Integrated Master in Bioengineering

Impact of biofilm infections and proposal of new approaches to counteract its recurrence and persistence

Dissertation for Master's degree in Biological Engineering

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Abstract

Biofilm associated infections (BAI) represent 65-80% of all bacterial infections. Bacterial biofilms are emerging as a significant global health concern due to their abilities to tolerate antibiotics and host defence. Only recently they were acknowledged as the main cause of complications adjacent to infections, namely chronicity, persistence, or recurrence. Bloodstream and urinary tract infections (BSI and UTI) are BAI and were chosen to evaluate its biofilm impact. This was achieved by performing separated systematic reviews and meta-analyses of observational studies. *In vitro* biofilm production (BFP) in BSI was highly related to resistant strains (odds ratio, OR: 2,82; 95% confidence interval, CI: 1,77-4,49; $p < 0,01$), especially for methicillin-resistant *Staphylococci.* BFP was also highly linked to BSI persistence (OR: 2,88; 95% CI: 1,69-4,93; $p < 0,01$) and even to mortality (OR: 2,05; 95% CI: 1,53-2,74; $p < 0,01$). Biofilm seems to impact BSI independently from clinical differences including treatment interventions, as statistical heterogeneities were lower than expected. In the case of UTI, multi-drug resistant (MDR) and extended spectrum β-lactamase (ESBL) producing strains of *Escherichia coli* (the main uropathogen), were linked to a great BFP prevalence (OR: 2,92; 95% CI: 1,30-6,54; $p < 0,01$ and OR: 2,80; 95% CI: 1,33-5,86; $p < 0,01$). More *in vitro* BFP was shown in catheter-associated UTI compared to non-catheter-associated but contrarily to what was expected, no statistically significant difference was verified (OR: 2,61; 95% CI: 0,67-10,17; $p = 0,17$). This may stand for an echo of the flaws intrinsic to BFP *in vitro* method characteristics. Statistical heterogeneities in UTI were higher than in BSI.

Based on the previously analysis and on literature data it is possible to ascertain that biofilm recovery ability is somehow overlooked. Persister cells are phenotypic variants whose function is survival: they can enter in a dormant state, easily surviving any current antibiotic treatment and can further re-colonize the biofilm. Lately, new agents targeting persisters (biofilm eradication agents) are being developed and revealed, such as phenazines, quinolines, nitroxide functionalized antibiotics, synthetic rhetinoids, repurposed anti-cancerous drugs, quaternary ammonium compounds, and antimicrobial peptides. These are very recent reports and depict a small niche within the biofilm research field; clinical approval remains distant to become a reality.

Phytochemicals have already been proven as excellent anti-biofilm agents, however they only have been tested alone when they may be much more potent when acting together due to synergistic effects that naturally occurs upon plant defence mechanism. Furthermore, studies of their capacity to inhibit biofilm regrowth or to fully eradicate biofilms at long-term are scarce. In this way, a new approach is proposed resorting to a case study. For this, cinnamic acid (CIN), citronellic acid (CITR) and the combination of the two phytochemicals (MIX) were evaluated for their biofilm regrowth inhibition activity in *E. coli*. Firstly, the minimum inhibitory concentration (MIC) of each compound was determined and pre-formed biofilms were exposed to the solutions for 24 h (t24) at MIC, 5 × MIC and 10 × MIC. Then, the compounds were removed and the ability to grow again for another 24h (t48) was evaluated. At each phase a biofilm characterization using several parameters were characterized: biomass removal (BR)/regrowth inhibition (BRI) (crystal violet staining), metabolic activity inhibition (MAI) (alamar blue staining) and colony forming

units (CFUs) quantification (plate count agar method). CIN reduced CFUs totally from 5 × MIC upon exposure but did not totally impede colonies formation upon providing regrowth conditions. CITR was never tested for antimicrobial activity and showed similar properties to CIN upon exposure. It fully inhibited biomass regrowth at 10 × MIC, yet MAI was far from total inhibition (53,8 ± 8,3%). Thus, and despite both compounds considerably inhibited biofilm recovery, the presence of dormant or persisters is very likely. Unfortunately, the main objective of this case study to assess MIX activities and interactions were not possible to carry out. Nevertheless, the potential for rational development of phytochemicals formulation was debated and must be explored in future studies.

Resumo

As infeções associadas a biofilmes (BAI) representam 65-80% de todas as infeções bacterianas. Devido à sua capacidade de tolerar os antibióticos e também a resposta imune do hospedeiro, os biofilmes bacterianos estão a emergir globalmente como uma preocupação global de saúde. Só mais recentemente têm sido reconhecidos como principais causadores de complicações adjacentes a infeções, tal como como a cronicidade, persistência ou recorrência. Neste estudo, as infeções da corrente sanguínea e do trato urinário (BSI e UTI) foram escolhidas para avaliar o impacto do biofilme. Para isso, realizaram-se duas revisões sistemáticas e meta-análises de estudos observacionais. A produção de biofilme *in vitro* (BFP) nas BSI foi altamente associada a estirpes resistentes (rácio de probabilidade, OR: 2,82; intervalo de confiança, 95% CI: 1,77-4,49; $p < 0,01$), e sobretudo a *Staphylococcus sp*. resistentes à meticilina. A BFP também foi significativamente associada à persistência da BSI (OR: 2,88; 95% CI: 1,69-4,93; $p < 0,01$) e até à mortalidade (OR: 2,05; 95% CI: 1, 53-2,74; $p < 0,01$). O biofilme parece impactar a BSI independentemente das diferenças clínicas, incluindo diferentes tratamentos, uma vez que as heterogeneidades estatísticas foram inferiores ao esperado. No caso das UTIs, as estirpes multirresistentes (MDR) e estirpes produtoras de β-lactamases de espetro alargado (ESBL) da *Escherichia coli* (principal uropatógeno), foram relacionadas a uma grande prevalência de BFP (OR: 2,92; 95% CI: 1,30-6,54; $p < 0, 01$ e OR: 2,80; 95% CI: 1,33-5,86; $p < 0,01$). Uma maior BFP foi verificada na UTI associada ao cateter em comparação com o não associado ao cateter, no entanto, ao contrário do esperado, não foi verificada diferença estatisticamente significativa (OR: 2,61; 95% CI: 0,67-10,17; $p = 0,17$). Isto pode representar um reflexo das falhas intrínsecas às características do método BFP *in vitro*. As heterogeneidades estatísticas na UTI foram maiores que na BSI.

Com base na análise anterior e nos dados da literatura, é possível verificar que a capacidade de recuperação de biofilme é de alguma forma negligenciada. As células persistentes são variantes fenotípicas cuja função é a sobrevivência: elas podem entrar em estado inativo e sobreviver facilmente a qualquer tratamento antibiótico atual, recolonizando posteriormente, o biofilme. Ultimamente, novos agentes que atuam nas células persistentes (agentes de erradicação de biofilme) têm sido desenvolvidos e revelados, como fenazinas, quinolinas, antibióticos funcionalizados com nitróxido, retinoides sintéticos, agentes anticancerígenos reaproveitados, compostos quaternários de amónio, e peptídeos. Estes representam soluções muito recentes e retratam um pequeno nicho da área de pesquisa dos biofilmes; a aprovação clínica permanece distante de uma realidade.

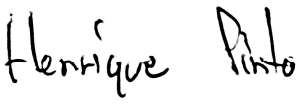
Os fitoquímicos já foram comprovados como excelentes agentes anti-biofilme, porém apenas foram testados individualmente quando podem ser muito mais potentes atuando em conjunto, devido aos efeitos sinérgicos que ocorrem naturalmente no mecanismo de defesa das plantas. Para além disso, estudos sobre as suas capacidades de inibir o recrescimento do biofilme ou de os eliminar totalmente a longo prazo são escassos. Desta forma, uma nova abordagem é proposta, recorrendo a um caso de estudo. Para isso, o ácido cinâmico (CIN), o ácido citronélico (CITR) e a combinação dos dois fitoquímicos (MIX) foram avaliados quanto à atividade de inibição do recrescimento do biofilme na *E. coli*. Primeiramente, a concentração

inibitória mínima (MIC) de cada composto foi determinada e os biofilmes pré-formados foram expostos às soluções fitoquímicas durante 24h (t24) na MIC, 5 × MIC e 10 × MIC. De seguida, os compostos foram removidos e a aptidão para crescer novamente por mais 24 h (t48) foi avaliada. Em cada fase, foi realizada uma caracterização do biofilme utilizando diferentes parâmetros: remoção de biomassa (BR) / inibição do recrescimento (BRI) (pela coloração do violeta de cristal), inibição da atividade metabólica (MAI) (pela coloração do azul de alamar) e quantificação de unidades formadoras de colónias (CFU) (método de contagem em placa de ágar). O CIN reduziu totalmente as CFUs de 5 × MIC após a exposição, mas não impediu totalmente a formação de colónias ao repor as condições de recrescimento. O CITR nunca foi testado quanto à atividade antimicrobiana e mostrou propriedades semelhantes ao CIN após exposição: inibiu completamente o crescimento de biomassa a 10 × MIC, apesar o MAI ter sido longe de inibição total (53,8 ± 8,3%). Assim, e apesar de ambos os compostos inibirem consideravelmente a recuperação do biofilme, a presença de células dormentes ou persistentes é provável. Infelizmente, o principal objetivo deste caso de estudo, avaliar as atividades e interações do MIX, não foi cumprido. No entanto, o potencial de desenvolvimento racional da formulação de fitoquímicos foi discutido e deve ser explorado.

Declaration

I hereby declare, on my word of honour, that this work is original and that all non-original contributions were properly referenced with source identification.

29th June, 2020



Content

Nomenclature

AMP	Antimicrobial peptide
BAI	Biofilm associated infection
BF	Biofilm
BFP	Biofilm production
BR	Biomass removal
BSI	Bloodstream infection
CA	Catheter associated
CAUTI	Catheter associated urinary tract infection
CFU	Colony forming unit
CI	Confidence interval
CIN	Cinnamic acid
CITR	Citronellic acid
CVC	Central venous catheter
DMSO	Dimethyl sulfoxide
DRBI	Non-device related biofilm infections
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
ESBL	Extended-spectrum β-lactamases
FDA	Food and drug administration
HCAI	Healthcare associated infection
MAI	Metabolic activity inhibition
MDR	Multidrug-resistant
MHB	Muller-Hinton broth
MIC	Minimum inhibitory concentration
MIX	Mixture
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
NDRBI	Non-device related infections
OD	Optical density
OR	Odds ratio
PCA	Plate Count Agar

QAC	Quaternary ammonium compound
RMA	Resistance-modifying agent
UTI	Urinary tract infections
VBNC	Viable but not cultivable cells
∑ CI	Combinatorial index

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Chapter 1: Introduction

1.1 Project presentation and objectives

Bacterial biofilms are typically pathogenic in nature and can cause serious nosocomial infections. Many of all microbial and chronic infections are greatly linked with biofilm formation. They offer enormous resistance against human immune system, and against antibiotics. Health related concerns are raised due to the biofilm potential to provoke infection chronicity, persistence, or recurrence. Biofilm associated infections are usually divided into device-related and non-device-related infections (Jamal et al., 2018).

There is currently a demand for the development of new drugs not as susceptible to bacterial and biofilm resistance as antibiotics. Plant products have demonstrated to be excellent compounds with unique properties, making them possible candidates for these therapeutics (Borges et al., 2016).

At the beginning of the project, the proposed objective was to experiment a new approach to combat biofilms, which would consist in the development of new formulations by testing combinations of phytochemicals to investigate synergistic or additive effects. Furthermore, *in vitro* methods would focus on biofilm regrowth after phytochemicals exposure to evaluate their impact on persistence. However, laboratorial work was interrupted by the struck of the SARS-CoV-2 pandemic. Preliminary results are presented, yet the main objective shifted into a deeper investigation of the problematic: Biofilm prevalence, its impact on resistant strains and clinical outcomes, were assessed in two common biofilm associated infections –bloodstream and urinary tract infections. This was accomplished by carrying out systematic reviews and meta-analysis to provide insightful data and clarifications. Furthermore, recent new approaches focused on counteract biofilm recurrence and persistence are debated.

1.2 Thesis organization

In addition to the introductory chapter (Chapter 1) that presents the motivation and the main goals of the study, this dissertation includes four additional chapters. Chapter 2 provides a brief review of the literature. The relevance of biofilm associated infection is discussed. It aims on the resistance that bacterial biofilms offer and on the various types of infections. At last, the benefits of a systematic review, as well as a meta-analysis are debated.

In Chapter 3 and 4, systematic reviews were performed on bloodstream infections (BSI) and urinary tract infections (UTI), respectively. For BSI, retrieved data yielded 4 meta-analysis with

subgroups by microorganism specie: biofilm production prevalence, its association to resistant strains, to persistence and to mortality. For UTI, 3 meta-analysis were performed: biofilm production prevalence, its association to resistant strains and to catheter associated infections.

Chapter 5 presents a brief discussion regarding new approaches to mitigate biofilm infections persistence or recurrence, and a proposal of a new approach with phytochemicals, resorting to a laboratorial case study where two phytochemicals were selected to primarily evaluate its action on biofilm recovery.

General conclusions and perspectives for further research are summarized in Chapter 6, giving an overview of the work developed.

Chapter 2: Literature Review

2.1 Sessile microbial communities

A biofilm is the predominating microbial lifestyle and is an example of a successful physiological adaptation as it thrives in most natural environments as well as in harsh conditions (Yin et al., 2019). The inventor of the Microscope, Anton Von Leeuwenhoek, described for the first time a biofilm in the 17th century. He observed microbial aggregates on scrapings of plaque from his own teeth. Back in the 1920s and 1930s, biofilms were already shown to be important for biofouling on submerged ship surfaces. However, in medicine, the concept of biofilm infections was initiated in the early 70s and the term biofilm was brought by J. W. Costerton in 1985 (Hoiby, 2017). Biofilms may impact health negatively as they are often associated with many pathogenic forms of human diseases (Yin et al., 2019). Currently, apart from medicine, natural unwanted or even deliberated engineered biofilms have an impact on several industries such as the pharmaceutical industry, food industry, agricultural production, environmental protection, energy utilization, scientific research, and others. On the flipside, unwanted biofilms can create many physical-chemical barriers to processes (Yin et al., 2019, Bryers, 2008). In bioprocesses, engineered biofilms can increase reactor productivity, system stability, and provide product separation.

Unlike in the planktonic state, sessile cells attach on a wide variety of biotic and abiotic surfaces and produce an extracellular polymeric substance (EPS) matrix that contains mainly polysaccharides, proteins, nucleic acids and lipids (Donlan, 2002). The attached three-dimensional polymer network provides many advantages for the microorganism community thriving within. Besides offering cohesion and stability that allows or facilitates cell-cell-recognition, cell-cell communication, and the exchange of genetic information via horizontal gene transfer. Biofilm matrix is often highlighted as a major physical and chemical protective barrier for microorganisms (Flemming and Wingender, 2010, Yin et al., 2019).

2.2 Biofilms and antibacterial resistance

Biofilm physiology, structure, composition and several interrelationship mechanisms are responsible for providing microbes up to 1000 times antimicrobial resistance (Shadia M and Abhinav, 2014, Borges et al., 2015). Therefore, it is crucial to understand biofilm complexity in order to be able to develop effective strategies to eradicate or control infections (Azeredo et al., 2017).

The main biofilm resistance mechanisms are (Borges et al., 2015): (1) The presence of EPS that confer protection as reduces the ability of penetration and diffusion of antimicrobial molecules;

(2) In the deeper areas of the matrix, the lack of diffusion leads to subinhibitory concentrations, which allows an activation of different phenotypes related to stress or survival responses and the emergence of genetic resistance, contributing to the biofilm community with greater adaptive resistance; (3) Just as there are low concentrations of antimicrobials, there are also some areas with low concentrations of nutrients, so it induces bacteria in a dormant state, that is, metabolically inactive. As a large majority of antimicrobials (especially antibiotics) act on the replication metabolism, these bacteria have enormous resistance (non-adaptive); (4) Programmed apoptosis consists of some cells that sacrifice themselves to serve as food for others, allowing them to multiply and quickly to reestablish the biofilm; (5) Although some antibiotics are able to kill dormant cells and spread easily throughout the matrix, their inability to completely eliminate biofilms has been demonstrated (Spoering and Lewis, 2001). The presence of phenotypic variants of the wild strain may be responsible for this tolerance, called persister cells, which survive lethal concentrations of antimicrobial agents without undergoing mutations that confer resistance. In fact, persister cells are a polemic subject to the scientific community as they are often associated and unassociated to dormant cells (Orman and Brynildsen, 2013, Wood et al., 2013). However, it has been demonstrated that the lack of significant growth or metabolic activity does not guarantee persistence, and that persistence is far more complex than dormancy (Orman and Brynildsen, 2013). This phenotypic variant can exist in planktonic and biofilm populations, yet as persister biofilm cells are protected by EPS, it is believed that these are the main responsible for the biofilm regrowth and for a great share in all biofilm antimicrobial resistance (Borges et al., 2015, Lewis, 2008).

2.3 Biofilm role on healthcare associated infections

A health care-associated infection (HCAI) or nosocomial infection is an infection that is acquired in a hospital or other healthcare facility. HCAI results in prolonged hospital stays, long-term disability, overcrowded community of patients treated together, enhanced resistance of microorganisms to antimicrobials, enormous additional costs for health systems, high costs for patients and their family, and unnecessary deaths. The prevalence of HCAI is estimated to be between 5.7% and 19.1%. Only in the United States, it is estimated that the mortality rate due to HCAI is at around 5,8% (WHO, 2016).

There are many routes of HCAI transmission whereas biofilms can play an important role. Bacteria can form persisting biofilms in healthcare units and medical devices, becoming more resistant to antibiotic and being responsible for HCAIs onset and spread. Indeed, among many factors that are currently increasing HCAI at a global level, including in developed countries, the rise of implantable device related infections and the negligence in control and cleaning procedures

of non-critical risk stands out (SCENIHR, 2009, WHO, 2016). Non-critical risk represents what encounters intact skin, such as stethoscopes, blood pressure cuffs and similar devices. Also included are surfaces that are close to patients: floor, walls, tables, railings, furniture, bed structure, among others (SCENIHR, 2009). Despite the low risk categorization, several studies suggest that biofilms (dry) can harbor pathogenic and multidrug-resistant bacteria in these surfaces and devices (Otter et al., 2015, Ledwoch et al., 2018).

2.4 Biofilm associated infections

A definition for biofilm associated infections (BAI) has already been proposed: " infections due to aggregated, pathogenic or opportunistic microorganisms encased in an exopolysaccharide matrix and recalcitrant to host defense mechanisms and antimicrobial treatment" (Hall-Stoodley et al., 2012). BAI can be caused by a single microorganism or by multiple species, being named polymicrobial biofilm infections.

According to some estimates, 65-80% of total human infections are associated with biofilm formation (Costerton et al., 1999, Jamal et al., 2018). However, planktonic antimicrobial susceptibility testing is often performed by clinics to assess antibiotic and treatment choice (EUCAST, 2000, Waters and Ratjen, 2017). Another problem related to microbiology clinical laboratories is that, even though having limitations, culture and microscopy are still two of the most utilized techniques (Franco-Duarte et al., 2019). These methods do not identify most bacteria in complex polymicrobial communities such as those found in biofilm associated infections (Rhoads et al., 2012, Wolcott and Ehrlich, 2008). Molecular techniques can overcome this challenge, however its implementation is not widespread due to higher costs and the degree of knowhow required (Bou et al., 2011). Thus, these clinical diagnostic and assessment tests can underperform and lead to unsuccessful infection treatments and further antibiotic resistance increase (Dias et al., 2018, Waters and Ratjen, 2017, Bou et al., 2011).

Running the following MeSH term search on PubMed: "biofilm infections OR biofilm associated infections", there are only 5 studies available on the database published until the end of 1984. From this date until 2019, the number of publications per 5 years have risen substantially (Figure 1). The growth tendency also matches to only "biofilm" search publications (Bjarnsholt, 2013). This may represent a recent and significant increase in the importance given to the role of biofilms on some infections as more and more they are acknowledged and understood.

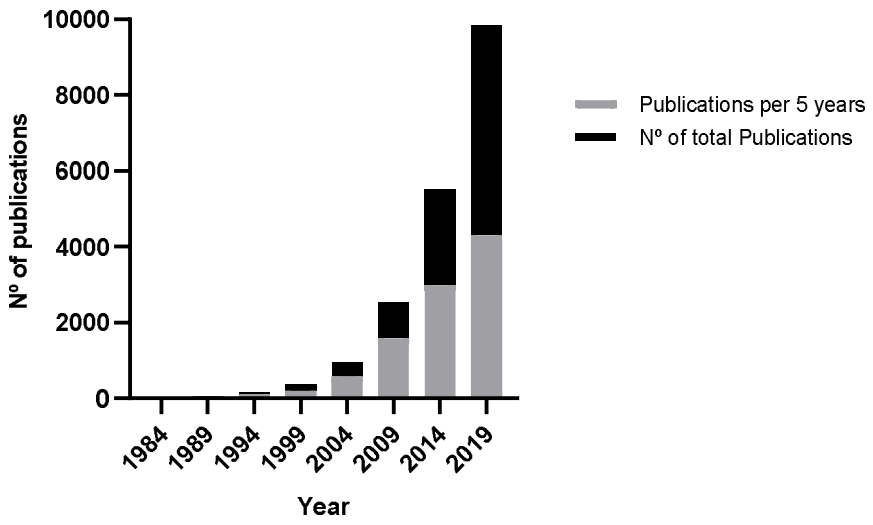

Figure 1 - Accumulated publications and per 5 years of biofilm associated infections on PubMed database.

Increasing evidence indicates that chronic or persistent bacterial infections are because of biofilm formation, differing with the planktonic bacteria found in acute infections which are in general, more easily treated. Moreover, a chronic infection cure with antibiotics, is very difficult and perhaps even impossible (Bjarnsholt, 2013). Alongside high frequency of infection, this generates substantial healthcare costs to the treatment (Lynch and Robertson, 2008). BAI are often divided into two categories, device-related biofilm infections (DRBI) and non-device related biofilm infections (NDRBI) (Jamal et al., 2018, Sun et al., 2013, Lynch and Robertson, 2008). Some types of infections can belong to device or non-device related biofilm infection depending on its cause.

NDRBI are native, mainly opportunistic, and often chronic infections associated with host tissues. Biofilm formation always occurs on biotic or natural surfaces of the human body and does not involve a foreign body (Lynch and Robertson, 2008, Sun et al., 2013).

DRBI includes all types of infections that can be caused by biofilm formation on indwelling medical devices whose innovations have prolonged and enhanced the quality of life for many patients. However, inserting a foreign body or material in a patient inevitably incomes high probability of microbial colonization. As a matter of fact, DRBI are the most common cause of HCAI: 50-70% can be attributed to medical devices. Even more worrying, they are likely to rise in number. The increasing types and device utilization rates, the aging of the population, and the rising frequency of comorbidities (leading to immunocompromised states), outstrips recent material science and implementation process advances. There are plenty of infections associated with the use

of medical devices in healthcare facilities and a list of them is presented in Table 1 (VanEpps and Younger, 2016)

Table 1 - Infections associated devices. Adapted from VanEpps and Younger 2016.

Vascular access devices	Cardiac devices	Prosthetic joints	Urinary devices	Other devices
- Peripheral intravenous catheter	- Intra-aortic ballon pump	- Shoulder	- Foley catheter	- Mesh for ventral hernia repair
- Arterial catheter	- Ventricular assist device	- Hip		- Ventriculoperitoneal shunt
- Central venous catheter	-Mechanical heart valve	- Knee		- Peritoneal dialysis catheter
- Peripherally inserted central catheter	- Cardiac implantable electronic devices	- Elbow		- Contact lenses[a] - Voice protheses[a] - Endotracheal tubes[b] - Intrateurine devices[b] - Fracture-fixation devices[c] -Dental implants[d] - Cochlear implant[c] - Breast implant[c] - Penile implant[c]
- Dialysis catheters	- coronary stents[c]			
- Venous access ports	- vascular grafts[d]			

a - (Jamal et al., 2018); b - (Sun et al., 2013); c - (Lynch and Robertson, 2008); d - (Hall-Stoodley et al., 2012)

Urinary catheters are the most used devices in hospitals followed by central venous catheters (CVC) (Darouiche, 2001). While urinary catheters are related to high infection rate and low mortality rate, CVC's are linked to low incidence rate, however mortality rate is moderate. On the other hand, mechanical heart valves are associated to high mortality despite a much less requirement use and low infection rate. The average rate of surgical implant-related infectious diseases ranges between 1% and 50% and it depends mainly on the type of material, implantation site and intended lifespan (Darouiche, 2001, VanEpps and Younger, 2016).

The most common and more important BAI, its main host microorganisms, and categorization into DRBI and/or NDRBI are presented in Table 2.

Table 2 - Most common BAI, main microorganisms and DRBI/NDRBI categorization.

Infection type	Main microorganism(s)	DRBI or NDRBI	Reference(s)
Cystic fibrosis lung infection	*Staphylococcus aureus; Pseudomonas aeruginosa; Haemophilus influenzae*	NDRBI	(Lyczak et al., 2002)
Periodontitis	*Streptococcus spp; Actinomyces spp* (early colonizers)	NDRBI	(Lasserre et al., 2018)
Dental caries/plaque	*Streptococci* (early colonizers)	NDRBI	(Marsh, 2010)
Peri-implantitis	*Streptococci* (early colonizers)	DRBI	(Dhir, 2013)
Chronic otitis media	*Staphylococcus aureus; Pseudomonas aeruginosa; Proteus mirabilis; Klebsiella sp.; Escherichia coli*	NDRBI and DRBI	(Obi et al., 1995, Adoga et al., 2011, Gu et al., 2014)
Infective nndocarditis	*Staphylococci; Streptococci; Enterococci*	NDRBI and DRBI	(Elgharably et al., 2016, Vincent and Otto, 2018)
Chronic osteomyelitis	*Staphylococci*	NDRBI and DRBI	(Masters et al., 2019)
Chronic sinusitis/rhinosinusitis	*Staphylococcus aureus; Pseudomonas aeruginosa; Haemophilus influenza*	NDRBI	(Fastenberg et al., 2016)
Chronic tonsillitis	*Staphylococcus aureus*	NDRBI	(Alasil et al., 2013, Torretta et al., 2013)
Chronic wounds	*Pseudomonas aeruginosa; Staphylococcus epidermidis; Staphylococcus aureus*	NDRBI	(Frykberg and Banks, 2015, Wolcott et al., 2016)
Periprosthetic joint infections	*Staphylococci; Streptococci;*	DRBI	(McConoughey et al., 2014)
Ventilated-associated pneumonia	*Pseudomonas aeruginosa; Staphylococcus aureus*	DRBI	(Fernandez-Barat and Torres, 2016)
Urinary tract infections	*Escherichia coli; Enterococcus spp.; Pseudomonas aeruginosa*	NDRBI and DRBI	(Hatt and Rather, 2008)
Chronic prostatitis	*Escherichia coli*	NDRBI and DRBI	(Delcaru et al., 2016)
Catheter-related bloodstream infections	*Staphylococcus aureus; Coagulase-negative staphylococci; Enterococci; aerobic Gram-negative bacilli: Candida*	DRBI	(Shah et al., 2013, Gahlot et al., 2014a)

It is important to mention that only most common or most cited host microorganisms are referred, although there can be many more involved in these types of infections. In fact, with the already mentioned advent of improved microbial identification methods, it is clear that BAI can be chronically colonized with complex, polymicrobial infections that otherwise were unidentified (Rhoads et al., 2012, Wolcott and Ehrlich, 2008). For instance, conventional culturing methodology only identifies around 1% of the bacteria in a chronic wound (Zhao et al., 2013). The emergence of resistant bacteria also contributes to an increase number of pathogens (Ventola, 2015). Moreover, there is other specific BAI to the various medical devices that are not as common or important and therefore, are not listed on table 2 such as tissue fillers infections, breast implants infections, ventricular assist devices infections and several others (VanEpps and Younger, 2016, Vinh and Embil, 2005).

To initially and rapidly comprehend a level of association of these infections to biofilms by the scientific community, another PubMed database study was performed through its search engine, as described: the number of publications related to each infection type MeSH terms, were retrieved from the end of 2009 till the end of 2019. Then, to assess the percentage of those publications that refers to the word "biofilm", the same search was performed but with the additional Boolean operator "AND", "biofilm" on the search box and in "All fields" category. The collected data is shown on the Table 3.

Table 3 - Number of publications, "biofilm" references and its ratio to each infection type.

Infection type	Nº of publications	"Biofilm" references	Ratio (%)
Chronic wounds	19581	108	0,6%
Dental caries/plaque	13793	1397	10,1%
Catheter associated urinary tract infections	10916	314	2,9%
Periodontitis	10638	676	6,4%
Infective endocarditis	6414	80	1,2%
Osteomyelitis	5136	121	2,4%
Chronic sinusitis/rhinosinusitis	4575	201	4,4%
Cystic fibrosis lung infection	4286	423	9,9%
Catheter-related bloodstream infections/bacteremia	3335	152	4,6%
Ventilated-associated pneumonia	3029	78	2,6%
Chronic otitis media	1596	92	5,8%
Periprosthetic joint infections	1517	109	7,2%
Peri-implantitis	1249	145	11,6%
Chronic prostatitis	1018	12	1,2%
Chronic tonsillitis	306	12	3,9%

Since urinary tract includes many types of infections, only catheter associated infections were included as they represent the most common type. According to the Central for Disease Control and Prevention, among urinary tract infections (UTI) acquired in the hospital, approximately 75% are associated with a urinary catheter (CDC, 2015).

The infections with the highest ratios are dental plaque/caries and peri-implantitis. Both infections belong to dentistry. Periodontitis also belongs to dentistry and although its ratio is not as high (6,4%), it is still one of the highest in the list. In fact, dentistry has somewhat successfully confronted biofilm disease as it is very common and professionals are able to manage it properly (Wolcott and Ehrlich, 2008). Nevertheless, even considering the method superficial and potentially flawed mainly due to search ambiguity, the ratios between the number of publications and "biofilm" references are surprisingly and worryingly low given the importance that biofilms can apparently have on infection recurrence and virulence. There is seems to exist a contrast between the degree of

association between all these infection types to biofilms, by the scientific/medical community overall and by specialists.

2.5 Systematic review and meta-analysis

A single study may fail to detect a true significant finding and consequently can end in a false negative result, especially if the sample size is small. Larger sample sizes are often not economically or logistically feasible. In addition, there is a massive abundance of published studies each year, with increasingly complexity and heterogeneity between them. However, keeping track of all relevant studies is almost impossible, but crucial to find an answer to a specific clinical problem (Tawfik et al., 2019, Garg et al., 2008).

A narrative review does not involve a systematic literature search, tends to be primarily descriptive, focusing on a subgroup of studies in an area chosen based on availability or author selection based on their point of view. It can also be confusing at times, particularly if similar studies have diverging results and conclusions. Thus, narrative method reviews can often lead to a poor-quality and/or biased review (Tawfik et al., 2019, Uman, 2011).

In contrary, systematic reviews involves a detailed plan, that identifies, combines, and evaluate all available quantitative or qualitative evidence to generate a robust and concrete answer to a focused research question (Mallett et al., 2012). Systematic reviews can include a meta-analysis, which requires using statistical techniques to synthesize the data from several studies into a single quantitative estimate or summary effect size. Traditional hypothesis testing can give us information about statistical significance but not necessarily clinical significance, this is, a sizeable or meaningful clinical data that strengths the relationship between two variables, providing more accurate information (Uman, 2011).

Systematic reviews and meta-analyses have become increasingly important in healthcare settings as they are on top of the quality of evidence publication pyramid (Figure 2). As a matter of fact, they are often used as a baseline for developing clinical practice guidelines (Tawfik et al., 2019, Gopalakrishnan and Ganeshkumar, 2013).

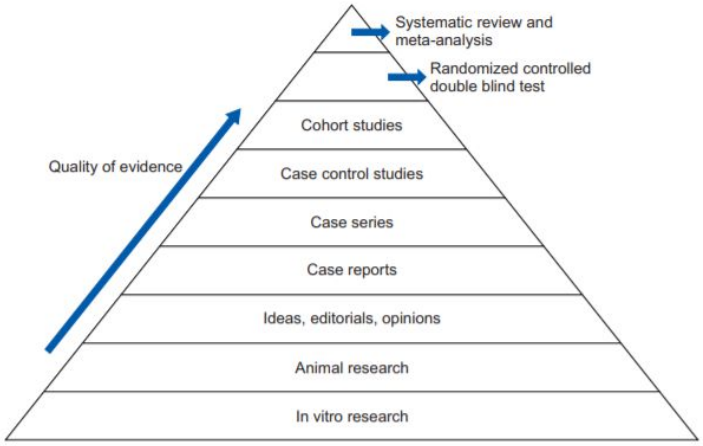

Figure 2 - Quality of publication type evidence pyramid. Retrieved from Tawfik et al. 2019.

When meta-analyses of observational studies are performed, there is an increased risk of biases and higher heterogeneity (clinical and methodologic) comparing to randomised controlled trials meta-analysis. Researchers should carefully consider whether included studies are able to answer the same clinical question, performing a quantitative report through examination of the amount of clinical and methodological heterogeneity and assessment of possible biases (Metelli and Chaimani, 2020).

Publication bias refers to journals publishing studies that are much more likely to report statistically significant results than studies reporting an insignificant or negative conclusion. When addressing a specific clinical question, published papers may report overestimated results in a meta-analysis. Therefore, it is important to address bias not only to assure the integrity of the individual meta-analysis, but also the integrity of the field (Sun et al., 2018, Rothstein et al., 2005).

Chapter 3: Biofilm Impact in Bloodstream Infections – A Systematic Review and Meta-Analysis

3.1 Introduction

Blood is normally a sterile environment and the presence of bacteria (bacteremia) or fungus (fungemia) in blood is defined as bloodstream infection (BSI). Bacteria and fungus can travel through the bloodstream to distant sites in the body, causing hematogenous spread. Thus, BSI can cause or be caused by localized infections such as endocarditis, pneumonia, UTI, meningitis, osteomyelitis, prosthetic infections, and others. Besides, catheter-related BSI is defined as the presence of bacteremia/fungemia originating from an intravenous catheter. It is the most common cause of nosocomial bacteremia and the main complication associated with catheterization (Viscoli, 2016, Franco-Paredes, 2016). BSIs have an estimated overall mortality rate of 15%-30% and was ranked as the 11th leading cause of death in the United States, in 2008 (Hattori et al., 2018, Miniño et al., 2011)

Resistance to antibiotic therapy due to biofilm formation has an important role in development of BSI and since it is present in most of the above mentioned infections and it can easily form after catheter insertion, it may represent a risk factor to patients (Gahlot et al., 2014b). The interest in focalizing on BSI, instead of a specific infection, is in the diagnostic certainty inherent to a positive blood culture. In this chapter, a systematic review and meta-analysis are presented in order to analyse biofilm *in vitro* production prevalence in different clinical outcomes, of isolates from BSI patients in healthcare settings.

3.2 Methods

PRISMA for systematic review protocols (PRISMA-P) served as a main guide for the approach of conducting a systematic review and meta-analysis (Shamseer et al., 2015).

3.2.1 Literature search

A systematic review was carried out by HP in both PubMed and Web of Science databases from January 2005 to May 2020, using a combination of Boolean operators (AND/OR/NOT), MeSH terms, publication types and other terms. Detailed search strategies are provided in Annex A.

3.2.2 Study selection

Papers were evaluated for eligibility, initially based on the title, then on the abstract and finally on the full text. Inclusion and exclusion criteria were predefined and are represented in Table 4.

Table 4 - Eligibility criteria - BSI.

Inclusion criteria
- Observational study and original research - Only Human BSI/bacteremia/fungemia/sepsis clinical isolates - Minimum of 15 clinical isolates (sample size) - Isolates from blood cultures and/or catheter tips - Reports on biofilm *in vitro* production prevalence - Reports on biofilm *in vitro* production prevalence related to clinical outcomes or to resistant vs susceptible strains - Healthcare settings - *In vitro* biofilm production/detection only - Crystal violet/safranin assay and on microtiter/tissue culture plates for biofilm production/detection * - Biofilm formation in 24h * - Results in categorical data (Optical density (OD) cut-offs) - OD cut-offs for negative/positive biofilm production * - Studies published in English, French or Portuguese and from January 1, 2005
Exclusion criteria
- Contaminant isolates - Results in OD mean values

*For biofilm production prevalence only (one-arm study)

Studies published before 2005 were not considered to ensure the focus on contemporary literature. To achieve in the most possible way standardized results, only studies that categorized data into positive/negative biofilm production (BFP), and performed crystal violet/safranin assay on microtiter/tissue culture plates with 24h incubation, were included on the BFP prevalence single-arm analysis (no comparison group). These criteria were left out for BFP association with clinical outcomes or with resistant vs susceptible strains (two-arm analysis) (see subsection 3.2.4).

3.2.3 Data extraction

Papers were retrieved from both databases and duplicates were removed using EndNote (X9.3.3, Clarivate Analytics). Data from eligible studies was extracted to a spreadsheet in Excel (Microsoft Office Excel 2016). The extracted data included first author, publication year, country, study type, bacteria, and sample size. Outcomes were divided into BFP prevalence, BFP in resistant vs susceptible strains, in persistent vs non-persistent BSI, and in survivors vs non-survivors patients. Some studies compared their isolates as high and/or moderate BFP vs low BFP in clinical outcomes or in resistant vs susceptible strains. For two-arm analysis purposes, higher BFPs were considered as the BFP overall outcome. Studies reporting BFP in multiple bacteria, only the group or specie with the largest sample size was included, whenever possible.Data manipulation was occasionally necessary and efforts were made to contact the authors when important data was

missing. In addition, if p-values were not available within the studies, they were calculated (see subsection 3.2.4).

3.2.4 Data analysis

Single-arm meta-analysis was conducted using Open Meta [Analyst] software to determine overall BFP prevalence. The results were presented in proportion values (0 to 1). Two-arm meta-analysis were executed using RevMan software (version 5.4, Cochrane) to determine BFP prevalence associated with resistance, persistence, and mortality. The estimates were presented in odds ratio (OR). For both types of analysis, forest plots were generated using 95% confidence intervals (CI) to assess the significance of the results. When possible (n > 1), sub-group analysis was always undertaken by microorganism group. Statistical heterogeneities were calculated as I^2 values, which were categorized as low (0-50%), moderate (50-75%), or high (>75%). Random-effects model was used to provide more confident data considering heterogeneity within and between reports. Studies were weighted in favor of those with thinner CIs. Publication bias was evaluated using the funnel plot, when number of studies were equal or higher than 10.

Missing p-values from data extraction were calculated as two tailed values in GraphPad website, using a 2x2 contingency table and Fisher´s exact test (GraphPad).

3.3 Results and discussion

3.3.1 Literature search and study selection

The systematic search on PubMed and Web of Science databases generated a total of 367 studies of which 40 were identified as eligible after duplicates removal, title, abstract and full text screening based on inclusion and exclusion criteria (Figure 3).

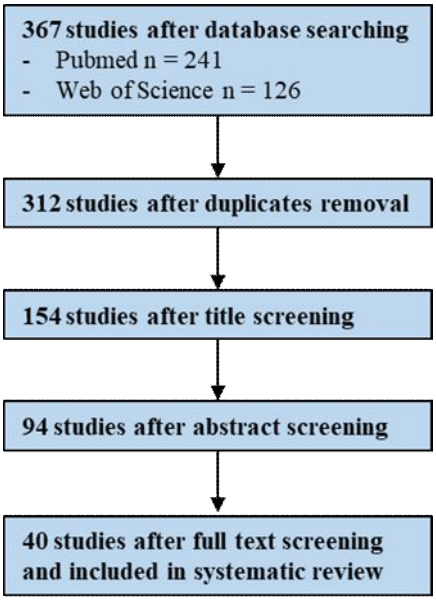

Figure 3 - Flowchart illustrating the study screening process - BSI

3.3.2 Study characteristics

Of the 40 eligible studies, BFP unrelated prevalence data were retrieved from 28 studies, 6 studies for BFP prevalence related to resistance, 5 to persistence and 10 to mortality. Only 1 study had data eligible for all analysis, another single study for BFP unrelated prevalence, related to resistance and mortality, 2 studies for prevalence and mortality and 5 studies shared data for prevalence and resistance (Annex A).

Candida sp. (n = 5), *Candida parapsilosis* (n=1), *Corynebacterium sp*. (n = 1), *Escherichia coli* (n = 4), *Enterococcus faecalis* (n = 1), *Staphylococcus sp.* (n = 4), *Staphylococcus aureus* (n = 4), *Staphylococcus epidermidis* (n = 5), *Staphylococcus haemolyticus* (n = 2) and *Streptococcus sp.* (n = 1) were the pathogens reported in BFP unrelated prevalence analysis. For prevalence related to resistance, the reported pathogens were *Staphylococcus sp.* (n = 1) *S. aureus* (n = 3), *S. epidermidis* (n = 1), and *E. coli* (n = 1). For BFP prevalence related to persistence: *Candida sp*. (n = 3), *Staphylococcus sp.* (n = 1), and *S. aureus* (n = 1). Finally, for prevalence related to mortality: *Candida sp.* (n = 5), C. *parapsilosis* (n=1), Chryseobacterium *meningosepticum* (n = 1), *E. coli* (n = 2), and *S. aureus* (n = 1) (Annex A).

3.3.3 BFP prevalence unrelated meta-analysis (Single-armed)

Combined results from all 28 studies are pooled in a forest plot presented in Figure 4 (proportion 0,59; 95% CI: 0,47-0,71; $p < 0,01$). Sub-groups are presented by group of bacteria. The highest proportion was estimated for other microorganism subgroup (proportion 0,64; 95% CI: 0,29-0,99; $p < 0,01$) and *Staphylococcus sp.* (proportion 0,63; 95% CI: 0,47-0,79; $p < 0,01$).

Candida sp subgroup. estimate was not much lower (proportion 0,57; 95% CI: 0,28-0,86; $p < 0,01$). The lowest estimate proportion is found in *E. coli* subgroup (proportion 0,41; 95% CI: 0,28-0,53; $p < 0,01$). Despite BFP proportion being considerable for all subgroups, estimates and 95% CIs varied deeply, which resulted in very high heterogeneities (all subgroups with $I^2 > 90\%$ and $p < 0,01$), making the meta-analysis unreliable and consequently inconclusive. Since a single-arm analysis does not include a comparison or control group, specific criteria were included towards BFP method to attempt diminishing heterogeneity (see subsection 3.2.2). But in fact, there are many other variables influencing outcomes within the BFP method, and that were not considered as criteria as an effort to obtain a considerable or minimum number of studies. These include culture media, concentrations and time procedures, optical density (OD) values and cut-offs, etc. Besides, the main purposes of most included papers were not to exclusively assess biofilm prevalence but to link it with other factors or outcomes. Moreover, a single-arm analysis is not only as valuable as two-arm, but it also exposes another problematic: the *in vitro* method. They are the most used techniques to detect biofilm capacity but do not accurately represent *in vivo* conditions and are not always demonstrative of the biofilms found in infections (Roberts et al., 2015).

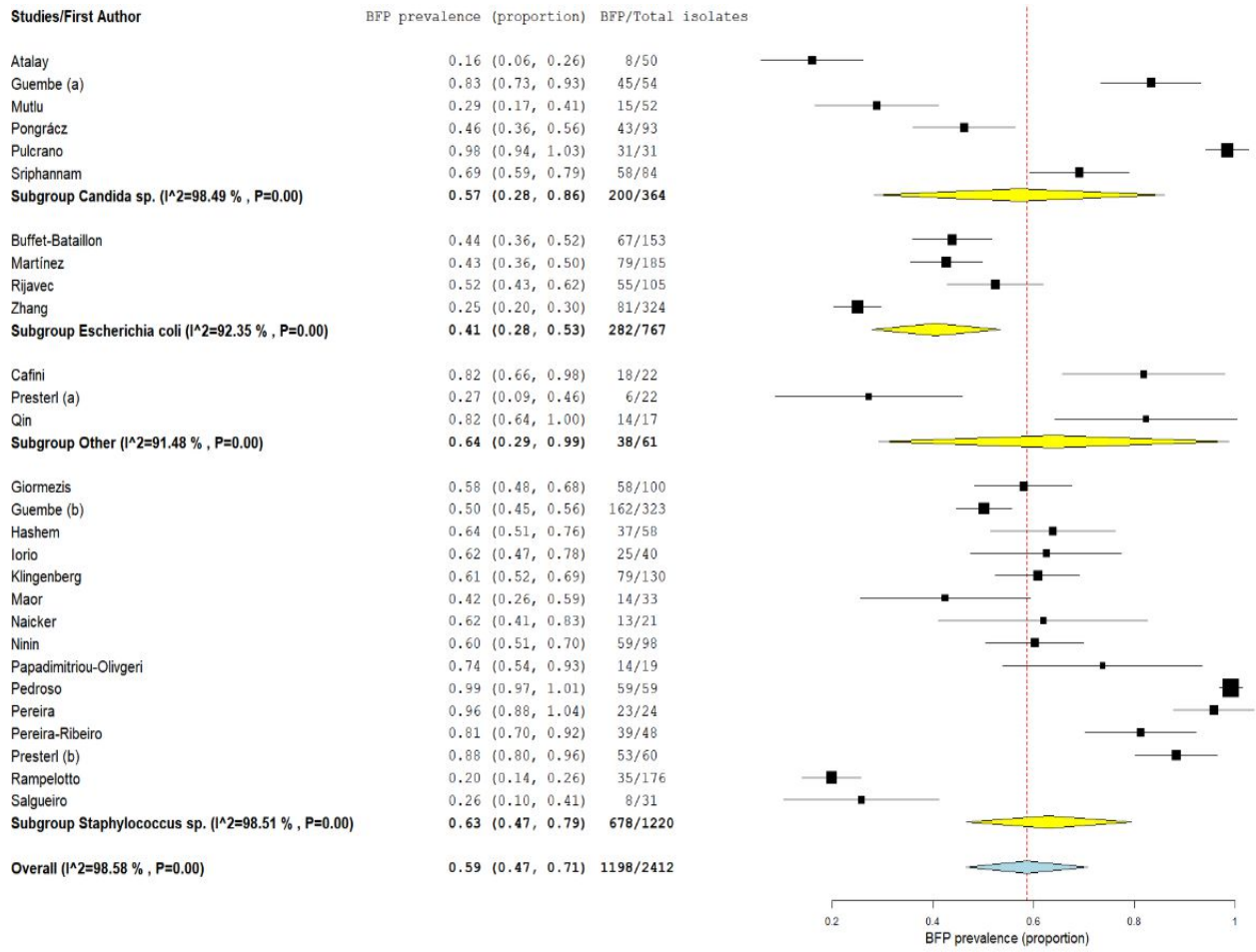

Figure 4 - Forrest plot of BFP prevalence and subgroup analysis by microorganism - BSI

3.3.4 BFP prevalence related to resistance meta-analysis (Two-armed)

Only one paper described high BFP in prevalence (Annex A, Table A.2). A higher BFP prevalence in resistant strains was observed with high statistical significance (OR: 2,82; 95% CI: 1,77-4,49; $p < 0,01$) (Figure 5). It is noticeable an overlap of CIs and OR estimates between all studies. This translates into a low statistical heterogeneity ($I^2 = 47\%$; $p = 0,10$) which is remarkable since there is a sizeable amount of study type heterogeneity (retrospective, retrospective cohort, prospective, etc.), and clinical heterogeneity between studies (i.e. demographics, comorbidities, severity of disease, treatment interventions, outcomes, etc.), as criteria towards those factors was minimal. This indicates that these factors do not seem to significantly affect the influence of biofilm on resistance. Additionally, there was not a single study with an OR < 1, which would indicate higher BFP in susceptible strains. Thus, there is substantial evidence that biofilm may play an important role in strains identified as resistant to antibiotics. Yet, since 5 out of 6 studies reports on methicillin-resistant *Staphylococcus sp.,* this is mainly applicable to that group of bacteria. Methicillin-resistant *S. aureus* (MRSA) is a major human pathogen and a public health problem,

with the ability to acquire resistance to most antibiotics (Klevens et al., 2007). Methicillin-resistant *Staphylococcus epidermidis* (MRSE) has emerged as a causative agent of infections often associated with implanted medical devices (Gill et al., 2005). The biofilm producing capacity of these microbes can very probably be associated to its virulence and resistance factors. The study from Zhang et al. (the 6th included study) reports on extended-spectrum β-lactamases (ESBL) *E. coli* and data shows potentially concerning findings on BFP impact, as it had a high OR, and a comparatively narrow CI (OR: 3,23; 95% CI: 1,88-5,54). Production of ESBLs is an important resistance mechanism that impedes the antimicrobial treatment of infections (Shaikh et al., 2015). Mortality following bacteraemia caused by ESBL producing *E. coli* has already been observed as significantly higher than non-ESBL producing *E. coli* (Melzer and Petersen, 2007). More data would be optimal to provide overall publications bias evaluation, as well as to provide more confidence to assumptions.

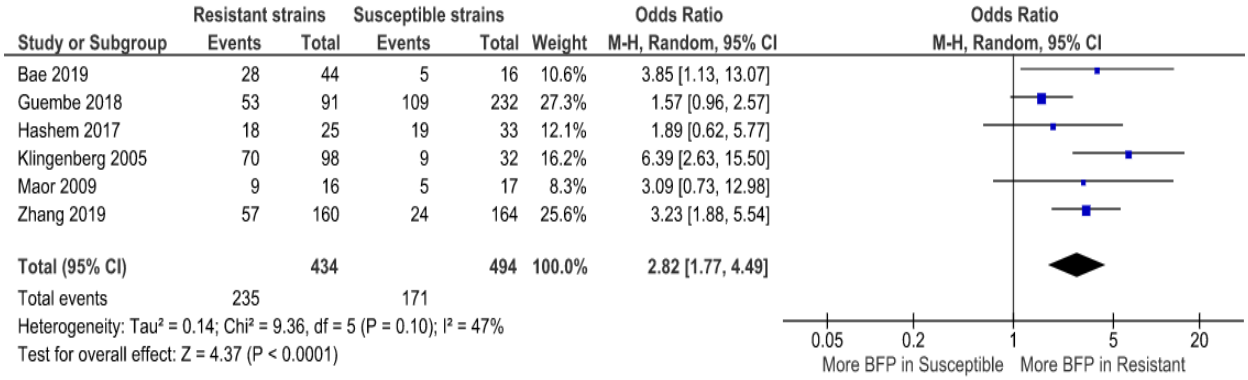

Figure 5 - Forrest plot of BFP prevalence in Resistant vs Susceptible strains - BSI

3.3.5 BFP prevalence related to persistence meta-analysis (Two-armed)

A total of 3 studies were retrospective and 1 was prospective, 2 were cohort and 1 multicenter. High BFP outcomes in prevalence were described in 3 studies, BF-positive (biofilm-positive) in 1 study and moderate/high metabolic activity in another one (Annex A, Table A.3). Overall, there is a high statistical significance indicating that BFP is related to persistent bacteremia/candidemia (OR: 2,88; 95% CI: 1,69-4,93; $p < 0,01$), and statistical heterogeneity was low ($I^2 = 40\%$; $p = 0,15$) (Figure 6). Once again, BFP impact on persistence can possibly be an independent factor from multiple study differences such as to resistance analysis (see subsection 3.3.4). Data from all studies had OR estimates higher than 1. Subgroup analysis wise, there is significantly more BFP production in persistent candidemia (OR: 4,88; 95% CI: 2,64-9,02; $p < 0,01$) than in persistent bacteremia from *Staphylococcus sp.* (OR: 1,94; 95% CI: 1,18-3,17; $p < 0,01$). Within both subgroups, there was no heterogeneity, however, heterogeneity between subgroups was high ($I^2 = 81,0\%$; $p = 0,02$), supporting the idea that *Candida sp.* biofilms can have

greater impact on infection persistence. Nevertheless, there is a substantial lack of studies, especially for subgroups analysis. Publication bias was not assessed.

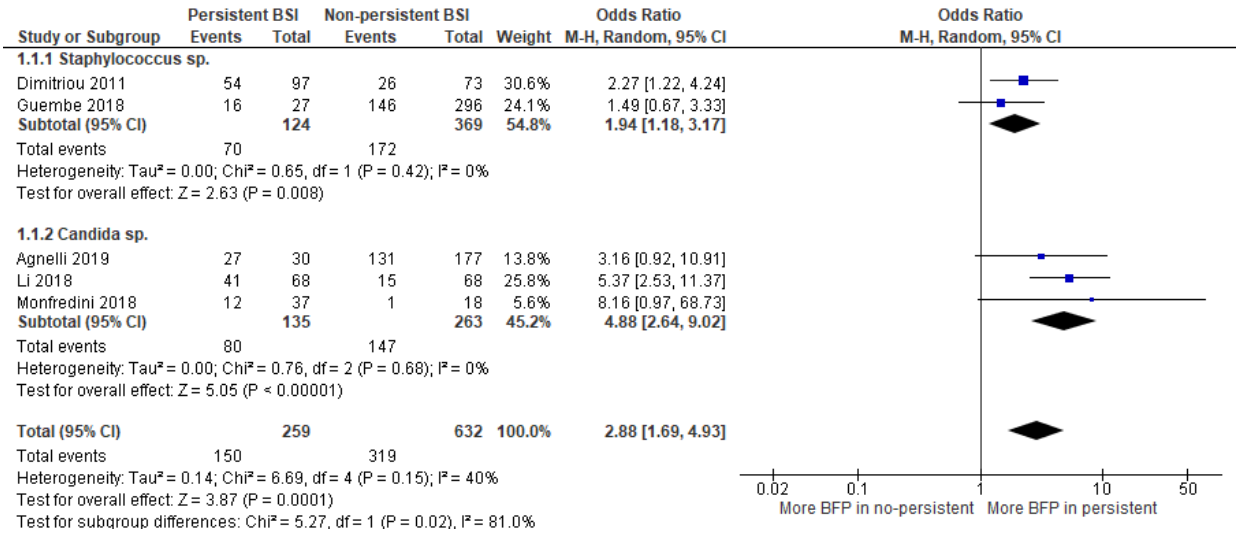

Figure 6 - Forrest plot of BFP prevalence in persistent vs non persistent BSI, and subgroup analysis by microorganism

3.3.6 BFP prevalence related to mortality meta-analysis (Two-armed)

Of the 10 observational studies included, the majority were retrospective (n = 9) and only 1 was prospective. 2 studies in total were cohort. 7 studies described mortality as 30-day mortality (after a defined event such as hospital admission), 1 paper as 14 day-mortality and 2 as in-hospital mortality. 5 studies defined BFP outcome in prevalence as BF-positive, 3 as high and moderate BFP and 2 as high BFP (Annex A, Table A.4). The following meta-analysis was divided into 3 subgroups (Figure 7). *E. coli* subgroup (OR: 1,70; 95% CI: 0,53-5,44; $p < 0,01$) and other bacteria (OR: 2,08; 95% CI: 0,48-9,03; $p < 0,01$), only had available data from 2 studies each. Although OR values were significant, statistical heterogeneity was high and moderate, respectively ($I^2 = 78\%$; $p = 0,03$ and $I^2 = 73\%$; $p = 0,05$). They had one study each where OR values were close to 1, which made estimates too uncertain (large CIs). Hence, these subgroups analysis are rather inconclusive due to insufficient data. However, 10 studies were included in the general meta-analysis (OR: 2,05; 95% CI: 1,53-2,74; p < 0,01). The outcomes highly suggest a significant association between biofilm and mortality from BSI/bacteremia/fungemia complications. Furthermore, high statistical significance indicates that BFP impacts candidemia mortality (OR: 2,05; 95% CI: 1,53-2,74) and there was no OR > 1. In Europe, the incidence of *Candida* BSI ranges from approximately 3 to 8,6 per 100 000 population per year, and 30-day mortality rate is extremely high at 35-40% (Puig-Asensio et al., 2014, Koehler et al., 2019). Overall statistical heterogeneity score was low ($I^2 = 42\%$; $p = 0,08$), though, it can be influenced by the 6 *Candida sp.* studies where heterogeneity was way

lower (I^2 = 17%; p = 0,08). Nonetheless, considering that random effects model tries to limit the influence of heterogeneity, and that 10 studies were included with several significant description differences (study types, mortality and outcomes, (Annex A, Table A.4), as well as with other clinical heterogeneities, these low statistical values are still remarkable. It may reveal that, BFP impact on mortality, aside from microorganism specie, as a very low dependence from those parameters, with treatment intervention included. This is particularly important to highlight because distinct contemporary treatment strategies or procedures do not seem to be tackling the biofilm issue.

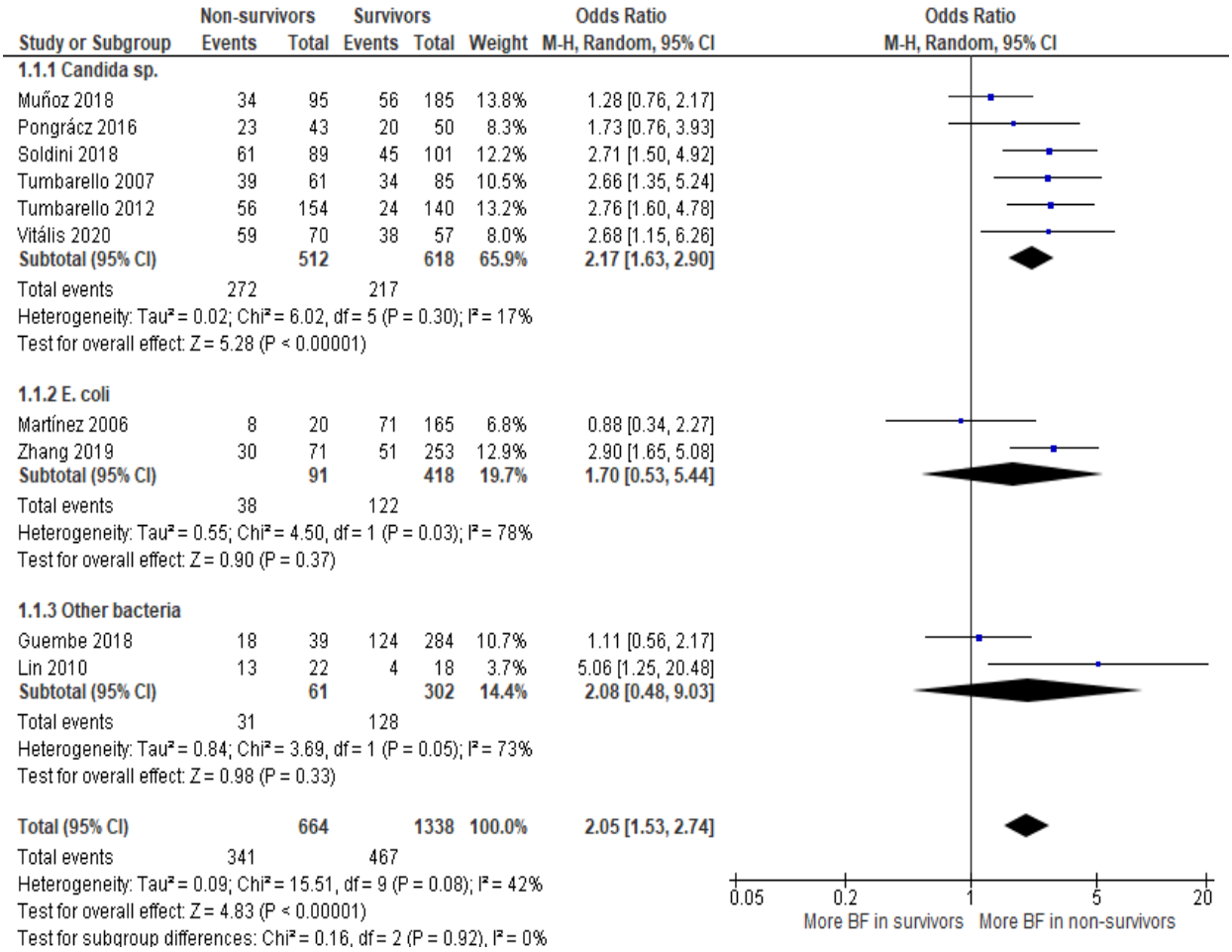

Figure 7 - Forrest plot of BFP prevalence in BSI non-survivors vs survivors, and subgroup analysis by microorganism.

Overall publication bias was evaluated as 10 studies is the minimum recommended number to pool studies outcomes on a funnel plot (Figure 8). When there are fewer studies, the power of the tests is too low to distinguish chance from real asymmetry (Sterne et al., 2008). No major publication bias was detected as an acceptable symmetry is observed. Therefore, the integrity of the meta-analysis is assured.

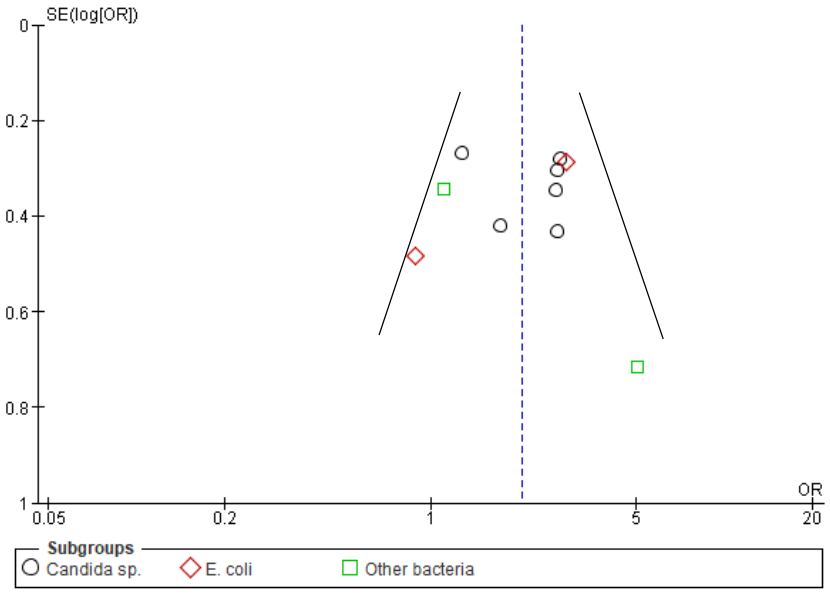

Figure 8 - Funnel plot of standard error by OR.

3.4 Conclusions

BFP prevalence in BSIs results were inconclusive as the single-arm meta-analysis revealed to be inadequate. However, two-arm meta-analysis adequately suggested high evidence of overall microbial species BFP impact on BSI resistance, persistence, and mortality. Regarding sub-groups analysis by microorganism specie, *Staphylococci* BFP had significantly higher prevalence in resistance strains. *Candida* species BFP highly impacted mortality. All other microorganism sub-group analysis showed potential disquieting findings, but there was not sufficient data to properly assume BFP impact. There was also a lack of data to evaluate publication bias, except for BFP impact on mortality whereas no major bias was detected.

In this chapter, the systematic review and meta-analysis demonstrated with multiple layers that biofilms must urgently be acknowledged as a BSI resistance and a virulence factor. A focus on biofilm in BSI treatment research is recommended to more efficiently tackle social and economic burdens caused by BSI, especially by candidemia and MR *Staphylococci* bacteremia.

Chapter 4: Biofilm Impact on Urinary Tract Infections – A Systematic Review and Meta-Analysis

4.1 Introduction

Urinary tract infections (UTIs) are a severe public health issue and are amongst the most common bacterial infection in humans. The societal costs of these infections, including health care costs, are around US$3.5 billion per year in the United States (Flores-Mireles et al., 2015). UTIs consists of many types of infections such as cystitis (bladder infection), pyelonephritis (kidney infection), prostatitis, urethritis, and bacteriuria (Najar et al., 2009). Catheter associated UTIs (CAUTIs) not only represents a major part of UTIs, but it also represents the most common type of nosocomial infection. Approximately 75% of UTIs are associated with a urinary catheter and 15-25% of patients receive urinary catheters during hospitalization (Delcaru et al., 2016, CDC, 2015). Additionally, CAUTIs are related with increased morbidity and mortality (Delcaru et al., 2016).

Bacterial biofilms play an important role in UTIs: uropathogens can form biofilms in the bladder and kidney reducing antibiotic susceptibility, causing this way infection relapse or recurrence. Catheters provides an ideal environment for the attachment and subsequent colonization of uropathogens. At some point of prolonged catheterization, large parts of biofilms or high concentrations of bacteria can detach from the catheter and enter the bladder leading to bacteriuria (Flores-Mireles et al., 2015). In this chapter, a systematic review and meta-analysis was achieved to investigate *in vitro* BFP prevalence in resistant strains and clinical outcomes, of isolates from UTI patients.

4.2 Methods

Methods used were very similar to those in Chapter 3, with some minor modifications.

4.2.1 Literature search

A systematic review was carried out by HP in both PubMed and Web of Science databases from January 2005 to May 2020, using a combination of Boolean operators (AND/OR/NOT), MeSH terms, publication types and other terms. Detailed searches strategies are provided in Annex B.

4.2.2 Study selection

Papers were evaluated for eligibility, initially based on the title, then on the abstract and finally on the full text. Inclusion and exclusion criteria were predefined and are represented in Table 5.

Table 5 - Eligibility criteria - UTI.

Inclusion criteria
- Observational study and original research - Only Human UTI clinical isolates - Minimum of 15 clinical isolates (sample size) - Isolates from urine or catheters - Reports on biofilm *in vitro* production prevalence - Reports on biofilm *in vitro* production prevalence related to clinical outcomes or to resistant vs susceptible strains - Healthcare settings (outpatients and inpatients) - *In vitro* biofilm production/detection only - Crystal violet/safranin assay and on microtiter/tissue culture plates for biofilm production/detection * - Biofilm formation in 24h * - Results in categorical data (OD cut-offs) - OD cut-offs for negative/positive biofilm production * - Studies published in English, French or Portuguese and from January 1, 2005
Exclusion criteria
- Contaminant isolates
- Results in OD mean values

*For biofilm production prevalence only (one-arm study)

Studies published before 2005 were not considered to ensure the focus on contemporary literature. To achieve in the most possible way standardized results, only studies that categorized data into positive/negative BFP, and performed crystal violet/safranin assay on microtiter/tissue culture plates with 24h incubation, were included on the BFP prevalence single-arm analysis (no comparison group). These criteria were left out for BFP association with clinical outcomes or with resistant vs susceptible strains (two-arm analysis) (see subsection 4.2.4).

4.2.3 Data extraction

Papers were retrieved from both databases and duplicates were removed using EndNote (X9.3.3, Clarivate Analytics). Data from eligible studies was extracted to a spreadsheet in Excel (Microsoft Office Excel 2016). The extracted data included first author, publication year, country, study type, bacteria, and sample size. Outcomes were divided into BFP prevalence, BFP in resistant vs susceptible strains, and CAUTI vs UTI non-CA (catheter associated). Some studies compared their isolates as high and/or moderate BFP vs low BFP. For two-arm analysis purposes, higher BFPs were considered as the BFP overall outcome. Studies reporting BFP in multiple bacteria, only data of the group or specie with the largest sample size was recovered, whenever possible. Data manipulation was occasionally necessary, and efforts were made to contact the authors when

important data was missing. In addition, if p-values were not available within the studies, they were calculated (see subsection 4.2.4).

4.2.4 Data analysis

Single-arm meta-analysis was conducted using Open Meta [Analyst] software to determine overall BFP prevalence. The results were presented in proportion values (0 to 1). Two-arm meta-analysis were executed using RevMan software (version 5.4, Cochrane) to determine BFP prevalence associated with resistance, persistence, and mortality. The estimates were presented in odds ratio (OR). For both types of analysis, forest plots were generated using 95% confidence intervals (CI) to assess the significance of the results. When possible (n > 1), sub-group analysis was always undertaken by microorganism group or resistance type. Statistical heterogeneities were calculated as I^2 values, which were categorized as low (0-50%), moderate (50-75%), or high (>75%). Random-effects model was used to provide more confident data considering heterogeneity within and between reports. Studies were weighted in favor of those with thinner CIs. Publication bias was evaluated using the funnel plot, when number of studies were equal or higher than 10.

Missing p-values from data extraction were calculated as two tailed values in Graphpad website, using a 2x2 contingency table and Fisher´s exact test (GraphPad).

4.3 Results and discussion

4.3.1 Literature search and study selection

The systematic search on PubMed and Web of Science databases generated a total of 454 studies of which 27 were identified as eligible after duplicates removal, title, abstract and full text screening based on inclusion and exclusion criteria (Figure 9).

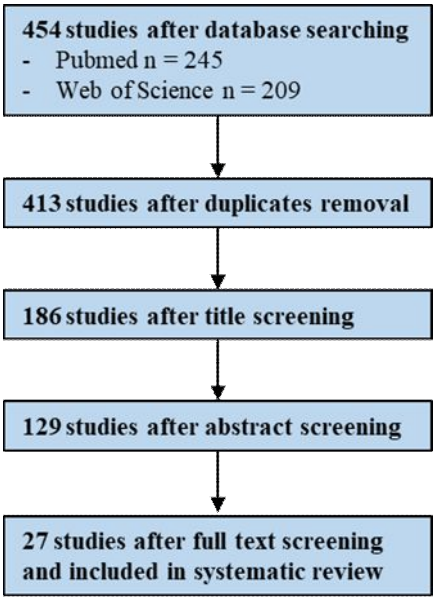

Figure 9 - Flowchart illustrating the study screening process – UTI.

4.3.2 Study characteristics

Of the 27 eligible studies, BFP unrelated prevalence data were retrieved from 16 studies, 13 studies reported for BFP prevalence related to resistance, and 7 to CAUTI. Only 1 study had data eligible for all analysis, another single study for BFP unrelated prevalence and related to CAUTI and a last single study for resistance and CAUTI. 5 studies shared data for prevalence and resistance (Annex B).

E. coli. (n = 9), *Enterococcus sp.* (n = 3), *Klebsiella sp.* (n = 1), MRSA (n = 1), *Proteus sp.* (n = 1), and *Acinetobacter baumanii.* (n = 1) were the pathogens reported in BFP unrelated prevalence analysis. For prevalence related to resistance, the reported pathogens were *E. coli* (n = 10), *Enterococcus sp.* (n = 1), *Klebsiella sp.* (n = 1), and MRSA (n = 1). For BFP prevalence related to CAUTI: *E. coli* (n = 4), *Enterococcus sp.* (n = 1), *E. faecalis* (n = 1), and, Gram-negative *bacilli* & Gram-positive *cocci* (n = 1) (Annex B).

4.3.3 BFP prevalence unrelated and meta-analysis (Single-armed)

Combined data from 16 studies are pooled in the forest plot presented in Figure 10. *Enterococci* subgroup had the lowest proportion estimate (proportion 0,63; 95% CI: 0,41-0,85; $p <$ 0,01), followed by *E. coli* subgroup (proportion 0,82; 95% CI: 0,74-0,89; $p < 0,01$) and by other bacteria species (proportion 0,84; 95% CI: 0,80-0,88; $p < 0,01$). Overall BFP prevalence estimate was significantly higher (proportion 0,79; 95% CI: 0,73-0,85; $p < 0,01$) than the estimate observed in BSI analysis (see subsection 3.3.3). However, statistical heterogeneity was very high ($I^2 = 92\%$; $p < 0,01$) just as it was in BSI meta-analysis. This is once more, mainly due to an absence of a fully standardized *in vitro* BFP method. In addition, the lack of a comparison group along with a

simplistic (compared to *in vivo*) *in vitro* method, leads once again to inconclusive results. Statistical heterogeneity from the latter subgroup (3 studies from different bacterial species) is inexistent (I^2 = 0%; p = 0,47), yet, this was no more than a coincidence.

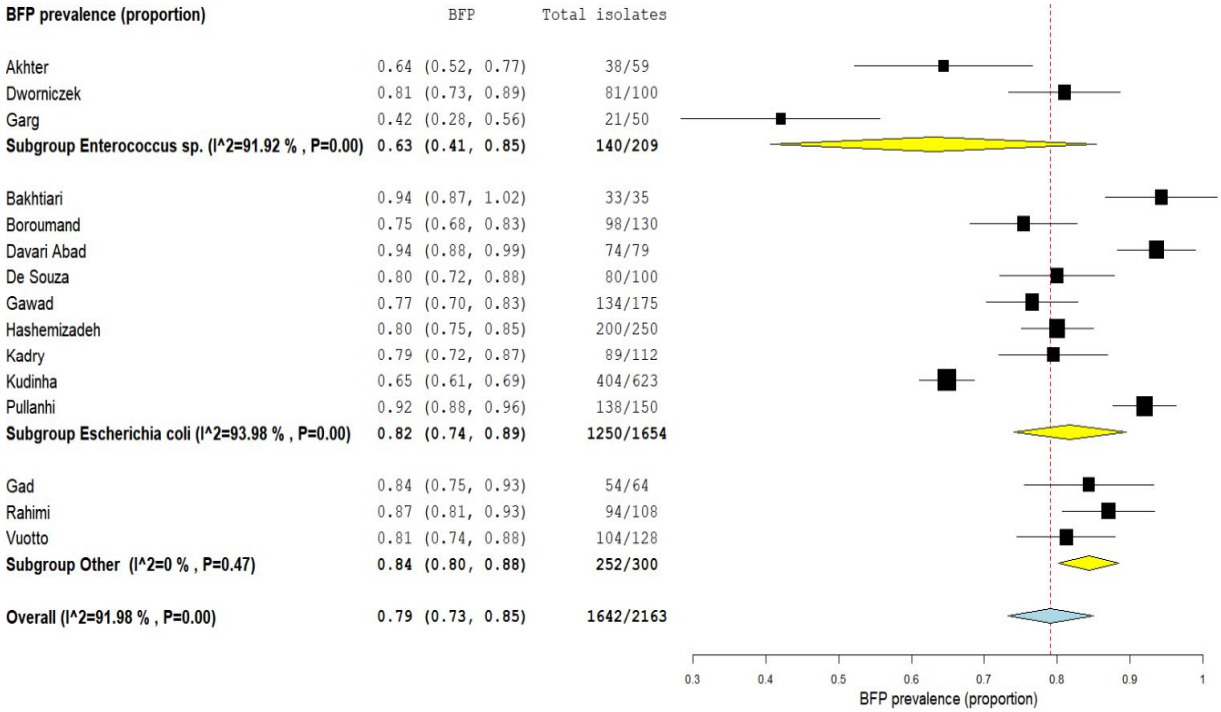

Figure 10 - Forrest plot of BFP prevalence and subgroup analysis by microorganism – UTI

4.3.4 BFP prevalence related to resistance meta-analysis (Two-armed)

One paper only described high/moderate BFP in prevalence (Annex B, Table B.2). With more papers included (n = 13) than in BSI meta-analysis (n = 6), high BFP prevalence in resistant strains was observed with slightly higher statistical significance (OR: 3,18; 95% CI: 1,88-5,39; p < 0,01) (Figure 11). But in contrary to BSI where overall statistical heterogeneity was low, UTI statistical heterogeneity was moderate, almost high (I^2 = 74%; p = 0,10). The reason for this divergence can be the higher number of studies, a higher difference in study designs, and/or superior sensibility to clinical heterogeneities. Multidrug-resistant (MDR) *E. coli* and ESBL *E. coli* had significant more BFP prevalence compared to non-MDR and non-ESBL, respectively (OR: 2,92; 95% CI: 1,30-6,54; p < 0,01 and OR: 2,80; 95% CI: 1,33-5,86; p < 0,01). *E. coli* is by far the most causative UTI specie and there are already suggestions for careful monitoring of antimicrobial use for UTI treatment is necessary (Kot, 2019, Mobley et al., 2009). In this study, it is proven that biofilms can play a major part on the resistance rise of uropathogenic *E. coli* to currently used antimicrobials. Both subgroup heterogeneities are also moderate, and therefore no differentiation between *E. coli* resistance types is detected.

It was observed one study with higher BFP prevalence in non-MDR E. *coli* with some statistical significance (OR: 0,60; 95% CI: 0,21-1,67). Authors argued that biofilms provide secondary protection and that the acquisition of resistance is not linked with biofilms (Kadry et al., 2020). Without ruling out the potential impact of other factors than biofilms, the results from the meta-analysis clearly seems to contradict the authors and, indicate that they are not a secondary protection mechanism.

The OR estimate for the "other" subgroup was extremely high (OR: 4,42; 95% CI: 0,96-20,35; $p < 0,01$). However, this value is leveraged by one study reporting ST131 *E. coli* vs non ST131 *E. coli* as the estimate (OR: 15,91; 95% CI: 6,88-36,78) and sample size (n = 623) were enormous (Kudinha et al., 2013). Although no more studies reported ST131, BFP was strongly associated with it. ST131 is the predominant *E. coli* clonal group among extraintestinal pathogenic *E. coli* isolates worldwide. They are frequently reported to produce ESBLs, and almost all are resistant to fluoroquinolones. Due to the spectrum of infections they cause, and the large number of virulence-associated genes they contain, ST131 *E. coli* isolates are considered to be truly pathogenic (Nicolas-Chanoine et al., 2014). In fact, in 2007, the clonal group was reported to probably cause the most significantly antimicrobial-resistant *E. coli* infections in the United States (Johnson et al., 2010).

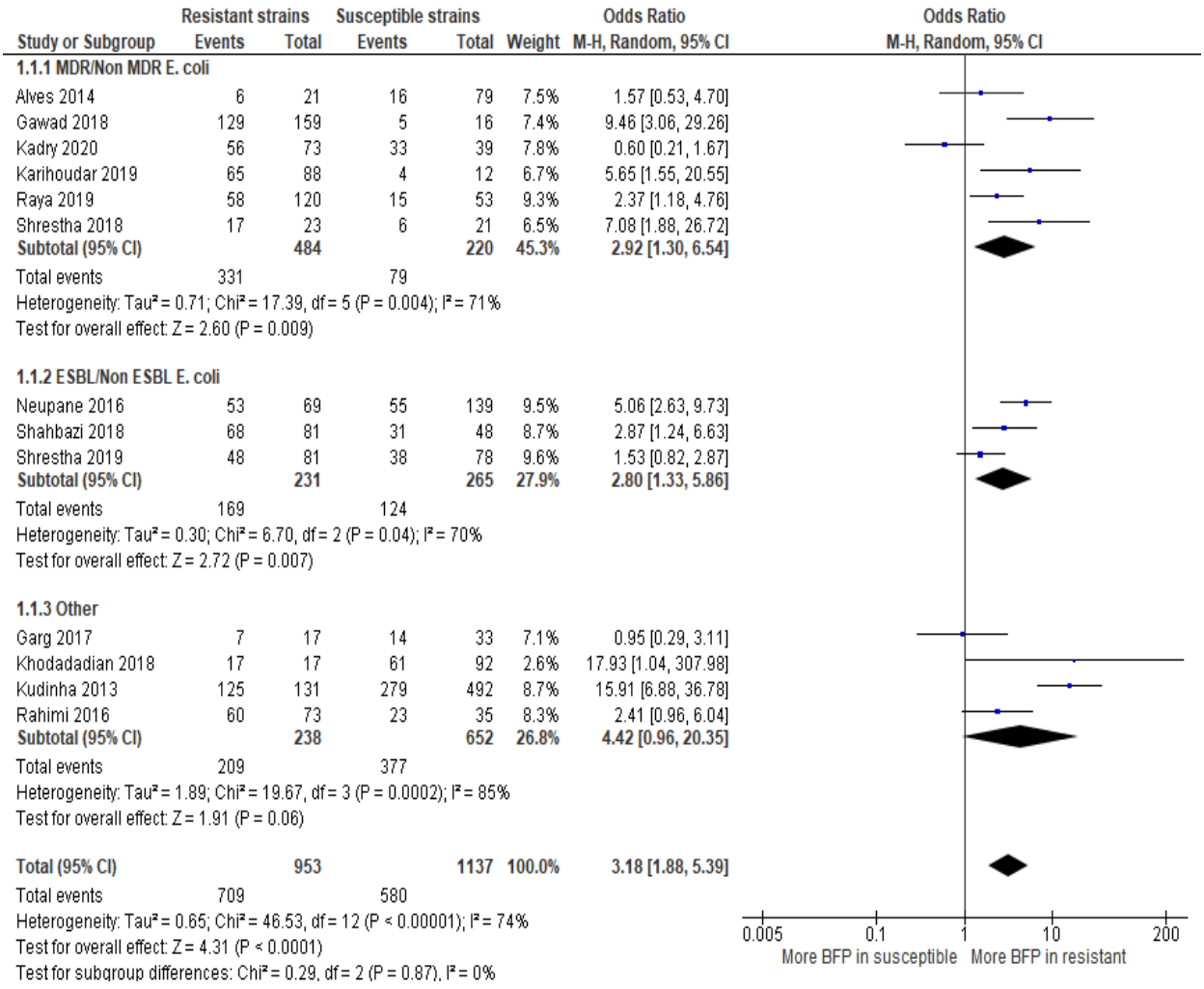

Figure 11 - Forrest plot of BFP prevalence in Resistant vs Susceptible strains – UTI

Overall publication bias was assessed, and the funnel plot is presented on Figure 12. A relative balanced symmetry is observed and thereby, bias is undetected, assuring the integrity of the meta-analysis.

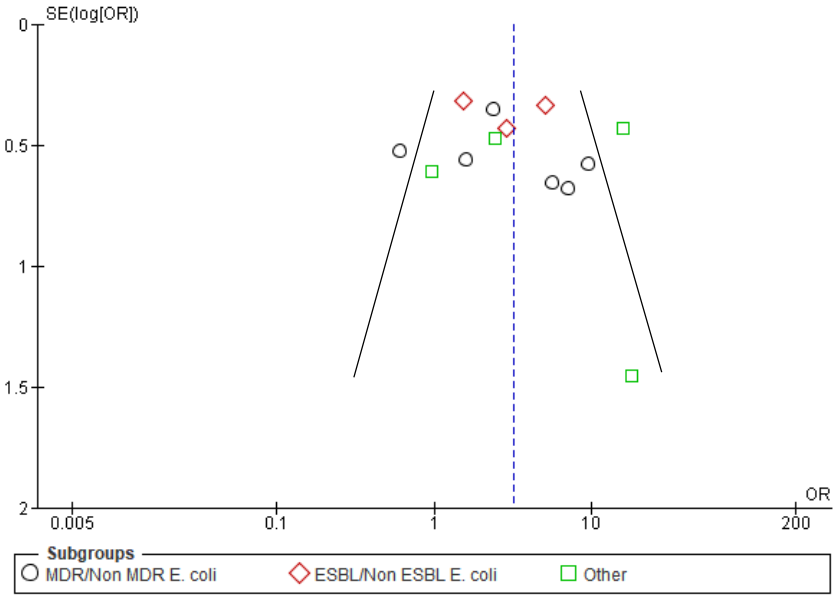

Figure 12 - Funnel plot of standard error by OR - UTI.

4.3.5 BFP prevalence related to CAUTI meta-analysis (Two-armed)

For BFP prevalence in CAUTI vs UTI non-CA, the meta-analysis was divided into 2 subgroups by bacteria specie, which left a study reporting BFP prevalence on Gram-negative *Bacilli* and Gram-positive *Cocci* (Figure 13). OR estimate from *Enterococci* subgroup indicates a slight prevalence of BFP in CAUTI, but there was not statistical significance to confidently confirm (OR: 1,20; 95% CI: 0,26-5,57; p = 0,81). However, only 2 studies were included. While one of them had a value under 1 OR, the other was too uncertain (large CI). *E. coli* subgroup included 4 studies, still, findings were not much different. Even though OR estimate was significantly higher, the CI is stretched towards < 1 OR, due to 2 reports with securely more BFP in UTI non-CA. The lone study has a sizeable sample (n = 471) and a confident estimate towards higher BFP in CAUTI. Consequently, it slightly weighted overall estimate into more prevalence in CAUTI, yet, confidence interval was not ideal (OR: 2,61; 95% CI: 0,67-10,17; p = 0,17).

As already mentioned in previously, CAUTI is the most common nosocomial infection type and, biofilms are long acknowledged as an important attributable cause (Trautner and Darouiche, 2004). It could be argued that findings were not expected to be this uncertain. However, the topology and characteristics of urinary catheters, the accumulation in the surface of fibrinogen following an immune response induced by catheterization, and the constant supply of nutrients from urine stream, favours an easy colonization of bacterial biofilms (Pelling et al., 2019). Thus, bacteria with less capacity to form *in vitro* biofilm, may be slightly more able to form on inserted catheters

and so, reducing the gap towards strong biofilm producers. Therefore, a non-statistically significant higher BFP prevalence in CAUTI is comprehensible.

Overall and *E. coli* statistical heterogeneities were high (I^2 = 91%; p < 0,01 and I^2 = 90%; p < 0,01; respectively). Since heterogeneities were not strongly observed in all BSI double-armed meta-analysis, it can possibly be largely due to clinical heterogeneities. As a matter of fact, UTIs comprise many women and men distinct infections, and in different locations of the tract. With such variations within study designs, it is normal to find various outcomes.

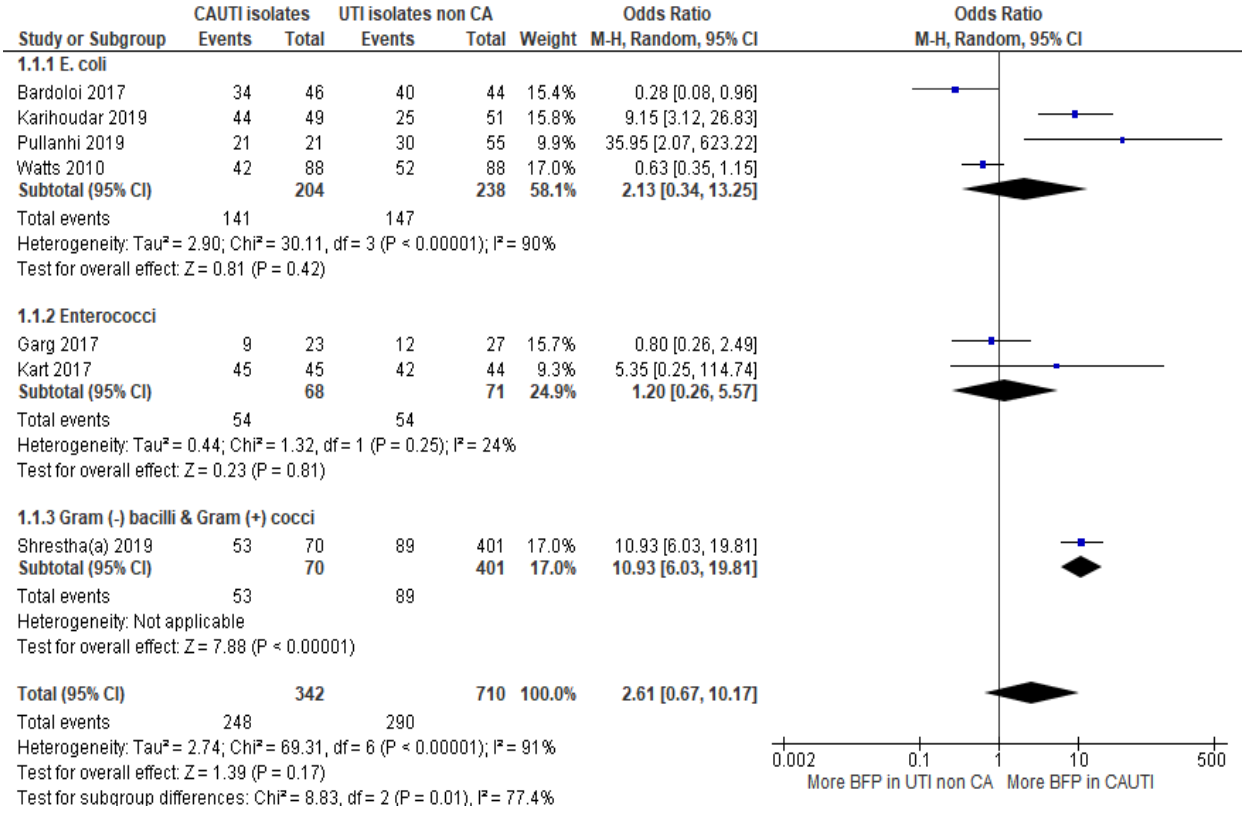

Figure 13 - Forrest plot of BFP prevalence in CAUTI vs UTI non-CA - UTI

4.4 Conclusions

BFP was substantially related to resistance, especially for ESBL and MDR *E. coli*, the main uropathogen. ST131 clonal group showed very worrying BFP prevalence despite data retrieved from only 1 study. Publication bias was not detected. On the other hand, BFP was not statistically significant related to CAUTI. A hypothesis given was that isolates with less capability to normally form biofilms, will more easily establish in the optimal environment of inserted catheters. Generally, higher heterogeneities were observed comparing to BSI study, possibly due to higher discrepancies in clinical characteristics inherent to UTIs.

In this chapter, the systematic review and meta-analysis demonstrated that biofilms seem responsible for resistance acquisition and clearly provides *E. coli* (the most responsible bacteria for UTI) resistance against antimicrobials. It is emphasized a necessity in thorough choice and monitoring of treatment strategies and development of new strategies. Although BFP was not statistically related to CAUTI, it unquestionably should not distract the current focus on the search for new prevention and anti-biofilm strategies against biofilm formation on catheters. Due to insufficient/absent eligible data, it was not possible to assess a relationship with other clinical outcomes such as persistent UTI and mortality.

Chapter 5: New Approaches to Counteract Recurrent and Persistent Biofilm Infections

5.1 Short overview about biofilm eradication compounds

The main mechanisms involved in recalcitrance of biofilms toward antimicrobials are multifactorial and complex (detailed description in chapter 2). Understanding biofilm processes is central to the development of effective strategies to eradicate and/or control BAI (Azeredo et al., 2017). To date there are none identified drugs directed to treat BAI. In addition, the uncontrolled and inappropriate use of antibiotics contributes to the emergence of multi-resistant bacteria. Pharmaceutical corporations are not prioritizing the discovery or invention of new antimicrobials, so in the last decade, only six antibiotics have been approved and commercialized (Borges et al., 2015). Furthermore, it is expected that in the coming years, serious public health issues will arise if there is no drastic change in the use and development of new antibiotics (Stanton, 2013). The need for new molecules and approach strategies in the treatment of BAI is urgent.

Although there is an emergence of biofilm prevention strategies to minimize BAIs impact, it is almost impossible to totally prevent formation, in a multitude of specific characteristics inherent to a vast type of BAIs.

Low metabolic activity, target inactivity, high efflux and low uptake of antibiotics are responsible for persisters high rate of survival. When aiming for killing persisters there two main different approaches: Killing persisters by re-sensitizing them to traditional antibiotics is a strategy being currently explored. Some compounds force growth or metabolic activity re-initiation exposing them to antibiotics. Saccharides and brominated furonones have been shown to activate persisters. Otherwise, using agents that do not require a metabolically active target to kill bacteria is another strategy that is being investigated (Fisher et al., 2017).

In this way, some classes of molecules presented below aims specially to face the persistence or recurrence of biofilms by targeting persisters and are named biofilm eradication agents.

5.1.1 Phenazines & Quinolines

Phenazines are redox-active secondary metabolites produced naturally by many bacterial species. Quinolines are heterocyclic aromatic compounds which hold some structural resemblance to phenazines. Many studies have reported impressive eradication activity and low cytotoxicity but appears to be limited to some Gram-positive pathogens. No *in vivo* analyses have been conducted,

yet, they are very promising in the development of next generation anti-biofilm therapeutics. (Verderosa et al., 2019b, Huigens, 2018)

5.1.2 Nitroxide functionalized antibiotics

Nitroxides are free radical species that contain a disubstituted nitrogen atom coupled to a univalent oxygen atom. They can be linked to other antimicrobials and are highly powerful, have low mammalian cytotoxicity, display a broad spectrum of activity, and are based on the structure of a well-established class of antibiotics (fluoroquinolones). (Verderosa et al., 2019b, Verderosa et al., 2019a)

5.1.3 Synthetic rhetinoids

Synthetic rhetinoids are a recent discovery of a new class of potential antibiotics with special anti-persister activity by disrupting lipid bilayers. The major obstacle for developing retinoids as therapeutic agents is their potential cytotoxicity. (Fauvart et al., 2018, Kim et al., 2018b)

5.1.4 Anti-cancerous drugs

Drug repurposing strategies are less timely and economically resourceful as Food and Drug Administration (FDA) approved drugs are repurposed to treat infections. Moreover, it has been demonstrated that MDR bacteria resistance is rarely crossed, usually because the target site is different from antibiotic target site. As surprising as it may seem, there are several similarities between cancer cells/tumors and bacterial infections, for instance drug-tolerant persisters also occur in cancer cell populations and are implicated in the recurrence of tumours. Many anti-cancerous drugs have already been shown as promising activity against persisters. However, the need for higher concentrations can be a limitation. (WC Soo et al., 2017, Le et al., 2020, Pacios et al., 2020)

5.1.5 Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are a large class of broad-spectrum bactericidal agents and have been used for a long time. Recently, it has been demonstrated as potent eradicators of pre-established bacterial biofilms by mimicking physic-chemical characteristics of antimicrobial peptides (AMPs). QACs structures are considerably less complicated and smaller than AMPs and so, there is a potential to modify their core structures to enhance activity. On the other hand, their toxicity is a major obstacle (Verderosa et al., 2019b, Jennings et al., 2014).

5.1.6 Peptides

Natural antimicrobial peptides (AMPs) have attracted considerable interest as a new class of anti-biofilm drugs for their versatility as they can be involved in all biofilm phases. AMPs may

inhibit the accumulation of bacteria on the surface by interacting with their adhesion proteins, can display an action against bacteria in their active state in the biofilm, and more importantly, they can also defeat persisters and dormant cells. They also interfere with EPS synthesis, signalling compounds, extracellular DNA, and proteins. Additionally, they do not show tendency to develop antimicrobial resistance (Galdiero et al., 2019, Batoni et al., 2011)

5.2 Proposal of a new strategic approach – combination of phytochemicals

Plants have been used for thousands of years for medicinal purposes and have the ability to produce a giant variety of secondary metabolites, named phytochemicals, many of which play a fundamental defence role that has evolved over millions of years to confer a selective advantage against various microorganisms, insects, nematodes and even other plants (Borges et al., 2015). Solid evidence has been demonstrated that plants are an excellent sustainable source (Green Status) to provide abundant natural compounds for the development of preventive and therapeutic agents against BAI (Borges et al., 2013). More than 90 phytochemicals and extracts from different parts of plants have been identified with anti-biofilm action. The mechanisms of some of these phytochemicals have been investigated, but most of these studies still represent very early stages in the development of new drugs (Song et al., 2018). In fact, phytochemicals exhibit incomparable structural diversity with various, complex and new mechanisms of action (Borges et al., 2015). It is recognized that the use of a single molecule capable of operating simultaneously on multiple targets can more advantageous for the treatment of infectious and complex diseases (Ramsay et al., 2018). It is theoretically more challenging for the pathogen to develop resistance when the active molecule has activity against multiple targets (Jayaraman et al., 2013). Therefore, the multifaceted mode of action of phytochemicals can probably impair the ability of pathogens to build resistance. Supporting this theory even more, there is no evidence about an emergence of bacterial resistance to phytochemicals (Borges et al., 2015). They can also be used as resistance modifying agents (RMAs), reducing the spread of antibacterial resistance and allowing the reuse of less effective, cheaper, and safer antibiotics (Abreu et al., 2012).

5.2.1 A case study

It is widely assumed that the actions of antioxidant phytochemicals do not explain the health benefits observed from diets rich in fruits and vegetables: the individual antioxidants studied in clinical trials do not appear to have consistent beneficial effects (Liu, 2004).

An analogy can be made towards antibacterial protection against infections and diseases as many secondary metabolites are released together. Thus, phytochemical combinations can have way

more potent antibacterial capacity, than acting alone. In fact, a vastly number of various molecules always act together and at low concentrations, in the self-protection mechanism of plants which can enhance a series of unknown synergistic and additive effects that can lead them to gain a competitive advantage. For instance, extracts and essential oils consist of several phytochemicals and are recognized as very effective antimicrobials with great potential for, in the recent future, to be used and marketed as preservatives in the food industry (Pandey et al., 2017).

The classical susceptibility tests usually used in clinic do not contemplate the bacteria in sessile state and consequently the treatment of some infection is particularly difficult to achieve. As the complete eradication of BAI based on conventional therapies is hard, they can become recurrent. One of the reasons is the altered microenvironment generated within the biofilm, leading to areas where the cells are in a dormant state. Taking into account that the effects of the majority of antimicrobial agents depends on the bacterial active metabolism and growth, this sessile population is unaffected. Therefore, it is possible to hypothesize that after the treatment phases, the cells that have resisted can recover and start to multiply, restoring the population. However, this occurrence continues to be overlooked and the dormant bacterial layers that may have an important role in biofilm regrowth are not considered.

In this way, the aim of this case study is the rational development of new formulations based in a mixture of plant natural compounds (phytochemicals). For these studies, cinnamic acid (CIN) was selected for their known anti-biofilm properties. Citronellic acid (CITR) was the other selected phytochemical. Firstly, their activity on the eradication of 24 h *E. coli* biofilms was evaluated alone and combined. Additionally, after phytochemicals exposure, the initial condition was restored and the ability of the *E. coli* biofilms to regrow was monitored to test their long-term biofilm control aptitude. CIN was never tested in this way. It was not possible to present combined results due to invalid/incoherent results.

5.3 Materials and methods

5.3.1 Bacterial strain and culture conditions

The bacterial specie *E. coli* CECT434 used in this work was obtained from the Spanish Type Culture Collection (CECT). A preculture was kept at 4ºC in a petri dish with the medium plate count agar (PCA) and working cultures were grown overnight (16-18 h) in batch culture by subculturing those cells in a 250 ml flask with 100 ml Muller-Hinton broth (MHB; Oxoid, England), through incubation at 37 ± 2°C and 160 rpm.

Phytochemicals

Citronellic acid (CITR) was supplied by Sigma-Aldrich. It is a carboxylic acid of the class of acyclic monoterpenes and is the component mostly isolated from the oil of the leaves of *Pelargonium* species (PubChem). Cinnamic acid (CIN) was obtained from Merck Kgaa and is a monocarboxylic acid that can be found in *Cinnamomum cassia* (cinnamon) (PubChem). Phytochemicals stocks, successive dilutions or solutions were prepared under sterile conditions in the dark with dimethyl sulfoxide (DMSO), a powerful organic solvent for therapeutic agents.

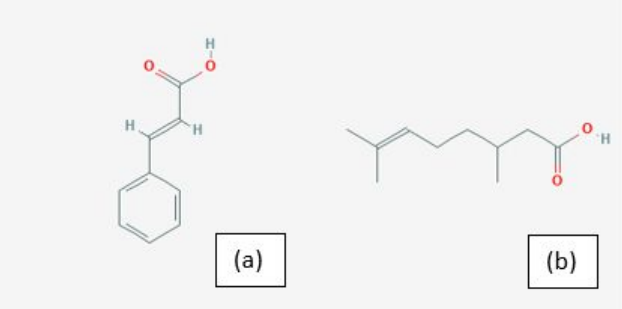

Figure 14 - Chemical structure of CIN (a) and CITR (b) retrieved from PubChem

5.3.2 Antimicrobial susceptibility assay

The minimum inhibitory concentration (MIC) of CIN was obtained from Malheiro et al., and the MIC of CITR was determined according to the CLSI guidelines, by the broth microdilution method in 96 wells plates with compound concentrations ranging from 6,25 to 1000 µg/mL. For this, an overnight bacterial culture was adjusted with fresh MHB to an OD (λ = 600 nm) of 0,132 ± 0,02. The negative controls were 200 µL of MHB, 200 µL of bacterial suspension, and 180 µL of bacterial suspension with 20 µL of DMSO. Remaining wells were filled with 180 µL of bacterial suspension (90% v/v) and 20 µL of compound (10% v/v) at each concentration (n = 6). Microplates were then incubated for 24 h at 37ºC and 160 rpm and the absorbance (λ = 600 nm) was read in a plate reader (SPECTROstar Nano, BMG-Labtech, Veldzigt-Netherlands) to analyse bacterial growth, prior and after incubation period. The MIC is the lowest concentration that inhibits bacterial growth, which is when OD after 24 h is equal or smaller than OD prior to incubation. To precisely confirm MIC value, a second assay was performed with concentrations ranging from 590 to 800 µg/mL.

5.3.3 Biofilm studies

For the biofilm studies three main approaches were applied: individualized tests for both CIN and CITR and another with the mixture of these compounds (MIX). For each compound, 3 concentrations were tested, MIC, 5 × MIC and 10 × MIC. For the MIX assay, these concentrations

of each compound were added together. In the first phase, *E. coli* biofilms were formed in 96 well microplates (24 h) (5.3.4). Then, the biofilms were exposed to the compound(s) at the specified concentrations (5.3.5). At the end of the exposure time (t24), 3 microplates were analysed and 1 microplate was used per assay, in order to analyse the compound activity on the biofilms: quantification of total biomass (5.3.6); quantification of metabolic activity (5.3.7); quantification of CFUs (colony forming units) (5.3.8). For the remaining 3 microplates, the microplate content was carefully removed, and fresh medium was added to allow the cells that survived to regrow. Finally, the same methods were repeated, but to assess the biofilm ability to regrow (t48). In figure 15, the methodology is outlined:

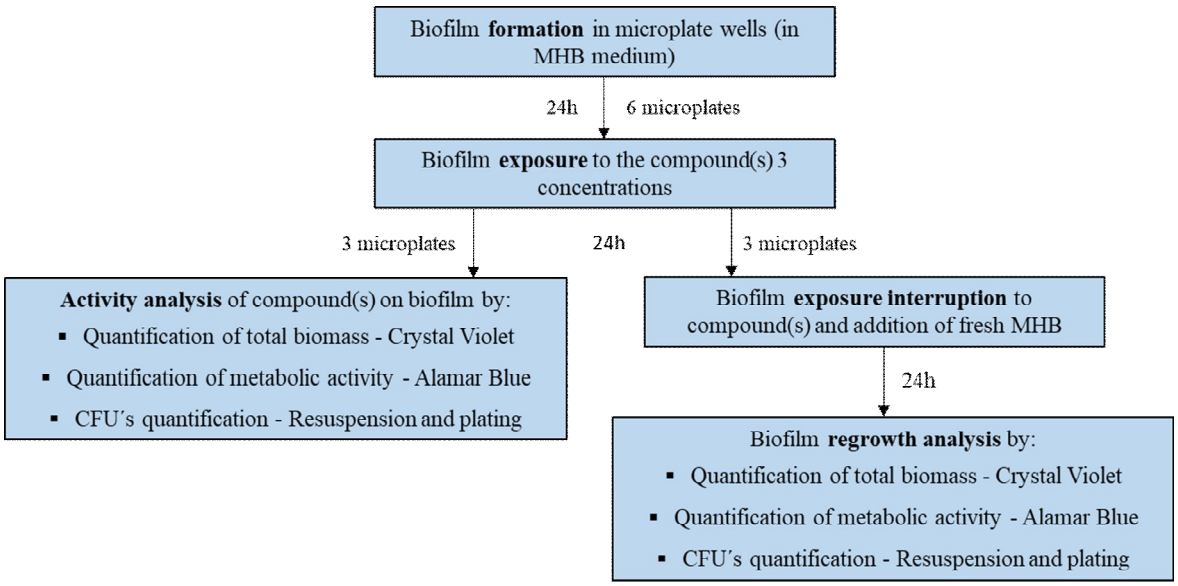

Figure 15 - Schematized methodology for biofilm studies.

5.3.4 Biofilm formation

To form biofilm in 96-wells flat-bottomed polystyrene tissue culture microtiter plates, bacterial cells were grown in batch culture with 100 ml of MHB at 37±2°C and 160 rpm. After growth, cultures OD at 620 nm was adjusted to 0.04 ± 0.02 with fresh medium. Negative control wells contain only MHB without bacterial cells and 180 µL of bacterial suspension with 20 µL of DMSO. Remaining wells contain only cells, 200 µL of bacterial suspension. After filling up the wells, microplates were covered and incubated for 24 h at 37 ± 2°C and 160 rpm.

5.3.5 Biofilm exposure to phytochemical(s) and subsequent regrowth

The content of each well was aspirated and washed with 200 µL sterile saline solution (0,85%) to remove non-adherent cells. Negative control contained only 200 µL of MHB and 180 µL MHB with 20 µL DMSO. Both phytochemical, in separate, were added at MIC, 5 × MIC and 10 ×

MIC in 20 µL with 180 µL MHB. The mixture of both was done with the same concentration but with 10 µL each, making up 200 µL total volume with 180 µL MHB. Microplates were covered and incubated for 24 h at 37 ± 2°C and 160 rpm. At this point, half of the plates were analyzed by 3 different assays. For the remaining plates, regrowth conditions were restored by adding fresh medium followed by an additional 24 h incubation period to finally assess the biofilm regrowth by the same assays.

5.3.6 Total biomass quantification

The biofilm total biomass quantification was done with crystal violet staining (n = 12). Upon 24 h of exposure to the compound(s) (t24) and after regrowth in fresh MHB (t48), the plate was turned upside down and the content of each well was removed with five hits. Then it was washed with 200 µL of sterile saline solution and the content was discarded by the same method. Remaining bacteria were fixed with 250 µL of 99% (v/v) ethanol for 15 min. Plates were again emptied and fixed bacteria were stained with 200 µL of 5% (v/v) crystal violet solution for 5 min. The content was discarded once more with five hits and the remaining stain was gently withdrawn. The dye bound to adherent biomass was resolubilized with 200 µL of 33% (v/v) glacial acetic acid. Finally, microplates absorbance was measured at 570 nm in a plate reader (Spectrostar).

With the OD values it was possible to obtain the percentage of biomass removal when exposed to phytochemicals (t24), according to Eq (1):

$$\%B_R = \frac{OD_{DMSO} - OD_{phy}}{OD_{DMSO}} \, x \, 100 \quad (1)$$

With $\%B_R$ as the percentage of biomass removal, OD_{DMSO} as optical density (570 nm) of biofilm exposed to DMSO and OD_{phy} as optical density (570 nm) of biofilm exposed to phytochemicals. After regrowth (t48) without the phytochemicals, it is calculated the percentage of biomass removal, according to Eq (2):

$$\%B_{RGI} - (1 - \frac{OD_{phy(t48)} - mean\ OD_{phy(t24)}}{mean\ OD_{DMSO(t48)} \quad mean\ OD_{phy(t24)}}) \, x \, 100 \quad (2)$$

$\%B_{RGI}$ represents the percentage of biomass regrowth inhibition (BRI), $OD_{phy(t48)}$ the optical density (570nm) of the regrowth after previous exposure to phytochemicals, $mean\ OD_{phy(t24)}$ the optical density (570 nm) mean of the biomass at the end of the exposure, and $mean\ OD_{DMSO(t48)}$ the optical density (570 nm) mean of the regrowth after previous exposure to DMSO.

5.3.7 Quantification of metabolic activity

The method used to assess biofilm metabolic activity was the alamar blue assay (n = 12). Upon 24 h of exposure to the compound(s) (t24) and after regrowth in fresh MHB (t48), the content of each well was removed and washed with 200 µL of sterile saline solution. For the staining procedure, 190 µL of fresh MHB were added to each well and then 10 µL of alamar blue solution (0,4 mM prepared with sterile distillate water) were applied with minimum exposure to light. Afterwards microplates were incubated for 20 min in darkness at 37 ± 2°C and 160 rpm. Following incubation, fluorescence at λexcitation = 570 ± 10 nm and λemission = 590 ± 10 nm with a microplate reader (Fluorstar). The percentage of biofilm metabolic activity inhibition after exposure to phytochemical (s) as well as after regrowth, was calculated according to Eq (3):

$$\%BF_{MAI} = \frac{F_{DMSO} - F_{phy}}{F_{DMSO}} \, x \, 100 \tag{3}$$

With $\%BF_{MAI}$ as the percentage of biofilm metabolic activity inhibition (MAI), F_{DMSO} as fluorescence intensity of biofilm exposed to DMSO and F_{phy} as fluorescence intensity of biofilm exposed to compounds and mixture.

5.3.8 Biofilm cultivable cells quantification (CFUs quantification)

Biofilm sessile cells culturability in solid medium (Plate Count Agar, PCA) was examined upon 24h of exposure to the compounds (n = 2). The content of each well was removed, 200 µL of sterile saline solution were added and the biofilm was scraped during 1 min to resuspend the attached sessile cells. The content was transferred to 1,5 mL Eppendorf. This procedure was done 3 times for each selected well resulting in 3 min of total scraping and 600 µL of saline solution with cells filling the Eppendorf. The first Eppendorf contained already 400 µL of sterile saline solution. To guarantee the determination of the number of biofilm cells, ten-fold serial dilutions in saline solution were performed. After obtaining the dilutions, 10 µL of each were plated in PCA plates following incubation at 37ºC for a minimum of 15 h. Each dilution plating was done in duplicate. Afterwards incubation time, the number of colony forming units (CFU) was visually counted with detection limits from 10 < CFU < 100 and expressed per square centimeter of the plates well (CFU/cm^2), according to Eq (4) and Eq (5):

$$\frac{CFU}{mL} = \frac{C}{SV \, x \, D} \tag{4}$$

With C as the number of CFU in solid medium, SV as sample volume in mL and D as dilution factor.

$$\frac{CFU}{cm^2} = \frac{\frac{CFU}{mL} x\ WV}{1{,}53} \quad (5)$$

With WV as the working volume in the well (0,2 mL) and 1,53 as the well area in cm^2. A logarithmic was applied to the $\frac{CFU}{cm^2}$ and results obtained were expressed as logarithmic reductions of $\frac{CFU}{cm^2}$.

5.3.9 Combinatorial Index

To evaluate the combined effect of CIN and CITR apart from comparing its results individually, their interaction was also classified due to a ∑ Combinatorial Index (∑ CI). The combinatorial index for phytochemicals was calculated, respectively, according Eq (6) and Eq (7):

$$CI_P = \frac{RE_{CIN}}{RE_{CIN|CITR}} \quad (6)$$

$$CI_A = \frac{RE_{CITR}}{RE_{CIN|CITR}} \quad (7)$$

With RE as the results obtained for phytochemicals alone for each method ($\%RE_{MAR}$; $\%B_R$; $\%B_{Ri}$; and log (CFU/cm^2) reduction). $RE_{CIN|CITR}$ represents the results for each method obtained from the combination of compounds.

The sum of CI_{CIN} and CI_{CITR} is the $\sum CI$, from Eq (8):

$$\sum CI = CI_{CIN} + CI_{CITR} \quad (8)$$

The combinatorial index values enable classifying the interaction between phytochemicals within synergistic (if $\sum CI < 0{,}5$) , additive ($0{,}5 < \sum CI < 2$), indifferent ($2 < \sum CI < 4$) and antagonistic ($\sum CI > 4$).

5.3.10 Statistical analysis

Data were examined by the one-way analysis of variance (ANOVA) using the statistical program GraphPad Prism version 8.0.1. The average and standard deviation within samples were calculated. Statistical calculations were based on confidence level ≥ 95% ($p < 0{,}05$) which was considered statistically significant.

5.4 Results and discussion

5.4.1 Inhibitory activity of CITR and ACIN on *E. coli* CECT434 planktonic cells

The MIC for CITR obtained was 800 μg/ml which is significantly lower than the MIC value of CIN reported as 2222.2 μg/mL (Malheiro et al., 2016).

5.4.2 Anti-biofilm properties of CIN

5.4.2.1 Biofilm analysis upon direct exposure (t24)

The percentage value of biomass removal (BR) at MIC was 23,9 ± 11,9%. At 5 × MIC, the percentage value was very similar and with a shorter standard mean (24,7 ± 3,2%; $p > 0,05$). Thus, the BR capacity of CIN seems to already have reached its maximum at MIC. The value of 10 × MIC would confirm it, but unfortunately, results were invalid. Ethylenediaminetetraacetic acid (EDTA), is considered an agent with a great capacity for disrupting biofilm matrix and exhibited 41% of BR under the same conditions, i.e., *E. coli* and 24h of exposure (Baptista et al., 2019). Compared to this value, CIN BR can be considered interesting.

The compound demonstrated a very considerable MAI at MIC (69,4 ± 4,1%), possibly indicating a great diffusion through the matrix. At the following increasing concentrations, values were very similar between them ($p > 0,05$) (76,0 ± 1,3% and 75,1 ± 1,89%; respectively). They were statistically significant higher ($p < 0,05$) than MIC value. By comparison, these values are almost identical to those presented by another phenolic, 4-tert-butylchatechol whose anti-biofilm properties were considered as very promising under the same conditions and against the same *E. coli* strain (MIC: 76 ± 2,0%; 5 × MIC: 77 ± 2,0%; 10 × MIC: 79 ± 1,0%) (Baptista et al., 2019).

Although CIN at MIC substantially inhibited metabolic activity, it did not affect much the biofilm cultivable cells count which is normal, since at this concentration CIN does not have biocidal properties and so, the slight reduction should be due to BR. Furthermore, it is observed a sudden and total log CFU/cm^2 reduction at 5 × MIC. The fact that the metabolic activity was not totally repressed (remained at around 25%), suggests the existence of viable but not cultivable cells (VBNC). For instance, multiple strains of *E. coli* have already shown the aptitude to enter a state of VBNC (Ding et al., 2017). This state is activated by the induction of multiple stress factors (such as the presence of antibiotics, biocides or phytochemicals) where the cells are characterized by being metabolically active, but do not multiply on the agar and can remain in this state for up to 1 year, so they are not detected by conventional techniques of counting CFU. There is then an underestimation of the total number of viable cells in environmental or clinical samples, representing a risk to public health (Li et al., 2014). There is also evidence that VBNC could be persister cells (Kim et al.,

2018a). On the other hand, it is known that CIN has a good antibacterial activity by disrupting membrane cells and thereby, it is possible that CIN killed a considerable share of cells.

CIN has already been reported as a good anti-biofilm agent on several bacteria. It was able to completely control adhered *S. aureus* and *E. coli*, which was even comparable to benchmark disinfectants action (Malheiro et al., 2016). In other studies, CIN inhibited both the production of the quorum-sensing dependent virulence factors and biofilm formation in *P. aeruginosa* but without affecting the viability of bacteria (Rajkumari et al., 2018, Ugurlu et al., 2016). In another study, by only measuring MAI of sessile cells upon exposure, it was concluded that CIN caused significant biofilm inactivation on *Streptococcus mutans*. On the other hand, it did not compromise fibroblast cell viability, suggesting that they may be new candidates for controlling oral infectious diseases. Moreover, molecular hydrophobicity seems to be responsible for higher antimicrobial effect (Ribeiro et al., 2018). These reports also led to antimicrobial evaluations against both planktonic and sessile cells of many CIN derivatives (Malheiro et al., 2019, De Vita et al., 2016).

5.4.2.2 Biofilm analysis upon regrowth conditions (t48)

Despite findings concerning CIN as an anti-biofilm agent are encouraging, no study has used methods to test its ability to tackle persistence or recurrence. BRI was detected at MIC (27,3± 18,2%). At 5 × MIC (67,6 ± 9,2%), BRI was significantly higher and suggests that this concentration may be reasonable against infection persistence. Outcome for 10 × MIC is too uncertain as a lot of variance was detected.

MAI at MIC, decreased almost by half but still to an acceptable and even surprising value since MIC is the minimum inhibition at planktonic state (34,9 ± 4,9%). Data from 5 × MIC and 10 × MIC shows impressively even higher ($p < 0,05$) MAI (87,1 ± 1,9% and 84,9 ± 0,95%). However, by analyzing CFUs quantification, the previous suspicion of VBNC induction is confirmed as there is no total reduction. Moreover, despite differences between the 2 highest concentrations were not observed until this stage, 10 × MIC reduced CFU count almost 5 times more (1,40 and 5,03 log CFU/cm^2, respectively). Thus, it is possible that higher concentrations than 5 × MIC only induces more VBNC or inhibits growth for additional periods, than it kills cells.

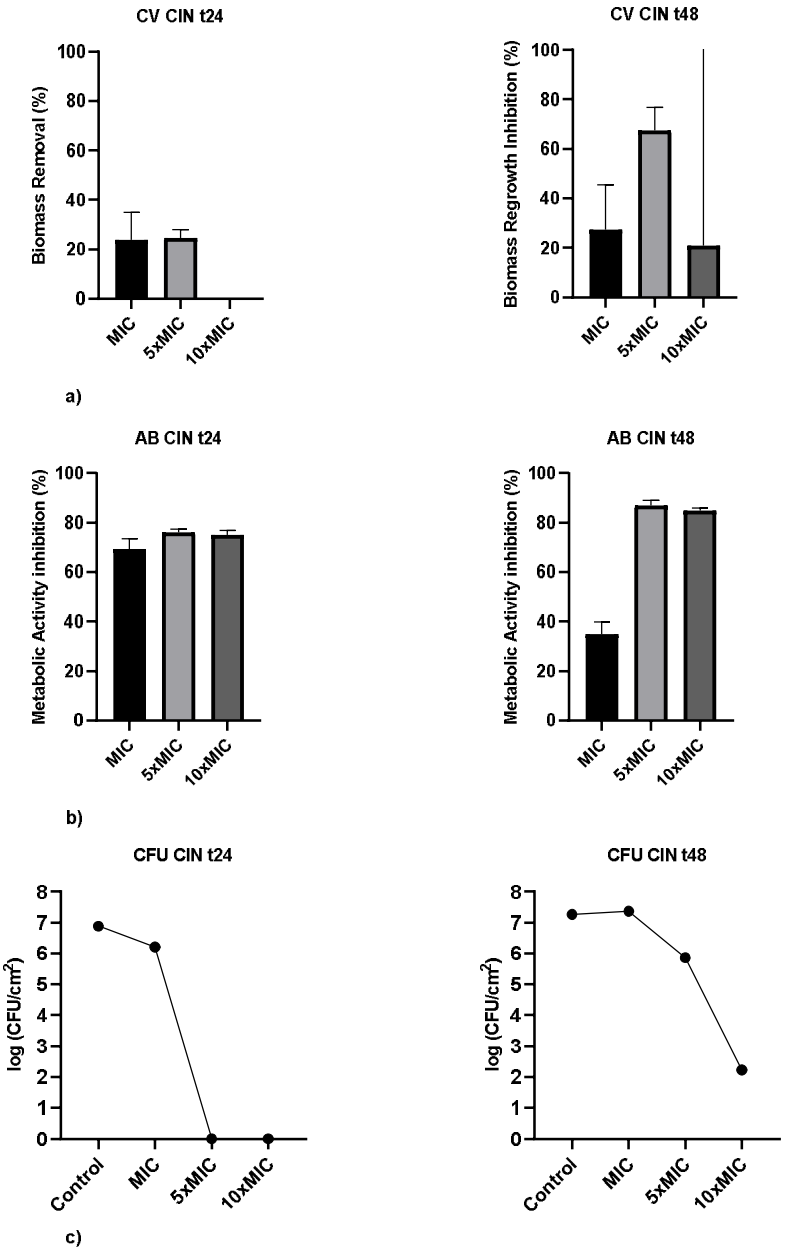

Figure 16 - CIN results. a) BR and BRI of CIN at t24 and t48. b) MAI of CIN at t24 and t48. c) CFU quantification at t24 and t48.

5.4.3 Anti-biofilm properties of CITR

5.4.3.1 Biofilm analysis upon direct exposure (t24)

At the lowest concentration (MIC), BR and MAI from CITR are residual or possibly non-existent (4,9 ± 6,8% and 2,3 ± 12,8%). Both highest concentrations (5 × MIC and 10 × MIC) significantly presented more BR and MAI, but just as in CIN, no statistical difference is detected

between them ($p > 0,05$). BR highest values between CITR and CIN were statistically similar ($p > 0,05$). The same does not apply to MAI ($p < 0,05$), with CIN inhibiting noticeably more.

No CFUs reduction is observed by CITR, leading to the impression of an absence of biocidal activity or even BR, which revealed to be considerable. Thereby, it is unlikely that CITR did not reduce cell count. Probably, something went wrong during procedure.

5.4.3.2 Biofilm analysis upon regrowth conditions (t48)

BRI was inexistent at MIC, but interestingly MAI increased significantly (33,4 ± 9,6%). At 5 × MIC, percentage value was half and more unsure (48,7 ± 29,7%) than BRI from 10 × MIC, which was total (100 ± 10,0), suggesting a great capacity of CITR to inhibit biofilm regrowth at high concentrations. However, MAI maintained from t24 contrasting to CIN, which not only increased from t48 but was also already more impressive (at t24). The incongruence between total BRI and not so great MAI (53,9 ± 8,3%), seems to indicate a large presence of VBNC or persisters. Once again, no CFUs reduction was observed.

It is the first time that both antimicrobial and anti-biofilm properties of CITR are tested and showed positive findings even in its action on 24 h persistence.

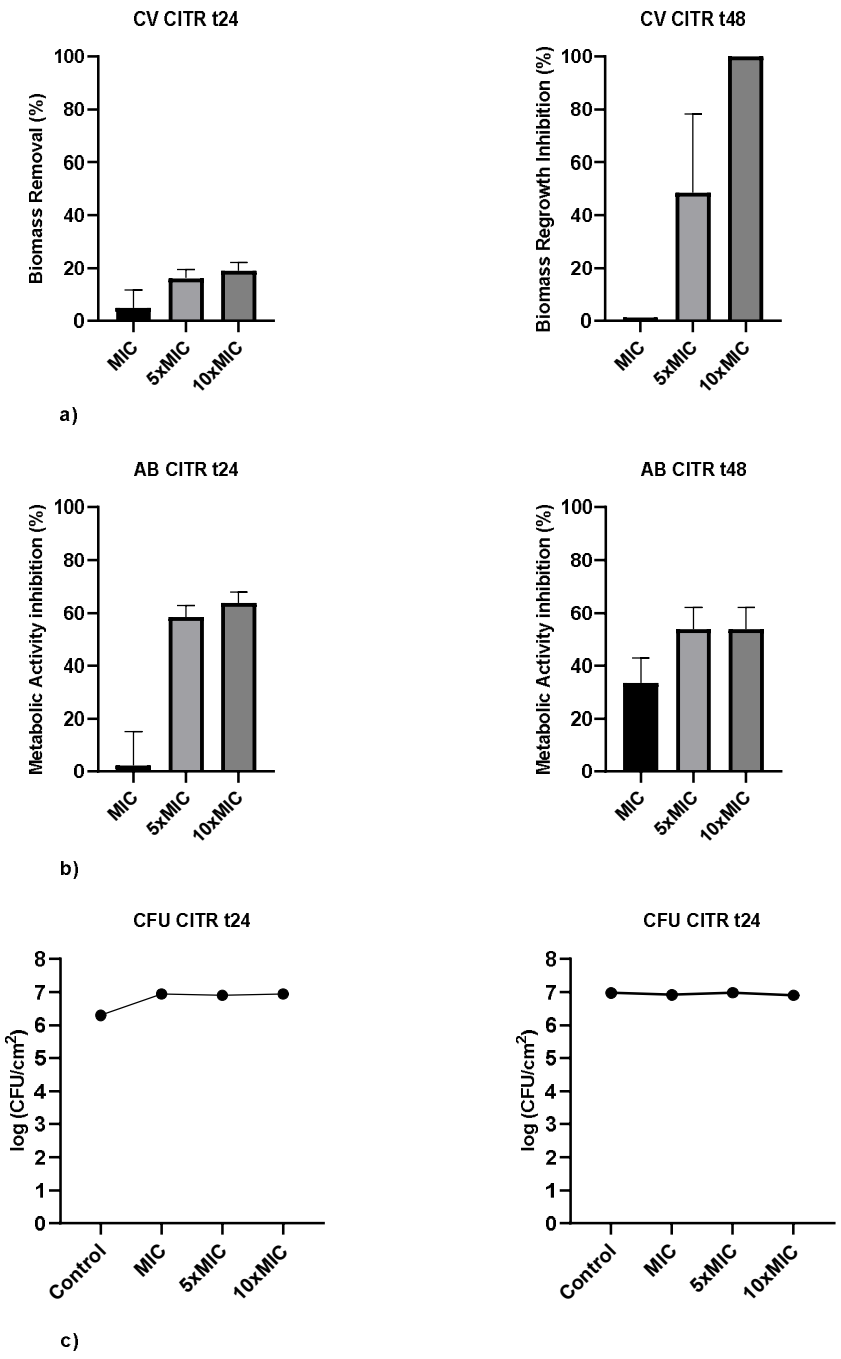

Figure 17 - CITR results. a) BR and BRI of CITR at t24 and t48. b) MAI of CITR at t24 and t48. c) CFU quantification at t24 and t48.

5.5 Conclusions

CIN confirmed its performance to eradicate already stablished *E. coli* biofilms (24 h) upon direct exposure and CITR showed its effect for the first time. Both CIN and CITR had both reasonable BR capacity when compared to EDTA while CIN presented more MAI. Upon regrowth conditions, both activities against persistence seemed to be encouraging at higher concentrations,

however, they did not seem to tackle VBNC/persisters effectively. CIN can represent an example that an agent normally seen as anti-biofilm does not mean that it will efficiently prevent the persistence of biofilms and consequently the infections recurrence.

Besides the existing negligence regarding antimicrobial susceptibility testing by clinical laboratories, even within biofilm research field, persistence and biofilm complexity are being overlooked. Favorably, over the last 5 years, an encouraging number of anti-persisters agents and treatments are emerging. Nevertheless, it represents very early stages of development and serious money and time investment for drug development is needed to convert these agents from laboratory testing to clinical trials and approval.

Even though it was not possible to assess the mixture activity, it is proposed a new approach towards biofilm regrowth inhibition. With a multidisciplinary approach assisted by appropriate methods, it is not only possible to properly assess recurrence or persistence but also to deeply understand the phytochemicals mechanisms of action, as well as their interactions with each other. This can be of great importance for discovering and learning several unknown synergistic, additive, and its interaction with biofilm, at different levels.

Chapter 6: Conclusions and Perspectives for Further Research

6.1 General conclusions

Antibiotic treatments are currently the main strategy to combat chronic, persistent, or recurrent infections, namely biofilm infections. However, they are long-term ineffective and contributes to increasing resistance. In this work, both systematic reviews and respective meta-analyses, showed with high level of evidence that biofilm have great impact on providing protection to microorganism strains, particularly to resistant ones from BSI and UTI; on persistent BSI; and more importantly, on BSI mortality.

In the recent past, new treatment approaches targeting persistence are being investigated and several promising biofilm eradication agents have already been identified but did not reach/surpass clinical trials. Here a new approach regarding a rational development of phytochemicals formulations was proposed and it is believed that could offer great benefits to incisively combat BAI and long-term biofilm regrowth. CIN and CITR can substantially inhibit regrowth but must be better investigated and tested together. Nonetheless, this approach and agents are still very far ahead from clinical approval.

Tangible and high-level of evidence collected from systematic reviews can be what is lacking to divert research focus on biofilm infections, especially on risks of biofilm recovery and its association with persistence, and to incentive investment from researchers and pharmaceuticals on new strategies and biofilm eradication agents.

On a side note, some observational studies included in the systematic reviews mentions culture identification of species which may lead to misleading biofilm impact data towards specie. Replacement of culture techniques by molecular ones are urgent to better identify causative specie and understand their interactions: biofilms may be often colonized by multiple species providing little known dynamics that impacts infections differently than single-specie biofilm (Peters et al., 2012). As previously mentioned, the use of culture techniques in the identification of infections microorganisms is much more common but way less sensitive than molecular techniques.

Likewise, the development and implementation in clinical settings of susceptible antimicrobial assays on sessile cells to accurately assess microorganism sensitivity is crucial.

6.2 Perspectives for further research

Further similar systematic reviews as presented in this work can be performed to other BAI, although, to provide reviews more status, it is recommended to conduct in addition, an assessment of individual studies quality via Newcastle-Ottawa scale. To better assess the risk of bias, the ROBINS-I tool (non-randomized studies) provided by Cochrane is proposed (Sterne et al., 2019). Furthermore, at least 2 or 3 reviewers should perform the selection and screening process independently to promote debate, minimize bias and errors.

Moreover, while conducting the selection process, some important aspects were noted. More observational studies are needed, especially relating biofilm prevalence to clinical outcomes, however, and more importantly, study designs and methods require standardization. This would enable not only a collection of more eligible data but also lead to less heterogeneities. Systematic reviews could also be performed to answer other specific biofilm implications. For instance, it was observed a great number of studies trying to relate virulence genes and other biological assays to biofilm-producing strains isolated from infections. The main objective is to set researchers on the same page in order to seek faster and efficiently more tangible evidence.

Conjugating systematic reviews of biofilm impact in a specific BAI with more socio-economic variables can be also interesting (healthcare costs, geographical contrasts, etc.).

Biofilms are very complex, and several methodologies have recently been developed that have contributed to a deeper understanding of its physiology (metabolome, proteome, and transcriptome), structure and composition. There is a great diversity of methods for the study of biomass, viability, structure, composition, and physiology of biofilm (Azeredo et al., 2017). To truthfully evaluate the anti-biofilm capacity of compounds, thorough study designs with multiple and pertinent interrelacional methods are required, particularly without neglecting biofilm recovery.

Taking the case study proposing a new approach as an example, with a greater number of more complex and more specific methods it would be possible to assess a greater number of details about the interaction of the compounds, individually with the biofilm, or together. For instance, to find out whether cells were killed or induced in VBNC, one could apply the LIVE/DEAD viability, flow cytometry method, or a PMA-qPCR (Azeredo et al., 2017). Ideally speaking, the ultimate objective of this approach is to create a database with phytochemicals modes of action on biofilm, individually and in mixture, as well as with the chemical interactions between compounds. In fact, the chemical stability is an important factor for the performance of any mixture. Therefore, before testing against biofilms, the compatibility of the mixtures in terms of chemical and structural changes should be firstly assessed by analytical techniques (DSC, FTIR and NMR spectroscopy). In this way, it will be possible to obtain a better understanding of all interactions and to develop

rationally combinations of phytochemicals, which effectively eliminate biofilms. However, there are relatively few phytochemicals with detailed mechanisms of action, and no combination has been recorded in literature. So, it is also proposed to perform screenings by the simple disk diffusion method prior to biofilm testing, with random combinations of phytochemicals to verify antibacterial activity. Moreover, strong antagonistic results should not be neglected given the relevance of understanding their interactions, to avoid similar combinations.

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Annexes

Annex A: Systematic review details – Bloodstream infections

PubMed database search:

"((((biofilm[MeSH Terms]) OR (biofilm[Title])) AND ((((((((((((mortality) OR (hospital stay)) OR (length of stay)) OR (virulence)) OR (persistence)) OR (recurrence)) OR (biofilm formation)) OR (biofilm presence)) OR (biofilm incidence)) OR (biofilm prevalence)) OR (outcome)) OR (impact)) OR (risk factor)) OR (antibiotic resistance))) AND ((((((((((((((((bacteremia[MeSH Terms]) OR (candidemia[MeSH Terms])) OR (bacteraemia[MeSH Terms])) OR (candidaemia[MeSH Terms])) OR (bloodstream infections[MeSH Terms])) OR (catheter associated bacteremia[MeSH Terms])) OR (catheter related bacteremia[MeSH Terms])) OR (catheter associated bloodstream infections[MeSH Terms])) OR (cathether related bloodstream infections[MeSH Terms])) OR (catheter bloodstream infections[MeSH Terms])) OR (catheter bacteremia[MeSH Terms])) OR (bacteremia[Title])) OR (bacteraemia[Title])) OR (candidemia[Title])) OR (candidaemia[Title])) OR (bloodstream infection[Title])) OR (bloodstream infections[Title]))) NOT ((((((((((Review[Publication Type]) OR (Letter[Publication Type])) OR (Case reports[Publication Type])) OR (Meta-analysis[Publication Type])) OR (Editorial[Publication Type])) OR (Conference abstract[Publication Type])) OR (Correspondence[Publication Type])) OR (Comment[Publication Type])) OR (Systematic review[Publication Type] OR (Clinical trial[Publication Type] OR (randomized controlled trial[Publication Type])))))))) AND (2005:2020[pdat]) AND (english[Filter] OR french[Filter] OR portuguese[Filter])"

Web of Science database search:

"TOPIC: (biofilm) *AND* **TITLE:** (mortality OR virulence OR recurrence OR persistence OR morbidity OR hospital stay OR length of stay OR antibiotic resistance) *AND* **TOPIC:** (bloodstream infections OR bacteremia OR bacteraemia OR candidemia OR candidaemia OR catheter related bloodstream infections OR catheter associated bloodstream infections) *NOT* **DOCUMENT TYPES:** (Review)

Refined by: DOCUMENT TYPES: (ARTICLE)"

Table A.1 – Studies describing in vitro BFP prevalence in isolates from BSI patients.

Study (Reference)	**Country**	**Study type**	**Microorganism**	**BFP prevalence** n/N (%)
(Atalay et al., 2015)	Turkey	NS	*Candida sp.*	8/50 (16%)
(Buffet-Bataillon et al., 2011)	France	Prospective cohort	*E. coli*	67/53 (43,8%)
(Cafini et al., 2015)	Spain	NS	*E. faecalis*	18/22 (81,8%)
(Giormezis et al., 2014)	Greece	NS	CoNS	58/100 (58,0%)
(Guembe et al., 2018)	Spain	Retrospective	*S. aureus*	162/323 (50,1%)
(Guembe et al., 2014)	Spain	Retrospective	*Candida sp.*	45/54 (83,3%)
(Hashem et al., 2017)	Egypt	NS	*Staphylococcus sp.*	37/58 (63,8%)
(Iorio et al., 2011)	Brazil	NS	*S. aureus*	25/40 (62,5%)
(Klingenberg et al., 2005)	Norway	NS	CoN *S. epidermidis*	79/130 (60,8%)
(Maor et al., 2009)	Israel	NS	*S. aureus*	14/33 (42,4%)
(Martínez et al., 2006)	Spain	Prospective	*E. coli*	79/185 (42,7%)
(Mutlu Sariguzel et al., 2015)	Turkey	NS	*Candida sp.*	15/52 (28,8%)
(Naicker et al., 2016)	South Africa	Prospective	MSSA	13/21 (61,9%)
(Ninin et al., 2006)	France	Retrospective	*S. epidermidis*	59/98 (60,2%)
(Papadimitriou-Olivgeri et al., 2016)	Greece	Retrospective	CoN *S. epidermidis*	14/19 (73,7%)
(Pedroso et al., 2016)	Brazil	NS	CoNS	59/59 (100%)
(Pereira et al., 2014)	Brazil	NS	MR *S.haemolyticus*	23/24 (95,8%)
(Pereira-Ribeiro et al., 2019)	Brazil	NS	*S. haemolyticus*	39/48 (81,3%)
(Pongrácz et al., 2016)	Hungary	Retrospective	*Candida sp.*	43/93 (46,2%)
(Presterl et al., 2005a)	Austria	NS	Viridians *Streptococci*	6/22 (27,3%)
(Presterl et al., 2005b)	Austria	Prospective	*S. epidermidis*	53/60 (88,3%)
(Pulcrano et al., 2012)	Italy	NS	*C. parapsilosis*	31/31 (100%)
(Qin et al., 2017)	Japan	NS	MR *Corynebacterium sp.*	14/17 (82,4%)
(Rampelotto et al., 2018)	Brazil	NS	CoNS	72/176 (40,9%)
(Rijavec et al., 2008)	Slovenia	NS	*E. coli*	55/105 (52,3%)
(Salgueiro et al., 2017)	Brazil	NS	*S. epidermidis*	8/31 (25,8%)
(Sriphannam et al., 2019)	Thailand	NS	*Candida sp.*	58/84 (69,0%)
(Zhang et al., 2019)	China	Retrospective	*E. coli*	81/324 (25,0%)

Notes: NS: Not specified; *E. coli*: *Escherichia coli*; *E. faecalis: Enterococcus faecalis*; CoNS: Coagulase-negative *Staphylococci*; CoN: Coagulase-negative; *S. aureus: Staphylococcus aureus*; *S. epidermidis*: *Staphylococcus epidermidis*; MSSA: Methicillin-susceptible *Staphylococcus aureus*; MR: Methicillin-resistant; *S. haemolyticus*: *Staphylococcus haemolyticus*; *C. parapsilosis*: *Candida parapsilosis*

Table A.2 - Studies describing in vitro BFP prevalence in resistant and susceptible strains of isolates from BSI patients.

Study (Reference)	**Country**	**Study type**	**Microorganism**	**BFP prevalence** n/N (%)	**p-value**
(Bae et al., 2019)	South Korea	NS	MRSA	28/44 (63,6%)	0,040*
			MSSA	5/16 (31,3%)	
(Guembe et al., 2018)	Spain	Retrospective	MRSA	53/91 (58,2%)**	0,080
			MSSA	109/232 (47,0%)**	
(Hashem et al., 2017)	Egypt	NS	MR *Staphylococcus sp.*	18/25 (72,0%)	0,285*
			MS *Staphylococcus sp.*	19/33 (57,6%)	
(Klingenberg et al., 2005)	Norway	NS	CoN MRSE	70/98 (71,4%)	<0,001
			CoN MSSE	9/32 (28,1%)	
(Maor et al., 2009)	Israel	NS	MRSA	9/16 (55,5%)	0,166*
			MSSA	5/17 (29%)	
(Zhang et al., 2019)	China	Retrospective	ESBL *E. coli*	57/160 (35,6%)	<0,001
			Non ESBL *E. coli*	24/164 (14,6%)	

Notes: *p-value calculated; **only High BFP; NS: Not specified; MRSA: Methicillin-resistant *Staphylococcus aureus;* MSSA: Methicillin-susceptible *Staphylococcus aureus;* CoN: Coagulase-negative; MRSE: Methicillin-resistant *Staphylococcus epidermidis;* MSSE: Methicillin-susceptible *Staphylococcus epidermidis;* ESBL: Extended-spectrum beta-lactamase; *E. coli*: *Escherichia col*.

Table A.3 - Studies describing in vitro BFP prevalence in isolates from persistent and non-persistent BSI.

Study (Reference)	Country	Study type	Microorganism	BFP outcome description	BFP prevalence n/N (%)		p-value
					Persistent BSI	Non-persistent BSI	
(Agnelli et al., 2019)	Spain	Retrospective cohort	*Candida sp.*	Moderate/high metabolic activity	27/30 (90,0%)	131/177 (74,0%)	0,064
(Dimitriou et al., 2011)	Greece	Prospective	CoN *Staphylococci*	BF positive	54/97 (55,7%)	26/73 (35,6%)	0,013
(Guembe et al., 2018)	Spain	Retrospective	*S. aureus*	Only High BFP	16/27 (59,3%)	146/296 (49,3%)	0,420
(Li et al., 2018)	Taiwan	Retrospective	*Candida sp.*	Only High BFP	41/68 (60,3%)	15/68 (22,1%)	<0,010
(Monfredini et al., 2018)	Brazil	Multicenter cohort	*Candida sp.*	Only High BFP	12/37 (32,4%)	1/18 (5,5%)	0,041*

Notes: *p-value calculated; CoN: Coagulase-negative; *S. aureus: Staphylococcus aureus*

Table A.4 - Studies describing in vitro BFP prevalence in isolates of BSI survivors and non-survivors

Study (Reference)	Country	Study type	Microorganism	Mortality description	BFP outcome description	BFP prevalence n/N (%)		p-value
						BSI non-survivors	BSI survivors	
(Guembe et al., 2018)	Spain	Retrospective	*S. aureus*	30-day mortality	Only High BFP	18/39 (46,1%)	123/284 (43,3%)	0,610
(Lin et al., 2010)	Taiwan	Retrospective	*C. meningosepticum*	14-day mortality	BF-positive	13/22 (59,1%)	4/18 (22,2%)	0,019
(Martínez et al., 2006)	Spain	Prospective	*E. coli*	In-hospital	BF-positive	8/20 (40,0%)	71/165 (43,0%)	0,800
(Muñoz et al., 2018)	Spain	Retrospective cohort	*Candida sp.*	30-day mortality	Only high BFP	34/95 (35,8%)	56/185 (30,3%)	0,418
(Pongrácz et al., 2016)	Hungary	Retrospective	*Candida sp.*	30-day mortality	BF-positive	23/43 (53,5%)	20/50 (40%)	0,216*
(Soldini et al., 2018)	Italy	Retrospective	*C. parapsilosis*	30-day mortality	High and moderate BFP	61/89 (68,5%)	45/101 (44,6%)	0,010
(Tumbarello et al., 2012)	Italy	Retrospective	*Candida sp.*	In-hospital	BF-positive	39/61 (63,9%)	34/85 (40,0%)	0,010
(Tumbarello et al., 2007)	Italy	Retrospective cohort	*Candida sp.*	30-day mortality	BF-positive	56/154 (36,3%)	24/140 (17,1%)	<0,001
(Vitális et al., 2020)	Hungary	Retrospective	*Candida sp.*	30-day mortality	High and moderate BFP	59/70 (84,3%)	38/57 (66,7%)	0,023
(Zhang et al., 2019)	China	Retrospective	*E. coli*	30-day mortality	High and moderate BFP	30/71 (42,2%)	51/253 (20,2%)	0,002

Notes: *p-value calculated; *C. meningosepticum: Corynebacterium meningosepticum*; *E. coli*: *Escherichia coli*; *C. parapsilosis*: *Candida parapsilosis*.

Annex B: Systematic review details – Urinary tract infections

PubMed database search:

"((((urinary catheter associated infections[MeSH Terms]) OR (urinary tract infections[MeSH Terms]) AND (english[Filter])) AND ((((biofilm[MeSH Terms]) OR (biofilm[Title])) OR (biofilms[MeSH Terms])) OR (biofilms[MeSH Terms]) AND (english[Filter]))) AND (((((((((mortality) OR (persistence)) OR (hospital)) OR (outcome)) OR (clinical)) OR (recurrence)) OR (risk factor)) OR (resistance) OR (impact) OR (epidemiology) OR (production) AND (english[Filter]))))) NOT ((((((((((((Review[Publication Type]) OR (Letter[Publication Type])) OR (Case reports[Publication Type])) OR (Meta-analysis[Publication Type])) OR (Editorial[Publication Type])) OR (Conference abstract[Publication Type])) OR (Correspondence[Publication Type])) OR (Comment[Publication Type])) OR (Systematic review[Publication Type] OR (Clinical trial[Publication Type] OR (randomized controlled trial[Publication Type])))))))) AND (english[Filter])) AND ((english[Filter]) AND (2005:2020[pdat])) AND (english[Filter]) AND (english[Filter]) Filters: English, French, Portuguese"

Web of Science database search:

"TOPIC: (biofilm) *AND* **TITLE:** (mortality OR recurrence* OR persist* OR hospital* OR outcome OR clinical OR resist* OR impact* OR epidemiology OR risk factors OR production) *AND* **TOPIC:** (urinary tract infections OR urinary catheter associated infections) *NOT* **DOCUMENT TYPES:** (Review)"

Table B.1 – Studies describing in vitro BFP prevalence in isolates from UTI patients.

Study (Reference)	Country	Study type	Microorganism	BFP prevalence n/N (%)
(Akhter et al., 2014)	Bangladesh	NS	*Enterococcus sp.*	76/118 (64,4%)
(Bakhtiari and Javadmakoei, 2017)	Iran	NS	*E. coli*	33/35 (94,3%)
(Boroumand et al., 2019)	Iran	Cross Sectional	*E. coli*	98/130 (75,4%)
(Davari Abad et al., 2019)	Iran	Cross Sectional	*E. coli*	74/79 (93,7%)
(De Souza et al., 2019)	Brazil	NS	*E. coli*	80/100 (80,0%)
(Dworniczek et al., 2014)	Poland	NS	*Enterococci*	81/100 (81%)
(Gad et al., 2009)	Egypt	NS	*Klesbiella sp.*	54/64 (84,4%)
(Garg et al., 2017)	India	Prospective	*Enterococcus sp.*	21/50 (42,0%)
(Gawad et al., 2018)	Egypt	NS	*E. coli*	134/175 (76,6%)
(Hashemizadeh et al., 2017)	Iran	NS	*E. coli*	200/250 (80,0%)
(Kadry et al., 2020)	Egypt	NS	*E. coli*	89/112 (79,5%)
(Kudinha et al., 2013)	Australia	Prospective	*E. coli*	404/623 (64,8%)
(Pullanhi et al., 2019)	India	NS	*E. coli*	138/150 (92,0%)
(Rahimi et al., 2016)	Iran	NS	MR *Staphylococcus sp.*	94/108 (59,3%)
(Shikh-Bardsiri and Shakibaie, 2013)	Iran	Retrospective	*Proteus sp.*	82/88 (93,2%)
(Vuotto et al., 2018)	Europe*	NS	*Acinetobacter baumannii*	104/128 (81,3%)

Notes: *5 countries; NS: Not specified; *E. coli*: *Escherichia coli*; MR: Methicillin-resistant.

Table B.2 - Studies describing in vitro BFP prevalence in resistant and susceptible strains of isolates from UTI patients.

Study (Reference)	Country	Study type	Microorganism	BFP prevalence n/N (%)	p-value
(Alves et al., 2014)	Portugal	Retrospective	MDR *E. coli*	6/21 (28,6%)	0,393*
			Non MDR *E. coli*	16/79 (20,2%)	
(Garg et al., 2017)	India	Prospective	VRE	7/17 (41,2%)	1,000*
			VSE	14/33 (42,4%)	
(Gawad et al., 2018)	Egypt	NS	MDR *E. coli*	129/159 (81,1%)	<0,001
			Non MDR *E. coli*	5/16 (31,3%)	
(Kadry et al., 2020)	Egypt	NS	MDR *E. coli*	56/73 (76,7%)	0,462*
			Non MDR *E. coli*	33/39 (84,6%)	
(Karigoudar et al., 2019)	India	NS	MDR *E. coli*	65/88 (73,9%)	0,001
			Non MDR *E. coli*	4/12 (33,3%)	
(Khodadadian et al., 2018)	Iran	Cross Sectional	MBL K. pneumoniae	17/17 (100%)**	0,003
			N-MBL K. pneumoniae	61/92 (66,3%)**	
(Kudinha et al., 2013)	Australia	Prospective	st131 *E. coli*	125/131 (95,4%)	<0,001
			Non st131 *E. coli*	279/492 (56,7%)	
(Neupane et al., 2016)	Nepal	Cross Sectional	ESBL *E. coli*	53/69 (76,8%)	0,050
			Non ESBL *E. coli*	55/139 (39,6%)	
(Rahimi et al., 2016)	Iran	NS	MDR MRSA	60/73 (82,2%)	0,086*
			Non MDR MRSA	23/35 (65,7%)	
(Raya et al., 2019)	Nepal	Cross Sectional	MDR *E. coli*	58/120 (48,3%)	0,015
			Non MDR *E. coli*	15/53 (28,3%)	
(Shahbazi et al., 2018)	Iran	NS	ESBL *E. coli*	68/81 (83,9%)	0,010*
			Non ESBL *E. coli*	31/48 (64,6%)	
(Shrestha et al., 2018)	Nepal	Prospective	MDR *E. coli*	17/23 (73,9%)	0,006*
			Non MDR *E. coli*	6/21 (28,6%)	
(Shrestha et al., 2019b)	Nepal	Cross Sectional	ESBL *E. coli*	48/81 (59,3%)	0,205*
			Non ESBL *E. coli*	38/78 (48,7%)	

Notes: *p-value calculated; **only High/moderate BFP; NS: Not specified; MRSA: Methicillin-resistant *Staphylococcus aureus;* ESBL: Extended-spectrum beta-lactamase; *E. coli*: *Escherichia coli*; MDR: Multi-drug resistant; VRE: Vancomycin-Resistant Enterococci; VSE: Vancomycin-Susceptible Enterococci; MBL: Metallo-Beta-Lactamase; K. pneumoniae: Klebsiella Pneumoniae.

Table B.3 - Studies describing in vitro BFP prevalence in isolates from CAUTI and UTI non-CAUTI.

Study (Reference)	Country	Study type	Microorganism	BFP prevalence n/N (%)		p-value
				CAUTI	UTI non-CA	
(Bardoloi and Yogeesha Babu, 2017)	India	Prospective	*E. coli*	34/46 (73,9%)	40/44 (90,9%)	0,052*
(Garg et al., 2017)	India	Prospective	*Enterococcus sp.*	9/23 (39,1%)	12/27 (44,4%)	0,779*
(Karigoudar et al., 2019)	India	NS	*E. coli*	44/49 (89,7%)	25/51 (49,0%)	<0,001*
(Pullanhi et al., 2019)	India	NS	*E. coli*	21/21 (100%)	30/55 (54,5%)	<0,001*
(Shrestha et al., 2019a)	Nepal	Prospective	Gram (-) *bacilli* & Gram (+) *cocci*	53/70 (75,7%)	89/401 (22,2%)	<0,001*
(Watts et al., 2010)	Australia	NS	*E. coli*	42/88 (47,7%)	52/88 (59,1%)	0,174

Notes: Only BF-positive; *p-value calculated; *E. coli*: *Escherichia coli;* Gram (-): Gram-negative; Gram (+): Gram-positive