U.PORTO
FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

Dissertation for Master's Degree in Bioengineering
Biological Engineering Branch

Horizontal gene transfer in bacterial biofilms: time-dependent interplay between surface material and hydrodynamics

Marta Moreira Ferreira

Supervisor: **Prof. Luciana Gomes**
Co-supervisors: **Dr. Rita Teixeira-Santos** and **Prof. Filipe Mergulhão**

ALiCE ASSOCIATE LABORATORY IN CHEMICAL ENGINEERING
lepabe Laboratory for Process Engineering, Environment, Biotechnology and Energy
Biofilm Engineering Lab

Department of Chemical Engineering

Porto, June 16th 2023

"Collaboration is key in achieving scientific breakthroughs. By pooling our knowledge and expertise, we can push the boundaries of what is possible."

Dr. Jane Goodall

Acknowledgments

Gostaria de expressar os meus mais sinceros agradecimentos a todas as pessoas que contribuíram para a realização desta tese e me acompanharam ao longo deste percurso.

Aos meus pais,
Que me proporcionaram a oportunidade de frequentar a faculdade e me apoiaram de forma incondicional ao longo de todo este percurso. Sem o seu amor, encorajamento e investimento, nada disto seria possível.

À minha restante família,
Expresso a minha gratidão por todo o apoio emocional e motivação transmitidos ao longo desta jornada.

Aos meus amigos,
Em especial à Joana e João, cujo apoio constante e amizade verdadeira foram fundamentais para ultrapassar os desafios académicos e pessoais. Vocês estiveram sempre ao meu lado, motivando-me e dando-me força nos momentos mais difíceis.

Ao Jobling,
Por toda a motivação e orgulho que me transmitiu ao longo desta jornada. O teu apoio constante deu-me a confiança necessária para enfrentar os desafios com determinação.

Aos meus colegas de curso que se tornaram meus amigos,
Em especial à Andreia, Joana, João, Matilde, Gonçalo, Maria, Teresa e Inês por toda a ajuda e amizade. O seu apoio, troca de ideias e companhia foram essenciais para enriquecer o meu trabalho e tornar esta jornada académica ainda mais gratificante.

À Ana e aos meus orientadores,
Ao Professor Filipe Mergulhão pela aceitação e sábias contribuições. À Luciana pelo apoio, aceitação em realizar esta dissertação, e por me ter guiado com sabedoria e conhecimento durante todo o desenvolvimento desta tese. À Rita por toda a ajuda e conhecimento. À Ana, que esteve sempre presente, oferecendo ajuda incondicional e pelas valiosas orientações e conhecimento ao longo do trabalho.

À Faculdade de Engenharia da Universidade do Porto,
Expresso a minha gratidão pelo corpo docente exemplar que colaborou ativamente para o meu desenvolvimento académico e pessoal.

Aos colegas e técnicas de laboratório do BEL (Biofilm Engineering Lab) e **E-101**
Agradeço-vos pelo ambiente acolhedor, pela convivência e pelo apoio oferecido.

Este trabalho foi financiado por LA/P/0045/2020 (ALiCE), UIDB/00511/2020 e UIDP/00511/2020 (LEPABE), e projeto SurfSAFE suportado pelo "European Union's Horizon 2020 Research and Innovation Programme" sob o contrato de concessão número. 952471.

Abstract

Horizontal gene transfer (HGT) in bacterial biofilms is a significant concern because of the dissemination of antibiotic resistance genes (ARGs), which hinders biofilm control in medical, industrial, and environmental fields. Microorganisms are nearby within biofilms, facilitating the exchange of genetic material. Several mechanisms, including conjugation, enable HGT within biofilms. Conjugation involves the direct transfer of genetic material from one bacterium to another through physical contact, and is mediated by a specialised genetic element called conjugative plasmid.

This study aimed to (1) explore the effects of various parameters, namely hydrodynamics, surface properties, and contact time, on the biofilm formation ability of recipient and donor *Escherichia coli* strains in pure and mixed cultures, and (2) gain a comprehensive understanding of how these variables influence the conjugation process in dual-species biofilms. For this purpose, *E. coli* carrying the conjugative IncP-1 plasmid pKJK5 was used as the donor strain, and biofilm formation assays were performed under two hydrodynamics (0 and 185 rpm of orbital shaking frequency), using three surface materials with distinct physicochemical properties (polyvinyl chloride, PVC; graphene sheet, GS; and glass, G), for 24 and 72 h. Cell quantification and plasmid transfer frequency were assessed by flow cytometry. Confocal laser scanning microscopy (CLSM) was used as a complementary method to evaluate the spatial organisation of representative biofilms.

Hydrodynamics had a significant impact on the maturation of donor and recipient single-species biofilms, with higher cell numbers detected under static conditions. In turn, surface properties were found to significantly affect biofilm density at the initial stage of biofilm formation. However, bacteria-surface interactions seem to be strain-dependent, with the GS displaying a higher number of recipient cells and G a higher number of donor cells.

In the co-culture, hydrodynamics affected the total number of biofilm cells for both incubation times. Concerning the transconjugant numbers and transconjugant/donor ratios, both hydrodynamics and incubation times influenced HGT, with no shaking conditions and longer bacteria contact time promoting plasmid transfer events. Additionally, a positive association between 0 rpm, G surface, number of donors and total biofilm cells, and number of transconjugants on dual-species biofilms was demonstrated using linear regression models. In addition to confirming that static conditions increased the number of transconjugants, confocal microscopy suggested that transconjugants are mainly located in the innermost regions of the biofilm.

Overall, the results showed that factors such as hydrodynamics and biofilm age can have a huge impact on plasmid transfer and potentially influence the dissemination of antibiotic resistance in microbial communities.

Keywords: biofilm; horizontal gene transfer; conjugation; plasmid; antibiotic resistance gene

Resumo

A transferência horizontal de genes (HGT) em biofilmes bacterianos é preocupante devido à disseminação de genes de resistência a antibióticos (ARGs), o que dificulta o controlo de biofilmes nas áreas médica, industrial e ambiental. Dentro dos biofilmes, os microorganismos estão próximos, facilitando a troca de material genético. Vários mecanismos, incluindo a conjugação, permitem a HGT dentro dos biofilmes. A conjugação envolve a transferência direta de material genético de uma bactéria para outra através de contacto físico e é mediada por um elemento genético especializado chamado plasmídeo conjugativo.

Este estudo teve como objetivos (1) explorar os efeitos de vários parâmetros, nomeadamente hidrodinâmica, propriedades de superfície e tempo de contacto, na capacidade de formação de biofilme de estirpes de *Escherichia coli* recetoras e doadoras em culturas puras e mistas, e (2) compreender de que modo estas variáveis influenciam o processo de conjugação em biofilmes duplos. Para este fim, *E. coli* portadora do plasmídeo conjugativo IncP-1 pKJK5 foi utilizada como estirpe doadora, e foram realizados ensaios de formação de biofilme a duas hidrodinâmicas (0 e 185 rpm de frequência de agitação orbital), utilizando três materiais de superfície com propriedades físico-químicas distintas (policloreto de vinil, PVC; folha de grafeno, GS; e vidro, G), durante 24 e 72 h. A quantificação das células e a frequência de transferência de plasmídeo foram avaliadas por citometria de fluxo. A microscopia confocal de varredura a laser (CLSM) foi utilizada como método complementar para avaliar a organização espacial de biofilmes representativos.

A hidrodinâmica teve um impacto significativo na maturação de biofilmes puros das espécies doadora e recetora, sendo detetado um maior número de células em condições estáticas. Por sua vez, verificou-se que as propriedades de superfície afetaram significativamente a densidade do biofilme na sua fase inicial da formação. No entanto, as interações bactéria-superfície parecem depender da estirpe, sendo que a superfície GS apresentou um maior número de células recetoras e a superfície G um maior número de células doadoras.

Em co-cultura, a hidrodinâmica afetou o número total de células do biofilme para ambos os tempos de incubação. No que diz respeito aos números de transconjugantes e aos rácios transconjugantes/doadoras, tanto a hidrodinâmica como os tempos de incubação influenciaram o HGT, sendo que as condições sem agitação e tempo de contacto mais longo promoveram a transferência de plasmídeo. Adicionalmente, uma associação positiva entre 0 rpm, superfície G, número de células doadoras e do biofilme, e número de transconjugantes em biofilmes mistos foi demonstrada através de modelos de regressão linear. Para além de confirmar que as condições estáticas aumentaram o número de transconjugantes, a microscopia confocal sugeriu que os transconjugantes estão principalmente localizados nas regiões mais internas do biofilme.

Em suma, os resultados mostraram que fatores como a hidrodinâmica e a idade do biofilme podem ter um grande impacto na transferência de plasmídeos e potencialmente influenciar a disseminação de resistência a antibióticos em comunidades microbianas.

Palavras-chave: biofilme; transferência horizontal de genes; conjugação; plasmídeo; gene de resistência a antibióticos.

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, 16th 2023

Marta Ferreira

(Marta Moreira Ferreira)

Contents

Abbreviations

AMR - Antimicrobial Resistance
ARB - Antibiotic Resistant Bacterial
ARG - Antibiotic Resistance Genes
CLSM - Confocal Laser Scanning Microscopy
CP - Coupling Protein
dsDNA - Double-Stranded DNA
EPS - Extracellular Polymeric Substances
G - Glass
GFP - Green Fluorescent Protein
GS - Graphene Sheet
GTAs - Gene Transfer Agents
HGT - Horizontal Gene Transfer
ICEs - Integrative and Conjugative Elements
IMDs - Implanted Medical Devices
LB - Luria-Bertani
MDR - Multidrug Resistant
MGE - Mobile Genetic Elements
MIC - Microbially Induced Corrosion
MVs - Membrane Vesicles
OD - Optical Density
oriT - Origin of Transfer
PVC - Polyvinyl Chloride
QS - Quorum-Sensing
ROS - Reactive Oxygen Species
SD - Standard Deviation
SE - Soil Extract
SE - Standard Error
SEM - Scanning Electron Microscopy
ssDNA - Single-Stranded DNA
SWW - Simulated Wastewater
UV - Ultraviolet
2D - Two-Dimensional

List of figures

List of tables

1. Introduction

1.1. Objectives

Horizontal gene transfer (HGT) within biofilms plays a significant role in microbial evolution, adaptation, and particularly in the dissemination of antibiotic resistance genes (ARGs) [1]. Biofilms are complex and structured communities of microorganisms that can form on a wide range of abiotic surfaces. They provide an ideal environment for HGT because they facilitate cell-to-cell contact and the establishment of physical connections between bacterial cells [2]. This enables the exchange of genetic material, including ARGs, allowing microorganisms to acquire new traits and enhance their survival capabilities [1].

Although there is a considerable body of research on biofilms and HGT, the transconjugation dynamics within biofilms is relatively less addressed. Transconjugation is a process by which bacteria transfer genetic material, typically in the form of plasmids, from a donor bacterium to a recipient bacterium [3]. Considering the importance of this phenomenon on bacteria evolution, preservation of natural ecosystems, and dissemination of ARG and resistant bacteria from the environment to humans, the main goals of this study were to (1) investigate the effects of three parameters – hydrodynamics, surface material, and bacteria contact time – on the biofilm formation ability of donor and recipient *Escherichia coli* strains in single- and dual-species cultures, and (2) unravel the complex interplay between these variables in the conjugation process within dual-species biofilms. Biofilm experiments were conducted in microplates under two different hydrodynamic conditions (static and 185 rpm of orbital shaking frequency), at two time intervals (24 and 72 h), and with three surface materials (polyvinyl chloride, PVC; graphene sheet, GS; and glass, G). Bacterial populations in single- and dual-species biofilms and plasmid transfer frequencies were assessed by flow cytometry. Additionally, confocal laser scanning microscopy (CLSM) was used to analyse the spatial distribution of biofilms, providing valuable information about the presence and preferential location of transconjugant cells within these biological structures.

1.2. Relevance of the work

Antimicrobial resistance (AMR) is widely recognised as a critical issue in the global health field, representing one of the foremost challenges faced during the 21st century [4]. Consequently, antibiotic-resistant bacterial (ARB) infections pose a major public health threat, leading to approximately 2.8 million infections and 35,000 deaths annually in the United States [5]. These infections also impose a substantial economic burden, with

estimated annual medical costs reaching 7.7 billion dollars [6]. HGT contributes to antimicrobial resistance through conjugation, which is considered its major mechanism. Bacteria possessing conjugative plasmids can transfer these plasmids to recipient bacteria, providing them with resistance genes. This process allows for the rapid spread of resistance within bacterial populations, potentially leading to the emergence of multidrug-resistant strains.

Biofilms are commonly formed on artificial surfaces found in medical devices, wastewater pipelines, food processing surfaces, and marine environments. It is well known that the adhesion of microorganisms and the consequent biofilm development are influenced by surface properties [7], hydrodynamics [8], and time [9]. However, few studies have specifically focused on the impact of these parameters on conjugation events within biofilms. Therefore, this thesis may contribute to the identification of factors that promote or hinder the transfer of genetic material between sessile cells, thereby enabling the development of targeted interventions to prevent or control HGT in various contexts. Ultimately, this knowledge can help mitigate the rise in antibiotic resistance in biofilms, safeguard public health, and reduce the burden of resistant infections.

1.3. Thesis outline

This thesis is structured into six distinct sections, each addressing different aspects of research. Section 1 provides the necessary context, outlining the main objectives, significance, and motivation behind the development of this study. In Section 2, the state of the art is presented, and the concepts relevant to the work are explained. Here, three key areas were explored: (1) horizontal gene transfer (HGT) and its association with antibiotic resistance, (2) biofilm characteristics, (3) antibiotic resistance in biofilms and (4) biofilms as hotspots for HGT. Section 3 provides a thorough description of the materials and methodology used in this study. Section 4 presents and discusses the obtained results. Section 5 summarises the primary conclusions drawn from this study, highlighting the main findings and their implications. Finally, Section 6 proposes potential avenues for future research.

2. State of the art

2.1. Horizontal gene transfer (HGT) and its association with antibiotic resistance

Horizontal Gene Transfer (HGT), also known as lateral gene transfer, refers to the transfer of genetic information between organisms, whether unicellular or multicellular. This process differs from "vertical" transmission, in which DNA is passed down from the parent to the offspring [10, 11]. HGT can occur through various mechanisms within or between species [12], playing a crucial role in the acquisition and spread of antibiotic resistance genes (ARGs) among bacteria.

Antibiotics are biologically active molecules that can exert toxic effects on the environment [13]. Although they are used to treat or prevent bacterial infections, they are also considered contaminants of increasing concern due to their common presence in different environmental contexts and the lack of specific regulations for monitoring their levels [13]. There are three different routes for releasing antibiotics into the environment: (1) feed additives for stockbreeding and fish aquaculture, (2) human and veterinary drugs, and (3) environmental release during production [1]. In the stockbreeding industry, where animals are often kept under small, crowded, and stressful conditions, there is an increased risk of infectious diseases, leading to the use of a variety of antibiotics [1, 14]. Additionally, the types and modes of action of antibiotics used in agriculture and veterinary practices are closely related or identical to those prescribed to humans [14]. Large quantities of antibiotics are also used in intensive aquaculture and released into aquatic environments [13].

Antibiotic resistance bacteria (ARBs) can transfer resistance genes to opportunistic animal or human pathogens [1]. The inadequate use of antibiotics has created selective pressure on bacteria, leading to the emergence of ARGs [7, 15]. When antibiotics are ingested, they are not fully metabolised and the remaining residues can contaminate urban wastewater, manure, and biosolids, thereby contributing to the spread of bacteria with ARGs [1]. The role of HGT in the exchange of ARGs is indeed significant in medical, environmental, and food-related scenarios [1], posing risks to public health. Urgent actions such as interventions and routine monitoring programmes are required to address this issue [1].

2.1.1. HGT mechanisms

Figure 1 shows six types of HGT mediators in prokaryotes. The three generally

known routes of HGT are natural transformation, transduction, and conjugation [16, 17]. Recent research has shown that HGT can also occur through a variety of mechanisms, including nanotubes, membrane vesicles (MVs), and gene transfer agents (GTAs) [18-20].

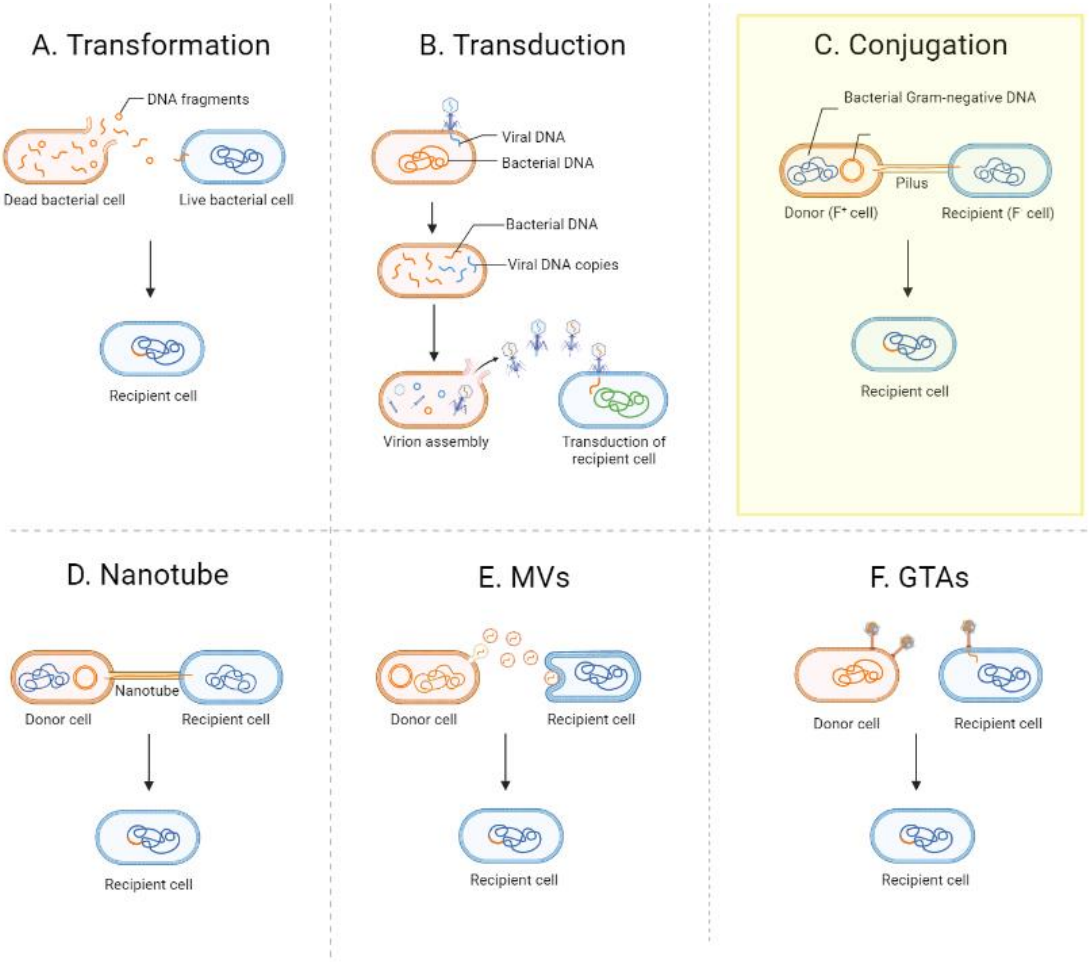

Figure 1. Six types of HGT mediators in prokaryotes. A. Transformation: this process involves the uptake of free DNA from the environment into the recipient cell. DNA is then integrated into the recipient cell genome. B. Transduction: this process involves the transfer of genetic material from one bacterial cell to another using a bacteriophage. C. Conjugation: this process involves the transfer of genetic elements, typically by cell-contact material from one bacterial cell to another. D. Nanotubes: are membranous structures that can be used by bacteria for direct cell-to-cell contact. E. MVs: this process involves the transfer of genetic material between bacteria using small membrane-bound vesicles that bud from the donor cell and are taken up by the recipient cell. F. GTAs: this process involves the transfer of genetic material between bacteria using small virus-like particles called gene transfer agents. Based on [7, 16, 20].

In 1928, Frederick Griffith injected mice with two strains of *Streptococcus pneumoniae*, a virulent strain (S strain) that could cause disease and a non-virulent strain (R strain) that did not cause disease [21]. As expected, mice injected with the S strain died,

while mice injected with the R strain survived. Griffith then heat-killed the virulent strain and injected the heat-killed bacteria along with a live non-virulent strain into mice. Unexpectedly, some mice died, and upon examination, he found that the R strain bacteria had been transformed into virulent S strain bacteria [21]. Later, Avery, MacLeod, and McCarty [22] showed that the transforming substance was DNA, providing the first evidence that DNA is the genetic material responsible for inheritance and genetic variation. Thus, natural transformation can be summarised as an active mechanism involving the uptake of naked extracellular DNA by competent cells [7].

Another mechanism of HGT is transduction, in which bacteriophages (viruses that can infect bacteria) transfer their genetic material into bacterial cells [23]. It was discovered by Lederberg and Zinder in 1951 [24]. They conducted investigations aimed at unravelling a system of conjugation and recombination in *Salmonella typhimurium*, ultimately leading to their discovery of transduction. Through the co-cultivation of two distinct mutant strains, one of which carried the lysogenic phage 22 (P22), they successfully retrieved infrequent prototrophs from the mixed culture. Their surprise arose when they observed that direct cell contact was unnecessary, as prototrophs persisted even when the strains were grown alongside each other but separated by an impermeable filter. This revelation led them to deduce that a filterable agent resistant to DNase facilitated this genetic exchange. Shortly thereafter, Zinder's subsequent findings demonstrated that the filterable agent was the temperate phage P22, capable of transferring fragments of genetic material from a donor strain to a recipient strain [25].

Discovered in 1946 by Tatum and Lederberg, conjugation is another mechanism of horizontal gene transfer [2]. It involves the transfer of genetic material, typically in the form of a plasmid, between two bacterial cells in physical contact. They designed an experiment using *Escherichia coli* mutants lacking the ability to synthesise growth factors. Single nutritional requirements were established through mutational steps, and two triple mutants were grown in a mixed culture. Different strains with varied nutritional needs were detected, including wild-type strains and single- or double-requirement mutants. Recombination between biochemical requirements and phage resistance was also observed, suggesting a sexual process involving gene assortment [26]. In bacteria, conjugation is a major mechanism that facilitates bacterial adaptation by allowing the genetic exchange of ARGs, resistance to heavy metals, virulence, and adaptive traits [3, 7, 17]. Particular emphasis is placed on the conjugation mechanism because it is the one studied in this thesis.

Conjugation can be divided into four stages [3]. In stage 1, transfer-competent donor cells select and attach to a suitable recipient cell, also known as mating pair formation,

involving surface-located proteins, including adhesion proteins that establish contact with the recipient cell. In stage 2, the conjugation element is processed to generate the DNA that is transferred to the recipient cell, which in most cases is single-stranded DNA (ssDNA). This DNA processing reaction requires a relaxase that forms a nucleoprotein complex called relaxosome, which introduces a site- and strand-specific nick within the origin of transfer (oriT). Host- and/or plasmid-encoded proteins, known as auxiliary proteins, often assist in the DNA processing reaction. During stage 3 of conjugation, a sophisticated membrane-embedded translocation machinery called transferosome is synthesised, through which DNA is transferred. This intercellular transferosome, a type IV secretion system, is composed of at least eight different proteins. Most of these proteins are present in multiple copies in the transferosome. The plasmid is recruited to the transferosome through interactions of an ATPase, named coupling protein (CP), which is located at the cytoplasmic entry side of the transferosome, and relaxosome proteins. Next, relaxase pilots transfer ssDNA into the recipient cell in an energy-consuming process that involves, besides CP, one or two additional ATPases. In stage 4, the DNA that entered the recipient cell was established. Once transferred, ssDNA is converted to double-stranded DNA (dsDNA). Many conjugative elements encode primases that are involved in the conversion of ss to dsDNA. Finally, conjugative elements encode proteins that transiently inactivate defence mechanisms, as most bacteria have evolved defence systems that inactivate incoming foreign DNA, including conjugative elements [3].

Gram-positive and Gram-negative bacteria differ in several ways, including their cell wall structure, staining characteristics, and susceptibility to antibiotics [27]. There are also some differences in the conjugation mechanisms between the two types of bacteria. The conjugation machines in Gram-positive species differ from those in Gram-negative species because they do not produce conjugative pili and instead rely on surface adhesins to mediate donor-recipient cell contacts [28]. Whereas the transport of single-stranded DNA has been found in both Gram-positive and Gram-negative systems, the transport of double-stranded DNA has been described for Gram-positive actinomycetes [29]. The conjugative pilus is a critical component of conjugation in Gram-negative bacteria. The pilus is thought to serve as an attachment device that mediates recognition of and attachment to recipient cells, as well as a conduit for the transport of relaxase and ssDNA. Some conjugative pili are capable of retraction, which brings donor and recipient cells together, thus resulting in proximity. Although tight conjugative junctions have been observed, suggesting that cell-to-cell contacts are required for conjugation to occur, the transfer has also been observed when cells are some distance apart [30].

Conjugation is a complex process that involves the transfer of genetic material between bacterial cells. The expression of conjugation genes in most systems is tightly regulated for two reasons. Firstly, the expression of numerous proteins involved in the process requires significant energy from the cell, which can strain the cell's resources. Second, the activation of the conjugation process can have major consequences for both the plasmid and host cells. Overall, the tight regulation of the expression of conjugation genes and the consequences of the conjugation process for both plasmid and host cells highlight the importance of maintaining a delicate balance between the benefits and costs of conjugation in bacterial populations [3].

Integrative and conjugative elements (ICEs) are mobile genetic elements (MGEs) that play a significant role in bacterial evolution. They reside in the host chromosome and can be transferred to recipient cells by conjugation. ICEs often carry additional genes that confer various traits to host cells, such as antibiotic resistance and pathogenicity. ICEs are not self-transmissible and require conjugation machinery to transfer themselves and their adjacent genes to recipient cells [31, 32].

Recent studies have shown that nanotubes can serve as another mechanism for HGT [16] in addition to the main types previously mentioned. Nanotubes are thin membranous structures that can be used by bacteria for direct cell-to-cell contact. They have been shown to transport a non-conjugative plasmid carrying antibiotic resistance genes between *Bacillus subtilis* cells. Nanotubes are distinguishable from conjugation pili and can transport not only DNA but also other cytoplasmic components, such as nutrients and fluorescent marker proteins. They have been observed in various bacterial species, including *E. coli* [33], suggesting that they may play a significant role in the distribution of biomaterials within bacterial communities, beyond ARGs alone [16].

Membrane vesicles (MVs) are small lipid-bilayer-enclosed particles that are released by both Gram-negative and Gram-positive bacteria, including those found in biofilms. MVs have been shown to mediate the transfer of ARGs between bacteria. Although MV-mediated HGT in natural environments still needs to be proven, numerous laboratory studies have demonstrated MV-mediated mobilisation of ARGs in a wide range of bacteria. MVs are also capable of conveying quorum-sensing signals, which are known to regulate conjugation, transformation, and phage induction [16]. MVs and GTAs are important HGT mechanisms in the marine environment. GTAs represent a relatively newly discovered mechanism that facilitates DNA transfer between bacterial cells [19]. They are phage-like particles containing DNA produced by some bacteria and archaea. These particles are particularly common in Alphaproteobacteria of the order Rhodobacterales, which are abundant in temperate oceans [34]. The role of MVs and GTAs in multistrain bacterial

biofilms remains unclear, and further investigations are needed to expand the understanding of HGT in biofilms [19].

2.2. Biofilm characteristics

Up to 80% of all bacteria live in biofilm communities [35]. Biofilms are highly structured multicellular communities of microorganisms, such as bacteria, fungi, and algae, embedded in a self-produced matrix of extracellular polymeric substances (EPS), mainly composed of polysaccharides, proteins, lipids, and nucleic acids [36-39]. The EPS work as a glue, “sticking” the microorganisms to the surface and providing them with a protective environment against dehydration and toxic components, a range of different nutrients, mechanical stability, and cell-to-cell communication [36, 37, 40-42]. Growing evidence has changed the definition of biofilms, which is not restricted to microbial layers on surfaces but includes various types of interfaces such as liquid-liquid, solid-liquid, solid-gas, liquid-gas, and even air-air (known as "bubble biofilms"). Therefore, biofilms do not necessarily require surface attachment to form [7, 43, 44].

A comparison of planktonic and biofilm studies showed that the frequency of plasmid transfer is higher in biofilms [2]. Studies have also shown that plasmid transfer through bacterial conjugation can occur in natural and artificial biofilms in various environments [2]. Most studies have been conducted at the population level and rely on cultivation-based assays, thus underestimating conjugation. To overcome this, researchers have used fluorescent markers to directly visualise plasmid transfer at the single-cell level within various biofilms, providing a more accurate assessment of a semi-solid agar surface, on a filter, in flow chambers, or in a more complex bioreactor [2]. The biofilm environment, with its high cell density [19] and proximity [16, 19], can facilitate HGT through bacterial conjugation [2]. Conversely, bacterial conjugation can also induce biofilm formation because the close contact established during gene exchange promotes the proximity necessary for biofilm formation [45, 46]. Furthermore, the released DNA enhances the stability of biofilms by synthesising EPS. Therefore, biofilms are hotspots for HGT, which can produce populations with antibiotic resistance or higher pathogenicity, thereby promoting biofilm homoeostasis [19].

2.2.1. Biofilm development models

Two models explain how biofilms develop: the classical five-step model and the “consensus model” [36, 47]. The classical model consists of attachment, cell-to-cell adhesion, proliferation, maturation, and dispersion [36]. Initially, microorganisms can be

reversibly attached to a surface, and most begin to produce EPS while remaining irreversibly attached. If the conditions are adequate, they can proliferate and mature, resulting in the formation of a biofilm with a complex three-dimensional structure, including channels and pores. The final stage of biofilm development is the detachment of cells from the biofilm structure (erosion and/or sloughing-off phenomena) and their dispersal into the environment to colonise new sites. This is a complex process involving numerous environmental conditions such as fluid shear forces, weak internal cohesion, and depletion of nutrients or oxygen supplied to the biofilm [36, 38, 42, 48]. Microorganisms can intercalate between planktonic and sessile stages depending on the specific environmental conditions [40]. However, this conventional biofilm development model has some limitations. For example, it has not yet been determined whether biofilm formation can be described as a true developmental process for biofilms formed outside a flow cell reactor and by species other than *Pseudomonas aeruginosa* and *Staphylococcus aureus* as model biofilm species. Also, it does not capture the wide variety of biofilm architectures observed in real-world systems, such as microbial mats, and does not consider the succession of events in biofilms formed in open systems with a continuous influx of new colonisers [47]. In contrast, the "cyclic model" or "three events model" represented in Figure 2 proposes that biofilm development occurs in a repeating cycle of three major events, independently of time, maturity and condition (*in vitro*, *in situ* or *in vivo*): (1) aggregation and attachment, (2) growth and accumulation, and (3) disaggregation and detachment. During aggregation and attachment event, bacteria aggregate to each other or attach to biotic and abiotic surfaces. In the growth and accumulation event, aggregated and attached bacterial colonies expand owing to the growth and recruitment of surrounding cells. In the disaggregation and detachment stage, bacteria can leave the biofilm as aggregates and single cells depending on the mechanism. Therefore, in this model, bacteria attach to biotic and abiotic surfaces and begin to grow, but after a certain amount of time, they detach and are carried away by the flow of liquid. However, some bacteria remain attached to the surface and serve as pioneers in the next cycle of attachment and growth [47].

In conclusion, both models have their strengths and weaknesses. The classical model provides a useful framework for understanding the basic stages of biofilm development but does not fully account for the dynamic nature of biofilms. The "cyclic model", on the other hand, provides a dynamic perspective on biofilm development overview that can be used to different scenarios.

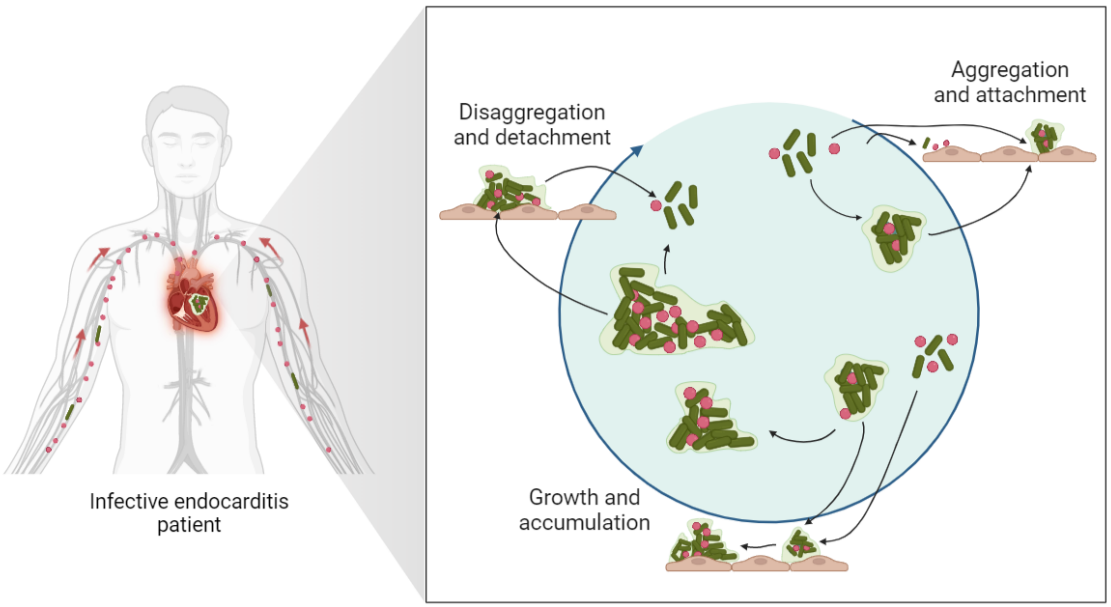

Figure 2. The "three events model" cyclic model of biofilm development in the exemplary context of *in vivo* biofilm formation in a medical scenario. This model proposes that biofilm development occurs in a repeating cycle of three major events: (1) aggregation and attachment, (2) growth and accumulation, and (3) disaggregation and detachment. In *in vivo* biofilm-infective endocarditis, bacteria attach to the heart valve surface and begin to grow, but after a certain amount of time, they detach and are carried away by the flow of blood. However, some bacteria remain attached to the surface and serve as pioneers in the next cycle of attachment and growth. Adapted from [47].

2.2.2. Biofilm occurrence

Biofilms are the most common way for microorganisms to live [41] and can be found in various areas, such as medicine, food, and the environment, including water systems [49]. Biofilms can cause significant problems, as they can infect and persist in biomedical devices [36, 50, 51], and induce product degradation in the food industry [52].

Biofilms pose a significant challenge to the prevention and treatment of medical infections. They are responsible for about 80% of hard and soft-tissue infections and can colonise mucosal surfaces, leading to recurrent inflammatory diseases. Consequently, they are responsible for a high number of hospital visits, surgical procedures, and deaths [53]. Microbial biofilms can also cause infections in implanted medical devices (IMDs) such as pacemakers, artificial hips, and urinary catheters and stents [53]. A weakened immune system around the implanted site and the formation of biofilms are the main causes of such infections. Biofilms can be comprised of Gram-negative or Gram-positive bacteria, and common bacteria that cause such infections include *S. aureus*, *Staphylococcus epidermidis*, *P. aeruginosa*, and *E. coli*. Device-related infections are associated with high morbidity and

mortality, leading to the complete removal of infected devices, which is costly and burdensome for the patient and the economy [53]. Therefore, understanding biofilm formation is crucial for preventing and managing these infections, improving patient outcomes, and reducing healthcare costs.

The occurrence of biofilms in the food industry is a significant concern because of their potential for foodborne illness outbreaks. Food processing lines offer an ideal environment for biofilm formation because of their complex manufacturing plants, long production periods, and large surface areas for biofilm growth. Bacteria in biofilms can bind to contact surfaces on different artificial substrates common in the food industry, such as stainless steel, polyethylene, wood, glass, polypropylene, and rubber, thereby increasing the risk of bacterial contamination of food products [52].

Biofilms can also cause several problems in water systems, including decreased water flow [54], increased pressure drop [54] and corrosion [55]. When microbes colonise various niches within biofilms, they promote a phenomenon known as microbially induced corrosion (MIC). MIC occurs when microbial activity alters the surface electrochemical properties of a metal or alloy, leading to an increased corrosion rate and high economic loss [55]. They can also act as reservoirs for pathogens that pose a risk to human health, such as *Legionella pneumophila* [56], *P. aeruginosa*, and nontuberculous *Mycobacterium* [55, 57]. This occurrence is influenced by a variety of factors, including temperature, nutrient availability, and surface material characteristics such as hydrophobicity and roughness [58, 59].

Therefore, understanding the dynamics of biofilm formation and growth in different scenarios is important for ensuring the safety and quality of food products and water supply, and for developing effective strategies to prevent and control biofilm-related infections.

2.3. Antibiotic resistance in biofilms

ARB infections are a significant public health concern, causing approximately 2.8 million infections and 35,000 deaths per year in the United States [5]. Apart from the devastating human toll, these infections impose a substantial economic burden, with an estimated annual medical cost of approximately 7.7 billion dollars [6]. The emergence of "superbugs", bacterial strains that are resistant to multiple antibiotics, has been attributed to the rise in ARBs and the prevalence of ARGs in the environment [60]. These bacteria can inactivate enzymes that cause antibiotic degradation, and their membranes can contain multidrug-resistant (MDR) efflux pumps that extrude antibiotics from the cell [60, 61].

Antimicrobial susceptibility tests are typically performed using planktonic cultures. However, biofilms respond differently to different antibiotic dosages, resulting in inadequate treatment or treatment failure. Compared to their planktonic counterparts, cells within a biofilm exhibit antibiotic resistance that can be up to 1,000 times greater [62]. Biofilm formation can promote important features of pathogenesis, such as significantly reduced susceptibility to antibiotics compared to susceptible non-biofilm producers [7].

Three mechanisms may contribute to the presence of ARB in biofilms. The first mechanism is based on the complex structure of biofilms that can create a barrier that hinders antibiotic penetration. The diffusion of antibiotics is slow and they are more likely to be deactivated at the biofilm surface before reaching their targets. Another mechanism is related to the biofilm microenvironment since deeper within the biofilm, oxygen levels may be significantly reduced and affect the bactericidal effects of specific antibiotics. The third resistance mechanism is related to the presence of small subpopulations of bacteria known as persister cells, which can enter a dormant state, rendering them resistant to antibiotics [63].

In addition to the described mechanisms, the close proximity of bacteria within the biofilm environment enables bacterial communication and facilitates the transfer of MGEs [7, 63]. The biofilm environment supports plasmid stability and enhances the transmission of resistance information among organisms [63]. Furthermore, transposable DNA elements transferred by bacteria often encode factors that promote biofilm formation, contributing to the sustainability of the biofilm and the persistence of infections[63].

2.4. Biofilms as hotspots for HGT

The combination of physical proximity, intercellular communication, presence of extracellular DNA, specialised genetic elements, genetic diversity, and stress-induced responses in biofilms collectively contribute to their status as hotspots for HGT.

Here, we describe some studies that have explored HGT through conjugation within biofilms. Røder et al. [64] conducted a study in which they developed a dual-labelled plasmid loss reporter system using the conjugative plasmid pKJK5. This system allowed for the analysis of plasmid loss at the cellular level, even at low frequencies. *Pseudomonas putida* KT2442 and an isogenic mutant MRB1 were used as models to investigate the influence of biofilm formation and dispersal on plasmid stability. The hypothesis was that the inability of MRB1 to actively disperse from the biofilm would lead to a higher plasmid retention rate. Two versions of the plasmid, one conjugative and one with a reduced rate of conjugation, were used to assess the impact of conjugation on plasmid maintenance. This

study revealed that MRB1 biofilms, which lacked active dispersal, exhibited a higher retention rate of the plasmid compared to the wild-type strain. Surprisingly, there was no significant effect of conjugation on plasmid maintenance in the biofilms. Laser scanning microscopy of the biofilms confirmed that plasmid loss was higher in the top layers where actively replicating cells were located. To investigate the relationship between plasmid loss and biofilm structure dynamics, both *P. putida* WT and MRB1 were grown in alginate beads, which restricted dispersal. The results showed similar levels of plasmid loss for both strains in the alginate beads, indicating that increased plasmid loss was associated with the dynamic development of the biofilm structure. Additionally, plasmid-free cells were found to grow faster than those carrying plasmids, suggesting a growth advantage in spatially structured communities when plasmid-encoded traits are not under selection [64].

Another study performed by Røder and colleagues [65] explored the influence of conjugative plasmids on biofilm formation in different bacterial hosts. The results revealed that the presence of plasmids had a significant impact on both the biomass and structure of biofilms. Interestingly, *P. putida* showed a reduced ability to form biofilms when carrying the plasmids pKJK5 and RP4. However, the results showed the introduction of the IncP-1 plasmid pKJK5 into *E. coli* did not significantly affect its ability to form biofilm compared to the plasmid-free variant. The same work also found synergistic interactions in multispecies biofilms, where the combined presence of three bacterial strains resulted in significantly more biofilm formation compared to individual strains [65]. These results challenge previous studies that suggested a beneficial role of conjugative plasmids in biofilm formation, emphasising the importance of considering different bacterial species.

Dionisio et. al [66] focused on the effects of donor-to-recipient (D/R) ratios, temperature, and nutrient concentrations on the conjugative transfer of an IncP-1 plasmid in three natural *E. coli* recipient strains. This study evaluated the D/R ratios and found that a higher proportion of donors to recipients resulted in a greater number of transconjugants. Under optimal conditions with a 1:1 D/R ratio, two out of three *E. coli* strains showed high transconjugant numbers and transfer frequencies of the IncP-1 plasmid. The transfer efficiency varied among strains, which could be attributed to strain-specific characteristics or the repression of silencing systems that limit the expression of acquired genes in the recipient cell. The temperature was found to significantly affect the conjugation events [66]. The highest number of transconjugants was observed at 37 °C, while lower temperatures led to a decrease in the transconjugant numbers. Nutrient concentration also played a role in plasmid transfer [66]. Lower nutrient concentrations in the medium led to a substantial decrease in conjugation events. Compared with rich nutrient media Luria-Bertani (LB), the use of common surrogates for natural conditions such as simulated wastewater (SWW) and

soil extract (SE) resulted in a reduction of conjugation events. Overall, the effects of temperature and nutrient concentration were strain-dependent, highlighting the complexity of plasmid transfer dynamics and the importance of recipient characteristics. These findings have implications for the understanding of the spread of resistance genes through conjugative plasmids in different environmental compartments.

These studies highlight the role of biofilms as plasmid reservoirs, the impact of conjugative plasmids on biofilm formation, and the influence of environmental factors on plasmid transfer dynamics.

2.4.1. Factors influencing biofilm formation and conjugation in biofilms

Several factors may influence biofilm formation and consequently, HGT in biofilms, such as the bacterial strains used as donors and recipients, cell physiology, environmental conditions (such as the substratum and hydrodynamics), and development stage.

It has been previously described that Gram-negative bacteria engage more frequently in HGT than Gram-positive bacteria [67]. Gram-positive species exhibit distinct conjugation machinery compared to Gram-negative species, as they lack the production of conjugative pili. Instead, they rely on surface adhesins as the means to facilitate contact between donor and recipient cells during conjugation [22]. However, both types of bacteria are largely involved in the transfer of conjugative plasmids within biofilms. For instance, pCF10, a conjugative plasmid in *Enterococcus faecalis*, carries genes for cell wall-anchoring proteins (PrgA, PrgB, and PrgC) that enhance cell-cell adhesion during the initial stages of biofilm formation. Another example is pOLA52, a plasmid found in *Klebsiella pneumoniae*, which harbours genes for type III fimbriae that facilitate cell attachment to surfaces. *E. coli* possesses various conjugative plasmids, such as the F plasmid, which promotes biofilm formation through a mechanism dependent on the presence of conjugation pili [16]. Qui et al. [68] found that Gram-negative bacteria, such as *Vitreoscilla* and *Citrobacter*, showed higher conjugative potential for the IncP1 plasmid. Additionally, this study found that Gram-positive receptors in *Vagococcus* and *Lactobacillus* were able to acquire plasmids from Gram-negative donors, suggesting that physical distance between donors and receptors may play a more significant role than their taxonomic distance [68].

Quorum sensing (QS) allows bacteria to communicate with each other, leading to changes in behaviour in response to variations in the density and composition of the microbial community in the surrounding environment [69]. The QS signalling pathway has a significant impact on various cellular processes, including the transfer of genetic material, biofilm formation, antibiotic resistance, virulence, metabolic activity, and EPS production.

QS may affect antibiotic resistance by altering the expression of efflux pumps and β-lactamases, modulating the membrane structure, and promoting biofilm formation [70]. A recent article also showed that electrochemical disinfection may increase the spread of ARGs by promoting conjugative plasmid transfer. Thus, sublethal electrochemical conditions can increase the transfer of ARGs encoding the conjugative plasmid pKJK5. Both active chlorine and superoxide radicals appear to contribute to reactive oxygen species (ROS) accumulation, thus triggering an oxidative stress response and the expression of conjugation-related genes [71].

Currently, the study of plasmid transfer efficiency in biofilms relies on static assays such as filter-mating or plate culture [72]. However, it is well known that fluid flow dynamics play a critical role in biofilm development. Microfluidics was recently employed by Li and colleagues [72] for the real-time monitoring of plasmid-mediated HGT in biofilms. They found that the routes for gene transfer strongly depend on the structure and composition of the biofilm. Indeed, the movement of fluid generates shear stress on the surfaces where biofilms form, which affects the transfer of nutrients, oxygen, and bacteria between the bulk medium and the biofilm. Faster flow rates enhance the external mass transfer, which increases the delivery of nutrients and oxygen to the biofilm surface. Hydrodynamics also influences the biofilm structure by modulating microbial adhesion and detachment [8]. High shear stress leads to thinner, more compact, and robust biofilms, whereas low shear stress results in thicker, multilayered biofilms. However, while faster flow rates may boost nutrient and oxygen supply, denser biofilms may hinder substrate diffusion into deeper layers [8]. This may have a direct influence on plasmid transfer rates in biofilms since it has been reported that HGT is affected by environmental abiotic factors such as oxygen concentration and availability [73, 74]. One study examined the horizontal transfer of ARGs in both growing and non-growing cultures of *E. coli* using a conjugative multi-antibiotic and metal-resistant plasmid [75]. They reported that the conjugation frequency was generally higher under shaking conditions than under non-shaking conditions.

Biofilm growth can also be influenced by the characteristics of the surface material [76]. Some studies have suggested that biofilm formation is promoted on hydrophilic surfaces. For example, hydrophilic surfaces such as glass favour the formation of larger biofilms by *S. aureus* compared with hydrophobic surfaces such as polypropylene and polystyrene [77, 78]. In contrast, other studies have suggested that adhesion and subsequent biofilm formation may occur more readily on hydrophobic surfaces than on hydrophilic ones [79]. Furthermore, Mazumder et al. [80] showed that biofilm formation

can alter the initial hydrophobic properties of the substratum. Biofilm formation is influenced by not only the hydrophobicity of the material but also its topography [76].

Biofilm initiation involves the production of cell appendages and adhesion factors by planktonic cells, and these accessory factors promoting attachment to surfaces are often encoded by conjugative plasmids, leading to enhanced biofilm formation by the host bacteria. Interestingly, the conjugative pilus of a derepressed F plasmid has been found to promote biofilm formation in initially non-biofilm-forming *E. coli* cells, with the pilin TraA identified as the main adhesion factor inducing biofilm formation. Analysis of derepressed IncF plasmid R1drd19- and F-carrying *E. coli* biofilms demonstrated the rapid development of a dense, mature 3D mushroom-type biofilm architecture similar to *P. aeruginosa* biofilms. Notably, this unique biofilm structure bypasses the need for certain cell surface appendages such as flagella, type 1 fimbriae, Ag43, or curli, which are typically essential for *E. coli* biofilm formation [2].

2.4.2. Experimental models for studying conjugation in biofilms

Researchers have employed various quantitative methods to measure the transfer rates of plasmids and other DNA elements involved in HGT in different biological contexts. These include *in vitro*, *in vivo*, and *in situ* approaches that provide valuable insights into the dynamics and frequency of MGE transfer.

In vitro experiments conducted under controlled laboratory conditions offer an ideal environment for measuring specific HGT rates and isolating the modulating factors. Several quantitative experimental techniques allow for the precise measurement and quantification of the biophysical transfer process, including fluorescence-based methods, quantitative PCR (qPCR), and real-time monitoring [81]. Fluorescence-based techniques use fluorescent markers or reporter genes to quantify the transfer of MGEs. For example, researchers may engineer MGEs with fluorescent protein markers, such as green fluorescent protein (GFP), and measure the appearance of fluorescent cells as an indicator of transfer. Flow cytometry or fluorescence microscopy can be employed to quantify the number of fluorescent cells and determine transfer rates. Quantitative PCR is a widely used technique for quantifying DNA and RNA molecules. In the context of MGE transfer, qPCR can be utilised to measure the abundance of specific MGEs in donor and recipient cells before and after co-culture. The transfer rates are estimated by comparing the relative changes in MGE abundance. Real-time monitoring techniques allow the continuous measurement of MGE transfer over time. For instance, researchers may employ time-lapse microscopy or automated imaging systems to track the transfer of MGEs in real-time. This enables the observation of transfer dynamics, including the rates and patterns of transfer within

biofilms. Researchers have used these techniques to explore the genetic space of bacterial strains, highlighting the impact of recipient strain characteristics on conjugation efficiency. Additionally, *in vitro* experiments have allowed the exploration of the impact of antibiotics on conjugation efficiency and population structure post-conjugation [81, 82]. Prensky et al. [83] presented experimental evidence that newly generated transconjugants exhibit reduced growth rates and/or prolonged lag times immediately following plasmid acquisition, indicating the presence of a plasmid acquisition cost. Although measuring these processes has improved our understanding of plasmid dynamics in simple *in vitro* settings, predicting plasmid fate in complex environments remains a challenge.

As indicated previously, the spread of ARGs through HGT is a major concern for global public health. However, much research on HGT has been conducted *in vitro* and little is known about the spread of resistance genes *in vivo*. Therefore, researchers are increasingly focusing on *in vivo* models to investigate the dissemination of clinically relevant ARGs under realistic conditions. This may help develop innovative strategies to reduce the spread of bacterial resistance [84]. *In vivo* approaches incorporate the complexity of natural systems but trade the degree of controllability found in *in vitro* approaches. In many cases, direct transfer rate quantification is complex, so researchers have used proxy measures to derive the underlying rate parameters. *In vivo* studies also enable the investigation of naturally occurring mechanisms and environmental factors that modulate and, in some cases, inhibit gene transfer [81]. For instance, in a mouse model of *Salmonella* infection treated with streptomycin, Devlin et al. [85] observed that the inflammation of the intestinal tract stimulated the growth and proliferation of both the donor and recipient bacteria present in the gut.

In silico studies involve mathematical and computational modelling of HGT. They provide insights into how mobile MGEs can affect bacterial population structure and phylogeny on evolutionary timescales; however, HGT is inferred and not directly observed. Studies have shown that high gene transfer rates increase the likelihood of pathogenicity and serve as a potential offensive mechanism against the human immune system [81]. The transfer of bacterial plasmids through conjugation is a complex process involving multiple factors, including DNA and protein regulation, intra and extracellular signalling, and interactions. Mathematical modelling has been used to understand the role and significance of these factors in conjugation in complex environments, such as the human gut. However, these models have limitations and are dependent on the quality and availability of data and metadata. Therefore, more effort is needed to identify and characterise important *in vivo* factors to achieve significant conclusions using *in silico* modelling [86].

In general, understanding the underlying mechanisms involved in the transfer of MGEs is vital for developing effective measures to prevent the dissemination of antimicrobial resistance and other harmful traits.

3. Materials and methods

3.1. Bacterial strains, plasmids, and growth medium

In this work, *E. coli* JM109(DE3) (Promega, Madison, WI, USA) was used as plasmid recipient [87], and *E. coli* MG1655::*laqI*^{q} carrying pKJK5, a conjugative, green fluorescent protein (GFP) tagged IncP1 plasmid served as the donor strain [65, 88].

The donor strain (kindly provided by Prof. Mette Burmølle, University of Copenhagen, Denmark) was chromosomally modified to include a *lacI*^{q}*-Lpp-mCherry-kan*^{R} gene cassette, which conferred red fluorescence, and a *lacI*^{q} repressor that hindered GFP expression from the plasmid pKJK5 when present in the donor strain because the *lac* promoter regulates GFP expression in the plasmid. To promote the removal of the *lac*I^{q} repressor from the lac operon site, isopropyl β-D-1-thiogalactopyranoside (1 mM IPTG; VWR International, Carnaxide, Portugal) was used, which acts as a lactose analogue and effectively displaces *lac*I^{q} from the lac operon, allowing T7 RNA Polymerase to bind to the T7 promoter and initiate gene transcription [89-91].

Overnight cultures were grown separately in Luria-Bertani (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} NaCl; MP Biomedicals, Solon, OH, USA) at 30 °C with orbital agitation at 120 rpm. The donor strain was selectively grown in LB supplemented with kanamycin (50 μg mL^{-1}; Eurobio, Courtaboeuf, France) and trimethoprim (20 μg mL^{-1}; Alfa Aesar, Ward Hill, MD, USA). Bacterial cells were washed once by centrifugation (3,202 × *g* for 10 min at 18 °C; Eppendorf Centrifuge 5810R; Eppendorf AG, Hamburg, Germany), and resuspended and diluted in LB to obtain cell suspensions with an optical density (OD) of 0.25 at 610 nm (approximately 1×10^{8} cell mL^{-1}).

3.2. Surface preparation

To assess biofilm development and plasmid transfer frequency, three different surface materials were used: polyvinyl chloride (PVC), graphene sheet (GS), and glass (G). PVC and G coupons were immersed in 96% (v/v) ethanol for 1 h with gentle shaking and rinsed with autoclaved ultrapure water. They were then autoclaved for 20 min at 70 °C and 120 °C, respectively. Subsequently, all three types of coupons were sterilised using ultraviolet (UV) light before and after gluing to the bottom of 12-well plates used as biofilm formation platform [79].

3.3. Surface characterisation

3.3.1. Contact angle measurements and hydrophobicity

The contact angles of the surfaces were determined in three independent experiments conducted at 25 ± 2 °C using the sessile drop method (Dataphysics OCA 15 Plus; Filderstadt, Germany) as described by Gomes et al. [79]. Water, formamide, and α-bromonaphthalene (Sigma-Aldrich Co., St. Louis, MO, USA) were used as reference liquids. At least 25 measurements were performed for each material in each experiment.

Surface hydrophobicity was determined through the subsequent application of the Van Oss approach [92-94]. According to this method, the total surface free energy of a pure substance (γ^{TOT}) results from the sum of the non-polar Lifshitz–van der Waals (γ^{LW}) and polar Lewis acid–base (γ^{AB}) free energy components (Equation (1)).

$$\gamma^{TOT} = \gamma^{LW} + \gamma^{AB} \quad (1)$$

In turn, the free energy component of γ^{AB} depends on the electron acceptor (γ^{+}) and electron donor (γ^{-}) parameters, as shown in Equation (2):

$$\gamma^{AB} = 2\sqrt{\gamma^{+}\gamma^{-}} \quad (2)$$

The surface free energy components can be determined by measuring the contact angles (θ) of a solid surface (s) with three different liquids (l) of known surface tension components. This process involves three equations, one for each reference liquid, that must be solved simultaneously (Equation (3)).

$$(1 + \cos\theta){\gamma_l}^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^{+}\gamma_l^{-}} + \sqrt{\gamma_s^{-}\gamma_l^{+}}\right) \quad (3)$$

The degree of hydrophobicity of a surface is expressed as the free energy of interaction (ΔG, mJ.m^{-2}) between two entities of that surface immersed in a polar liquid (water (w) as a model solvent). This was determined using Equation (4).

$$\Delta G = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^{+}\gamma_w^{-}} + \sqrt{\gamma_s^{-}\gamma_w^{+}} - \sqrt{\gamma_s^{+}} - \sqrt{\gamma_w^{+}\gamma_w^{-}}\right) \quad (4)$$

If the interaction between the two entities is stronger than the interaction of each

entity with water (ΔG < 0 mJ.m^{-2}), the material is hydrophobic (free energy of interaction is attractive); in contrast, if ΔG > 0 mJ.m^{-2}, the material is hydrophilic (free energy of interaction is repulsive) [95].

3.3.2. Optical profilometry

Optical profilometry was used to determine the average roughness (S_a) of the three surface materials, as previously performed by Whitehead et al. [96, 97]. A MicroXAM surface mapping microscope (ADE Corporation, XYZ model 4400 mL system, Tucson, AZ, USA), with an objective of 50× and connected to an AD phase shift controller (Omniscan, Wrexham, UK), was used to image five areas (167 μm × 167 μm) of at least four different coupons of each surface material. S_a values were obtained through extended range vertical scanning interferometry using MapView AE 2.17 software (Omniscan, Wrexham, UK).

3.3.3. Scanning electron microscopy (SEM)

The surface morphology of the three surfaces was examined using SEM. The coupons were placed on SEM stubs (Agar Scientific, Stansted, UK) and sputter-coated with gold for 30 s in an SEM coating system (Polaron, London, UK). The secondary electron detector of a Supra 40VP scanning electron microscope (Carl Zeiss Ltd., Cambridge, UK) was used to obtain images of at least three coupons for each surface at an accelerating voltage of 2 kV.

3.4. Biofilm formation assays

Biofilms were formed in 12-well microtiter plates (VWR International, Carnaxide, Portugal) where *E. coli* JM109(DE3) (recipient strain) and *E. coli* MG1655 (donor strain) were individually and co-incubated in mixed cultures on three different surface materials (PVC, GS, and G), under static (0 rpm) and orbital shaking conditions (185 rpm).

The wells were filled with a total volume of 3 mL of cell suspension (adjusted to 0.25 $OD_{610\ nm}$ as described in Section 2.1.). Single- and dual-species biofilms (1:1 ratio, maintaining the final cell concentration of 1 × 10^8 cells mL^{-1}) of *E. coli* JM109(DE3) and *E. coli* with plasmid pKJK5 were then grown for 24 and 72 h at 25 °C under static and dynamic conditions in a shaker with a 25 mm orbital diameter (Agitorb 200ICP; Norconcessus, Ermesinde, Portugal). The choice of the shaking condition was based on a previous study from the group that reported a shaking frequency of 185 rpm in this incubator corresponding to an average shear rate of 40 s^{-1} on the bottom of the microplate [98]. Two

independent experiments with triplicate sets of coupons were performed for each condition (n = 6).

3.5. Biofilm analysis

3.5.1. Flow cytometry

Flow cytometry was used to evaluate the total cell number of single- and dual-species biofilms and monitor plasmid transfer within the dual-species biofilms of *E. coli* JM109(DE3) + *E. coli* MG1655 through the identification of donor and recipient cells. Due to the lack of the *lacI*^{q} gene in the recipient cells of *E. coli* JM109(DE3), GFP is expressed if the conjugative plasmid is transferred into these cells (transconjugants); on the other hand, the donor cells of *E. coli* MG1655 correspond to the mCherry-expressing cells. This information allows for the calculation of conjugation efficiency as the number of transconjugants per number of donors.

Biofilm cell suspensions were obtained by vortexing each coupon in 3 mL of sterile saline solution (8.5 g L^{-1} NaCl) at full speed (ZX4; VELP Scientifica Srl, Usmate, Italy) for 3 min [99]. Then, bacterial cells were analysed in a CytoFLEX flow cytometer model V0-B3-R1 (Beckman Coulter, Brea, CA, USA) using the CytExpert software version 2.4.0.28 (Beckman Coulter, Brea, CA, USA). All biofilm samples were diluted in saline solution before acquisition to assure accurate analysis. Bacterial cells were gated based on the forward scatter (FSC) and side scatter (SSC) signals. For dual-species biofilms, the number of mCherry-positive events (donor cells) was registered at FL2 (Fluorescence detector; BP 585/42 nm), while the number of GFP-positive events (transconjugant cells) was recorded at FL1 (BP 525/40 nm). Samples were acquired at a flow rate of 10 µL min^{-1} and results were presented as cells.cm^{-2}.

3.5.2. Confocal laser scanning microscopy (CLSM)

The spatial organisation and presence of transconjugants in mixed biofilms were assessed using CSLM. Biofilm samples were mounted on a coverslip and scanned using a 40× water objective (Leica HCX PL APO CS; Leica Microsystems GmbH, Wetzlar, Germany) in an inverted microscope (Leica DMI6000-CS) with 488 nm argon and 633 nm helium-neon lasers. The emitted fluorescence was simultaneously recorded within the ranges of 480–580 nm and 610–700 nm to monitor the GFP and mCherry proteins, respectively. A minimum of four stacks of horizontal plane images (512 × 512 pixels, corresponding to 387.5 µm × 387.5 µm) with a z-step of 1 µm was acquired for each coupon.

Top view and vertical cross-sections through the biofilms (two-dimensional (2D) images) were generated from the CLSM acquisitions by using the "Section" function of IMARIS 9.3 software (Bitplane AG, Zurich, Switzerland).

3.6. Statistical analysis

Descriptive statistics were used to calculate the mean and standard deviation (SD) or standard error (SE) for the number of biofilm total cells and transconjugant/donor ratios.

Differences between the number of biofilm cells obtained for three tested surfaces and two hydrodynamics were evaluated using Kruskal-Wallis and Mann-Whitney tests since the variables were not normally distributed. Statistically significant differences were considered for p-values < 0.05 (confidence level of 95 %).

Linear regression models were performed to estimate the effect of the independent variables - tested surfaces, hydrodynamic conditions, number of biofilm donor cells, and number of biofilm total cells - on transconjugant cells. PVC and 0 rpm were used as the reference conditions for the surfaces and hydrodynamics variables, respectively. Models were adjusted for the incubation time. Results were presented as mean estimates of effect (β) and the corresponding 95% confidence intervals (95% CI). Significant results were considered for p-values < 0.05.

Data analysis was performed using the IBM SPSS Statistics version 27.0 for Windows (IBM SPSS, Inc., Chicago, IL, USA).

4. Results and discussion

The main purpose of the present study was to investigate the impact of bacteria contact time, surface properties, and hydrodynamics on plasmid conjugation in defined bacterial biofilms using flow cytometry and confocal laser scanning microscopy as complementary method. Two different hydrodynamic conditions, – static (0 rpm) and shaking (185 rpm) –, and three different surface materials – polyvinyl chloride (PVC), graphene sheet (GS), and glass (G) – were chosen to carry out this study. The ability of the donor and recipient *E. coli* strains to form single- and dual-species biofilms was evaluated under the conditions aforementioned, and the efficiency of plasmid transfer within dual-species biofilms was estimated for bacterial strains co-cultured for 24 and 72 h.

4.1. Surface material analysis

Since it is known that surface properties influence cell adhesion and subsequent biofilm formation [100], the three materials tested for biofilm growth and conjugation efficiency were evaluated for their hydrophobicity (by contact angle measurements using the sessile drop method), roughness (by optical profilometry), and morphology (by scanning electron microscopy).

The hydrophobicity of the PVC, GS, and G surfaces was determined based on the contact angle values and further surface energy calculations (Table 1). Glass had a hydrophilic character ($\Delta G > 0$ mJ.m^{-2}), whereas the remaining materials (PVC and GS) were hydrophobic ($\Delta G < 0$ mJ.m^{-2}). Among the hydrophobic materials, GS was the most hydrophobic (p-value < 0.05). This surface parameter is of critical significance in biofilm studies since a substantial body of scientific research has consistently demonstrated that the hydrophobic characteristics of substratum surfaces, along with the presence of surface charge and roughness, exert a profound influence on the bacterial adhesion process and consequent biofilm development on solid surfaces [101]. Several studies have demonstrated that the extent of bacterial adhesion generally increases with increasing hydrophobicity and decreasing surface energy of abiotic surfaces for hydrophilic bacteria like *E. coli* [102, 103]. Thus, it is expected that the attachment of *E. coli* cells is higher on PVC and GS surfaces. However, the existing literature presents conflicting findings regarding the impact of hydrophobicity on biofilm formation [101]. Lorenzetti et al. [104] showed that increased hydrophilicity of treated surfaces reduced bacterial adhesion. Also, Verhorstert et al. [105] observed that polymeric films exhibited hydrophilic characteristics and showed a considerable decrease in the growth of *E. coli* compared to hydrophobic polypropylene

films. However, the presence of superhydrophobic surfaces resulted in a substantial reduction in bacterial adhesion [106]. Nevertheless, Mazumder et al. [80] suggested that the process of biofilm formation can bring about changes in the hydrophobic characteristics of substrate surfaces, implying that bacterial cells that have already attached themselves can actively modify the properties of the surface.

The attachment of bacteria at the early stages of biofilm formation and the subsequent growth of biofilms are also influenced by surface roughness and topography [101]. A notable difference in the average surface roughness (S_a) of three tested materials was observed (Table 1). The roughest surface was GS (S_a = 5.5 μm), followed by PVC (S_a = 1.4 μm) and G (S_a = 0.7 μm), which was the smoothest surface tested. According to Whitehead and Verran [107], surface roughness has the potential to strengthen the overall binding energy between cells and the surface by promoting a greater microorganism/material interface. Therefore, the roughness analysis predicts that *E. coli* biofilm development will be higher on GS and PVC surfaces than on G surface.

Table 1. Surface properties of PVC, GS, and G. Contact angles with water (θ_W), formamide (θ_F), and bromonaphthalene (θ_B), hydrophobicity (ΔG), and average roughness (S_a). Values represent mean ± standard deviation (SD) of three independent experiments.

Surface material	**Contact angles (°)**			**Hydrophobicity (mJ.m^{-2})**	**Roughness (μm)**
	θ_W	θ_F	θ_B	ΔG	S_a
PVC	79.3 ± 0.9	79.4 ± 0.6	40.3 ± 0.5	-12.3	1.4 ± 0.4
GS	75.2 ± 7.6	62.6 ± 7.7	37.6 ± 5.3	-32.8	5.5 ± 1.2
G	47.0 ± 0.4	49.1 ± 0.5	63.4 ± 0.9	19.3	0.7 ± 0.2

By employing SEM, it was possible to assess the morphological features of surfaces with exceptional resolution at the micrometre and nanometre scales [108]. SEM micrographs (Figure 3b) showed that glass (right image) had a very smooth surface compared to PVC (left) and GS (middle). While PVC shows some pits and bumps along the surface, the graphene sheet reveals the typical layered structure of some graphene materials [109]. These findings corroborated the profilometry analysis. Surface roughness plays a crucial role in bacterial attachment by increasing the available surface area and providing a supportive structure cell for adhesion. Additionally, the presence of rough surfaces can offer protection to bacteria against shear forces, thereby hindering the detachment of bound bacteria [101].

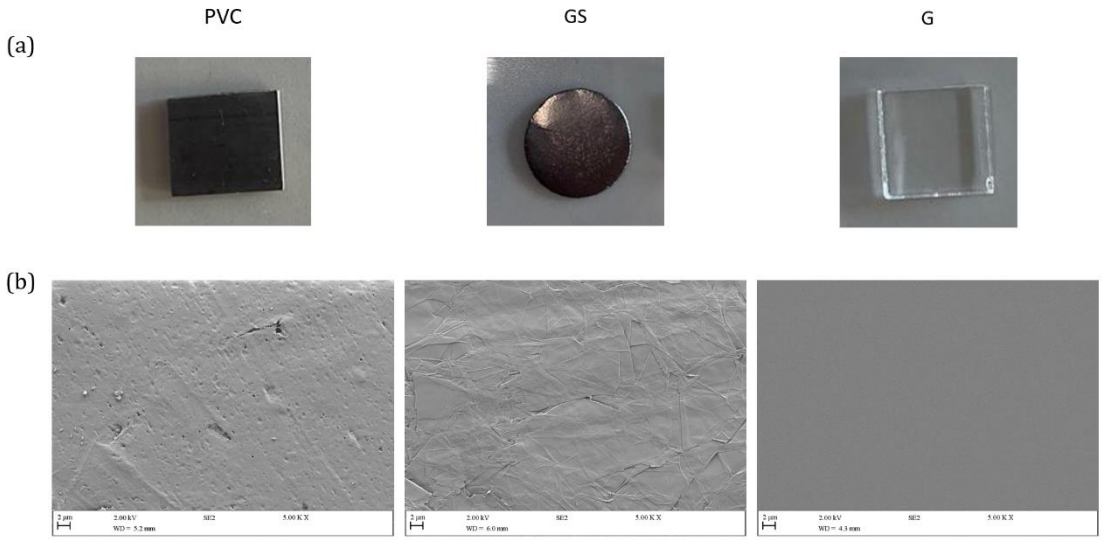

Figure 3. (a) Photos and (b) SEM images of PVC, GS and G surfaces. The micrographs have a magnification of 5,000×. The scale bars correspond to 2 µm.

4.2. Effect of hydrodynamics and surface material on *E. coli* JM109(DE3) biofilm formation (recipient strain)

To understand the biofilm-forming ability of *E. coli* recipient strain on different hydrodynamic conditions and surface materials, single-species biofilms were formed for 24 and 72 h, and the total number of sessile cells was determined using flow cytometry (Figure 4).

The number of *E. coli* JM109(DE3) biofilm cells exhibited significant variation when subjected to different hydrodynamic conditions (0 rpm vs. 185 rpm), with a p-value < 0.001 (Figure 4a). For 24 h, *E. coli* JM109(DE3) biofilms formed at 0 rpm displayed a slightly lower number of total cells than those formed at 185 rpm (1.0×10^8 cells.cm^{-2} vs. 1.3×10^8 cells.cm^{-2}; p-value = 0.153). Conversely, for 72 h, the number of cells was significantly higher for biofilms formed at 0 rpm (2.8×10^8 cells.cm^{-2}) than those formed at 185 rpm (5.7×10^7 cells.cm^{-2}) (p-value < 0.001).

Indeed, hydrodynamics plays a crucial role in *E. coli* biofilm development [8], which can be attributed to several factors. In the absence of agitation, the transfer of nutrients and oxygen to the biofilm may have been reduced [8], thus explaining the slightly lower number of biofilm cells for 0 rpm, at an initial biofilm stage (24 h). On the other hand, at a shaking frequency of 185 rpm (corresponding to an average shear rate of 40 s^{-1} [98]), the shear stress may contribute to the detachment of sessile cells, limiting their accumulation and the

formation of a denser biofilm, at a mature stage [110, 111]. This phenomenon may explain the lower number of cells in biofilms formed at 185 rpm for 72 h compared to those formed at 0 rpm.

Moreover, although shear forces can initially boost bacteria's metabolic activity and biofilm growth, it has been shown that sustaining higher metabolic activity over extended periods is not feasible [112]. This can explain the lowest difference between hydrodynamic conditions in the number of biofilm cells at 24 h (1.0 x 10^8 cells.cm^{-2} at 0 rpm vs. 1.3 x 10^8 cells.cm^{-2} at 185 rpm) when compared to 72 h of incubation (2.8 x 10^8 cells.cm^{-2} at 0 rpm vs. 5.7 x 10^7 cells.cm^{-2} at 185 rpm; Figure 4a).

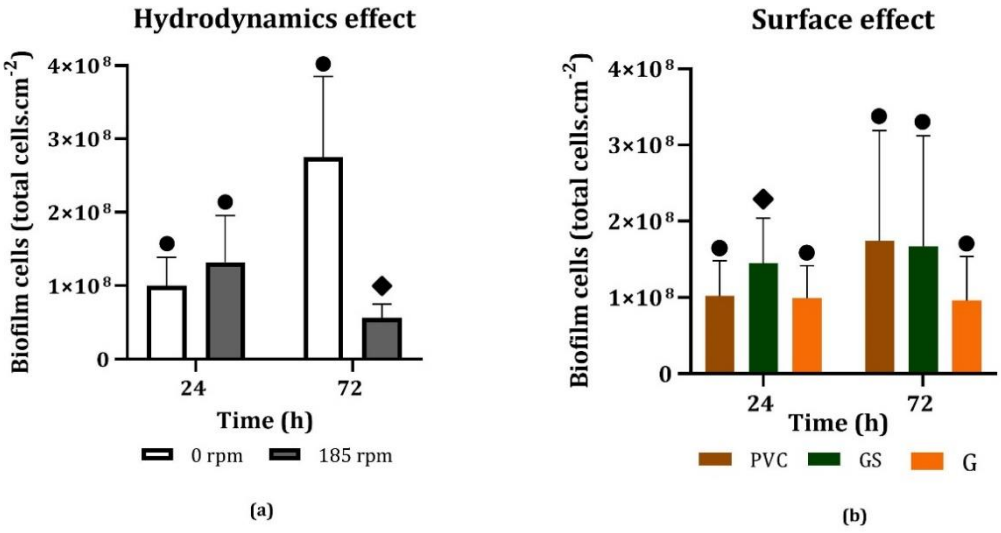

Figure 4. (a) Hydrodynamics and (b) surface effects on *E. coli* JM109(DE3) biofilm formation. Data are presented as the mean of total cell number ± SD. Different symbols(● and ◆) indicate significant differences between the number of biofilm cells in the tested conditions for the same time point (*p*-values < 0.05).

In general, no statistically significant differences were found between the number of biofilm cells at 24 and 72 h (*p*-value = 0.70), indicating that other factors such as surface properties may be influencing *E. coli* JM109(DE3) biofilm growth.

Regarding the surface materials used (PVC, GS, and G; Figure 4b), results indicated that, for biofilms of 24 h, the GS surface showed a significantly higher number of cells than the PVC and G surfaces (*p*-values < 0.05). In turn, for biofilms formed for 72 h, there were no significant differences between the number of cells obtained for the three tested surfaces (Figure 4b). Considering that biofilm formation is initiated by bacterial attachment to a surface, surface properties and bacterial-surface interactions play a major role in the initial stage of biofilm formation [101], thus explaining the differences in the number of cells

registered only for 24 h of incubation. The higher number of biofilm cells registered for GS can be attributed to the higher hydrophobicity level of this surface compared to PVC and G reported in Section 4.1 (Table 1). Furthermore, the higher surface roughness of graphene when compared to the other tested materials (Table 1) may have increased the contact area between the bacteria and this surface and enhanced biofilm formation during the first 24 h of the biofilm assay. Nevertheless, both surface properties (hydrophobicity and roughness) showed no influence after 72 h of biofilm formation.

4.3. Effect of hydrodynamics and surface material on *E. coli* MG1655 biofilm formation (donor strain)

To gain insight into the biofilm-forming ability of the *E. coli* donor strain under the tested hydrodynamic and surface conditions, the same type of quantitative analysis was performed (Figure 5).

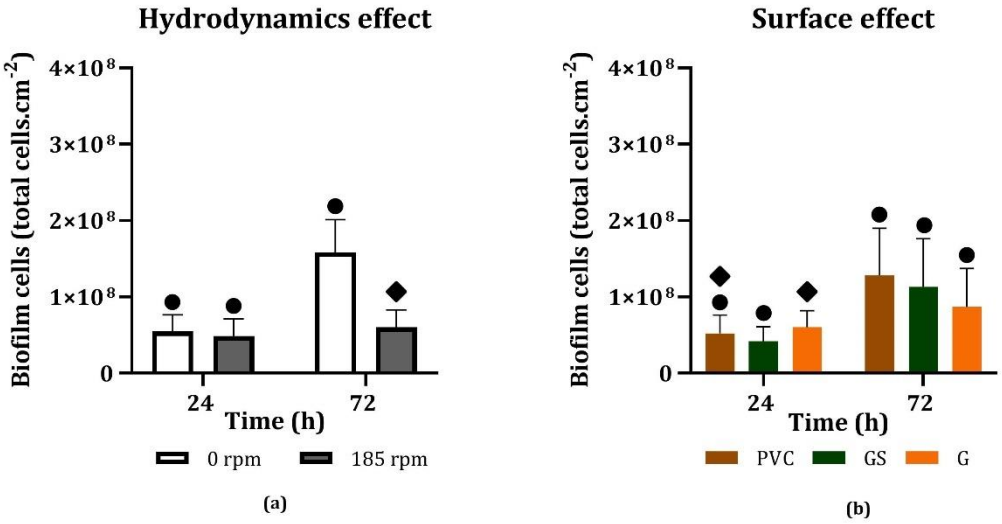

Figure 5. (a) Hydrodynamics and (b) surface effects on *E. coli* MG1655 biofilm formation. Data are presented as the mean of total cell number ± SD. Different symbols(● and ◆) indicate significant differences between the number of biofilm cells in the tested conditions for the same time point (*p*-values < 0.05).

Concerning hydrodynamics, there were no significant differences between the number of cells of biofilms formed at 0 and 185 rpm for 24 h (Figure 5a). In turn, for 72 h, biofilms formed at 0 rpm displayed a significantly higher number of cells than those formed at 185 rpm (1.6 x 10^8 cells.cm^{-2} vs. 6.0 x 10^7 cells.cm^{-2}; *p*-values < 0.001). This tendency was also observed for *E. coli* JM109(DE3) biofilms formed under the same conditions.

Additionally, the comparison between the 24-h and 72-h incubation periods revealed a highly significant difference in biofilm cell count (*p*-value < 0.001). This

highlights the significant impact of the incubation time on the biofilm growth of this specific *E. coli* strain.

Considering the surface materials used (PVC, GS, and G; Figure 5b), results indicated that, for biofilms formed for 24 h, the G surface displayed a higher number of cells compared to GS (p-value < 0.05), but similar to PVC (p-value > 0.05) surfaces. In opposition, for biofilms formed for 72 h, there were no significant differences between the number of cells obtained for the three tested surfaces (Figure 5b). Overall, the single-species biofilm analysis suggested that bacteria-surface interactions seem to be dependent on the tested strain.

Interestingly, compared to the *E. coli* JM109(DE3) strain discussed in the previous section, *E. coli* MG1655 formed, in general, less dense biofilms with a maximum of 1.7×10^8 total cells.cm^{-2} (p-value < 0.001). Plasmids are usually described as being capable of promoting biofilm formation [46, 113]. However, it has also been reported that biofilm phenotypes are due to molecular crosstalk between plasmids and chromosomes, which was also shown to be specific to plasmid-host combinations [66]. For instance, the same plasmid used in the present study (plasmid pKJK5) decreased *Pseudomonas putida* ability to form biofilms and increased that of *Kluyvera sp.* but did not affect *E. coli* [65]. In addition, conjugative pili (such as the one of IncP-1 plasmid pKJK5) may decrease the ability of conjugative plasmids to enhance biofilm formation, and even non-conjugative plasmids may enhance biofilm formation despite not expressing sex pili, as shown by our research group [87]. To conclude, the lower biofilm formation of the donor strain appears to be related to its intrinsic characteristics.

4.4. Effect of hydrodynamics and surface material on transconjugation

The biofilm-forming ability of *E. coli* JM109(DE3) and *E. coli* MG1655 in co-culture was also assessed under different hydrodynamics and surfaces materials (Figures 6 and 7), even before analysing the effect of these parameters on plasmid transfer ratio within mixed biofilms.

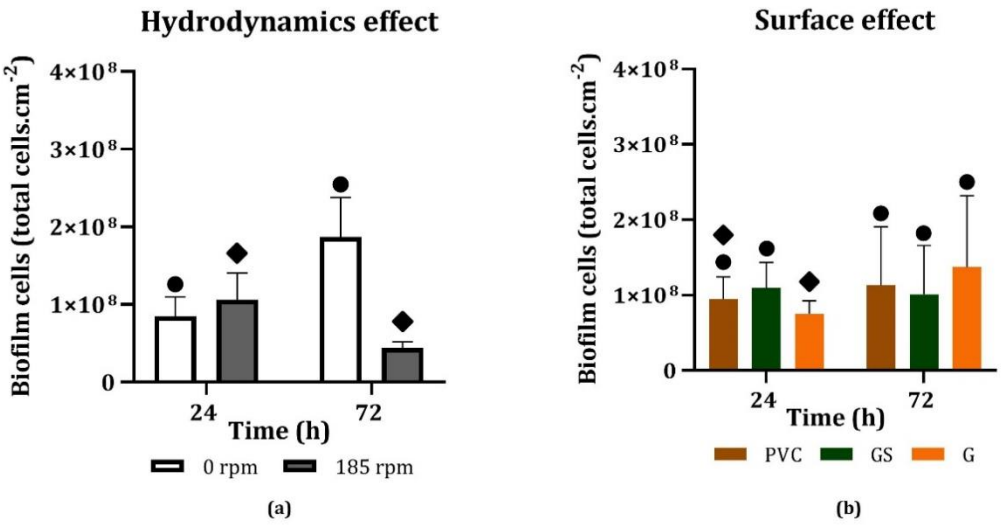

Figure 6. (a) Hydrodynamic and (b) surface effect on dual-species biofilms (total cells.cm^{-2}). Data are presented as the mean ± SD. Different symbols(● and ◆) indicate significant differences between the number of biofilm cells in the tested conditions for the same time point (*p*-values < 0.05).

In general, for both incubation times, the comparison between 0 and 185 rpm showed a highly significant difference in the total number of biofilm cells (*p*-value < 0.05; Figure 6a). This suggests that the hydrodynamic conditions impact the development of dual-species biofilms of *E. coli* JM109(DE3) (recipient strain) and *E. coli* MG1655 (donor strain). In contrast, the incubation time did not have a significant effect on the total cell amount of multi-species biofilms (*p*-value > 0.05).

In turn, the comparison of three surfaces at 24 h showed statistically significant differences in the total number of biofilm cells (*p*-value < 0.05; Figure 6b). More specifically, the GS surface displayed a significantly higher number of biofilm cells compared to the G surface (1.1 x 10^8 cells.cm^{-2} vs. 7.5 x 10^7 cells.cm^{-2}; *p*-value < 0.05). This surface effect can be explained by the aforementioned factors, such as the higher hydrophobicity of GS compared to G, as well as the higher roughness and porosity, which can create more favourable conditions for biofilm formation on carbon-based materials [114]. Similar to what happens

in single-species biofilms, there were no significant differences in the number of total cells of dual-species biofilms formed on different surfaces for 72 h (Figure 6b).

The previous results (Figure 6) considered all biofilm cells, including those that did not acquire conjugative plasmids. Thus, it is important to perform the same type of analysis to evaluate only the influence of hydrodynamics and surface type on the number of transconjugants (i.e. bacterial cells that incorporate the plasmid pKJK5 and consequently express GFP).

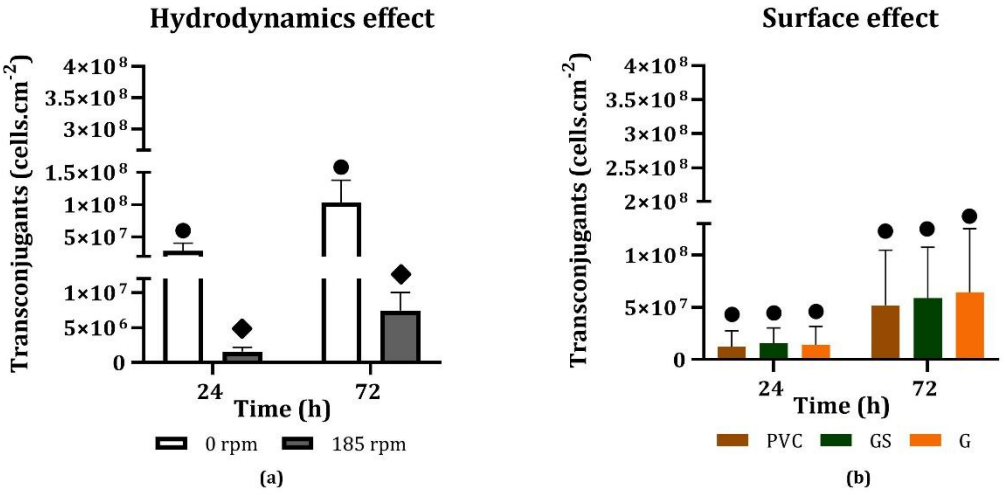

Figure 7. (a) Hydrodynamic and (b) surface effects on transconjugant cell number within dual-species biofilms (transconjugant cells.cm^{-2}). Data are presented as the mean ± SD. Data are presented as the mean ± SD. Different symbols(● and ◆) indicate significant differences between the number of biofilm cells in the tested conditions for the same time point (p-values < 0.05).

For both incubation times, the number of transconjugant cells was significantly higher for biofilms formed at 0 rpm compared to those formed at 185 rpm (1.26 Log higher at 24 h and 1.14 Log higher at 72 h; p-value < 0.05) (Figure 7a), suggesting that hydrodynamic conditions have a substantial impact on the process of transconjugation. A recent study found that shear forces associated with fluid flow induce bacterial stress [115]. Probably, under shaking conditions, *E. coli* cells suffered various forms of stress, such as mechanical stress imposed by shear forces caused by the oscillation of the liquid inside the wells of the microplate, oxidative stress resulting from changes in bacteria metabolism to adapt to environmental conditions [116], and detachment stress, causing cell dispersion and reduced biofilm cell density. It is believed that the combination of these factors may impact biofilm growth, affecting the dynamics of *in situ* plasmid transfer.

Regarding the contact time between donor and recipient cells (Figure 7a), it can be concluded that an increase from 24 to 72 h leads to a significantly higher number of transconjugant cells (approximately 4-fold higher, p-value < 0.001). These results suggest that a longer contact time has a positive influence on the transconjugation phenomenon between sessile cells [117].

Concerning the surface effect (Figure 7b), statistical analysis conducted for both time points indicated that there were no significant differences in the number of transconjugants among the three tested surfaces (p-value > 0.05).

Therefore, based on a complete statistical analysis, hydrodynamics and bacteria contact time were found to significantly influence the process of transconjugation during biofilm development, whereas the type of surface material did not exhibit a statistically significant effect. It is important to note that this pioneering study addressing three parameters simultaneously (hydrodynamic, surface, and time) did not have any other studies for comparison.

The ratio of transconjugants to donor cells is a valuable metric for understanding the efficiency of genetic material transfer during biofilm formation under different conditions. A higher ratio signifies more effective dissemination of genetic traits, potentially leading to increased dissemination of resistance factors. On the other hand, a lower ratio indicates limitations or barriers in the acquisition of genetic material. Figure 8 illustrates the ratio of transconjugants to *E. coli* MG1655 cells (donors) under different hydrodynamic conditions (0 rpm and 185 rpm) and at two different time points (24 h and 72 h) across three different surfaces (PVC, GS, and G).

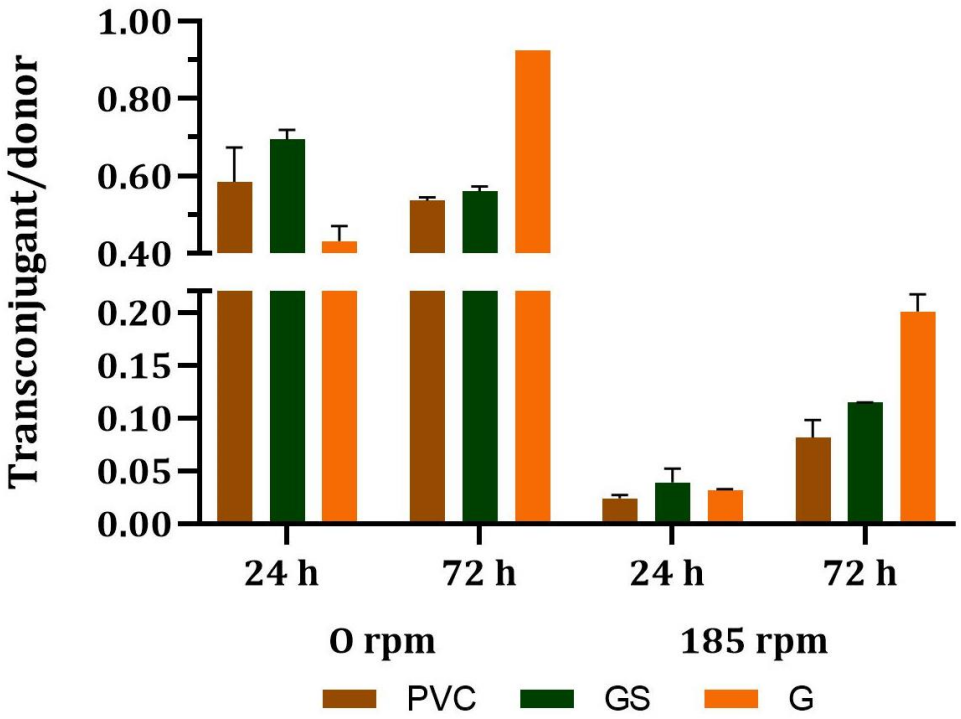

Figure 8. Transfer frequencies of plasmid pKJK5 from *E. coli* MG1655 (donor strain) to *E. coli* JM109(DE3) (recipient strain) within dual-species biofilms formed at 0 and 185 rpm on PVC, GS, and G surfaces for 24 and 72 h. Mean value ± standard error (SE) is shown.

In terms of hydrodynamics, it is evident that transconjugant/donor ratios were higher under static conditions than under dynamic conditions. Indeed, the ratios varied between 0.43 and 0.93 for the static condition, while they do not exceed 0.20 for the shaking condition. The main factor responsible for this behaviour is probably the much higher number of transconjugants detected in biofilms formed without agitation (Figure 7). The lower number of transconjugant cells on dual-species biofilms formed at 185 rpm can be explained by the energy demands associated with the conjugation process. The cells need to allocate their energy resources towards adapting to agitation [115], which leaves them with limited energy available for the conjugation process.

Regarding the incubation time, an overall increase in the ratio values was observed from 24 to 72 h, being this increase particularly evident for all surface materials under dynamic conditions (Figure 8). This can be explained by the fact that extended periods of co-incubation lead to the transmission of extra or distinct plasmids due to prolonged cell-to-cell interactions [118].

At 24 h, for both hydrodynamic conditions, GS seems to promote plasmid transfer. In contrast, after 72 h, the glass surface presented the highest number of conjugation events,

whether with or without shaking. This indicates that the surface properties seem not to be responsible for the different levels of transconjugation observed in the dual-species biofilms studied.

In order to understand the impact of the independent variables - hydrodynamic conditions, tested surfaces, number of biofilm donor cells, and number of biofilm total cells - on transconjugant events, linear regression models were applied.

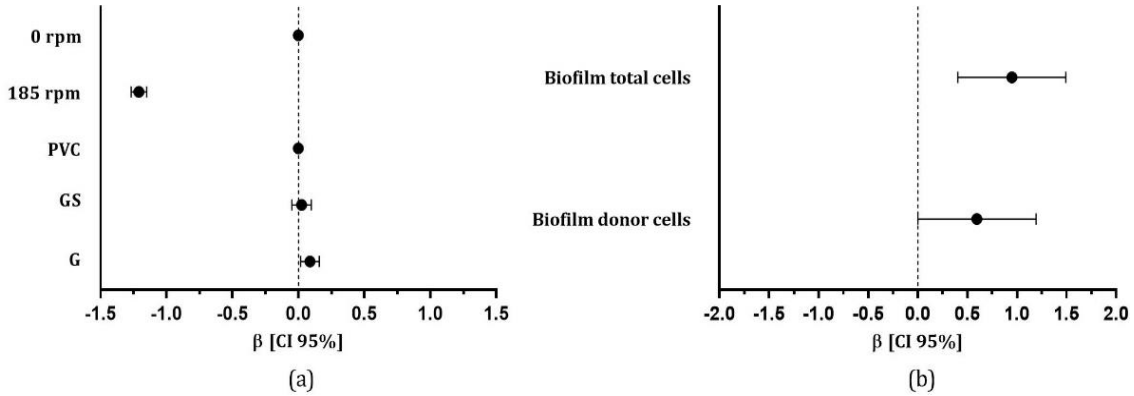

Figure 9. Association between the number of transconjugant cells and (a) the tested surfaces (PVC, polyvinyl chloride; GS, graphene; and G, glass) and hydrodynamics (0 and 185 rpm); and (b) the number of biofilm total cells and donor cells (presented as Log cell.cm^{-2}). PVC and 0 rpm were used as the reference conditions in (a). Linear regression models were adjusted for incubation time. Results were presented as mean estimates of effect (β) and the corresponding 95% confidence interval (CI 95%).

In general, higher shear forces (185 rpm of orbital shaking) were significantly associated with a lower number of transconjugant cells (-1.21 Log cells.cm^{-2} [-1.27: -1.15]) when compared to lower shear forces (0 rpm; Figure 9a). In turn, for the glass surface, the number of transconjugant cells was significantly higher (0.09 Log cells.cm^{-2} [0.02: 0.16]) than for the PVC surface. The GS surface was also associated with a higher number of transconjugant cells (0.02 Log cells.cm^{-2} [-0.05: 0.10]), although this association was not significant (Figure 9a). It is important to highlight that, in general, the conditions that led to a higher number of transconjugant cells were the same which resulted in a higher number of biofilm donor (*E. coli* MG1655), recipient (*E. coli* JM109(DE3)), and total cells (Figures 4, 5 and 6).

Concerning the association between the number of transconjugant cells and the number of biofilm total and donor cells, data indicated there is a significant association between these variables (Figure 9b). For 1 Log cells.cm^{-2} increment of biofilm total cells, the number of transconjugant cells increases, on average, by 0.95 Log cells.cm^{-2} [0.41:1.49]. Similarly, the number of transconjugant cells increases, on average, by 0.60 Log cells.cm^{-2}

[0.01:1.20] for each 1 Log cells.cm^{-2} of biofilm donor cells. Altogether, these results suggested that the efficiency of transconjugation depends on the number of donor and recipient cells present in the biofilm.

The spatial distribution of mixed biofilms and the formation of transconjugants were assessed by confocal laser scanning microscopy (CLSM) under both static and orbital shaking conditions (Figure 10), as the flow cytometry results demonstrated that the hydrodynamics had a strong influence on the conjugation efficiency (Figures 8 and 9a). *E. coli* cells that constitutively express mCherry (*E. coli* MG1655 strain) should be red fluorescent, whereas the transconjugants should appear green fluorescent.

A lower number of transconjugants was observed in the biofilm formed under shaking conditions (Figure 10b) than under non-shaking conditions (Figure 10a). These microscopic images corroborated the analyses discussed before. Additionally, it was found that regardless of the tested hydrodynamics, transconjugants became stably established in the biofilm, being preferentially located in the innermost regions close to the surface.

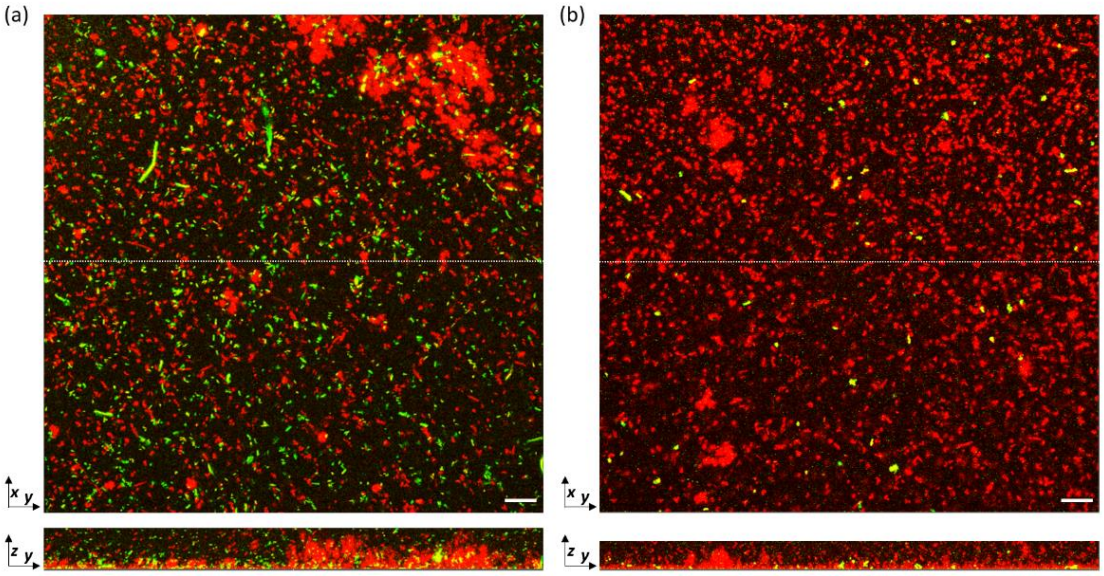

Figure 10. Microscopic visualisation of pKJK5 plasmid transfer within biofilms of *E. coli* JM109(DE3) + *E. coli* MG1655 formed on graphene sheet (GS) after 24 h under (a) 0 rpm and (b) 185 rpm. Donor cells are identified as red fluorescent cells, whereas the transconjugants appear in green. The top micrographs correspond to the top view of the biofilm, while the micrographs shown below are vertical sections of the biofilm collected at the positions indicated by the white dots. Representative images were obtained from confocal z-stacks using IMARIS software. White scale bars correspond to 100 μm.

5. Conclusions

In this work, the influence of bacteria contact time, hydrodynamics, and surface properties on plasmid conjugation in bacterial biofilms were evaluated using PVC, graphene and glass surfaces, at two shaking frequencies, 0 and 185 rpm.

Surface analysis showed that G exhibited a hydrophilic behaviour, while PVC and GS were hydrophobic, with GS being the most hydrophobic material. In addition, GS displayed a rougher surface than PVC and G, which had smoother surfaces.

In the single-species biofilms of *E. coli* JM109(DE3) and *E. coli* MG1655, hydrodynamics had a significant influence at 72 h of incubation, with a great number of biofilm cells being observed when there was no agitation. In turn, the surface properties significantly influenced biofilm density at the initial stage of biofilm formation (24 h). However, cell-surface interactions seem to be strain-dependent, with the GS displaying a higher number of *E. coli* JM109(DE3) cells and G a higher number of *E. coli* MG1655 cells.

In dual-species biofilms, hydrodynamics appeared to impact the total number of biofilm cells for both incubation times, while, once again, surface properties exerted a significant effect at 24 h of incubation, with GS showing a higher number of biofilm cells.

Concerning the number of transconjugant cells and the ratio of transconjugant/donor cells, both bacteria contact time and hydrodynamics had a significant influence on the transconjugation process, with the absence of agitation and the 72 h of bacteria contact time promoting it. In general, the conditions that promoted biofilm formation were the same which led to a higher number of transconjugant cells. Linear regression models demonstrated that there was a positive association between the 0 rpm, G surface, number of donor cells and total biofilm cells, and number of transconjugants on dual-species biofilms. Confocal microscopy corroborated the flow cytometry data and suggested that the transconjugants tended to be located in the innermost layers of the biofilm.

Overall, these findings emphasise the importance of considering hydrodynamics, incubation time, and surface properties when studying biofilm formation and, in particular, the transconjugation mechanism. Understanding these factors can help researchers design experiments, optimise conditions, and ultimately identify potential intervention points to reduce the dissemination of antibiotic resistance in diverse biological systems such as healthcare settings, environmental reservoirs, and agricultural practices.

6. Future work

In future work, it might be interesting to analyse the structure and composition of the biofilm matrix, since it has been described that extracellular polymeric substances (EPS) play an important role in biofilm formation and can influence horizontal gene transfer (HGT) through conjugation within biofilms. Although EPS can create physical barriers and limit diffusion, it can also concentrate cells and genetic material, promote intercellular communication, and serve as a reservoir for the uptake of genetic material.

Experimental studies varying the donor-to-recipient ratio can also be conducted to evaluate its impact on biofilm formation and plasmid conjugation, in an attempt to understand the role of donor and recipient strains on HGT within biofilms.

Exploring plasmid transfer between Gram-positive and Gram-negative species is another study that can provide valuable insights into conjugation mechanisms and transferability of genetic material across different bacterial groups.

As most conjugation studies have focused on plasmids, it would be interesting to further expand research into other mobile genetic elements (MGEs), such as integrative and conjugative elements (ICEs) and integrons.

Considering that flow cell systems and microfluidic devices offer powerful tools for studying HGT in biofilms under defined flow conditions, their application may provide a comprehensive analysis of the impact of hydrodynamics on HGT in different real-life scenarios. In fact, their ability to create controlled and reproducible environments, provide real-time imaging and observation, offer a high spatial and temporal resolution, facilitate high-throughput screening, and allow manipulation of environmental parameters make them valuable platforms for investigating the mechanisms and dynamics of HGT within biofilm communities.

References

[1] L. Serwecińska, "Antimicrobials and Antibiotic-Resistant Bacteria: A Risk to the Environment and to Public Health," *Water,* vol. 12, no. 12, p. 3313, 2020, doi: 10.3390/w12123313.

[2] C. Virolle, K. Goldlust, S. Djermoun, S. Bigot, and C. Lesterlin, "Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level," *Genes,* vol. 11, no. 11, p. 1239, 2020, doi: 10.3390/genes11111239.

[3] W. J. J. Meijer *et al.*, "Multiple Layered Control of the Conjugation Process of the Bacillus subtilis Plasmid pLS20," *Frontiers in Molecular Biosciences,* Review vol. 8, 2021, doi: 10.3389/fmolb.2021.648468.

[4] T. M. Coque, R. Cantón, A. E. Pérez-Cobas, M. D. Fernández-de-Bobadilla, and F. Baquero, "Antimicrobial Resistance in the Global Health Network: Known Unknowns and Challenges for Efficient Responses in the 21st Century," *Microorganisms,* vol. 11, no. 4, p. 1050, 2023, doi: 10.3390/microorganisms11041050.

[5] "About Antibiotic Resistance." https://msdh.ms.gov/msdhsite/_static/14,23756,228.html (accessed on 15/06/2023).

[6] R. B. Slayton *et al.*, "Vital Signs: Estimated Effects of a Coordinated Approach for Action to Reduce Antibiotic-Resistant Infections in Health Care Facilities — United States," *American Journal of Transplantation,* vol. 15, no. 11, pp. 3002-3007, 2015, doi: 10.1111/ajt.13555.

[7] C. Michaelis and E. Grohmann, "Horizontal Gene Transfer of Antibiotic Resistance Genes in Biofilms," *Antibiotics,* vol. 12, no. 2, p. 328, 2023, doi: 10.3390/antibiotics12020328.

[8] A. Soares, L. C. Gomes, G. A. Monteiro, and F. J. Mergulhão, "Hydrodynamic Effects on Biofilm Development and Recombinant Protein Expression," *Microorganisms,* vol. 10, no. 5, p. 931, 2022, doi: 10.3390/microorganisms10050931.

[9] S. Karačić, O. Modin, P. Hagelia, F. Persson, and B.-M. Wilén, "The effect of time and surface type on the composition of biofilm communities on concrete exposed to seawater," *International Biodeterioration & Biodegradation,* vol. 173, p. 105458, 2022, doi: 10.1016/j.ibiod.2022.105458.

[10] S. M. Soucy, J. Huang, and J. P. Gogarten, "Horizontal gene transfer: building the web of life," *Nature Reviews Genetics,* vol. 16, no. 8, pp. 472-482, 2015, doi: 10.1038/nrg3962.

[11] D. G. Quispe-Huamanquispe, G. Gheysen, and J. F. Kreuze, "Horizontal Gene Transfer Contributes to Plant Evolution: The Case of *Agrobacterium* T-DNAs," *Frontiers in Plant Science,* vol. 8, p. 2015, 2017, doi: 10.3389/fpls.2017.02015.

[12] V. Daubin and G. J. Szöllősi, "Horizontal Gene Transfer and the History of Life," *Cold Spring Harbor Perspectives in Biology,* vol. 8, no. 4, p. a018036, 2016, doi: 10.1101/cshperspect.a018036.

[13] M. Pepi and S. Focardi, "Antibiotic-Resistant Bacteria in Aquaculture and Climate Change: A Challenge for Health in the Mediterranean Area," *International Journal of Environmental Research and Public Health,* vol. 18, no. 11, 2021, doi: 10.3390/ijerph18115723.

[14] C. Manyi-Loh, S. Mamphweli, E. Meyer, and A. Okoh, "Antibiotic Use in Agriculture and Its Consequential Resistance in Environmental Sources: Potential Public Health Implications," *Molecules,* vol. 23, no. 4, 2018, doi: 10.3390/molecules23040795.

[15] W. Xiong, Y. Sun, X. Ding, M. Wang, and Z. Zeng, "Selective pressure of antibiotics on ARGs and bacterial communities in manure-polluted freshwater-sediment microcosms," *Frontiers in Microbiology,* vol. 6, p. 194, 2015, doi: 10.3389/fmicb.2015.00194.

[16] K. Abe, N. Nomura, and S. Suzuki, "Biofilms: hot spots of horizontal gene transfer (HGT) in aquatic environments, with a focus on a new HGT mechanism," *FEMS Microbiology Ecology,* vol. 96, no. 5, 2020, doi: 10.1093/femsec/fiaa031.

[17] L. C. Beltran *et al.*, "Archaeal DNA-import apparatus is homologous to bacterial conjugation machinery," *Nature Communications,* vol. 14, no. 1, p. 666, 2023, doi: 10.1038/s41467-023-36349-8.

[18] I. P. A. Lee, O. T. Eldakar, J. P. Gogarten, and C. P. Andam, "Bacterial cooperation through horizontal gene transfer," *Trends in Ecology and Evolution,* vol. 37, no. 3, pp. 223-232, 2022, doi: 10.1016/j.tree.2021.11.006.

[19] A. Luo, F. Wang, D. Sun, X. Liu, and B. Xin, "Formation, Development, and Cross-Species Interactions in Biofilms," *Frontiers in Microbiology,* Mini Review vol. 12, 2022, doi: 10.3389/fmicb.2021.757327.

[20] M. Emamalipour *et al.*, "Horizontal Gene Transfer: From Evolutionary Flexibility to Disease Progression," *Frontiers in Cell and Developmental Biology,* Review vol. 8, 2020, doi: 10.3389/fcell.2020.00229.

[21] M. Blokesch, "Natural competence for transformation," *Current Biology,* vol. 26, no. 21, pp. R1126-R1130, 2016, doi: 10.1016/j.cub.2016.08.058.

[22] O. T. Avery , C. M. MacLeod , and M. McCarty "Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types: Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type III," *Journal of Experimental Medicine,* vol. 79, no. 2, pp. 137-158, 1944, doi: 10.1084/jem.79.2.137.

[23] M. E. Olson and A. R. Horswill, "Bacteriophage Transduction in *Staphylococcus epidermidis*," *Methods in Molecular Biology,* vol. 1106, pp. 167-72, 2014, doi: 10.1007/978-1-62703-736-5_15.

[24] J. Lederberg, E. M. Lederberg, N. D. Zinder, and E. R. Lively, "Recombination analysis of bacterial heredity," *Cold Spring Harbor Symposia on Quantitative Biology,* vol. 16, pp. 413-43, 1951, doi: 10.1101/sqb.1951.016.01.030.

[25] P. F. Smith-Keary, "Transduction and Transformation," in *Genetic Elements in Escherichia coli*, P. F. Smith-Keary Ed. London: Macmillan Education UK, 1988, pp. 27-53.

[26] J. Lederberg and E. L. Tatum, "Gene recombination in *Escherichia coli*," *Nature,* vol. 158, no. 4016, p. 558, 1946, doi: 10.1038/158558a0.

[27] M. Varghese and M. Balachandran, "Antibacterial efficiency of carbon dots against Gram-positive and Gram-negative bacteria: A review," *Journal of Environmental Chemical Engineering,* vol. 9, no. 6, p. 106821, 2021, doi: 10.1016/j.jece.2021.106821.

[28] E. Grohmann, P. J. Christie, G. Waksman, and S. Backert, "Type IV secretion in Gram-negative and Gram-positive bacteria," *Molecular Microbiology,* vol. 107, no. 4, pp. 455-471, 2018, doi: 10.1111/mmi.13896.

[29] V. Kohler, W. Keller, and E. Grohmann, "Regulation of Gram-Positive Conjugation," *Frontiers in Microbiology,* vol. 10, p. 1134, 2019, doi: 10.3389/fmicb.2019.01134.

[30] G. Waksman, "From conjugation to T4S systems in Gram-negative bacteria: a mechanistic biology perspective," *EMBO Reports,* vol. 20, no. 2, 2019, doi: 10.15252/embr.201847012.

[31] E. L. Bean, C. Herman, M. E. Anderson, and A. D. Grossman, "Biology and engineering of integrative and conjugative elements: Construction and analyses of hybrid ICEs reveal element functions that affect species-specific efficiencies," *PLOS Genetics,* vol. 18, no. 5, p. e1009998, 2022, doi: 10.1371/journal.pgen.1009998.

[32] C. M. Johnson and A. D. Grossman, "Integrative and Conjugative Elements (ICEs): What They Do and How They Work," *Annual Review of Genetics,* vol. 49, pp. 577-601, 2015, doi: 10.1146/annurev-genet-112414-055018.

[33] S. Pande *et al.*, "Metabolic cross-feeding via intercellular nanotubes among bacteria," *Nature Communications,* vol. 6, no. 1, p. 6238, 2015, doi: 10.1038/ncomms7238.

[34] P. Bárdy, T. Füzik, D. Hrebík, R. Pantůček, J. Thomas Beatty, and P. Plevka, "Structure and mechanism of DNA delivery of a gene transfer agent," *Nature Communications,* vol. 11, no. 1, p. 3034, 2020, doi: 10.1038/s41467-020-16669-9.

[35] H.-C. Flemming and S. Wuertz, "Bacteria and archaea on Earth and their abundance in biofilms," *Nature Reviews Microbiology,* vol. 17, no. 4, pp. 247-260, 2019, doi: 10.1038/s41579-019-0158-9.

[36] Y. Jiang *et al.*, "Biofilm application in the microbial biochemicals production process," *Biotechnology Advances,* vol. 48, p. 107724, 2021, doi: 10.1016/j.biotechadv.2021.107724.

[37] M. Germec, A. Demirci, and I. Turhan, "Biofilm reactors for value-added products production: An in-depth review," *Biocatalysis and Agricultural Biotechnology,* vol. 27, p. 101662, 2020, doi: 10.1016/j.bcab.2020.101662.

[38] L. C. Gomes and F. J. M. Mergulhão, "A Selection of Platforms to Evaluate Surface Adhesion and Biofilm Formation in Controlled Hydrodynamic Conditions," *Microorganisms,* vol. 9, no. 9, 2021, doi: 10.3390/microorganisms9091993.

[39] A. R. Tomé, F. M. Carvalho, R. Teixeira-Santos, M. Burmølle, F. J. M. Mergulhão, and L. C. Gomes, "Use of Probiotics to Control Biofilm Formation in Food Industries," *Antibiotics,* vol. 12, no. 4, p. 754, 2023, doi: 10.3390/antibiotics12040754.

[40] M. Edel, H. Horn, and J. Gescher, "Biofilm systems as tools in biotechnological production," *Applied Microbiology and Biotechnology,* vol. 103, no. 13, pp. 5095-5103, 2019, doi: 10.1007/s00253-019-09869-x.

[41] J. Azeredo *et al.*, "Critical review on biofilm methods," *Critical Reviews in Microbiology,* vol. 43, no. 3, pp. 313-351, 2017, doi: 10.1080/1040841x.2016.1208146.

[42] H. C. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S. A. Rice, and S. Kjelleberg, "Biofilms: an emergent form of bacterial life," *Nature Reviews Microbiology,* vol. 14, no. 9, pp. 563-75, 2016, doi: 10.1038/nrmicro.2016.94.

[43] H.-C. Flemming *et al.*, "Who put the film in biofilm? The migration of a term from wastewater engineering to medicine and beyond," *npj Biofilms and Microbiomes,* vol. 7, no. 1, p. 10, 2021, doi: 10.1038/s41522-020-00183-3.

[44] S. Sjöberg *et al.*, "Bubble biofilm: Bacterial colonization of air-air interface," *Biofilm,* vol. 2, p. 100030, 2020, doi: 10.1016/j.bioflm.2020.100030.

[45] S. Águila-Arcos, I. Álvarez-Rodríguez, O. Garaiyurrebaso, C. Garbisu, E. Grohmann, and I. Alkorta, "Biofilm-Forming Clinical *Staphylococcus* Isolates Harbor Horizontal Transfer and Antibiotic Resistance Genes," *Frontiers in Microbiology* vol. 8, p. 2018, 2017, doi: 10.3389/fmicb.2017.02018.

[46] J.-M. Ghigo, "Natural conjugative plasmids induce bacterial biofilm development," *Nature,* vol. 412, no. 6845, pp. 442-445, 2001, doi: 10.1038/35086581.

[47] K. Sauer *et al.*, "The biofilm life cycle: expanding the conceptual model of biofilm formation," *Nature Reviews Microbiology,* vol. 20, no. 10, pp. 608-620, 2022, doi: 10.1038/s41579-022-00767-0.

[48] J. M. R. Moreira, L. C. Gomes, K. A. Whitehead, S. Lynch, L. A. Tetlow, and F. J. Mergulhão, "Effect of surface conditioning with cellular extracts on *Escherichia coli* adhesion and initial biofilm formation," *Food and Bioproducts Processing,* vol. 104, pp. 1-12, 2017, doi: 10.1016/j.fbp.2017.03.008.

[49] J. D. Bryers and C.-T. Huang, "Recombinant plasmid retention and expression in bacterial biofilm cultures," *Water Science and Technology,* vol. 31, no. 1, pp. 105-115, 1995, doi: 10.1016/0273-1223(95)00159-K.

[50] K. C. Cheng, A. Demirci, and J. M. Catchmark, "Advances in biofilm reactors for production of value-added products," *Applied Microbiology and Biotechnology,* vol. 87, no. 2, pp. 445-56, 2010, doi: 10.1007/s00253-010-2622-3.

[51] L. C. Cook and G. M. Dunny, "The influence of biofilms in the biology of plasmids," (in eng), *Microbiology Spectrum,* vol. 2, no. 5, p. 0012, 2014, doi: 10.1128/microbiolspec.PLAS-0012-2013.

[52] J. M. R. Moreira *et al.*, "Evaluation of SICAN performance for biofouling mitigation in the food industry," *Food Control,* vol. 62, pp. 201-207, 2016, doi: 10.1016/j.foodcont.2015.10.023.

[53] M. A. Rather *et al.*, "Microbial biofilm: A matter of grave concern for human health and food industry," *Journal of Basic Microbiology,* vol. 61, no. 5, pp. 380-395, 2021, doi: 10.1002/jobm.202000678.

[54] L. Javier, L. Pulido-Beltran, J. S. Vrouwenvelder, and N. M. Farhat, "Permeation Increases Biofilm Development in Nanofiltration Membranes Operated with Varying Feed Water Phosphorous Concentrations," *Membranes,* vol. 12, no. 3, p. 335, 2022, doi: 10.3390/membranes12030335.

[55] F. Di Pippo, L. Di Gregorio, R. Congestri, V. Tandoi, and S. Rossetti, "Biofilm growth and control in cooling water industrial systems," *FEMS Microbiology Ecology,* vol. 94, no. 5, 2018, doi: 10.1093/femsec/fiy044.

[56] A. Pereira, A. R. Silva, and L. F. Melo, "*Legionella* and Biofilms—Integrated Surveillance to Bridge Science and Real-Field Demands," *Microorganisms,* vol. 9, no. 6, p. 1212, 2021, doi: 10.3390/microorganisms9061212.

[57] I. B. Gomes, J.-Y. Maillard, L. C. Simões, and M. Simões, "Emerging contaminants affect the microbiome of water systems—strategies for their mitigation," *npj Clean Water,* vol. 3, no. 1, p. 39, 2020, doi: 10.1038/s41545-020-00086-y.

[58] S. Kerdi, A. Qamar, J. S. Vrouwenvelder, and N. Ghaffour, "Effect of localized hydrodynamics on biofilm attachment and growth in a cross-flow filtration channel," *Water Research,* vol. 188, p. 116502, 2021, doi: 10.1016/j.watres.2020.116502.

[59] Q. T. Trinh, K. C. Bal Krishna, A. Salih, A. Listowski, and A. Sathasivan, "Biofilm growth on PVC and HDPE pipes impacts chlorine stability in the recycled water," *Journal of Environmental Chemical Engineering,* vol. 8, no. 6, p. 104476, 2020, doi: 10.1016/j.jece.2020.104476.

[60] M. Hamad, F. Al-Marzooq, G. Orive, and T. H. Al-Tel, "Superbugs but no drugs: steps in averting a post-antibiotic era," *Drug Discovery Today,* vol. 24, no. 12, pp. 2225-2228, 2019, doi: 10.1016/j.drudis.2019.08.004.

[61] P. Blanco *et al.*, "Bacterial Multidrug Efflux Pumps: Much More Than Antibiotic Resistance Determinants," *Microorganisms,* vol. 4, no. 1, 2016, doi: 10.3390/microorganisms4010014.

[62] H. Gu, K. W. Kolewe, and D. Ren, "Conjugation in *Escherichia coli* Biofilms on Poly(dimethylsiloxane) Surfaces with Microtopographic Patterns," *Langmuir,* vol. 33, no. 12, pp. 3142-3150, 2017, doi: 10.1021/acs.langmuir.6b04679.

[63] A. Prinzi and R. E. Rohde, "The Role of Bacterial Biofilms in Antimicrobial Resistance," *American Society for Microbiology (ASM),* 2023, doi: 10.1155/2020/1705814.

[64] H. L. Røder *et al.*, "Biofilms can act as plasmid reserves in the absence of plasmid specific selection," *npj Biofilms and Microbiomes,* vol. 7, no. 1, p. 78, 2021, doi: 10.1038/s41522-021-00249-w.

[65] H. L. Røder, L. H. Hansen, S. J. Sørensen, and M. Burmølle, "The impact of the conjugative IncP-1 plasmid pKJK5 on multispecies biofilm formation is dependent on the plasmid host," *FEMS Microbiology Letters,* vol. 344, no. 2, pp. 186-92, 2013, doi: 10.1111/1574-6968.12175.

[66] J. A. Gama, E. G. A. Fredheim, F. Cléon, A. M. Reis, R. Zilhão, and F. Dionisio, "Dominance Between Plasmids Determines the Extent of Biofilm Formation," *Frontiers in Microbiology,* Original Research vol. 11, 2020, doi: 10.3389/fmicb.2020.02070.

[67] M. Groussin *et al.*, "Elevated rates of horizontal gene transfer in the industrialized human microbiome," *Cell,* vol. 184, no. 8, pp. 2053-2067.e18, 2021, doi: 10.1016/j.cell.2021.02.052.

[68] Y. Qiu, J. Zhang, B. Li, X. Wen, P. Liang, and X. Huang, "A novel microfluidic system

enables visualization and analysis of antibiotic resistance gene transfer to activated sludge bacteria in biofilm," *Science of The Total Environment,* vol. 642, pp. 582-590, 2018, doi: 10.1016/j.scitotenv.2018.06.012.

[69] K. Papenfort and B. L. Bassler, "Quorum sensing signal-response systems in Gram-negative bacteria," *Nature Reviews Microbiology,* vol. 14, no. 9, pp. 576-88, 2016, doi: 10.1038/nrmicro.2016.89.

[70] R. V. Sionov and D. Steinberg, "Targeting the Holy Triangle of Quorum Sensing, Biofilm Formation, and Antibiotic Resistance in Pathogenic Bacteria," *Microorganisms,* vol. 10, no. 6, p. 1239, 2022, doi: 10.3390/microorganisms10061239.

[71] H. Li, A. Dechesne, Z. He, M. M. Jensen, H. L. Song, and B. F. Smets, "Electrochemical disinfection may increase the spread of antibiotic resistance genes by promoting conjugal plasmid transfer," *Science of The Total Environment,* vol. 858, p. 159846, 2023, doi: 10.1016/j.scitotenv.2022.159846.

[72] B. Li, Y. Qiu, J. Zhang, X. Huang, H. Shi, and H. Yin, "Real-Time Study of Rapid Spread of Antibiotic Resistance Plasmid in Biofilm Using Microfluidics," *Environmental Science & Technology,* vol. 52, no. 19, pp. 11132-11141, 2018, doi: 10.1021/acs.est.8b03281.

[73] R. Pinilla-Redondo, L. Riber, and S. J. Sørensen, "Fluorescence Recovery Allows the Implementation of a Fluorescence Reporter Gene Platform Applicable for the Detection and Quantification of Horizontal Gene Transfer in Anoxic Environments," *Applied and Environmental Microbiology,* vol. 84, no. 6, pp. e02507-17, 2018, doi: doi:10.1128/AEM.02507-17.

[74] M. Skolimowski *et al.*, "Microfluidic dissolved oxygen gradient generator biochip as a useful tool in bacterial biofilm studies," *Lab on a Chip,* vol. 10, no. 16, pp. 2162-9, 2010, doi: 10.1039/c003558k.

[75] B. Headd and S. A. Bradford, "Physicochemical Factors That Favor Conjugation of an Antibiotic Resistant Plasmid in Non-growing Bacterial Cultures in the Absence and Presence of Antibiotics," *Frontiers in Microbiology,* Original Research vol. 9, 2018, doi: 10.3389/fmicb.2018.02122.

[76] E. N. Hayta, M. J. Ertelt, M. Kretschmer, and O. Lieleg, "Bacterial Materials: Applications of Natural and Modified Biofilms," *Advanced Materials Interfaces,* vol. 8, no. 21, p. 2101024, 2021, doi: 10.1002/admi.202101024.

[77] J. S. Lee, Y. M. Bae, S. Y. Lee, and S. Y. Lee, "Biofilm Formation of *Staphylococcus aureus* on Various Surfaces and Their Resistance to Chlorine Sanitizer," *Journal of Food Science,* vol. 80, no. 10, pp. M2279-86, 2015, doi: 10.1111/1750-3841.13017.

[78] S. I. Faria, R. Teixeira-Santos, M. J. Romeu, J. Morais, V. Vasconcelos, and F. J. Mergulhão, "The Relative Importance of Shear Forces and Surface Hydrophobicity on Biofilm Formation by Coccoid Cyanobacteria," *Polymers,* vol. 12, no. 3, p. 653, 2020, doi: 10.3390/polym12030653.

[79] L. C. Gomes, L. N. Silva, M. Simões, L. F. Melo, and F. J. Mergulhão, "*Escherichia coli* adhesion, biofilm development and antibiotic susceptibility on biomedical materials," *Journal of Biomedical Materials Research Part A,* vol. 103, no. 4, pp. 1414-23, 2015, doi: 10.1002/jbm.a.35277.

[80] S. Mazumder, J. O. Falkinham, A. M. Dietrich, and I. K. Puri, "Role of hydrophobicity in bacterial adherence to carbon nanostructures and biofilm formation," *Biofouling,* vol. 26, no. 3, pp. 333-339, 2010, doi: 10.1080/08927010903531491.

[81] J. Moralez, K. Szenkiel, K. Hamilton, A. Pruden, and A. J. Lopatkin, "Quantitative analysis of horizontal gene transfer in complex systems," *Current Opinion in Microbiology,* vol. 62, pp. 103-109, 2021, doi: 10.1016/j.mib.2021.05.001.

[82] C. Kessler, J. Hou, O. Neo, and M. M. C. Buckner, "*In situ*, *in vivo*, and *in vitro* approaches for studying AMR plasmid conjugation in the gut microbiome," *FEMS Microbiology Reviews,* vol. 47, no. 1, 2022, doi: 10.1093/femsre/fuac044.

[83] H. Prensky, A. Gomez-Simmonds, A.-C. Uhlemann, and A. J. Lopatkin, "Conjugation

dynamics depend on both the plasmid acquisition cost and the fitness cost," *Molecular Systems Biology,* vol. 17, no. 3, p. e9913, 2021, doi: 10.15252/msb.20209913.

[84] S. Tao, H. Chen, N. Li, T. Wang, and W. Liang, "The Spread of Antibiotic Resistance Genes *In Vivo* Model," *Canadian Journal of Infectious Diseases and Medical Microbiology,* vol. 2022, p. 3348695, 2022, doi: 10.1155/2022/3348695.

[85] J. R. Devlin *et al.*, "*Salmonella enterica* serovar Typhimurium chitinases modulate the intestinal glycome and promote small intestinal invasion," *PLOS Pathogens,* vol. 18, no. 4, p. e1010167, 2022, doi: 10.1371/journal.ppat.1010167.

[86] L. C. Ott and M. Mellata, "Models for Gut-Mediated Horizontal Gene Transfer by Bacterial Plasmid Conjugation," (in English), *Frontiers in Microbiology,* Review vol. 13, 2022, doi: 10.3389/fmicb.2022.891548.

[87] J. S. Teodósio, M. Simões, and F. J. Mergulhão, "The influence of nonconjugative *Escherichia coli* plasmids on biofilm formation and resistance," *Journal of Applied Microbiology,* vol. 113, no. 2, pp. 373-382, 2012, doi: 10.1111/j.1365-2672.2012.05332.x.

[88] G. Sengeløv, K. J. Kristensen, A. H. Sørensen, N. Kroer, and S. J. Sørensen, "Effect of Genomic Location on Horizontal Transfer of a Recombinant Gene Cassette Between *Pseudomonas* Strains in the Rhizosphere and Spermosphere of Barley Seedlings," *Current Microbiology,* vol. 42, no. 3, pp. 160-167, 2001, doi: 10.1007/s002840010197.

[89] L. C. Gomes and F. J. Mergulhão, "Effects of antibiotic concentration and nutrient medium composition on *Escherichia coli* biofilm formation and green fluorescent protein expression," *FEMS Microbiology Letters,* vol. 364, no. 5, 2017, doi: 10.1093/femsle/fnx042.

[90] A. Soares, L. C. Gomes, G. A. Monteiro, and F. J. Mergulhão, "The Influence of Nutrient Medium Composition on *Escherichia coli* Biofilm Development and Heterologous Protein Expression," *Applied Sciences,* vol. 11, no. 18, p. 8667, 2021, doi: 10.3390/app11188667.

[91] Q. Wang, A. K. Olesen, L. Maccario, and J. S. Madsen, "An easily modifiable conjugative plasmid for studying horizontal gene transfer," *Plasmid,* vol. 123-124, p. 102649, 2022, doi: 10.1016/j.plasmid.2022.102649.

[92] C. J. van Oss, M. K. Chaudhury, and R. J. Good, "Monopolar surfaces," *Advances in Colloid and Interface Science,* vol. 28, pp. 35-64, 1987, doi: 10.1016/0001-8686(87)80008-8.

[93] C. J. Van Oss, R. J. Good, and M. K. Chaudhury, "Additive and nonadditive surface tension components and the interpretation of contact angles," *Langmuir,* vol. 4, no. 4, pp. 884-891, 1988, doi: 10.1021/la00082a018.

[94] C. J. Van Oss, L. Ju, M. K. Chaudhury, and R. J. Good, "Estimation of the polar parameters of the surface tension of liquids by contact angle measurements on gels," *Journal of Colloid and Interface Science,* vol. 128, no. 2, pp. 313-319, 1989, doi: 10.1016/0021-9797(89)90345-7.

[95] S. Bayoudh, A. Othmane, F. Bettaieb, A. Bakhrouf, H. B. Ouada, and L. Ponsonnet, "Quantification of the adhesion free energy between bacteria and hydrophobic and hydrophilic substrata," *Materials Science and Engineering: C,* vol. 26, no. 2, pp. 300-305, 2006, doi: 10.1016/j.msec.2005.10.045.

[96] K. A. Whitehead, P. J. Kelly, H. Li, and J. Verran, "Surface topography and physicochemistry of silver containing titanium nitride nanocomposite coatings," *Journal of Vacuum Science & Technology. B* vol. 28, pp. 180-187, 2010, doi: 10.1116/1.3293232.

[97] A. Skovager, K. Whitehead, H. Siegumfeldt, H. Ingmer, J. Verran, and N. Arneborg, "Influence of flow direction and flow rate on the initial adhesion of seven Listeria monocytogenes strains to fine polished stainless steel," *International Journal of Food Microbiology,* vol. 157, no. 2, pp. 174-81, 2012, doi:

10.1016/j.ijfoodmicro.2012.04.028.

[98] M. J. Romeu *et al.*, "Biofilm formation behaviour of marine filamentous cyanobacterial strains in controlled hydrodynamic conditions," *Environmental Microbiology,* vol. 21, no. 11, pp. 4411-4424, 2019, doi: 10.1111/1462-2920.14807.

[99] I. M. Oliveira, M. Gomes, L. C. Gomes, M. F. R. Pereira, O. S. G. P. Soares, and F. J. Mergulhão, "Performance of Graphene/Polydimethylsiloxane Surfaces against S. aureus and *P. aeruginosa* Single- and Dual-Species Biofilms," *Nanomaterials,* vol. 12, no. 3, p. 355, 2022, doi: 10.3390/nano12030355.

[100] C. Spengler *et al.*, "Strength of bacterial adhesion on nanostructured surfaces quantified by substrate morphometry," *Nanoscale,* 10.1039/C9NR04375F vol. 11, no. 42, pp. 19713-19722, 2019, doi: 10.1039/C9NR04375F.

[101] S. Zheng *et al.*, "Implication of Surface Properties, Bacterial Motility, and Hydrodynamic Conditions on Bacterial Surface Sensing and Their Initial Adhesion," *Frontiers in Bioengineering and Biotechnology,* Review vol. 9, 2021, doi: 10.3389/fbioe.2021.643722.

[102] R. M. Goulter, I. R. Gentle, and G. A. Dykes, "Issues in determining factors influencing bacterial attachment: a review using the attachment of *Escherichia coli* to abiotic surfaces as an example," *Letters in Applied Microbiology,* vol. 49, no. 1, pp. 1-7, 2009, doi: 10.1111/j.1472-765X.2009.02591.x.

[103] J. K. Oh *et al.*, "The influence of surface chemistry on the kinetics and thermodynamics of bacterial adhesion," *Scientific Reports* vol. 8, no. 1, p. 17247, 2018, doi: 10.1038/s41598-018-35343-1.

[104] M. Lorenzetti *et al.*, "The Influence of Surface Modification on Bacterial Adhesion to Titanium-Based Substrates," *ACS Applied Materials & Interfaces,* vol. 7, no. 3, pp. 1644-1651, 2015, doi: 10.1021/am507148n.

[105] K. W. J. Verhorstert, Z. Guler, L. de Boer, M. Riool, J. W. R. Roovers, and S. A. J. Zaat, "*In Vitro* Bacterial Adhesion and Biofilm Formation on Fully Absorbable Poly-4-hydroxybutyrate and Nonabsorbable Polypropylene Pelvic Floor Implants," *ACS Applied Materials & Interfaces,* vol. 12, no. 48, pp. 53646-53653, 2020, doi: 10.1021/acsami.0c14668.

[106] Z. Montgomerie and K. C. Popat, "Improved hemocompatibility and reduced bacterial adhesion on superhydrophobic titania nanoflower surfaces," *Materials Science and Engineering: C,* vol. 119, p. 111503, 2021, doi: 10.1016/j.msec.2020.111503.

[107] K. A. Whitehead and J. Verran, "The Effect of Surface Topography on the Retention of Microorganisms," *Food and Bioproducts Processing,* vol. 84, no. 4, pp. 253-259, 2006, doi: 10.1205/fbp06035.

[108] W. Zhou, R. Apkarian, Z. L. Wang, and D. Joy, "Fundamentals of Scanning Electron Microscopy (SEM)," in *Scanning Microscopy for Nanotechnology: Techniques and Applications*, W. Zhou and Z. L. Wang Eds. New York, NY: Springer New York, 2007, pp. 1-40.

[109] C. Singh, A. K. Mishra, and A. Paul, "Highly conducting reduced graphene synthesis via low temperature chemically assisted exfoliation and energy storage application," *Journal of Materials Chemistry A,* 10.1039/C5TA04655F vol. 3, no. 36, pp. 18557-18563, 2015, doi: 10.1039/C5TA04655F.

[110] S. Wang, H. Zhu, G. Zheng, F. Dong, and C. Liu, "Dynamic Changes in Biofilm Structures under Dynamic Flow Conditions," *Applied and Environmental Microbiology,* vol. 88, no. 22, pp. e01072-22, 2022, doi: 10.1128/aem.01072-22.

[111] J. M. R. Moreira, J. S. Teodósio, F. C. Silva, M. Simões, L. F. Melo, and F. J. Mergulhão, "Influence of flow rate variation on the development of *Escherichia coli* biofilms," *Bioprocess and Biosystems Engineering,* vol. 36, no. 11, pp. 1787-1796, 2013, doi: 10.1007/s00449-013-0954-y.

[112] M. Krsmanovic, D. Biswas, H. Ali, A. Kumar, R. Ghosh, and A. K. Dickerson, "Hydrodynamics and surface properties influence biofilm proliferation," *Advances*

in Colloid and Interface Science, vol. 288, p. 102336, 2021, doi: 10.1016/j.cis.2020.102336.

[113] A. Reisner, B. M. Höller, S. Molin, and E. L. Zechner, "Synergistic Effects in Mixed *Escherichia coli* Biofilms: Conjugative Plasmid Transfer Drives Biofilm Expansion," *Journal of Bacteriology,* vol. 188, no. 10, pp. 3582-3588, 2006, doi: 10.1128/jb.188.10.3582-3588.2006.

[114] F. Sousa-Cardoso *et al.*, "Graphene-Based Coating to Mitigate Biofilm Development in Marine Environments," *Nanomaterials,* vol. 13, no. 3, p. 381, 2023, doi: 10.3390/nano13030381.

[115] G. C. Padron *et al.*, "Shear rate sensitizes bacterial pathogens to H_2O_2 stress," *Proceedings of the National Academy of Sciences,* vol. 120, no. 11, p. e2216774120, 2023, doi: 10.1073/pnas.2216774120.

[116] M. E. McBee, Y. H. Chionh, M. L. Sharaf, P. Ho, M. W. L. Cai, and P. C. Dedon, "Production of Superoxide in Bacteria Is Stress- and Cell State-Dependent: A Gating-Optimized Flow Cytometry Method that Minimizes ROS Measurement Artifacts with Fluorescent Dyes," *Frontiers in Microbiology,* Methods vol. 8, 2017, doi: 10.3389/fmicb.2017.00459.

[117] M. Hausner and S. Wuertz, "High rates of conjugation in bacterial biofilms as determined by quantitative *in situ* analysis," *Applied and Environmental Microbiology,* vol. 65, no. 8, pp. 3710-3, 1999, doi: 10.1128/aem.65.8.3710-3713.1999.

[118] T. S. Darphorn, B. B. Koenders-van Sintanneland, A. E. Grootemaat, N. N. van der Wel, S. Brul, and B. H. Ter Kuile, "Transfer dynamics of multi-resistance plasmids in *Escherichia coli* isolated from meat," *PLOS ONE,* vol. 17, no. 7, p. e0270205, 2022, doi: 10.1371/journal.pone.0270205.