

REVIEW ARTICLE

Impact of polymicrobial biofilms in catheter-associated urinary tract infections

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ABSTRACT

Recent reports have demonstrated that most biofilms involved in catheter-associated urinary tract infections are polymicrobial communities, with pathogenic microorganisms (e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) and uncommon microorganisms (e.g. *Delftia tsurhatensis*, *Achromobacter xylosoxidans*) frequently co-inhabiting the same urinary catheter. However, little is known about the interactions that occur between different microorganisms and how they impact biofilm formation and infection outcome. This lack of knowledge affects CAUTIs management as uncommon bacteria action can, for instance, influence the rate at which pathogens adhere and grow, as well as affect the overall biofilm resistance to antibiotics. Another relevant aspect is the understanding of factors that drive a single pathogenic bacterium to become prevalent in a polymicrobial community and subsequently cause infection. In this review, a general overview about the IMDs-associated biofilm infections is provided, with an emphasis on the pathophysiology and the microbiome composition of CAUTIs. Based on the available literature, it is clear that more research about the microbiome interaction, mechanisms of biofilm formation and of antimicrobial tolerance of the polymicrobial consortium are required to better understand and treat these infections.

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Introduction

The microorganisms found in diverse environments, including aquatic, soil, industrial and clinical settings, are able to alter their state between a planktonic and sessile mode of life. Typically, more than 90% of them live in the sessile state, which is induced when microorganisms are exposed to changing environmental conditions (Costerton et al., 1987). This can result in a microbial community, known as a microbial biofilm, that involves one or more species of microorganisms adhered to an inert or living surface, enclosed in an extracellular polymeric substance (EPS) matrix containing nucleic acids, proteins and polysaccharides (Costerton et al., 1999; Davey & O'Toole, 2000). The ecological advantages of microorganisms in forming biofilms include protection from hostile environmental conditions (e.g. pH, chemical exposure, radiation and phagocytosis), acquisition of biofilm-specific antibiotic-resistant phenotypes and expanded metabolic cooperation (Costerton et al., 1987; Davey & O'Toole, 2000). In addition, individual cells embedded in microbial biofilms display an altered phenotype, which is associated with a reduced growth

rate, higher tolerance to antimicrobial agents or to host defenses, altered expression of specific genes and secretion of molecules and virulence factors (Costerton et al., 1987; Donlan, 2002; Donlan & Costerton, 2002; Hall-Stoodley & Stoodley, 2009; Stewart & Franklin, 2008).

Microbial biofilms play an important role in about 80% of human microbial infections (Davies, 2003; Römling & Balsalobre, 2012). Common human infectious diseases involving biofilm formation in body tissues include chronic airway infections in cystic fibrosis patients (Høiby et al., 2010), chronic otitis (Wessman et al., 2015), chronic sinusitis (Jain & Douglas, 2014), chronic (diabetes) wound infection (James et al., 2008), periodontitis (Socransky et al., 1998) and urinary tract infection (UTI) (Soto et al., 2006). In addition, due to recent advances in medical science, indwelling medical devices (IMDs) have been widely used in hospitals. Their use may result in IMD-related infections involving biofilms (Wu et al., 2015), a situation that has been described for intravenous catheters (Smith & Nolan, 2013), prosthetic heart valves (Murdoch et al., 2009), urinary catheters (Hola et al., 2010), orthopedic devices

Table 1. Microorganisms commonly found in indwelling medical devices-associated biofilm infections.

Indwelling medical devices	Prevalent causative microorganisms	Refs.
Urinary catheters	<i>Escherichia coli</i> , <i>Proteus mirabilis</i> , <i>Enterococcus faecalis</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i>	Hola et al., 2010; Hooton, 2010; Nicolle, 2005
Central venous catheter	Coagulase-negative staphylococcus, <i>Staphylococcus aureus</i> , <i>Enterococcus faecalis</i> , <i>P. aeruginosa</i> , <i>Candida albicans</i> , <i>K. pneumoniae</i>	Costerton et al., 1999; Lynch & Robertson, 2008; Smith & Nolan, 2013
Prosthetic heart valve	<i>S. aureus</i> , <i>Streptococcus</i> sp., coagulase-negative staphylococcus, <i>Enterococcus</i> sp.	Lynch & Robertson, 2008; Murdoch et al., 2009
Orthopedic prosthesis	Staphylococci, <i>Streptococcus</i> sp., <i>Staphylococcus pneumoniae</i>	Campoccia et al., 2006; Costerton et al., 1999; Lynch & Robertson, 2008
Contact lenses	<i>P. aeruginosa</i> , <i>S. aureus</i> , <i>Staphylococcus epidermidis</i> , <i>E. coli</i> , <i>Proteus</i> sp., <i>Candida</i> sp.	Costerton et al., 1999; Lynch & Robertson, 2008; Sankaridurg et al., 2000; Willcox, 2007
Intrauterine devices	<i>S. epidermidis</i> , <i>S. aureus</i> , <i>C. albicans</i> , <i>Micrococcus</i> sp., <i>Lactobacillus plantarum</i> , <i>Enterococcus</i> sp., <i>Prevotella</i> sp., <i>E. coli</i>	Auler et al., 2010; Lynch & Robertson, 2008; Pal et al., 2005
Voice prosthesis	<i>C. albicans</i> , <i>Candida tropicalis</i> , <i>Candida glabrata</i> , <i>S. epidermidis</i> , <i>Streptococcus salivarius</i> , <i>P. aeruginosa</i> , <i>K. pneumoniae</i> , <i>S. aureus</i> , <i>Klebsiella oxytoca</i>	Kania et al., 2010; Sayed et al., 2012; Tićac et al., 2010; van der Mei et al., 2014
Cardiac pacemakers	Coagulase-negative staphylococcus, <i>Streptococcus</i> sp., <i>S. aureus</i> , <i>S. epidermidis</i>	Chu et al., 2015; Inacio et al., 2015; Oliva et al., 2013
Biliary tract stents	<i>Enterococcus</i> sp., <i>E. coli</i> , <i>Klebsiella</i> sp., <i>Clostridium</i> sp., <i>Streptococcus</i> sp., <i>Candida</i> sp.	Basioukas et al., 2014; Donelli et al., 2007; Schneider et al., 2014
Breast implants	<i>E. coli</i> , <i>S. epidermidis</i> , <i>Propionibacterium acne</i> , coagulase-negative staphylococcus	Del Pozo et al., 2009; Karau et al., 2013; Lynch & Robertson, 2008; Rieger et al., 2013

(Campoccia et al., 2006), cardiac pacemakers (Inacio et al., 2015), intrauterine devices (Auler et al., 2010), biliary tract stents (Schneider et al., 2014), breast implants (Karau et al., 2013), contact lenses (Willcox, 2007) and voice prosthesis (van der Mei et al., 2014).

The surface of IMDs offers a favorable environment for the colonization and growth of a large number of microorganisms, with a predominance of Gram-negative and Gram-positive bacterial species, as shown in Table 1. The source of these microorganisms might be the skin of hospitalized patients or health-care workers and the hospital environment (Prüss et al., 1999).

IMDs-associated biofilm infections have a great impact in public health. For instance, higher healthcare costs are related with these infections (Donlan, 2001), due to the prolonged stay in hospitals and the continued use of antimicrobials. Another worrying feature is that these types of infections are difficult to eradicate, due to the presence of polymicrobial communities involving multi-drug resistant pathogens (Francolini & Donelli, 2010). In fact, polymicrobial biofilms are in general more resistant to antibiotic treatment than the corresponding single-species biofilms (Al-Bakri et al., 2005; Burmølle et al., 2006; Kara et al., 2006; Leriche et al., 2003) and corresponding planktonic cells (Ceri et al., 1999). In most cases, the only way to treat them is through the removal or substitution of the IMD (Donlan, 2001).

Catheter-associated urinary tract infections

The European Center for Disease Prevention and Control reported that, annually, approximately 4.1 million

patients are estimated to acquire a nosocomial infection (infections acquired in hospitals and other healthcare facilities) in European hospitals (Wolfe et al., 2012). Among these infections, UTIs are a frequent nosocomial infection (Stamm & Norrby, 2001). The major risk factor for UTIs is the use of urinary catheters. It was reported that about 70–80% of nosocomial infections are related with its use (Lo et al., 2014; Zarb et al., 2012).

The urinary catheter is inserted in patients through the urethra and into the bladder, in order to measure the urine output and to prevent/control urine retention or incontinence (Hall-Stoodley et al., 2004; Warren, 2001). Despite careful aseptic management, the risk of developing catheter-associated urinary tract infections (CAUTI) increases 3–7% with each day of catheterization (Hooton, 2010). This risk is higher for women, older patients and diabetic patients (Hooton, 2010). It was estimated that approximately \$3790 is the minimum amount spent in the treatment and diagnostic of each episode of CAUTI (Friedman, 2014), including antimicrobial therapy, increased length of hospitalization, physician visits and morbidity. In addition, it has been reported that patients with CAUTIs might develop other complications such as cystitis, pyelonephritis, Gram-negative bacteremia, prostatitis, epididymitis, endocarditis, vertebral osteomyelitis, septic arthritis, endophthalmitis and meningitis (Denstedt et al., 2000).

The presence of microbial biofilms on the surface of urinary catheters removed from patients has long been documented (e.g. Ganderton et al., 1992). In fact, the replication of microorganisms in the presence of a continuous or intermittent flow of a warm nutritious medium such as urine plays a crucial role on the

establishment and development of a biofilm community on the surface of urinary catheters (Tenke et al., 2006). Additionally, the lumen of the urinary catheters is characterized by the absence of inherent defense mechanisms, which makes the microorganism less prone, for example, to detachment by the urine flow, to phagocytosis and to the action of antimicrobials agents (Trautner & Darouiche, 2004). Also, the normal defenses of the bladder might be weakened when the urinary catheter is used (Warren, 2001).

The recent use of advanced molecular technologies has revealed the polymicrobial nature of UTIs/CAUTIs (Fouts et al., 2012; Frank et al., 2009; Lewis et al., 2013; Wolfe et al., 2012). The microbial communities in CAUTIs can be shaped according to the host immune defenses of the patient, prophylaxis measures and administered antibiotics. The way different microorganisms involved in a polymicrobial community interact (synergistically or antagonistically) will also have an impact on the microbial diversity, virulence and response to therapy.

Pathogenesis of uropathogenic biofilms

As demonstrated in Figure 1(a), CAUTIs can develop in several ways. After the insertion of the urinary catheter, some urine components (proteins and other organic molecules, including magnesium and calcium ions) form a conditioning film along the urinary catheter surface. Such phenomenon alters the characteristics of the catheter surface and allows the adhesion of uropathogenic microorganisms (microorganisms that colonize, persist and cause infection in the urinary tract) (Hatt & Rather, 2008). The planktonic microorganisms adhere to the surface either by physical forces (e.g. van der Waals forces) or by specific adhesion molecules such as adhesins (Gupta et al., 2016; Marić & Vraneš, 2007) (Figure 1(bI, II)). Flagella and pili are also well-known virulence factors, expressed by uropathogenic microorganisms, which help the initial attachment of microorganisms to the uroepithelial cells and to the urinary catheter surface (Flores-Mireles et al., 2015; Nicolle, 2014b; O'Toole & Kolter, 1998). Then, reversely attached microbial cells become strongly adhered to the surface (irreversible attachment) (Hall-Stoodley et al., 2004). In the next stage, microorganisms firmly attached to the urinary catheter surface interact with each other (Gupta et al., 2016) and start to produce EPS (Busscher et al., 2008; Jacobsen et al., 2008; Tenke et al., 2006) (Figure 1(bIII)). EPS is mainly constituted by polysaccharides, proteins, lipids and nucleic acids (DNA and RNA); EPS protects the microorganisms within the biofilm, favoring the mechanical stability of the biofilm and the adhesion of the microorganisms to the surface (Flemming &

Wingender, 2010) (Figure 1(bIII)). The constant replication of microorganisms and EPS production results in the formation of three-dimensional structures with channels between them that are filled with urine and enable the transport of essential nutrients and oxygen to the microbial community (Denstedt et al., 2000; Jacobsen et al., 2008; Tenke et al., 2006) (Figure 1(bIV)).

Lastly, microorganisms present within the biofilm community firmly adhered to the surface of urinary catheter can detach and return to the planktonic form (Hall-Stoodley et al., 2004). At this stage, some microorganisms are able to produce enzymes that destabilize and breakdown the biofilm matrix; for example, *E. coli* produces *N*-acetyl-heparosan lyase (Sutherland, 1999). If these microorganisms are able to move against the urine flow, they may colonize other sites of the urinary tract such as the bladder and the kidneys, and may even reach the bloodstream (Figure 1(bIV)) (Jacobsen et al., 2008; Tenke et al., 2006).

Once a mature biofilm is formed, the uropathogenic microorganisms have to further adapt to the conditions found in the urinary tract environment. This can be accomplished by the expression of genes responsible for the capsular polysaccharide and lipopolysaccharide synthesis, iron acquisition systems, antibiotics resistance mechanisms, nitrogen and oxygen levels adaptation (Snyder et al., 2004), and toxins production (e.g. hemolysins and cytotoxic necrotizing factor 1) (Jacobsen et al., 2008).

Nitrogen, iron and amino acids are essential nutrients for the survival of uropathogens during CAUTI development (Alteri et al., 2009; Subashchandrabose & Mobley, 2015). However, iron concentration in urine is too low (Jacobsen et al., 2008; Shand et al., 1985). To overcome this, uropathogens are able to upregulate genes encoding molecules capable of recruiting the iron, called siderophores (Demir & Kaleli, 2004; Snyder et al., 2004). Consequently, the survival and growth of the uropathogenic microorganisms during CAUTIs development, even at low iron concentrations, might be guaranteed when these microorganisms produce high concentrations of siderophores, or at least, when they are able to use the siderophores produced by neighboring microorganisms (Jacobsen et al., 2008; Shand et al., 1985).

In addition, some bacteria, including *P. mirabilis*, *Proteus vulgaris* or *Providencia rettgeri*, convert urea, found at high concentrations in urine, to ammonia and carbon dioxide through the use of the urease enzyme (Donlan & Costerton 2002; Jacobsen et al., 2008). As an alternative, microorganisms such as the urease-negative *E. coli* use glutamine synthetase, an enzyme involved in glutamine synthesis and in ammonia metabolism (Hagan et al., 2010; Snyder et al., 2004). As a result of

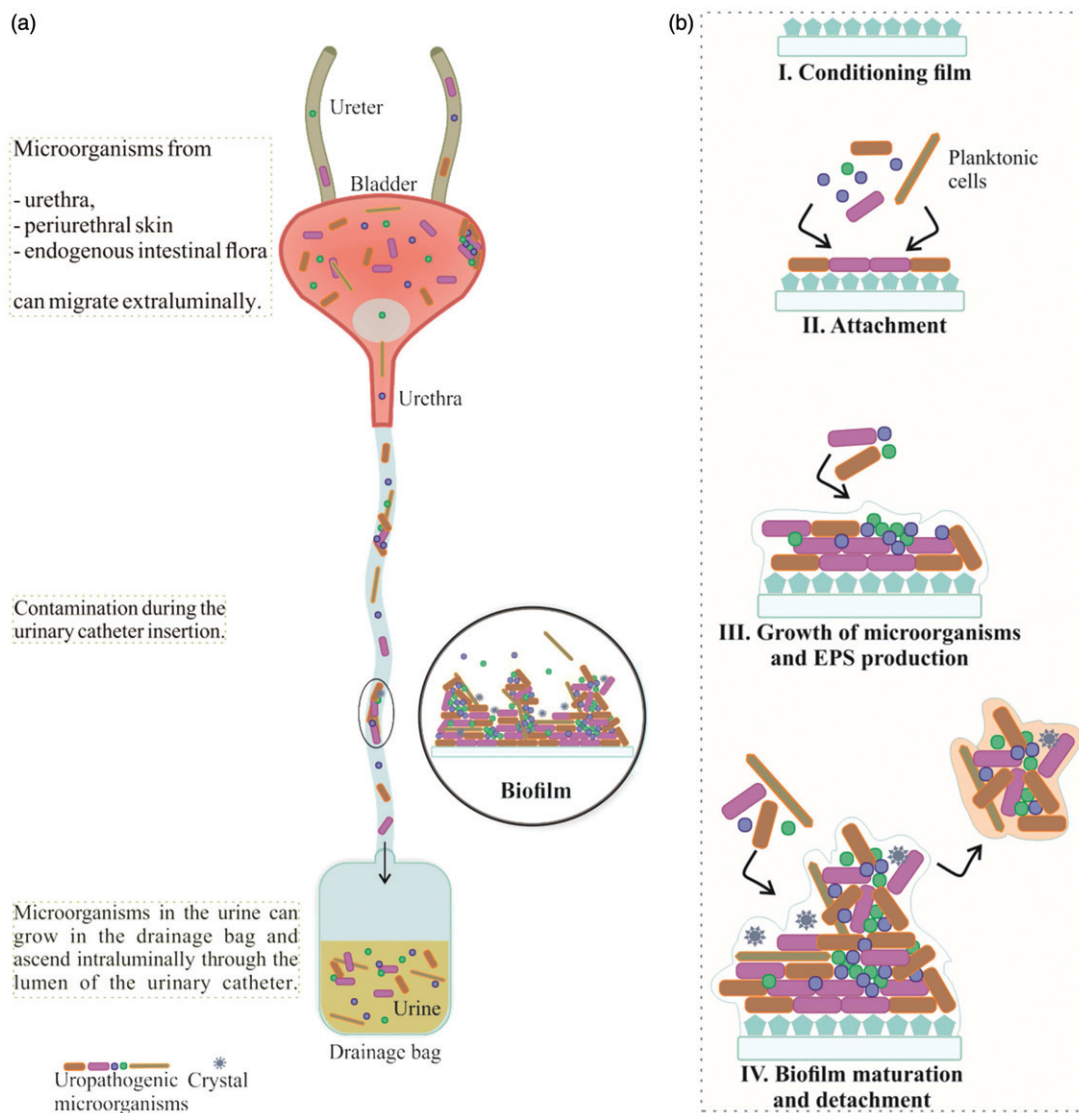


Figure 1. Pathogenesis of biofilm formation on urinary catheters during catheter-associated urinary tract infections development. (a) Catheter-associated urinary tract infections can develop in several ways: i) microorganisms present in the urethra, periurethral skin or endogenous intestinal flora can migrate around the catheter extraluminally; ii) microorganisms can grow in the interior of the drainage bag and ascend intraluminally through of the urinary catheter; iii) environmental and common skin microorganisms can be directly introduced at the time of the catheter insertion due to inadequately decontaminated equipment or improper practices of the healthcare workers (Stamm, 1991; Tambyah et al., 1999); (b) Concerning CAUTIs, the first step involved in the formation of a catheter-associated biofilm is the deposition of a conditioning film on the surface of the urinary catheter, which facilitates the binding of microorganisms (I, II). The next step involves the division of microorganisms and EPS production (III). Consequently, a mature biofilm with a 3-dimensional structure is formed, with spaces between the aggregates that are filled with fluid (urine). Finally, microorganisms or aggregates of these microorganisms can detach from the mature biofilm, colonizing other sites (IV).

urea conversion into ammonia and carbon dioxide, the pH of the local environment becomes alkaline, which causes the precipitation of some minerals present in the urine, including calcium phosphate and magnesium ammonium phosphate (Broomfield et al., 2009; Stickler, 2014). This represents a frequent problem associated with the formation of crystalline biofilms (as represented in Figure 1), which may have severe consequences, including trauma of the bladder and the urethral epithelia. Also, the deposition of the crystalline material

on the surface of urinary catheters is frequently responsible for the blockage of the urine flow (Hatt & Rather, 2008; Stickler, 2008; Tenke et al., 2006).

The microbiome diversity: traditional and uncommon microorganisms

In short-term catheterization (up to 7 days), the urinary catheter is frequently colonized by a single species. In long-term catheterization, the catheter is placed for

several weeks and months and, in this case, a polymicrobial infection is inevitable (Jacobsen et al., 2008; Nicolle, 2001; Warren, 1991, 2001), with a predominance of Gram-negative bacteria (Hola et al., 2010; Hooton, 2010; Nicolle, 2005). Using standard culture methods, it has been shown that most of the catheters are infected by three or more microorganisms, and that only 12.5% of the infections are monomicrobial (Hola et al., 2010).

Traditionally, the diagnosis of CAUTIs is based on symptoms that patients present in combination with microbiological culture of urine. Urine collected from the catheter or from the bladder is cultured on agar medium plates to detect the microorganisms involved in the infection (Hooton, 2010; Nicolle, 2014a). The infection is diagnosed when patients have a positive urine culture of $\geq 10^5$ CFU ml⁻¹ ($\geq 10^4$ CFU ml⁻¹ in children) in association with other symptoms (e.g. dysuria, urinary urgency, fever) (Burckhardt & Zimmermann, 2011). However, the microbiological culture of urine from catheters might not reflect the microorganisms present in the biofilm formed on the surface of the catheter. In fact, microorganisms in biofilms have a different phenotype and behavior comparing with the planktonic microorganisms present in urine. Typically, biofilm populations grow slowly or poorly on agar medium plates (Costerton, 2007). In addition, a viable but nonculturable state of some microorganisms and the antibiotic administration to prevent the infection might cause a false-negative result (Zimmerli et al., 2004). Thus, to improve the recovery and the quantification of the microorganisms attached to the urinary catheter, methodologies based on sonication have been recommended (Hola et al., 2010). As the microorganisms attached extra- or intraluminally to the catheter can be different, a sonication step before the culture might allow the identification and quantification of microbial population located on both the outer and inner surface of the urinary catheter (Frank et al., 2009). Then, the analysis of the microbial composition can be performed by microscopy, cultivation or culture independent techniques (Hoiby et al., 2015).

Recently, many culture-independent techniques have been widely used to identify and quantify the microbial diversity found on biofilm infection (Wolcott et al., 2013). Previous studies (Davies et al., 2004; Dowd et al., 2008; Guembe et al., 2012; Khot et al., 2009; Price et al., 2009; Thomsen et al., 2010; Tuttle et al., 2011) showed that some molecular techniques, including the denaturant gradient gel electrophoresis (DGGE), cloning, pyrosequencing, 16S ribosomal ribonucleic acid (rRNA) gene polymerase chain reaction (PCR) for bacteria or 18S and 28S rRNA PCR for fungi and terminal restriction fragment length polymerase (TRFP)

seem to be more reliable to assess the microbiota present in polymicrobial biofilms comparing with the conventional culture methods. Using these techniques, it would be likely that the 12.5% of monomicrobial infections found by Hola et al. (2010) in long-term catheterization could be even lower. In addition to the quantification of diversity, microscopy analysis in combination with fluorescent *in situ* hybridization (FISH) has been used as a way to locate microorganisms within the biofilm (e.g. Almeida et al., 2011; Azevedo et al., 2015; Cerqueira et al., 2013; Malic et al., 2009). FISH is based on phylogenetic markers found in 16S or 23S rRNA sequences of microorganisms, as ribosomes are particularly abundant and relatively stable in viable cells (Amann et al., 2001). While each urinary catheter displays a unique biofilm community (Frank et al., 2009), a list of the most prevalent microorganisms that are recurrently recovered from urinary catheters is presented in Figure 2. The European Center for Disease Prevention and Control (ECDC, 2015), in an annual epidemiological report from 2014, reported that the most frequently isolated microorganisms were *E. coli* (28%), *Candida* sp. (18%), *Enterococcus* sp. (17%), *P. aeruginosa* (14%) and *Klebsiella* sp. (8%) (Figure 2(a)). Other studies reported a clear prevalence of *E. coli*, *P. aeruginosa*, *E. faecalis* and *P. mirabilis* (Hola & Ruzicka, 2011; Matsukawa et al., 2005; Ohkawa et al., 1990). The antimicrobial resistance of these microorganisms requires attention due to the continuous use of antimicrobial agents. In 2012, the ECDC reported that the most common isolates from CAUTIs were resistant to, at least, one of the antimicrobial agents used in clinical practice. Among the most prevalent microorganisms, 26.3% of *E. coli* isolates are resistant to third-generation cephalosporins; 26.6% of *P. aeruginosa* isolates are resistant to ceftazidime; and, 9.5% of *Enterococcus* sp. isolates are resistant to vancomycin (ECDC, 2015).

Other microorganisms less commonly found on polymicrobial catheter biofilms, and designated here as uncommon species, have also been isolated and identified (Figure 2(b)). The pathogenic potential of most of these microorganisms remains poorly studied. The presence of these types of microorganisms in biofilm-associated infections has been reported not only for CAUTIs (e.g. Frank et al., 2009; Hola et al., 2010; Xu et al., 2012), but also for cystic fibrosis (e.g. Bittar et al., 2008; Coenye et al., 2002). For example, in a study performed by Frank et al. (2009), it was reported that the frequency in urinary catheters-associated biofilms of *D. tsuruhatensis* and *A. xylosoxidans* was 25% for each bacterium, while *B. fungorum* was present in 13% of the samples. These uncommon bacteria appeared in CAUTIs in combination with well-established pathogenic bacteria such as *E. coli*, *K. pneumoniae* and *P. aeruginosa* (Frank et al., 2009).

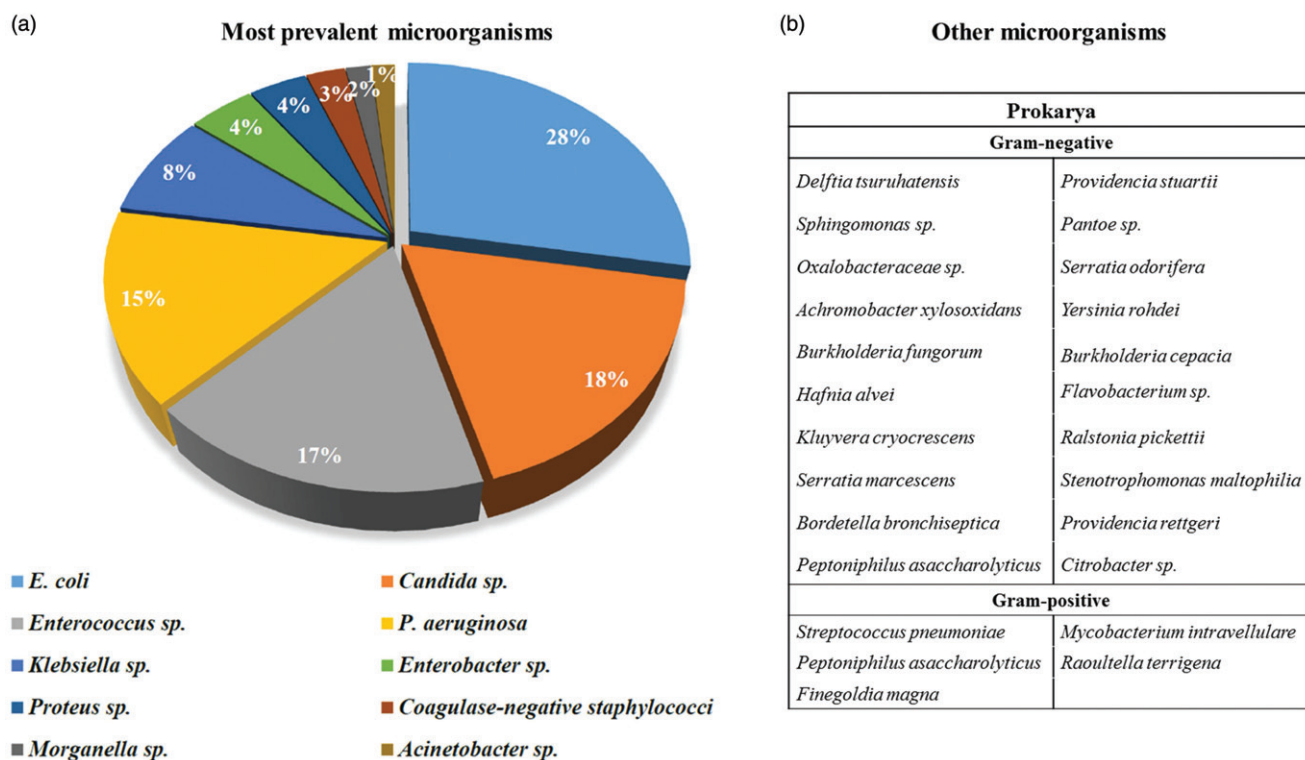


Figure 2. Microbial diversity found in catheter biofilms. (a) Percentages of the ten most frequently isolated microorganisms from catheter-associated urinary tract infections; (b) List of some other microorganisms less frequently found in patients with catheter-associated urinary tract infections (Choe et al., 2012; Hola et al., 2010; Hola & Ruzicka, 2011; Xu et al., 2012).

The microbial interactions between uncommon and pathogenic bacteria and how these types of bacteria adapt to the urinary catheter niche and influence the disease outcome remain unclear. Nonetheless, the potential implication and contribution of these uncommon bacteria in nosocomial human infections has been receiving more attention (Azevedo et al., 2014, 2016; Lopes et al., 2012, 2014, 2015).

Microbial ecology – microbial interactions between microorganisms

Given that the outcome of a polymicrobial infection depends on how microbial communities interact, the research community rapidly recognized the urgency to study polymicrobial biofilms.

When the interactions are antagonistic, at least one of the microorganisms will be negatively affected by the presence of another. Antagonistic microbial interactions might be mediated by different mechanisms (Figure 3(a)). In a competition relationship, microorganisms may occupy the same niche and compete for nutrients and physical resources (Faust & Raes, 2012; Harrison, 2007; Rastall et al., 2005; Song et al., 2014).

Adding to this competition, microorganisms may also produce toxins such as bacteriocins (Michel-Briand

& Baysse, 2002), that when secreted kill the other members of the microbial community (Chang et al., 2005). In this latter case, a microorganism might be able to affect negatively the other without being affected by the interaction.

Other mechanisms have been recently described that, instead of releasing a toxin into the extracellular space, require a close contact between cells in order for the producer to be able to deliver the effector into the target cell milieu. More specifically, a competition mechanism, named type VI secretion system (T6SS), found in Gram-negative bacterial species consists in delivering proteins and toxins into a target cell in order to kill all susceptible neighbors in a contact-dependent manner (Filloux et al., 2008; Ho Brian et al., 2014; Hood et al., 2010). The most important components of the T6SS are the Hcp and VgrG, which assemble to form a membrane puncturing device that resembles an inverted phage tail and tube. It is thought that VgrG forms the puncturing tip, while Hcp forms a contractile tube. Together they make contact between the producer and the target cell membranes, delivering the T6SS effectors (Alteri & Mobley, 2016).

Another mechanism dependent on cell-to-cell contact, referred to as contact-dependent growth inhibition (CDI) system, has also been described in bacteria (Ruhe

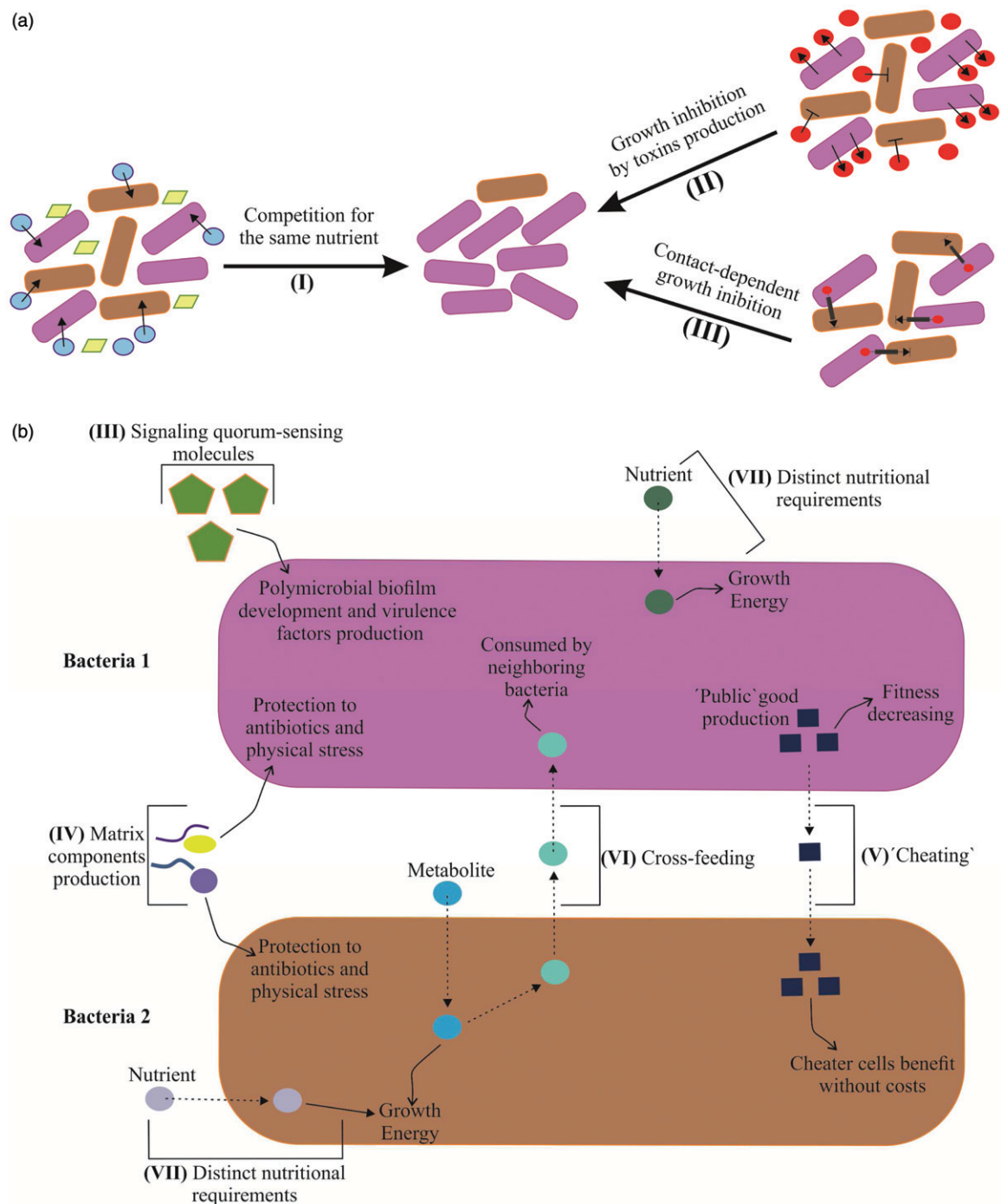


Figure 3. Schematic illustration of potential bacterial interactions within biofilm communities. (a) Antagonistic interactions among bacteria might involve a competition for nutrients (I), of toxins that kill the neighboring members (II). (b) On the other hand, bacteria within biofilms might cooperate by producing cell-signaling molecules (III), matrix components (IV), public good metabolites (V) and cross-feeding metabolites (VI). In addition, they might use different nutritional sources and in this case the fitness of one species is not affected by the other (VII). These interactions result in the growth of bacteria within biofilms and polymicrobial biofilm development with high resistance to stress conditions.

et al., 2013; Willett et al., 2015). While CDI is widely distributed in Gram-negative bacteria, this mechanism has also been identified in some Gram-positive bacteria (Braun & Patzer, 2013). CDI systems are composed of the CdiA toxin secreted by CdiB (both part of the Two-Partner Secretion system family), an outer

membrane β -barrel transporter, and an immunity protein. CdiA exhibits a modular design consisting of a very large and conserved N-terminal domain exposed at the cell surface and acting as an adhesin; and a shorter highly variable C-terminal domain carrying toxin activity that is delivered into the

cytoplasm of the recipient cell, causing cell inhibition or death (Braun & Patzer, 2013).

While these mechanisms are understudied in biofilm populations, their role on driving microbial ecology is clear. For instance, there is evidence for transcriptional regulation of the T6SS during biofilm formation, either by promoting biofilm formation or by competing with neighboring bacteria (Alteri & Mobley, 2016; Aubert et al., 2008; Moscoso et al., 2011; Russell et al., 2014). The same happens with CDI systems. They are involved in adhesion and biofilm formation in different species, such as *E. coli*, *Burkholderia thailandensis* and *P. aeruginosa* (Anderson et al., 2012, 2014; Garcia et al., 2013; Mercy et al., 2016; Ruhe et al., 2015). In fact, it has also been shown that CDI might function in both cooperative and competitive ways to build microbial communities with some type of kind-selective social behavior (Anderson et al., 2014; Garcia et al., 2013; Ruhe et al., 2015). While these works have provided valuable information, studies into UTI related settings are inexistent so far, which limits the transposition of this information to the clinical field.

Regarding synergistic interactions, both microorganisms might benefit from the presence of each other (Figure 3(b)). This type of interaction might occur, for instance, when the microorganisms cooperate in order to increase the overall resistance to antimicrobial agents of all members involved in a microbial community (Rodríguez-Martínez & Pascual, 2006). Another example of this behavior occurs when two microorganisms exchange metabolic products, known as the cross-feeding phenomenon (Woyke et al., 2006). Metabolic cooperation is an example observed when certain bacterial species modify the environmental conditions within biofilms (e.g. pH, oxygen concentration) to favor the growth of neighbors. For example, oxygen consumption by aerobic microorganisms could be beneficial for the growth of anaerobic microorganisms (Stewart & Franklin, 2008).

In other situations, one of the microorganisms benefits from the presence of another without affecting it. An example of this behavior happens when one microorganism metabolizes a compound produced by other community members (Faust & Raes, 2012) or receives a plasmid carrying genes that promote antibiotic resistance or virulence factors expression (Ghigo, 2001).

The increase of fitness of one member might also occur at a cost of others (West et al., 2006). In particular, secondary metabolites produced with a cost to microorganisms might be used by neighboring members (cheaters) of the biofilm without any cost (Xavier, 2011). Examples, of this type of molecules, called “public goods”, involve quorum sensing signaling molecules

(Diggle et al., 2007), iron scavenging siderophores (Brockhurst et al., 2008; Cordero et al., 2012; Harrison & Buckling, 2009; Jiricny et al., 2010) and antibiotic-degradation enzymes (Ciofu et al., 2000; Dugatkin et al., 2005). “Public goods” producers spend energy in its production and the cheaters exploit the “public goods” without cooperating; hence, cheaters are able to out-compete the “public good” producers (Nadell et al., 2009). For example, a group of bacteria might be able to produce antibiotic-degrading enzymes that will help all microbial members resist to antibiotic agents. This phenomenon is crucial to the β -lactam antibiotics inactivation in which the presence of β -lactamases in the biofilms matrix might inactivate them (Ciofu et al., 2000; Dugatkin et al., 2005; Gould et al., 2008).

Briefly, considering that the polymicrobial biofilms exist in most human infections (Elias & Banin, 2012), it is highly probable that the synergistic interactions among microorganisms occur, enabling the coexistence of microorganisms (Periasamy & Kolenbrander, 2009). In this way, inter-species cooperation increases the fitness and the resistance of the whole biofilm community even if any detrimental environmental conditions occurs (Giaouris et al., 2015; Kara et al., 2006; Luppens et al., 2008). In some situations, microorganisms co-inhabit the same environment without interacting (neutralism). For instance, microorganisms might share the same space, but have different nutritional requirements. Thus, the microorganisms live together without any beneficial or harmful effect on each other (Song et al., 2014).

Ecological perspective in the context of CAUTIs polymicrobial communities

Most, if not all types of interactions described above also occur under the context of CAUTIs (Table 2). These interactions are very diverse, as the same species present distinct behaviors when co-cultured with different bacteria.

For instance, the *in vitro* study performed by Cerqueira et al. (2013) has shown that despite *E. coli* being the most prevalent causative agent of CAUTIs, it turned out to be the less well-adapted to dual-species biofilms, in contrast with *P. aeruginosa* that seemed to persist better within the microbial consortia. By contrast, Croxall et al. (2011) demonstrated that *E. coli* from polymicrobial UTI samples showed more resistance to antibiotics and was more invasive in *in vitro* epithelial cell infection studies. These are two good examples of *E. coli* populations being “shaped” by the complex consortium and surrounding microenvironment in which they are inserted.

The same happens for other UTI-associated species. Antagonistic interactions between *P. aeruginosa* and *P. mirabilis* were observed in urinary catheter biofilms (Lehman & Donlan, 2015; Macleod & Stickler, 2007). However, other studies have shown that the virulence of *P. aeruginosa* and *S. aureus* biofilms is clearly stimulated when both bacteria are grown in a consortium in conditions mimicking CAUTIs (Goldsworthy, 2008). Similar observations were made by Korgaonkar et al. (2013) when it was demonstrated that the virulence of *P. aeruginosa* was enhanced when co-cultured with Gram-positive bacteria; for example, *P. aeruginosa* is

able to use peptidoglycan shed by Gram-positive bacteria to produce virulence factors with lytic functions. However, in the context of cystic fibrosis, the interaction between these two bacteria may be different. Mashburn et al. (2005), Barnabie & Whiteley (2015) reported that the presence of *P. aeruginosa* affects negatively *S. aureus*; in fact, *P. aeruginosa* was able to kill *S. aureus* to use the siderophores of this microorganism. These studies highlight the need for a tight control of the biofilm conditions, as small changes might have a great impact on ecological interactions. While mixed biofilm studies in CAUTIs have mainly focused on

Table 2. Different types of potential microbial interactions described in the literature among pathogenic bacteria occurring in CAUTIs.

Pathogen	Experimental conditions	Microbial interaction	Explanation	Refs.
<i>E. coli</i> with <i>P. aeruginosa</i>	Six-well tissue culture plates containing silicone coupons immersed in AUM at 37 °C and 120 rpm	<i>P. aeruginosa</i> dominated over <i>E. coli</i> when co-cultured	<i>E. coli</i> presented lower growth rate (0.20 h ⁻¹) when compared to <i>P. aeruginosa</i> (0.30 h ⁻¹) under the conditions at which the biofilm was formed. The extracellular production of virulence factors by <i>P. aeruginosa</i> , such as N-acyl-L-homoserine lactones, can negatively regulate biofilm formation by <i>E. coli</i> in mixed biofilms.	Cerqueira et al., 2013
<i>P. aeruginosa</i> with <i>S. aureus</i>	Glass flow cells supplied with a constant flow of AUM (30 ml h ⁻¹) at 37 °C	Virulence of <i>P. aeruginosa</i> and <i>S. aureus</i> was stimulated when both bacteria grown in consortium	Production of <i>P. aeruginosa</i> exotoxin A was increased nearly 2000-fold when <i>P. aeruginosa</i> and <i>S. aureus</i> were grown in a mixed biofilm.	Goldsworthy, 2008
<i>P. mirabilis</i> with <i>M. morgani</i> , <i>K. pneumoniae</i> , <i>E. coli</i> , <i>E. cloacae</i> or <i>P. aeruginosa</i>	Bladder model constituted by a glass chamber maintained at 37 °C. Silicone catheters were inserted into the chamber. The catheter retention balloons were inflated with 10 ml sterile water and the catheters were connected to drainage bags. Sterile AUM was pumped into the chambers	Impact on the ability of <i>P. mirabilis</i> to encrust and block urinary catheters	Co-infection of <i>P. mirabilis</i> with <i>M. morgani</i> , <i>K. pneumoniae</i> or <i>E. coli</i> had no effect on the ability of <i>P. mirabilis</i> to encrust and block catheters. Co-infection with <i>E. cloacae</i> or <i>P. aeruginosa</i> significantly increased the time that catheters took to block. A pre-inoculation with <i>E. cloacae</i> , <i>M. morgani</i> , <i>K. pneumoniae</i> or <i>E. coli</i> significantly delayed catheter blockage. However, <i>P. mirabilis</i> was able to colonize the biofilms and block the urinary catheters.	Macleod & Stickler, 2007
<i>P. mirabilis</i> with <i>P. stuartii</i>	Mice were inoculated with 50 µL of a 1:1 mixture of both bacteria	High incidence of urolithiasis and bacteremia	Total urease activity was increased during co-culture. A synergistic induction of urease activity might explain in part the high incidence of <i>P. mirabilis</i> and <i>P. stuartii</i> in polymicrobial CAUTIs.	Armbruster et al., 2014
<i>P. mirabilis</i> with <i>P. aeruginosa</i>	96-well microplates in human urine, at 37 °C with 100 rpm	Antagonistic interaction between <i>P. aeruginosa</i> and <i>P. mirabilis</i>	The elimination of <i>P. aeruginosa</i> at 72 h was probably due to the increase of pH between 48 and 72 h as a result of <i>P. mirabilis</i> urease activity.	Lehman & Donlan, 2015
<i>E. coli</i> with <i>P. mirabilis</i>	Mouse model of ascending UTI	<i>E. coli</i> and <i>P. mirabilis</i> do not directly compete for nutrients	<i>E. coli</i> and <i>P. mirabilis</i> have different central metabolic pathways despite have access to the same nutrients in the urine.	Alteri et al., 2015

bacterial interactions, a few studies have investigated cross-kingdom interactions, especially between bacteria and *C. albicans* species. For instance, *C. albicans* and *E. coli* exhibit a cooperative interaction in UTI-related settings. *E. coli* has proved to enhance adhesion of *C. albicans* to the bladder mucosa, increasing the likelihood of fungal urinary tract infections (Levison & Pitsakis, 1987). de Pádua et al. (2008) have also shown that *P. aeruginosa* adhesion to urinary catheters is enhanced in the presence of *C. albicans*, while *C. albicans* adhesion was not significantly affected. This phenomenon was probably due to the *P. aeruginosa* selective attachment to the fungus filamentous form (Hogan & Kolter, 2002). On the other hand, it should be noticed that *C. albicans*-*P. aeruginosa* biofilms have also been associated with antagonistic interactions mainly due to the toxic effect of some *P. aeruginosa* metabolites on *C. albicans* (Morales et al., 2013). However, those observations were not made on UTI-related settings. *Candida*-bacteria interactions seem to be common in human-related biofilms, especially with the oral or vaginal microflora (for a review on *Candida* interactions with bacteria see Morales & Hogan, 2010), but information on urinary catheter-associated biofilm is limited and simulation of biofilm real conditions is still poor or inexistent in the majority of the studies. The complexity of interactions is even higher when the factor “time” is taken into account.

For instance, Macleod & Stickler (2007) reported that any antagonistic effect of four other urinary tract pathogens (*Enterobacter cloacae*, *Morganella morganii*, *E. coli* and *K. pneumoniae*) against *P. mirabilis* in catheter biofilms is minimal and temporary.

Also, co-infection of *P. mirabilis* with *E. cloacae* or *P. aeruginosa* significantly increased the time that catheters took to block (Macleod & Stickler, 2007).

Recent data have also suggested the importance of studying bacterial metabolism during infection development to better characterize the microbial interactions (Alteri et al., 2009, 2015). In fact, bacterial metabolism seems to contribute to the persistence and pathogenesis of bacteria within biofilms as much as their virulence abilities (Alteri et al., 2009). For instance, the catabolism of amino acids present in urine generates tricarboxylic acid cycle (TCA; also known as citric acid cycle or Krebs cycle) intermediates and gluconeogenesis substrates, allowing uropathogenic *E. coli* to infect more efficiently the urinary tract (Alteri et al., 2009). The TCA cycle involves a series of chemical reactions used by all aerobic organisms to generate energy through the oxidation of acetyl-CoA derived from carbohydrates, fats and proteins into carbon dioxide and chemical energy in the form of guanosine triphosphate

(Kim et al., 2008). Gluconeogenesis is a metabolic pathway that results in the production of glucose from certain non-carbohydrate carbon substrates such as lactic acid, glycerol, glycine, serine, aspartate, and others (Berg & Stryer, 2002). Based on this, a recent study analyzed the central metabolism of *E. coli* and *P. mirabilis* to explain their ability to co-infect the same niche (urinary tract). Firstly, it could be assumed that both bacteria have the same nutritional preferences since they colonize the same environment; however, results showed that *E. coli* and *P. mirabilis* use different central metabolic pathways despite having access to the same nutrients in urine (e.g. amino acids, peptides and urea). This suggests that co-infecting bacteria might not necessarily compete for nutrients, hence increasing their fitness during UTI development (Alteri et al., 2015).

Altogether, these studies have focused on the ability of pathogenic bacteria commonly found on biofilm catheters to change their behavior when living in a consortium. While all these studies have revealed some aspects of interactions and persistence displayed by known CAUTIs pathogens, the role of uncommon bacteria on CAUTIs development or the interactions between uncommon bacteria and CAUTIs-associated pathogenic bacteria is poorly described.

Potential role of uncommon bacteria over pathogenesis of CAUTIs

In the literature, there are already some indications about the possible contributions of uncommon microorganisms on the pathophysiology and on the antimicrobial susceptibility pattern of biofilms-associated with CAUTIs (Azevedo et al., 2016; Lopes et al., 2014). Recently, it was reported that despite the unknown pathogenic nature of *D. tsuruhatensis* and *A. xylosoxidans*, they might interact synergistically with *E. coli*, developing well-organized microbial consortia with increased tolerance to several antibiotics commonly prescribed in CAUTIs (Azevedo et al., 2016; Lopes et al., 2014).

In addition, it was demonstrated that a residual concentration of uncommon bacteria was enough to contribute to *E. coli* survival. The specific mechanism of this shared protection is currently unknown; however, three theories were proposed: (1) transfer of genetic material from the uncommon bacteria to *E. coli*; (2) induction of a different physiological state in the susceptible species due to antibiotic uptake; (3) degradation of the antibiotic in the biofilm matrix, through the action of the enzymes produced by uncommon bacteria. In this case, uncommon bacteria have their metabolism directed to

the secretion of β -lactamases and *E. coli* (the susceptible cells) benefits from the action of the secreted β -lactamases, predominating in the microbial consortium.

In conclusion, these observations suggested that the surface of urinary catheter and urine is a favorable habitat for polymicrobial biofilms involving *E. coli* and uncommon bacteria. In these conditions, *E. coli* and uncommon bacteria, with a previous unrecognized role, appear to be able to persist and survive, adjusting their cell concentrations and metabolism to get maximum benefits from living together. Even when outnumbered, the presence of uncommon bacteria showed to have a protective impact on the whole community. For instance, this could be crucial if any stress condition (e.g. use of antibiotic agents) occurs. Other possible contributions of uncommon bacteria to the development of CAUTIs can be found in Figure 4.

CAUTIs treatment – importance of shaping polymicrobial interactions

New lessons in polymicrobial infections should be taken into consideration, especially in the context of strategies used for the treatment of CAUTIs. Currently, the therapeutic strategies to prevent or treat CAUTIs involve the use of renally excreted antibiotics in combination with a periodic replacement of the urinary catheter. To minimize the chances of a new re-infection, it is

recommended to replace the urinary catheter after 48 h of antibiotic treatment (Wu et al., 2015).

Antibiotics (e.g. trimethoprim/sulfamethoxazole, nitrofurantoin, ciprofloxacin, gentamicin, amoxicillin/clavulanic acid) are administered during 7 days in patients who have relief of symptoms and 10–14 days for patients who do not respond to the antibiotic therapy (Hooton, 2010). However, it has been described that bacterial uropathogens isolated from patients with CAUTI or UTI revealed high resistance to some antibiotic agents used in clinical practice, such as ampicillin, trimethoprim sulfamethoxazole, ciprofloxacin, amoxicillin/clavulanic acid, and others (Croxall et al., 2011; Kazi et al., 2015; Zhanel et al., 2000). In addition, if the urinary catheter is not removed or replaced, re-infection can occur after the end of an antibiotic treatment.

The different behavior of complex communities associated with CAUTIs led scientists to speculate on their ability to control biofilm by interfering on their species composition; or on the possibility of anticipating the possible clinical outcome based on the biofilm composition. An *in vivo* study performed by Armbruster et al. (2014) showed that the co-infection of *P. mirabilis* and *P. stuartii*, also a common co-colonizer of urinary catheters, resulted in a higher incidence of urolithiasis and bacteremia due to an increased activity of total urease (Armbruster et al., 2014). This might indicate that the simultaneous presence of these species in CAUTI-associated biofilms represents an additional risk for the

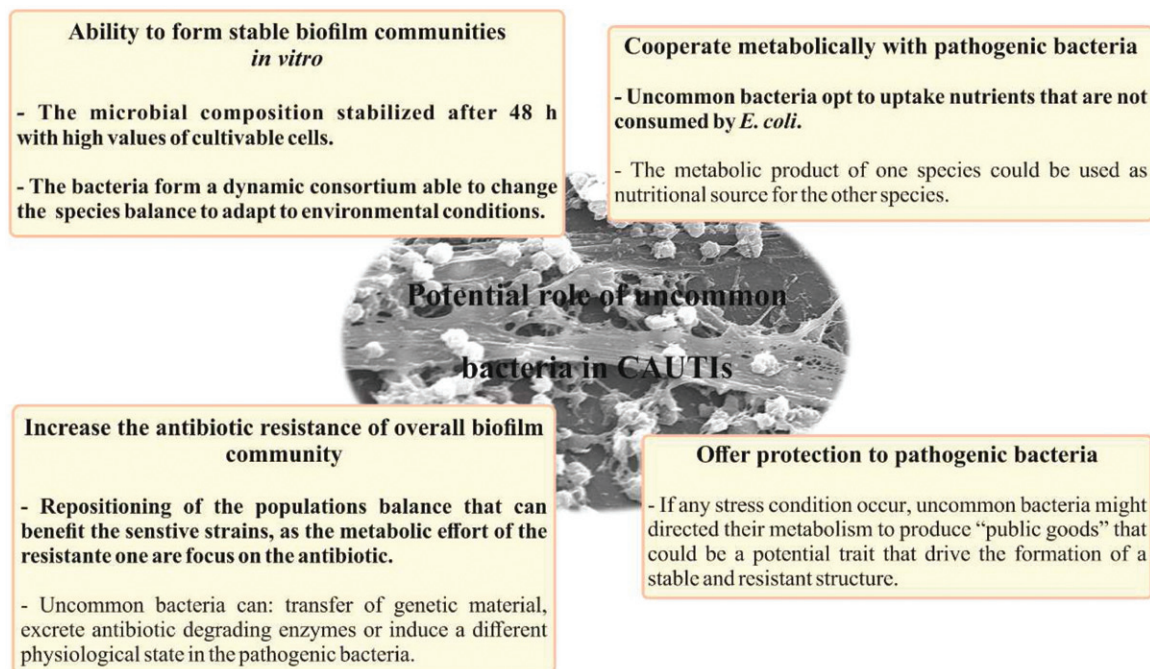


Figure 4. Potential contribution of uncommon bacteria to a more stable polymicrobial biofilms development in CAUTIs. They were observed in the studies found in literature (Azevedo et al., 2016; Lopes et al., 2014).

patient. If this proves to be true, those clinical situations should be handled with extra attention. Hence, it becomes imperative that the clinicians know the biofilm composition in order for a more adequate therapy selection for an effective prevention or treatment of infections.

Concluding remarks

Understanding how polymicrobial biofilm communities behave and how uncommon bacteria and pathogenic bacteria interact might have further implications in the control and treatment of CAUTIs, since bacterial behavior cannot be predicted from studies of single-species biofilms. In addition, it is also important to note that the role of uncommon bacteria is underestimated probably due to the absence of commercial media and kits to detect these bacteria in hospitals.

The topic of this review raises several clinically-relevant questions: Is the composition of a consortium indicative of a potential harmful biofilm? Which antibiotic should be prescribed for a particular consortium? The medical community has recognized the urgency to better understand the clinical significance of species involved in polymicrobial infections and how they might affect the disease outcome. This could constitute the basis for a new strategy in the control and treatment of CAUTIs that uses the knowledge on the microbiome of each patient for a personalized therapy selection.

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