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Probiotics: A novel approach to fight biofilms in urinary tract devices

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"Success is not final, failure is not fatal.
It is the courage to continue that counts."

Winston Churchill

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ABSTRACT

Device-associated infections are a critical problem in the medical field due to the high propensity of clinical settings to microbial colonization. Previous studies have proposed the use of probiotics as useful microorganisms to control pathogenic biofilms and/or prevent microbial adhesion to several materials. Therefore, part of this work consisted of studying the interactions of two probiotic strains (*Lactobacillus plantarum* and *Lactobacillus rhamnosus*) with bacteria commonly found in biofilms developed in urinary catheters (*Escherichia coli* and S*taphylococcus aureus*) by two different approaches: displacement of pre-formed biofilms of pathogens (displacement strategy) and inhibition of pathogens adhesion to the surface (exclusion strategy). Moreover, the growth conditions for lactobacilli biofilm development were optimized by testing different hydrodynamic, nutritional and temporal conditions. Colony-forming unit (CFU) counts and crystal violet (CV) staining method were used to determine biofilm cell culturability and total biomass, respectively. Another goal of this work was to review the relevant literature about the potential use of probiotics to fight biofilm formation in medical devices using a PRISMA-oriented (Preferred Reporting Items for Systematic reviews and Meta-Analyses) systematic search and meta-analysis.

This study showed that the individual activity of each probiotic caused a reduction up to 60 and 63% in the culturability of 24-h biofilms of *E. coli* and *S. aureus* on silicone, respectively. This antimicrobial effect is probably associated with the release of harmful substances by *Lactobacillus* strains that affect pathogenic bacteria, and with the integration of probiotic cells into the pre-established biofilms. Regarding the biofilm-forming capacity of probiotics, both strains were able to form robust and stable biofilms on silicone surfaces; however, *L. rhamnosus* demonstrated more difficulty in maintaining its culturability. The replacement of culture medium every day enhanced probiotic biofilm formation, and the use of a low shaking frequency during biofilm growth led to more robust biofilms. Therefore, 24- and 48-h biofilms of *L. plantarum* were used in the exclusion assays, inhibiting the adhesion of *E. coli* by 94 and 97%, respectively. These preliminary results suggest that the developed probiotic biofilms have a good potential to be used as biocontrol agents against pathogenic bacteria, which will pave the way to further experiments on exclusion mechanisms.

The systematic review and meta-analysis revealed that the use of probiotics and their metabolites is a promising approach to hinder biofilm formation in medical devices by a broad spectrum of pathogenic microorganisms. Although their efficacy seems to be independent of the adopted anti-biofilm strategy, the prevention of initial cell attachment is a more promising strategy than battle pre-formed uropathogenic biofilms. This review highlights the need to properly analyze and report data, as well as the importance of standardizing the culture conditions in order to facilitate the comparison between studies. This is essential to increase the studies' predictive value and translate these *in vitro* findings into clinical applications.

Keywords: Probiotics, Biofilms, Pathogen, Medical Devices, Urinary Tract Devices

RESUMO

As infeções associadas a dispositivos médicos são um problema crítico na área médica devido à sua alta propensão à colonização microbiana. Estudos anteriores propuseram o uso de probióticos como microrganismos úteis para controlar biofilmes patogénicos e/ou impedir a adesão microbiana a vários materiais. Deste modo, parte deste trabalho consistiu em estudar as interações de duas estirpes de probióticos (*Lactobacillus plantarum* e *Lactobacillus rhamnosus*) com bactérias comumente presentes em biofilmes desenvolvidos em cateteres urinários (*Escherichia coli* e *Staphylococcus aureus*) por duas abordagens distintas: dispersão de biofilmes de patogénicos pré-formados (estratégia de disrupção) e inibição da adesão de patogénicos à superfície (estratégia de exclusão). Além disso, as condições de crescimento para o desenvolvimento dos biofilmes de *Lactobacillus* foram otimizadas, testando-se diferentes condições hidrodinâmicas, nutricionais e temporais. A contagem de unidades formadoras de colónias (UFC) e o método de coloração por cristal de violeta (CV) foram utilizados para determinar a culturabilidade e a biomassa total dos biofilmes, respetivamente. Outro objetivo deste trabalho consistiu numa revisão sistemática sobre o potencial uso de probióticos para combater a formação de biofilmes em dispositivos médicos, realizando uma pesquisa orientada pela declaração PRISMA (Principais Itens para Reportar Revisões Sistemáticas e Meta-Análises) e meta-análise.

Este estudo mostrou que a atividade individual de cada probiótico causou uma redução até 60 e 63% na culturabilidade nos biofilmes de 24 h de *E. coli* e *S. aureus* em silicone, respetivamente. Este efeito antimicrobiano está provavelmente associado à libertação de substâncias nocivas que afetam o crescimento das bactérias patogénicas e à integração de células de probióticos nos biofilmes pré-estabelecidos. Em relação à capacidade de formação de biofilmes dos probióticos, ambas as estirpes formaram biofilmes robustos e estáveis em silicone; no entanto o *L. rhamnosus* demonstrou maior dificuldade em manter a culturabilidade. A substituição diária do meio de cultura e o uso de uma baixa frequência de agitação levaram à formação de biofilmes mais robustos. Assim, os biofilmes de 24 e 48 h do *L. plantarum* foram utilizados nos ensaios de exclusão, inibindo a adesão de *E. coli* ao silicone em 94 e 97%, respetivamente. Estes resultados preliminares sugerem que os biofilmes de probióticos têm potencial para serem usados como agentes de biocontrolo contra bactérias patogénicas, o que abrirá caminho para futuras experiências relativas aos mecanismos de exclusão.

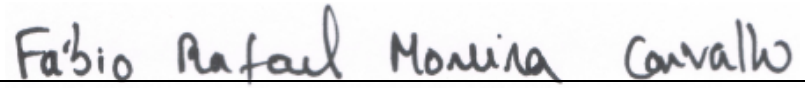
A revisão sistemática e a meta-análise revelaram que o uso de probióticos e os seus metabolitos é uma abordagem promissora para impedir a formação de biofilmes em dispositivos médicos. Embora a sua eficácia pareça ser independente da estratégia aplicada, a prevenção da adesão inicial dos patogénicos surge como a estratégia mais promissora, ao invés de dispersar os biofilmes pré-formados. Esta revisão destaca a importância de padronizar as condições experimentais e a necessidade de analisar e reportar adequadamente os dados, de modo a facilitar a comparação entre os estudos. Isso é essencial para aumentar o valor preditivo dos estudos e traduzir as descobertas *in vitro* em aplicações clínicas.

Palavras-chave: Probióticos, Biofilmes, Patogénico, Dispositivos Médicos, Dispositivos do Trato Urinário

DECLARATION

Declaro, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

Declare, under oath, that this work is original and that all non-original contributions were properly referenced with the source identification.

June 29th 2020 

(Fábio Rafael Moreira Carvalho)

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ABBREVIATIONS

AUM – Artificial Urine Medium

CAUTI – Catheter-Associated Urinary Tract Infections

CFU – Colony-Forming Unit

CI – Confidence Interval

CV – Crystal Violet

ECDC – European Centre for Disease Prevention and Control

EcN – *E. coli* Nissle 1917

EHEC – Enterohemorrhagic *E. coli* O157:H7

EPS – Exopolysaccharides

FOS – Fructo-Oligosaccharides

GRAS – Generally Regarded As Safe

HCAI – Healthcare-Associated Infections

LAB – Lactic Acid Bacteria

LBA – Luria-Bertani Agar

LTA – Lipoteichoic Acid

MDAI – Medical Device-Associated Infections

MINORS – Methodological Index for Non-Randomized Studies

MRSa – De Man, Rogosa, Sharpe Agar

MRSA – Methicillin-Resistant *Staphylococcus aureus*

MRSB – De Man, Rogosa, Sharpe Broth

OD – Optical Density

PDMS – Polydimethylsiloxane

PRISMA – Preferred Reporting Items for Systematic reviews and Meta-Analyses

PVC – Polyvinyl Chloride

QS – Quorum-Sensing

SD – Standard Deviation

SSI – Surgical Site Infections

UPEC – Uropathogenic *E. coli*

UTI – Urinary Tract Infections

UV – Ultraviolet

VBNC – Viable But Non-Culturable

1. INTRODUCTION

1.1. Objectives

Urinary catheters are very susceptible to bacterial contamination, which can lead to the development of biofilms on the intra and extraluminal surfaces of the devices [1]. There are several problems associated with biofilm-based catheter-associated urinary tract infections (CAUTI), such as a poor response to the classical antibiotic therapy, which has led to a growing resistance of pathogenic microorganisms, resulting in recurrent infections [2][3]. Although numerous strategies have been developed and are currently used to control biofilm formation, the pursuit of novel and effective anti-biofilm strategies continues. Since *Lactobacillus* strains gained importance in the medical field due to their ability to produce antimicrobial compounds to control microflora [4], we decided to study the potential action of probiotics against biofilm formation in urinary catheters.

The original aim of this work was to study the interaction of two probiotic strains (*Lactobacillus plantarum* and *Lactobacillus rhamnosus*) with bacteria commonly found in biofilms developed in urinary catheters (*Escherichia coli* and S*taphylococcus aureus*) by two different approaches. First, the ability of probiotics to displace pre-formed biofilms – displacement strategy – was assessed, and then the ability of biofilms of probiotics to inhibit the adhesion of uropathogenic bacteria to surfaces – exclusion strategy – was evaluated. Also, the conditions for the growth of probiotic biofilms that will cover the silicone surfaces before contact with pathogenic strains were optimized. Different hydrodynamic, nutritional and temporal conditions were tested using 12-well plates. The Crystal violet (CV) staining method was used to quantify the total biofilm amount, whereas the number of biofilm culturable cells was determined from the colony-forming units (CFU) counts.

Due to the COVID-19 lockdown, the work plan was changed and focused on systematically review the relevant literature about the potential of using probiotics to fight biofilm formation in medical devices using a PRISMA-oriented systematic search. The quality of the selected studies was assessed according to an adapted Methodological Index for Non-Randomized Studies (MINORS) scale and a meta-analysis was carried out.

1.2. Relevance of the work

CAUTI are one of the most common hospital-acquired infections worldwide [2], with a huge negative impact on patients' safety and healthcare systems [5]. Thus, due to the lack of an adequate response with conventional approaches, there is an urgent need to find novel strategies to reduce CAUTI rates. CAUTI are mostly originated from the formation of pathogenic biofilms on the device surface [6], so this study is important to assess if probiotics are an effective solution to control and prevent catheter colonization and subsequent biofilm growth.

In the last years, the use of probiotics, including *Lactobacillus* species, has received much attention to prevent and treat urogenital disorders. Among several studies conducted to investigate the effect of probiotics and their products against biofilms associated with urinary catheters, some studies

showed promising results in displacing adhering uropathogens and inhibiting bacterial adhesion to catheter materials [6]–[8]. However, most of the anti-adhesion and anti-biofilm assays were performed under static conditions, while just a small number of studies used physiologically relevant flow conditions [9][10]. On the other hand, most studies have been carried out with buffer or nutrient-rich culture media, but rarely in urine; only a couple of studies used nutritional conditions similar to those found in human urine [11][12]. Therefore, to the best of our knowledge, this is the first study on the ability of probiotics to control and prevent biofilm formation combining the effect of nutritional conditions (artificial urine medium, AUM), hydrodynamics and surface material (silicone rubber). In this way, we intend to be as close as possible to the *in situ* conditions that better predict how probiotics will perform *in vivo*.

Biofilm-related infections have been a major clinical problem in medical devices due to the increasingly widespread ability of pathogens to generate persistent biofilms and the low efficiency of human immune system and antibiotics to counteract biofilm development. New evidence supporting the effect of probiotics on the prevention and treatment of device-associated biofilms and an increasing interest in promoting a natural approach to health have intensified the research in the field of probiotics and their metabolites to battle pathogenic biofilms. The systematic review takes a close look at the effectiveness of probiotics in inhibiting biofilm formation by different approaches. To the best of our knowledge, this is the first systematic review with meta-analysis about the anti-adhesive and antimicrobial activity of probiotics against medical device-associated infections.

1.3. Thesis outline

This thesis is divided into seven sections. Section 1 presents the context, main objectives, relevance and motivation for the development of this work. Section 2 encloses the literature review, starting with the discussion of the problem, a detailed description of the biofilm formation process with a close look at the biofilm formation in urinary catheters, and ending with the presentation of the main characteristics of probiotics. Since part of this work consists of a systematic review focused on the use of probiotics to fight biofilm formation in medical devices, these results are presented in Section 4 of Results and Discussion. Sections 3 and 4 are divided into two independent subsections: the experimental work and the systematic review. In Section 3, the materials and methodologies used in both parts of this work are fully described. In Section 4, the results are presented and discussed. First, the effect of probiotics in pathogenic biofilms and the ability of probiotics to form biofilms were experimentally evaluated. Then, the results of the systematic review were discussed, and the quality assessment and meta-analysis of the included studies were performed. Section 5 presents the main conclusions drawn from this work. Finally, Section 6 lists the scientific events in which this work was presented, and Section 7 presents some ideas and suggestions for future work.

2. STATE OF THE ART

2.1. Medical device-associated infections (MDAI)

Medical devices have been widely used in the prevention, diagnosis, treatment and mitigation of some diseases, improving the healthcare and life quality of patients. However, the aging population, the growing prevalence of diseases and deteriorating lifestyle led to a fast growth in the use of medical devices [3][13]. Indwelling medical devices and implants, such as mechanical heart valves, artificial veins, catheters, prosthetic joints and pacemakers, are particularly vulnerable to microbial contamination [14][15]. Thus, colonization of implants and prosthetic medical devices poses a critical problem and plays a key role in the increasing number of potentially life-threatening healthcare-associated infections (HCAI) [16]. HCAI are infections that patients acquire while receiving treatment for medical or surgical conditions in care homes, hospitals or in patient's own homes, and are the most frequent adverse events during care delivery [17][18]. HCAI are a major problem for patient safety since they are associated with high mortality and morbidity rates, and increased length of hospital stay, posing a huge financial burden on healthcare systems [3][17]. The European Centre for Disease Prevention and Control (ECDC) estimated that approximately 4.1 million patients are affected by HCAI every year in Europe [18][19] with a mean HCAI prevalence of 7.1% [18], causing 16 million extra days in the hospital and leading to approximately €7 billion of direct costs [20]. In 2002, the estimated HCAI incidence rate in the United States was 4.5% and about 1.7 million cases were reported, resulting in 99 000 deaths [21]; in 2015, approximately 700 000 cases of HCAI in the United States acute care hospitals were estimated, resulting in 72 000 deaths [22]. The overall annual direct costs of HCAI in the United States hospitals ranges from $28 to $45 billion [23]. In the United States and Europe, urinary tract infections (UTI) were the most commonly reported type of HCAI (36% and 27%, respectively), followed by surgical site infections (SSI) (20%), bloodstream infection and pneumonia (both 11%) in the United States, and lower respiratory tract infection (24%) and SSI (17%) in Europe [18]. Medical device-associated infections (MDAI) comprise 50-70% of all HCAI [3][24]. In fact, 83% of patients in a hospital who acquired pneumonia were being ventilated, 87% of patients with a bloodstream infection had a device inserted in their circulatory system, and 97% of patients with a UTI had a urinary tract device in place [20].

The location of a device in the body can affect the degree of colonization. Indwelling devices act as a bridge between the nonsterile outside environment and the sterile inside of the patient [13]. They disturb the local host defense mechanisms, thereby facilitating the access of bacteria to vital organs [20]. Despite the efforts to maintain sterility, the initial contamination of medical devices occurs by infectious agents from endogenous sources, such as the skin, nose, mouth, gastrointestinal tract, or vagina (which are normally colonized by local microbial flora), or exogenous sources, such as the surgical or clinical staff, contaminated water or other external environmental sources [25][26].

When an indwelling medical device is contaminated, several variables like cell adherence, rate of cell attachment, the fluid flow rate through the device and physicochemical properties of the surface,

have an impact on the progress of the infection, which, in most cases, lead to the formation of biofilms [26]. Microbial biofilms play an important role in about 80% of human microbial infections [19], being a critical issue in the medical field, interfering with clinical therapy of chronic and wound-related infections, as well as persistent and recurrent infections involving various indwelling medical devices [3]. Biofilms may contain many different types of microorganisms, and single or multiple species, depending on the device duration into the patient – the longer a device remains in a patient, the more likely the device will be colonized, often by multiple species [13].

Biofilms formed on medical devices may be composed of gram-positive and gram-negative bacteria, and yeasts. Microorganisms commonly isolated from these devices include the gram-positive bacteria *Enterococcus faecalis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, the gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*, and yeasts, particularly *Candida* species [3][13][17][26]. Among them, *S. aureus* and *S. epidermidis* are described as the most common bacteria found in device-associated infections, being the leading cause of prosthetic heart valve infections (40-50%), catheter biofilm infections (50-70%) and bloodstream infections (87%) [3][14][15]. More than two-thirds of MDAI are suggested to be caused by the staphylococcal species [3].

Thus, the development of biofilms constitutes a challenge in treating HCAI. Novel technologies to prevent biofilm formation on medical devices, such as drug-eluting coatings, bactericidal coatings and adhesion-resistant surfaces are being developed, but it is not clear how these technologies work *in vivo* or how they affect clinical outcomes. Therefore, the clinical field warrants further investigation to find new alternatives that prevent the establishment of biofilms [13].

2.1.1. Catheter-associated urinary tract infections (CAUTI)

Urinary tract infections, caused by an invasion of any part of the urinary system by a pathogen [1], are considered the most common bacterial infections with an estimated annual worldwide incidence of 250 million cases [5] and a mortality rate of 2.3% [21], having a great impact on patients and healthcare systems. Among UTI acquired in hospitals, about 75-80% are associated with catheter insertion [24][27][28]. CAUTI cause high morbidity and mortality, increased length of hospital stay, and increased cost of treatment. The annual treatment costs of CAUTI are over $451 million in the United States [29] and £1–2.5 billion in the United Kingdom [30]. These infections pose a serious concern since, in general hospitals, 15–25% of hospitalized patients have been estimated to have a urethral catheter at some time of their stay [1][27][28] and it is suggested that an episode of CAUTI can extend a hospital stay from 0.5 to 1 day [29]. Moreover, the daily risk of getting a CAUTI is reported to be between 3–7% when a catheter is in place and increases with the duration of catheterization [29]. In short-term catheterization (< 7 days), the incidence of CAUTI is reported to be between 10 and 50%, however, in long-term catheterization (> 28 days), almost all patients develop bacteriuria [26][31]. Clinically, the presence of microorganisms in urine is diagnosed as UTI only if other symptoms are

present at the same time. If no symptoms are occurring, the diagnosis is limited to candiduria or bacteriuria [20]. Criteria for diagnosis of CAUTI include signs or symptoms such as fever, rigors and pain, and more than 10^5 CFU/mL of one or several bacterial species detected in a urine sample [1][20].

Urinary catheters are considered the most common indwelling devices. In the United States, over 30 million urinary catheters are inserted annually with an infection incidence between 10 to 30% [20][24][32]. Urinary catheters are tubular latex, polyurethane, or silicone devices [24][26] used to prevent urine retention in surgical procedures, to measure urine output and in cases of urinary incontinence and treatment of prostate hyperplasia or cancer [17][31]. An added concern related to urinary catheters is the presence of microbial biofilms on the surfaces due to the high susceptibility of their materials to be colonized. The pathogens may enter into the closed catheter system by transferring the patient's own fecal or skin microbiota during insertion, through microbial migration along the device, or when the collecting tube or drainage bag becomes contaminated by bacteria, which can lead to the development of biofilms in the intra and extraluminal surfaces of the devices [31].

2.2.Biofilms

Biofilms can be defined as agglomerates of microorganisms protected by a self-synthesized matrix of extracellular polymeric substances [33]. These compounds are secreted into the environment and usually include exopolysaccharides (EPS, high molecular-weight polymers that are composed of sugar residues), proteins and nucleic acids [33], depending on the species and environmental conditions (shear forces, temperature and nutrient availability) [13]. The extracellular matrix serves as a platform for surface attachment and facilitates communication among the cells through biochemical signals, a phenomenon called quorum-sensing (QS) [34]. The matrix protects the pathogen against host defense and antimicrobial agents by limiting the diffusion of antibiotics [33], enhancing horizontal transmission of plasmid-associated antibiotic-resistant genes, and creating an altered microenvironment [27]. Channels in the biofilm architecture allow for water, air and nutrients to access all parts of the structure [3].

Theoretically, biofilms can establish on any biotic or abiotic surface comprising a wide spectrum of environments. Bacterial adherence is important for the successful biofilm formation, especially when surfaces are exposed to hydrodynamic forces such as the urinary tract. The capacity of microorganisms to adhere to certain substrata is affected by many factors such as the physicochemical properties of the surface (e.g. surface chemistry, topography, charge and hydrophobicity), the compounds and conditions of the surrounding environment (e.g. pH, flow rate, medium composition and temperature), the cell surface characteristics of organisms (e.g. flagella), the number and type of microorganisms and the host's immune system [13][35][36].

Microorganisms can switch between planktonic (suspension) and sessile state (biofilm) [3]. While planktonic organisms are described as having a relatively high cell growth and cell division rate, the sessile state appears to be the natural and predominant state of bacteria [33]. Cells in biofilms are 10

to 1000-times more resistant to antimicrobial treatments than their planktonic counterparts [25]. The advantages of microorganisms in forming biofilms include protection from hostile environmental conditions (pH, chemical agents or phagocytosis), acquisition of biofilm-specific antibiotic-resistant phenotypes and expanded metabolic cooperation [20].

Biofilm formation is a dynamic process that involves four main stages, including reversible attachment, irreversible attachment, maturation and detachment. When body fluids, such as blood, saliva or urine, contact with a device, they may be adsorbed on the surface, forming a conditioning film composed mainly by proteins (such as albumin, fibrinogen, collagen or fibronectin) and polysaccharides [14][37]. This conditioning film plays an important role in helping the planktonic cells to adhere to the surface via physical forces or bacterial appendages (e. g. flagella or pili) [24][38]. Therefore, the initial contact of cells with a surface is mediated by weak and reversible interactions via van der Waals forces, hydrophobic interactions, steric interactions and electrostatic interactions [3][13][39]. Once in contact, the bacterial appendages overcome the physical repulsive forces and anchor on the surface, immobilizing the reversibly attached microorganisms [39]. Also, the first cells adhered start to produce the extracellular polymeric matrix, which makes the biofilm to become irreversibly attached to the underlying surface, providing adhesion sites to other cells [13][39]. During growth and differentiation, cells start to communicate with each other via QS [3] by producing signaling molecules (e. g. autoinducers) which stimulate the expression of specific genes related to biofilm formation [35]. Then, biofilm progressively spreads over the surface by exponential growth of its population and its morphology may vary according to the environmental conditions [39]. Finally, in the detachment phase, biofilm cells begin to disperse and colonize new surfaces [40].

The presence of biofilms causes numerous problems in the biomedical field, interfering with the clinical therapy of several infections, including the persistent infections involving indwelling medical devices. Although numerous strategies have been established and are currently used to control biofilms, the pursuit of novel and effective anti-biofilm strategies continues.

2.2.1. Biofilms in urinary catheters

The establishment and growth of biofilms on the surface of urinary catheters are strongly affected by the presence of a continuous or intermittent flow of a nutritive medium like urine [19]. Additionally, the catheter lumen does not have inherent defense mechanisms, such as phagocytosis or the action of antimicrobials agents, which makes biofilm harder to treat [41]. Urinary catheter biofilms are commonly composed of one species at the beginning, but long periods of catheterization rapidly lead to the development of multi-species biofilms. The most common microorganisms contributing to CAUTI are *E. coli* (57%), followed by *K. pneumoniae* (15%), *P. aeruginosa* (12%), *S. aureus* (8%), *Enterobacter* spp. (3%), and *E. faecalis*, *Acinetobacter* spp. and *P. mirabilis* (1.5% each) [27]. A clear prevalence of *E. coli*, *P. aeruginosa*, *E. faecalis* and *P. mirabilis* in biofilm-associated UTI was also reported [19][26][42][43].

Figure 1 presents the biofilm formation process in a urinary catheter. As mentioned above, when biological fluids contact with materials, a conditioning layer of adsorbed biological substances is formed, altering the surface properties (e. g. chemistry, roughness and topography) [20]. This phenomenon may allow the adhesion of uropathogenic microorganisms. Then, the microorganisms attached to the catheter interact with each other and start to produce extracellular polymeric substances forming a biofilm. Finally, sessile cells can detach from the biofilm and move against the urine flow, colonizing the bladder or kidneys [19]. In addition, some urease-positive pathogens, including *P. mirabilis*, *Proteus vulgaris*, *Providencia rettgeri*, *P. aeruginosa* and *K. pneumoniae* convert urea to ammonia and carbon dioxide through the urease enzyme, increasing the pH of the local environment, thereby causing the precipitation of some minerals, including calcium phosphate and struvite [26][44]. The deposition of mineral salts on both surfaces of catheters can cause encrustation, which may completely block the flow. This might result in device failure and have harmful consequences in the bladder and urethral epithelia [45].

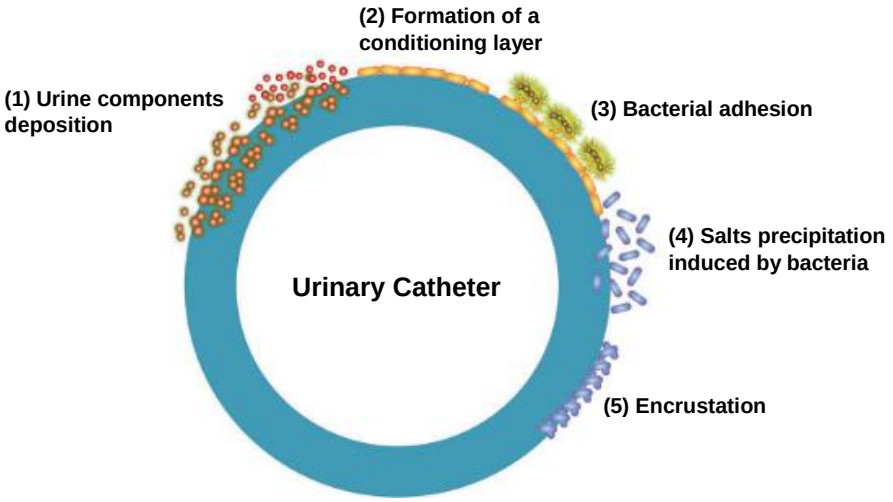

Figure 1. Biofilm formation process in a urinary catheter (Adapted from: Ramstedt *et al*. [20]).

The primary treatment of CAUTI includes the use of antibiotics, but the growing resistance of uropathogens has led to their poor response to antibiotic therapy [26]. Thus, new approaches are needed to treat and prevent CAUTI.

2.3.Probiotics

2.3.1. Definition and properties

Probiotics have been with us for as long as people have eaten fermented milk, but their association with health benefits dates only from the turn of the last century [46]. Probiotics are defined as viable microorganisms (bacteria or yeasts) that, when ingested in an appropriate concentration, have beneficial effects on the host [47]. They have received substantial attention regarding their potential health-promoting properties, so some of them have the status of Generally Regarded as Safe (GRAS).

The most commonly used bacterial probiotics are species of lactic acid bacteria (LAB) which include *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Lactococcus* [48][49]. Other LAB genera with proven probiotic action include *Enterococcus*, *Pediococcus*, and *Leuconostoc* [50]. Likewise, the nonpathogenic *Saccharomyces boulardii* is currently the only yeast recognized as probiotic [48][49]. LAB are generally gram-positive, non-sporing, catalase-negative, aerotolerant, acid-tolerant, and strictly fermentative bacteria [51][52]. Metabolically, LAB are known to produce lactic acid as a major end-product of carbohydrate fermentation and other metabolites [51]–[53]. Thus, probiotics are able to grow in different habitats using different types of carbohydrates such as fructo-, galacto-, gluco-, xylo- or oligosaccharides [53]. From glucose metabolism, LAB are classified as homofermentative, producing entirely lactic acid through the Embden-Meyerhof pathway, or heterofermentative, producing several metabolites in addition to lactic acid, including ethanol, acetic acid, and carbon dioxide via pentose monophosphate pathway [50][54]. These substances contribute to a physiologically restrictive environment, inhibiting pathogens adherence [55]. LAB can also produce secondary metabolites, including bacteriocins, EPS, and enzymes [56]. Among LAB, *Lactobacillus* and *Bifidobacterium* are known to resist gastric acid, bile salts and pancreatic enzymes and to colonize the intestinal tract [48]. *Lactobacillus* is a genus of gram-positive facultative anaerobic or microaerophilic rod-shaped bacteria [57][58]. This genus comprises 183 recognized species, including *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. casei*, *L. reuteri* and *L. rhamnosus*, which have beneficial effects in the health of the animal and human gastrointestinal and digestive systems [50]. *Bifidobacterium* are heterofermentative, non-motile, catalase-negative, and anaerobic bacteria, with the ability to metabolize glucose, galactose, lactose, and fructose and produce lactic and acetic acids as by-products [50][52][58]. *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* have been reported for their diverse probiotic effects and are widely used in yogurts, milk, cheese and other dairy product [50]. Two of the most commercial LAB with an important role in the food industry are *Streptococcus thermophilus* and *Lactococcus lactis*, however, there is little known about their probiotic properties [51][52].

These microbial groups possess the ability to withstand unfavourable conditions of the human body and contribute to the health of the host, regulating microbes and exerting biological functions [50]. There is substantial evidence from *in vitro* and *in vivo* studies that both live and dead probiotic cells can act as biological response modifiers [59]. Therefore, a new approach to the treatment and prevention of infectious diseases may involve the use of probiotics.

2.3.2. Mechanisms of action

The effectiveness of probiotics is strain-specific, and each strain has multiple and diverse impacts on the host through different mechanisms [55]: (1) pathogen growth inhibition by the production of antimicrobial substances and selective metabolites creating a hostile microecology; (2) competitive exclusion of pathogens by blocking adhesion sites; (3) increased adhesion to the intestinal mucosa and subsequent inhibition of pathogen adhesion; (4) competition for nutrients; (5) influence of mucosal cell–

cell interactions and cellular stability by the enhancement of intestinal barrier function; (6) aggregation between probiotics of the same strain (auto-aggregation) or between genetically different strains (co-aggregation); and (7) modulation of the immune system [46][60]–[62].

In terms of antimicrobial activity, probiotics are able to modify the environment to make it less suitable for competitors by the production of antimicrobial substances and a physiologically restrictive environment with respect to pH, redox potential and hydrogen sulfide production [55][58][60]. The production of hydrogen peroxide is very important because it has bactericidal effects on most pathogens [62]. Many strains of lactobacilli and bifidobacteria produce other antimicrobial substances like organic acids (lactic, acetic, propionic, succinic), biosurfactants and bacteriocins [46][58][60]. Among these, bacteriocins, defined as proteins with antimicrobial activity, have received increasing attention due to several advantages over most of the antibiotics [53]. Bacteriocins have not been associated with side effects on humans since they are rapidly broken down in the organism [53][58]. On the other hand, probiotics can induce intestinal epithelial cells to produce antimicrobial peptides like defensins and cathelicidins [49].

Another mechanism of action of probiotics is the competition for adhesion sites. Probiotics compete with invading pathogens for binding sites on epithelial cells and the overlying mucus layer in a strain-specific manner [49]. The adhesion of probiotics to intestinal mucosa prevents pathogens from effectively colonize the gastrointestinal tract [58][60].

Competition for limiting resources, such as nutrients, is another way by which probiotics limit the growth of pathogens [60]. A good example is iron, a limited element in the host and essential for almost all bacteria. However, lactobacilli do not need iron in their natural habitat, which might be a critical advantage in competition with other microorganisms [63].

Probiotics can influence mucosal cell–cell interactions and cellular stability by influencing many of the components of the epithelial barrier function either by decreasing apoptosis of intestinal cells or increasing mucin production [49].

Auto- and co-aggregation between probiotics are of great importance once bacterial aggregates may achieve the appropriate conditions to form biofilms, or adhere to the mucosal surfaces of the host and thus be functional [46]. Also, it was demonstrated that probiotics may secrete molecules that interact with the transcription of genes involved in biofilm formation or inhibit the QS signaling [49].

It has also been observed that probiotics can exert an immunomodulatory effect. Probiotics have an influence on numerous cell types involved in innate and adaptive immune responses (monocytes, epithelial, dendritic, natural killer, B and T cells), enhancing phagocytic activity of macrophages or increasing the secretion of immunoglobulin-A [51][55][58][60]. Immunomodulation can be performed by probiotics through metabolites, cell wall components and DNA. Specialized membranous cells (M cells) are responsible for the detection of probiotics and their products by recognition receptors. These interactions work through the expression of pattern-recognition receptors, which will recognize pathogen-associated molecular patterns, triggering an immune response [63].

2.3.3. Main characteristics of probiotic strains

The selection of probiotic organisms requires a systematic approach to infer the functional/beneficial properties that are usually associated with probiotics. In order to be effective, probiotic bacteria must: (1) tolerate a wide range of pH (2-8) and grow well at acidic pH (2-5); (2) survive in gastrointestinal tract environments (e.g. have excellent bile tolerance and withstand high salt concentration in the human gut); (3) produce antimicrobial substances; (4) adhere to the intestinal mucosa, which is one of the most important factors for colonization; (5) adapt to the intestinal microflora without displacing the native bacteria; (6) compete for the nutrients found on the intestinal epithelium; (7) have intrinsic antibiotic resistance, which is considered a safety issue when the risk of gene transfer is present; (8) be genetically stable, have good growth properties *in vitro* and *in vivo*, and maintain their high viability at processing, lyophilization and storage [46][50][51][62].

When selecting probiotics, it is necessary to take into account their ability to adhere to the biotic and/or abiotic surfaces and to produce the inhibitory substances, as well as their ability to survive and grow in the respective ecological unit. Finally, other important criteria are the need to be non-pathogenic and possess GRAS status.

Inhibition of biofilm formation through the use of probiotics is an attractive strategy that has received significant attention from the clinical field in the last years [64]–[66]. In controlled clinical trials, probiotic bacteria have demonstrated to be beneficial in the treatment of gastrointestinal diseases and some inflammatory bowel diseases [55]. The increasing evidence that probiotics can inhibit and prevent device-associated infections, together with an increasing interest in promoting a natural approach to health, have intensified the research in the field of probiotics and led to the proof of principle that probiotic bacteria can be used as a therapeutic strategy. The complications associated with indwelling devices have been the main driving force for the development of novel alternatives like probiotics.

3. MATERIALS AND METHODS

3.1. Experimental work

3.1.1. Surface material for biofilm formation

Urinary catheters are tubular devices commonly made from silicone or latex [67]. In order to mimic the surface material of that medical device, biofilm formation assays were performed on silicone rubber coupons (1 x 1 cm). Silicone coupons were first washed by immersion in 70% (v/v) ethanol solution for 1 h and then allowed to dry [68]. After that, they were subjected to ultraviolet (UV) sterilization for 30 min. Double-sided adhesive tape was placed in each plate well to fix the coupons to the bottom, with subsequent UV sterilization for more 30 min and gluing of sterile coupons.

3.1.2. Microorganisms and culture conditions

3.1.2.1. Probiotic strains

Two probiotic strains were tested, *Lactobacillus plantarum* and *Lactobacillus rhamnosus* (Biomodics ApS, Denmark). These bacteria were preserved at -80 ºC in 30% (v/v) glycerol cryovials, streaked on De Man, Rogosa, Sharpe Agar (MRSa; Sharlab, Spain), and incubated for 48 h at 37 ºC. Lactobacilli are primarily facultative or strict anaerobes, so generally, they have fastidious growth requirements [46]. *Lactobacillus* inocula were prepared by collecting bacterial cells from MRSa plates into 250 mL of De Man, Rogosa, Sharpe Broth (MRSB; Merck, Spain) and incubation for 18 ± 2 h at 37 ºC in an orbital shaker at 120 rpm (Agitorb 200, Aralab, Portugal). MRS was used because it supports the lactobacilli growth [69].

3.1.2.2. Uropathogenic strains

Escherichia coli CECT 434 and *Staphylococcus aureus* CECT 976 were chosen as model microorganisms of biofilm-based UTI. Bacteria were preserved at -80 ºC in 30% (v/v) glycerol cryovials, streaked on Luria-Bertani Agar (LBA; Thermo Fisher Scientific, USA), and incubated for 24 h at 37 ºC. Single colonies were collected from LBA plates, inoculated in 250 mL of AUM and incubated at 37 °C for 18 ± 2 h at 120 rpm to prepare a starting culture. AUM is an artificial urine medium which provides nutrients similar to that found in human urine. This medium supports the growth of uropathogenic bacteria at concentrations of up 10^8 CFU/mL, and it was used in a wide range of experiments modelling the growth and attachment of urinary pathogens in the clinical environment like *E. coli* and *S. aureus* [70]. AUM was prepared as described by Brooks and Keevil [70] using the formulation presented in Table A-1 in Appendix A.

3.1.3. Anti-biofilm activity of probiotics

3.1.3.1. Displacement strategy – Influence of probiotics on pre-formed pathogenic biofilms

The displacement strategy consisted in the formation of *E. coli* and *S. aureus* single-species biofilms during two different periods (24 and 48 h), after which the biofilms were inoculated with the *Lactobacillus* strains separately to evaluate biofilm cell inactivation and biomass reduction.

After overnight incubation, pathogens and probiotics were harvested by centrifugation at 3202 g for 10 min at 25 ºC (5810 R Centrifuge, Eppendorf) and washed twice with AUM. Then, cell concentration was assessed by optical density (OD) at 610 nm in AUM for all strains in order to obtain a final concentration of 10^8 CFU/mL. OD was adjusted at $OD_{610\ nm} \approx 0.15$ for *E. coli*, $OD_{610\ nm} \approx 0.2$ for *S. aureus* and $OD_{610\ nm} \approx 0.7$ for both *Lactobacillus* strains. This was confirmed by CFU counts after plating serial dilutions of *E. coli* and *S. aureus* on LBA and of *Lactobacillus* on MRSa.

Uropathogenic biofilms were formed on silicone coupons placed inside 12-well polystyrene plates (VWR, USA), where each well was filled with 3 mL of the respective bacterial suspension (*E. coli* or *S. aureus*). The plates were incubated for 24 and 48 h at 37 ºC at 140 rpm (Agitorb 200, Aralab, Portugal). After the formation of uropathogenic biofilms, cell suspensions were removed from each well and 3 mL of probiotic suspension was added for periods of contact of 6 and 24 h. The 6 h contact period was used to evaluate the short-term activity of *Lactobacillus* strains on the biofilms, while 24 h of exposure allowed to evaluate their prolonged action. Control wells were prepared with 3 mL of sterile AUM instead of probiotic suspension.

3.1.3.2. Exclusion strategy – Influence of pre-adhered probiotics on *E. coli* colonization

The exclusion strategy consisted in the formation of single-species biofilms of probiotics, after which the biofilms were exposed to *E. coli* in order to estimate their ability to prevent adhesion to substratum. An initial screening of the biofilm-forming capacity of both probiotics was performed with different culture media, periods of biofilm formation and hydrodynamic conditions in order to determine the cultivation conditions that form a more robust lactobacilli biofilm. The optimal cultivation conditions were then used for the exclusion assays.

For the initial screening, after overnight incubation, probiotics were harvested and suspensions were prepared in AUM and MRSB as previously described. The wells were loaded with 3 mL of the respective probiotic suspensions and the plates were incubated for 24, 48 and 72 h at 37 ºC at two hydrodynamic conditions: static and 140 rpm. A second screening experiment was performed to evaluate the effect of medium replacement on *Lactobacillus* biofilm formation. The culture medium was replaced every day in order to overcome possible nutritional limitations. Plates were incubated for 24, 48, 72 and 96 h in MRSB at two hydrodynamic conditions: a low shaking frequency to stimulate biofilm formation (40 rpm) and a high shaking frequency (140 rpm).

Due to the lower ability of *L. rhamnosus* to form biofilms, only *L. plantarum* was used for the exclusion strategy. *L. plantarum* biofilms were formed first and then inoculated with *E. coli* to estimate their adhesion to the pre-conditioned surfaces. *L. plantarum* biofilms were formed in MRSB as presented above: plates were incubated for 48 and 72 h at 37 ºC under low shaking conditions and the culture medium was replaced every day. After formation of *L. plantarum* biofilms, cell suspensions were removed from each well and 3 mL of *E. coli* suspension in AUM was added for 6, 24 and 48 h. Medium was replaced daily. Two negative controls were prepared: the first by adding MRSB without *L. plantarum* to a well containing a silicone coupon, and the second by adding AUM without *E. coli* to pre-established *L. plantarum* biofilms. However, due to limitations in performing laboratorial work imposed by the current pandemic situation, it was only possible to form *L. plantarum* biofilms for 24 and 48 h and expose them to *E. coli* for 6 h.

3.1.4. Biofilm analysis

The biofilm amount and culturability were assessed by CV staining and CFU counts, respectively. The medium was removed from the wells and the non-adherent cells were washed with 8.5 g/L NaCl.

3.1.4.1. Crystal violet (CV) staining method

Crystal violet staining is one of the most popular biofilm quantification methods. It is based on the ability of the CV dye to color some components present in the biofilm matrix [34] and be retained by the peptidoglycan wall of both live and dead bacterial cells [71][72].

After washing, the silicone coupons were transferred to 24-well plates (Thermo Fisher Scientific, USA) to quantify only the amount of biofilm formed on the silicone coupon. Biofilms were fixed with 1 mL of 100% (v/v) ethanol (VWR, USA), which was removed after 15 min of contact. The microtiter wells were allowed to dry at room temperature, and 1 mL of 1% (v/v) CV (Merck, Germany) solution was added to each well and incubated for 5 min. The dye attached to the biofilm was solubilized by adding 1 mL of 33% (v/v) acetic acid (VWR, USA). Finally, 200 μL of each well was transferred to a 96-well plate (VWR, USA) and the absorbance at 570 nm was read in a microtiter plate reader (SpectroStar Nano, BMG LABTECH). When absorbance values exceeded 1, samples were diluted in 33% (v/v) acetic acid, and the resulting measurements were corrected for the dilution factor and considering the absorbance of the control sample. The biofilm amount was expressed as Abs (570 nm) values. Higher absorbance values correspond to higher biofilm amounts.

3.1.4.2. Colony-forming unit (CFU) counts

Coupons were transferred to 15 mL Falcon tubes filled with 2 mL of 8.5 g/L NaCl solution. Biofilm cells were detached from the coupons by vortexing (ZX4, Velp Scientifica) for 2 min [73]. Then, serial dilutions in NaCl solution were performed, plated on LBA (for *E. coli* and *S. aureus* enumeration) and MRSa (for *L. plantarum* and *L. rhamnosus* enumeration), and incubated at 37 °C for 24 and 48 h, respectively.

To estimate the percentage of CFU reduction of pathogens exposed to probiotics, the following formula was applied:

$$\text{Reduction (\%)} = [(CFU_{Control} - CFU_{Experimental}) / CFU_{Control}] \times 100$$

where $CFU_{Control}$ corresponds to culturable cells of control biofilms (in CFU/cm^2) and $CFU_{Experimental}$ to culturable cells of treated biofilms (in CFU/cm^2).

3.1.5. Statistical analysis

All assays corresponded to at least three independent biological experiments performed with duplicate replicates. Statistical analysis was made using Excel to perform Student's t-test and single factor ANOVA (analysis of variance). The single factor ANOVA was used to test the null hypothesis. The null hypothesis in ANOVA is valid when all the sample means are equal or do not have any significant difference. On the other hand, the alternate hypothesis is valid when at least one of the sample means is different from the rest of the sample means. *p* -values < 0.1 and < 0.05 were considered as statistically significant (90% and 95% confidence interval, respectively). For each parameter, the mean and standard deviation (SD) were calculated, and data were presented as mean ± SD.

3.2.Systematic review

A systematic review consists on a review of a formulated question using systematic and explicit methods to provide a complete summary of current literature relevant to that question [74].

3.2.1. Search strategy, study eligibility and data extraction

Previously published studies concerning the use of probiotics for control and prevention of biofilm formation in medical devices were systematically reviewed according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) Statement [74]. It consists of a checklist with 27 items to include when reporting a systematic review or meta-analysis and a four-phase flow diagram that indicates how the search should be performed, which helps authors to improve and standardize the structure of systematic reviews and meta-analyses [74].

The initial search was carried out until 7 April 2020 using the following electronic databases: PubMed, ScienceDirect, Cochrane Library and Compendex. The search strategy combined a set of central keywords – Probiotic, Biofilm, Surface and Medical devices – with a wide range of terms and their combinations that were adapted for each database (the full search strategy for each database is described in Table B-1 in Appendix B). Also, the reference sections of all included articles and screened reviews were hand-searched for additional articles that were not identified through the database search. The search was limited to articles published from 1980 to April 2020 in English language only.

Peer-reviewed full-text articles related to studies where probiotics are used to control and prevent biofilm formation in medical devices were assessed for eligibility. The first screening to identify eligible studies was based on the titles of the selected articles, according to the eligibility criteria. The abstract was evaluated whenever the title was ambiguous. When the information obtained through the title and abstract was inconclusive, the full text of the article was briefly read. In case the full text was not available, the study was excluded. Any disagreement regarding study inclusion was resolved by discussion and consensus between two reviewers. After screening, all full-text articles assessed for eligibility were analyzed and evaluated by one reviewer according to the defined selection criteria.

The inclusion criteria for qualitative synthesis were: (1) studies where probiotic cells and/or substances resulting from their metabolism are used as a way to control pathogens; (2) inhibition strategies of pathogens including displacement, exclusion and competition. The exclusion criteria consisted in: (1) studies focused on the antimicrobial effect of probiotics and/or substances isolated from them, while not assessing their anti-biofilm potential; (2) studies where biotic surfaces such as epithelial tissues are used as substratum; and (3) non-original articles (including reviews or reports).

Information regarding the inhibition strategy of pathogens, probiotic strains and/or their anti-biofilm substances, biofilm-forming pathogens, biofilm substratum or surface, used methodologies (including culture conditions, biofilm platforms and biofilm analysis techniques) and obtained outcomes were extracted from each included study and inserted in an electronic spreadsheet by one reviewer. Posteriorly, this data was confirmed by another reviewer. Moreover, the percentage of reduction of

biofilm formation was retrieved whenever possible for meta-analysis. If this result was not described, an estimate was made with the values obtained from graphs and tables, comparing the appropriate values with control.

3.2.2. Quality assessment

The quality assessment of the selected studies was conducted according to an adapted Methodological Index for Non-Randomized Studies (MINORS) scale [75]. MINORS is a validated instrument designed to assess the methodological quality and potential bias of non-randomized surgical studies [75]. Although there are no methodological indices to measure the risk or the quality of laboratory-based studies, similarly to other authors [76], we adapted the MINORS scale to our specific context to assess whether the studies are representative of real conditions, evaluating their predictive value. Even if *in vitro* studies cannot provide direct knowledge regarding the effectiveness of some treatment in humans, they are a simplified representation of reality and may be useful as a preliminary screening method to identify promising clinical applications. Because of that, we considered the evaluation of the methodological quality of the *in vitro* studies a critical step in this review.

Thus, an adapted MINORS scale [75] for *in vitro* experiments was developed based on previous studies [76] to assess the methodological quality of the included studies. The following parameters were considered: a clearly stated aim; detection of bias; an adequate control group; appropriate methodology; description of pathogens, anti-biofilm substances, culture conditions, biofilm formation period, and surface substratum; the predictive value of study; clarity of results and adequate statistical analyses (the full description of each parameter is presented in Table B-2 in Appendix B). Like the original MINORS scale, the modified scale consists of 12 items scored on a 3-point scale: 0 (not reported), 1 (inadequately reported), or 2 (adequately reported), where the ideal global score would be 24. Studies were scored by one reviewer.

3.2.3. Meta-analysis

The meta-analysis was performed according to Harrer and co-workers methodology for meta-analysis [77]. The packages “meta” and “metafor” for R programming language were used to estimate the pooled effect sizes, heterogeneity testing and funnel plotting. The individual studies’ effect estimates were retrieved from the reported proportions of biofilm reduction. The heterogeneity between studies was evaluated using the I^2 and τ^2 tests. A p-value < 0.05 was considered statistically significant, and $I^2 \geq 50\%$ indicates the existence of significant heterogeneity, while τ^2 equal zero or close zero indicates that there is no variance between studies [77]–[79]. The publication bias of the selected studies was assessed using Begg’s funnel plot and Egger’s test [80].

4. RESULTS AND DISCUSSION

4.1.Experimental results

4.1.1. Displacement strategy – Influence of probiotics on pre-formed pathogenic biofilms

This strategy consisted in the formation of *E. coli* and *S. aureus* single-species biofilms during 24 and 48 h, after which they were exposed to *Lactobacillus* strains to evaluate their capacity to disrupt the pre-formed uropathogenic biofilms.

4.1.1.1. *E. coli* and *S. aureus* biofilm formation

In order to have an idea of the biofilm amount formed by *E. coli* and *S. aureus* before probiotic application and confirm their ability to adhere to silicone and form biofilm in AUM, biofilm growth was assessed after 24 and 48 h of incubation.

Both strains demonstrated to be able to grow in AUM and survive under the hydrodynamic conditions used, as well as to adhere and form stable biofilms on silicone rubber, as showed by the results of biofilm culturability (around 7 Log CFU/cm^2) and biomass amount (see Appendix C, Figure C-1). Comparing 24 and 48 h biofilms, there was an increase in the number of both *E. coli* and *S. aureus* culturable cells in about 5% ($p > 0.1$; see Appendix C, Figure C-1 A) and 71% ($p < 0.01$; see Appendix C, Figure C-1 B), respectively, which was expected since bacteria had more time to replicate. For *E. coli*, this difference was not statistically significant, which may be due to nutritional limitations inside the wells. Moreover, results of *E. coli* biofilm amount are in agreement with those of culturability assay once no difference between the two time points was observed ($p > 0.1$; see Appendix C, Figure C-1 C). The ability of *E. coli* to adhere to silicone and form stable biofilms on this surface material was previously reported by our research group [73][81].

4.1.1.2. Effect of probiotics on pre-formed biofilms

In what concerns the ability of probiotics to displace pre-formed biofilms, both probiotics showed promising results against both pathogens. The culturability of *E. coli* biofilms after exposure to probiotics (Figure 2 A and 2 B), decreased when compared to the control samples (untreated biofilms), mainly in the 24-h biofilms. The highest reductions occurred when 24-h *E. coli* biofilms contacted with probiotics for 6 h (24+6 h biofims), with reductions of 60% for *L. plantarum* and 58% for *L. rhamnosus* ($p < 0.01$; Figure 2 A). Also, reductions of 25% for *L. plantarum* and 38% for *L. rhamnosus* were obtained for 24+24 h biofilms. Nevertheless, *L. plantarum* exerted a significant anti-biofilm activity against 48+24 h biofilms of *E. coli*, reducing their culturability in 50% ($p < 0.01$; Figure 2 B). Regarding *S. aureus* culturability after probiotic addition (Figure 2 C and 2 D), there was a reduction in both 24- and 48-h biofilms, being the most significant decreases in 24+24 h biofilms (with reductions of 63% for *L. plantarum* and 47% for *L. rhamnosus*), and in 48+6 h biofilms (with reductions of 53% for *L. plantarum* and 40% for *L. rhamnosus*). For both pathogens, 48-h biofilms seemed to be less

susceptible to the antimicrobial action of probiotics (Figure 2 B and 2 D). This result was expected since longer periods of incubation usually lead to the formation of more robust and difficult to remove biofilms because of the higher complexity that biofilms acquire over time, for instance, by creating communication channels for cross-feeding metabolites to get into biofilm or by increasing the production of matrix components that confer protection against external agents [3][19][33]. All percentages of culturability reduction are summarized in Table C-1 in Appendix C.

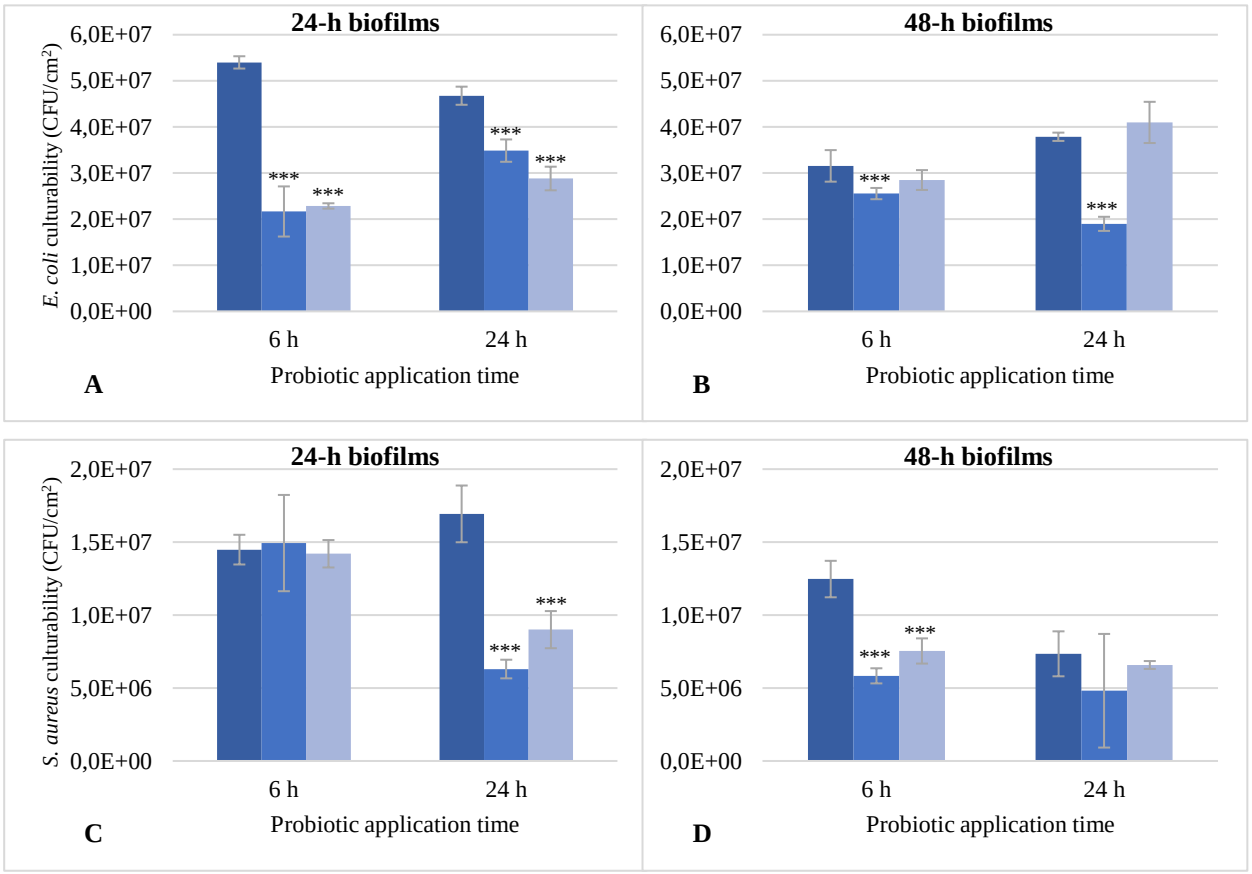

Figure 2. Culturability of 24- and 48-h biofilms of *E. coli* (A and B) and *S. aureus* (C and D) after contact with probiotics for 6 and 24 h. *E. coli* or *S. aureus* biofilm (negative control); *E. coli* or *S. aureus* biofilm + *L. plantarum*; *E. coli* or *S. aureus* biofilm + *L. rhamnosus*. Symbol *** indicates statistically different values for $p < 0.01$ when compared to control.

The activity of probiotics is directly related to the production and release of antimicrobial metabolites, competition for adhesion sites in the surface and competition for nutrients with the pathogens [82]. Therefore, the decrease of *E. coli* and *S. aureus* cells in the biofilms may occur by replacement with probiotic cells and competition with probiotics for limited resources. The reduction of *E. coli* culturable cells may be associated with the lactic acid and other organic acids resulting from probiotics metabolism, which have been shown to effectively kill gram-negative bacteria like *E. coli* by causing cell membrane damage and consequence leak of intracellular material [65]. Likewise, Fayol-Messaoudi *et al.* [83] suggested that the bactericidal activity of *Lactobacillus* strains may be due to the synergistic action of lactic acid and secreted bacteriocins, where lactic acid acts as a permeabilizer of

the outer membrane of gram-negative bacteria, thus increasing their susceptibility to antimicrobial molecules as bacteriocins. In agreement, Cadieux *et al*. [84] showed that *L. rhamnosus* strongly inhibited the growth of uropathogenic *E. coli* by producing bacteriocins, hydrogen peroxide and lactic acid. Regarding the mechanisms by which probiotics counteract *S. aureus* biofilms, Melo *et al.* [85] found that the supernatant of *L. plantarum* had a strong inhibitory effect on *S. aureus* growth. Also, Klaenhammer [86] described that *L. plantarum* bacteriocins exhibited different properties and a high spectrum of inhibition against the growth of *S. aureus*. In addition, it has been reported that *L. rhamnosus* can produce biosurfactants with antiadhesive, antimicrobial and anti-biofilm activities against several bacteria, including *E. coli* and *S. aureus* [64][87].

A higher reduction in *S. aureus* biofilm culturability compared to *E. coli* was expected due to the differences in membrane composition of both bacteria. Since *S. aureus* is a gram-postive bacteria, it lacks the outer cell membrane, thus it would have less protective barriers against external agents as probiotics. However, in this work (Figure 2), the 24-h *S. aureus* biofilms were less susceptible than *E. coli* biofilms to the action of both probiotics since a reduction in *S. aureus* culturability was only obtained after 24 h of exposure to probiotics, whereas for *E. coli* a decrease was immediately observed after 6 h of contact.

Batch fermentation profiles of *Lactobacillus* strains indicated that bacteriocin production occurs during the exponential growth phase and declines during the stationary growth phase (24 to 30 h after the start of the fermentation) [88]. Since *Lactobacillus* have been active for 42 h (18 h for the inoculum plus 24 h of exposure to *E. coli*), the reduction of probiotics activity in 24+24 h *E. coli* biofilms when compared to 24+6 h can be related to protein aggregation or proteolytic degradation of bacteriocins.

The culturability of probiotics in the sessile state was also evaluated in this study (see Appendix C, Figure C-2). It can be seen from Figure C-2 A in Appendix C that *E. coli* culturability reduction in 24-h biofilms was accompanied by the presence of probiotics in the biofilms, which suggests that the adhered *Lactobacillus* cells may have contributed to the reduction of *E. coli* culturability. However, *L. rhamnosus* lost culturability in the biofilms after 24 h of interaction with *E. coli.* For 48-h biofilms, the reduction in *E. coli* culturability was accompanied by the presence of probiotics in biofilms (see Appendix C, Figure C-2 B). In the case of *S. aureus*, the culturability reduction was not accompanied by the presence of probiotics in biofilms (see Appendix C, Figure C-2 C and C-2 D). *L. rhamnosus* lost its culturability after interaction with *S. aureus* biofilms for 24 h in both 24- and 48-h pre-formed biofilms, and *L. plantarum* was not present in any biofilm. These findings suggest that, beyond the anti-biofilm action by integration of probiotics cells into the biofilms, they may act through the release of antimicrobial substances from planktonic cells. Further assays were performed to analyze the presence of *L. rhamnosus* in the planktonic fraction (data not shown) and it was detected in an amount of $2.5x10^5$ ($\pm$ $4.3x10^3$) CFU/mL for *E. coli* and $1.6x10^4$ ($\pm$ $4.17x10^3$) CFU/mL for *S. aureus*, which helps to justify the decrease in pathogens culturability, even in absence of *Lactobacillus* cells within the biofilm. Thus, it can be suggested that *L. rhamnosus* (and *L. plantarum*) acted on the sessile cells through the release

of harmful substances into the surrounding environment. Other possible explanation for the lack of culturability of *L. rhamnosus* may be that cells were in a viable but non-culturable (VBNC) state. VBNC cells are living cells that have transiently lost their ability to grow on routine media [89][90]. VBNC cells continue to maintain membrane integrity and undamaged genetic information, while dead cells have a damaged membrane that is unable to retain chromosomic and plasmidic DNA; while dead cells are metabolically inactive, VBNC cells are metabolically active and carry out respiration; moreover, dead cells do not express genes, while VBNC cells continue protein synthesis [89][90].

Figure C-3 in Appendix C presents the results of biofilm quantification using CV staining. For *E. coli*, it was noticed that probiotics maintained or increased the biofilm amount, except the *L. rhamnosus* which decreased the total biomass of 48-h biofilms after 6 h of contact ($p < 0.01$; see Appendix C, Figure C-3 B). Regarding *S. aureus*, probiotics maintained or increased the biofilm amount, except *L. plantarum* which decreased the total biomass of 24-h biofilms after 6 h ($p = 0.04$; see Appendix C, Figure C-3 C). Therefore, there is a poor correlation between the CV staining and cell culturability methods. Since the CV assay is used for the quantification of total biofilm mass (it binds non-specifically to cells, as well as matrix components), the discrepancy between CV staining results and cell counts could be due to cell death and lysis, followed by the release of DNA. Biofilm matrix-associated DNA, EPS and proteins and/or proteinaceous material may explain the variation between absorbance reads [91]. The biofilms in contact with probiotics that present low culturability and high biomass amount probably created defense mechanisms against external stimuli that include the production of extracellular polymeric substances.

A major effectiveness of probiotics in biofilm inactivation could be achieved by adding some important nutrients for probiotics growth in the AUM used. Although this medium is the closest approach to the nutritional conditions in which probiotics are intended to be applied, the lack of some nutrients (such as glucose) does not allow probiotics to ferment in large scale, which can decrease the pH and affect the growth of pathogens. Moreover, if probiotics were under anaerobic conditions, higher rates of biofilm reduction could maybe be obtained. Fructo- (FOS) and galacto-oligosaccharides have been used to promote the growth of probiotics, mainly *Lactobacillus* genus [53]. Muñoz *et al*. [53] demonstrated that the production of acetic acid was favored in medium with FOS and lactic acid production decreased in MRSB. Moreover, FOS allowed the production of protein extracts of *Lactobacillus* strains with antimicrobial activity against important pathogens. Therefore, the supplemention of AUM with FOS may contribute to a higher inhibition of biofilm formation.

4.1.2. Exclusion strategy – Influence of pre-adhered probiotics on *E. coli* colonization

This strategy consists in the formation of *L. plantarum* and *L. rhamnosus* single-species biofilms, after which they will be exposed to pathogen suspensions in order to evaluate the ability of *Lactobacillus* strains in conditioning the silicone surface, behaving as a protective barrier to fence off and retard pathogen colonization. This strategy is relevant since various exometabolites of *Lactobacillus* strains, such as EPS and biosurfactants, may inhibit biofilm formation by interfering with the initial attachment of pathogens [65][92].

4.1.2.1. Probiotic biofilm formation – Optimization of growth conditions

Given the lack of information about the optimal growth conditions for probiotics, the first assignment related to this strategy was to optimize the cultivation conditions for the formation of robust probiotic biofilms on polymeric surfaces. These preliminary tests were essential to establish the ideal conditions for the growth of *Lactobacillus* biofilms that will cover the silicone surfaces before contact with uropathogenic strains. For this purpose, two different culture media (AUM and MRSB) were tested at different incubation times (24, 48 and 72 h) and under static and dynamic conditions. Figure 3 presents the results obtained for culturability and total biomass of *L. plantarum* and *L. rhamnosus* biofilms in all tested conditions.

Concerning culture media, MRSB was used because it is an appropriate growth medium for *Lactobacillus* strains [69]. On the other hand, AUM was used for probiotic biofilm growth since it is expected that *Lactobacillus* strains are in contact with urine after the catheters are inserted into the patients, reducing the risk of a nutrient shock which may compromise their antimicrobial action. Regarding the agitation conditions, it is expected that the static state allows the formation of biofilms with higher biomass amounts since the gravitational force can favor cell deposition [93]. On the contrary, we believe that biofilms formed under dynamic conditions are thinner, but more cohesive, since cells are probably metabolically more active and focused on trying to overcome the adverse conditions created by the fluid flow [94]. Thus, it is expected that the biofilms formed in MRSB have a higher amount of culturable cells, whereas the biofilms formed in static conditions have more biomass due to sedimentation.

Regarding *L. plantarum* biofilm culturability (Figure 3 A), the presence of culturable cells on silicone coupons was confirmed in 83% of the cases, indicating its ability to survive and form biofilms in the tested conditions. Looking at the effect of incubation time on cell culturability, *L. plantarum* culturability decreased over time, regardless of the growth medium and hydrodynamic conditions tested. The exception was the static biofilm formed in MRSB, which exhibited a slight increase in culturability (about 0.6 Log CFU/cm^2) from 48 to 72 h ($p < 0.01$). Thus, it can be concluded that longer incubation times do not ensure more culturable cells in the biofilm, which may be due to nutritional limitations inside the wells. Since the culture medium was not replaced, most of the nutrients may have been

depleted, leading to limitations in the probiotic growth. For static conditions using AUM, the culturable cells disappeared after 48 and 72 h of incubation. In what concerns to the culture medium, biofilms formed in MRSB exhibited higher culturability than those formed in AUM, as expected and previously explained (except in the case of biofilms formed under dynamic conditions, where no difference between the two media was observed at 24 h (p = 0.29), and an increase of around 0.8 Log CFU/cm^2 in the number of culturable cells was registered at 48 h (p < 0.01)).

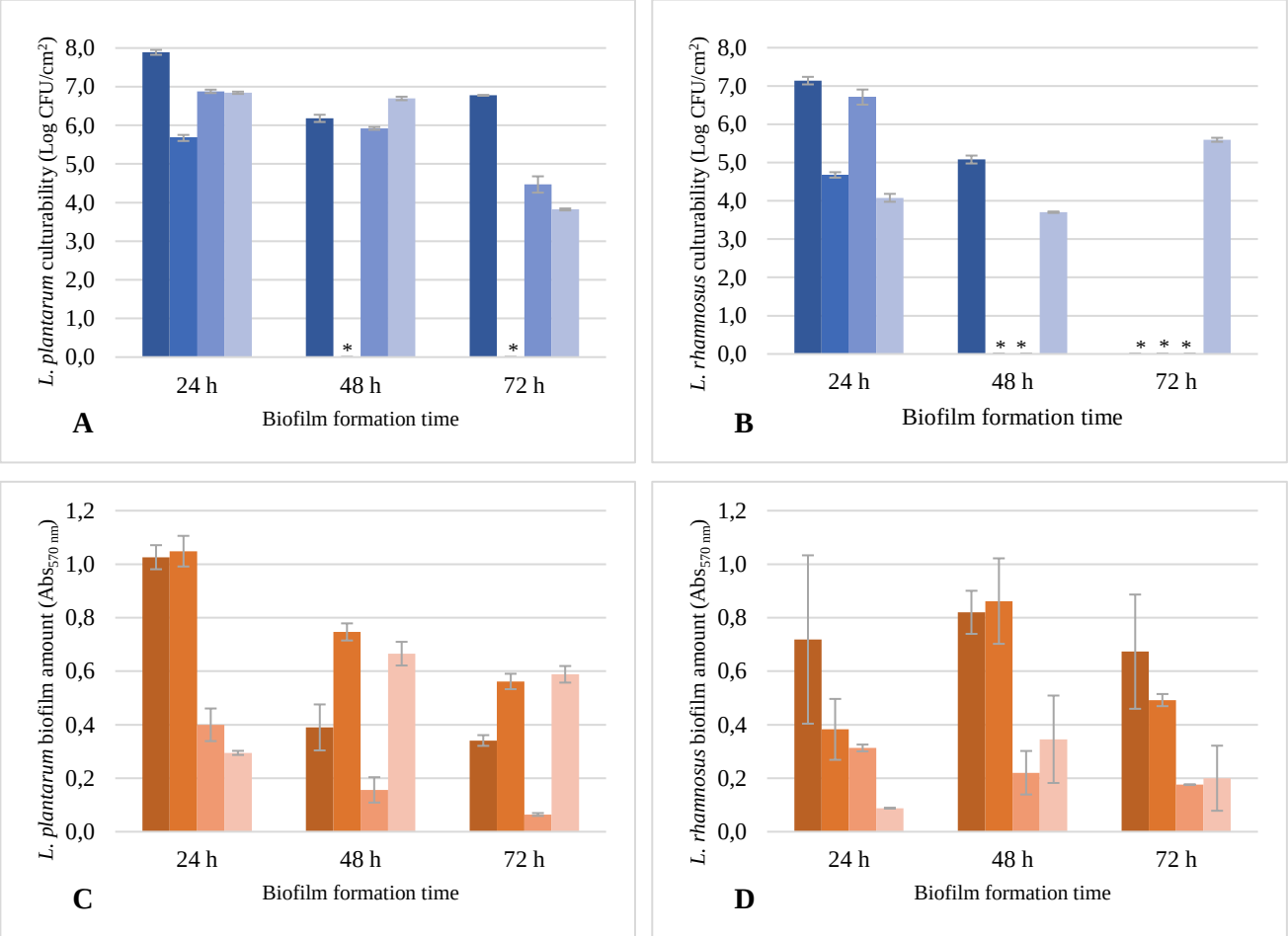

Figure 3. Culturability (A and B) and total biomass (C and D) of *L. plantarum* and *L. rhamnosus* biofilms, respectively, after 24, 48 and 72 h of development. Biofilms were formed in two different media (AUM and MRSB) and in dynamic and static conditions. MRSB - Static state; AUM - Static state; MRSB - Dynamic state; AUM - Dynamic state. * No colonies were detected.

The ability of *L. plantarum* to form biofilm on the silicone coupons under the tested conditions was confirmed by CV staining (Figure 3 C). Regarding the effect of incubation time on biofilm formation, the CV results are in agreement with those of the culturability assay, once there was a marked reduction in biofilm biomass over time (p < 0.1); the only exception was the biofilm formed under dynamic conditions in AUM, which suffered an increase in biomass between 24 and 48 h (p < 0.01). Note that for 48 and 72-h biofilms formed under static conditions in AUM, the loss of *L. plantarum* culturability (Figure 3 A) was accompanied by a higher amount of biofilm (Figure 3 C). This could be explained by a VBNC state of *L. plantarum* cells [89] or by the existence of cell death and lysis, followed

by the release of intracellular material [91]. Concerning the hydrodynamic conditions, biofilms formed under static state presented significantly higher biomass amounts than the biofilms grown under shaking conditions, regardless of the incubation time and culture medium ($p < 0.1$; Figure 3 C). This was not verified only for the 72-h biofilms formed in AUM, where a similar amount of biofilm was found for both fluid conditions ($p = 0.34$).

Other research groups evaluated the biofilm-forming capacity of *L. plantarum* and showed that different strains of *L. plantarum* were able to grow as a biofilm in MRS broth on abiotic surfaces, such as polystyrene and glass [57][95][96].

Regarding *L. rhamnosus* biofilm culturability (Figure 3 B), the presence of culturable cells on silicone coupons was confirmed in 58% of the cases, but such biofilm formation was inconsistent. It can be observed an enormous difficulty of probiotic biofilm cells in maintaining their culturability for 48 and 72 h. As observed for *L. plantarum*, culturability decreased over time, except in the case of dynamic biofilm formation in AUM, which resulted in a significant increase of cell culturability from 48 to 72 h ($p < 0.01$). The 24-h biofilms presented higher culturability values, with those formed in MRSB having the highest number of culturable cells (on average 6.9 Log CFU/cm^2 against 4.4 Log CFU/cm^2 for biofilms formed in AUM).

The CV results (Figure 3 D) confirmed the ability of *L. rhamnosus* to form biofilms on the silicone coupons under the tested conditions. For the biofilms that have lost culturability, the high absorbance values obtained through the CV method were possibly associated with cells in a VBNC state, dead cells and/or production of extracellular polymeric substances, as mentioned for *L. plantarum*. It is important to note that the biofilms formed in static conditions had a higher biomass amount than the biofilms developed under shaking conditions, regardless of the growth medium. This was probably favored by the gravitational force, which contributes to cell deposition, as previously referred. Concerning the growth medium, for the same incubation time and hydrodynamic condition, there were no statistical significant differences between the biofilms formed in MRSB and AUM ($p > 0.1$). The exception was the biofilms formed under dynamic conditions during 24 h, where the biofilms formed in MRSB presented higher biomass content than those formed in AUM.

L. rhamnosus was described as able to form *in vitro* biofilms on polystyrene, a characteristic strongly influenced by the culture medium used [97] and pH, where low pH values decrease the *L. rhamnosus* capacity to develop biofilms [98]. Other *L. rhamnosus* strains have shown a strong capacity to form biofilms in MRSB also on polystyrene surfaces [95][98]. However, some authors showed that *L. rhamnosus* had a poor ability to grow in mono-species biofilms, but adhered and developed better in presence of other microorganisms in multi-species biofilms [99].

In this work, *L. plantarum* seems to be the probiotic with higher ability to form biofilms. The higher propensity of *L. plantarum* to form biofilms compared to *L. rhamnosus* can be justified by the higher production of extracellular polymeric substances [13][17][91][100][101]. Fernández *et al.* [91] reported that *L. plantarum* capacity to form biofilms is highly affected by the composition of the culture

medium, growth temperature and time of incubation. They found that *L. plantarum* biofilms typically contain proteins and/or proteinaceous material cementing the biofilm cells to the surface. Also, the auto-aggregation is a key mechanism in biofilm formation and the well-known ability of probiotic strains to auto-aggregate might promote adhesion to host cells and displacement of pathogens [65][102][103]. The auto-aggregation capability and the degree of hydrophobicity of the bacterial outer surface influence the adhesive properties of lactobacilli. In fact, microorganisms' ability to adhere to certain substrata depends on van der Waals attraction forces, gravitational forces, steric interactions, protein adhesion and electrostatic interactions, but one of the more important factors is the hydrophobicity of the cells [3][8] [104]. Despite the natural hydrophobicity of each species, several compounds are described to be directly related to the cell surface characteristics, such as lipoteichoic acid (LTA), outer membrane proteins and lipids, surface fibrils, various fimbriae or polysaccharides [104]. Some authors suggested the existence of an S-layer, a mono-layer composed of identical proteins or glycoproteins, on the surface of specific *Lactobacillus* strains that are involved in the adhesion phenomenon [92]. However, the hydrophobic character of microorganisms may not be immutable since it was reported that microorganisms can switch between hydrophobic and hydrophilic phenotypes, depending on the environmental conditions and growth phases, and consequently attach to a wide range of surfaces [105]. Besides cell surface properties, the characteristics of the surface material play an important role in bacterial adhesion, including the chemical composition, physicochemical properties (surface charge, hydrophobicity, topography and roughness), and physicomechanical parameters (elastic modulus and hardness) [20]. The relatively hydrophilic nature of *L. rhamnosus* compared with *L. plantarum* [87] may be a possible explanation for the less amount of *L. rhamnosus* biofilm in the hydrophobic silicone. Thus, the hydrophobic nature, auto-aggregation capacity, and efficient production of extracellular polymeric substances and other cell components may be responsible for maintaining *L. plantarum* population adhered to the silicone coupons [106]. Another factor that could have influenced the adhesion of these strains is the production of biosurfactants, which may alter the surface wettability and energy.

L. plantarum seems to be the most promising probiotic strain both in counteracting the pre-formed biofilms of pathogens and in forming stable biofilms on silicone surfaces, so its use was proposed for the exclusion strategy. As mentioned before, medium replacement may interfere with the extent of biofilm formed and we believe that more probiotic biofilm can be obtained by adding fresh medium to each well every day. Hence, a second screening was performed to evaluate the effect of medium replacement on *L. plantarum* biofilm formation. We also decided to extend the period of biofilm formation until 96 h (24, 48, 72 and 96 h time points) to confirm the effect of incubation time on biofilm formation. Since MRSB was the medium that exhibited higher culturability values and biomass amount and it is an appropriated growth medium for *Lactobacillus* strains, these assays were performed only with MRSB. Regarding the hydrodynamic conditions, although the biofilms formed without shaking had higher culturability and biomass amount, this condition will not be used for the exclusion assays once we believe that these biofilms consisted of poorly adhered cells as a result of sedimentation. This

characteristic was observed while unsticking the silicone coupons from the 12-well plates, when the static-formed biofilm often disintegrated. Instead of that, we decided to test a low shaking frequency in order to try to get the high biomass content characteristic from static conditions and the cohesive character of biofilms formed under more intense shaking conditions. Figure 4 presents the results obtained for culturability and total biomass of *L. plantarum* in the tested conditions.

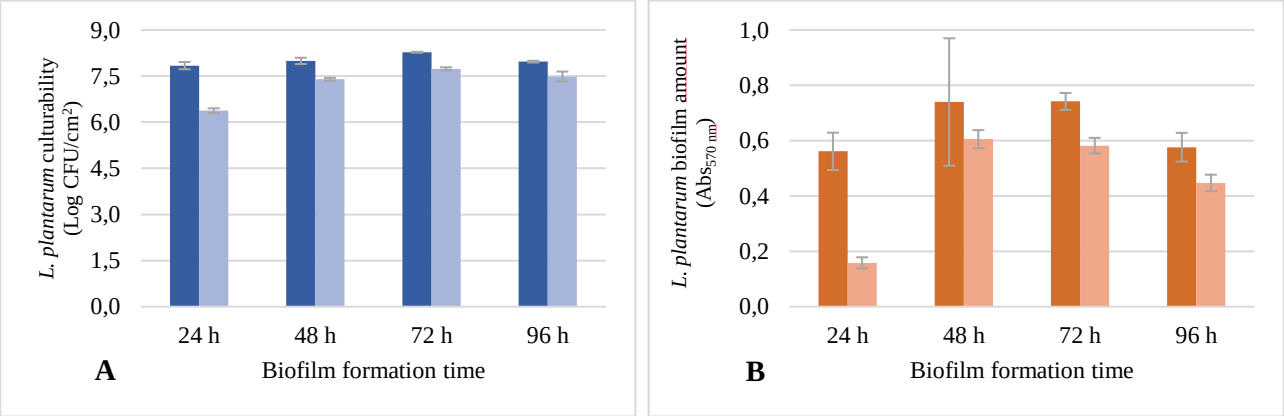

Figure 4. Culturability (A) and total biomass (B) of *L. plantarum* biofilms after 24, 48, 72 and 96 h of development in MRSB with medium replacement at low and high shaking frequencies. 40 rpm; 140 rpm.

Comparing the results obtained for *L. plantarum* in Figure 3 and 4, it is clear that replacing the culture medium every day enhanced biofilm formation. All biofilms had more than 6.4 Log CFU/cm^2, with an average of 8.0 Log CFU/cm^2 for low shaking conditions and 7.3 Log CFU/cm^2 for high shaking conditions (Figure 4 A). Additionally, the results of biofilm biomass (Figure 4 B) are in agreement with culturability. Thus, the nutritional limitations in wells appear to have been overcome and the *L. plantarum* biofilms for exclusion assays were formed for 48 and 72 h (higher culturability and biomass amount) in MRSB under low shaking conditions (40 rpm).

4.1.2.2. Effect of probiotics on biofilms

For the exclusion assays, since *E. coli* seemed to be the most vulnerable pathogen to the action of probiotics in the displacement strategy, only this bacterium was used as model uropathogen. This strategy consisted in the formation of *L. plantarum* biofilms for 48 and 72 h, after which they were exposed to *E. coli* for periods of contact of 6, 24 and 48 h. However, due to the events caused by the COVID-19 pandemic, it was only possible to form biofilms of *L. plantarum* for 24 and 48 h, and expose them to *E. coli* for 6 h, as presented in Figure 5.

For the incubation period tested, *L. plantarum* biofilms were able to inhibit *E. coli* adhesion to silicone. In fact, significant reductions in *E. coli* adhesion were caused by both 24-h (94%, $p < 0.01$; Figure 5 A) and 48-h biofilms (97%, $p < 0.01$; Figure 5 B). These preliminary results demonstrate that prevent the initial attachment of pathogens by coating the surface with probiotic biofilms seems to be a promising strategy to reduce the incidence of CAUTI.

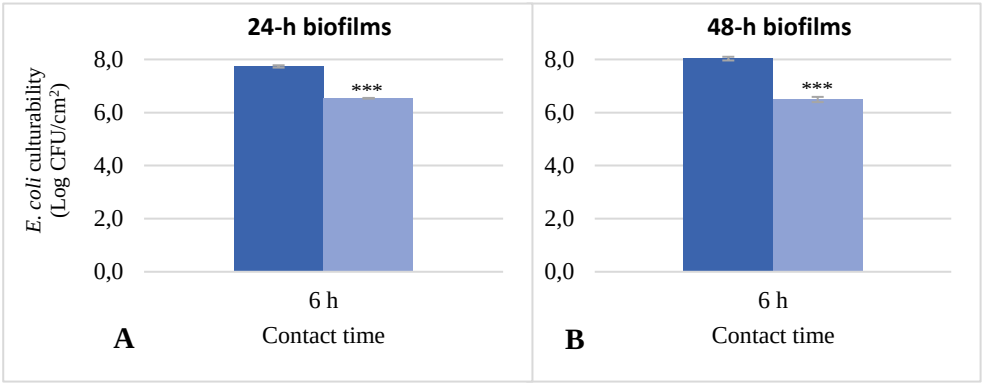

Figure 5. *E. coli* culturability after 6 h of contact with 24- (A) and 48-h (B) biofilms of *L. plantarum*. *E. coli* biofilm (negative control); *E. coli* biofilm + *L. plantarum*. Symbol *** indicates statistically different values for $p < 0.01$ when compared to control.

4.2.Systematic review

4.2.1. Study selection and characterization

The search resulted in a total of 188 articles identified through database searching using the described methodology. This number was increased upon inclusion of 17 additional records identified through other sources (previous searches and references of selected articles), resulting in a total of 205 studies. After duplicates removal, 165 records proceeded to the screening phase. From these, 111 records were excluded based on title and abstract, since they did not fulfill the pre-determined criteria for eligibility. Further examination of the remaining 54 full-text articles resulted in the exclusion of 9 articles according to the exclusion criteria: 2 studies were focused on the antimicrobial effect of probiotics, not performing biofilm assays; 3 studies used epithelial tissues as substrata; 1 study used anti-biofilm substances not resulted from probiotics metabolism; 3 studies correspond to non-original articles. Of the 45 studies eligible for qualitative synthesis, 36 presented the required data for meta-analysis. All this information is schematized in Figure 6.

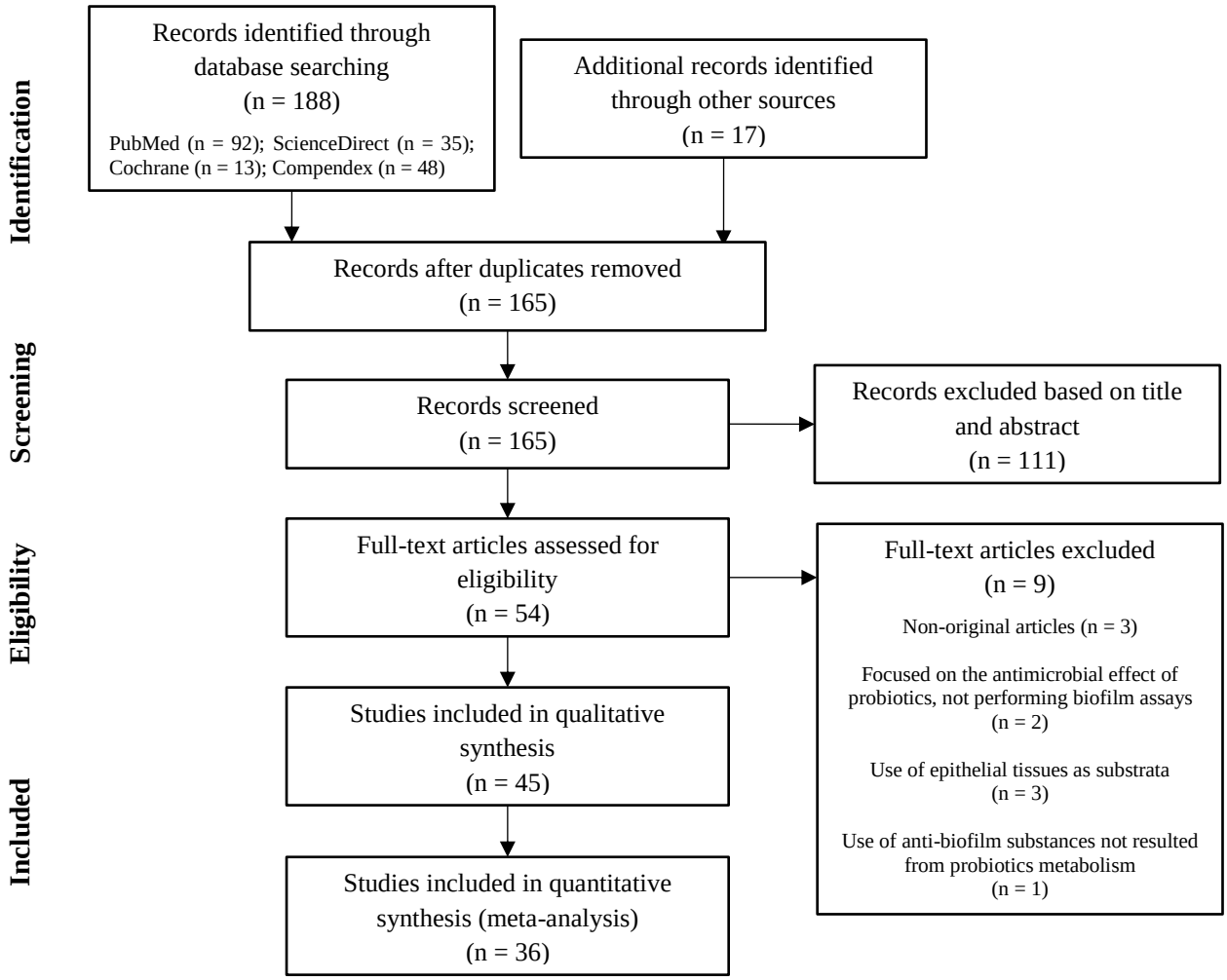

Figure 6. Summary of the literature search based on the PRISMA flow-chart.

Currently, the treatment of biofilm-related infections still depends on conventional antimicrobial therapies, which increase the selective pressure in favor of antibiotic-resistant strains, posing a threat for patients' health [3][15]. Although in the last decades the advances in the development of new effective antimicrobial therapeutics have reduced the incidence of device-associated infections and improved the understanding of biofilm development on medical devices, probiotics were recently introduced as a potentially reliable option to inhibit or delay the onset of biofilm formation in medical devices [107].

In this context, the main advances on probiotics and their metabolites for preventing and eradicating biofilms from the surfaces of medical devices are reviewed and discussed. Tables 1, 2 and 3 describe the anti-biofilm strategies and the potential of probiotics and their metabolites against several bacterial and fungal species (Tables D-1, D-2 and D-3 in Appendix D present the extended analysis of each individual study). Anti-biofilm strategies were grouped into three categories as represented in Figure 7: (1) displacement, (2) exclusion and (3) competition. Displacement strategy consists in the disruption of the architecture of pre-formed pathogen biofilms through the addition of probiotics and/or their metabolites; exclusion resides in pre-coat a surface with probiotics and/or their metabolites in order to inhibit pathogens adhesion; and competition consists in the co-culture of planktonic probiotics and/or their metabolites and pathogen cells. The division into different strategies intends to find out at which stage of biofilm development is most promising and advantageous to act, once each strategy represents a possible approach. Besides, some anti-biofilm substances may be more compatible with a particular strategy due to its characteristics, as further explained. Thus, studies were grouped according to the anti-biofilm substance, and all strategies included the use of biosurfactants, bacteriocins, EPS, cell-free supernatants (except in the exclusion strategy), cells, and other less representative substances isolated from probiotics. Because one study can hold more than one strategy and/or anti-biofilm substance, a total of 22 experiments were included for displacement, 23 for exclusion, and 33 for the competition strategy. The most used methodologies for biofilm examination were CFU counting and CV staining, which were used in 23 and 22 studies of the total 45, respectively. Polystyrene and silicone-based surfaces were the biofilm substratum most used (33 and 31% of the studies, respectively). Moreover, this review addresses the microbial biofilms developed on a wide range of indwelling medical devices, such as central venous catheters [108], urinary tract devices (catheters and stents) [6][7][109], voice prostheses [110]–[112] and dental prostheses [99][113][114]. Regarding the anti-biofilm substances, probiotic cells (44% of the studies) were the most used, followed by biosurfactants (24% of the studies), and *Lactobacillus* were the dominant probiotic genus.

Table 1. Characteristics of displacement studies in medical devices.

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Ref.
Biosurfactants	*L. brevis* *L. gasseri* *L. jensenii* *L. rhamnosus*	*A. baumannii* *C. albicans* *C. krusei* *C. tropicalis* *E. aerogenes* *E. coli* *K. pneumoniae* MRSA *S. aureus* *S. saprophyticus*	Polystyrene Silicone elastomeric discs	Biosurfactants disrupted the biofilms of all tested bacteria by 16-65%, depending on the tested concentrations. For yeasts, a biofilm reduction of about 35% was achieved.	[64], [108], [115]
Bacteriocins	*L. acidophilus* *L. plantarum*	*P. aeruginosa* *S. marcescens*	Foley silicone catheter pieces Polystyrene	Bacteriocins showed inhibitory activity against biofilm formation of *P. aeruginosa* (59%) and biofilm-living cells of *S. marcescens* (48%).	[116], [117]
EPS	*Leu. citreum* *Leu. mesenteroides* *Leu. pseudo-mesenteroides* *Ped. pentosaceus*	*E. coli* *E. faecalis* *S. aureus*	N. A.	The capacity of EPS to disrupt the pre-formed biofilms increased when increasing its concentration, and it was lower than its capacity to prevent biofilm adhesion. Biofilm formation was reduced in 53-77%.	[118]
Cell-free supernatants (crude and/or neutralized)	*L. fermentum* *L. gasseri* *L. helveticus* *L. pentosus* *L. plantarum* *L. rhamnosus* *S. salivarius*	*C. albicans* *C. krusei* *C. parapsilosis* *C. tropicalis* *E. coli* *K. pneumoniae* *P. aeruginosa* *S. aureus*	Glass Polystyrene Polyurethane PVC	Cell-free supernatants significantly induced biofilm disruption on the different surfaces by 38-80%, depending on the species. The neutralized supernatants showed good anti-biofilm activity, inhibiting up to 74% of *P. aeruginosa* and 78% of *K. pneumoniae* biofilm formation.	[82], [87], [119], [120], [121]
Cells	*B. infantis* *B. longum* *E. faecium* *L. acidophilus* *L. casei* *L. casei rhamnosus* *L. casei shirota* *L. fermentum* *L. helveticus* *L. paracasei* *L. plantarum* *L. reuteri* *L. rhamnosus* *L. rhamnosus* GG *Lact. lactis* *Lact. lactis cremoris* *S. cremoris* *S. salivarius* *S. thermophilus*	*A. vaginae* *C. albicans* *C. tropicalis* *E. coli* *G. vaginalis* *S. aureus* *S. mutans* *S. oralis* Staphylococcal strains Streptococcal strains	Bovine enamel saliva-coated Denture surface Glass Polyurethane Saliva-conditioned titanium discs Silicone latex Silicone rubber	Probiotics overlaid on pre-formed biofilms reduced biofilm culturable cells of gram-positive bacteria by 79-99% and biofilm formation by 89-94%. Biofilm culturable cells of yeasts were significantly reduced by more than 63%. *B. infantis* or *E. faecium* did not significantly reduce the number of yeasts in biofilms. *L. rhamnosus* microcapsules significantly reduced *E. coli* culturable cells in the biofilm up to 80% in a dose-dependent manner.	[8], [87], [110], [114], [121], [122], [123], [124], [125]
Lipoteichoic acid (LTA)	*L. plantarum*	*A. naeslundii* *E. faecalis* *L. salivarius* *S. mutans*	Glass Polystyrene	LTA activity was inconsistent once in one study it did not affect the established biofilm, and in another study the pre-formed biofilms were disrupted in a dose-dependent manner.	[126], [127]

Notes: EPS – Exopolysaccharides; MRSA – Methicillin-Resistant *Staphylococcus aureus*; PVC – Polyvinyl Chloride; N. A. – Not Available.

Table 2. Characteristics of exclusion studies in medical devices.

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Ref.
Biosurfactants	*L. acidophilus* *L. brevis* *L. casei* *L. delbrueckii* *L. fermentum* *L. helveticus* *L. paracasei* *L. plantarum* *L. reuteri* *L. rhamnosus* *Lact. lactis* *S. thermophilus*	*B. cereus* *B. subtilis* *C. albicans* *C. tropicalis* *E. coli* *E. faecalis* *K. pneumoniae* *L. innocua* *L. monocytogenes* *P. aeruginosa* *P. mirabilis* *P. putida* *P. stuartii* *P. vulgaris* *R. dentocariosa* *S. aureus* *S. epidermidis* *S. flexneri* *S. marcescens* *S. salivarius* *S. typhi*	PDMS discs Polystyrene Silicone elastomeric discs Silicone rubber	Pre-adsorbed biosurfactants displayed high anti-adhesive activity against both gram-positive (61-97%) and gram-negative (40-75%) bacteria. Also, pre-adsorbed biosurfactant significantly reduced the adhesion of yeasts to silicone by 50-85%. Biosurfactants demonstrated anti-biofilm and anti-adhesive potential against *P. vulgaris* and *B. subtilis* on PDMS discs.	[9], [12], [108], [111], [112], [128], [129], [130]
Bacteriocins	*L. fermentum* *L. plantarum*	*P. aeruginosa* *S. aureus*	Foley silicone catheter pieces Polystyrene	Pre-coating with bacteriocins reduced the number of biofilm culturable cells in 99%.	[109], [131]
EPS	*L. fermentum* *Leuc. citreum* *Leuc mesenteroides* *Leuc pseudo-mesenteroides* *Ped. pentosaceus*	*E. coli* *E. faecalis* *P. aeruginosa* *S. aureus*	Polystyrene	Pre-coating with EPS reduced the number of biofilm culturable cells of *P. aeruginosa* by 96% and inhibited the adhesion of bacteria in a dose-dependent manner by 87-90%.	[118], [131]
Cells	*E. coli* Nissle 1917 *L. acidophilus* *L. casei* *L. casei rhamnosus* *L. fermentum* *L. paracasei* *L. rhamnosus* *Lact. lactis* *Lact. lactis* ssp. *lactis* *S. thermophilus*	*A. naeslundii* *C. albicans* *E. coli* *E. faecalis* *F. nucleatum* *Klebsiella* ssp. *S. mutans* and non-*mutans* streptococci strains *P. aeruginosa* *S. aureus* *S. mutans* *S. oralis* *S. sobrinus* UPEC *V. dispar*	Denture surface Foley silicone catheter pieces Glass Saliva-coated hydroxyapatite discs Polystyrene Saliva-conditioned titanium discs Silicone Silicone latex	Probiotics reduced the adhesion of pathogens up to 3 Log CFU, depending on the species, and biofilm biomass by 8-30%. Pre-coating with EcN biofilms reduced the adherence of *E. faecalis* on silicone up to 2 Log CFU.	[6], [8], [114], [124], [132], [133], [134], [135]
Collagen-binding protein (p29)	*L. fermentum*	*E. coli* *E. faecalis*	Polyisobutylene-polystyrene (PIB-PS) copolymer Silicone rubber	Coating with p29 resulted in a reduction of 34 and 75% in *E. coli* adhesion and 47 and 18% in *E. faecalis* adhesion to silicone rubber and PIB-PS, respectively.	[7]
Lipoteichoic acid (LTA)	*L. plantarum*	*S. mutans*	Polystyrene	Biofilm formation was inhibited, but to a lesser degree in comparison with co-incubation (40% of reduction).	[126]

Notes: CFU – Colony-Forming Units; EcN – *E. coli* Nissle 1917; EPS – Exopolysaccharides; PDMS – Polydimethylsiloxane; UPEC – Uropathogenic *E. coli*.

Table 3. Characteristics of competition studies in medical devices.

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Ref.
Biosurfactants	*L. acidophilus* *L. brevis* *L. helveticus* *L. jensenii* *L. paracasei* *L. reuteri* *L. rhamnosus*	*A. baumannii* *B. cereus* *C. albicans* *E. coli* MRSA *P. aeruginosa* *S. aureus* *S. marcescens* *S. mutans* *S. oralis*	Medical grade silicone tubes Polystyrene Polystyrene pre-coated with human plasma Silicone elastomeric discs Saliva-conditioned titanium discs	Biosurfactants displayed high anti-adhesive activity and inhibited biofilm formation of all pathogens by a remarkable decrease in biomass production (60-100%) and biofilm culturable cells (90-99%). The inhibitory effect showed a dose-dependence.	[9], [64], [108], [113], [129]
Bacteriocins	*L. fermentum* *L. plantarum*	*P. aeruginosa* *S. aureus*	Polystyrene	Co-incubation with bacteriocins reduced the number of *P. aeruginosa* biofilm culturable cells in 93% and biofilm formation of both pathogens in 56-62%.	[109], [131]
EPS	*L. delbrueckii* ssp. *bulgaricus* *L. fermentum* *L. rhamnosus*	*B. cereus* *E. faecalis* *L. monocytogenes* *P. aeruginosa*	Polystyrene	Co-incubation with EPS reduced the number of *P. aeruginosa* biofilm culturable cells in 97% and inhibited biofilm formation between 74 and 90%, depending on the species, in a dose-dependent manner.	[10], [131]
Cell-free supernatants (crude, acid, neutralized, proteinase K-treated, heat-treated and/or octyl-sepharose beads-treated)	*B. subtilis* *L. acidophilus* *L. fermentum* *L. gasseri* *L. helveticus* *L. paracasei* *L. plantarum* *L. rhamnosus* *S. salivarius*	*C. albicans* *C. krusei* *C. parapsilosis* *C. tropicalis* *E. coli* *E. faecalis* *K. pneumoniae* ssp. *pneumoniae* *P. aeruginosa* *S. aureus* *S. mutans* *S. oralis*	Glass Polystyrene Polyurethane PVC Saliva-conditioned titanium discs Silicone	Cell-free supernatants were able to reduce the number of biofilm culturable cells by more than 81% and inhibit the ability of pathogens to adhere to the different surfaces by 39-99%. Generally, neutralized supernatants had less effect on biofilm formation.	[5], [82], [92], [119], [121], [124], [126], [136], [137]
Cells	*E. coli* Nissle 1917 *L. acidophilus* *L. casei* *L. casei rhamnosus* *L. fermentum* *L. helveticus* *L. paracasei* *L. plantarum* *L. rhamnosus* *L. rhamnosus* GG *L. salivarius* *Lact. lactis* ssp. *lactis* *S. thermophilus*	*A. naeslundii* *C. albicans* *E. coli* EHEC *F. nucleatum* *K. pneumoniae* ssp. *pneumoniae* *S. mutans* and non-*mutans* streptococci strains *P. aeruginosa* *S. aureus* *S. epidermidis* *S. mutans* *S. oralis* *S. sanguinis* *S. sobrinus* *V. díspar*	Bovine enamel saliva-coated Glass Polystyrene Polyurethane Polypropylene Saliva-coated hydroxyapatite discs Saliva-conditioned titanium discs Silicone latex Silicone rubber	The adhesion of pathogens was significantly reduced by the presence of probiotic cells (11-93%) and their culturability decreased up to 7.2 Log CFU. *L. rhamnosus* microcapsules significantly reduced biofilm formation up to 82% in a dose-dependent manner. *Lactobacillus* strains showed ability to inhibit the growth of an uropathogenic biofilm on silicone rubber for at least 8 days. EcN was able to outcompete pathogenic strains during biofilm formation, inhibiting biofilm culturability up to 4 Log CFU.	[8], [11], [92], [99], [121], [123], [124], [125], [132], [133], [136], [138], [139]
Lipoteichoic acid (LTA)	*L. plantarum*	*A. naeslundii* *E. faecalis* *L. salivarius* *S. mutans*	Glass Human dentin slices Polystyrene Saliva-coated hydroxyapatite discs	LTA inhibited single- and multi-species biofilm formation by 75 and 57%, respectively, in the different surfaces.	[126], [127]

Notes: CFU – Colony-Forming Units; EcN – *E. coli* Nissle 1917; EHEC – Enterohemorrhagic *E. coli* O157:H7; EPS – Exopolysaccharides; MRSA – Methicillin-Resistant *Staphylococcus aureus*; PVC – Polyvinyl Chloride.

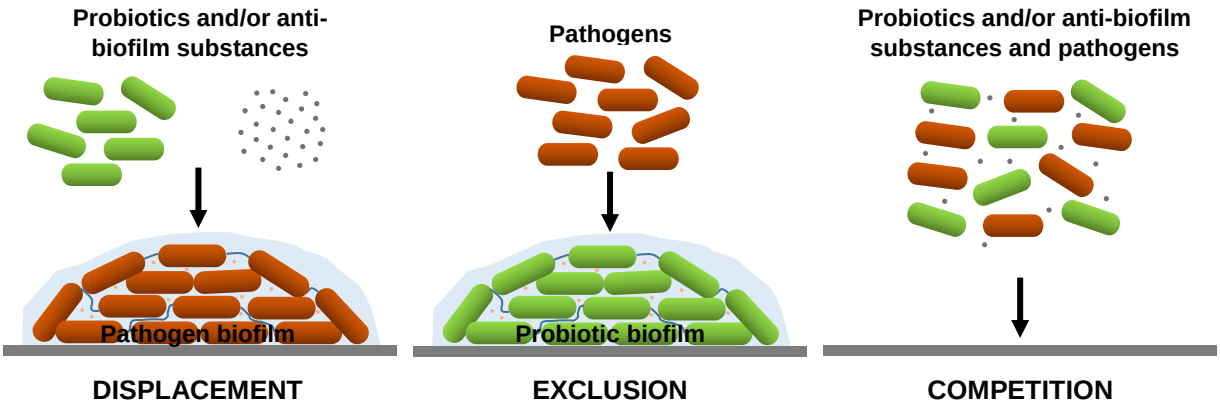

Figure 7. Scheme of the inhibition strategies of pathogens by probiotics and/or anti-biofilm substances isolated from them. Green rods represent probiotic cells, brown rods represent pathogen cells, and small points represent all anti-biofilm substances. Displacement – probiotics and/or anti-biofilm substances are added to pre-formed pathogen biofilm; Exclusion – pathogen cells are added to pre-formed probiotic biofilm or pre-coated surface with anti-biofilm substances; Competition – planktonic probiotics and/or anti-biofilm substances and pathogen cells are co-cultured. Adapted from Pérez-Ibarreche *et al*. [140].

Biosurfactant production is a mechanism by which probiotics interfere with pathogens. It has been proved that the adsorption of biosurfactants to a substratum surface may interfere with microbial adhesion and desorption processes [141][142]. Biosurfactants are a structurally diverse group of surface-active compounds released or associated to the cell wall of a wide variety of microorganisms [143]–[145], with amphipathic properties (both hydrophilic and hydrophobic moieties within the same molecule) [108][146][147]. Recently, biosurfactants have received special attention due to their advantages over synthetic surfactants, such as higher biodegradability, lower toxicity and effectiveness at extreme environments [142][144][145][148]. Although the mechanisms of action of biosurfactants are not fully elucidated, due to their amphipathic nature they can form an interfacial film that affects the properties (surface energy and wettability) of the original surface, modifying its hydrophobicity and reducing the surface and interfacial tensions, thus affecting the adhesion of pathogens [141][142][149]. Beyond that, biosurfactants were reported to disrupt the cytoplasmic membrane, leading to cell lysis and metabolite leakage, by inducing changes in physical membrane structure or by disrupting protein conformations changing important membrane functions [108][129][150].

Up to date, several studies have reported the effectiveness of probiotics' biosurfactants in antagonizing microbial biofilm formation on abiotic surfaces. Biosurfactants were studied on different surfaces, including silicone-based surfaces, polystyrene, polydimethylsiloxane (PDMS), and titanium discs, and tested against a broad spectrum of gram-positive and gram-negative bacteria, and yeasts. The anti-biofilm properties of biosurfactants were examined in the three strategies, however, due to the tendency of these molecules to accumulate at the interfaces and change the surface tension and hydrophobicity, the majority of the studies inspected the pre-conditioning of the surface materials with these substances.

Regarding to the displacement strategy (Table 1), data showed that different concentrations of biosurfactants were able to disrupt the biofilms of all tested bacteria on polystyrene at different levels (16-65%) [64][115]. For yeasts, a biofilm reduction of about 35% was achieved [115]. When the same studies compared the effect of biosurfactants in displacement and other strategies, a lower reduction in biofilm formation was observed for the displacement strategy. Sambanthamoorthy *et al.* [64] demonstrated that biosurfactants reduced initial adherence and disrupted pre-formed biofilms of clinical multidrug-resistant strains, however their action was more pronounced on pre-coated surfaces than on pre-formed biofilms. Likewise, Ceresa *et al*. [108] demonstrated that biosurfactants decreased the initial deposition and biofilm growth of *Candida albicans* on silicone surfaces, but on pre-formed biofilms, no significant inhibitory activity was observed. This suggests that biosurfactants are more suitable for the pre-coating and co-incubation approaches than for attacking pre-formed biofilms.

Pre-adsorbed biosurfactants (Table 2) displayed high anti-adhesive activity against both gram-positive (61-97%) and gram-negative (40-75%) bacteria. Biosurfactants produced by *Lactobacillus* spp. strains showed good results in reducing biofilm formation of both gram-positive (61-87%) and gram-negative (40-75%) bacteria on polystyrene [9][128]–[130]. Biosurfactants also demonstrated anti-adhesive potential against *P. vulgaris* and *Bacillus subtilis* on PDMS discs [130]. Anti-adhesive experiments on silicone rubber indicated their use as a promising strategy for the development of anti-adhesive biological coatings on urinary catheters [12] and voice prostheses [111][112]. Surlactin, a biosurfactant isolated from *L. acidophilus* RC-14, caused a marked reduction in deposition rates and adhesion on silicone rubber after 4 h, with particularly effect against *E. faecalis*, *E. coli* and *S. epidermidis* [12]. Biosurfactants isolated from *S. thermophilus* A and *Lact. lactis* 53 significantly reduced the adhesion of *S. epidermidis* and *Streptococcus salivarius* to silicone by more than 90% and of yeasts by 50-85% [111][112].

Similarly, the results obtained when the competition strategy was chosen (Table 3) were very promising. All biosurfactants isolated from *Lactobacillus* spp. displayed high anti-adhesive activity and inhibited biofilm formation by a remarkable decrease of biomass production (60-100%) and culturable cells (90-99.9%), depending on pathogen species. Biosurfactants were successfully tested on silicone-based surfaces, reducing almost completely the microbial adhesion after 3 days [108][129]. Likewise, biosurfactants displayed high anti-adhesive activity against biofilm formation of *Serratia marcescens* (73%), *Acinetobacter baumannii* (76%), *E. coli* (79%) and *S. aureus* (88%) in polystyrene surfaces [9][64]. Sambanthamoorthy *et al.* [64] suggested that the structural differences in cell wall and membranes exhibited between treated and untreated cells may be due to biosurfactants interference in the cell division process. Additionally, Ciandrini *et al.* [113] showed that biosurfactants inhibited the adhesion and biofilm formation of *Streptococcus mutans* (77-99.9%) and *Streptococcus oralis* (66-98%) in saliva-conditioned titanium discs. Several studies demonstrated that this inhibitory effect in different biomedical scenarios is dose-dependent.

Recently, bacteriocins have been explored to combat several microbial infections in the biomedical field and are used to prevent microbial contamination in the food industry [151]. Bacteriocins are a heterogeneous group of ribosomal synthesized proteins or peptides that show both bactericidal and bacteriostatic activities against other bacteria [151]–[154], and are classified in three classes (Type I, II or II) based on their structural, physiochemical, molecular characteristics and antibacterial activity [109][153]. Bacteriocins are one of the most interesting alternatives to antibiotics due to their high stability, low toxicity, significant potency and both broad and narrow spectra of activity [109][152][154]. There are several proposed mechanisms of biofilm inhibition by bacteriocins, including i) pore formation in target-cell wall leading to leakage of cellular content; ii) inhibition of cell wall synthesis; iii) depolarization of the cytoplasmic membrane; and iv) render target-cell membrane permeable to small molecules and thereby disrupt the proton motive force resulting in cell death [4] [60][151].

Up to date, few studies reported the *in vitro* effectiveness of bacteriocins produced by probiotics to inhibit biofilm formation in medical surfaces. Anti-biofilm experiments were performed on silicone-based surfaces and polystyrene, and no significant differences between strategies were observed. Bacteriocins decreased the amount of pre-formed biofilms of *P. aeruginosa* on Foley catheters by 59% [117] and of *S. marcescens* on polystyrene by 48% (Table 1) [116]. Likewise, bacteriocins-coated Foley catheters prevented the adhesion of *P. aeruginosa* and *S. aureus* (Table 2) [109]. Sharma *et al*. [131] compared the ability of bacteriocins to reduce the biofilm formation of *P. aeruginosa* PAO1 through pre-coating and co-incubation on polystyrene and, in both experiments, the number of biofilm culturable cells were reduced in more than 93%. The same authors also demonstrated synergic associations between bacteriocins and EPS, which enhanced cell death of *P. aeruginosa* (Tables 2 and 3) [131]. Likewise, Ray Mohapatra and Jeevaratnam [109] demonstrated that the co-incubation of bacteriocins and pathogens prevented microbial adhesion of *P. aeruginosa* and *S. aureus* by 56 and 62%, respectively (Tables 2 and 3). Although bacteriocins isolated from probiotics inhibited microbial adhesion and biofilm formation, their ability to weaken biofilms formed on medical devices warrants further investigation.

In recent years, some bacterial EPS were proposed to regulate biofilm formation and inhibit microbial adhesion. EPS are a large group of long-chain high-molecular-mass biopolymers that are produced by the metabolic pathways of various microorganisms and differ in terms of monomer composition, molecular mass, degree of branching and structure [10][155]–[157]. Among the diversity of EPS-producing microorganisms, EPS of LAB have several applications due to their potential biological activities, namely antioxidant, immunomodulating, anti-tumor or antimicrobial properties [155][156]. Although EPS produced by probiotics have many industrial applications, such as food products, bioemulsifiers or bioflocculants [156][157], their application as an anti-biofilm agent, particularly in medical devices, has been barely explored and the EPS mechanisms to prevent biofilm formation are not well understood.

EPS isolated from *Lactobacillus* spp. and *Leuconostoc* spp. presented anti-adhesive and antimicrobial activities against some gram-positive and gram-negative bacteria in polystyrene. Their capacity to disperse pre-formed biofilms was demonstrated against *E. coli*, *E. faecalis* and *S. aureus* (53 to 77% of biomass reduction) (Table 1) [118]. However, the ability of EPS to disrupt pre-formed biofilms is lower than its capacity to prevent biofilm adhesion. In fact, in both exclusion and competition strategies, EPS showed excellent anti-adhesive activity against all tested pathogenic biofilms, reducing biofilm formation by about 90% in a dose-dependent manner [10][118] and the number of *P. aeruginosa* biofilm culturable cells up to 97% (Tables 2 and 3) [131]. EPS might have decreased QS signals needed for biofilm formation, acting through the inhibition of initial attachment and auto-aggregation of cells by affecting the bacterial surface properties and restricting cell-surface interactions [118][131].

Probiotics are known to produce many metabolites with antimicrobial and anti-biofilm activities which are frequently secreted for the surrounding medium, such as bacteriocins and antimicrobial peptides, diverse organic and fatty acids, biosurfactants and hydrogen peroxide [4][60]. These exometabolites are often collected from the cell-free supernatant to assess their combined effect on biofilm formation of pathogens. The mechanism of action of supernatants is directly related to the antimicrobial metabolites produced by probiotics, which may differ between species since different probiotics release different amounts and types of metabolites.

In the last years, several studies using cell-free supernatants were performed in order to determine their anti-biofilm activity in medical devices. The influence of cell-free supernatants (mainly those isolated from *Lactobacillus* spp.) on biofilm formation in different surfaces was studied, including silicone, glass, polystyrene, polyurethane, polyvinyl chloride (PVC) and saliva-conditioned titanium discs against a wide range of pathogens. Supernatants were not used in the exclusion strategy, probably due to limitations in forming a coating with some of their antimicrobial substances.

Cell-free supernatants significantly induced biofilm disruption on different surfaces, between 38 and 80% for *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *Candida* spp. [82][119]–[121] (Table 1). *E. coli* biofilms challenged with supernatants also decreased cell density in glass [87]. The activity of neutralized supernatants was assessed by Poornachandra *et al.* [120] to exclude the activity of organic acids. Indeed, the supernatants showed good anti-biofilm activity, inhibiting biofilm formation of *P. aeruginosa* and *K. pneumoniae* up to 74% and 78%, respectively (Tables 1 and 3), suggesting the presence of bioactive substances such as bacteriocins or biosurfactants. However, the ability of supernatants to prevent biofilm adhesion seems to be slightly higher than to disrupt pre-formed biofilms. James *et al.* [121] and Varma *et al.* [119] compared the displacement and competition strategies in polystyrene and polyurethane, and PVC, respectively, and demonstrated that supernatants had a slightly higher effect on biofilm formation in co-incubation assays (Tables 1 and 3). Regarding to competition strategy (Table 3), cell-free supernatants reduced the number of biofilm culturable cells by more than 81% [92][121][124][137] and inhibited pathogens adhesion to different surfaces by 39-99% [5][82][92] [119][121][124][126][136][137], depending on the species (Tables 1 and 3). Also, *Candida* spp. multi-

species biofilm formation was reduced by 67% on silicone surface [82]. Likewise, supernatants were effective in reducing the number of culturable cells of oral biofilms of *S. oralis* and *S. mutans* by more than 5 Log CFU on saliva-conditioned titanium discs [124]. In addition, Ahn *et al.* [126] co-incubated *S. mutans* cells with proteinase K-, heat- and octyl-sepharose beads-treated supernatants to characterize molecules that might be involved in inhibiting *S. mutans* biofilm formation, and demonstrated that proteinase K- or heat-treated supernatants decreased biofilm formation up to 70%, while octyl-sepharose beads-treated supernatant had less effect (Table 3).

Probiotic cells have been widely studied over the years and proposed as a promising protection against pathogen colonization on medical devices. Similarly to supernatants, the activity of probiotic cells is directly related to the produced antimicrobial metabolites, and also with the competition for nutrients and adhesion sites on the surface [82].

The antagonistic effect of probiotics, mainly *Lactobacillus* spp., was studied on several surfaces, including silicone-based surfaces, glass, polystyrene, polyurethane, polypropylene, saliva-conditioned titanium and hydroxyapatite discs, and denture surface against a wide range of bacteria and yeasts. Comparing all the strategies, the action of probiotics was independent of the strategy adopted, since no significant differences were observed between them. When the same articles compared the effect of probiotic cells between the strategies, an equal reduction was achieved either by pre-coating the surface with probiotics, co-incubation or disruption of pre-formed biofilms [8][121][124][125][132][133]. The most tested probiotic species were *L. rhamnosus, L. plantarum, L. acidophilus* and *L. fermentum*.

Probiotics were successfully tested on silicone-based surfaces, resulting in inhibition of biofilm culturable cells of *S. aureus* (99.9%), *E. faecalis* (99.9%) and multi-species biofilms (83-95%) (Tables 1, 2 and 3), thereby revealing their potential application in urinary catheters [6][8][11][134] and voice prostheses [110][122]. Moreover, probiotics exerted anti-biofilm activity on polystyrene (Tables 2 and 3), reducing the biofilm culturability of *E. coli* [125][135] and *K. pneumoniae* [92] up to 99.9%, and the biofilm amount of *S. mutans* strains between 28 and 70% [133][136]. Probiotics inhibited biofilm development in other polymeric surfaces, reducing the number of biofilm culturable cells of *C. albicans* up to 80% in polyurethane (Tables 1 and 3) [121] and *E. coli* O157:H7, *S. aureus* and *S. epidermidis* up to 99.9% in polypropylene (Table 3) [138]. In glass, the introduction of *L. rhamnosus* and *L. reuteri* into pre-formed biofilms resulted in a significant killing of pathogens (Table 1) [87][125], while *L. acidophilus* coating demonstrated high resistance to bacterial adhesion (Table 2) [134]. Another application where probiotics have been gaining interest is in oral biofilm treatment or caries prevention. Therefore, *L. rhamnosus* GG, *S. thermophilus* and *Lact. lactis* ssp. reduced bacterial adhesion by decreasing biofilm culturable cells of several pathogens up to 85% in saliva-coated hydroxyapatite discs (Tables 2 and 3) [99][132]. In the same way, *L. paracasei* and *L. rhamnosus* evidenced a reduction between 2 and 8 Log CFU of *S. mutans* and *S. oralis* in saliva-conditioned titanium discs depending on the strategy used (Tables 1, 2 and 3) [124]. Likewise, *L. rhamnosus* GG and *L. casei* significantly disrupted and inhibited the adhesion of *C. albicans* biofilms on the denture surface by 99.9% (Tables 1

and 2) [114]. Song *et al.* [125] demonstrated that the use of microcapsules containing *L. rhamnosus* GG cells disrupted the architecture of *E. coli* biofilm and impeded biofilm formation in approximately 80%. Data indicated that *L. rhamnosus* GG microcapsules decreased the transcriptional activity of numerous virulence-related genes that are involved in QS, thereby inhibiting biofilm formation [125]. *E. coli* Nissle 1917 (EcN) has been widely characterized and used for many years as a probiotic. It was found that this strain was able to outcompete *E. coli*, *S. aureus* and *S. epidermidis* during co-incubation [138][139], via the extracellular function of DegP, a bifunctional periplasmic protein [138]. Lastly, Chen *et al*. [6] demonstrated that pre-coating with EcN biofilms reduced the adherence of *E. faecalis* to silicone up to 2 Log CFU for 11 days (Table 2). Therefore, probiotics are able to antagonize the growth and development of potentially pathogenic biofilms, which makes them a relevant solution to fight many biofilm-based infections. It can be concluded that the inhibition of pathogens is dependent on the *Lactobacillus* strains involved.

Other substances are emerging to fight biofilm formation on medical surfaces. One of them is a collagen-binding protein (p29) isolated from *L. fermentum* RC-14. Cadieux *et al.* [7] demonstrated that coating silicone rubber and polyisobutylene-polystyrene (PIB-PS) copolymer with p29 inhibited attachment of *E. coli* in 34 and 75% and *E. faecalis* in 47 and 18%, respectively (Table 2).

Another molecule that recently attracted attention due to its capacity to interfere with pathogens infection and inhibit inflammatory responses is the LTA [126][127]. LTA is an amphiphilic glycolipid commonly present in the cell wall of bacteria which is involved in cell adhesion and biofilm formation [126][127]. Ahn *et al.* [126] and Kim *et al.* [127] evaluated the potential application of LTA isolated from *L. plantarum* against *S. mutans* and multi-species biofilms (*Actinomyces naeslundii*, *L. salivarius*, *S. mutans* and *E. faecalis*) on polystyrene, hydroxyapatite discs, human dentin slices and glass (Tables 1, 2 and 3). The displacement of pre-formed biofilms was the less effective strategy (Table 1), but co-incubation reduced biofilm formation in 75% for *S. mutans* and 57% for multi-species cultures (Table 3) [126][127]. LTA probably interferes with sucrose decomposition, which is required for the production of EPS [126]. These results encourage future investigation of LTA on other surfaces.

Thus, based on reviewed studies, independently of the strategy used, the different anti-biofilm substances showed a promising effect in both prevention and control of biofilms, suggesting the use of probiotics to counteract pathogenic biofilms in medical devices. Nevertheless, avoiding the initial attachment of pathogens by coating the surfaces seemed to be the best approach to fight biofilm-based infections instead of removing established biofilms, which, theoretically, is a much harder task.

Overall, these data provide important clues that should be considered upon the development of new antimicrobial treatments to eradicate biofilm-related diseases.

4.2.2. Quality assessment

As mentioned above, it is important to analyze qualitatively the methodologies and procedures used in reviewed studies in order to guarantee the validity of the results and their predictive power.

The 45 included studies were scored according to the adapted MINORS scale presented in Table B-2 in Appendix B. Since the maximum score assigned by criterion is 2, the ideal global score would be 24. The studies score varied between 14 and 24, and the mean was 19.7 ± 2.7. The mean of each criterion is presented in Table B-2 in Appendix B.

All articles clearly stated the aim of the work (criterion 1, mean=1.93) and the used methodologies (criterion 4, mean=1.84). Additionally, 44 of the 45 articles had an adequate control group corresponding to untreated biofilms (criterion 3, mean=1.93). All articles described the biofilm platforms used, while 7 studies did not mention the surface for biofilm formation (criterion 9, mean=1.84). Moreover, in 87% of the studies, the concentration of the anti-biofilm substances was reported (criterion 6, mean=1.87) and only 1 study did not report the biofilm formation period (criterion 8, mean=1.76). Therefore, the high score attributed to these criteria indicates the methodological quality of the eligible studies.

Although these results are very encouraging, there is a lack of detail in the description of some methodological aspects that may contribute to the high heterogeneity among the efficacy of the different anti-biofilm substances. About 31% of the studies did not mention the sample size of replicates or independent assays (criterion 2, mean=1.64), thus decreasing the validity of their results. One-third of the studies did not report the concentration of the pathogenic microorganisms used for biofilm inoculation (criterion 5, mean=1.64), which is crucial to replicate the experiments, and in 51% of the studies the cell density used was not representative of an ideal clinical scenario (criterion 10, mean=1.07), decreasing the studies' predictive value. The different concentrations used through the studies may also contribute to increased heterogeneity, since starting from different cell concentrations will affect differently the biofilm treatment. Also, culture conditions were not properly reported in 73% of the studies (criterion 7, mean=1.27). About 64% of the studies did not report the hydrodynamic conditions, but of the studies in which this parameter was mentioned, 24% were performed under agitation and 11% in static conditions. Since shear forces affect the formation and structure of biofilms on medical devices [72][158], it is important to conveniently describe the hydrodynamic conditions used for biofilm assays.

In turn, the temperature is mainly 37 ºC over the studies (differs in 13% of the studies). For the biofilm inhibition period, 6 h was assumed as the minimum reasonable period to evaluate the short-term action of probiotics; 20% of the anti-biofilm studies were performed in less than 6 hours. This may be another factor contributing to studies' heterogeneity since biofilm formation varied from hours to several days.

Moreover, about 36% of the included studies reported the effect of anti-biofilm substances as the proportion of biofilm reduction, without specifying cell concentrations either for control or treatment after biofilm incubation (criterion 11, mean=1.58). Although the ratio depends on the reduction between control and treated biofilm, it does not illustrate the real degree of biofilm inhibition. Also, it should be

noted that 42% of the studies either did not perform any statistical analysis or the statistic tests were not adequate to validate the main outcomes (criterion 12, mean=1.31).

Although indwelling medical devices may differ in design and surface characteristics, the rate and extent of biofilm formation are affected by the physicochemical properties of the surface (such as hydrophobicity, charge and energy [3]), concentration, genus and species of microorganisms initially contaminating the device, biofilm formation period, flow rate and composition of the culture medium [13][26]. Therefore, despite the promising results of probiotics against microbial biofilms in medical devices, it is essential to highlight the importance of standardizing the *in vitro* testing conditions in order to facilitate the comparison between studies, and properly reporting and analyzing the results as a way to increase the studies' predictive value.

4.2.3. Meta-analysis

Thirty-six of the 45 selected studies were included in the meta-analysis. The standard mean proportions of biofilm reduction and the respective standard deviation were retrieved from these studies and grouped according to the anti-biofilm substance used either to inhibit or control microbial biofilms, including the biosurfactants, cells, EPS, and cell-free supernatants. Since the number of studies demonstrating the efficacy of bacteriocins and other anti-biofilm substances was reduced, they were not included in the meta-analysis. Additionally, the methodology of analysis used for the biofilm quantification (CFU counting, CV method for biomass quantification, or other) was not discriminated because it only represents different means to evaluate the efficacy of anti-biofilm substances.

The pooled effect estimates and respective 95% confidence interval were calculated for the four anti-biofilm substances. Figure 8 represents the forest plot of the pooled effect estimates for the proportion of biofilm reduction induced by biosurfactants. Heterogeneity in the mean proportion of biofilm reduction was not observed among the 10 included studies ($I^2 = 0\%$; $\tau^2 = 0$; $p = 0.61$). The pooled results showed a mean proportion (95% CI) of 70% (62-78%) and a predictive interval of 61-80%.

In turn, the heterogeneity in the mean proportion of biofilm reduction induced by cells (Figure 9) among the 17 included studies was statistically significant ($I^2 = 96\%$; $\tau^2 = 0.0169$; $p < 0.01$). This heterogeneity can be justified by great variability in the growth conditions (e. g. media, hydrodynamic conditions, cell concentration and inoculation periods) used among the selected studies. For example, Stepanović *et al*. [159][160] showed that the biofilm formation of some microorganisms was remarkably reduced under dynamic conditions. Since most of the studies did not indicate shear stress or shear rate values, it was not possible to fully compare the effectiveness of the different substances. Also, heterogeneity can be justified by the test of different species of probiotics, whose amount and diversity of metabolites will vary according to the species, as well as their action. For this substance, the pooled results showed a mean proportion (95% CI) of 77% (68-87%) and a predictive interval of 46-100%.

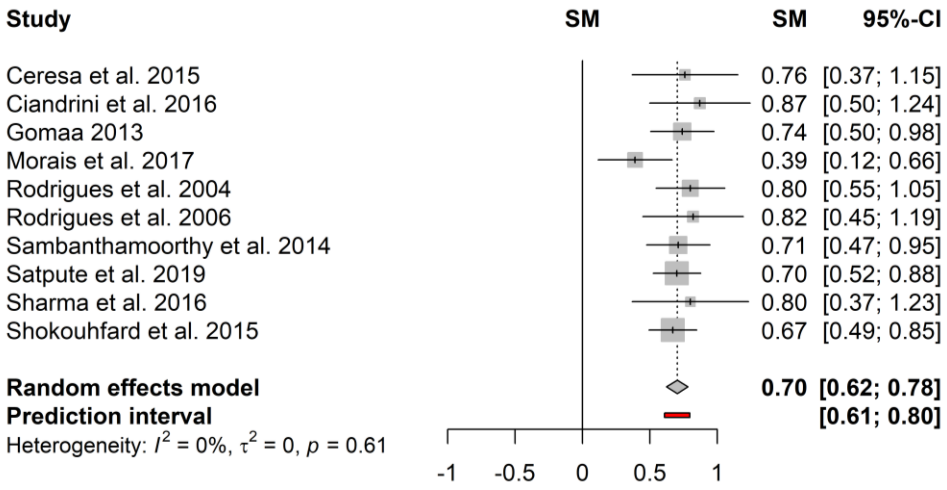

Figure 8. Forest plot of the pooled effect estimates for the proportion of biofilm reduction induced by biosurfactants. The vertical dashes represent the effect estimate and the horizontal line is the respective confidence interval at 95% (95%-CI) obtained for each study. Grey squares represent the standard deviation of each study, while the grey diamond represents the pooled effect estimates. The red bar represents the predictive interval. Heterogeneity test: I^2 – I^2 test; τ^2 – Tau-squared test.

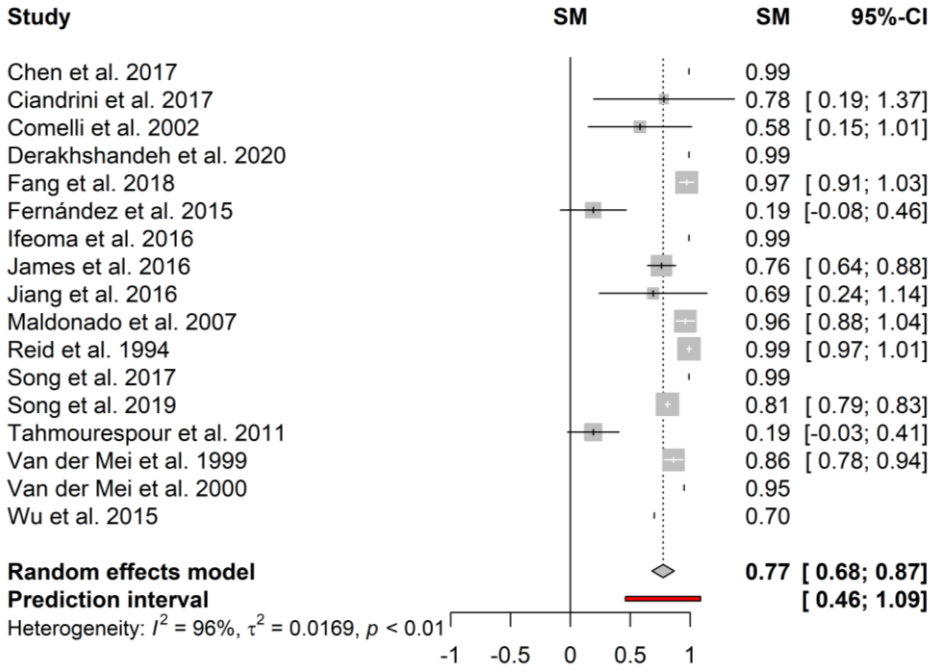

Figure 9. Forest plot of the pooled effect estimates for the proportion of biofilm reduction induced by cells. The vertical dashes represent the effect estimate and the horizontal line is the respective confidence interval at 95% (95%-CI) obtained for each study. Grey squares represent the standard deviation of each study, while the grey diamond represents the pooled effect estimates. The red bar represents the predictive interval. Heterogeneity test: I^2 – I^2 test; τ^2 – Tau-squared test.

Regarding the biofilm reduction induced by EPS, only 3 studies were included in the meta-analysis (Figure 10). Although the heterogeneity in the mean proportion was not statistically significant (I^2 = 0%; τ^2 = 0; p = 0.58), these could have been caused by the reduced number of studies included. The

pooled results showed a mean proportion (95% CI) of 88% (86-90%) and a predictive interval of 76-100%.

Lastly, Figure 11 represents the forest plot of the pooled effect estimates for the proportion of biofilm reduction induced by cell-free supernatants. Heterogeneity in the mean proportion of biofilm reduction among the 10 included studies was statistically significant (I^2 = 99%; τ^2 = 0.05; p < 0.01). Studies' heterogeneity can be justified by great variability in the growth media used, which would influence the expression of the several metabolic pathways and, consequently, the quantity and diversity of produced metabolites, thereby affecting the ability of probiotics to inhibit biofilm formation. Kimelman and Shemesh [137] studied the influence of growth medium on the anti-biofilm activity of supernatants produced by *B. subtilis* and found that growth of the *Bacillus* cells in the MRS led to a significantly higher inhibition on *S. aureus* biofilm formation when compared to that produced in the LB medium, proving the growth media impact. Furthermore, the versatility of LAB to use a wide range of substrates by several pathways may enhance the heterogeneity [161]. On the other hand, the supernatants are submitted to a purification step in which, depending on the adopted procedure (speed and time of centrifugation, type of filters), different compounds will be obtained in each pool, where there may be a higher prevalence of a given compound. Chapman *et al.* [5] obtained the supernatant only by a centrifugation step at 2050 g for 10 min, while Ciandrini *et al*. [124] centrifuged the bacterial cultures at 176 400 g for 15 min and filtered the supernatant through a 0.22 µm pore size filter, and Kimelman and Shemesh [137] used a 0.45 µm filter. Thus, small variations in the growing environment or purification procedures can have significant impacts on the quantity and diversity of metabolites produced by probiotics and this may contribute to the high heterogeneity of the studies.

In addition, the pooled results displayed a mean proportion (95% CI) of 76% (59-93%) and a predictive interval ≥16%.

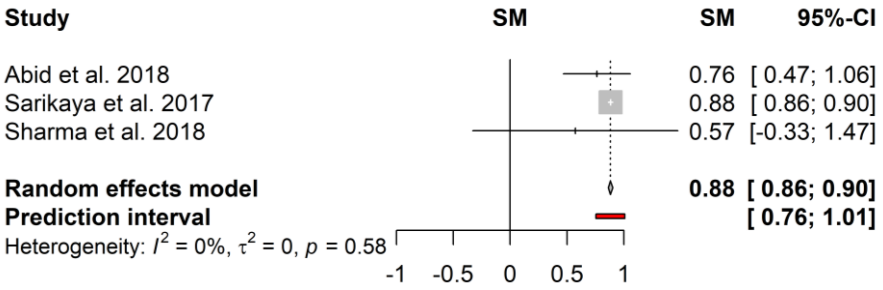

Figure 10. Forest plot of the pooled effect estimates for the proportion of biofilm reduction induced by EPS. The vertical dashes represent the effect estimate and the horizontal line is the respective confidence interval at 95% (95%-CI) obtained for each study. Grey squares represent the standard deviation of each study, while the grey diamond represents the pooled effect estimates. The red bar represents the predictive interval. Heterogeneity test: I^2 – I^2 test; τ^2 – Tau-squared test.

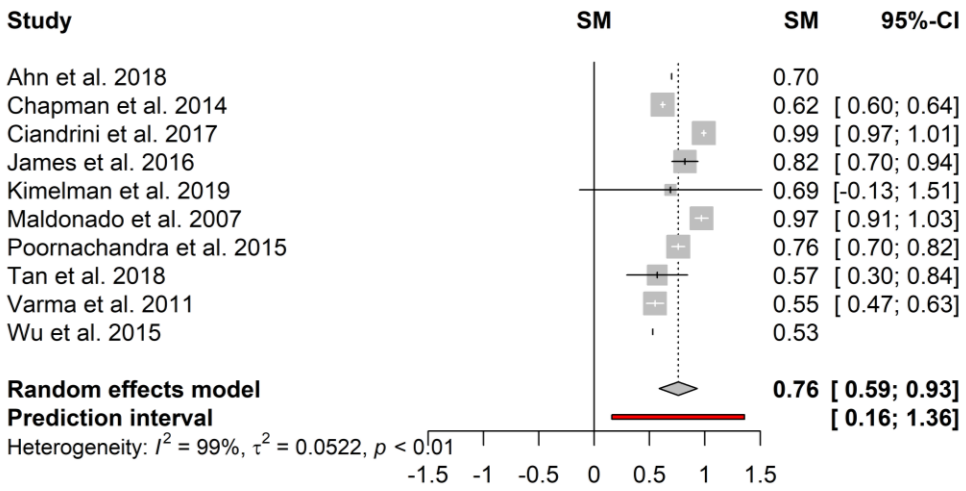

Figure 11. Forest plot of the pooled effect estimates for the proportion of biofilm reduction induced by cell-free supernatants. The vertical dashes represent the effect estimate and the horizontal line is the respective confidence interval at 95% (95%-CI) obtained for each study. Grey squares represent the standard deviation of each study, while the grey diamond represents the pooled effect estimates. The red bar represents the predictive interval. Heterogeneity test: $I^2 – I^2$ test; τ^2 – Tau-squared test.

4.2.3.1. Publication bias

For the assessment of publication bias, the Begg's funnel plot and the Egger's test were used (see Appendix E, Figure E-1). Both the funnel plot and the Egger's test for the analysed substances, including the biosurfactants, cell, EPS, and cell-free supernatants were not statistically significant (p = 0.412, p = 0.321, p = 0.072, p = 0.875, respectively), suggesting no publication bias in sample size.

Overall, the systematic review and meta-analysis showed that the use of probiotics is a promising approach to prevent biofilm formation by a broad spectrum of pathogenic microorganisms and their efficacy seems to be independent of the anti-biofilm strategy applied: displacement, exclusion or competition. The meta-analysis results showed a pooled effect estimate for the proportion of biofilm reduction of 70% for biosurfactants, 76% for free-cell supernatants, 77% for cells, and 88% for EPS. Nevertheless, in the case of cells and free-cell supernatants, significant heterogeneity was observed among the selected studies that must be considered when interpreting these results. Although EPS have a greater proportion of biofilm reduction, it is premature to consider that this is the most effective substance on the inhibition and control of biofilms, because only 3 studies were included, requiring further research about its application in control of microbial biofilm formation in medical devices.

In general, probiotics proved to be one effective approach against both gram-negative and gram-positive bacteria, as well as yeasts, which might be an advantage for their further application in the clinical field to control and prevent nosocomial infections associated to medical devices.

4.2.4. Limitations and strengths

A limitation of this meta-analysis resides in the fact that the studies were grouped regardless of the anti-biofilm strategy employed (competition, displacement, or exclusion) due to the high heterogeneity between the efficacy of the different anti-biofilm substances. Additionally, the proportion

of biofilm reduction was analysed without considering the biofilm-forming pathogens (gram-positive and gram-negative bacteria, yeast, or multi-species biofilms). However, according to linear regression models, the effect estimate for the proportion of biofilm reduction was not significantly influenced by the anti-biofilm strategy or the biofilm forming pathogen (see Appendix E, Figure E-2), and according to Begg's funnel plot and Egger's test, there was no publication bias in sample size, not influencing the meta-analysis results. Moreover, the meta-analysis results should be interpreted with caution since about one-third of the included studies reported the effect of anti-biofilm substances as the proportion of biofilm reduction, without specifying cell concentrations either for control or treated samples. Therefore, despite the present meta-analysis supports the use of probiotics against microbial biofilms, it is essential to rigorously report the obtained results, as well as the statistical analysis.

To the best of our knowledge, this is the first systematic review and meta-analysis about the anti-adhesive and antimicrobial activity of probiotics against device-associated infections. The effectiveness of probiotics and their metabolites to inhibit and control biofilm formation was critically discussed regarding the anti-biofilm strategy, surface material, and pathogenic microorganisms. Additionally, it was possible to ascertain the pooled estimate effect of each anti-biofilm agent. These findings may be helpful to arise new research questions or guide future investigations. Moreover, this review highlights the need to properly analyze and report data, since some experimental procedures lack the detail, difficulting their repetition and comparison of results. Furthermore, it is essential that further studies provide a better insight into the interactions between biofilms and probiotics, and closely simulate the *in situ* conditions on the various indwelling devices, providing reproducible and accurate results and allowing to translate experimental knowledge into clinical applications.

5. CONCLUSIONS

In this work, the interaction of two probiotic strains (*L. plantarum* and *L. rhamnosus)* with bacteria commonly found in biofilms developed in urinary catheters (*E. coli* and *S. aureus*) was investigated. Regarding to the displacement strategy, probiotics showed promising results against pathogenic biofilms developed in a polymeric surface. Each probiotics caused a reduction up to 60 and 63% in the culturability of 24-h biofilms of *E. coli* and *S. aureus*, respectively, whereas 48-h biofilms seemed to be less susceptible to the antimicrobial activity of probiotics. The suggested mechanisms of action of probiotics are the release of harmful substances that affect pathogen growth and the integration of probiotic cells into the biofilm. In what concerns the screening of biofilm-forming capacity of probiotics, both *Lactobacillus* strains were able to form stable biofilms on silicone surfaces. However, *L. rhamnosus* demonstrated more difficulty in maintaining culturability. Cell culturability in biofilms decreased over time, regardless of the growth medium and hydrodynamic conditions tested, possibly due to nutritional limitations. Also, biofilms formed in MRSB had more culturable cells and biomass amount than those formed in AUM. The replacement of culture medium every day enhanced biofilm formation and the number of biofilm culturable cells over time, overcoming the nutritional limitations initially found. Although biofilms formed in static conditions presented a higher amount of biomass, the use of a low shaking frequency to stimulate biofilm formation led to the formation of more robust biofilms. Concerning the exclusion strategy, *L. plantarum* demonstrated a good short-term activity in inhibiting the adhesion of *E. coli* to silicone. The culturability of *E. coli* biofilms after exposure to probiotics for 6 h was reduced in 94 and 97% for 24- and 48-h biofilms, respectively. Thus, these preliminary results indicate that coating of the surface with probiotics may be a promising strategy to prevent the initial attachment of pathogens. Additionally, probiotics showed potential to act against uropathogenic biofilms developed on polymeric surfaces, which will pave the way to further experiments on the topic. Furthermore, *L. plantarum* seemed to be the most promising probiotic since it was able to reduce the culturability of pathogens and form robust/stable biofilms on silicone. Although the percentages of reduction were not very high (corresponding to about 0.5 and 1.5 Log CFU/cm^2 for displacement and exclusion strategies, respectively), this is a proof of principle study to demonstrate the ability of probiotics to control and prevent biofilm formation, combining the effect of nutritional conditions, agitation and surface material in order to better predict how probiotics will perform *in vivo*.

Finally, the systematic review and meta-analysis showed that the use of probiotics and their metabolites is a promising approach to restrain biofilm formation in medical devices by a broad spectrum of pathogenic microorganisms. Although their efficacy seems to be independent of the anti-biofilm strategy applied, the prevention of initial cell attachment by coating the surfaces with probiotic biofilms is more effective than battle pre-formed pathogenic biofilms. Moreover, this review highlights the need to properly analyze and report data, as well as the importance of standardizing the culture conditions in order to facilitate the comparison between studies. This is crucial to increase the studies' predictive value and translate these findings into clinical applications.

6. OUTPUTS

The current project is being developed since last year in collaboration with Prof. Mette Burmølle from the Department of Biology of the University of Copenhagen (Denmark). An abstract entitled "The potential use of probiotics to control biofilm formation in urinary catheters" was accepted for poster presentation in the 6th Eurobiofilms ESGB meeting in Glasgow (Scotland, 2019). Later, in February 2020, the current project was accepted for oral presentation in the 13th Meeting of Young Researchers of U. Porto (IJUP) with the title "Use of probiotics in the control of biofilm formation in urinary catheters" and the respective book of abstracts was published (ISBN: 978-989-746-253-5). In April 2020, this project was accepted for oral presentation in the 11th Symposium on Bioengineering at Faculty of Engineering of U. Porto with title "Probiotics: A new way to fight urinary catheter infections", achieving the 3rd place on the Science Under 5' scientific pitch contest. Also, a review article addressing the topics covered in the systematic review is currently being prepared to be submitted to a peer-reviewed journal.

7. FUTURE WORK

Due to the limitations in performing laboratorial work imposed by the current pandemic situation, it was only possible to form *L. plantarum* biofilms for 24 and 48 h and expose them to *E. coli* for 6 h. Therefore, in what concerns to future work, the exclusion assays need to be repeated by forming *L. plantarum* biofilms on silicone coupons for 48 and 72 h and exposing these potentially beneficial biofilms to *E. coli* for longer periods (24 and 48 h).

In addition, other techniques will be used to complete the study of the interactions between uropathogenic bacteria and probiotics, namely the confocal laser scanning microscopy. This microscopic technique, combined with the Live/Dead staining, will allow us to evaluate the spatial distribution of the strains within the biofilm and check the biofilm viability.

Also, the roughness and hydrophobicity of *L. plantarum* biofilms will be determined to conclude about the changes in topography and physicochemical properties of silicone rubber induced by the adhesion of probiotics.

The use of other biofilm platforms for initial adhesion and biofilm formation studies under controlled hydrodynamic conditions will also be considered in future work. A parallel-plate flow-chamber (PPFC) was fully characterized by our research group [162], so it offers the opportunity to mimic the *in vivo* hydrodynamic forces of the attached bacteria. Indeed, by regulating the applied fluid flow, the bacterial adhesion and subsequent biofilm growth can be studied under conditions that are more similar to those find in several biomedical scenarios.

Additionally, probiotic supernatants can be isolated and used to study their inhibitory potential against pathogenic biofilms. Supernatants can also be subjected to several treatments (neutralized and heat- and protease-treated) in order to know which molecules are responsible for biofilm inhibition.

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APPENDICES

APPENDIX A: Composition of artificial urine medium

Table A-1. Composition of AUM (in 1L of distilled water) [70].

Peptone (Oxoid, England)	1 g
Yeast extract (Oxoid, France)	0.05 g
Lactic Acid 1.1 mmol/L (Fluka, Spain)	82 μL
Citric acid (Scharlab, Spain)	0.4 g
Sodium bicarbonate (Merck, Germany)	2.1 g
Urea (Fisher Scientific, Belgium)	10 g
Uric acid (VWR, Belgium)	0.07 g
Creatinine (Acros, USA)	0.8 g
Calcium chloride.$2H_2O$ (Merck, Germany)	0.37 g
Sodium chloride (VWR, Belgium)	5.2 g
Iron II sulfate.$7H_2O$ (VWR, Belgium)	0.0012 g
Magnesium sulfate.$7H_2O$ (Labkem, Spain)	0.49 g
Sodium sulfate.$10H_2O$ (Merck, Germany)	3.2 g
Potassium dihydrogen phosphate (Fisher Scientific, UK)	0.95 g
Di-potassium hydrogen phosphate (Panreac, Spain)	1.2 g
Ammonium chloride (Merck, Germany)	1.3 g

The pH was adjusted to 6.5 and AUM was sterilized by passing through a 0.2 μm nylon membrane filter, since autoclaving caused precipitation.

APPENDIX B: Methods associated with the systematic review

Table B-1. Databases used in the search and respective keywords.

PubMed:
((probiotic*[Title/Abstract] OR lactobacillus[Title/Abstract] OR lactobacilli[Title/Abstract] OR lactic acid bacteria[Title/Abstract]) AND (biofilm*[Title/Abstract]) AND (surface[Title/Abstract] OR coat*[Title/Abstract]) AND (pathogen*[Title/Abstract] OR medical device*[Title/Abstract] OR catheter*[Title/Abstract]))
Cochrane library:
((probiotic*[Title/Abstract] OR lactobacillus[Title/Abstract] OR lactobacilli[Title/Abstract] OR lactic acid bacteria[Title/Abstract]) AND (biofilm*[Title/Abstract]) AND (uropathogen*[Title/Abstract] OR pathogen*[Title/Abstract]) AND (device*[Title/Abstract] OR medic*[Title/Abstract] OR catheter*[Title/Abstract] OR urin*[Title/Abstract] OR health*[Title/Abstract] OR silicone[Title/Abstract] OR surface*[Title/Abstract] OR coat*[Title/Abstract]))
ScienceDirect:
((probiotic OR lactobacillus OR lactobacilli OR lactic acid bacteria) AND (biofilm) AND (surface) AND (pathogen OR medical device OR catheter))
Compendex:
((probiotic OR lactobacillus OR lactobacilli OR lactic acid bacteria) AND (biofilm) AND (medical device OR health OR surface OR coating))

Table B-2. Methodological index for *in vitro* studies and respective mean of all studies.

Criterion	**Mean**
1. A clearly stated aim: The hypothesis/aim of the study is explicitly stated and testable by statistical means.	1.93
2. Detection of bias: Data were collected according to an established protocol. At least 3 independent experiments were performed for each assay.	1.64
3. An adequate control group: There is a control group corresponding to untreated biofilms.	1.93
4. Appropriate methodology: Description and explanation of the methods in accordance with the outcomes you want to obtain. The used methods are the same for control and exposure treatment.	1.84
5. Pathogens description: The pathogens species and quantity used for inoculation are described. 0: not reported 1: organism species OR organism quantity 2: organism species AND organism quantity	1.64

6. Anti-biofilm substances: Description of substances used to control/prevent biofilm formation, including identity/origin and concentration. 0: not reported 1: description of origin OR concentration 2: description of origin AND concentration	1.87
7. Culture conditions: Description of how assays were performed in sufficient detail to repeat (or detailed methodology is referenced), including culture medium, hydrodynamic conditions and temperature. 0: not described 1: sufficient detail to repeat OR a description of culture medium OR hydrodynamic conditions OR temperature 2: sufficient detail to repeat AND a description of culture medium AND hydrodynamic conditions AND temperature	1.27
8. Biofilm formation period: Because some microorganisms may grow/act slower, longer incubation periods may be needed to ensure successful biofilm inhibition. 0: duration of exposure not reported 1: culture of < 6 h 2: culture of ≥ 6 h	1.76
9. Surface: Description of substratum for biofilm formation. 0: not described 1: description of surface OR biofilm platform 2: description of surface AND biofilm platform	1.84
10. Predictive value: *In vitro* studies may use inoculum concentrations exceeding those encountered in a clinical scenario. 0: not described 1: inoculation with flora at the same concentration as that found in clinical scenario (< 10^5 CFU/mL) 2: inoculation with a concentration of bacteria which exceeds that found in clinical scenario (> 10^5 CFU/mL)	1.07
11. Results clarity: The results of the study are presented in a clear and organized way. 0: results are not clear 1: results are clear 2: results are clear and easy to understand AND cell concentrations or optical density values either for control or treatment experiments were reported	1.58
12. Adequate statistical analyses: Description and implementation of statistical tests appropriate to the dataset with the calculation of confidence intervals and *p* values.	1.31

CFU – Colony-Forming Units.
The items are scored 0 (not reported), 1 (inadequately reported) or 2 (adequately reported).

APPENDIX C: Other results regarding the displacement strategy

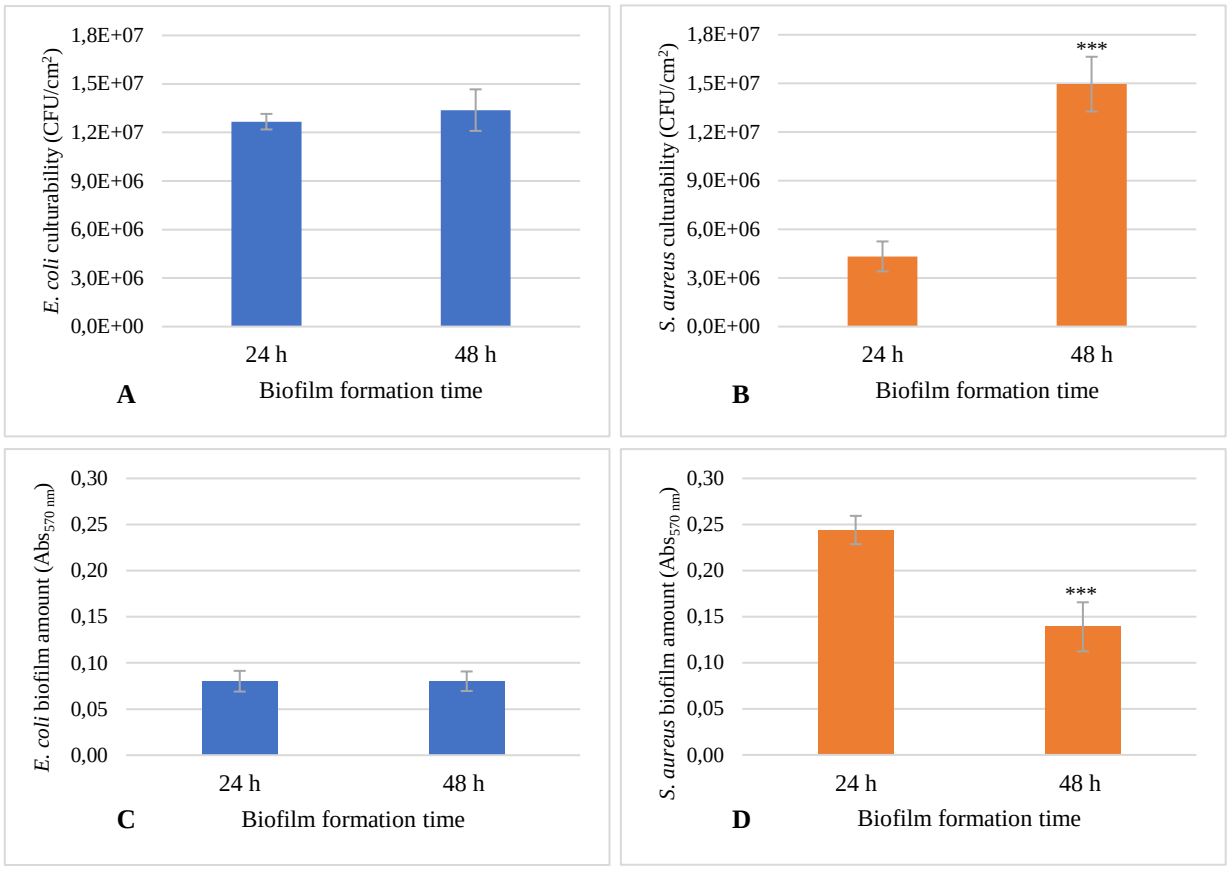


Figure C-1. *E. coli* and *S. aureus* biofilm culturability (A and B, respectively) and total biomass (C and D, respectively) after 24 and 48 h of biofilm development. Symbol *** indicates statistically different values for $p < 0.01$ between the 24- and 48-h biofilms.

Table C-1. Percentages of reduction of *E. coli* and *S. aureus* biofilms culturability after probiotic exposure for 6 and 24 h.

		Time of exposure to probiotics (h)	Percentage of reduction	
			L. plantarum	***L. rhamnosus***
E. coli	24-h biofilms	6	60%	58%
		24	25%	38%
	48-h biofilms	6	19%	10%
		24	50%	-
S. aureus	24-h biofilms	6	-	2%
		24	63%	47%
	48-h biofilms	6	53%	40%
		24	34%	10%

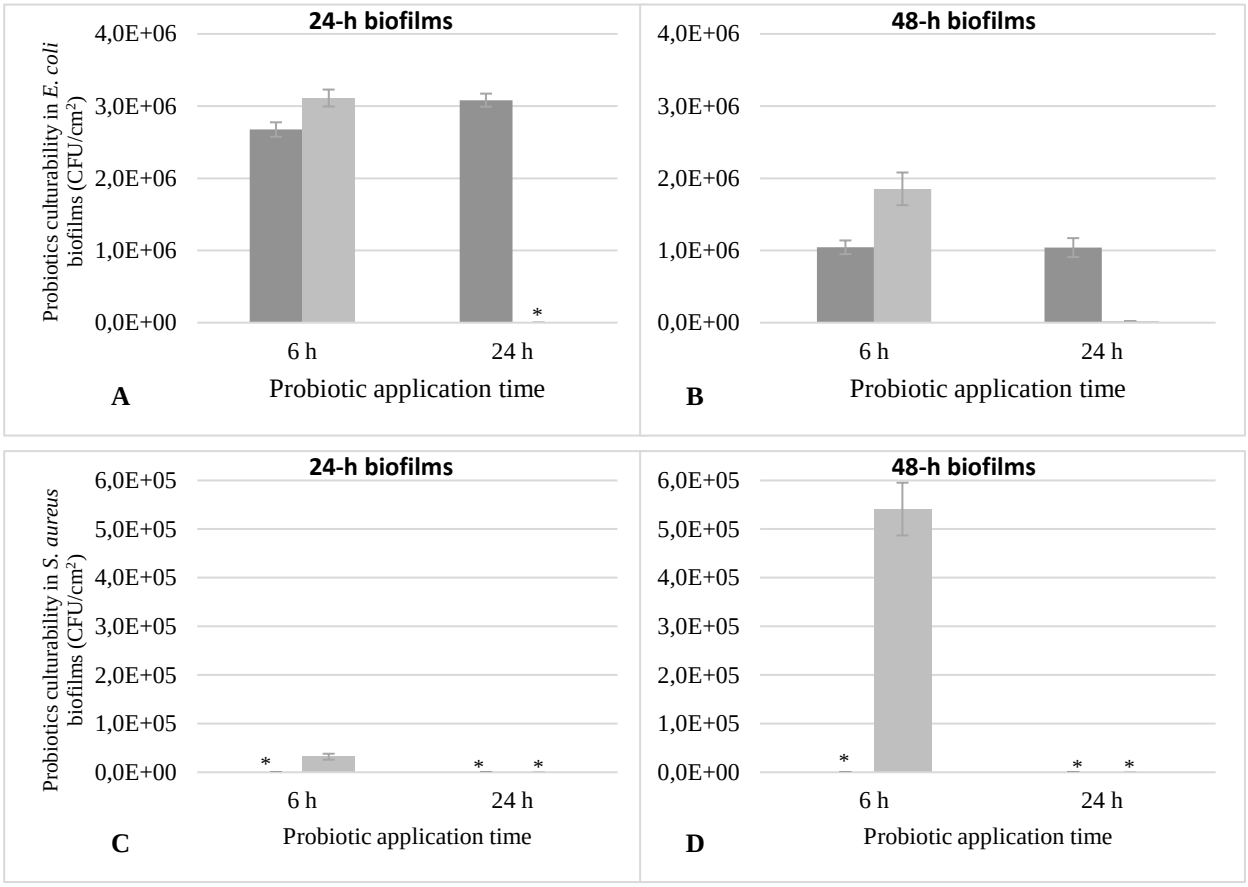


Figure C-2. Probiotic culturability after 6 and 24 h contact with 24- and 48-h *E. coli* (A and B, respectively) and *S. aureus* (C and D, respectively) biofilms. *E. coli* or *S. aureus* biofilm + *L. plantarum*; *E. coli* or *S. aureus* biofilm + *L. rhamnosus*. * no colonies were detected.

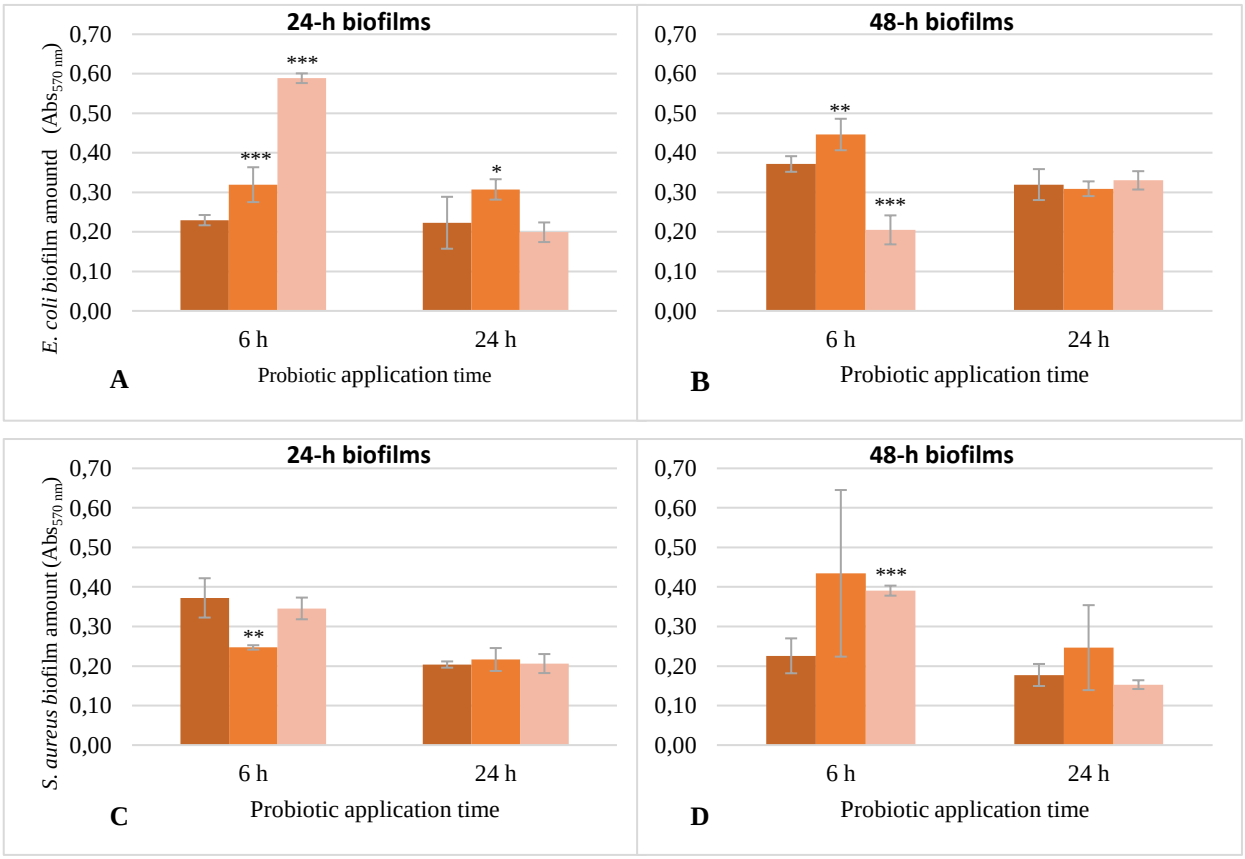


Figure C-3. Total biomass of 24- and 48-h biofilms of *E. coli* (A and B) and *S. aureus* (C and D) after contact with probiotics for 6 and 24 h. *E. coli* or *S. aureus* biofilm (negative control); *E. coli* or *S. aureus* biofilm + *L. plantarum*; *E. coli* or *S. aureus* biofilm + *L. rhamnosus*. Symbols *, ** and *** indicate statistically different values for $p < 0.1$, $p < 0.05$ and $p < 0.01$, respectively, when compared to control.

APPENDIX D: Individual description of all studies included in the systematic review

Table D-1. Characteristics of displacement studies in medical devices.

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Year	Ref.
Biosurfactants	*L. jensenii* *L. rhamnosus*	*A. baumannii* *E. coli* MRSA *S. aureus*	Polystyrene	Both biosurfactants disrupted pre-formed biofilms of *A. Baumannii* (≈ 58%), *E. coli* (≈ 65%) and *S. aureus* (≈ 61%).	2014	[64]
	L. brevis	*C. albicans*	Silicone elastomeric discs	No significant activity (either inhibitory or stimulating) could be established.	2015	[108]
	L. gasseri *L. jensenii*	*C. albicans* *C. krusei* *C. tropicalis* *E. aerogenes* *E. coli* *K. pneumoniae* *S. saprophyticus*	Polystyrene	Biosurfactants disrupted the biofilms of all tested microorganisms at different levels at varying concentrations being more efficient against *E. aerogenes* (64%), *E. coli* (46%) and *S. saprophyticus* (39%). For yeasts, it was achieved about 35% of biofilm reduction.	2017	[115]
Bacteriocins	*L. acidophilus*	*P. aeruginosa*	Foley silicone catheter pieces	Both crude and purified bacteriocins showed marked inhibitory activity against biofilm formation (27 and 59% of reduction, respectively) and biofilm culturable cells of *P. aeruginosa*.	2011	[117]
	L. acidophilus *L. plantarum*	*S. marcescens*	Polystyrene	Bacteriocins produced by both *Lactobacillus* showed a significant inhibitory effect on the biofilm of *S. marcescens*.	2016	[116]
EPS	*Leu. citreum* *Leu. mesenteroides* *Leu. pseudo-mesenteroides* *Ped. pentosaceus*	*E. coli* *E. faecalis* *S. aureus*	N. A.	The capacity of EPS to disrupt the pre-formed biofilms increased when increasing its concentration and was lower than its capacity to prevent the biofilm adhesion. The highest biofilm reduction was observed against *S. aureus* (77%).	2018	[118]
Cell-free supernatants (crude and/or neutralized)	*L. rhamnosus*	*E. coli*	Glass	*E. coli* biofilms challenged with supernatant of *L. rhamnosus* caused a marked decrease in cell density, and increased cell death.	2011	[87]
	L. fermentum	*S. aureus*	PVC	Supernatants significantly induced biofilm disruption of *S. aureus* resulting in 50% dispersion.	2011	[119]
	L. pentosus *L. plantarum*	*K. pneumoniae* *P. aeruginosa*	N. A.	The neutralized supernatants showed good anti-biofilm activity, inhibiting up to 74% of *P. aeruginosa* and 78% of *K. pneumoniae* biofilm formation.	2015	[120]
	Single- and multi-species: *L. helveticus* *L. plantarum* *S. salivarius*	*C. albicans*	Polystyrene Polyurethane	The combined supernatants significantly reduced the amount of pre-formed *C. albicans* biofilm on polystyrene and polyurethane by > 65%.	2016	[121]
	L. gasseri *L. rhamnosus*	Single- and multi-species: *C. krusei* *C. parapsilosis* *C. tropicalis*	N. A.	Supernatants disrupted single- and multi-species pre-formed biofilms (up to ≈ 65 and ≈ 40%, respectively).	2018	[82]
Cells	*L. casei rhamnosus* *L. acidophilus* *L. fermentum* *L. casei*	*S. aureus*	Silicone latex	Reductions between 79 and 98% in displacing of *S. aureus*.	1994	[8]

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Year	Ref.
Cells	*L. rhamnosus* *Lact. lactis* Buttermilk containing *Lact. lactis and S. cremoris* Yoghurt containing *B. longum, L. acidophilus and S. thermophilus*	*C. albicans* *C. tropicalis* Staphylococcal strains Streptococcal strains	Silicone rubber	The yoghurt had a small effect on the bacteria and stimulated yeast growth, while buttermilk yielded a strong inhibition on both. *Lact. lactis* had the greatest effect in reducing the number of adhered yeast on the number of adhering yeasts and no effect on the adhering bacteria, while the effects of *L. rhamnosus* were virtually absent.	1999	[110]
	B. infantis *E. faecium* *L. casei shirota* *L. fermentum* *L. rhamnosus* *Lact. lactis* *Lact. lactis cremoris* *S. thermophilus*	*C. albicans* *C. tropicalis* Staphylococcal strains Streptococcal strains	Silicone rubber	Exposure of biofilms to *B. infantis* or *E. faecium* did not significantly reduce the number of yeasts in the biofilm. *L. fermentum*, *L. rhamnosus* and *Lact. lactis cremoris* led to a reduction in the number of yeasts. *L. casei shirota* and *S. thermophilus* significantly reduced the number of yeasts in ≈ 61 and ≈ 66%, respectively. *Lact. lactis* had the greatest effect on the number of adhering yeasts (≈ 96% of reduction).	2000	[122]
	L. reuteri *L. rhamnosus*	*A. vaginae* *E. coli* *G. vaginalis*	Glass	Introduction of lactobacilli into biofilm resulted in the almost complete killing of the pathogens.	2011	[87]
	L. rhamnosus	*S. mutans*	Bovine enamel saliva-coated	Lack of activity of *L. rhamnosus* on biofilms where no statistical differences in biofilm biomass and viable cells of *S. mutans* were observed.	2015	[123]
	Single- and multi-species: *L. helveticus* *L. plantarum* *S. salivarius*	*C. albicans*	Polyurethane	The combination of probiotics overlaid on pre-formed *C. albicans* biofilms reduced biofilm culturable cells by > 63 %.	2016	[121]
	L. casei *L. rhamnosus* GG	*C. albicans*	Denture surface	*C. albicans* biofilm on the denture surface was significantly reduced by both probiotics (≈ 99%).	2017	[114]
	L. rhamnosus *L. paracasei*	*S. mutans* *S. oralis*	Saliva-conditioned titanium discs	*Lactobacillus* strains remarkably decreased both oral streptococci biofilms in about 8 Log CFU, regardless of the presence of live or heat-killed cells, and decreased biomass production.	2017	[124]
	L. rhamnosus GG microcapsules	*E. coli*	Glass	*L. rhamnosus* microcapsules significantly reduced *E. coli* culturable cells in the biofilm up to ≈ 80%, in a dose-dependent manner.	2019	[125]
Lipoteichoic acid (LTA)	*L. plantarum*	*S. mutans*	Polystyrene	LTA did not affect the established biofilm.	2018	[126]
	L. plantarum	Multi-species biofilms: *A. naeslundii* *E. faecalis* *L. salivarius* *S. mutans*	Glass	Pre-formed multi-species biofilms were disrupted by treatments with LTA in a dose-dependent manner.	2019	[127]

Notes: CFU – Colony-Forming Units; EPS – Exopolysaccharides; MRSA – Methicillin-Resistant *Staphylococcus aureus*; PVC – Polyvinyl Chloride; N. A. – Not Available.

Table D-2. Characteristics of exclusion studies in medical devices.

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Year	Ref.
Biosurfactants	*L. acidophilus*	*C. albicans* *E. coli* *E. faecalis* *K. pneumoniae* *P. aeruginosa* *P. mirabilis* *P. stuartii* *S. epidermidis*	Silicone rubber	Biosurfactant (Surlactin) layers caused a marked reduction in adhesion after 4 h for the majority of the bacteria. Inhibition was particularly effective against *E. faecalis*, *E. coli* and *S. epidermidis*.	1998	[12]
	Lact. lactis	*C. albicans* *C. tropicalis* *R. dentocariosa* *S. aureus* *S. epidermidis* *S. salivarius*	Silicone rubber	The number of bacterial and yeast cells adhering to the silicone rubber with pre-adsorbed biosurfactant was significantly reduced by 78-90% and 56-78%, respectively.	2004	[112]
	S. thermophilus	*C. albicans* *C. tropicalis* *R. dentocariosa* *S. aureus* *S. epidermidis* *S. salivarius*	Silicone rubber	The number of bacterial and yeast cells adhering to the silicone rubber with pre-adsorbed biosurfactant was significantly reduced by 89-97% and 67-70%, respectively.	2006	[111]
	L. acidophilus *L. brevis* *L. casei* *L. delbrueckii* *L. fermentum* *L. paracasei* *L. plantarum* *L. reuteri* *L. rhamnosus*	*C. albicans* *P. vulgaris* *S. aureus*	Polystyrene	Biosurfactants exhibited considerable anti-adhesive activity against *S. aureus* (61%), *P. vulgaris* (75%) and *C. albicans* (85%).	2013	[128]
	L. brevis	*C. albicans*	Silicone elastomeric discs	Biosurfactant reduced *C. albicans* adhesion and biofilm formation to pre-coated silicone by 62, 53, 50 and 44% after 1.5, 24, 48 and 72 h.	2015	[108]
	L. acidophilus	*S. marcescens*	Polystyrene	Biosurfactants displayed high anti-adhesive activity against *S. marcescens* biofilm formation, reducing cell adhesion up to 60%, depending on the concentration.	2015	[9]
	L. helveticus	*B. cereus* *E. coli* *L. innocua* *L. monocytogenes* *P. aeruginosa* *S. typhi* *S. flexneri* *S. aureus* *S. epidermidis*	Polystyrene	The biosurfactant showed anti-biofilm activity against all the pathogens (from 40 to 87 %) with the highest biosurfactant concentration assayed.	2016	[129]
	L. acidophilus	*B. subtilis* *E. coli* *P. aeruginosa* *P. putida* *P. vulgaris* *S. aureus*	PDMS discs Polystyrene	Biosurfactants demonstrated anti-biofilm and anti-adhesive potential against *P. vulgaris* and *B. subtilis* on PDMS discs. On polystyrene, biosurfactants displayed anti-adhesive and anti-biofilm activities with adhesion inhibition of 81 and 79% for *S. aureus* and *B. subtilis*, and in a range of 59 to 65% for the remaining bacteria.	2019	[130]
Bacteriocins	*L. fermentum*	*P. aeruginosa*	Polystyrene	Pre-coating with bacteriocins reduced the number of biofilm culturable cells in 99%.	2017	[131]
	L. plantarum	*P. aeruginosa* *S. aureus*	Foley silicone catheter pieces	Catheters coated with bacteriocins prevented bacterial colonization.	2019	[109]

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Year	Ref.
EPS	*L. fermentum*	*P. aeruginosa*	Polystyrene	Pre-coating with EPS reduced the number of biofilm culturable cells in 96%.	2017	[131]
	Leu. citreum *Leu. mesenteroides* *Leu. pseudo-mesenteroides* *Ped. pentosaceus*	*E. coli* *E. faecalis* *S. aureus*	N. A.	EPS inhibited the adhesion of pathogenic biofilms in a dose-dependent manner and it was able to inhibit biofilm formation by a maximum of 90% for *E. coli*.	2018	[118]
Cells	*L. acidophilus* *L. casei* *L. casei rhamnosus* *L. fermentum*	*S. aureus*	Silicone latex	Data demonstrated reductions between 70 and 99% in adhesion of *S. aureus*.	1994	[8]
	Lact. lactis ssp. *lactis* *S. thermophilus*	Multi-species biofilm: *A. Naeslundii* *F. nucleatum* *S. oralis* *S. sobrinus* *V. dispar*	Saliva-coated hydroxyapatite discs	*S. thermophilus* caused a slight decrease in the oral pathogens counts, except of *F. nucleatum*. This decrease was stronger with *Lact. lactis*. The bacterial consortium was reduced up to ≈ 80%.	2002	[132]
	L. acidophilus	*S. mutans* and non-*mutans* streptococci strains	Polystyrene	*L. acidophilus* reduced the streptococcal adhesion with more effect on *S. mutans* (30%) than non-*mutans* streptococci (8%).	2011	[133]
	L. acidophilus immobilized on sodium alginate	*E. coli* *Klebsiella sp.* *P. aeruginosa* *S. aureus*	Foley silicone catheter pieces	The results showed significant reduction in the number of *S. aureus* and *Klebsiella* cells, however, *E. coli* and *P. aeruginosa* showed no reduction.	2016	[134]
	L. acidophilus	*E. coli* *Klebsiella sp.* *P. aeruginosa* *S. aureus*	Glass	*L. acidophilus* reduced the attachment and growth of all pathogens. The effect was more pronounced against *E. coli*, *Klebsiella* and *S. aureus*.	2016	[134]
	L. casei *L. rhamnosus* GG	*C. albicans*	Denture surface	Adhesion of *C. albicans* was significantly reduced (≈ 99%).	2017	[114]
	L. rhamnosus *L. paracasei*	*S. mutans* *S. oralis*	Saliva-conditioned titanium discs	Both *Lactobacillus* strains reduced about 2 Log CFU the adhesion of both oral streptococci, regardless of the presence of live or heat-killed cells, and decreased biomass production.	2017	[124]
	Recombinant *Lact. lactis* expressing FimH	UPEC	Polystyrene	*Lact. lactis* resulted in a significant reduction in UPEC biofilm cells after 24 and 48 h of exposure (almost 3 and 2 Log CFU, respectively).	2020	[135]
	E. coli Nissle 1917	*E. faecalis*	Silicone	Pre-coating with EcN biofilms reduced the adherence of the *E. faecalis* on all silicone modified surfaces (up to ≈ 2 Log CFU).	2017	[6]
Collagen-binding protein (p29)	*L. fermentum*	*E. coli* *E. faecalis*	Polyisobutylene-polystyrene (PIB-PS) copolymer Silicone rubber	Coating with p29 resulted in reductions of 34 and 75% in *E. coli* adhesion and 47 and 18% in *E. faecalis* adhesion to silicone rubber and PIB-PS, respectively.	2003	[7]
Lipoteichoic acid (LTA)	*L. plantarum*	*S. mutans*	Polystyrene	Biofilm formation was inhibited, but in a lesser degree in comparison with co-incubation (≈ 40% of reduction).	2018	[126]

Notes: CFU – Colony-Forming Units; EcN – *E. coli* Nissle 1917; EPS – Exopolysaccharide; PDMS – Polydimethylsiloxane; UPEC – Uropathogenic *E. coli*; N. A. – Not Available.

Table D-3. Characteristics of competition studies in medical devices.

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Year	Ref.
Biosurfactants	*L. jensenii* *L. rhamnosus*	*A. baumannii* *E. coli* MRSA *S. aureus*	Polystyrene pre-coated with human plasma	Both biosurfactants significantly reduced initial adherence of *A. Baumannii* (≈ 76%), *E. coli* (≈ 79%) and *S. aureus* (≈ 88%).	2014	[64]
	L. brevis	*C. albicans*	Silicone elastomeric discs	Biofilm formation was reduced on silicone discs by 89, 90 and 90% after 24, 48 and 72 h of incubation, respectively.	2015	[108]
	L. acidophilus	*S. marcescens*	Polystyrene	Biosurfactants displayed high anti-adhesive activity against *S. marcescens*, depending on the concentration, reducing cell adhesion up to 73%.	2015	[9]
	L. acidophilus *L. paracasei* *L. reuteri* *L. rhamnosus*	*S. mutans* *S. oralis*	Saliva-conditioned titanium discs	Biosurfactants inhibited the adhesion and biofilm formation *of S. mutans* (77-99%) and *S. oralis* (66-98%) by a remarkable decrease in biomass production and biofilm culturable cells. The inhibitory effect showed a dose-dependence.	2016	[113]
	L. helveticus	*B. cereus* *C. albicans* *E. coli* *P. aeruginosa* *S. aureus*	Medical grade silicone tubes	Biofilm development of all pathogens was diminished absolutely on silicone tubes.	2016	[129]
Bacteriocins	*L. fermentum*	*P. aeruginosa*	Polystyrene	Co-incubation with bacteriocins reduced the number of biofilm culturable cells by 93%.	2017	[131]
	L. plantarum	*P. aeruginosa* *S. aureus*	N. A.	Bacteriocins resulted in significant reductions in biofilm formation in 56% for *P. aeruginosa* and 62% for *S. aureus*.	2019	[109]
EPS	*L. fermentum*	*P. aeruginosa*	Polystyrene	Co-incubation with EPS reduced the number of biofilm culturable cells in 97%.	2017	[131]
	L. delbrueckii ssp. *bulgaricus* *L. fermentum* *L. rhamnosus*	*B. cereus* *E. faecalis* *L. monocytogenes* *P. aeruginosa*	Polystyrene	The EPS indicated potent anti-biofilm activities by inhibiting all the pathogens between 74 and 90%, in a dose-dependent manner.	2017	[10]
Cell-free supernatants (crude, acid, neutralized, proteinase K-treated, heat-treated and/or octyl-sepharose beads-treated)	*L. fermentum*	*K. pneumoniae* ssp. *pneumoniae*	Polystyrene	Acid supernatant inhibited *K. pneumoniae* biofilm formation in ≈ 95% after 24 h of incubation and decreased their culturability in ≈ 6.5 Log CFU. Neutralized supernatant inhibited the biofilm formation at a lower degree.	2007	[92]
	L. fermentum	*P. aeruginosa* *S. aureus*	Glass PVC	Adhesion of both pathogens was inhibited up to ≈ 60% and the thickness of biofilm decreased from 20 to 6 μm when grown in the presence of supernatant.	2011	[119]
	Single- and multi-species: *L. acidophilus* *L. fermentum* *L. plantarum* *L. rhamnosus*	*E. coli* *E. faecalis*	N. A.	Neutralized supernatants did not cause significant inhibition of biofilm formation, unlike non-neutralized supernatants which significantly inhibited biofilm formation by both pathogens up to 63%.	2014	[5]
	L. salivarius	*S. mutans*	N. A.	*L. salivarius* supernatant inhibited *S. mutans* biofilm formation in a contact-independent manner by approximately 53%.	2015	[136]
	Single- and multi-species: *L. helveticus* *L. plantarum* *S. salivarius*	*C. albicans*	Polystyrene Polyurethane	Supernatants significantly reduced the ability of *C. albicans* to adhere to polystyrene and polyurethane, reducing biofilm formation by more than 75%.	2016	[121]
	L. paracasei *L. rhamnosus*	*S. mutans* *S. oralis*	Saliva-conditioned titanium discs	The undiluted supernatants were able to completely inhibit the biofilm formation of both oral streptococci (reduction > 5 Log CFU).	2017	[124]

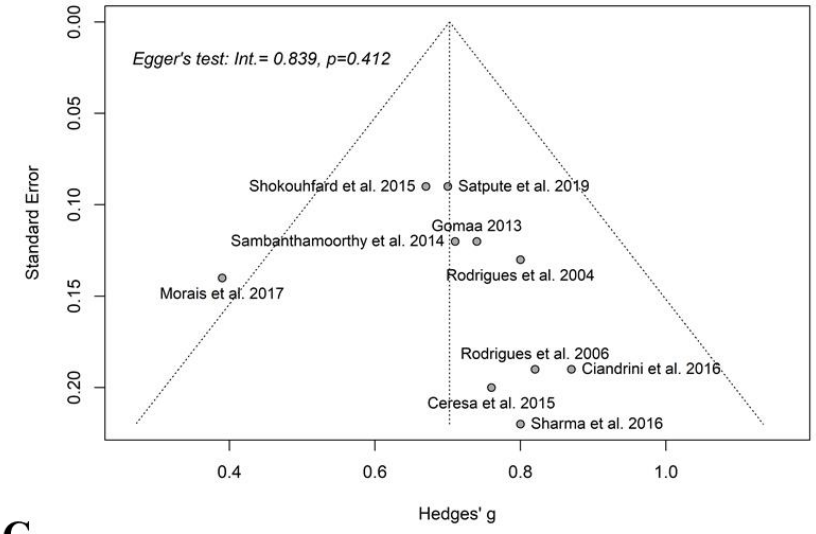
Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Year	Ref.
Cell-free supernatants (crude, acid, neutralized, proteinase K-treated, heat-treated and/or octyl-sepharose beads-treated)	*L. plantarum*	*S. mutans*	Polystyrene	Crude supernatant inhibited *S. mutans* biofilm formation in a dose-dependent manner. Similarly, proteinase K or heat treated supernatants inhibited the biofilm formation up to ≈ 70%. Supernatant treated with octyl-sepharose beads had less effect on biofilm formation.	2018	[126]
	L. gasseri *L. rhamnosus*	Single- and multi-species: *C. krusei* *C. parapsilosis* *C. tropicalis*	Silicone	*Candida* biofilms were reduced significantly in the presence of supernatant (up to 71%). Supernatant reduced up to 67% the amount of mixed biofilms on silicone surface.	2018	[82]
	Mono- and co-culture: *B. subtilis* *L. plantarum*	*S. aureus*	N. A.	The supernatants strongly inhibited biofilm formation by *S. aureus*. A major impact was related to *B. subtilis* supernatants (around 2.5 Log CFU reduction), although there was a modest contribution by *L. plantarum* (around 0.5 Log CFU reduction).	2019	[137]
Cells	*L. acidophilus* *L. casei* *L. casei rhamnosus* *L. fermentum*	*S. aureus*	Silicone latex	The adhesion of *S. aureus* was significantly reduced (from 84 to 99%) by the presence of lactobacilli.	1994	[8]
	L. casei rhamnosus *L. fermentum*	Naturally occurring uropathogens - not identified	Silicone rubber discs	*Lactobacillus* had the ability to inhibit the growth of an uropathogenic biofilm on silicone rubber for at least 8 days. Both *Lactobacillus* markedly inhibited uropathogens growth up to 100%.	2000	[11]
	L. fermentum	*K. pneumoniae* ssp. *pneumoniae*	Polystyrene	*L. fermentum* inhibited *K. pneumoniae* biofilm formation in ≈ 93% after 24 h of incubation and decreased their numbers in ≈ 6.5 Log CFU.	2007	[92]
	L. acidophilus	*S. mutans* and non-*mutans* streptococci strains	Polystyrene	*L. acidophilus* reduced the streptococcal adhesion with more effect on *S. mutans* (28%) than non-*mutans* streptococci (11%).	2011	[133]
	L. rhamnosus	*S. mutans*	Bovine enamel saliva-coated	Lack of activity of *L. rhamnosus* on *S. mutans* biofilms where no statistical differences in biofilm biomass and viable bacteria of *S. mutans* were observed.	2015	[123]
	L. rhamnosus GG *L. salivarius*	*S. mutans*	Glass Polystyrene	*L. salivarius* significantly reduced the number of attached bacteria and network-like structures comprising EPS, reducing *S. mutans* biofilm formation up to 70%.	2015	[136]
	L. rhamnosus GG	*C. albicans* *S. mutans* *S. sanguinis*	Saliva-coated hydroxyapatite discs	*L. rhamnosus* integrated into all oral biofilms and reduced the counts of *C. albicans* and *S. mutans*.	2016	[99]
	L. paracasei *L. rhamnosus*	*S. mutans* *S. oralis*	Saliva-conditioned titanium discs	Both *Lactobacillus* strains evidenced a significant decrease of adhesion of both oral streptococci in a range from 5.6 to 7.2 Log CFU.	2017	[124]
	L. rhamnosus GG microcapsules	*E. coli*	Polystyrene	*L. rhamnosus* microcapsules significantly reduced biofilm formation up to ≈ 82% in a dose-dependent manner.	2019	[125]
	Single- and multi-species: *L. helveticus* *L. plantarum* *S. salivarius*	*C. albicans*	Polyurethane	The combination of probiotics cells was able to significantly reduce adherence to polyurethane, reducing biofilm formation of *C. albicans* by more than 67%.	2016	[121]

Anti-biofilm Substances and Probiotic strains		Biofilm Forming Pathogens	Abiotic Surface	Major Conclusions	Year	Ref.
Cells	*Lact. lactis* ssp. *lactis* *S. thermophilus*	Multi-species biofilm: *A. Naeslundii* *F. nucleatum* *S. oralis* *S. sobrinus* *V. dispar*	Saliva-coated hydroxyapatite discs	*S. thermophilus* caused a decrease in all oral pathogens and that reduction was significantly increased by *Lact. lactis*. The bacterial consortium was reduced up to ≈ 50%.	2002	[132]
	E. coli Nissle 1917	*E. coli*	N. A.	EcN was able to compete with pathogenic strains during biofilm formation.	2010	[139]
	E. coli Nissle 1917	EHEC *P. aeruginosa* *S. aureus* *S. epidermidis*	Polypropylene	EcN inhibited the EHEC, *S. aureus* and *S. epidermidis* biofilm culturability by 1, 3 and 4 Log CFU, respectively. No effect on *P. aeruginosa* biofilms was observed.	2018	[138]
Lipoteichoic acid (LTA)	*L. plantarum*	*S. mutans*	Human dentin slices Polystyrene Saliva-coated hydroxyapatite	LTA inhibited biofilm formation of *S. mutans* in polystyrene, saliva-coated hydroxyapatite discs and human dentin slices in a dose-dependent manner (up to ≈ 75%).	2018	[126]
	L. plantarum	Multi-species biofilms: *A. naeslundii* *E. faecalis* *L. salivarius* *S. mutans*	Glass Human dentin slices	LTA inhibited multi-species biofilm formation on culture plates and dentin slices up to ≈ 55% in a dose-dependent manner.	2019	[127]

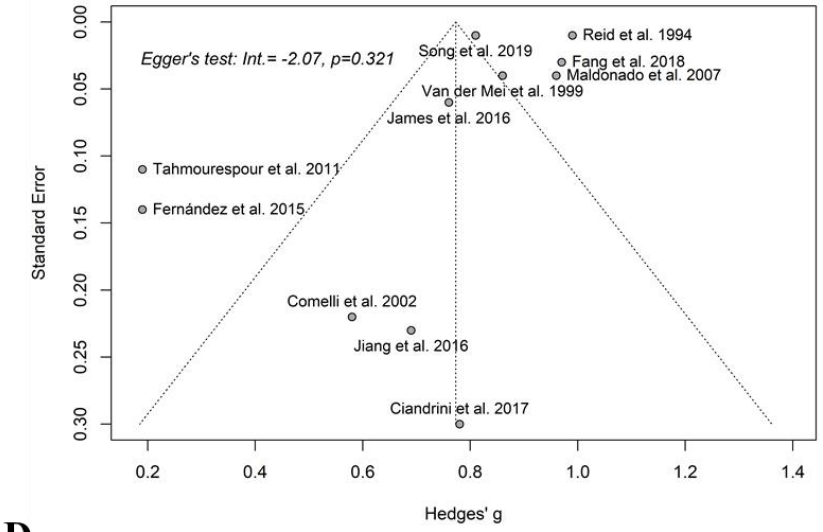
Notes: CFU – Colony-Forming Units; EcN – *E. coli* Nissle 1917; EHEC – Enterohemorrhagic *E. coli* O157:H7; EPS – Exopolysaccharides; MRSA – Methicillin-Resistant *Staphylococcus aureus*; PVC – Polyvinyl Chloride; N. A. – Not Available.

APPENDIX E: Supplementary material regarding meta-analysis

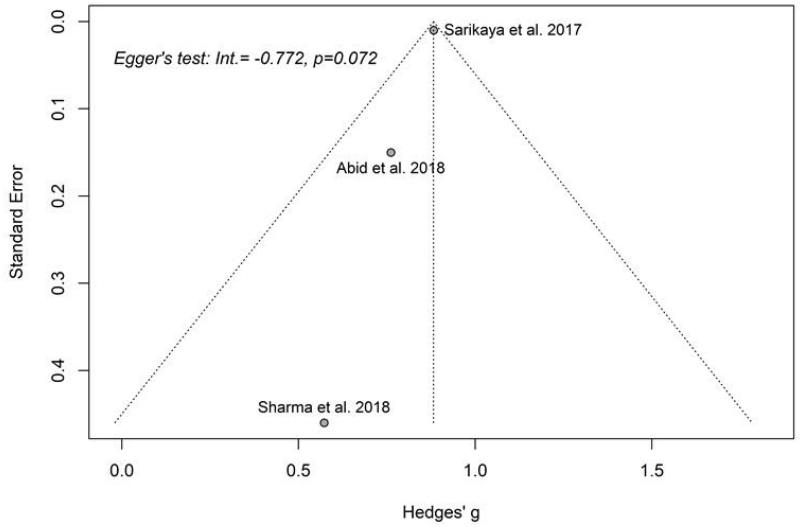
A.



B.



C.



D.

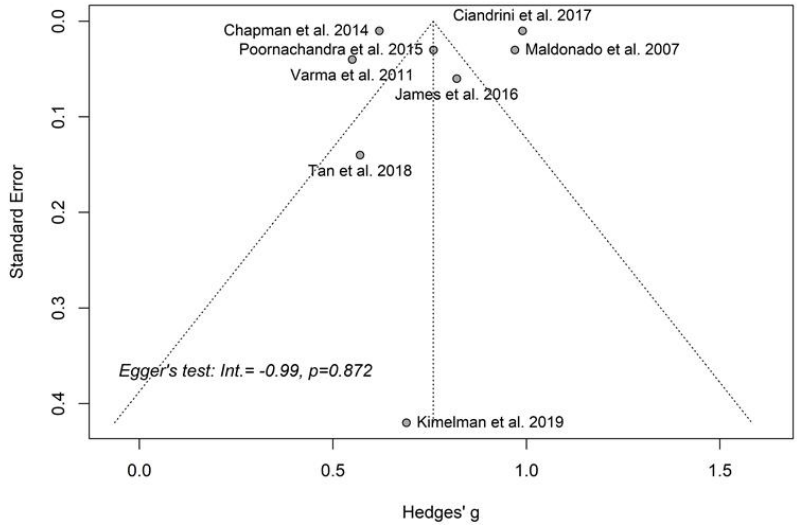


Figure E-1. Begg's funnel plot for evaluation of publication bias in the selected studies for the four anti-biofilm substances: (A) Biosurfactants, (B) Cells, (C) EPS and (D) Cell-free supernatants. The funnel graph plots the Hedges' g (standard mean of proportion of biofilm reduction) against the standard error. The Egger test for publication bias was not statistically significant for the four represented substances (p = 0.412, p = 0.321, p = 0.072, p = 0.875, respectively), suggesting that there was no significant publication bias associated with different sample sizes. The dashed line represents Egger's test regression.

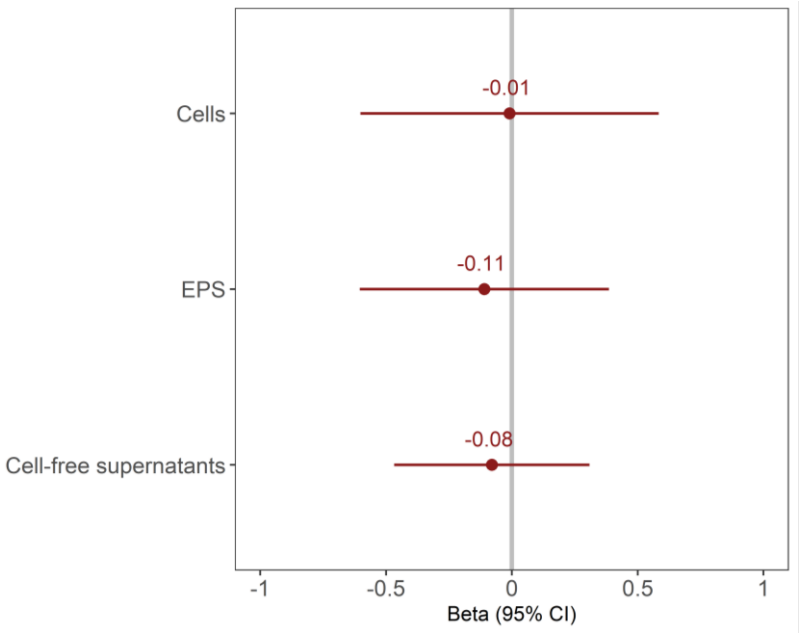


Figure E-2. Linear regression model between the proportion of biofilm reduction and the type of anti-biofilm substance. Biosurfactants were used as the reference category. Models were adjusted for strategy and the biofilm-forming pathogen.