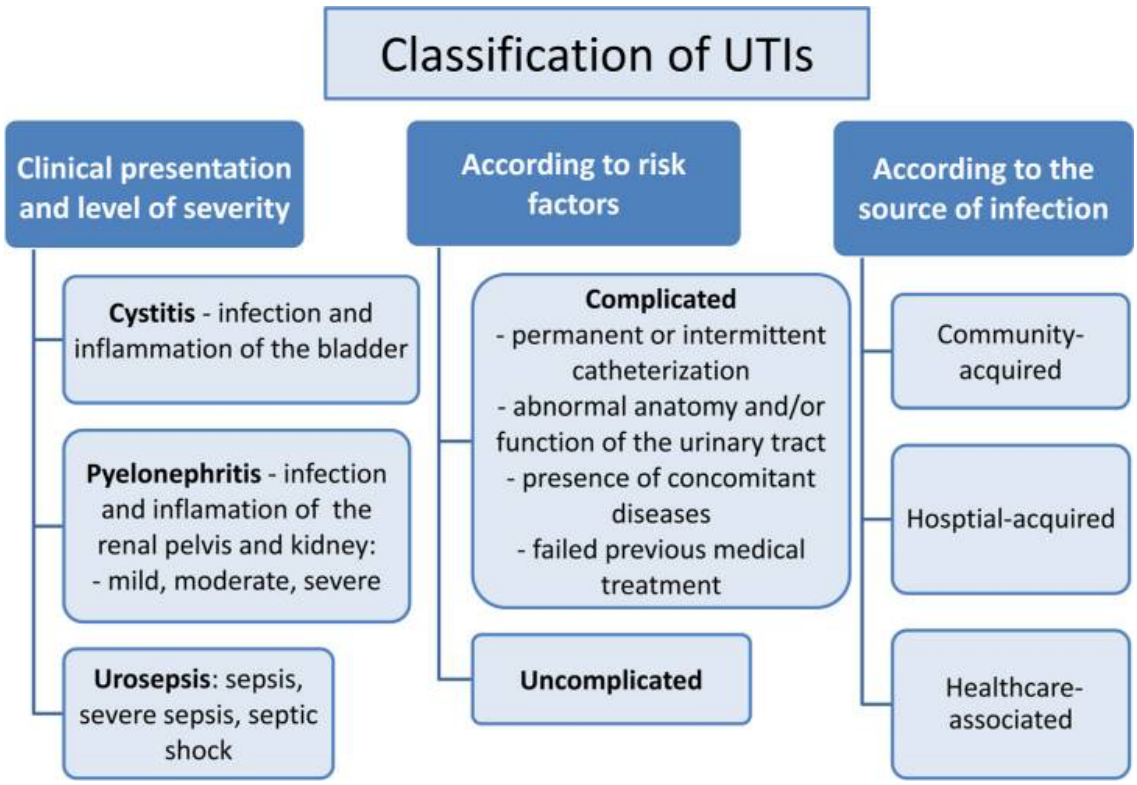

Fig. 1 Classification of UTIs [Kot, 2019]

Following an initial UTI, recurrence is not uncommon. About 25% of women and 20–40% of children who have had a UTI may experience a recurrent episode. Recurrent UTIs (RUTIs), defined as at least three UTI episodes occurring within 12 months, can pose challenges in terms of management and may require preventive strategies [*Gonzàlez et al., 2020*]. Individuals with RUTIs often receive antibiotic treatment, either as a therapeutic approach during active infections or as a prophylactic measure to prevent future episodes. However, the overuse of antibiotics is a concern due to the potential development of antibiotic resistance. In addition, long-term antibiotic treatment can disrupt the normal microbial communities in the vagina and gastrointestinal tract, leading to imbalances in the microbial microbiome, and secondary health issues [*Wawrysiuk et al., 2019*].

RUTIs can be a relapse or a reinfection. A relapse occurs when a UTI is caused by the same microorganism that was responsible for a previous infection, and this recurrence happens after what was considered adequate treatment. In other words, the bacteria were not completely eradicated during the initial treatment, leading to a resurgence of the same

infection. Reinfection, on the other hand, involves a UTI caused by a different microorganism or by the same microorganism that was previously isolated but after treatment and a period of negative urine cultures. Reinfections are often attributed to new exposures to potential pathogens, and the source may be extra urinary [*Caretto et al., 2017*]. UTIs can be caused by both Gram-negative and Gram-positive bacteria, as well as certain fungi. For uncomplicated UTIs, the most common causative agent is uropathogenic *E. coli* (UPEC), followed by other bacteria such as *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus faecalis*, group B *Streptococcus* (GBS), *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Candida* spp. In the case of complicated UTIs, the most prevalent microorganisms are UPEC, *Enterococcus* spp., *K. pneumoniae*, *Candida* spp., *S. aureus*, *P. mirabilis*, *P. aeruginosa*, and GBS. Treatment strategies for UTIs depend on the specific pathogen involved and the nature of the infection [*Flores-Mireles et al. 2015*].

1.3 Uropathogenic *E. coli* (UPEC)

UPEC are the most frequent microorganisms responsible for UTIs. Long considered extracellular pathogens, UPECs are, however, capable of adhering to and invading the cells of the genitourinary tract. The first indications that UPEC could occupy intracellular niches within the urinary tract came from observations dating to the late 1970s [*Fukushi et al., 1979*]. UPEC typically enters the urinary tract through an ascending route, transiting across the urethral opening and up the urethra before colonizing the bladder. Once in the bladder, UPEC can use peritrichous filamentous adhesive organelles known as type 1 pili for colonization, that are composed of repeating FimA subunits linked to a distal 3-nm-wide tip fibrillum containing the FimH adhesin. FimH adhesin in the type 1 pili can bind to mannose-containing glycoprotein receptors, including uroplakin 1a (UP1a), which is a tetraspanin membrane protein. Uroplakin complexes and specialized tight junctions link the umbrella cells in the bladder, forming a strong permeability barrier [*Lewis et al., 2016*]. UPEC can invade bladder cells through the endocytic machinery of phagocytosis or pinocytosis. In the case of phagocytosis, specific cell surface receptors on the bladder cell

membrane recognize and bind to UPEC. Rho family GTPases, such as Cdc42 and Rac, are activated in response to the binding of UPEC. Activation of these GTPases is a key step in initiating the signalling cascade that leads to actin assembly. The polymerization of actin filaments leads to the formation of cell surface extensions, such as filopodia and lamellipodia, that zipper up around the invading bacterium to execute the uptake.

As the cell surface extensions close in, they eventually fuse together, encapsulating the UPEC bacterium within a membrane-bound vesicle known as a phagosome.

The phagosome undergoes a series of maturation steps, including fusion with lysosomes, leading to the formation of a phagolysosome. This acidic and enzymatic environment aids in the degradation of the engulfed UPEC [*Martinez et al., 2000*].

UPEC invasion of epithelial cells by pinocytosis may occur via caveolae and clathrin-coated pit portals. Clathrin-coated pits are invaginations in the plasma membrane that have a lattice-like coat made primarily of clathrin protein. Clathrin-coated pits also involve assembly proteins that facilitate the formation of the clathrin lattice and cargo selection. In this mechanism, specific receptors on the cell surface recognize and bind to UPEC or other cargo. This binding triggers the assembly of clathrin around the cargo, forming a coated pit. The coated pit then invaginates and pinches off from the plasma membrane, forming a clathrin-coated vesicle. This vesicle containing UPEC is then transported into the cell's interior [*Kim et al., 2018*]. Most of the internalized bacteria are expelled out of urothelial cells by Rab27b-mediated exocytosis [*Song et al., 2009*].

Cell-based imaging studies have revealed that some bacteria escape their enclosing vesicle, and they may be found free in the cytosol or within membrane-enclosed vesicles. Free cytosolic UPEC rapidly multiplies, and forms structures known as Intracellular Bacterial Communities (IBCs) [*Anderson et al, 2004; Justice et al., 2004*]. These communities represent clusters of bacteria that reside and replicate within the host cell [*Conover et al., 2016*].

Invaded UPEC encased in lipid-enclosed vesicles could serve as portals to traffic the extracellular bacteria to the cytosol. Here, UPEC enters a dormant state establishing quiescent intracellular reservoirs (QIRs) [*Flores-Mireles et al., 2015*]. As a host countermeasure, infected epithelial cells undergo an apoptotic-like cell death, detaching from the urothelium to be expelled in the urine with the internalized bacteria (exfoliation).

Although the presence of QIR has yet to be observed in humans, these models suggest that exfoliation may be a double-edged sword because this expose deeper layers of the urothelium to infection, which is thought to lead to QIR formation [*Dikshit et al., 2015*].

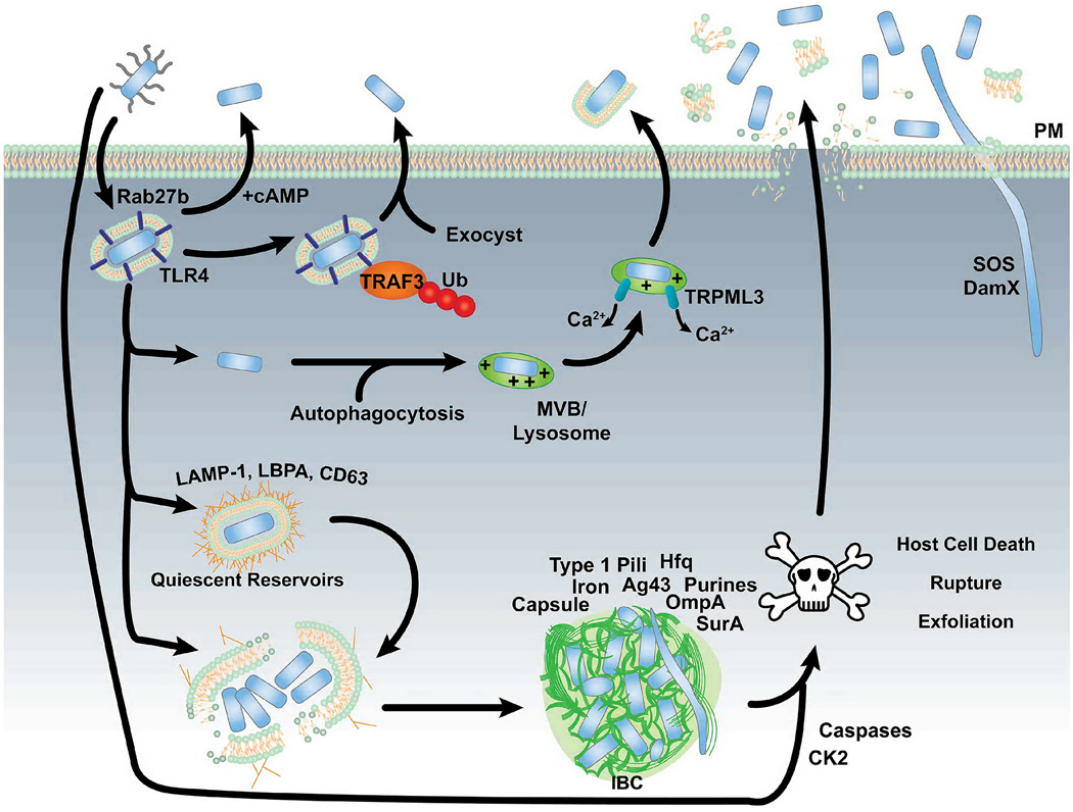

Fig. 3 The intracellular outcome of UPEC strains [*Lewis et al., 2016*]

Chronic bladder infection is common and can be either latent, in the form of the QIR, or active, in the form of asymptomatic bacteriuria (ASB/ABU) or chronic cystitis. In mice, as suggested by Hannan et al., 2012, the fate of bladder infection is determined within the first 24 hours of infection and constitutes a putative host-pathogen mucosal checkpoint that contributes to susceptibility to RUTIs [*Hannan et al., 2012*]. Many works show that the upstream movement of bacteria may transport persistent UPEC isolates from the skin, vagina or gut into the urinary tract, resulting in a repeated accumulation of UPEC in the urinary tract [*Brannon et al., 2020; Landry et al., 2022; Mulvey et al., 2001*].

1.4 UPEC Virulence Factors (VFs)

Unlike commensal isolates, UPEC harbour specialised virulence factors (VFs) closely related with colonization and persistence of bacteria in the urinary tract. These include surface structural components, such as lipopolysaccharide (LPS), polysaccharide capsule, flagella, outer-membrane vesicles, pili, curli, non-pilus adhesins, outer-membrane proteins (OMPs), as well as secreted toxins, secretion systems, and TonB-dependent iron-uptake receptors, including siderophore receptors [*Terlizzi et al., 2017*]. They are often encoded by genes grouped within pathogenicity-associated islands (PAIs) [*Mobley et al., 2019*]. The capsule is made of different chains of polysaccharides and covers lipopolysaccharide (LPS), which represents the O antigen. LPS and capsular polysaccharides are involved in the evasion of the host immune system [*Whitfield, 2009*]. Furthermore, urine composition also restricts bacterial growth. It represents a poor medium, frequently changing osmolarity and having a low pH. For this reason, UPEC synthesises metabolic factors, such as guanine, adenine and glutamine to overcome the issue. In addition, they also can evade the host's defences; in this regard the O-antigens contribute to bacterial resistance by forming a thick layer, thus avoiding the activity of the membrane attack complex (MAC) of the complement system [*Landraud et al., 2004*].

Among all the VFs expressed by UPECs, those that seem most involved in the adhesion, colonization, and invasion of the urothelium are [*Dhakal et al., 2008*]:

- The adhesin encoded by the *FimH* gene associated with type 1 pilus
- The adhesin encoded by the *PapG* gene associated with P-type fimbriae
- The adhesin encoded by the *sfa* gene associated with S-type fimbriae
- The cytotoxic necrotizing factor 1 (CNF1)
- α-hemolysin

90% of UPEC isolates express type 1 fimbriae or pilus encoded by the *fimA – fimH fimB* operons [*Abdul Raheem Hasan et al., 2021*]. Binding of FimH to its mannose receptor causes

transcriptional activation of the *fimB* gene leading to increased expression of type 1 pili. The type 1 pilus is a highly conserved protein structure among *Enterobacteriaceae*. From a structural point of view, the type 1 pilus appears as a thick helical rod 7 nm thick composed of multiple FimA subunits associated with an apical fibrillar structure made up of the FimH subunit, a mannose-binding fragment, and two adapter proteins FimF and FimG. The ability of FimH to bind mannose and mannosylated protein provides the bacterium with the ability to adhere to various cell types, including those of the epithelium of the urinary bladder and intestine [*Spaulding et al., 2017*]. The P-type pilus, encoded by the *pap* genes, has the shape of a rod at the end of which an adhesin (PapG protein) is linked, which mediates contact with the eukaryotic membrane. This also interacts with Toll-like receptor 4 (TLR4), reducing the expression of the polymeric immunoglobulin receptor (PIGR). This results in impaired transport of immunoglobulin A (IgA) across the epithelium, thus modulating the local immune response and preventing UPEC opsonization and clearance [*Du et al., 2021*].
S-type pili are characterized by a structure like that of type 1 and P pili, they are made up of a major subunit, SfaA and three minor subunits SfaG, SfaH, SfaS [*Kline et al.,2010*].
Hemolysin has been reported to be central for bladder and kidney tissue colonization in mice and it has a cytotoxic activity on a wide range of mammalian cell types. Hemolysin is able to bind to the receptor on the surface of the host cell and induce its lysis. This large toxin of 110 kDa, encoded by the *hly* operon, is secreted into the extracellular medium by a direct crossing of the two-membranes mechanism defining the type I secretion system [*Welch, 2015*].
The CNF1 toxin is commonly found in 30% of UPEC strains, always associated with HlyA within a PAI [*Mobley et al., 2009*]. Within host cells, CNF1 functions as a deamidase, converting specific glutamine residues on the Rho GTPases RhoA, Rac, or Cdc42 to glutamic acid. This amino acid change inhibits both the target proteins' intrinsic GTP hydrolytic activity and GTP hydrolysis induced by GTPase-activating proteins (GAPs), thus rendering Rho GTPases constitutively active. These GTPases are involved in a variety of cellular processes, including reorganization of the actin cytoskeleton [*Bower et al., 2005*].
Intracellular iron is crucial for various cellular processes, including metabolism and the synthesis of essential biomolecules. UPEC responds to the iron limitation encountered

within the mammalian urinary tract by using different iron acquisition mechanisms. First, UPECs produce and use siderophores, small molecules with high affinity for iron, to capture ferric iron, which is a form of iron commonly found in the extracellular environment. In addition, UPECs upregulate the expression of genes encoding iron transporters. These transporters are likely involved in the uptake of ferrous iron from the environment. Furthermore, UPECs utilize outer-membrane receptors to capture iron from heme molecules [*Subashchandrabose and Mobley, 2015*].

Bacteria with increased numbers of VFs, were more likely to be associated with same-strain RUTI episodes than strains with fewer virulence factors [*Silverman et al., 2013*].

Despite these evidences, it has also been suggested that no single VFs is sufficient for UPEC to cause UTI and no distinct virulence profile can predict RUTIs [*Hunstad and Justice, 2010*].

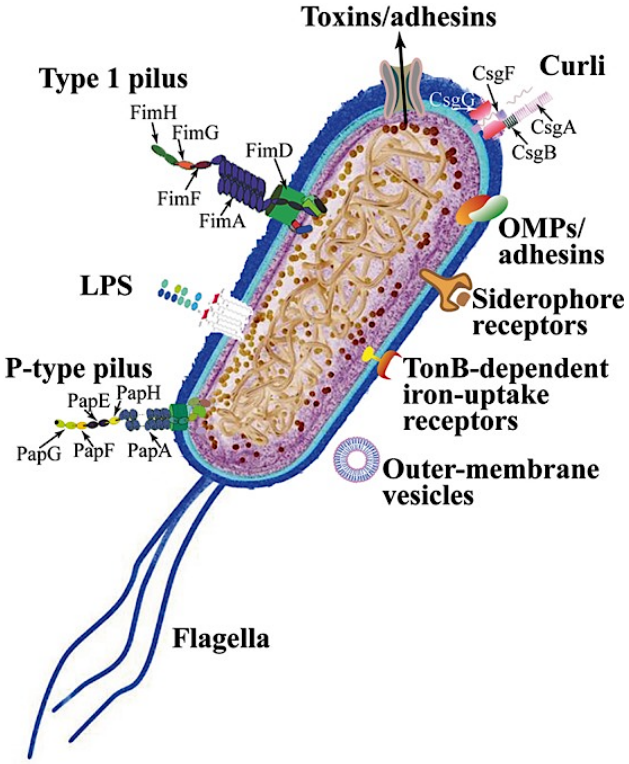

Fig. 4 E. coli virulence factors [Terlizzi et al., 2017]

1.4.1 Biofilm

The microbial biofilm is defined as a structured community, enclosed in a matrix of extracellular polymeric substances (EPS). EPS is composed of polysaccharides, proteins, and extracellular DNA (eDNA) and represents more than 80% of the overall structure of the biofilm; inside numerous channels allow the passage and exchange of water, air, and nutrients [*Hibbing et al., 2010*].

Biofilm formation is an important factor in bacterial virulence. The biofilm matrix serves as a protective shield, helping bacteria withstand environmental stressors such as changes in temperature, pH, and nutrient availability [*Flemming et al., 2016*]. EPS provides protection against host immune responses and bactericidal substances. This protection is crucial for the bacteria to evade the host's defence mechanisms, including phagocytosis and the activity of antimicrobial peptides. In fact, biofilm formation is strongly associated with increased resistance to antibiotic chemotherapy. In addition, biofilm formation is often linked to chronic infections, as bacteria in biofilms can persist for extended periods [*Alhariri et al., 2017*]. Biofilm formation consists of three consecutive steps: 1) adhesion; 2) accumulation and maturation; 3) detachment and dispersion [*Sharma et al., 2016*]. The initial adhesion and reversible attachment to the surface are controlled by environmental signals and characteristics, such as osmolarity, pH, temperature and the availability of nutrients, oxygen, and iron. Subsequently during the accumulation and maturation phase, the bacteria multiply and form aggregates organized into microcolonies incorporated into a self-produced polymeric matrix; when a certain cell density has been reached, the disintegration begins and the bacteria were released and dispersed [*Flemming et al., 2021*].

In vitro studies suggest that certain antibiotics, such as some cephalosporins, amikacin, and Ciprofloxacin (CIP) have been found to reduce UPEC-produced biofilms. This finding implies that specific antibiotics, when used at appropriate concentrations, may have the potential to disrupt biofilm formation by UPEC [*González et al., 2017*].

Indeed, biofilm-related infections pose a significant challenge in clinical settings. Developing advanced drug delivery systems that can effectively target and penetrate biofilms is a possible solution for improving the efficacy of treatments.

1.5 Immune response and ROS production

The body's initial defence mechanism against UPEC infection is the innate immune system. Innate immune responses involve various components such as macrophages, neutrophils, and natural killer cells that recognize and respond to pathogens in a non-specific manner. The infected host produces proinflammatory cytokines and chemokines as part of the immune response [*Kim et al., 2010*]. Activation by UPEC of pathogen associated molecular pattern (PAMP)-receptors, such as Toll-like receptor 4 (TLR4), Toll-like receptor 5 (TLR5), Toll-like receptor 11 (TLR11), induces an inflammatory response and cytokine secretion. When UPEC activates these receptors, it triggers a signaling cascade that ultimately leads to the activation of nuclear factor-kappa B (NF-κB) and the production of pro-inflammatory cytokines. Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, IL-8 and interferon- gamma (IFN-γ) are some of the major cytokines being released during UTI [*Spencer et al., 2014*]. Bladder epithelial cells express IL-1β [*Nagamatsu et al., 2015*], that is important for the clearance of UPEC strains [*Ambite et al., 2016*]. UPEC isolates can induce the secretion of IL-6 from the urothelium. IL-6 is present in the urine of patients with cystitis or pyelonephritis, suggesting a potential role in UTI pathogenesis [*Samuelsson et al., 2004*], as is IL-8. IL-8 is crucial for recruiting neutrophils during a UTI [*Godaly et al., 2000*]. The rapid recruitment of neutrophils into the bladder lumen is a protective mechanism to combat UPEC and limit the spread of infection. The inflammatory response may lead to the exfoliation (shedding) of infected bladder epithelial cells. This shedding helps eliminate the reservoir of bacteria within the bladder, contributing to a decrease in bacterial burden and reducing the risk of reinfection [*Hayes and Abraham, 2016*]. The bladder epithelium normally has an exceptionally slow turnover rate of approximately 40 weeks in both mice and humans. However, large numbers of exfoliated bladder epithelial cells can often be found within urine from human patients with UTIs [*Kim et al., 2010*].

As is known in literature, bacteria that do not stimulate a strong immune response, which involves TLR4, often underlie Asymptomatic Bacteriuria (ASB); while a strong immune response causes symptomatic UTIs [*Morales et al., 2023; Ragnarsdóttir et al., 2010*].

Elevated reactive oxygen species (ROS) levels are often associated with tissue damage, and this phenomenon has been observed in various organs [*Mittal et al., 2014*]. The presence of

malondialdehyde, a byproduct of oxidative stress and a marker of lipid peroxidation, in the urine of patients with acute UTIs suggests increased ROS during these infections [*Kurutas et al., 2005*]. ROS can be generated by different sources, including mitochondrial ROS formation, xanthine oxidase (XO), uncoupled nitric oxide (NO) synthase and NADPH oxidases (NOX). One of the most important sources of ROS is the family of NOX, widely distributed in the body, including various types of smooth muscle and epithelium [*Dao et al., 2020*]. SOD is a crucial enzyme that helps maintain cellular redox potential by neutralizing excess superoxide radicals (O_2-).

It converts superoxide radicals into less harmful hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). SOD ability to neutralize superoxide radicals is emphasized as a vital protective mechanism for normal cells during infections caused by intracellular pathogens [*Losada-Barreiro et al., 2022*].

1.6 Antimicrobial Resistance (AMR)

In the field of infectious diseases, antimicrobial resistance (AMR) represents one of the most worrying global emergencies. Numerous factors have contributed to the environmental spread of multiple resistances, including hospital infections, the emergence of zoonotic pathogens, and poor medical practices. Inappropriate and extensive use of conventional antibiotics has reduced their effectiveness against most pathogens [*Ruiz et al., 2017*]. WHO has declared that AMR is one of the top ten global Public Health threats facing humanity. In addition to death and disability, prolonged illness results in longer hospital stays and higher costs. For common bacterial infections, including UTIs, high rates of resistance against frequently used antibiotics, such as fluoroquinolones, have been observed. For example, the rate of resistance to CIP, varied from 8.4% to 92.9% for *E. coli.* Bacteria resistant to colistin have also been detected in several countries and regions, causing infections for which there is no effective antibiotic treatment at present [*Gogry et al., 2021*].

AMR and the emergence of multi-drug resistant (MDR) pathogens in UTIs are linked to the high rates of inadequate antibiotic empirical therapies. A study by *Wagenlehner et al., 2016* involving 27,542 patients from 856 urology units in 70 countries revealed that 56% of

hospitalized patients were treated with antimicrobials. Among these patients, 46% received prophylactic antibiotic treatment, 26% for microbiologically proven UTIs, 21% for suspected UTIs, and 7% for other infections. The study also found that broad-spectrum antibiotics were commonly applied, including fluoroquinolones (35%), cephalosporins (27%), and penicillins (16%) [*Wagenlehner et al., 2016*]. There is a lot of evidence of the correlation between the increased use of broad-spectrum antibiotics and the subsequent rise in AMR and MDR bacteria [*Kot, 2019*]. As suggested by *Bartoletti et al., 2016,* antibiotic treatment is recommended against symptomatic UTIs [*Bartoletti et al., 2016*]. The *European Association of Urology* recommends fosfomycin trometamol, pivmecillinam, nitrofurantoin, trimethoprim-sulfamethoxazole (TMP-SMZ) and aminoglycosides for this purpose. Additionally, the use of fluoroquinolones as first-line antibiotics is discouraged due to relevant negative side effects. Fluoroquinolones are known for their broad-spectrum activity and ability to penetrate various tissues, including the kidneys, so the treatment with these antibiotics is limited to acute pyelonephritis and complicated UTIs [*Zagaglia et al., 2022*]. Among aminoglycosides, Gentamicin (GM) is used for the treatment of many types of bacterial infection, but the limit for its use is due to the well-known toxicity. The main adverse effect of aminoglycosides is ototoxicity, neuromuscular blockade, and nephrotoxicity [*Lopez-Novoa et al., 2011*]. Intravesical instillation of GM can overcome systemic toxicity and can be used for treating RUTIs and lower UTIs in patients with neurogenic bladders [*Andretta et al., 2022*]. It must also be considered that due to its high hydrophilicity, GM diffuses very slowly and poorly through cell membranes. Due to its weak basic properties, after reaching the interior of the cell, GM is confined within the lysosomes, where the acidic pH may suppress its activity [*Tulkens and Trouet, 1978*].

The rise in antimicrobial resistance among UPEC isolates, especially those producing extended-spectrum beta-lactamases (ESBL) enzymes and exhibiting multidrug resistance (MDR), is indeed a significant global Public Health concern. The challenges posed by drug-resistant UPEC strains have implications for treatment outcomes, patient well-being, and the overall burden on healthcare systems [*de Souza da-Silva et al., 2020*].

Addressing the issue of antimicrobial resistance in UPEC requires a multifaceted approach, including the development of new antibiotics or new strategies, implementation of effective

infection prevention and control measures, and global collaboration to combat the spread of resistant strains.

1.7 Alternative strategies

In addition to the common antibiotic therapy used for the treatment of UPEC infections, there are currently many innovative strategies introduced.

Medicinal plants, such as cranberry, have been traditionally used for the prevention and treatment of UTIs. The protective effect of cranberry is often attributed to its polyphenolic compounds, particularly proanthocyanidins. These compounds are believed to act as anti-adhesive agents, preventing the adherence of UPEC to the uroepithelial cell receptors [*Head, 2008*].

Research in animal models have explored the potential of vaccines in reducing the occurrence and severity of urinary tract infections (UTIs). Vaccines targeting virulence factors of uropathogenic bacteria, such as pili and flagella, have shown promise in preclinical studies [*Langermann et al., 2000*].

Other promising strategies are the microbiota transplantation, the use of probiotics and anti-adhesive therapeutics, such as d-mannose, and the phage therapy, which is based on the ability of phages to enter and lyse bacteria [*Zagaglia et al., 2022*]. Furthermore, in order to improve the therapeutic characteristics of antimicrobial agents, the use of nanotechnology has received particular attention; this has emerged as a promising approach for the treatment of intracellular infections and infections related to biofilm formation.

1.7.1 Nanocarriers

Nanocarriers, such as nanoparticles, niosomes (Nio), nanoemulsions and liposomes, can be designed with precise control over their size, shape, and surface properties. This level of control allows for improved drug solubility, stability, and bioavailability. Furthermore, the small size of nanoparticles can enable better tissue penetration and cellular uptake.

Nanocarriers can protect the encapsulated drug from degradation, enzymatic breakdown, or premature release in the body [*Marianecci et al., 2014*]. Some examples of the use of nanocarriers in the treatment of UTIs can be found in the literature. For example, a gel consisting of phytocompounds derived from cranberries loaded into nanoemulsions used intravaginally for the treatment of UTI [*Kaur et al.,2017*]. Another example is carbon dots (CDs) coated calcium carbonate nanocarriers (CCNC) from organic chicken eggshells conjugated with levofloxacin against multidrug resistance (MDR) *E. coli* having Extended-Spectrum Beta-Lactamase (ESBL) genes [*Kanwal et al., 2022*].

Among the nanocarriers, Nio are vesicular structures constituted by non-ionic surfactants which, in contact with an aqueous medium, form bilayer structures. Nio are highly versatile structures as they allow the delivery of different substances: water-soluble ones which are preferentially localized within the aqueous compartment and between the interlamellar aqueous spaces; the lipophilic ones, on the other hand, which intercalate between the hydrophobic tails constituting the double layer. Furthermore, the delivery of drugs within Nio could increase their stability, efficacy, therapeutic index, and reduce their toxicity [*Yeo et al., 2017*]. Nio can be classified based on: lamellarity (number of double layers that characterize them), size, preparation method used. Nio can be divided into three groups based on the size of the vesicles: small unilamellar vesicles (0.025–0.05 mm), multilamellar vesicles (>0.05 mm), and large unilamellar vesicles (>0.10 mm) [*Kazi et al., 2010*]. The stability of Nio is mainly influenced by the surfactant used, the properties of the encapsulated drug, the hydration temperature, the detergent, the polymerization of surfactant monomers and the charged molecules [*Diljyot, 2012*].

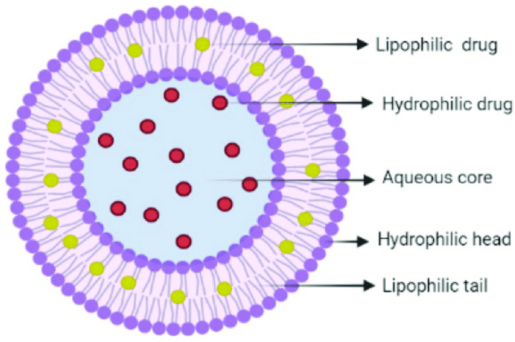

Fig. 5 Niosome structure [Milan et al., 2022]

2. AIM OF THE STUDY

Urinary tract infections (UTIs) represent an important chapter in the field of general medicine, ranking among the main causes of morbidity, outpatient visits and healthcare costs. Uropathogenic *Escherichia coli* (UPEC) are the main causes of UTIs. Several studies have shown that UPEC, long considered extracellular pathogens, can invade epithelial cells of the genitourinary tract both *in vitro* and *in vivo*. Factors, such as invasion of host cells and biofilm formation, provides UPECs with a survival advantage and significantly contributes to the pathogenesis of UTIs. Due to these characteristics, the treatment of UTIs may require long antibiotic therapies.

It therefore appears clear that the optimization of antibiotic therapy strategies for the treatment of this pathological state represents a priority.

The study was conducted in the two following steps:

FIRST STEP

The study of UPEC strain characteristics is crucial in understanding the mechanisms that enable these bacteria to persist and cause UTIs. The factors that contribute to the ability of UPEC to survive, adhere, colonize, damage, invade host cells, and evade host defences are multifaceted. As suggested by some authors, the complex interplay between bacterial infection capabilities, treatment success and host factors are likely to determine recurrence rather than specific genetic characteristics of the *E. coli* bacteria itself [*Ikäheimo et al.,1996; Murray et al., 2021*]. Numerous evidences in the literature have shown that not a single virulence factor (VF) is sufficient for UPEC to cause UTIs and no distinct virulence profile can predict recurrent UTIs (RUTIs) [*Hunstad and Justice, 2010*].

For these reasons, the focus of the first step of this study was to identify bacterial characteristics possibly associated with recurrence of the infection. In this study, UPEC strains, isolated from patients with RUTIs were sequenced, phenotypically and genotypically characterized, and assayed for invasion and survival ability in cell models.

SECOND STEP

UTIs are associated with significant use of antibiotics that cause implications for bacterial ecology and spread of resistance to antibiotics, especially when it stems from the empirical antimicrobial treatment of recurrent UTIs. Due to the limitation of the conventional approaches to combat microbial infections, the use of drug-delivery systems has been hypothesized for the controlled and localized release of drugs even in the case of multi-resistant bacteria and strong biofilm formers. Recently, advances in the field of nanotechnology have provided a promising tool for improving the activity of antimicrobial agents. The use of nanocarriers is useful to make the drug reach the release site more quickly and efficiently [*Mansouri et al., 2021*]. Nanocarriers have the great advantage, compared to conventional therapies, of protecting the active substances conveyed from enzymatic degradation, making them more available at the site of action and improving their solubility thanks to their inclusion in the carrier itself.

Therefore, the second step of this project was to study new alternative strategies, in the field of nanocarriers, to counteract recurring UTIs.

In particular:

1. The formation of intracellular structures provides UPEC with a great advantage and leads to the development of relapses and the failure of antibiotic therapies with an increase in the phenomenon of antibiotic resistance [*Sharma et al., 2021*]. Among aminoglycoside antibiotics, Gentamicin (GM) shows a concentration-dependent bactericidal activity and a prolonged post-antibiotic effect against a broad spectrum of bacteria including UPEC. Due to its polar nature, however, the rate of oral absorption and tissue penetration are poor in addition to its poor penetration into cells. In order to improve its therapeutic characteristics, the use of nanotechnology has received particular attention; this has emerged as a promising approach to enhance the activity of conventional antimicrobial agents and for the treatment of intracellular infections by allowing drug release within infected cells [*Malik et al., 2023*]

For this reason, the aim of this part of the study was to evaluate the activity of GM-loaded niosomes (GM-Nio) against UPEC strains isolated from patients with recurrent UTI.

2. In the field of infectious diseases, antibiotic resistance represents one of the most worrying global emergencies, with important implications both from a clinical point of view and in terms of economic impact. Another phenomenon that leads to the increase in antibiotic resistance is linked to the ability of bacteria to form biofilm [*Flemming et al., 2016*]; in fact, approximately 60% of infections are associated with microorganisms capable of forming biofilms [*Ciofu et al., 2022*]. The formation of this structure represents an excellent strategy for bacterial survival upon exposure to antimicrobial compounds. Slow growth, and EPS, which prevents compound molecules from diffusing and reaching the bacteria, are responsible for the development of antibiotic tolerance. Various studies described the antimicrobial and antibiofilm activity of peculiar CIP-loaded noisome formulations (CIP-Nio) against Gram-negative and Gram-positive bacteria [*Kashef et al., 2020; Mirzaie et al., 2020 ; Tyagi et al., 2017*]; so another goal of this research was to evaluate antibiofilm activity of CIP-Nio against UPEC strains.

3. MATERIALS AND METHODS

3.1 Bacterial strains

The strains used in this study were:

- *E. coli* LC2 and *E. coli* LC3 were two UPEC strains, collected over a 10 years span from a patient with recurrent UTI (cystitis pyelonephritis).
- *E. coli* EC73 was one of 3 UPEC strains, isolated over 2 years from a male patient with recurrent UTIs, already characterized and able to invade and persist in prostatic cells [*Conte et al., 2016*].
- *E. coli* K-12 MG1655 was a weak biofilm producer and a non-invasive strain.
- *E. coli* ATCC 700928 (CFT073) (ATCC, Manassas, VA, USA) was a reference strain isolated from the blood of a patient with acute pyelonephritis.

Bacterial strains were grown in Brain Heart Infusion broth (BHI) (Oxoid Ltd., Basingstoke, UK) and stored in BHI with glycerol at −80 °C.

3.1.1 GFP plasmid transformed strains

E. coli EC73 was transformed with the GFP-expressing plasmid pFPV25.1 as described by *Valdivia & Falkow, 1996* [*Valdivia & Falkow, 1996*] using the calcium chloride method. Transconjugants were selected on Luria-Bertani (LB) agar plates (Oxoid Ltd., Basingstoke, UK) with ampicillin (100 μg/ml), incubated overnight at 37°C. Green fluorescent transconjugant colonies were picked and stored in glycerol at - 80 °C.

3.2 Cell line

The human bladder cancer cell line (T24) was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Corning, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; SAFC Biosciences Inc., Lenexa, KS, USA), 100 IU/L penicillin and 100

mg/L streptomycin. Cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

3.3 Characterization of *E. coli* strains

3.3.1 Identification and susceptibility testing

The isolates were tested on the VITEK 2 (BioMérieux, France) platform for identification at the Microbiology laboratory of the Policlinico "Umberto I" in Rome. The Vitek 2 compact Automated Expert System (AES) (BioMérieux, France) was used for metabolic profiling. Using the Vitek 2 ID-GNB card (BioMérieux, France), identification of Gram-negative bacilli occurs through testing the organism's metabolic activity in biochemical tests designed to measure carbon source utilization and enzymatic activity. The Biochemical tests used are shown in Table 1.

Results were interpreted by the Advanced Expert System software (AST-N202) using EUCAST breakpoints [*The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of mics and zone diameters, version 10.0, 2020; http://www.eucast.org/ clinical_breakpoints/*]

The tested antibiotics, representing several antibiotic families, were as follows: 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.

Adonitol	ADO
Beta-galactosidase	BGAL
D-Glucose	dGLU
Gamma-glutamyl-transferase	GGT
Fermentation/glucose	OFF
D-Maltose	dMAL
D-Mannitol	dMAN
D-Mannose	dMNE
L-Proline arylamidase	ProA
Tyrosine arylamidase	TyrA
Urease	URE
D-Sorbitol	dSOR
Saccharose/sucrose	SAC
D-Tagatose	dTAG
D-Trehalose	dTRE
Malonate	MNT
5-Keto-D-gluconate	5KG
L-Lactate alkalinization	ILATk
Succinate alkalinization	SUCT
Alpha-galactosidase	AGAL
Phosphatase	PHOS
Glycine arylamidase	GlyA
Ornithine decarboxylase	ODC
Lysine decarboxylase	LDC
Coumarate	CMT
Beta-glucoronidase	BGUR
O/129 resistance	O129R
L-Malate assimilation	IMLTa
Ellman	ELLM
L-Lactate assimilation	ILATa

a Abbreviation.

Tab. 1 Biochemical tests used to differentiate between UPEC isolates

3.3.2 Swimming motility assay

Swimming motility assay was performed on soft LB-agar plates containing 0.25% agar. The plates were allowed to dry overnight at 4 °C before use. Swimming plates were seeded below the agar surface with 2 μL bacterial culture. The plates were incubated at 37 °C for 24 h after which the diameters of swimming zones were measured [*Dusane et al., 2014*].

3.3.3 Hemolysis

Hemolysin production was assessed using plates containing 5% defibrinated sheep blood (Oxoid Ltd., Basingstoke, UK). The plates were examined after up to 48 h of incubation at 37 °C for the presence of a hemolysis area around colonies.

3.3.4 Biofilm production

E. coli strains were tested for their ability to form biofilm, using the model described by *Stepanovic et al., 2007* [*Stepanovic et al., 2007*]. Biofilm assays were conducted in 96-well polystyrene microplates, inoculating 20 μL of each bacterial strain (1–2 × 10^8 CFU/mL) in a well filled with 180 μL of Tryptic Soy Broth (TSB). Plates were incubated at 37 °C for 24 h. After the incubation time, microplates were washed twice with phosphate-buffered saline (PBS) and fixed with methanol (99.8% *v*/*v*) for 15 min. Subsequently, wells were stained for 20 min with 1% w/v crystal violet (Sigma-Aldrich, USA), then rinsed three times with H_2O, and eluted with 95% ethanol.

The biofilm formation was quantified through the measurement of the optical density (OD) at 570 nm with a microplate reader (Tecan Sunrise, *X*-fluor). Based on the cut-off OD, defined as three standard deviations 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

3.3.5 Adhesion, invasion, and survival assay on T24 cells

Adhesiveness of *E. coli* strains was assayed to T24 cells, by culturing the cells in a 24-well plate at a density of 2 × 10^5 cells/ml for 24 h at 37°C in 5% CO_2. T24 monolayers were infected with bacterial suspension at a multiplicity of infection (MOI) of 10 or 1 in the case

of *E. coli* ATCC 700928 (CFT073), then centrifuged twice at 500 *g* for 2.5 min, to synchronize infection, and incubated at 37 °C in 5% CO_2.

Regarding adhesion, after 30 min, cells were extensively washed with PBS, then lysed adding 0.1% *(v/v)* Triton X-100 and plated on Tryptic soy agar (TSA) plates for 24 h at 37 °C, to determine the number of adherent bacteria, as colony forming unit (CFU). *E. coli* strains were considered adherent when the mean adhesion index (number of adherent bacteria/initial inoculum) was ≥ 0.8%.

For invasion assay, 1h post infection, T24 cells were washed three times with PBS and then incubated for 1h at 37 °C in 5% CO_2 in growth medium containing gentamicin (GM) (100 μg/ml) (Thermo Fisher Scientific, Waltham, MA, USA), to kill extracellular bacteria.

For survival assay, after the incubation time, cell monolayers were incubated again with a growth medium containing 50 μg/ml GM, for 24 h at 37 °C in 5 % CO_2. Survival was assessed at 24h, 48h and 72h. After each incubation time, cells were washed three times with PBS and lysed in 0.1 % *(v/v)* Triton X-100. The number of viable bacteria was determined by plating the dilutions of cell lysates in TSA agar. A strain was considered invasive when the ratio between the number of intracellular bacteria and the initial inoculum was ≥ 0.1 %. Strains were considered able to survive when the ration was ≥100% and to replicate intracellularly when the ratio was ≥200%. *E. coli* K12 MG1655 was used as negative control in the invasion and survival assays.

3.3.6 Bacterial DNA extraction

E. coli strains were grown on TSA plates for 24 h at 37°C. Then, a single colony was inoculated in TSB and incubated overnight at 37°C. After the incubation time, whole-DNA bacterial extracts, from each culture, were prepared using "QIAamp DNA Mini Kit" (Qiagen, Germany), as the manufacturer's instruction. The qualitative and quantitative analysis of DNA extraction was assessed with the NanoDrop™ spectrophotometer (Thermo Scientific, USA).

3.3.7 Phylogenetic group belonging

Belonging to a specific phylogroup was assessed by a 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* [*Clermont et al., 2000*] and based on the obtained amplification, *E. coli* strains are assigned to phylogroup A, B1, B2 or D.

The primers used were:

- ChuA.1 (5′-GACGAACCAACGGTCAGGAT-3′)
- ChuA.2 (5′-TGCCGCCAGTACCAAAGACA-3′),
- YjaA.1 (5′-TGAAGTGTCAGGAGACGCTG-3′)
- YjaA.2 (5′-ATGGAGAATGCGTTCCTCAAC-3′),
- TspE4C2.1 (5′-GAGTAATGTCGGGGCATTCA-3′)
- TspE4C2.2 (5′-CGCGCCAACAAAGTATTACG-3′)

Primers generated 279-, 211-, and 152-bp fragments, respectively.

Amplifications were carried out in 25 μL reaction mixtures composed by:

- 1X PCR reaction buffer (Biolabs Inc., New England, UK)
- 0.2 mM of dNTPs (Biolabs Inc, New England, UK)
- 0.5 μM of primers (Sigma-Aldrich, Milan, Italy)
- 1.25 U Taq DNA polymerase (Biolabs Inc., New England, UK).
- 50 ng/μL of DNA bacterial extracts

Each cycle consisted of a denaturing step of 30 s at 94 °C, followed by an annealing step of 30 s at 56 °C and an extension step at 72 °C for 30 s. After 30 cycles of PCR, amplicons were

separated by 2.0% agarose gel electrophoresis in 45 mM Tris-borate, 1 mM EDTA buffer (pH 8.0) containing 5 μL of Midori Green Advance DNA stain (Nippon Genetics Europe GmbH, Germany). Amplifications were performed in triplicate and their size was determined by comparison with a 100-bp DNA ladder (Biolabs Inc., New England, UK).

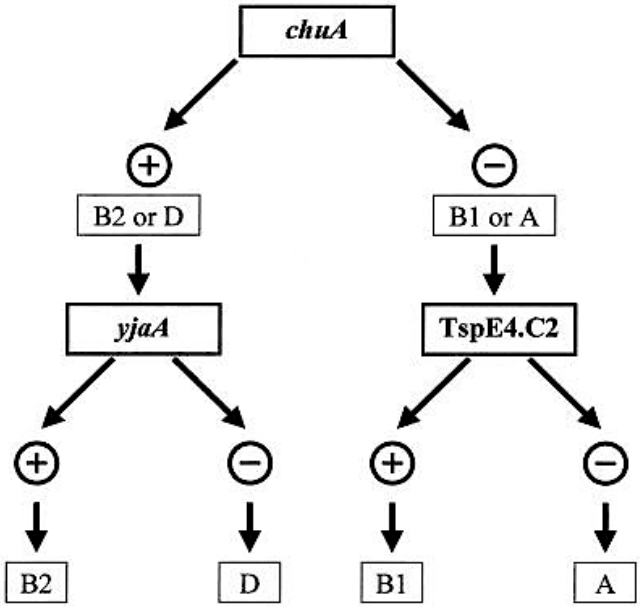

Fig. 6 Dichotomous decision tree to determine the phylogenetic group of an E. coli strain [*Clermont et al., 2000*]

3.3.8 Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR)

UPEC strains were genotyped by *Random Amplified Polymorphic DNA- Polymerase Chain Reaction* (RAPD-PCR), a molecular epidemiology technique used to type bacterial isolates on the base of the analysis of bacterial polymorphisms [*Wolska and Szweda, 2012*]. RAPD-PCR involves the use of short non-specific primers and low stringency reaction conditions, to amplify random and numerous genome sections. The amplification result represents a genetic fingerprint of each bacterial strain. Bacterial isolates with similar genomes shared similar amplification profiles.

In this case the primer used were:

- 3EC: 5′-GTAGACCCGT-3′

- 4EC: 5'-AAGAGCCCGT-3'

For each isolate 25µl of reaction mix was prepared. The mix contained: 3mM $MgCl_2$ (Bio Line, London, UK), 0.5 mM $dNTP_s$ (New England BioLabs, UK), 1 µM of each primer, 1 U of Taq DNA polymerase (Bio Taq DNA polymerase, Meridian Bioscience, USA) in 1X PCR buffer (Meridian Bioscience, USA) and 3 µl of template dsDNA. PCR reaction was performed, with a GeneAmp PCR System 2400 (Perkin Elmer) thermocycler, as follows: 1 cycle at 95°C for 5 min, 45 cycles at 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. Amplification products were solved by electrophoresis on 1% agarose-tris-borato-EDTA (TBE) gel, containing 5µl of "Midori Green Advance DNA Stain" (Nippon Genetics Europe GmbH). 1kb DNA Ladder (Meridian Bioscience, USA) was used as a DNA molecular weight marker. The electrophoresis was carried out at 90 V for 90 minutes in 1X TBE buffer. The gel was visualized under UV transilluminator (UVP, Cambridge). The DNA banding patterns of RAPD-PCR assays were analysed using TotalLab TL120 Trace version 2006 (Nonlinear Dynamics). The Dice coefficient of similarity was calculated on the base of presence/absence of bands while the unweighted pair group method with arithmetic averages (UPGMA) as agglomerative method. Cluster analysis has been carried out by using the statistical excel plugin XLstat 7.5 (Addinsoft, USA). The similarity percentage cut-off was set at 80%.

3.3.9 *In silico* virulence genotyping

The presence of 26 virulence-associated factors of ExPEC was determined by in silico analysis. Gene sequences were taken from GenBank and tested against the whole genomes of the *E. coli* ATCC 700928 (CFT073), *E. coli* UTI89 and *E. coli* 83972 strains using the BLASTn algorithm included in BLAST+ v.2.4.0. The VFGs analysed were represented by six functional groups:

1. Adhesins: *fimH* (type 1 fimbriae), *focG* (F1C fimbriae), *papA-H* (P fimbriae), *sfaS* (S fimbriae);
2. Biofilm related: *agn43* (Antigen 43)

3. Iron uptake: *chuA* (heme receptor), *fyuA* (yersiniabactin siderophore receptor), *iroN* (salmochelin siderophore receptor), *ireA* (iron-regulated element, siderophore receptor), *iutA* (aerobactin siderophore receptor);
4. Protectins: *iss* (increased serum survival), *kpsM II* (group II capsule), *kpsM II K2* (group II capsule, K2), *kpsMT III* (group III capsule), *traT* (serum resistance);
5. Toxins: *cnf1* (cytotoxic necrotizing factor 1), *hlyA*/ *hlyE* (hemolysin A and E), *sat* (secreted autotransporter toxin);
6. Miscellaneous: *ibeA* (invasion of brain endothelium), *malX* (pathogenicity-associated island marker of CFT073), *usp* (uropathogenic specific protein).

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 *E. coli* strains, the presence of virulence genes was tested on genomic data produced by next-generation sequencing. Briefly, 2 ml of an overnight bacterial culture was used for total genomic DNA extraction using the GenElute bacterial genomic DNA kit (Sigma-Aldrich, Italy) according to the manufacturer's instructions. DNA final elution was performed in water. DNA was sequenced using 250-bp paired-end reads on the Illumina 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. Filtered data were mapped to reference sequences using Bowtie2 v.2.2.9. Hits presenting a query coverage of ≥95% were considered positive.

3.3.10 Interleukins evaluation

Interleukins were evaluated after 2 h of infection on human T24 cells with *E. coli* strains. After the infection time, the cells were washed, detached and each Sample was processed for total RNA extraction using TRizol reagent (Invitrogen, Carlsbad, CA). 1 μg of RNA was retro transcribed into cDNA and Real-Time qPCR was performed by power up SYBR green master mix (Applied Biosystems, Waltham, Massachusetts, USA). Samples were run 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 levels were calculated using the ΔΔCT method [Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.)*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262]. Primer sequences are shown in Table 2.

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′

Tab. 2 Primers used in RT-qPCR for the detection of interleukins

3.3.11 Reactive Oxygen Species (ROS) production

Reactive oxygen species (ROS) production was evaluated after 24 h of infection on human T24 cells with *E. coli* strains.

3.3.11.1 Evaluation of ROS generation

The generation of ROS was monitored using a peroxide-sensitive fluorescent compound, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). H_2DCFDA is a chemically reduced form of fluorescein which, after penetrating inside the cells, is deacetylated by intracellular esterases into a non-fluorescent compound. This product is subsequently oxidized by ROS into a highly fluorescent compound, 2', 7' - dichlorofluorescein (DCF). The infected monolayers were washed with 1X PBS and incubated with H2DCFDA (10 μM) for 30 min at 37°C. After a further 3 washes in PBS 1X, the fluorescence was measured with a flow cytometer (Perkin-Elmer, Massachusetts, USA) at a wavelength of 488/535 nm. Untreated

and hydrogen peroxide-treated cells were used as negative and positive controls, respectively.

3.3.11.2 Western Blotting

After the infection time, the cells were detached with trypsin (Corning, NY, USA). 1 mL of RPMI medium, supplemented with 10% FBS, was then added to the cells, to deactivate the trypsin action. The cells were centrifuged for 10 min at 5000 rpm at 4°C. Once the supernatant was removed, the cell pellets were treated as follows. Preparation of total extracts was performed by adding 1% TEEN triton buffer (10 mM tris HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, protease inhibitor cocktail dissolved 1:100) to the cell pellets. Samples were kept at 4°C for 20 minutes and shaked every minute. Subsequently, samples were centrifuged at 14000 rpm, 4°C for 20 minutes and then the supernatant containing the cellular proteins was removed and stored in aliquots at -80°C. The total protein content was determined according to Bradford method [*Bradford, 1976*].
Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose (GE Healthcare, Life Sciences, Little Chalfont, Buckinghamshire, UK). After the transfer, each membrane was incubated for 1h at room temperature in Tris Buffer Saline 20 mM (TBS-T Tris-HCl pH 7.4; 137 mM NaCl; 0.1% Tween 20) containing 5% non-fat dried milk powder (Blotting-Grade Blocker, PanReac AppliChem, ITW reagents). Subsequently, the membrane was incubated overnight at 4 °C with the primary antibody dissolved in TBS-T containing 5% milk.
The primary antibodies used were:

- polyclonal anti-actin (A2066 Sigma-Aldrich; Milan, Italy) (1:1000)
- polyclonal anti-System Xc^- (Ab175186 Abcam, Milan, Italy) (1:1000)
- polyclonal anti-Nrf2 (16396-1-AP Protein Tech; Manchester, United Kingdom) (1:1000)
- polyclonal anti-Ftn (sc25617, Santa Cruz, CA, USA) (1:1000)
- polyclonal anti-HCP (A0031, Dako, Santa Clara, CA, USA) (1:1000)
- polyclonal anti-lamin A (Ab26300 Abcam; Milan, Italy) (1:1000)
- monoclonal anti-vinculin (sc-73614, Santa Cruz, CA, USA) (1:1000)

- monoclonal anti-ARA70 (NCOA4) (sc-373739, Santa Cruz, CA, USA) (1:1000)
- monoclonal anti-bLf (sc-53498, Santa Cruz CA, USA) (1:1000)
- monoclonal anti-Fpn 31A5 (1:1000), generously provided by T. Arvedson (Amgen)
- monoclonal anti-TfR1 (sc-32272, Santa Cruz, CA, USA) (1:1000)
- monoclonal anti- p-Histone H2A.X (Ser 139) (sc-517348, Santa Cruz, CA, USA) (1:1000).

The day after, the membrane was then incubated with the appropriate HRP-conjugated secondary antibody (Bio-Rad, Hercules, California, USA) (1:1000) in TBS-T containing 2.5% milk for 1 h at room temperature. The reagent used for detection was Clarity Western ECL substrate (170-5061, Bio-Rad, Hercules, California, USA). Differences between samples with respect to the presence of the proteins of interest were normalized using, as reference, actin protein for total extracts and lamin A protein for nuclear extracts.

3.4 Niosomes (Nio) preparation and characterization

All Nio formulations used in this work were prepared and characterized by the "Nanomedicine Lab" of the Department of Chemistry and Drug Technology of "Sapienza" University of Rome.

Nio were prepared using a technique called "*Thin Layer Evaporation*" or "*Film Formation Technique*": the surfactants (Tween 85 and Span 80), appropriately weighed, are solubilized using a methanol/chloroform mixture. The solution obtained was subjected to the action of a Rotavapor (Heidolph Type VV2000), at room temperature for approximately 1 h, to evaporate the organic solvent and form a thin layer of film on the walls of the test tube. Any traces of organic solvent were then eliminated with an oil pump (Büchi-Italia S.r.l., Assago, MI). The film was rehydrated with Hepes buffer (pH=7.4, 0.01 M) or with a solution containing GM or CIP. To obtain a unilamellar suspension, an ultrasonic disintegrating sonicator (Vibracell-VCX 400, Sonics, Taunton, MA, USA) is used by setting the following parameters:

- Amplitude: 25%
- Temperature: 60°C
- Time: 5 minutes

Purification was then carried out using a chromatographic column. Subsequently the samples were subjected to a 0.22 μm membrane filtration to obtain monodisperse samples of optimal size and to guarantee the sterility of the sample (Table 3).

Samples	Tween 85 (mM)	Span 80 (mM)	GM (mg/mL)	CIP (mg/mL)
Nio	22.5	22.5	-	-
GM-Nio	22.5	22.5	10.0	-
CIP-Nio	22.5	22.5	-	2.0

Tab 3. Nio used in this study

These samples were then characterised in terms of sizing and ζ potential using Dynamic Light Scattering (DLS, Malvern NanoZetaSizer apparatus, Worcestershire, UK) which provides information on the diameter of the particles present in suspension and on the presence of particles with a surface electrical charge.

The nanosystems were also characterised in terms of microviscosity, polarity and fluidity of the niosomal bilayer using different lipophilic probes: the preparation method of these formulations is the same as previously reported, to which it was however, an appropriate quantity of pyrene or 1,6-Diphenyl-1,3,5-hexatriene (DPH) is added.

Fluorimetric analysis was performed using a spectrofluorimeter (PerkinElmer, LS5013, Waltham, MA, USA).

Stability studies were subsequently performed over time for 90 days at different temperatures (25°C and 4°C) and in different media, monitoring the variations in the hydrodynamic diameter and ζ potential.

Stability studies of niosomal preparation were performed in simulated biological fluids (Artificial Urine, pH 6.6). Artificial urine was prepared according to *Monika Pietrzyńska et al., 2017* [*Pietrzyńska and Voelkel, 2017*] and the composition is reported in Table 4.

Reagent	Dosage (g)
Urea	25.0
NaCl	9.0
NH_4Cl	3.0
Creatinine	2.0
Na_2HPO_4	2.5
KH_2PO_4	2.5
Na_2SO_3	3.0
Distilled water	Total 1.0 L

Tab. 4 Artificial body urine (pH 6.6): chemical composition

To evaluate the release capacity of the described samples, diffusion studies were carried out, monitoring the release over time of GM or CIP, using a dual-beam spectrophotometer (Perkin-Elmer, Lambda 25 UV-vis, Waltham, MA, USA), which allows the simultaneous reading of the sample and a reference standard. The absorbance obtained is directly related to the concentration of the drug present in the analyzed phase. Using the Lambert-Beer law, the real concentration can therefore be traced using a calibration line, previously constructed by carrying out measurements of the absorbance of solutions with a known concentration. These release experiments were conducted at a temperature of 37°C for a duration of 48 hours.

3.4.1 Transmission Electron Microscopy (TEM) analysis

Microscopy experiments were performed at "National Center of Innovative Technologies in Public Health", National Institute of Health, Rome, Italy.

TEM was used to visualize morphology of Nio. One drop of each niosomal sample was placed into a formvar carbon-coated grid. After 2 min adsorption, the preparations were

negatively stained with 2% (*v*/*v*) filtered aqueous sodium phosphotungstate acid (PTA) and examined by a FEI 208S transmission electron microscope (FEI Company, Hillsboro, OR, USA) with an accelerating voltage of 100 kV. Adobe Photoshop software was used for the image editing.

3.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC determination of different Nio was performed by the microdilution method and carried out in triplicate. 10 μL of exponentially growing bacterial cultures diluted to 0.5 McFarland were added to 190 μL of BHI (Oxoid Ltd., Basingstoke, UK) containing the preparations at concentrations ranging from 125 μg/mL to 7.8 μg/mL. Free antibiotics were diluted and tested similarly. After the incubation at 37 °C for 24 h, the bacterial growth was evaluated by measuring the optical density at 595 nm with a microplate reader (Tecan Sunrise, *X*-fluor). MBC was determined by sub-culturing on Tryptic Soy Agar (TSA, Oxoid, Basingstoke Hampshire, UK) for 24 h 10 μL from each well with no visible growth. MBC is defined as the lowest drug concentration that kills 99.9% or more of the bacterial inoculum.

3.6 Evaluation of cytotoxicity

Niosomal preparation cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay. The MTT assay is a standard colorimetric assay that allows the measurement of mitochondrial enzymatic activity involved in the reduction reaction of MTT to formazan. This reaction is catalyzed by the mitochondrial enzyme succinate dehydrogenase, active only in live cells, that has the function of cutting the tetrazolium ring of MTT (yellow coloured substance) leading to the formation of formazan (a blue salt).

T24 cells (5 × 10^5 cells/mL) were plated in a 96 well microplate and cultured for 24 h at 37 °C with 5% CO_2. The cells were, then, treated for 24 h with different concentrations of Nio,

starting from 250 to 31.2 μg/mL. After the incubation time, 100 μL of 0.5 mg/mL of MTT was added to each well and plates were incubated at 37 °C for an additional 4 h. Subsequently, the supernatants were removed and 100 μL of dimethyl sulfoxide (DMSO) were added to each well for 10 min at room temperature, to solubilize the formazan. Finally, the optical density at 568 nm was measured using a microplate reader (PerkinElmer, Boston, MA, USA). The viability percentage (%) was calculated as: Mean OD treatment/Mean OD control ×100.

3.7 Nio cell interaction

In order to visualize Nio cell interaction, T24 cells were seeded on 8-well chamber-slides (Falcon, Corning, NY, USA) for 24 h at 37 °C with 5% CO_2. After 24 h, the monolayers were exposed to niosomes loaded with Nile Red dye, for different times. Cells treated with free Nile Red, prepared as 1 mg/mL stock solution in acetone, and used at a final concentration of 100 ng/mL, were used as control. After the incubation times, cells were washed with PBS (pH 7.4) and fixed in methanol/acetone (1:1) solution for 5 min at -20°C. Slides, extensively washed with PBS, were mounted with 0.1% (*w*/*v*) p-phenylenediamine in 10% (*v*/*v*) PBS, 90% (*v*/*v*) glycerol. The images were taken using fluorescence microscopy using a Leica DM4000 fluorescence microscope (Leica Microsystem, Wetzlar, Germany) equipped with an FX 340 digital camera. After, they were processed with Adobe Photoshop CS4 software (Adobe Systems, San Jose, CA, USA).

3.8 Bacterial and Nio interactions with T24 cells

In order to study both niosomal cell and niosome-bacteria interaction, T24 cells were seeded on 8-well chamber-slides (Falcon, Corning, NY, USA) for 24 h at 37 °C. After this time, monolayers were exposed to GFP-*E. coli* EC073 strain in presence of niosomes loaded with Nile Red (NR) dye (a hydrophobic fluorophore dye molecule) or GM-NR co-loaded niosomes for 1 h at 37 °C. After this incubation time the samples were treated as described before. Fluorescent images were taken using a Leica DM4000 fluorescence microscope

(Leica Microsystem, Wetzlar, Germany) equipped with an FX 340 digital camera and processed with Adobe Photoshop CS4 software (Adobe Systems, San Jose, CA, USA).

3.9 Anti-invasive activity of Nio

The anti-invasive ability of GM carried by Nio were also tested. In particular, the effect of GM-Nio and Nio, on the adherent and invasive behaviour of *E. coli* strains on bladder cells, was assayed. T24 cells were cultivated in a 24-well plate at a density of 2 × 10^5 cells/mL. To verify the putative inhibitory effect of empty Nio and GM-Nio on intracellular *E. coli* strains in the infected T24 cells, 50 μg/mL of Nio were put in contact with the bladder monolayers during the infection period. Then, cells were centrifuged twice at 500 *g* for 2.5 min, to synchronize infection, and incubated for 1h at 37 °C in 5% CO_2. After eliminating the extracellular bacteria, by incubating T24 cells with growth medium containing gentamicin (100 μg/mL) for 1h at 37 °C in 5% CO_2, the number of intracellular bacteria was determined as described above.

3.10 Evaluation of biofilm inhibition

In order to evaluate the biofilm inhibition induced by CIP-Nio, 20 μL of each bacterial strain (1–2 × 10^8 CFU/mL) was inoculated into wells of a 96 well plate containing 180 μL of TSB supplemented with these substances at a sub-MIC concentration (3.9 μg/mL). The inhibition of cell attachment was evaluated after 24 h incubation at 37 °C. The percentage of biofilm inhibition by the preparations has been calculated using the following formula [*Lagha et al., 2019*]:

$$Biofilm\ inhibition\ (\%) = 100 - (OD570\ sample/OD570\ control \times 100)$$

Values higher than 40% were considered relevant in biofilm inhibition. Uninoculated TSB broth was used as a negative control.

3.10.1 Scanning Electron Microscopy (SEM) biofilm analysis

SEM analysis was performed in the "Department of Anatomy, Histology, Forensic Medicine and Orthopedics" at "Sapienza" University of Rome

Biofilms grown on aluminium stubs were washed and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for at least 48 h. After washing overnight with phosphate buffer (pH 7.4), the samples were postfixed with OsO_4 1.33 % in H_2O for 1 h at room temperature. The biofilms were, subsequently, washed for 20 min with H_2O, and treated for 30 min with tannic acid 1% in H_2O. After another 20 min wash, the excess of water was dried carefully with filter paper, and then the samples were mounted on the specimen holder and observed with Hitachi SU3500 microscope (Hitachi, Japan) at variable pressure conditions of 5 kV and 30 Pa as described by *Relucenti et al., 2021* [*Relucenti et al., 2021*]. Three-dimensional reconstruction of images was undertaken by Hitachi Map 3D Software (v.8.2., Digital surf, Besançon, France) and, for each image, a selected area was extracted for the 3D image reconstruction procedure. The surface topography of the extracted area is shown in false colours, and it was handled by the particle count procedure to assess the size of extracellular matrix granules.

3.11 Statistical Analysis

All experiments were conducted in triplicate. One-way and repeated-measures analyses of variance (ANOVA) followed by post hoc Student's unpaired and paired t tests, as needed, were used to assess statistical significance. A P value of ≤ 0.05 was considered statistically significant.

4. RESULTS

FIRST STEP OF THE STUDY

4.1 Characterization of *E. coli* strains

Recently, a study recruiting women with sporadic infection and recurrent cystitis, demonstrated among uropathogenic *Escherichia coli* (UPEC) isolates an overall low rate of resistance to some antibiotics [*Vautrin et al., 2023*].

The preliminary screening based on antibiotic resistant patterns was performed on UPEC strains. Antimicrobial assay showed that both *E. coli* LC2 and *E. coli* LC3 strains possessed the same fully susceptible profile to fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole, penicillins, and tetracycline. Similarly, *E. coli* EC73 presented only an intermediately-resistance to ciprofloxacin.

The ability of UPEC to thrive in diverse environments within the host, such as the gut, urine, bladder, kidneys, and bloodstream, is crucial for its pathogenesis.

The metabolic flexibility of UPEC is a key factor in its success as a pathogen. UPEC has evolved mechanisms to sense and respond to changes in nutrient availability within these different host environments. This adaptability allows the bacterium to survive and persist in the face of dynamic and competitive conditions [*Mann et al., 2017*]. In our study UPEC isolates showed biochemical profiles similar to those of the general *E. coli* population. The metabolic reactions, such as alkalinization of lactate, was observed in *E. coli* LC2, *E. coli* LC3 and *E. coli* EC73 strains.

The ability of UPEC to orchestrate both adhesion and motility is critical for its successful colonization and ascending infection in the urinary tract [*Lane et al., 2007; Subashchandrabose and Mobley, 2015*]. Furthermore, motility enables UPEC to migrate to a new area facilitating biofilm expansion [*Pratt and Kolter, 1998*].

Swimming motility of UPEC was evaluated by measuring the diameters of the swimming zone. As already demonstrated for *E. coli* CFT073 strain [*Yang et al., 2016*] also *E. coli* LC2 and *E. coli* LC3 showed a high motility (Table 5).

Biofilm production may be the key determinant for the persistence of UPEC in reservoirs [*Soto et al., 2006*].

This ability was quantitatively assessed according to the method described by Stepanovic and collaborators [*Stepanović et al., 2007*]. The layer of adherent bacteria was evaluated by staining with crystal violet and measuring the optical density (OD) at 590 nm. Based on OD readings, the positive strains were classified into strong, moderate and weak biofilm producers. In these experimental conditions *E. coli* EC73, as already demonstrated [*Conte et al., 2016*] confirmed its strong ability to form biofilm, differently to *E. coli* LC2 and *E. coli* LC3 strains that were moderate biofilm producers. As in literature, *E. coli* CFT073 *E. coli* MG1655 were confirmed as strong and weak biofilm producers respectively (Table 5).

In order to classify *E. coli* strains into one of the major phylogenetic classes of A, B1, B2, or D, a rapid and easy phylogenetic grouping technique based on triplex PCR has been developed to detect the genes *chu*A, yjaA, and TspE4. Phylogenetic analysis indicated that the majority of UPEC isolates belonged to phylogroup B2, followed by group D, group A, and group B1 [*Halaji et al., 2022*]. According to Clermont et al., in this study, the PCR based phylogenetic analysis was carried out for all *E. coli* isolates [*Clermont et al., 2000*]. *E. coli* LC2 and *E. coli* LC3 strains belonged to D phylogroup, wherease, as already demonstrated, *E. coli* EC73 and *E. coli* CFT073 was B2 [*Conte et al., 2016*].

	Strains				
Characteristics	***E. coli* LC3**	***E. coli* LC2**	***E. coli* EC73**	***E. coli* ATCC 700928 (CFT073)**	***E. coli* K12 MG1655**
Phylogenetic group	D	D	B2	B2	A
Hemolysis	-	-	-	+	-
Motility	+++	+++	+	+++	+
Biofilm production	MODERATE	MODERATE	STRONG	STRONG	WEAK

Tab.5 UPEC features. Motility was expressed by mean diameter (centimetres) of the motility zone of each group, based on duplicate measurements obtained from two independent experiments performed on each isolate tested. +: < 1; ++: 1 > cm < 2; +++: > 2 cm

UTIs usually start with UPEC contamination, the colonization of the urethra, and the migration into the bladder lumen. Most of the characterized strains of UPEC invade the bladder epithelium and undergo an intracellular infection cycle and there is evidence that this occurs in most human UTIs. The infection cycle is a complex pathway involving epithelial attachment, invasion of host cells, and intracellular proliferation, leading to the eventual rupture of the bladder epithelial cell, dissemination, and reinfection of surrounding epithelial cells. The adhesive, invasive, and intracellular survival abilities of UPEC strains were assayed by infecting T24 monolayers, as described above. All isolates were found to adhere to cell monolayers (Table 6).

The gentamicin protection assay was used to assess the ability of the strains to invade and to survive within the infected cells. As shown in Table 7, compared to the non-invasive control *E. coli* K-12 strain MG1655, *E. coli* LC2, LC3, EC73 and *E. coli* CFT073 were able to invade bladder cells. Concerning intracellular survival, at 24 post infection, all strains were not able to efficiently survive into the cells (Table 8).

Strains	Adhesion (CFU/mL ± SD)	Adhesion (%± SD)
***E. coli* LC2**	1.40E+05 ± 228.30	**7.80** ± 0.35
***E. coli* LC3**	1.80E+05 ± 123.80	**7.16** ± 0.13
***E. coli* EC73**	1.00E+05 ± 675.99	**6.40** ± 2.74
***E. coli* ATCC 700928 (CFT073)**	1.13E+05 ± 197.65	**10.7**± 1.54
***E. coli* K12 MG1655**	9.10E+04 ± 229.07	**2.27**± 0.80

Tab. 6 Adhesion ability of E. coli strains. The results were expressed as CFU/mL or as percentage (%) respect to the initial inoculum. A strain was considered adherent (in bold) when the ratio was ≥ 0.8 %. Data were expressed as mean ± SD.

Strains	Invasion (CFU/mL ± SD)	Invasion (%± SD)
***E. coli* LC2**	3.10E+03 ± 1048.80	**0.34** ± 0.25
***E. coli* LC3**	5.23E+03 ± 899.10	**0.41** ± 0.11
***E. coli* EC73**	1.43E+04 ± 16780.50	**0.90** ± 0.55
***E. coli* ATCC 700928 (CFT073)**	3.67E+02 ± 237.60	**0.70** ± 0.20
***E. coli* K12 MG1655**	0	0

Tab. 7 Invasion ability of E. coli strains, evaluated by gentamicin protection assay. The results were expressed as CFU/mL or as percentage (%) respect to the initial inoculum. A strain was considered invasive (in bold) when the ratio was ≥ 0.1 %. Data were expressed as mean ± SD.

Strains	Survival 24h (CFU/ml ± SD)	Survival 24h (%± SD)
***E. coli* LC2**	2.35E+03 ± 120.2	75.8± 35.9
***E. coli* LC3**	1.43E+03 ± 134.5	27.3 ± 11.8
***E. coli* EC73**	4.33E+03 ± 369.2	30.3± 29.0
***E. coli* ATCC 700928 (CFT073)**	0	0
***E. coli* K12 MG1655**	0	0

Tab. 8 Survival ability of E. coli strains, evaluated by gentamicin protection assay. The results were expressed as CFU/mL or as percentage (%) respect to the intracellular bacteria. The survival ability was defined as the ≥ 100% of the ratio between the number of surviving bacteria and the intracellular bacteria. Data were expressed as mean ± SD.

The two isolates belonging to the same phenotypic group (LC2 and LC3) were subjected to RAPD analysis. Genotype comparisons between *E. coli* isolates from the patient showed that the original and recurrent strain were genetically similar and belonged to the same clonal group (data not shown).

UPEC are equipped with a diverse repertoire of virulence factors (VFs, which play a crucial role in the manifestation of urinary tract infections (UTIs). The ability of UPEC to cause UTIs is not only dependent on the presence of these virulence genes but also on the regulation of their expression levels. The dynamic interplay of these factors contributes to the severity and outcome of the infection [*Tseng et al., 2002; Pitout, 2012*].

The primary infecting *E. coli* was screened for the presence of the VFGs reported in Table 9. The *in silico* analysis reveals that *E. coli* LC2 strain shares with *E. coli* EC73 strain some virulence factors. Neither *E. coli* LC2 nor *E. coli* EC73 had HlyA (present in *E. coli* CFT073, *E. coli* UTI 89 e *E. coli* 83972) and CNF1 (present in *E. coli* UTI 89 e *E. coli* 83972). Haemolytic activity study demonstrated that only CFT073 expressed lysis ability in blood agar plates.

Interestingly LC2 and MG1655 possessed HlyE. Hemolysin E is a cytolytic protein that potentially can destroy host cells. HlyE (also known as clyA or sheA) encodes a secreted, pore-forming protein (hemolysin E/cytolysin A) that is active against mammalian erythrocytes and other cells; *E. coli* K-12 MG1655 does not express HlyE under normal laboratory conditions however a hemolytic phenotype can be induced by experimentally manipulating expression levels [*del Castillo et al., 2001*].

Concerning the iron uptake systems, *E. coli* LC2 strain share with *E. coli* EC73 only few genes: *ent* which encodes siderophore enterobactin, *chu*, which encodes heme transport system, *sit*, which encodes a permease involved in the uptake of iron and manganese, and *fyuA*, which encodes the yersiniabactin receptor, indicating that multiple iron acquisition systems are required to survive and grow within infected tissues.

Factor type or characteristic	***Gene***	*E. coli* LC2	*E. coli* EC73	*E. coli* ATCC 700928 (CFT073)	*E. coli* K12 MG1655	*E. coli* UTI89	*E. coli* 83972
Adhesin	*fimH*	+	+	+	+	+	+
	focG	-	-	+	-	-	+
	sfaS	-	-	+	-	+	-
	pap A-H	-	+	+	-	+	+
Biofilm related	*agn43*	+	-	+	+	-	-
Iron acquisition system	*chuA*	+	+	+	-	+	+
	fyuA	-	+	+	-	+	+
	iroN	-	+	+	-	+	+
	ireA	-	+	+	-	-	-
	iutA	-	-	+	-	-	+
Toxins	*CNF1*	-	-	-	-	+	+
	sat	-	-	+	-	-	+
	hlyA	-	-	+	-	+	+
	hlyE	+	-	-	+	-	-
Protectins	*iss*	+	+	+	+	+	+
	kpsMT II	+	+	+	-	+	+
	traT	+	+	-	-	+	-
Invasin	*ibeA*	-	-	-	-	+	-
Pathogenicity-associated Island marker of CFT073	*malX*	-	+	+	-	+	+
Uropathogenic specific protein	*usp*	-	+	+	-	+	+
Sequence Type		ST394	ST95	ST73	ST10	ST95	ST73

Tab. 9 In silico analysis of VFs and ST of UPEC strains

The genome plasticity of *E. coli* is indeed remarkable, and this adaptability has led to the emergence of various pathogenic strains with diverse VFGs arrangements. Some strains of *E. coli* that are typically associated with intestinal infections have been found to possess virulence genes associated with extraintestinal infections. Considering molecular markers from all diarrheagenic *E. coli* (DEC) pathotypes, enteroaggregative *E. coli* (EAEC) virulence genes are among the most frequent markers of DEC strains reported in extraintestinal pathogenic *E. coli* (ExPEC) strains isolated from sporadic cases of extraintestinal infections and outbreaks [*Toval et al., 2014*].

The *aap* gene, a EAEC genetic determinant, is present only in LC2 strain among the analyzed isolates. The *aap* gene codes for dispersin, a protein which acts as an anti-aggregation and prevents bacterial accumulation [*Asadi Karam et al., 2017*].

The outer membrane invasion/intimin-like protein *eaeX* gene, was also present only in *E. coli* LC2. *EaeX* was predicted to have 22 immunoglobulin-like repeats and to bear significant homology to the outer membrane adhesins invasin of *Yersinia* spp. and intimin of enteropathogenic *E. coli* [*Sheikh et al., 2006*].

The activation of some specifically receptors by UPEC triggers a signaling cascade that ultimately leads to the production of pro-inflammatory cytokines. Tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), IL-6, IL-8 and interferon- gamma (IFN-γ) are some of the major cytokines being released during UTI.

We investigated the ability of UPEC to modulate epithelial cytokine signalling by examining the *in vitro* responses of bladder epithelial cell line to the strains. Interleukin expression was evaluated by Real- Time qPCR after 2 h post infection. In our study, EC73 strain, compared to uninfected sample, could efficiently induce all the assayed interleukins in T24. On the contrary LC2 strains caused IL-6, IL-8, IL-1β and TNF-α production but did not reach statistical significance respect to the control. Furthermore, LC2 strain induced less cytokine response than did the laboratory *E. coli* strain MG1655 from cultured bladder epithelial cells (Figure 7).

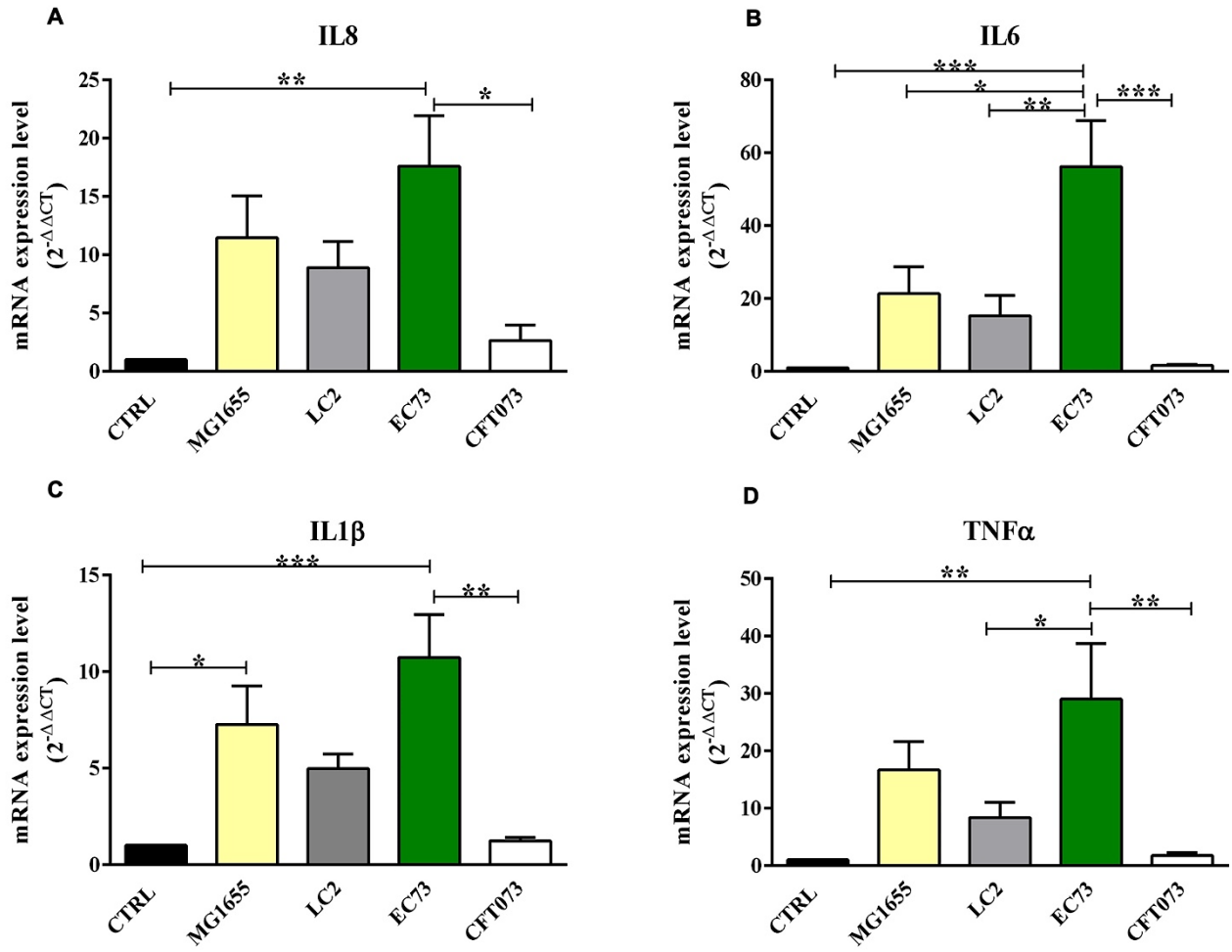

*Fig. 7 Interleukin analysis based on mRNA expression level. Values were reported as mean ± SD. **** P-value<0.0001; ** P-value <0.01; * P-value <0.05.*

In our experiments, infected cells were stained with the ROS indicator dye 2',7'-dichlorodihydrofluorescein diacetate (H2DCF); at 24 h post infections UPEC strains caused a significant increase in ROS production in comparison with non-infected cells (Figure 8).

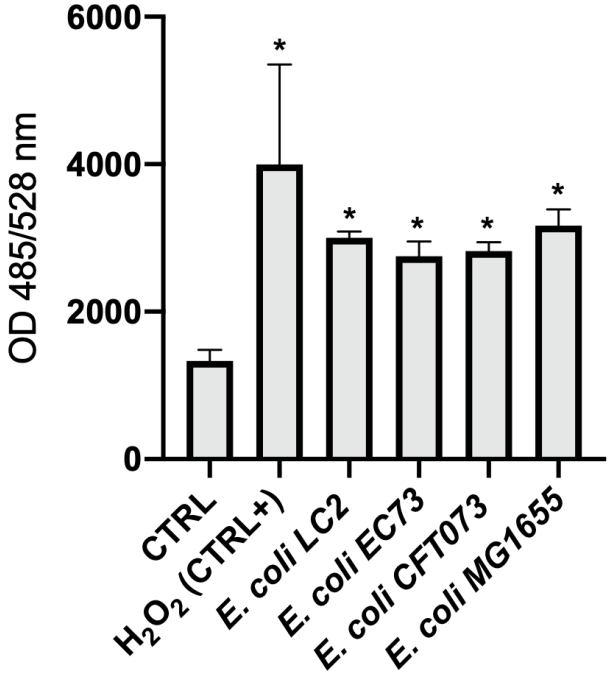

Fig. 8 Evaluation of oxidative stress of T24 cells after 24h of bacterial infection. Values were reported as mean ± SD. All considered conditions were compared to untreated control.

** p value ≤ 0.05.*

We performed immunoblot analyses to compare the expression levels of the antioxidative enzymes superoxide dismutase (SOD)-1, (SOD)-2, and catalase. The expression level of SOD-1 and SOD-2 was high for both *E. coli* LC2 and *E. coli* EC73 strains whereas the expression level of catalase was very high only for *E. coli* CFT073 strain. Our result indicated that the high basal expression of catalase, SOD-1 and SOD-2 in bladder cells could detoxify excessive ROS production by UPEC infection (Figure 9).

The NOX2 NADPH oxidase (NOX2) plays a key role in modulating immune responses to infection. This multimeric enzyme is composed of both membranes bound (gp91phox/p22phox) and cytosolic subunits (p40phox/ p47phox/p67phox/Rac), which once assembled mediates the generation of reactive oxygen species (ROS) in the lumen of phagosomes to directly kill engulfed microorganisms [*Rybicka et al., 2010*]. In our results, a

higher expression of NOX-2 was observed in cells infected by *E. coli* LC2 and *E. coli* EC73 strains (Figure 10). It has been reported that an impact of NOX2-derived sROS on the endosomal environment relates to the process of acidification [*Bode et al., 2023*]

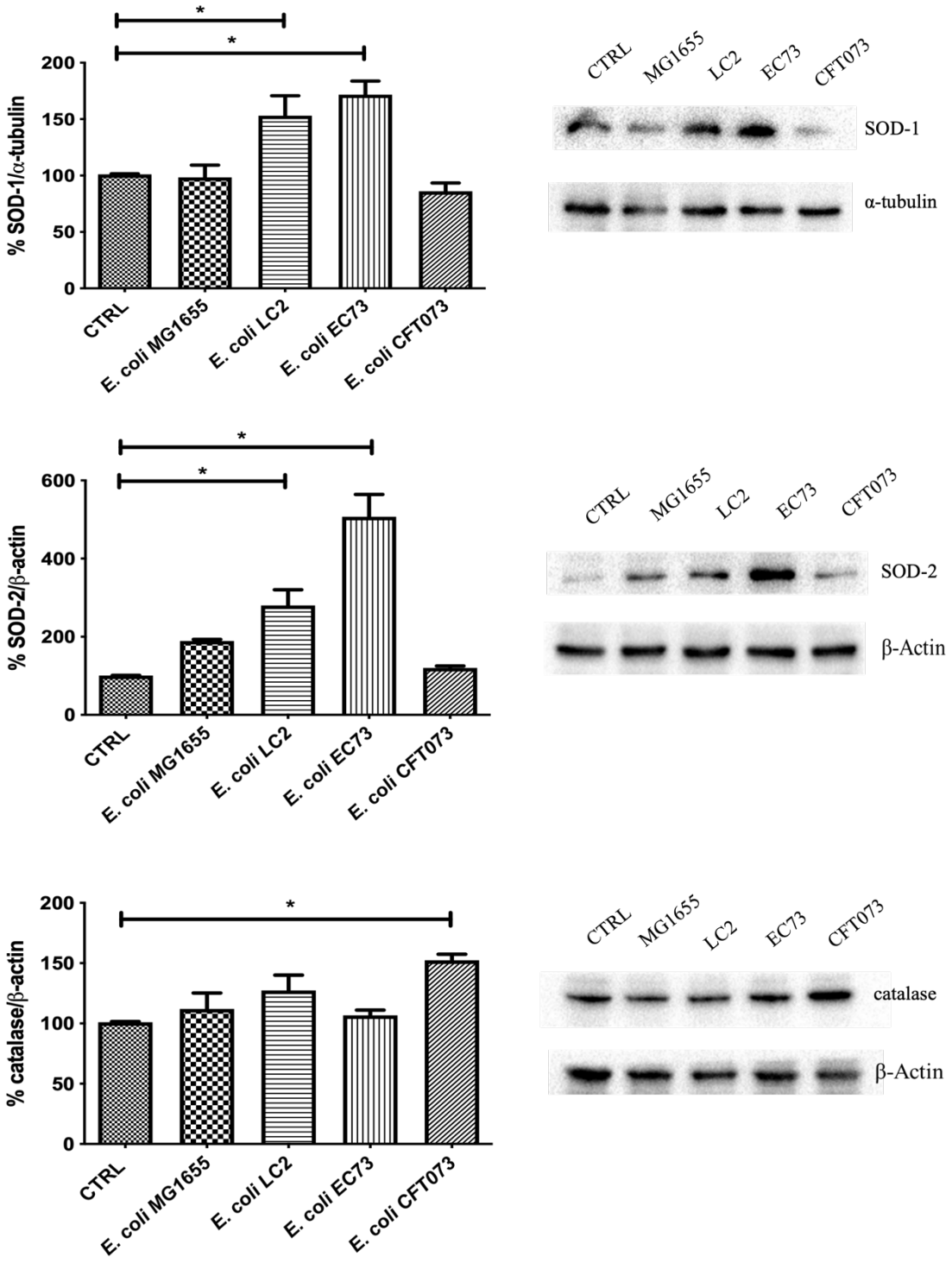

Fig. 9 Evaluation of the expression of proteins with antioxidant action. Results are expressed as percentages (%). All considered conditions were compared to untreated control (CTRL).

** p value ≤ 0.05.*

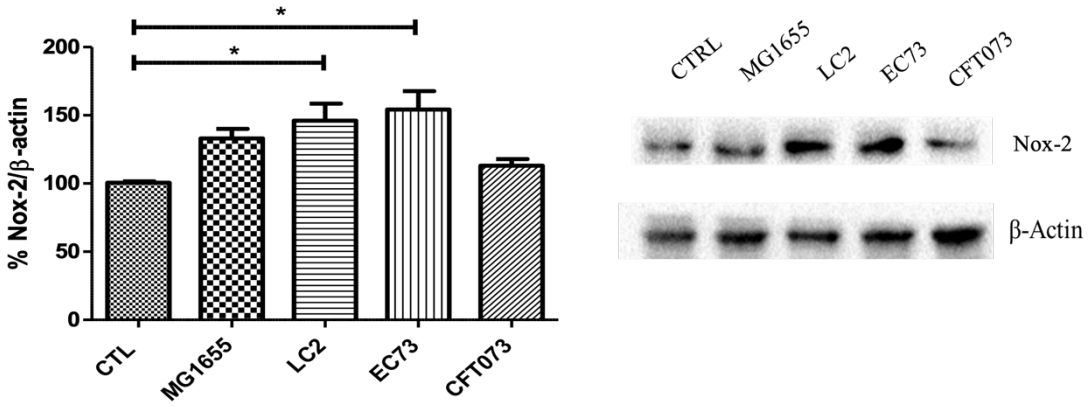

Fig. 10 Evaluation of the expression of proteins with pro-oxidant action. Results are expressed as percentages (%). All considered conditions were compared to untreated control (CTRL).

** p value ≤ 0.05.*

SECOND STEP OF THE STUDY

4.2 Nio preparation and characterization

All the prepared samples, by the "Nanomedicine Lab" of "Sapienza" University of Rome, were found to have nanometric dimensions below 300 nm and showing a good stability profile, especially if stored at a temperature of 4°C. The samples utilised in this study were resumed in table 9, with some essential characteristics.

Sample	Hydrodynamic diameter (nm) ± SD	Potential ζ (mV) ± SD	PDI ± SD	[Antibiotic] (mg/mL)
Nio	113.10 ± 3.17	-34.80 ± 1.91	0.2	-
GM-Nio	225.90 ± 1.52	-47.81 ± 1.12	0.2	0.9
CIP-Nio	260.41 ± 4.03	-37.50 ± 2.12	0.2	0.4

Tab. 9 Niosomal sample utilised in this study: empty niosomes (Nio), GM loaded niosomes (GM-Nio) and CIP loaded niosomes (CIP-Nio).

The characterization studies of the vesicular bilayer, conducted using pyrene and DPH, have shown "structural" differences between empty niosomes and niosomes loaded with different antibiotics. In both analyses, there was a change in both the pyrene spectrum and the anisotropy value: in fact, the vesicular bilayer of the niosomes in which GM and CIP was conveyed was found to be more rigid and polar and less micro viscous.

Sample	Polarity	Microviscosity	Fluidity
Nio	0.96	1.01	0.22
GM-Nio	1.11	0.37	0.39
CIP-Nio	0.97	0.46	0.35

Tab. 10 Bilayer characterization. (SD values are all in the range ± 0.01–0.02).

Finally, the samples were characterized by the same release profile for both antibiotics: approximately 15% within the first hour, 40% within the eighth hour and 50% within the

forty-eighth hour (Figure 11). Both preparations appeared to be stable in the artificial urine (Figure 12).

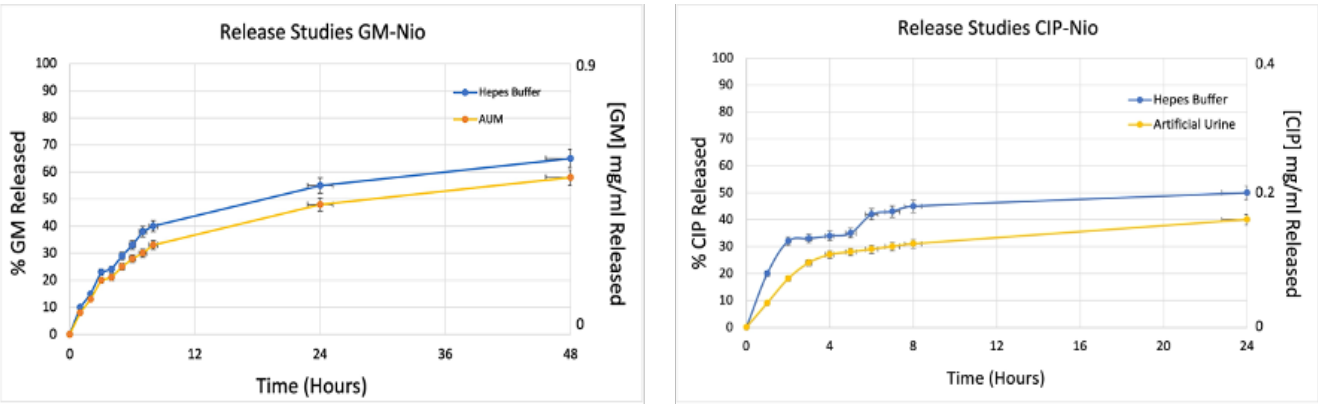

Fig. 11 GM and CIP release profile in Hepes buffer (blue) and artificial urine (yellow). Data were obtained as the mean of three independent experiments.

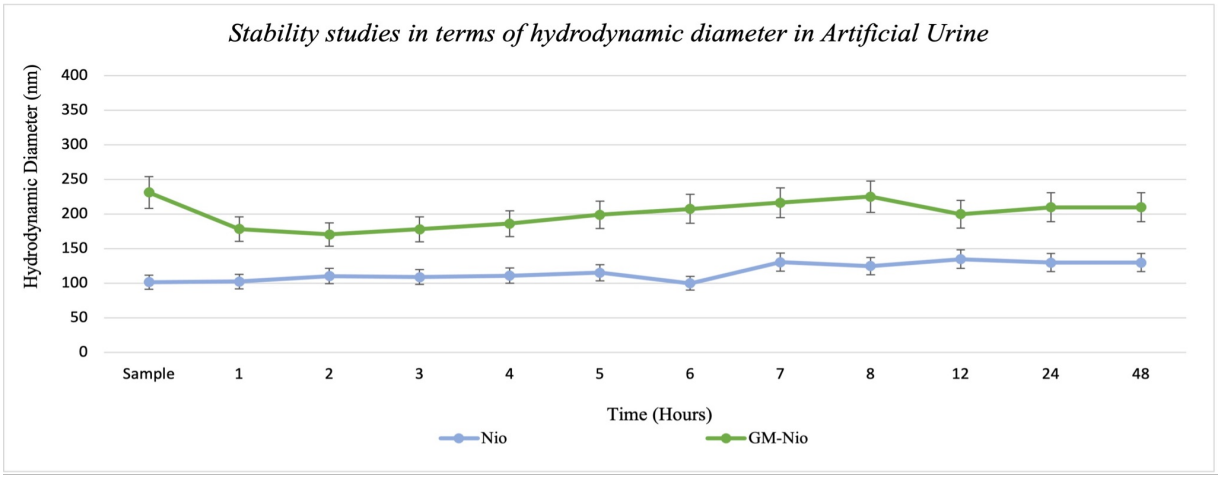

Fig. 12 Stability of empty Nio and GM-Nio in artificial urine. Data were obtained as the mean of three independent experiments.

Moreover, empty and loaded Nio were visualised by TEM. The TEM analysis of empty and CIP-loaded Nio showed spherical vesicles with size corresponding approximately to dimensions revealed by DLS. The microscopy observation confirmed the increase of the size of CIP-loaded Nio with respect to empty Nio reported by DLS (Figure 13)

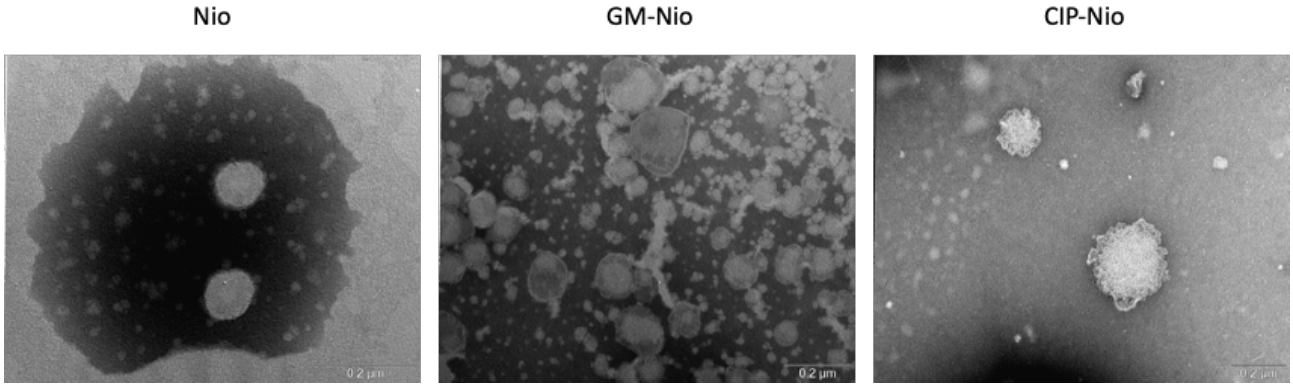

Fig. 13 TEM images of Nio, GM-Nio and CIP-Nio.

4.3 Determination of MIC

After 24 h of incubation at 37 °C, MIC of the various preparations was determined by the broth microdilution method. In our study, the empty niosomal preparations showed no or lower antimicrobial activity compared to antibiotics-loaded niosomes (Figure 14). GM-loaded niosomes showed more action than empty niosomes but only against the reference strains (Figure 15) while CIP-loaded niosomes significantly inhibited the growth of all *E. coli* strains (Figure 16). Probably, niosomal vesicles, thanks to specific surfactants employed, are able to perturb microbial phospholipid membranes of the bacteria, allowing the interaction with the antibiotic. Furthermore, since fluoroquinolones can cross the cytoplasmic membrane by simple diffusion, any condition which creates a concentration gradient towards the bacterial cell outer membrane could improve drug permeation [*Satish et al., 2012*]

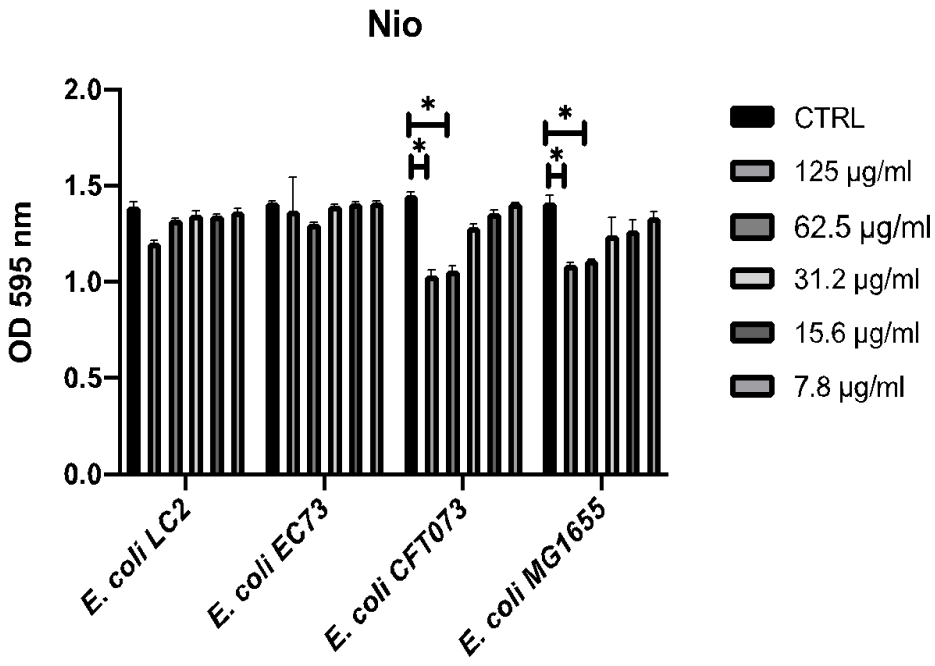

*Fig. 14 Susceptibility test with empty niosomes Data were expressed as mean ± SD. All considered conditions were compared to untreated control. * p value ≤ 0.05.*

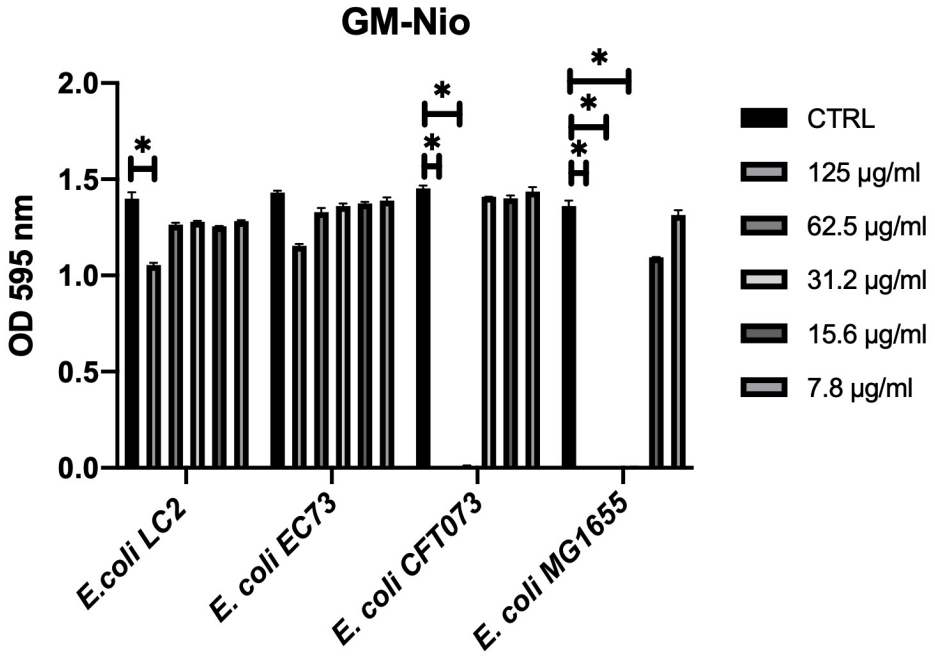

*Fig. 15 Susceptibility test with GM-loaded niosomes. Data were expressed as mean ± SD. All considered conditions were compared to untreated control. * p value ≤ 0.05.*

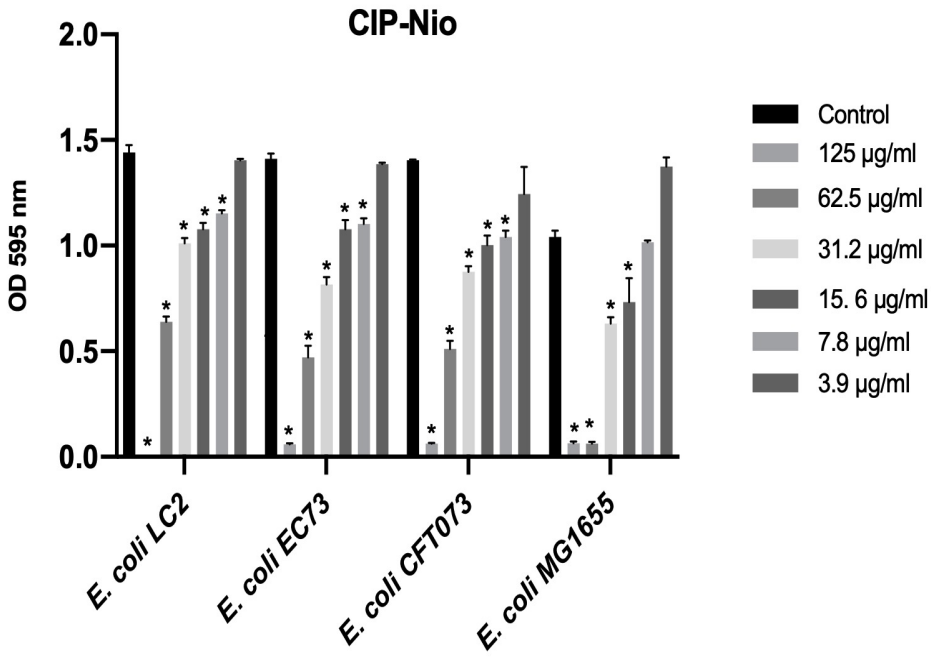

*Fig. 16 Susceptibility test with CIP-loaded niosomes. Data were expressed as mean ± SD. All considered conditions were compared to untreated control. * p value ≤ 0.05.*

4.4 Evaluation of Nio cytotoxicity

The growth inhibitory activity of niosomal formulation against T24 cells was assessed by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Niosomes were put in contact with bladder cell monolayers and cytotoxic effects evaluated after 24 hours. As shown in Figure 13, treatment with empty niosomes resulted in inhibition of cell proliferation only at highest concentration. This cytotoxicity shown a slight increase when niosomes were loaded with antibiotics (Figure 17).

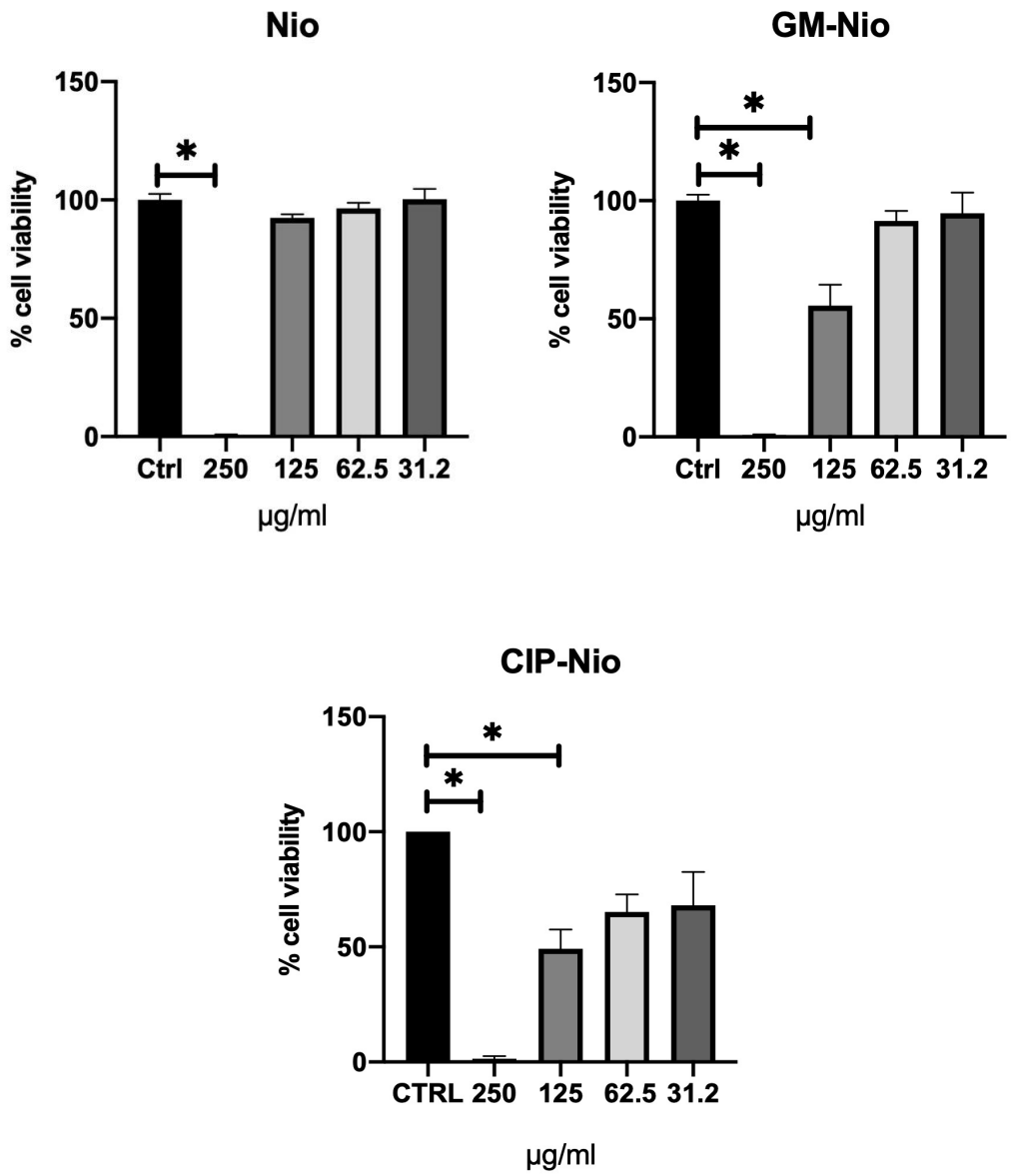

*Fig. 17 Cytotoxic activity of empty, GM-loaded and CIP- loaded niosomes (% of cell viability). MTT assay on human bladder cancer cells (T24). Data were expressed as mean ± SD. All considered conditions were compared to untreated control. * p value ≤ 0.05.*

4.5 Bacterial and Nio interactions with T24 cells

In order to verify the ability of niosomes to interact with bladder cells, the niosomes were probed by following the aggregation of the Nile Red (NR). A fluorescence microscope was used to assess interaction efficiency and the samples analysed were: NR loaded niosomes, NR-GM and NR-CIP co-loaded niosomes. To better understand the interaction of UPEC strains and niosomes with bladder cells, the early step of cell-bacteria interaction in presence

of empty or GM niosomes loaded with NR by using GFP-EC73 was visualised. NR delivered by empty or GM loaded Nio showed a rapid and massive interaction with the cells. Bacteria expressing GFP seemed to attach to cells, primarily at basolateral edges. However, no discernible distinctions were observed between cells treated with free NR, NR-Nio, or NR-GM-Nio, and there was no colocalization with either empty niosomes or niosomes loaded with GM (Figure 18).

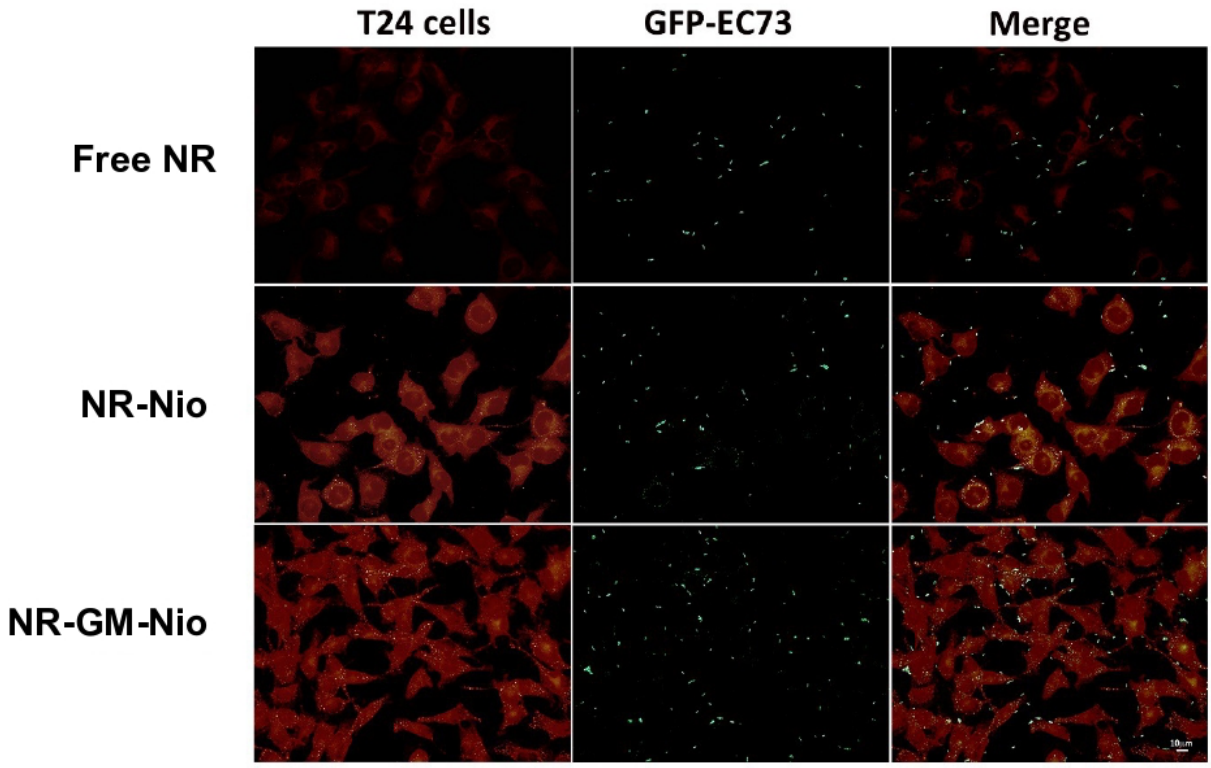

Fig.18 T24 cells treated with free Nile Red (NR) or Nile Red loaded niosomes (NR-Nio) or Nile Red and GM co-loaded niosomes (NR-GM-Nio). Colocalization of E. coli EC73 transformed with the GFP-expressing plasmid pFPV25.1.

Concerning NR-CIP-Nio, an increased fluorescence in the bladder cells, with respect to Nile Red niosomes, was detected (Figure 19).

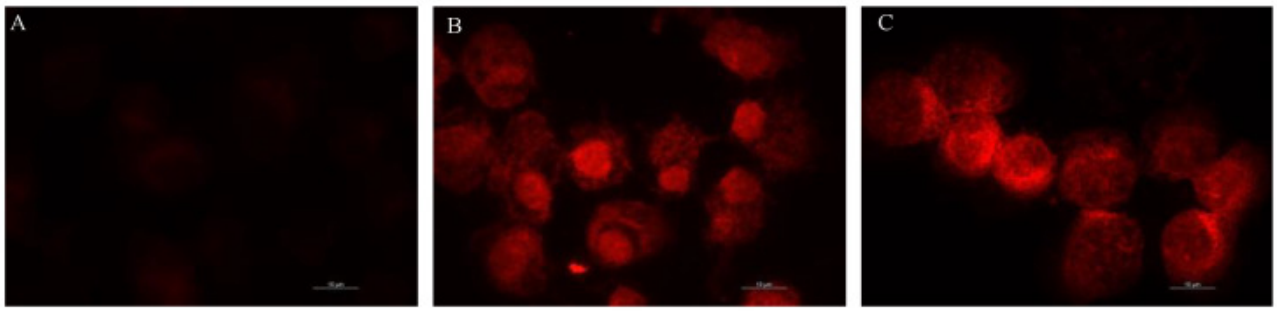

*Fig.19 T24 cells treated with free NR (**A**), cells treated with NR-Nio (**B**) and Nile Red and CIP co-loaded niosomes (NR-CIP-Nio) (**C**).*

4.6 Anti-invasive activity of Nio

Gentamicin (GM) is an aminoglycoside antibiotic with a broad spectrum of antibacterial activity; however, its bioactivity is hampered by its poor penetration into cells [*Chaves and Tadi, 2023*]. In order to improve its therapeutic characteristics, the use of nanotechnology has received particular attention; this has emerged as a promising approach to enhance the activity of conventional antimicrobial agents and for the treatment of intracellular infections by allowing drug release within infected cells [*Alaoui et al., 2022*].

To determine the effect of empty and GM-Nio on the bacterial invasion, the monolayers were treated with 50 μg/ml of different preparations during the infection phase. Following treatment with empty Nio, there is no significant decrease in invasion rates (data not shown). Instead, invasion assay results suggested that the treatment with 50 μg/ml of GM-Nio formulation significantly inhibited the invasion of EC73 strain, resulting in about one logarithm decrease in bacterial counts (Figure 20).

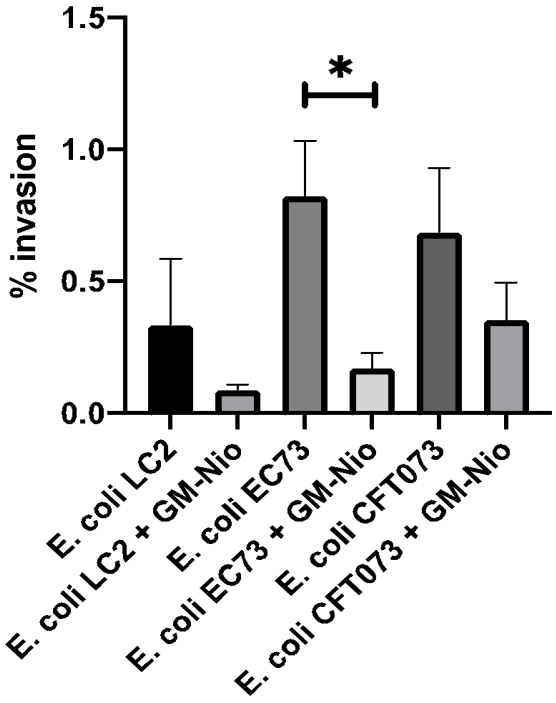

*Fig. 20 Evaluation of the anti-invasive activity of GM-Nio. The results are expressed as the mean ± SD of the invasion percentage, defined as the ratio between the number of intracellular bacteria and the initial inoculum. * p value ≤ 0.05.*

4.7 Evaluation of biofilm inhibition

Biofilm formation represents an excellent strategy for the survival of microorganisms upon exposure to antimicrobial compounds. Slow growth, typical of bacteria that grow in a sessile form, and EPS, which prevents compound molecules from diffusing and reaching the bacteria, are responsible for the development of antibiotic tolerance. Antimicrobial resistance can also be promoted by the spread of resistance genes via horizontal gene transfer, facilitated by the proximity of bacterial cells in the biofilm [*Flemming et al., 2016*]. CIP, a broad-spectrum fluoroquinolone, has been widely used against various bacterial infections, however, an increasing proportion of clinical isolates have proven resistant, contributing to increasing rates of hospitalisation and length of hospital stay [*Fasugba et al., 2015*]. Transport studies of CIP with nanocarriers showed highly reduced dosages and side effects, greater stability, release control and decreased antibiotic resistance even in the case of multiresistant bacteria and biofilm formers [*Mansouri et al, 2021; Yayehrad et al., 2022*].

In order to verify the effect of CIP-loaded niosomes on biofilm production, we treated bacterial strains with niosome preparations for 24 h. The results obtained showed that the sub-MIC concentration (3.9 μg/ml) of niosome-encapsulated CIP significantly inhibited biofilm formation when compared with empty niosomes or sub-MIC concentrations of free CIP (Table 11).

Strains	**Biofilm Formation Abilty**	**Nio (%)**	**CIP-Nio (%)**	**Free CIP ½ MIC (%)**	**Free CIP ¼ MIC (%)**
E. coli LC2	MODERATE	8.4	**47.0**	1.7	3.4
E. coli EC73	STRONG	31.6	**52.1**	2.8	29.2
E. coli ATCC 700928 (CFT073)	STRONG	12.1	**64.6**	3.8	0.4
E. coli K-12 MG1655	WEAK	**77.3**	**56.0**	0	37.2

Tab. 11 Percentage of biofilm inhibition by sub-MIC concentration (3.9 μg/ml) of Nio, CIP-Nio and free CIP. Values higher than 40% were considered relevant in biofilm inhibition (in bold).

As reported by *Dong et al*., 2019 treatment with sub-MICs of CIP for 24h inhibited biofilm formation and reduced the expression of virulence genes and biofilm formation genes in *E. coli*.

For this purpose, SEM visualisation of biofilm structure of *E. coli* CFT073, with or without the treatment with CIP loaded Nio, was performed. From our results, SEM analyses confirmed that the biofilm structure of the bacteria changed significantly after treatment with sub-MIC concentration (3.9 μg/ml) of CIP-loaded Nio.

The observation of untreated samples (Figure 21 A-B) showed that the sample was characterized by the presence of an abundant extracellular matrix (ECM), whose surface appears irregular, being in some areas smooth and compact (indicated in the Figure with the letter "c"), while in others it appears spongy and rough (indicated in the Figure with the letter "s"). The ECM ultrastructure consists of a 3D network of trabeculae showing a

globular aspect. A labyrinthic system of narrow channels develops in the ECM, giving it a spongy appearance.

In the case of treated samples, ultrastructural modifications appeared (Figure 21 D-E). The picture at low magnification (Figure 21 D) showed the sample presenting a spongy appearance (s) with also compact and smooth areas (c). However, the image at higher magnification (Figure 21 E) clarified that the spongy areas have a different meshes structure with respect to untreated sample. The treatment affected the trabecular structure disassembling the globular structures into fine filamentous structures.

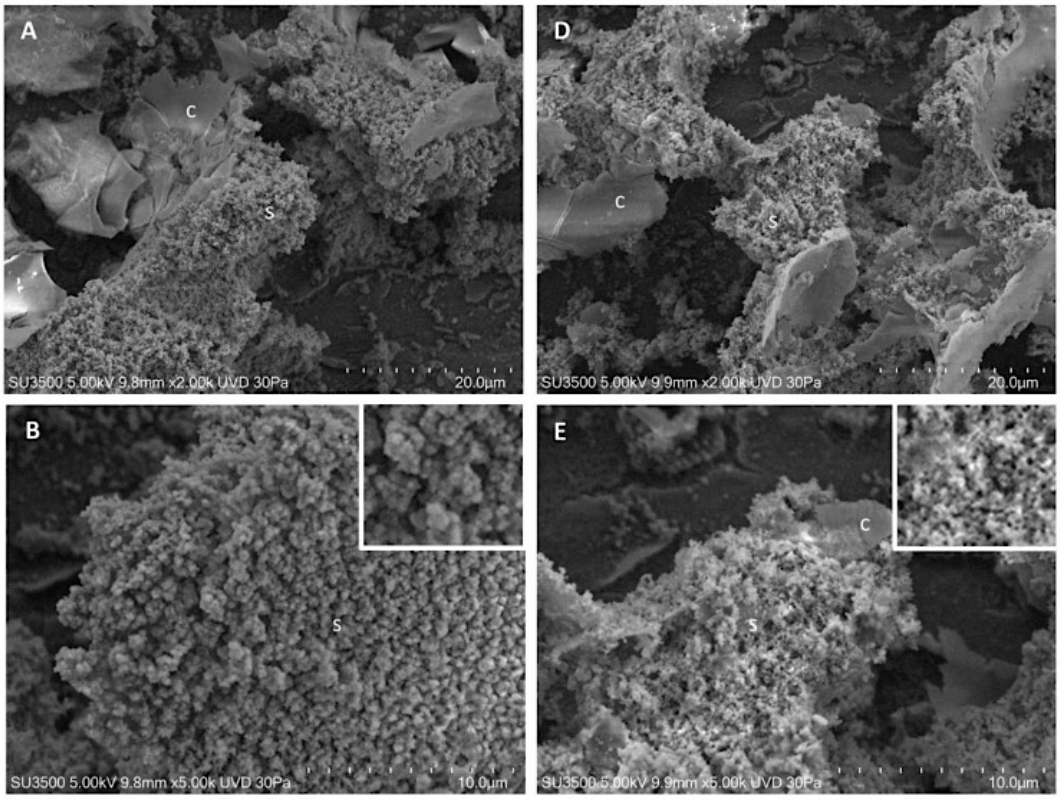

Fig. 21 SEM analysis of biofilm structure when not treated (A-B) or treated with CIP-Nio (D-E).

5. DISCUSSION AND CONCLUSION

Urinary Tract Infections (UTIs) and associated bladder diseases are a major problem, particularly in the case of recurrent UTIs (RUTIs), when the consequences are often chronic and debilitating. Previously thought to be an exclusively extracellular disease, bladder infections caused by uropathogenic *E. coli* (UPEC) are now recognized to be complex pathogenic events with distinct acute and chronic phases of infection. Molecular details regarding UPEC infection are lacking due to the complex range of syndromes associated with UTIs. Several studies have investigated the virulence of recurrent *E. coli* without identification of any traits associated to recurrence and as suggested by some authors, the complex interplay between bacterial infection capabilities, treatment success and host factors are likely to determine recurrence rather than specific genetic characteristics of the *E. coli* bacteria itself [*Thänert et al., 2019; Karami et al.,2020; Soto et al., 2007*].

For this reason, the primary aim of this study was to characterize UPEC strains from patients with recurrent UTIs, in order to understand the possible factors associated with the recurrence of the infection.

E. coli LC2 and *E. coli* LC3 strains were two UPEC strains, collected over 10 years span from a patient with recurrent UTI whereas *E. coli* EC73 was one of 3 UPEC strains, isolated over 2 years from a male patient with recurrent UTIs, already characterized and able to invade and persist in prostatic cells [*Conte et al., 2016*]. *E. coli* LC2 and *E. coli* LC3 strains shared a same phenotypic profile and, as evidenced by RAPD-PCR, were suspected to be closely related clones. Antimicrobial assay showed that both *E. coli* LC2 and *E. coli LC*3 strains possessed the same fully susceptible profile to fluoroquinolones, aminoglycosides, trimethoprim-sulfamethoxazole, penicillin, and tetracycline. Similarly, EC73 presented only an intermediately-resistance to ciprofloxacin. Recently, a study recruiting women with sporadic infection and recurrent cystitis, demonstrated among UPEC isolates an overall low rate of resistance to fosfomycin, nitrofurantoin, pivmecillinam, amoxicillin-clavulanic acid, and third-generation cephalosporin. In contrast, resistance was common and significantly higher among recurrent than sporadic cystitis patients for amoxicillin, trimethoprim-sulfamethoxazole, and quinolones [*Vautrin et al., 2023*]. Our results were in good agreement

with a study of UPEC isolates that showed little effect of the antimicrobial susceptibilities of UPEC isolates on the development and progression of RUTIs [*Luo et al.,2012*].

UPEC appears to utilize amino acids and peptides as a nutrient source during its growth in human urine. The breakdown of peptides into amino acids provides UPEC with intermediates that can be further used in metabolic pathways like the TCA cycle and gluconeogenesis, contributing to its overall fitness and ability to thrive in the urinary tract [*Alteri et al. 2009*]. As suggested by some authors, the metabolic reactions, such as alkalinization of lactate, observed in *E. coli* LC2, *E. coli* LC3 and *E. coli* EC73 strains, could be related to bacterial efforts to relieve acid stress generated by amino acid metabolism. It has recently been suggested that bacterial metabolic capability enhances fitness and contributes to pathogenesis [*Gibreel et al., 2012*].

Generally, the ability of UPEC to colonize and cause infection in the urinary tract results from the cumulative action of VFs. However, these VFs are not sufficient to explain the success of some UPEC clones, which shows moderate VF profiles. Some studies have analyzed the presence of VFGs in relation to RUTIs in women with contrasting results. According with our evidences, 3 VFs were associated significantly with absence of recurrence, including CNF1, hly, and sfa/foc [*Foxman et al., 1995*].

On the contrary *Ejrnaes et al., 2011,* demonstrated that *E. coli* causing persistence or relapse were characterized by a higher prevalence of hemolysis and 12 VFGs (*sfa/focDE, papAH, agn43, chuA, fyuA, iroN, kpsMTII, kpsMTII K2, cnf1, hlyD, malX and usp*). *KpsMTII* K2 and *agn43a*CFT073 were independently associated with persistence or relapse. As suggested by the authors, although no single VF is sufficient for UPEC to cause infections, timely and stepwise expression of multiple, potentially redundant VFs can significantly contribute to UTI development [*Ejrnæs et al., 2011*].

A study indicated a key role of the high carriage rate of iron capture systems in the development and progression of RUTIs persistence [*Luo et al., 2012*]. In this study, *E. coli* EC73 is equipped with genes with this function, while *E. coli* LC2 is less rich in it. The genome of UPEC shows a high degree of plasticity, which leads to the emergence of 'intermediary strains' with different traits from the parental pathotypes.

The *aap* gene codes for dispersin, a protein which acts as an anti-aggregation and prevents bacterial accumulation. The relationship between *aap* gene and biofilm formation has been reported in some studies. *Moazeni et al*., 2023 demonstrated that 88.9% of hybrid strains which carried *aap* gene was a biofilm producer from weak to strong [*Moazeni et al., 2023*]; in addition, *Cordeiro et al*., 2008 suggested that this gene can be considered a molecular marker for EAEC detection [*Cordeiro et al., 2008*]. In our results, the *aap* gene was present only in LC2 strain that is a moderate biofilm producer, suggesting a hybrid nature of this strain or an intestinal reservoir.

Evidences showed that *E. coli* isolates causing persistence or relapse belonged mainly to phylogenetic groups D and B2 [*Ejrnæs et al., 2011; Kao et al., 2023; Luo et al., 2012*]. *E. coli* EC73 strain belonged to B2 phylogroup whereas *E. coli* LC2 belonged to D phylogroup and was ST394. It has been demonstrated that phylogroup D EAEC strains mainly belong to ST131 and ST394 complexes [*Okeke et al., 2010*].

Reservoirs within the host, as well as the ability to invade cells of the genitourinary tract, provide UPEC with a major advantage in pathogenesis. The UPEC strains analysed in this study were able to adhere and invade T24 cells but they didn't survive up to 24 h. UPEC invades superficial bladder epithelial cells via a type 1 pilus-dependent mechanism. Inside these epithelial cells, UPEC organisms multiply to high numbers to form intracellular bacterial communities, allowing them to avoid immune detection.

Bladder epithelial cells produce IL-6 and IL-8 in response to *E. coli* infection. In our study, compared to uninfected samples, T24 cells infected with *E. coli* EC73 strain released increased amounts of IL-6 and IL-8, 2 h post-infection. On the contrary, low levels of cytokine production were detected in cells infected with *E. coli* LC2 and *E. coli* CFT073. These data are consistent with previous reports indicating that some UPEC strains, in order to persist within host urothelial cells, can modulate the release of proinflammatory cytokines. The authors investigated the ability of UPEC to alter epithelial cytokine signalling by examining the *in vitro* responses of bladder epithelial cell lines to the cystitis strains *E. coli* UTI89 and *E. coli* NU14 [*Hunstad et al., 2005*]. The UPEC strains induced significantly less IL-6 than did the laboratory *E. coli* strain MG1655 from T24 bladder epithelial cells. They

found that insertional mutations in the *rfa* and *rfb* operons and in the *surA* gene all abolished the ability of *E. coli* UTI89 to suppress cytokine induction. The *rfa* and *rfb* operons encode LPS biosynthetic genes, while *surA* encodes a periplasmic cis-trans prolyl isomerase important in the biogenesis of outer membrane proteins. The suppressive effect of UPEC on bladder epithelial cytokine responses was not observed at lower UPEC doses; a MOI of approximately 40 was required.

As suggested, the ability of the cystitis strains to dampen the cytokine response of the epithelium may give the bacteria an advantage in invading the epithelium and escaping early innate defences. The early events of cystitis could offer an array of opportunities for uropathogenic *E. coli* to interact with host epithelia, soluble factors, and immune effector cells. UPEC is able to subvert host defences by invasion into facet cells and by eventual formation of a quiescent reservoir, critical steps that may be aided by its modulation of host innate responses.

It has been demonstrated that UPEC pore-forming toxin HlyA triggered an increase in mitochondrial Ca2+ levels and manipulated mitochondrial dynamics by causing fragmentation of the mitochondrial network. Alterations in mitochondrial dynamics resulted in severe impairment of mitochondrial functions by loss of membrane potential, increase in reactive oxygen species production, and ATP depletion [*Lu et al., 2018*]. In our case, only *E. coli* CFT073 and *E. coli* UTI89, as yet described in literature, possessed HlyA. These data are in agreement with that described by Joshi et al., 2021 showing that UPEC infection promotes the production of ROS in bladder cells infected by UTI 89 UPEC strain. In this study the authors verify whether the transcription factor NF-E2-related factor 2 (NRF2) plays a role in managing ROS and UPEC in bladder epithelial cells. They demonstrated that the treatment with the NRF2 pathway activator dimethyl fumarate (DMF) leads to increased expression of antioxidant response genes, decreased expression of inflammatory cytokines IL-6 and IL-1β and reduced immune cell influx to the mucosa, indicative of reduced overall inflammatory response along with decreased bacterial burden in urine and tissue [*Joshi et al., 2021*].

To conclude, rather than a common adaptation mechanism to the urinary tract, our results suggest a diversity of mechanisms leading to host-specific adaptation and thus to recurrence. Further studies exploring host-pathogen relationships and impact of QIR formation with organoid models in RUTI pathogenesis should be performed to contribute to translation of these results into innovative treatments.

Invasion of host cells provides UPECs with a survival advantage and significantly contributes to the pathogenesis of UTIs. They determine both quiescent intracellular reservoirs (QIRs) [*Mysorekar and Hultgren, 2006*] and biofilm-like intracellular bacterial communities (IBCs) containing large numbers of bacteria [*Mulvey et al., 2001*]. QIRs and IBCs allow evasion of the host immune response resulting in persistent infection, contributing to pathogenesis and antibiotic resistance. Furthermore, the intracellular environment protects the pathogens against the antibiotics because of poor drug penetration. Moreover, efficacy of antibiotics such as GM, decreases in acidic organelles such as lysosomes [*Tulkens, 1991*]

In order to improve therapy against urinary infections and prevent recurrent infections, antibacterial niosomal formulations were designed, prepared and characterized, obtaining stable nanocarriers with the ability to load and release GM, to rapidly enter bladder cells and exert effective antibacterial activity against UPEC strains. The characterization results of niosomes (Nio) collectively suggest that the formulated nanovesicles effectively interact with cells. Notably, a substantial fluorescence signal was observed in cells exposed to either empty or GM-Nile Red (NR) loaded Nio after just 1 hour of treatment. The intracellular fluorescence patterns indicated the presence of NR within the cytoplasm, displaying both a punctate pattern with a predominant perinuclear localization and a diffuse staining throughout the entire cell. It is likely that GM, carried by the Nio, was internalized by the cells, potentially disrupting bacterial entry and survival. The results of colony-forming unit (CFU) counts strongly support the efficacy of GM-Nio in reducing intracellular bacterial load, with varying degrees of effectiveness observed among UPEC strains. Notably, GM-Nio exhibited a greater inhibitory activity against the intracellular EC73 strain in comparison to the CFT073 strain, indicating distinct intracellular behaviours. Furthermore,

the inhibitory impact of niosomal GM seemed to manifest after bacterial entry into cells, as demonstrated by co-localization experiments and CFU counts, which did not reveal a difference between empty and GM-Nio during bacterial adhesion.

Many studies have shown the successful internalization of negative surface nanocarriers with different mechanisms: cationic drug delivery systems commonly use the clathrin pathways, whereas anionic ones could undergo internalization via caveolae pathways [*Murugan et al., 2015*] GM-Nio are characterized by a negative surface and, for this reason, we can hypotize that can follow caveolae internalization route. This hypothesis will be investigated and will be the focus of the future studies.

UPEC strains infiltrate bladder epithelial cells using both endocytosis and pinocytosis mechanisms, which involve the formation of caveolae and clathrin-coated pits on the plasma membrane. The transportation of UPEC in these structures may guide them into distinct pathways, influencing their ultimate fate. For instance, Afa/Dr adhesins and UPEC strains expressing type 1 pili can enter host cells through caveolae-like lipid raft domains, potentially evading prompt fusion with lysosomes [*Kim et al., 2018*].

Additionally, studies have indicated that when caveolae separate from plasma membranes, they merge with a cellular compartment known as the caveosome, which operates under neutral pH conditions. Caveosomes have the capability to circumvent lysosomes, thus shielding their contents from the effects of hydrolytic enzymes and lysosomal degradation [*Foroozandeh and Aziz, 2018*]. Nevertheless, a substantial body of evidence suggests that major endocytosis pathways can direct their contents to early endosomes. Once in the early endosomes, cargo may undergo recycling back to the cell surface or persist within the early endosome as it transitions into a late-endosomal compartment, ultimately being directed toward lysosomes [*Rennick et al., 2021*].

The different extent of intracellular inhibition against both *E. coli* CFT073 and *E. coli* EC73 strains suggested that both bacterial structural and genotypic features, and the endocytic pathways they follow to invade bladder cells are probably responsible for the different efficacy of GM-Nio.

Concerning *E. coli* CFT073, recent studies have revealed that its survival within lysosomes is attributed to its capacity to evade acidification of LAMP1+ compartments through the

expression of HlyA. Disrupting microtubule formation in infected bladder cells facilitates intracellular bacterial survival by hindering the acidification of lysosomes containing bacteria [*Naskar et al., 2023*]. In contrast, the *E. coli* EC73 strain, lacking the *HlyA* gene as reported by our studies and already demonstrated by *Conte et al.*, 2016 [*Conte et al., 2016*] may employ a distinct entry pathway to infect bladder cells compared to *E. coli* CFT073. Regardless of the mechanism through which Nio or UPEC enter cells, it is essential for the GM delivered by Nio to retain its activity and produce the observed inhibitory effects. Given the nearly neutral pH values found in caveosomes, caveolae, and early endosomes, coupled with UPEC's strategies to avoid endosome-lysosome fusion, it is reasonable to hypothesize that intracellularly delivered GM can maintain its activity.

These findings lead to the conclusion that the delivery of GM-Nio could serve as a potential approach for treating intracellular UPEC infections and preventing intracellular persistence. Further comprehensive studies will be conducted to investigate the interaction between GM-Nio and cells, aiming to comprehend and elucidate the obtained results and anticipate the *in vivo* behaviour of the loaded nanocarriers. Utilizing a multidisciplinary approach to comprehend the physical-chemical characteristics of both empty and loaded Nio is crucial for substantiating their suitability for the intended application. Notably, this study underscores the excellent stability of GM-Nio over time and shows that GM-Nio exhibits noteworthy efficacy against intracellular UPEC strains, making them a promising candidate for targeted enrichment within the infection microenvironment. Future investigations will delve into unraveling the mechanism of nanocarrier cellular internalization.

In addition to the formation of intracellular structures, another phenomenon that leads to the increase in antibiotic resistance is the formation of biofilm. Also in this case, nanotechnology appears to be a promising solution. Nio could have an excellent chance of penetrating the biofilm thanks to the surfactants used and their "*penetration enhancer*" activity, which facilitates the interaction and absorption of the nanocarriers with the substrate. For this reason, Nio are highly studied drug-delivery systems for the controlled and localized release of drugs even in the case of multi-resistant bacteria and strong biofilm formers [*Mansouri et al., 2021*]. For this purpose, empty and CIP loaded niosomal formulations were prepared and deeply characterized. Nio exhibited: good stability over

time and in different media, the capability to protect the entrapped drug by degradation phenomena. CIP- Nio, at sub-MIC concentration, showed a significant antibiofilm effect against bacterial strains with respect to free CIP. In addition, the Nio showed ultrastructural modifications on the bacterial biofilm, these results are in accordance with literature where some authors demonstrated antibiofilm activity of antibiotic-loaded Nio preparations against a wide variety of pathogens [*Abdelaziz et al., 2015; Barakat et al., 2014*]. Some authors suggested the adsorption of the nanocarriers on the biofilm surface with subsequent delivery of the encapsulated drug to the bacterial cells. Moreover, they revealed the down-regulation of *ica*B biofilm gene expression compared to the free CIP; they postulated that this reduction could be associated with the inhibition of transcription of bacterial genes by reactive oxygen species (ROS) and/or direct interaction of the Nio-encapsulated CIP with the transcription factors [*Mirzaie et al., 2020*].

The results of this study will contribute to develop niosomal formulations that, employed at the appropriate concentration, could represent a promising drug delivery system, stable and inexpensive, to counteract intracellular and biofilm producer bacteria. Further investigations will be necessary to assess the safety and efficacy of the Nio for a potential application in clinical trials.

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7. DECLARATION OF AUTHORSHIP

All of the work reported in this thesis was designed, conducted, analyzed and written up by the author.

The following publication has been prepared from material contained in this thesis:

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