Unveiling the Role of Free DNA in Biofilm Formation; Potential Relevance in Cholera Epidemiology

By

Abir Hassan Afrid
19136005

&

Mohammad Rafiue Rahman
19136049

A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfilment of the requirements for the degree of
B.Sc. in Biotechnology

Mathematics and Natural Sciences
BRAC University
October 2023

DECLARATION

It is hereby declared that

1. The thesis submitted is my/our own original work while completing a Bachelor of Science degree at Brac University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. We have acknowledged all main sources of help.

Student's Full Name & Signature:

_____ _____

Abir Hassan Afrid
19136005

Mohammad Rafiue Rahman
19136049

APPROVAL

The thesis/project titled “Unveiling the Role of Free DNA in Biofilm Formation; Potential Relevance in Cholera Epidemiology” submitted by

1. Abir Hassan Afrid (19136005)
2. Mohammad Rafiue Rahman (19136049)

of Spring, 2023 has been accepted as satisfactory in partial fulfilment of the requirement for the degree of B.Sc. in Biotechnology on October 19, 2023.

Examining Committee:

Supervisor:
(Member)

_____

Dr. Iftekhar Bin Naser
Associate Professor, Microbiology Program,
Department of Mathematics and Natural Sciences
BRAC University

Program Director:
(Member)

_____

Dr. Munima Haque
Associate Professor, Biotechnology Program,
Department of Mathematics and Natural Sciences
BRAC University

Departmental Head:
(Chair)

_____

Dr. A.F.M. Yusuf Haider
Professor, Department of Mathematics and Natural Sciences
BRAC University

ABSTRACT

This study meticulously probes the impact of both isogenic and heterogenic genomic DNA, in its entirety and fragmented states, on the density of biofilms formed by distinct bacterial strains, spanning both gram-positive and gram-negative classifications. Utilising samples from two distinct strains of *Vibrio cholerae* (WT346 and 1877), *Escherichia coli* (0157:H7), and *Staphylococcus aureus* (ATCC 25923), which were procured from the Life and Natural Sciences Laboratory at BRAC University, the formation and robustness of biofilms were quantitatively analysed via the crystal violet assay technique. Notably, biofilm density exhibited pronounced variations when exposed to isogenic genomic DNA as opposed to its heterogenic counterpart. Moreover, intact genomic DNA consistently elicited enhanced biofilm thickness relative to its fragmented variant, which elicited a more heterogeneous and attenuated response. Among the bacterial exemplars scrutinised, strain 1877 exhibited the most pronounced percentage augmentation in biofilm density in the presence of both fragmented (150%) and whole (125%) ATCC 25923 genomic DNA. This investigation underscores the nuanced interplay between genomic DNA and bacterial biofilm dynamics, offering pivotal insights for microbial ecology.

Keywords: Biofilm Formation; Genome DNA; Whole; Fragmented; Isogenic; Heterogenic

DEDICATION

To global researchers, whose dedication and pursuit of knowledge enrich academia and inspire future scholars, thank you for advancing our collective understanding.

ACKNOWLEDGEMENT

First and foremost, all praises to Allah who has blessed us with abundant blessings, dedication, perseverance, guidance, protection, wisdom, and strength for the timely and successful completion of our research thesis. We will be forever indebted to our parents for the constant love, support, guidance, and encouragement they have provided every step of the way.

Our deepest gratitude to our supervisor **Dr. Iftekhar Bin Naser**, Associate Professor at the Department of Mathematics and Natural Sciences, BRAC University for his unconditional support and invaluable assistance during our entire journey.

We would also like to convey our sincere gratitude to **Dr. Munima Haque**, Program Director of Biotechnology Program & Associate Professor, Department of Mathematics & Natural Sciences, BRAC University, for her wholehearted and unwavering support whenever we sought her assistance.

We would like to express our heartfelt gratitude to Professor **Dr. A F M Yusuf Haider**, Chairperson and Professor, Department of Mathematics & Natural Sciences, BRAC University, for his active support, encouragement and contribution. We sincerely thank everyone who has contributed to this project, whether directly or indirectly. It is because of their thoughtfulness and compassion that We were able to complete our research for our dissertation.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

CVEC	Conditionally Viable Environmental Cells
VBNC	Viable But Nonculturable Cells
LBR	Laboratory Biofilm Reactor
EPS	Extracellular Polymeric Substances
QS	Quorum Sensing
ECM	Extracellular Matrix
GTF	Glycosyltransferase
VPS	Vibrio Polysaccharide
PNAG	Poly-N-acetylglucosamine
ATCC	American Type Culture Collection
APW	Alkaline Peptone Water
LB	Luria Bertani Broth
TSB	Tryptone Soy Broth
TCBS	Thiosulfate-Citrate-Bile Salts-Sucrose
LA	Luria Bertani Agar
MSA	Mannitol Salt Agar
TSA	Tryptone Soy Agar
SDS	Sodium Dodecyl Sulfate
PCI	Phenol:chloroform:isoamyl alcohol
CI	Chloroform:isoamyl alcohol
TE	Tris-EDTA
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric acid
OD	Optical Density

CHAPTER 1 INTRODUCTION

Biofilms, structured communities of microbial cells, epitomise the adaptability of microorganisms. These communities adhere to surfaces and become encapsulated within a self-produced extracellular matrix. While they are found ubiquitously across diverse environments – from aqueous habitats to host organisms – they exhibit unique characteristics distinct from their planktonic counterparts. These include alterations in growth rates, gene expression, and notably, an increased resistance to antimicrobial agents. The evolutionary significance of biofilms underlines their adaptability and resilience across these diverse environments.

The matrix-mediated adhesion is not just a passive mechanism; it actively facilitates the establishment of micro-niches within the biofilm. These niches enable synergistic interactions among microbial cells and offer a robust defence against external perturbations. Comprising a rich blend of polysaccharides, proteins, lipids, and extracellular DNA (eDNA), this matrix is the linchpin of biofilm architecture and functionality. While the roles of many matrix components have been extensively studied, the contribution of eDNA, both intra- and inter-species, is a recently burgeoned focal point of interest.

Cholera's prevalence in regions like Bangladesh highlights the dangerous liaison between pathogenic biofilms and public health. Biofilm-associated infections, especially in clinical settings, pose significant challenges due to their heightened tolerance to standard antimicrobial treatments and evasion of host immune responses. However, the implications of biofilms are not solely pathological. In industrial and environmental domains, biofilms have both beneficial roles, as seen in bioremediation processes, and detrimental impacts, such as biocorrosion.

Given the vast clinical, environmental, and industrial ramifications of biofilms, a deep E. understanding of the nuances underpinning their formation, structure, and stability is indispensable. Although examining the biofilm-forming capabilities of individual species provides foundational insights, true ecological contexts often encompass multispecies

communities. Within these communities, diverse microbial interactions can lead to both competitive and cooperative outcomes, thereby shaping the final biofilm phenotype.

With this backdrop, the present investigation seeks to elucidate the role of eDNA in the biofilm dynamics of three bacterial species: *Vibrio cholerae*, *Eshcherichia coli*, and *Staphylococcus aureus*. The selection of these organisms is rooted in their clinical significance and their well-established biofilm-forming aptitudes. By delving into the contribution of eDNA to their biofilm phenotypes, this research aims not only to broaden the current understanding but also to identify potential avenues for targeted interventions.

1.1 Background

Cholera Epidemiology: Delving Deeper into the Role of Phages, Genetics, and Environment

Cholera, a formidable disease predominantly caused by the bacterium *Vibrio Cholerae*, continues to present considerable challenges to global public health systems. Beyond the immediate symptoms and distress it causes, the underlying intricacies of its epidemiology are a web of interactions between genetic changes within the bacterium, the environmental reservoirs it resides in, and the ever-present influence of bacteriophages. These factors don't operate in isolation; rather, they continually interact, influence, and shape the landscape of cholera outbreaks. This review seeks to provide a deeper understanding of these multifaceted interactions and their roles in sustaining cholera in various regions worldwide.

Continual Genomic Adaptations of *V. cholerae*

The recent identification of the BD-1.2 subclade of *Vibrio cholerae* during Bangladesh's 2022 cholera epidemic, as presented by Monir et al. (2023), underscores the bacterium's continuous genomic adaptation. *V. cholerae*, the etiological agent behind cholera, remains a critical health threat globally, especially in regions persistently grappling with its endemicity. Monir and colleagues, through meticulous genomic monitoring amidst the 2022 outbreak, unveiled the rise of the BD-1.2 subclade, characterised by distinctive genetic markers that suggest an evolutionary

divergence from known strains. This discovery not only accentuates the genomic malleability inherent to cholera but also underscores the relentless evolutionary progression of *V. cholerae* in adapting to diverse environmental and host-specific pressures.

Environmental Dynamics and Cholera Transmission

Faruque et al. (2005) conducted a comprehensive study to investigate the relationship between cholera and aquatic ecosystems. Their study illuminated a pattern where cholera outbreaks mirrored the concentrations of *V. cholerae* in water bodies, suggesting that environmental factors in these ecosystems may steer cholera dynamics. One pivotal environmental determinant highlighted in the study is water temperature. *V. cholerae* displays optimal growth in warm aquatic conditions, with elevated temperatures correlating with enhanced bacterial proliferation and survival. The investigation revealed that during thermally elevated periods, concentrations of *V. cholerae* in aquatic systems experienced a surge, aligning with heightened cholera incidences. Such findings accentuate the significance of water temperature in fostering *V. cholerae* expansion, subsequently influencing cholera outbreak patterns. Another salient environmental parameter emphasised is salinity. *V. cholerae* exhibits a predilection for brackish waters characterised by intermediate salinity. Regions with pronounced salinity exhibited elevated *V. cholerae* concentrations, correlating with a heightened propensity for cholera outbreaks. This intimates that salinity potentiates the growth and resilience of *V. cholerae*, fostering cholera manifestation in specific locales. The investigation further illuminated the intricate role of microbial ecology in modulating cholera dynamics. The microbial milieu in aquatic systems can sway the trajectory of *V. cholerae* survival and propagation. While specific microbial entities might offer a conducive ambiance or essential nutrients for *V. cholerae*, others could antagonise or impede its proliferation. Alterations in this microbial assemblage were concomitant with fluctuations in *V. cholerae* concentrations, underscoring the paramount influence of aquatic microbial ecology on cholera's epidemiology.

Bacteriophages: Key Modulators in Cholera Epidemiology

Faruque and Mekalanos (2012) elucidated the intricate dynamics between bacteriophages and toxigenic *V. cholerae*. Their findings illuminated the indispensable role bacteriophages assume in guiding *V. cholerae* evolution by modulating its population dynamics and fostering genetic versatility. Bacteriophages can mediate genetic exchange amongst diverse *V. cholerae* strains, culminating in the emergence of novel virulent variants. This accentuates the criticality of bacteriophages in shaping the evolutionary trajectory and virulence of *V. cholerae*. Hoque et al. (2016) demystified *V. cholerae's* defensive mechanisms against environmental bacteriophages, unveiling the exploitation of quorum sensing—a sophisticated bacterial communication mechanism—to thwart bacteriophage incursions. This coordinated, collective response fortifies *V. cholerae's* resilience against bacteriophage predation, offering a glimpse into the sophisticated interaction dynamics between the bacteria and bacteriophages. Naser et al. (2017) delved into the influence of the bacteriophage's CRISPR-Cas system on the evolution of toxigenic *V. cholerae*. Recognized as a bacterial and archaeal adaptive immune mechanism, the CRISPR-Cas system defends against bacteriophage invasions. The system enables *V. cholerae* to assimilate and integrate bacteriophage DNA sequences, conferring immunity against subsequent invasions. Such insights exemplify the evolutionary tug-of-war between *V. cholerae* and bacteriophages, underscoring the CRISPR-Cas system's centrality in sculpting *V. cholerae's* genetic diversity and adaptability.

V. cholerae Dormancy and Autoinducer Dynamics

Vibrio cholerae, the pathogen responsible for the gastroenteric ailment cholera, remains a perennial global health conundrum. Contemporary research accentuates the profound impact environmental bacteriophages exert on cholera epidemiology, particularly concerning the bacterium's biofilm-associated and free-swimming manifestations (Gallego-Hernández et al., 2020). Biofilms are complex, matrix-enclosed communities of bacteria. For *V. cholerae*, biofilm formation not only amplifies its persistence in natural habitats but also its infectivity (Gallego-Hernández et al., 2020). These biofilms act as defensive fortresses against numerous environmental stressors, most notably predation by both protozoa and bacteriophages. In regions where cholera is endemic, the eradication of these biofilms can drastically reduce the incidence of the disease (Gallego-Hernández et al., 2020). This significance is further emphasised by

patient stool samples, which have been observed to contain *V. cholerae* in both free-floating (planktonic) cells and as biofilm aggregates. Intriguingly, the infectivity of these biofilm aggregates is notably higher than their planktonic counterparts (Gallego-Hernández et al., 2020). Transitioning to a state known as Conditionally Viable Environmental Cells (CVEC) or the more commonly recognized term 'viable but nonculturable' (VBNC) cells, presents another dimension to the bacterium's environmental survival strategy (Kamruzzaman et al., 2010). These cells, though dormant, are believed to play a vital role in cholera transmission. Despite their recognized importance, the exact triggers prompting *V. cholerae* to adopt this dormant state and the broader implications of such a transition remain largely enigmatic (Kamruzzaman et al., 2010). However, existing research does suggest that this transition is inextricably linked to the formation of biofilms and is governed by quorum sensing, a system where bacterial behaviours are regulated based on population density (Kamruzzaman et al., 2010). Elaborating on this dormancy phenomenon, Naser et al. (2019) unveiled the role of autoinducers, specific signalling molecules produced by *V. cholerae*, in orchestrating the revival of CVEC (Naser et al., 2019). Acting as cellular messengers, autoinducers facilitate intrapopulation communication, enabling coordinated behaviour among *V. cholerae* communities. Within the context of CVEC, these molecules serve as environmental indicators, hinting at the opportune moments for dormant cells to resuscitate (Naser et al., 2019). A follow-up study in 2021 delved deeper into the precise autoinducers involved, identifying AI-2 and CAI-1 among others, and the associated signalling pathways that mediate this revival (Naser et al., 2021). Through these revelations, it becomes evident that autoinducers are instrumental in the bacterium's adaptive strategies, ensuring its survival and amplifying its resilience against environmental adversities. In summation, understanding the nuances of *V. cholerae's* interactions with its environment, its ability to form protective biofilms, and the triggers that influence its transition between active and dormant states is pivotal for shaping effective interventions and control measures against cholera.

Forward-Looking Control Measures

Cholera's epidemiology, multifaceted and deeply entwined with environmental and genetic dynamics, calls for an integrative approach to devise effective containment measures. While traditional methods have rendered varying degrees of success, innovative interventions,

particularly the use of bacteriophages, emerge as promising alternatives. However, it's worth noting that while bacteriophages may aid in controlling *V. cholerae* populations, the aftermath of their action presents another layer of complexity. As phages lyse bacteria, they release a deluge of genetic material into the environment. This free DNA, rich in genetic information, may inadvertently serve as a reservoir of genetic material that dormant *V. cholerae* cells can uptake, potentially leading to their resuscitation or even acquiring new traits. Such interactions amplify the importance of understanding the broader ecological consequences of any intervention. In essence, a holistic grasp of these dynamics can pave the way for more strategic, informed, and sustainable measures to manage and possibly preempt future cholera outbreaks.

CHAPTER 2 LITERATURE REVIEW

Biofilms represent one of the most intricate and diverse forms of microbial existence. Beyond their biological intrigue, they have profound implications for human health, industry, and the environment. These dynamic microbial communities, both in their architecture and function, showcase the adaptability and resilience of microorganisms across diverse settings. Whether it's the nuanced hierarchical construct they exhibit or the multitude of factors influencing their formation and stability, biofilms warrant comprehensive academic scrutiny.

The following literature review delves deep into various facets of biofilm research. From foundational concepts that define these complex communities to a focused examination of their core components, this review strives for a holistic understanding. A particular emphasis is placed on the role of DNA—both intra and inter-species—and its significance in biofilm dynamics. By anchoring our discussion within the context of key bacterial species—*V. cholerae*, *E. coli*, and *S. aureus*—and employing insights from laboratory biofilm reactor (LBR) analyses, we aim to bridge traditional understanding with contemporary findings.

Engage with the subsequent sections to unravel the complexities of biofilms, appreciate their hierarchical design, discern the multifactorial influences on their formation, and comprehend the pivotal role of DNA in shaping these microbial fortresses.

2.1 Biofilms: Defining Complex Microbial Communities and Their Importance

Biofilm development signifies an orchestrated transition from free-living planktonic states to structured multicellular communities adhering to surfaces. Within the academic community, defining "biofilm" has seen its evolution over the years, reaching a consensus captured in the guide “Research on Microbial Biofilms," released on December 20, 2002. According to this seminal guide, a biofilm is delineated as "an accumulation of microorganisms, encompassing bacteria, fungi, and/or protozoa, along with associated bacteriophages and other viruses, embedded within a polysaccharide matrix, adhering either to biologic or nonbiologic solid surfaces."

In parallel to the term "biofilm", the descriptor "biofouling" has emerged in the scientific lexicon to signify attached microbial growth on surfaces. The genesis of the term "biofouling" is derived from "fouling," which traditionally pertains to the contamination of surfaces predominantly by mineral deposits. While "fouling" and "biofouling" converge in their reference to surface contamination, the latter accentuates the amalgamation of mineral deposits and biological entities - living microorganisms, macroorganisms, and the extracellular polymeric substances (EPS) excreted by these microorganisms. It's worth noting that "biofouling" engenders lesser contention than "biofilm" within scholarly circles. This is perhaps attributed to the connotation carried by "fouling," which evades implying any specific geometrical configuration of the deposits, an implication inherently suggested by the term "film."

In microbial research, the term "biofilm" maintains its prominence. Defined as a structured community of microbial cells anchored to a surface and enveloped in an extracellular polymeric substance (EPS) matrix (Karygianni, et al. 2020), biofilms have garnered significant attention. Their adaptability and resilience open doors for innovations spanning healthcare, agriculture, and environmental conservation. Their ubiquitous presence, play a multifaceted role that touches various sectors. Ecologically, they are integral to natural environments, fostering essential nutrient cycling processes that underpin ecosystem functions. In the medical realm, biofilms are often a bane, shielding pathogens and making infections notoriously hard to treat due to their resilience against antibiotics and immune defences. On the industrial front, their impact is

twofold: while they are harnessed for vital processes like wastewater treatment and bioremediation, they can also lead to biofouling, causing operational challenges and potential equipment damage.

Biofilms, as structured microbial communities, are pervasive in various natural habitats, significantly influencing key ecological processes such as nutrient cycling, which, in turn, reinforces the functioning of ecosystems. The formation of biofilms on surfaces offers multiple advantages to microbial communities. Apart from providing a stable growth environment and potential catalytic benefits due to the close proximity of cells, biofilms confer a heightened level of protection to the resident microorganisms against a plethora of environmental challenges. For instance, biofilms shield their inhabitants from harmful ultraviolet radiation (Espeland & Wetzel, 2001), metal toxicity (Teitzel & Parsek, 2003), acid stress (McNeill et al., 2003), and even extremes of dehydration and salinity (Magrex-Debar et al., 2000). Moreover, the protective nature of biofilms extends to thwarting predatory threats like phagocytosis (Leid et al., 2002) and offers resistance against a myriad of antibiotics and other antimicrobial substances (Stewart et al., 2001; Gilbert et al., 2002; Mah et al., 2001). This elevated resistance to biocidal agents exhibited by biofilms can be attributed to three proposed mechanisms, underscoring the intricate and robust nature of biofilm structures. In essence, biofilms play a pivotal role in ecological dynamics, offering microorganisms a fortified habitat against diverse environmental adversities. Their ubiquity across natural settings underlines their ecological significance, while their intrinsic resistance to external threats, from ultraviolet radiation to antibiotics, highlights their robust and adaptable nature. The complex protective mechanisms inherent to biofilms emphasise their evolutionary advantage and the need for continued research into understanding and harnessing their potential.

Medically, biofilms often correlate with tenacious infections, as they can provide refuge to pathogens that are recalcitrant to antibiotics and the host's immune defence. Biofilms are implicated in a wide range of diseases, including chronic rhinosinusitis, urinary tract infections, dental caries, and medical device-associated infections (Foreman et al., 2011; Domenico et al., 2022). In cases of chronic rhinosinusitis, bacterial biofilms found on the mucosa of the sinus and nasal passages have been linked to more persistent and severe symptoms (Foreman et al., 2011).

Similarly, when biofilms develop on medical equipment like catheters and implants, they can perpetuate infections and render treatments ineffective (Domenico et al., 2022). These biofilms grant microorganisms the ability to initiate prolonged infections and shield them from the host's immune response (Domenico et al., 2022). Beyond the realm of chronic infections, biofilms also hold implications in the food industry. Foodborne pathogens within biofilms exhibit enhanced resistance to external stressors, including cleaning agents, leading to persistent diseases that are hard to treat (Liu et al., 2023). Furthermore, the emergence of biofilms in water supply systems and machinery used for food processing jeopardizes both public health and economic stability (Maes et al., 2019). In conclusion, biofilms have significant implications for health consequences. Their innate resistance to antibiotics, disinfectants, and host defences makes biofilm-associated infections difficult to treat. Biofilms are associated with chronic and recalcitrant diseases, and their presence on medical devices and in food processing environments poses risks to public health.

Biofilms display a dual nature in industries. While instrumental in wastewater treatment and bioremediation, they can also instigate biofouling, leading to operational inefficiencies and potential equipment degradation. In water treatment and cooling systems, biofilm formation is a major concern. Biofilms can contaminate water systems and pose a threat to public health (Wang, 2023). They adhere to surfaces and create a protective environment for bacteria, viruses, and other harmful organisms to grow and proliferate (Wang, 2023). Controlling biofilm growth in these systems is essential to maintain water quality and prevent the spread of waterborne diseases. In biotechnology and manufacturing, biofilms can have both positive and negative implications. Biofilms can be exploited for their ability to enhance microbial growth and productivity in bioprocesses, such as wastewater management, bioremediation, and the production of valuable chemicals (Funari & Shen, 2022; Chen et al., 2019). However, excessive biofilm growth can be problematic and lead to reduced process efficiency, equipment damage, and increased energy costs (Coughlan et al., 2016). Managing and controlling biofilm formation is crucial in these industries to optimise production processes and prevent adverse effects. In conclusion, biofilms have significant industrial implications in various sectors. They can impact food safety, water treatment, biotechnological processes, and medical device manufacturing.

Given the multi-faceted roles and implications of biofilms, they aren't just a subject of academic interest. Their relevance spans health, environment, and industry. Understanding their dynamics, structure, and functions is crucial, not just for the microbial communities they harbour but also for the larger systems they impact.

2.2 Biofilm Systems: An In-Depth Exploration of Fundamental Components

A biofilm system refers to the organised arrangement and environment in which biofilms develop, function, and interact. Central to the study of biofilms is the concept of a biofilm system, which comprises a constellation of compartments delineating both the biofilm's structure and its activity. This system includes the surface, which serves as the anchoring surface for microorganisms, and the biofilm matrix, marked by microorganisms enveloped in the protective extracellular polymeric substance (EPS) matrix. Moreover, a vital component to consider is the nutrient solution, providing essential growth stimulants. In particular contexts, a gaseous phase might also be incorporated. The constituents within these compartments can vary, contingent on the specific characteristics and needs of the targeted biofilm system.

The initial adherence of microbes to surfaces represents a cardinal phase in the biofilm developmental process. In such scenarios, microorganisms often exhibit a predilection towards two predominant types of surfaces. The primary, termed the substratum, serves as the initial site of microbial adhesion and subsequent aggregation. The intricacy of this adhesion process is modulated by the inherent attributes of the substratum, encompassing its surface topography and the molecular constituents it embodies. As underscored by O'Toole et al. (2000), the inception stages of adhesion to the substratum might exhibit a transient nature. However, as the biofilm undergoes evolution and consolidation, these interconnections solidify. Post this foundational adhesion, ensuing layers of microbial cells congregate upon these primary settlers, culminating in a stratified biofilm configuration. The exteriors of these microbial entities are endowed with adhesive molecules, facilitating adherence to both non-living surfaces and fellow microbes. The nuanced interplay governing intercellular interactions in the orchestration of elaborate biofilm formations has been expounded upon by Hori and Matsumoto (2010).

Central to the biofilm's structural and functional integrity is the biofilm matrix. This matrix is predominantly comprised of extracellular polymeric substances (EPS). The EPS matrix is a dynamic amalgamation of polysaccharides, proteins, nucleic acids, and lipids. It acts as a shield, offering cells within the biofilm protection against various environmental threats, including antibiotic agents and the immune system's phagocytic cells. A deeper insight into the roles and significance of the biofilm matrix is provided by Flemming and Wingender (2010).

For the sustenance and growth of biofilms, a continuous supply of nutrients is imperative. The solution of these nutrients offers the vital elements that underpin the growth and metabolic undertakings of the biofilm. The diffusion rate of nutrients into the biofilm can markedly influence its overall thickness and activity. In this vein, Stewart and Franklin (2008) expound upon the ramifications of nutrient limitations on biofilm structure and behaviour. Additionally, in certain biofilm systems, especially those found in bioreactors or wastewater treatment setups, the presence and role of a gas phase cannot be overlooked. This gas phase can supply essential gases, such as oxygen for aerobic organisms or methane for methanogenic microbes. In contexts like wastewater treatments, this phase aids in expelling undesired gases from the waste. The intricate interactions of the gas phase with biofilms, especially within bioreactor settings, have been discussed in depth by Rittmann and McCarty (2001).

In conclusion, biofilms represent a sophisticated and intricately structured microbial system, embedded within a myriad of environments ranging from natural ecosystems to industrial apparatuses. The delineation of the biofilm system into distinct compartments — from the anchoring surface to the nutrient-rich environment — provides a comprehensive blueprint of its structural and functional dynamics. Understanding the intricacies of biofilm development and maintenance is vital due to their significant impact on areas like healthcare, industry, and environmental studies. Persistent exploration in this field is crucial to maximise the advantages of biofilms while mitigating their potential issues.

2.3 Biofilm Structure: A Hierarchical Construct

The formation of biofilms is a sophisticated and multi-stage process resulting in the creation of organized microbial assemblages on surfaces. The progression of biofilm formation involves several phases such as initial adhesion, maturation, and eventual dispersal. These stages, which may not be strictly sequential and can often intersect, are defined by specific structural and behavioural characteristics, modulated by the production of extracellular polymeric substances (EPS) and microbial-environment interactions (Wei & Yang, 2023).

Biofilms are intricate microbial communities bound to surfaces and encased within a self-generated extracellular matrix (Peterson et al., 2015). This matrix, composed of polysaccharides, proteins, lipids, and nucleic acids, furnishes structural integrity to the biofilm, facilitating its formation and maintaining its stability (Peterson et al., 2015; Drago et al., 2011).

Environmental factors and microbial composition dictate the biofilm's architecture (Bridier et al., 2017). Manifesting in various morphologies - be it mushroom-like formations, towering structures, or intricate three-dimensional assemblages - biofilms' spatial configuration relies on continuous structural adjustments. These adjustments involve matrix synthesis, ecological reconfigurations, generation of varied physiological regions, motility regulation, and enzymatic activity (Bridier et al., 2017). Furthermore, the biofilm's microbial makeup can profoundly impact its structure and overall function. Interactions between diverse microbial species within the biofilm can influence species distribution, growth patterns, resilience, and the potential for new organism invasions (Baliarda et al., 2021).

Biofilms exhibit adaptability, with their structures being dynamic, continually adjusting to environmental changes (Bridier et al., 2017). They transition through stages from initial surface adherence, intensified attachment through EPS synthesis, architectural development, maturation, to eventual cellular dispersal (Pang et al., 2023). These transitions are orchestrated by the production of extracellular matrix components and interspecies interactions (Tolker-Nielsen, 2015). Fundamentally, biofilm processes represent a myriad of interconnected interactions and activities governing biofilm inception, sustenance, and eventual disintegration. These processes,

combining physical, chemical, and biological facets, are inherently tied to biofilm growth rates and the myriad microbial functions within the biofilm ecosystem.

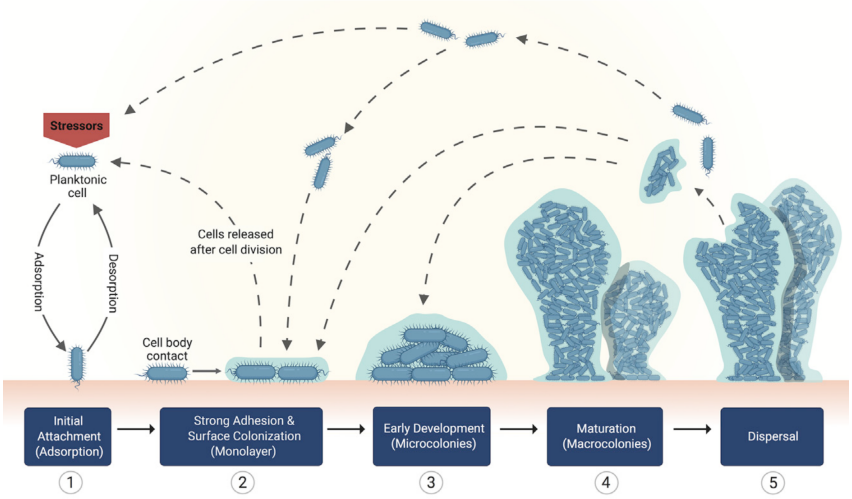


Figure 1: Biofilm Development Stages

Figure 1 illustrates the intricate progression of biofilm formation, which can be delineated into five pivotal stages: (1) Initial Reversible Attachment (2) Permanent Adhesion and Colonisation (3) Preliminary Biofilm Architecture (4) Maturation Phase (5) Dispersal. Image courtesy of Guzmán-Soto et al. (2021).

2.3.1 Initiation of Biofilm Formation: Adsorption, Attachment, and Early Development

The intricate process of biofilm formation commences when individual microbial cells, originally in a planktonic or free-floating state, make their initial contact with a surface. This primary interaction sets the foundation for what eventually becomes a mature, multi-layered biofilm. The early stages of this developmental journey are characterised by a series of tightly regulated events, each crucial for biofilm establishment and progression.

The initial phase involves the passive transport of these microbial cells towards surfaces, largely facilitated by the liquid flow above the substrate. Upon nearing their destination, these cells encounter what is often described as a conditioning film – a layer rich in proteins, polysaccharides, and other organic materials that pre-exist on many submerged surfaces (Costerton et al., 1995). This conditioning film, acting as an intermediary, plays a pivotal role in microbial interactions, influencing the efficiency with which cells adhere, or 'stick' to the surface. The likelihood of a bacterium achieving stable and permanent attachment is influenced by a

myriad of factors. Surface properties, such as its inherent hydrophobicity and roughness, the physiological state of the bacteria, and local hydrodynamic conditions, all play determinative roles. Three primary mechanisms oversee the transport of these cells to their target surfaces: diffusive transport, dictated by concentration gradients; convective transport, powered by liquid flow; and the active movement, exhibited by bacterial motility structures such as flagella. Among these, convective transport stands out as the most dominant, often overshadowing the efficiencies of the other mechanisms (Hinsa et al., 2003).

Upon reaching the surface, bacteria undergo reversible attachment, a phase where they can sample various surfaces without permanent commitment (Razatos et al., 1998). Establishing this initial contact isn't straightforward. Bacteria need to overcome specific energy barriers to achieve direct contact with the surface, a process vital to solidify their presence (Razatos et al., 1998). Post this preliminary exploration, bacteria cement their relationship with the surface via irreversible attachment, fostering more potent interactions between themselves and the substrate (Petrova & Sauer, 2012). This transition is mediated by bacterial adhesins like fimbriae and lipopolysaccharides which target specific receptors on the surface, playing an instrumental role in fostering stable attachment and initiating biofilm development (Berne et al., 2018; Li et al., 2007).

As they acclimate to their new surface-attached lifestyle, there's an uptick in the secretion and sensing of autoinducers, signalling molecules crucial for quorum sensing (Waters & Bassler, 2005). Concurrent with these attachment processes, bacteria commence the secretion of EPS – intricate concoctions of polysaccharides, proteins, and nucleic acids. These substances not only anchor the biofilm but also provide its architectural backbone, crucial for structural stability (Muhammad et al., 2020). The synthesis of EPS, governed by intricate signalling pathways, is indispensable during the attachment phase (Petrova & Sauer, 2009).

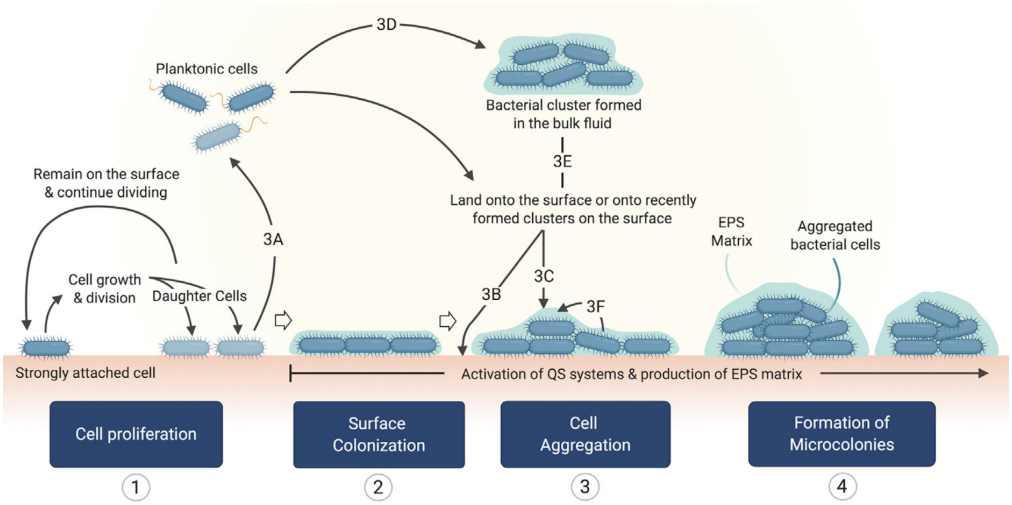


Figure 2: Microcolony Formation Pathways

This figure illustrates the key stages involved in microcolony formation during biofilm development: (1) Cell Proliferation: Initial bacterial attachment leads to rapid cell proliferation. (2) Surface Colonisation: Bacterial cells attached to surfaces undergo continuous growth and division. Released cells (3A) may either colonise new surfaces (3B) or integrate into existing bacterial clusters (3C, 3D). (3) Formation of Microcolonies: In the absence of a solid substrate, bacterial clusters can colonise new surfaces or integrate into developing biofilms (3E). High cell density prompts bacterial aggregation (3F), culminating in the formation of microcolonies (4). Image courtesy of Guzmán-Soto et al. (2021).

The attachment phase isn't solely about bacterial-surface interactions. Bacteria often engage with host extracellular matrix components like fibronectin, fibrinogen, or vitronectin. Armed with specific factors, some bacterial species form bonds with these components, enhancing attachment and propelling biofilm accumulation (Christner et al., 2010). This intimate contact instigates changes within the bacterial community. The intricacies of the attachment phase are further nuanced by environmental variables such as temperature, nutrient availability, and the physicochemical characteristics of the surface (Landini & Zehnder, 2002; Li et al., 2011). Depending on these conditions, bacteria might adopt varied attachment behaviours, with mechanisms such as electrostatic interactions, hydrophobicity, or charge playing pivotal roles.

In conclusion, the attachment phase in biofilm formation is a choreographed ensemble of reversible and irreversible attachment, EPS synthesis, interactions with host matrix components,

and responses to environmental cues. Collectively, these processes facilitate bacterial adhesion, laying the foundational blueprint for the ensuing growth and maturation of biofilms.

2.3.2 Biofilm Maturation: Structural Evolution and Functional Dynamics

The maturation phase signifies a pivotal juncture in a biofilm's life cycle, embodying a continuum of profound structural and functional metamorphoses. From the preliminary cellular interactions with the substrate to the sculpting of an intricate, multi-tiered microbial society, the evolution of a biofilm is a testament to microbial adaptability and resilience.

Following their initial anchoring, cells waste no time in stagnation. Following the attachment, biofilms embark on a phase of clonal growth, setting the stage for microcolony formation (Reisner et al., 2003). These microcolonies, aggregates of cells, act as the foundational building blocks of biofilms (Donlan, 2001). A rapid secretion of extracellular polymeric substances (EPS) ensues. This matrix, abundant in polysaccharides and proteins, multi-tasks. Its role during the inception of attachment might be a topic of academic discourse, but its instrumental function in ensuring the structural coherence and protective sheathing of the biofilm during maturation is well-accepted (Flemming and Wingender, 2010). Post the attachment milestone, the biofilm experiences a crescendo in microbial activity. This leads to the thickening of the biofilm and an elaboration of its structural and functional blueprint. Predominantly governed by nutrient availability during this phase, the growth contrasts the initial stages which are swayed significantly by surface characteristics (Donlan, 2002).

As the biofilm's girth expands and its density intensifies, it grapples with pronounced nutrient gradients. The outer echelons of the biofilm bask in nutrient abundance, whereas the inner sanctum might teeter on deprivation. This dichotomy is further complicated by accumulating metabolic by-products (Stewart & Franklin, 2008). Innovatively, the biofilm architecture evolves to counter these challenges. Water channels emerge, becoming lifelines that ferry nutrients inwards and transport waste outwards. The EPS matrix takes centre stage during this evolution. It not only holds the fort, providing structural integrity, but its production sees amplification, modulated in part by the regulation of cyclic di-GMP levels through enzymes like

phosphodiesterases (PDEs) (Katharios-Lanwermeyer et al., 2021). Extracellular DNA (eDNA), another key component, amplifies its role in maturation, often leading to the formation of biofilm streamers, structures that amplify biofilm dimensions (Pakkulnan et al., 2019; Xia et al., 2021).

The extracellular milieu is rife with conversations. Quorum sensing, a form of microbial dialogue, modulates cell-cell interactions and signalling within this thriving community (Verma-Gaur et al., 2015). Such synchronised actions streamline the exchange of genetic material, metabolic products, and also manage waste. With maturation, microcolonies flourish, evolving into intricate three-dimensional structures. Their intricate design, replete with channels and void spaces, optimises nutrient and waste exchange, underscoring their vital role in sustaining the biofilm's health and vitality (Tolker-Nielsen et al., 2000; Xia et al., 2021). Some trace elements, like iron, further augment this development, with chelated iron transport or internal iron levels acting as functional signals for biofilm progression (Banin et al., 2005). Alongside these structural shifts, the biofilm exhibits functional dynamism. They adapt by expressing genes that are precisely tailored to their immediate environmental conditions, reflecting the biofilm's inherent capability to respond and adapt to its surroundings (Karatan & Watnick, 2009).

In conclusion, the maturation of a biofilm is a harmonious blend of structural evolutions and functional adaptations, encompassing polysaccharide production, architectural innovations, cellular communications, and metabolic adjustments. Collectively, these nuanced processes forge a biofilm that's not just structurally sound but also functionally adept. Each of these processes culminates to ensure the mature biofilm's stability, architecture, and functional efficiency.

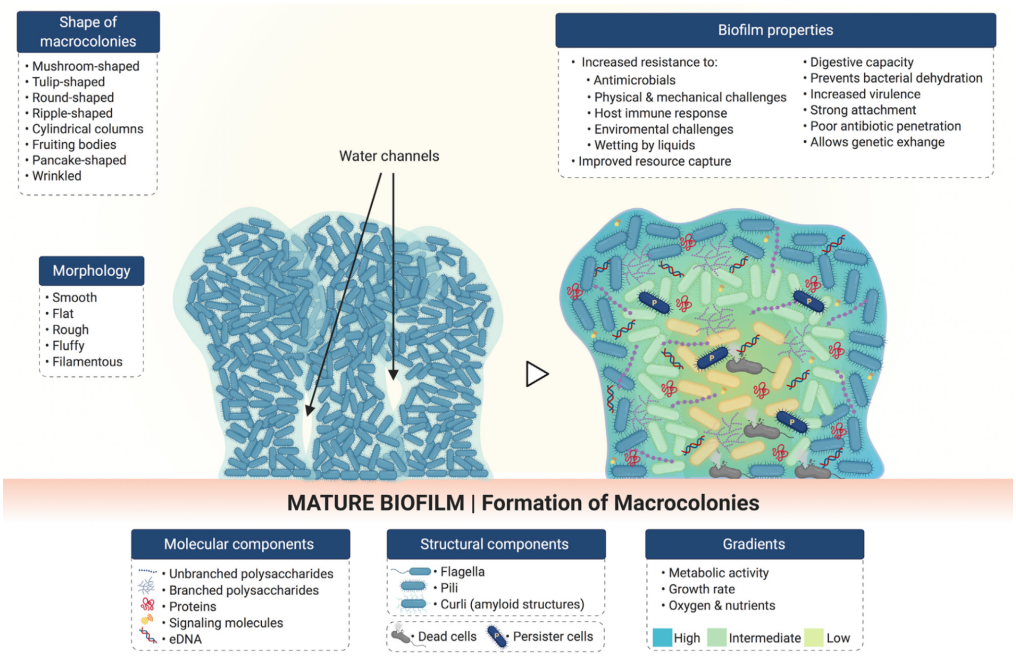


Figure 3: Morphology and Architecture of Mature Biofilms

Figure 3 illustrates the morphology and architecture of mature biofilms, highlighting the pivotal role of molecular and structural components, including proteins, extracellular DNA (eDNA), pili, fimbriae, curli, etc. These elements collectively contribute to the 3D spatial organisation and physiological dynamics of biofilms. Additionally, mature biofilms exhibit phenotypic diversity correlated with gradients within their structure. Image courtesy of Guzmán-Soto et al. (2021).

2.3.3 Biofilm Development: Detachment and Renewal

Biofilm development, contrary to a mere path of unending growth, represents a cyclical process. The distinct phases of growth are punctuated by periods of dispersion, setting the stage for its perpetual renewal. As a biofilm matures, it undergoes significant internal environmental changes. One of the key shifts is the depletion of nutrients, an inevitable consequence of the intensive metabolic activity of the dense microbial community. The depletion is paired with a concomitant accumulation of metabolic waste, including the remnants of dead bacterial cells, which can potentially reach toxic levels within the biofilm microenvironment (Stewart & Franklin, 2008).

While internal factors drive changes, external pressures are not to be ignored. Biofilms, particularly mature ones, are often subjected to external physical forces. Notably, fluid shear forces arising from surrounding fluid movements can pose a significant mechanical challenge,

potentially compromising the biofilm's structural integrity (Hall-Stoodley et al., 2004). Environmental cues, such as carbon starvation, can similarly act as triggers, compelling biofilms to revert to a planktonic state for survival (Nair et al., 2021).

In response to both intrinsic and extrinsic shifts, biofilms exhibit passive and active dispersal mechanisms. Passive dispersion occurs naturally, often driven by external factors such as fluid shear, abrasion, and even human interventions, resulting in the detachment of microbial cells or small biofilm chunks (Sauer et al., 2022). This can be further delineated into erosion, the consistent detachment of cells or small biofilm portions, and sloughing, which refers to a sudden and extensive biofilm loss (Stoodley et al., 2001).

Active dispersal, on the other hand, is a strategic response of the biofilm to environmental signals. Here, specific enzymes are produced by bacterial cells, targeting and degrading the Extracellular Polymeric Substances (EPS) matrix that serves as the biofilm's backbone (Kaplan, 2010). With the weakening of this structural foundation, dormant motility structures, like flagella, might be re-expressed, giving cells the required apparatus to break away and initiate movement. This active detachment is often facilitated by bacterial species producing surfactants, molecules that cut down the adhesive properties of the matrix, aiding in the detachment process (Boles et al., 2005). These now free-floating, or planktonic, cells can then initiate colonisation elsewhere, continuing the microbial community's cyclical pattern of growth and dispersion.

Further complexity to this dispersal process is introduced by signalling molecules such as Nitric oxide (NO), which has been identified to prompt biofilm dispersal across different microbial species (Barraud et al., 2014). Another molecule, cyclic di-GMP (c-di-GMP), has been identified as a crucial determinant in the biofilm dispersal sequence (Chua et al., 2015). Delving more into the molecular intricacies of biofilm dispersal reveals significant roles for specific genetic markers and proteins. The serine protease HtrA stands out as a key protein involved in the dispersal of pneumococcal biofilms, acting as a catalyst that promotes detachment and safeguards bacterial colonies (Chao et al., 2020).

Furthermore, Biofilms demonstrate a profound capability for intercellular communication through quorum sensing, an essential synchronised communication mechanism crucial for their growth and development. Disrupting this pathway can effectively curb biofilm proliferation and spur its dispersal (Jiang et al., 2020). Simultaneously, the biofilm's structural foundation, the Extracellular Matrix (ECM), composed of extracellular DNA (eDNA), proteins, and polysaccharides (Pang et al., 2023), serves as a protective shield against external adversities. Yet, this very structure is susceptible to dispersal mechanisms. Enzymatic agents, including proteases, DNases, and glycoside hydrolases, target and break down the matrix elements, facilitating biofilm detachment (Fleming & Rumbaugh, 2017).

Conclusively, understanding the detachment and dispersal step in biofilm formation is paramount, considering its implications in biofilm control strategies, infection pathogenesis, and biofilm reactor operations. The mechanisms, whether genetic, molecular, or environmental, give insights into the holistic understanding of biofilm biology, their survival tactics, and potential interventions for their control (McDougald et al., 2011). The multifaceted nature of biofilm dispersal draws from a combination of signalling molecules, environmental triggers, molecular dynamics, cellular communication pathways, and structural interactions.

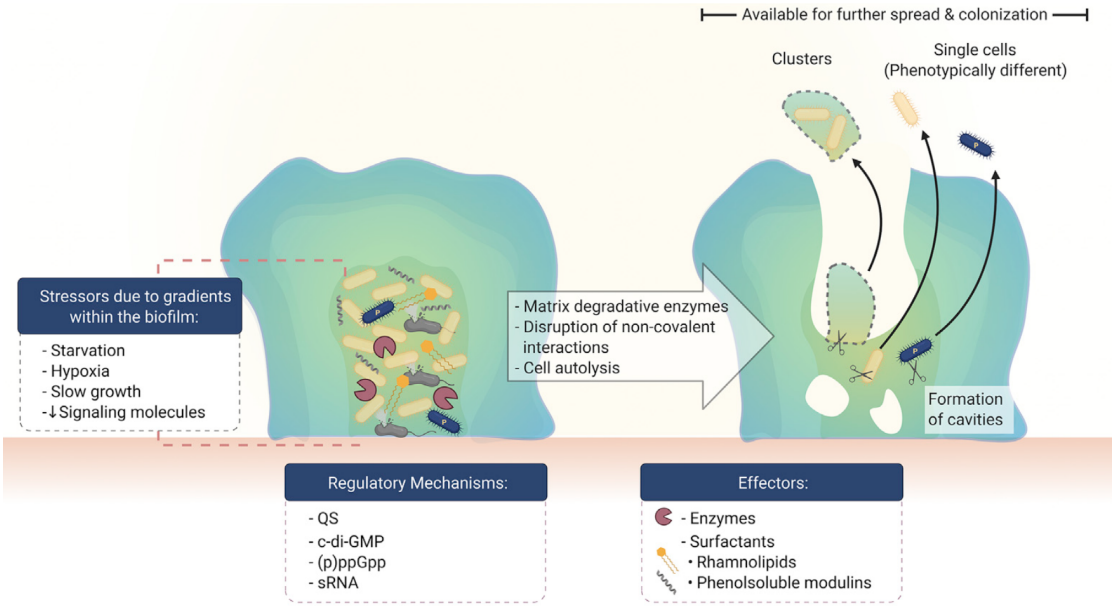


Figure 4: Biofilm Dispersal

Figure 4 illustrates the process of biofilm dispersal, driven by bacterial regulatory mechanisms, such as quorum sensing, c-di-GMP, as well as responses to environmental stressors and internal effectors. These mechanisms collectively lead to the disruption of biofilms, releasing individual cells and bacterial clusters into the bulk fluid, making them available for further spread and colonisation. Image courtesy of Guzmán-Soto et al. (2021).

In conclusion, biofilms, representing structured microbial assemblies, exhibit a sophisticated developmental trajectory characterised by distinct phases: attachment, maturation, and detachment. The initial attachment phase is marked by both reversible and irreversible bacterial adhesions, setting the stage for subsequent biofilm elaboration. As the biofilm progresses to maturation, the extracellular polymeric substances (EPS) play an indispensable role in conferring structural integrity and resilience against environmental perturbations. This resilience is further accentuated by intricate cellular interactions and molecular pathways that regulate the synthesis and organisation of the matrix components. The concluding detachment phase facilitates the biofilm's dynamic nature, allowing microbial cells to colonise novel environments, thereby ensuring biofilm perpetuation and dissemination. The multifaceted processes intrinsic to each phase, underscored by myriad molecular mechanisms and environmental influences, highlight the complexity inherent to biofilm biology. An in-depth understanding of these processes is imperative for harnessing biofilm-associated benefits in various applications while devising strategies to mitigate their potential adversarial impacts in clinical and industrial contexts.

2.3.4 Biofilm Architecture: A Nuanced Conceptual Exploration

The realm of microbial ecology has perennially been enriched by the captivating study of biofilms. Historically, academic discourse depicted biofilms with a sense of deceptive simplicity, suggesting an almost monolithic congregation of microbial cells embedded within the Extracellular Polymeric Substances (EPS) matrix (Costerton et al., 1995). However, the continuous evolution of scientific methodologies, fortified by advances in microscopy and molecular approaches, illuminated the intricate tapestry that truly defines a biofilm's architecture (Lawrence et al., 1991). Modern interpretative frameworks juxtapose biofilms as entities of profound heterogeneity, emblematic of the intricate dance of life at microscopic scales. This revised perspective elegantly harmonises previously disparate observations, elucidating:

The orchestrated manoeuvrability of specific small molecules or particulates within the biofilm's matrix, a phenomenon expounded in seminal studies (Davies et al., 1998). The nuanced mosaic of oxygen gradients within biofilms, which attests to the micro-habitats and ecological niches formed, allowing microbial residents to adapt to varied aerobic exigencies (Stewart and Franklin, 2008). The sophisticated nutrient stratification across biofilm strata, emphasising the spatiotemporal dynamics of microbial sustenance and competition (Beyenal et al., 2004). The biofilm's fluidic architecture, crafted with an innate wisdom that ensures optimal material exchange and response to environmental vicissitudes (Stoodley et al., 1999).

Drawing a parallel with urban landscapes, biofilms unfold as bustling metropolises of microbial activity. The cornerstone 'microcolonies', ensconced in protective EPS, emerge as crucibles of fervent metabolic processes, reminiscent of urban cores (Watnick and Kolter, 2000). Interspersed among these are the interstitial boulevards - channels facilitating fluid movement, nutrient transit, and waste egress. Central to this intricate urbanism are:

Base Sublayer: The foundational precinct, occasionally revealing glimpses of the underlying substrate, anchoring the biofilm's integrity and fostering initial microbial alliances (O'Toole and Kolter, 1998).

Microcolonies: These vibrant nodes, awash with microbial congregations, manifest the biofilm's metabolic vigour and collective resilience (Wimpenny and Colasanti, 1997).

Interstitial Voids: Far from mere voids, these channels epitomise the biofilm's circulatory system, underscoring its adaptive dynamism in response to external stimuli (Donlan, 2002).

An aesthetically and functionally intriguing manifestation in certain biofilms is the emergence of EPS streamers. Particularly dominant in fluidic terrains, these streamers, tethered to microcolonies, exemplify the biofilm's adaptive prowess, serving multifaceted roles from nutrient sequestration to microbial recruitment (Hall-Stoodley et al., 2004).

In synthesis, the intricate architecture of biofilms, while revealing the marvels of microbial ingenuity, underscores an imperative. It beckons a deeper, more humane engagement with this microscopic realm. For in understanding and respecting these diminutive life forms and their habitats, we not only enrich our academic repositories but also cultivate a more empathetic relationship with the microbial world. In doing so, we pave the way for informed interventions, be they in the realms of medical therapeutics, environmental stewardship, or industrial applications.

2.4 Biofilm Formation: An Interplay of Multifaceted Factors

Biofilm assembly is a multifaceted process orchestrated by an intricate interplay of environmental, genetic, and molecular dynamics. Historically, the primary understanding of biofilm formation was simplistically viewed through a limited lens. However, with the emergence of dedicated research, a far richer tapestry of interdependent factors has emerged, illuminating the complexity and adaptability of biofilm structures.

Environmental Conditions: Extreme environments, with heightened salinity or temperature metrics, actively foster biofilm genesis. Such formations serve dual purposes: protecting microorganisms from external environmental assaults and acting as ecological bastions for microbial survival (Yin et al., 2019). Phylogenetic history and microbial coexistence further accentuate these environmental dynamics, with biofilms emerging as unique microbial ecosystems (Madsen et al., 2016).

Genetic Underpinnings: Biofilm formation's genetic modulation is intricate. Specific gene expressions underpin the biofilm's development, evolution, and stability. Recognizing these genetic pathways is paramount, especially in realms such as sustainable agriculture where the prevention or promotion of biofilms could herald ecological breakthroughs (Velmourougane et al., 2017).

Quorum Sensing (QS): A salient mechanism, QS acts as a bacterial social network, allowing coordinated behaviour premised on population densities. Its modulatory influence over biofilm

formation is evident in many bacterial species, with cyclic-di-GMP and QS mechanisms acting as biofilm promoters (Morinaga et al., 2020). However, nuanced exceptions exist, such as in Paracoccus denitrificans, where these mechanisms inhibit biofilm development, highlighting the diverse roles QS can play (Morinaga et al., 2020).

Iron Bioavailability: Iron's role in biofilm dynamics can't be overstated. While iron limitation might encourage biofilm formation in certain species, it conversely inhibits it in others, evidencing a species-specific response mechanism (Wu & Outten, 2009).

Biochemical Pathways and Mitigation Approaches: Within the protective enclave of a biofilm, microbial colonies exhibit adaptive behaviours that bolster resistance against external adversities like antibiotics and immune responses (Liu et al., 2020). This adaptability has spurred research into devising countermeasures, encompassing physical, chemical, and biological strategies, aiming to curtail biofilm proliferation (Liu et al., 2020).

The Extracellular Matrix: The EPS matrix, an intricate amalgam of macromolecules, lends structural and functional integrity to biofilms. This matrix, a medley of polysaccharides, proteins, lipids, and nucleic acids (eDNA), is diverse, mirroring the metabolic potential of the resident microbes and their environmental backdrop (Frantz et al., 2010).

In encapsulation, the formation and sustainability of biofilms are orchestrated by an ensemble of determinants ranging from environmental variables to intricate genetic pathways. Unravelling these influences is not merely academically vital; it's pivotal for strategizing interventions across diverse sectors. As research continually enriches our understanding of biofilms, the quest for mastery over these microbial fortresses persists.

2.5 The Multifaceted Role of DNA in Biofilm Formation: An Academic Exploration

Biofilms, the structured communities of microorganisms encapsulated within a self-produced extracellular matrix (ECM), represent a ubiquitous form of microbial existence. These intricate assemblies offer protection, facilitate nutrient acquisition, and provide bacteria with a means to

persist in often hostile environments. One of the foundational components of this matrix, extracellular DNA (eDNA), emerges as a key player in the orchestration of biofilm formation and maintenance. Its ubiquitous presence across diverse bacterial genera, from the notorious *Pseudomonas aeruginosa* to the health-impacting *Streptococcus pneumoniae*, accentuates its pivotal role in biofilm dynamics. Indeed, the very act of biofilm formation, from the initial microbial adherence to the maturation and structural robustness of the community, hinges significantly on the properties and functionalities endowed by DNA. This section delves into the multifaceted role of DNA, particularly eDNA, in biofilm development, elucidating its significance in bacterial adherence, structural integrity, and overall biofilm resilience. Drawing from empirical research and comprehensive studies, we aim to present a holistic understanding of DNA-mediated biofilm processes, providing invaluable insights for potential biofilm management strategies and therapeutic interventions.

Extracellular DNA (eDNA) is an integral component in biofilm formation, exhibiting significant influence across a myriad of bacterial species. Biofilms represent complex microbial assemblages that anchor tenaciously to various substrates. Integral to these assemblages is the extracellular matrix (ECM), a self-secreted amalgamation of proteins, polysaccharides, and notably, DNA (Fazli et al., 2014). The ECM is pivotal, conferring structural integrity and architectural sophistication to the biofilm while simultaneously serving as a defensive bulwark against environmental perturbations and antimicrobial interventions. Within this milieu, DNA, especially eDNA, is indispensable. It furnishes a scaffold that facilitates bacterial cell attachment and cohesion, both quintessential for biofilm inception and subsequent maturation phases (Ma et al., 2009). In certain microorganisms, such as Pseudomonas aeruginosa, eDNA acts as a vital connector between cells, accentuating its paramount significance within the biofilm construct (Ma et al., 2009). Beyond structural contribution, DNA's influence extends to the modulation of ECM components, fortifying biofilm robustness, and playing a pivotal role in the nascent colonisation phases. Hence, a profound comprehension of DNA's intricate roles in biofilm formation is imperative for future scholarly pursuits, especially when strategizing interventions for biofilm-linked afflictions.

Bacterial species, including prominent ones like *Pseudomonas*, *Burkholderia*, *Bacillus*, and *Staphylococcus*, are known to incorporate DNA into their biofilm matrices, demonstrating the universality of this phenomenon across various genera (Fazli et al., 2014; Vilain et al., 2009; Ma et al., 2009; Huseby et al., 2010). The process of biofilm development and the synthesis of essential components such as exopolysaccharides, lectins, and biosurfactants are intricately controlled at the molecular level. In these bacterial species, small non-coding RNAs (sRNAs) play a pivotal role in directing and regulating the production of these biofilm matrix components (Fazli et al., 2014). Notably, the biofilms formed by *Pseudomonas aeruginosa* and *Bacillus subtilis* showcase the presence of extracellular DNA (eDNA) as a significant component of their extracellular polymeric substances (EPS), emphasising the role of eDNA in biofilm structure and function (Vilain et al., 2009; Ma et al., 2009). For instance, the eDNA found in *P. aeruginosa* biofilms is not merely a by-product but originates from specific chromosomal segments and has a clear functional role. The eDNA in biofilms serves multifunctional roles: it imparts structural rigidity to the biofilm and functions as an adhesive, promoting cellular interactions and preserving biofilm cohesion (Ma et al., 2009). The revelation that eDNA, derived from random chromosomal fragments of *P. aeruginosa*, contributes to biofilm stability points to a potential evolutionary strategy harnessed by bacteria to enhance the sturdiness and resilience of their communities. Such insights into the mechanics of biofilm formation across diverse bacterial species provide foundational knowledge, instrumental for devising strategies against biofilm-related infections and leveraging biofilms' advantageous properties in multiple domains.

Moreover, the significance of unfragmented DNA in biofilm genesis and maintenance isn't confined to a handful of bacterial species; it's a widespread bacterial strategy. For example, research on *Streptococcus pneumoniae* has unveiled the pivotal role that intact DNA plays in its biofilm behaviour. Specifically, the presence of undisturbed, long-chain DNA has been observed to markedly amplify both the biomass and the survival rates of its biofilms. These observations underscore that DNA, far from being a mere passive constituent, is an active agent determining biofilm matrix dynamics. Instead, lengthy DNA chains play an active role in mediating cellular interactions, leading to a higher degree of cell cohesion within the biofilm. As a direct consequence of this increased cohesion, the stability and resilience of the biofilm are markedly enhanced (Carrolo et al., 2010). Similarly, the biofilms of *Campylobacter jejuni*, a bacterium

associated with gastrointestinal infections, further exemplify the ubiquity of this phenomenon. Investigations into the biofilm matrix of *C. jejuni* have revealed that extracellular DNA (eDNA) isn't just a minor or incidental component. Instead, it features prominently as one of the major constituents of the extracellular matrix (ECM) that envelopes and protects the bacterial community within the biofilm (Brown et al., 2015). Taken together, these findings from different bacterial species, spanning *Streptococcus pneumoniae* to *Campylobacter jejuni*, underscore the universal and pivotal role of eDNA in biofilm formation and stabilisation. This understanding can pave the way for novel therapeutic approaches targeting biofilm-associated infections, by potentially targeting the eDNA component of the biofilm matrix.

Delving into the intricacies of biofilm formation, especially during its nascent stages, unveils fascinating dynamics that are crucial to microbial colonisation on various surfaces. *Streptococcus* mutans, a notorious bacterium implicated in dental caries, offers a compelling case in point. Research by Senpuku et al. (2019) has shed light on the interplay between DNA, glycosyltransferases (GTFs), and vesicles during the initial colonisation of the human tooth surface. Specifically, it isn't just any generic form of DNA that contributes to this process, but a distinct type that, when synergistically combined with GTFs and vesicles, amplifies the biofilm's early establishment. GTFs are essential enzymes secreted by *Streptococcus* mutans that catalyse the conversion of sucrose to extracellular polysaccharides, which aid in the adherence of bacteria to the tooth enamel. The partnership between these GTFs and specific DNA fragments can be seen as a foundational aspect of the biofilm matrix, establishing a sticky platform that allows the bacterium to effectively adhere to and proliferate on the tooth surface. Moreover, vesicles, which are small, membrane-bound structures released by bacteria, further contribute to this dynamic. Acting as carriers for various molecular components, including proteins, lipids, and genetic material, vesicles facilitate intercellular communication and material transfer. When these vesicles are in concert with the specialised DNA fragments and GTFs, they potentially enhance the efficiency of bacterial adherence, establishing a fortified front for the burgeoning biofilm. Thus, understanding the roles and interrelationships of smaller DNA fragments, GTFs, and vesicles in the early stages of biofilm development not only illuminates the sophisticated mechanisms employed by bacteria such as *Streptococcus* mutans but also underscores potential

targets for therapeutic interventions aimed at preventing dental biofilm formation and the subsequent onset of dental diseases.

In essence, the quintessential role of DNA, particularly its extracellular form (eDNA), in biofilm formation underscores a complex interplay of molecular and cellular processes. Its multifarious functions encompass the nuanced regulation of matrix components, facilitation of initial colonisation, and enhancement of the structural fortitude of biofilms. Consequently, eDNA emerges as a lynchpin in myriad stages of biofilm development: from the incipient stages of microbial attachment, the establishment of structural robustness, augmentation of cellular cohesiveness, to the modulation of antibiotic resistance mechanisms. Such profound understanding of the DNA-centric mechanisms in biofilm physiology is paramount for the academic community. It offers not merely a microscopic view of microbial behaviours but also presents a humane opportunity to craft nuanced strategies aimed at ameliorating the morbidity associated with biofilm-induced infections. By delving into the DNA-mediated intricacies of biofilm dynamics, the scientific community is poised to unearth targeted interventions that can significantly reduce biofilm-associated complications, thereby benefiting a vast swath of humanity afflicted by these persistent microbial communities.

2.6 Uncharted Territory: gDNA's Role in Laboratory Biofilm Reactor Analysis

The biofilm realm, with its intricate and multi-dimensional microbial interactions, has garnered substantial research attention over the past decades. Much of this research has been centred on understanding the role of extracellular DNA (eDNA) in biofilm formation, given its established contributions to biofilm matrix stability, structure, and antibiotic resistance (Fazli et al., 2014; Ma et al., 2009). However, despite the wealth of knowledge surrounding eDNA, there remains a glaring gap in the exploration and utilisation of genomic DNA (gDNA) — both in its whole and fragmented forms — for the analysis of biofilm activity. This distinction between eDNA and gDNA is pivotal; while eDNA primarily concerns the DNA present in the extracellular matrix (often released due to cell lysis), gDNA pertains to the entire genetic material within a cell, providing a much more comprehensive perspective on microbial dynamics and interactions.

Delving deeper into the intricacies of biofilm formation, researchers have also elucidated the regulatory mechanisms, such as small non-coding RNAs (sRNAs), that guide the synthesis of biofilm matrix components in various bacterial species (Fazli et al., 2014). Yet, the role of gDNA — in both its fragmented and whole forms — as a potential regulatory agent or structural component remains insufficiently investigated. Furthermore, interspecies and intraspecies variations in gDNA can offer novel insights into the heterogeneity and adaptability of biofilms in response to varying environmental stimuli (Vilain et al., 2009; Huseby et al., 2010).

A direct testament to the role of eDNA in biofilm formation emerges from the utilisation of DNase. This enzyme, by degrading DNA, has been shown to significantly impact the biofilm structure and function (Petersen et al., 2005; Tetz et al., 2009; Conover et al., 2011). Such DNase interventions have rendered disruptions in biofilm formation across a spectrum of bacteria, irrespective of their gram-negative or gram-positive classifications. Delving into specific instances, Petersen et al. (2005) showcased how DNA release mechanisms in *Streptococcus pneumoniae* biofilms are intricately tied to a quorum-sensing circuit integral for competence development in natural transformation. Tetz et al. (2009), while discussing the universality of eDNA's function, pointed out that DNase treatments can modify biofilm biomass across disparate bacterial species, both gram-positive and gram-negative. A notable observation by Conover et al. (2011) pertains to the differential effect of DNase I based on biofilm maturity, where its influence is markedly prominent on mature biofilms in gram-positive bacteria. Nevertheless, a caveat to the efficacy of DNase treatment emerges with biofilm age. Conover et al. (2011) elucidated that the susceptibility of biofilms to DNase diminishes as they age, leading to a reduced response in older biofilm structures. So, it is vital to recognise the distinctions between gDNA and eDNA for a nuanced understanding of biofilm mechanisms and for the development of innovative strategies in biofilm management and utilisation.

The potential of gDNA in offering a nuanced, molecular insight into biofilm dynamics is largely untapped. By harnessing the information encapsulated within gDNA, there's the possibility of delving into microbial interactions, evolutionary adaptability, structural dynamics, and biofilm resilience with an unprecedented level of detail. Quantifying the impact of various gDNA forms in controlled laboratory biofilm reactors, for instance, could offer a standard, replicable

methodology for assessing biofilm activity, interactivity, and resilience (Ma et al., 2009). This could, in turn, lead to improved strategies for biofilm management, enhanced reactor designs, and even the potential to exploit biofilm benefits in a myriad of applications, from wastewater treatment to bioremediation.

Given these potential advantages, there's an urgent need to pivot research focus towards gDNA-centric analysis in biofilm studies. This would not only elucidate novel aspects of biofilm behaviours in laboratory settings but also bridge a crucial knowledge gap in the comprehensive understanding of biofilm research. Thus, for a field that continually evolves and holds promise in various applied sectors, addressing this research gap is paramount for further advancements and innovations.

All in all, biofilms, which are communities of microorganisms embedded within an extracellular matrix, have been extensively studied for their role in bacterial persistence and resistance. The importance of extracellular DNA (eDNA) in biofilm structure and integrity is well-established. However, there is a gap in our understanding of the role of genomic DNA (gDNA), particularly when sourced directly from bacteria, in inducing or enhancing biofilm production.

Studies have shown that eDNA is a major structural component of many different microbial biofilms. It is released through autolysis, active release through membrane vesicles and nanofibers, and other mechanisms. eDNA plays a crucial role in the formation, mechanical stability, and maturation of bacterial biofilms. It provides mechanical stability to biofilms and protects bacterial cells from physical stress, antibiotics, and detergents. Additionally, eDNA chelation of divalent cations induces genes involved in modifying bacterial cell surface properties, favouring biofilm resistance to antimicrobial agents and detergents.

The distinction between eDNA and gDNA and their specific impact on biofilm formation is underexplored. It is unclear whether the introduction of gDNA, particularly in whole or fragmented form, promotes biofilm formation. Additionally, the differential response of biofilm initiation due to intra- and inter-species genomes remains inadequately explored.

The dynamics of *Vibrio cholerae* amidst cholera outbreaks serve as a contextual foundation for this investigation. In environments abundant with phages, which induce substantial bacterial mortality and consequent eDNA discharge, *V. cholerae* often assumes a latent state encapsulated within biofilms. Notably, biofilms developed in such phage-saturated environments exhibit enhanced resilience, implying a potential role of eDNA in amplifying biofilm establishment and acting as a shield against phage predation.

By addressing these knowledge lacunae, this study aspires to illuminate the intricate interactions between gDNA integration and bacterial biofilm kinetics. This could unravel bacterial survival mechanisms and pave the way for potential therapeutic interventions targeting bacterial pathologies.

2.7 Laboratory Biofilm Reactors (LBRs): An In-depth Analysis

Laboratory Biofilm Reactors (LBRs) have emerged as pivotal tools in advancing biofilm research. These reactors present a meticulously controlled milieu, facilitating a deeper understanding of biofilm genesis, architecture, and functionality. LBRs, being a niche category, are meticulously designed for in-depth analyses of specific biofilm-associated phenomena, offering a granular perspective into the multifaceted biofilm ecosystem.

Utilising LBRs, scholars can modulate and scrutinise the impact of determinants such as nutrient concentration, flow dynamics, thermal conditions, and pH on the attributes of biofilms, as elucidated by Xavier et al. (2004). Moreover, LBRs have been instrumental in delving into the interplay between biofilms and diverse substrates, as showcased by Kanematsu et al. 's (2016) research on urological stents and catheters. In the domain of environmental technology, LBRs have been invaluable for refining biofilm-centric wastewater treatment methodologies (Chaali et al., 2018) and have also been applied to biotechnological pursuits, such as recombinant protein production (Gomes et al., 2016).

It's imperative to differentiate between 'biofilm reactors' and 'biofilm systems.' While they may seem interchangeable, they have distinct connotations. A biofilm system alludes to the natural

habitats fostering biofilm growth, while a biofilm reactor, epitomised by LBRs, is an intentionally devised infrastructure tailored for controlled biofilm studies.

Quantifying Biofilm Dynamics in Reactors:

Biofilm reactors enable the measurement of essential parameters like biofilm thickness, microbial density, nutrient diffusion rates, and metabolic activity. These parameters shed light on biofilm dynamics, providing a comprehensive view of their formation, maintenance, and dissolution.

Exploring Biofilm Activity:

Central to biofilm research is understanding 'biofilm activity' – a reflection of the nutrient utilisation rate within the biofilm. This rate serves as an indicator of a biofilm's metabolic health and its efficiency. Researchers often measure biofilm activity by tracking the consumption of growth-limiting nutrients. The choice of nutrient to monitor is influenced by the specific biofilm process in question and the ease of measurement. In essence, biofilm activity provides insights into the metabolic pulse of biofilms, bridging the theoretical with practical application.

To conclude with, biofilm reactors, especially Laboratory Biofilm Reactors (LBRs), have revolutionised our approach to studying the multifaceted world of biofilms. They have not only granted researchers the precision to delve deep into biofilm intricacies but have also enabled an enhanced understanding of how biofilms interact with varying environments and stimuli. This comprehensive insight is instrumental in devising effective biofilm-based applications, from wastewater treatment to biotechnological innovations. The distinction between biofilm reactors and systems underscores the importance of controlled research environments. As our grasp on biofilm activity sharpens, we find ourselves better equipped to harness the potential of biofilms, making strides in both theoretical understanding and practical advancements. The journey through biofilm research exemplifies the delicate dance between observation, experimentation, and application, and LBRs serve as a testament to the significance of precision tools in scientific exploration.

2.8 Insights into *V. Cholera, E. coli*, and *S. aureus* Biofilms

2.8.1 *Vibrio Cholerae*

Vibrio Cholerae biofilms exhibit distinctive features that set them apart from other bacterial biofilms, such as those of *Staphylococcus aureus* and *Escherichia coli*. This differentiation stems largely from their unique molecular architecture and principles guiding their assembly (Berk et al., 2012). Specifically, the biofilm development in *V. cholerae* revolves around the synthesis of Vibrio polysaccharide (VPS) and an ensemble of matrix proteins: RbmA, RbmC, and Bap1 (Berk et al., 2012). These proteins, armed with carbohydrate-binding domains, orchestrate the intricate biofilm development processes, steering the directionality of the rod-shaped bacterial proliferation, a feature regulated by the rbmA gene (Yan et al., 2016). Such a dense, ordered growth instils superior mechanical properties within the biofilm, making it a robust microbial fortress (Yan et al., 2016).

Diving deeper into the molecular mechanics, extracellular DNA and nucleases emerge as pivotal players in *V. cholerae* biofilm genesis (Seper et al., 2011). This bacterial pathogen has evolved to utilise nucleosides, especially during nutrient scarcity, potentially by derepressing genes involved in nucleoside catabolism through the repressor CytR (Seper et al., 2011). Further enriching its adaptability, *V. cholerae* can leverage O-antigen polysaccharides for Ca^2+-dependent biofilm development in marine settings, revealing its adaptability and multifaceted biofilm-forming strategies (Kierek & Watnick, 2003).

Notably, the signalling molecules cyclic AMP (cAMP) and cyclic di-GMP modulate biofilm formation in *V. cholerae*, emphasising the significance of extracellular matrix components, including the pivotal VPS, in maturing biofilms (Fong & Yildiz, 2008). Moreover, the VPS-dependent biofilm exhibits heightened activation within the arthropod intestine, solidifying its role in colonising environmental hosts like arthropods (Purdy & Watnick, 2011).

Contrasting with the pathogenic traits, certain *lactobacilli* isolates display anti-biofilm properties against the *Vibrio cholerae* species, indicating potential therapeutic interventions using probiotic strains (Kaur et al., 2018). Still, the challenge remains substantial. Modern studies have reported

the virulent biofilm-forming capabilities of *V. cholerae*, coupled with a plethora of virulence phenotypes and antimicrobial resistances (Awuor et al., 2022). Adding to its repertoire of biofilm-related genes, a conserved regulatory circuit has been identified that controls essential adhesins in *V. cholerae*, instrumental for cell attachment and biofilm development (Kitts et al., 2019).

On the biomechanical front, *V. cholerae* biofilms exhibit distinctive mechanical properties at the air-liquid interface, further highlighting their complex nature (Hollenbeck et al., 2014). The culprits behind these mechanical properties are the extracellular matrix components, which include the already discussed matrix proteins, VPS, and even DNA (Hollenbeck et al., 2014).

In summation, while biofilms of *E. coli* and *S. aureus* have their own idiosyncratic features, the ones formed by *V. cholerae* are distinct in molecular composition, architecture, and mechanics. Understanding these differences is pivotal for devising strategies against biofilm-associated infections and for leveraging the potential benefits of biofilms in various applications.

2.8.2 *Escherichia coli*

Biofilms, or microbial communities adhering to surfaces, are vital survival strategies for many bacteria, including the often-studied *Escherichia coli (E. coli)*. These biofilms and their unique characteristics have become subjects of extensive research due to their implications in various fields.

Unique Characteristics and Formation: *E. coli* biofilms exhibit distinctive features that set them apart from other bacterial biofilms. Studies by Reisner et al. (2006) highlighted the significant genetic diversity among *E. coli* strains and its profound impact on biofilm formation. They observed the growth medium's influential role in promoting increased biofilm development. Notably, they also explored the synergistic effects in mixed *E. coli* biofilms, emphasising that the model system provides insight into the mechanisms of biofilm development in natural *E. coli* populations.

The role of DNA, particularly extracellular DNA, has been recognized in biofilm structural stability across bacterial species. *E. coli* distinctly uses DNA in biofilm formation, with certain genes and efflux pumps playing pivotal roles (Ballén et al., 2022). Gene deletions, such as tolC and emrD, have demonstrated a decrease in biofilm formation capability. These genetic nuances underpinning biofilm formation are unique to *E. coli*, differentiating it from others like *S. aureus* and *V. cholerae*.

Culotti & Packman (2014) delved into *E. coli's* interactions with another bacterium, *Pseudomonas aeruginosa*. They underscored the complex dynamics during biofilm formation and how competitive interactions shape their growth. In more nutrient-rich environments, *E. coli* colonises the interior of *P. aeruginosa* clusters, illustrating the competitive nature of biofilm microbiomes. Mathlouthi et al. (2018) investigated the influence of environmental parameters like temperature and pH on *E. coli* biofilm formation. The study found significant variances in biofilm-forming abilities among strains under varying conditions. Furthermore, the presence of plasmids augments biofilm formation.

The polymicrobial nature of many biofilms brings about intriguing interactions. Brucker et al. (2015) examined the effect of fungal β-1,3-glucan on *E. coli's* resistance to ofloxacin when co-existing in a biofilm with *Candida albicans*. Their results provide fascinating insights into the biofilm's matrix and its role in increased antibiotic resistance.

In conclusion, *E. coli* biofilms, with their unique genetic components, interactions, and adaptive strategies, differentiate themselves considerably from biofilms of other organisms like *S. aureus* and *V. cholerae*. The studies mentioned provide just a glimpse into the world of *E. coli* biofilms, highlighting the vast research avenues still left to be explored.

2.8.3 *Streptococcus aureus*

Staphylococcus aureus biofilms are intricate assemblies that significantly underpin the infections caused by this bacterium. Their uniqueness lies not just in their complex architecture but also in the molecular constituents that compose them. *S. aureus* biofilms, both MSSA and MRSA

strains, are constructed on various surfaces and prominently feature extracellular DNA (eDNA) and Poly-N-acetylglucosamine surface polysaccharide (PNAG) in their matrix, setting them apart from biofilms of other species like *V. cholerae* and *E. coli* (Izano et al., 2008). These components serve dual purposes; while PNAG provides structural resilience, eDNA fosters biofilm formation and ensures its stability, underscoring its distinctive role compared to other bacterial biofilms (Izano et al., 2008).

Furthermore, the biofilm-associated DNA doesn't merely offer structural benefits. It acts as a dynamic reservoir of genetic material. This facilitates horizontal gene transfer within the biofilm, spawning a hotbed for the dissemination of antibiotic-resistance genes, accentuating the adaptability and resilience of *S. aureus* biofilms in varied environments (Izano et al., 2008).

Biofilm formation is pivotal to the virulence of *S. aureus*. Within the protective cocoon of the biofilm, the bacteria gain an enhanced defence against host immune responses and are better positioned to resist antimicrobial agents. This formidable barrier is further reinforced in polymicrobial environments. In co-existing biofilms with *Candida albicans*, *S. aureus* showcases heightened resistance to antibiotics, such as vancomycin. This is credited to unique adhesins produced by *C. albicans*, fortifying the bacterial-fungal interaction and shielding the bacteria from antimicrobial action (Harriott & Noverr, 2009).

When it comes to tackling biofilm-associated *S. aureus* infections, clinicians face an uphill battle. The natural defences of the biofilm, combined with factors such as the presence of eDNA, make them notoriously resistant to conventional treatments. The biofilm matrix operates as a formidable physical shield, limiting the infiltration of antimicrobial agents and, in essence, creating sanctuaries for bacteria where they can thrive unchallenged (Gao et al., 2018). The therapeutic conundrum is further compounded by *S. aureus's* propensity to exist intracellularly, explaining the often chronic nature of biofilm-linked infections (Gao et al., 2018).

In summation, *S. aureus* biofilms represent a complex microbiological entity with distinctive features that set them apart from biofilms of other species. Their molecular complexity and

adaptive capacity make them formidable opponents in clinical settings, necessitating continued research efforts to develop more effective countermeasures.

2.9 Research Objectives

1. Elucidation of Genomic DNA (gDNA) in Biofilm Formation Dynamics:

Objective 1.1: Conduct preliminary characterizations of biofilm for the designated bacterial strains to establish control parameters, comprising biofilm thickness, microbial density, metabolic flux, and structural taxonomy.

Objective 1.2: Expose biofilms to both whole and fragmented configurations of gDNA. Document shifts in biofilm structural tenacity, microbial synchrony, and overall biofilm vitality. The intent is to discern whether the configurational state of gDNA modulates its interaction potency with established biofilm components.

Objective 1.3: Harness quantitative assays, such as crystal violet staining techniques, to ascertain the magnitude and significance of gDNA-induced variations in biofilm accrual relative to controls.

2. Probing Intra- and Inter-species gDNA Modulations in Biofilm:

Objective 2.1: Administer conspecific gDNA to the biofilm and meticulously document biofilm architectural and functional pivots. Question under scrutiny: Does isogenic gDNA integration within biofilms differ from heterogenic gDNA admittance?

Objective 2.2: Incorporate heterospecific gDNA into the biofilm milieu and chart resulting dynamical shifts. The investigative trajectory is to decipher whether alien gDNA assumes a disruptive, neutral, or symbiotic role within the biofilm ecosystem.

Objective 2.3: Upon data collation from the intra- and inter-species gDNA engagements, proffer comparative analyses elucidating pronounced and subtle distinctions in biofilm reciprocation to gDNA sources.

3. Comparative Analysis of gDNA Effects in Gram-Positive vs. Gram-Negative Biofilms:

Objective 3.1: Prior to gDNA administration, characterise inherent biofilm genesis disparities between chosen gram-positive and gram-negative strains, furnishing a foundational reference for subsequent analyses.

Objective 3.2: Facilitate the interaction of gDNA with biofilms from both bacterial classifications. Systematically record alterations in parameters like biofilm stratification, microbial liaisons, matrix componential distribution, and environmental resilience.

Objective 3.3: Drawing from the innate cellular wall diversities between gram-positive and gram-negative phenotypes, dissect the modus operandi of gDNA vis-à-vis these walls, extrapolating potential variances in biofilm interaction paradigms.

4. Comparative Framework of eDNA and gDNA Influences on Biofilm Morphogenesis:

Objective 4.1: Coalesce your empirical revelations on gDNA-induced modulations with extant literature delineating eDNA-mediated biofilm transformation.

Objective 4.2: Advocate hypothetical molecular or cytological pathways underpinning the contrastive or analogous biofilm trajectories instigated by eDNA and gDNA.

5. Explore the potential correlation between the presence of eDNA and the evolutionary outbreak of cholera.

Objective 5.1: Integrate all research results to discern the role of eDNA in influencing the structural integrity and resilience of biofilms.

CHAPTER 3 MATERIALS AND METHODS

3.1 Bacterial Strains

The main bacterial strains used in this study were obtained from the Life Science Laboratories (Department of Mathematics and Natural Sciences, BRAC University, BD) and the details are as listed below in Table 3-1. Four bacterial strains were meticulously selected based on their biofilm-forming capabilities, given the primary objective to elucidate the influence of free DNA on biofilm development. Specifically, two strains were identified as *Vibrio cholerae*, while the remaining strains were classified as *Staphylococcus aureus* and *Escherichia coli*. Notably, strains lacking the ability to produce biofilms were excluded from the selection criteria to ensure relevance and focus in the study's aim.

Table 1 Bacterial strain and their growth media

Strain	General growth media	
	Liquid Broth	Agar
V. cholerae WT 346	APW[b], LB[c]	TCBS[e], LA[f]
V. cholerae 1877	APW[b], LB[c]	TCBS[e], LA[f]
E. coli 0157:H7	LB[c]	MacConkey, LA[f]
S. aureus ATCC®[a] 25923	TSB[d], LB[c]	MSA[g], TSA[h]

a ATCC, American Type Culture Collection

b APW, Alkaline Peptone Water

c LB, Luria Bertani Broth

d TSB, Tryptone Soy Broth

e TCBS, Thiosulfate-Citrate-Bile Salts-Sucrose

f LA, Luria Bertani Agar

g MSA, Mannitol Salt Agar

h TSA, Tryptone Soy Agar

3.1.1 Revival of Bacteria

In this study, bacterial strains were sourced from the Life Science Laboratories at Brac University. Initially, all bacterial strains were revived from a -20°C glycerol T1N1 stock by streaking onto LB agar (LA) petri dish plates using an inoculating needle. The plate was incubated overnight at 37°C in an Incucell General incubator. Following revival, specific growth and confirmation media were utilised for each bacterial species.

3.1.2 Routine bacteria working plate preparation

Vibrio cholerae strains: Cultivated on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar for identification. For routine maintenance and working stock preparation, they were cultured on Luria Bertani Agar (LA). Inoculum was prepared using Alkaline Peptone Water (APW).

Escherichia coli strain: Identified on MacConkey Agar and subsequently maintained on Luria Bertani Agar (LA) for working stocks. Luria Bertani (LB) Broth was used for inoculum preparation.

Staphylococcus aureus strain: Initially grown on Mannitol Salt Agar (MSA) for identification and subsequently cultured on Tryptone Soy Agar (TSA) for working stocks. Tryptic Soy Broth (TSB) was employed for inoculum preparation.

3.1.3 Routine bacteria stock preparation

Bacterial stocks for all strains utilised in this research were routinely prepared as follows:

A single bacterial colony was carefully picked using an inoculating needle from a fresh agar plate. The selected colony was then stabbed into 2 ml of soft agar contained within a sterile microcentrifuge tube. This culture was incubated overnight at 37°C in an Incucell incubator. After incubation, the culture was stored at -20°C for future use.

For long-term preservation, glycerol stocks were prepared:

A single bacterial colony from a fresh agar plate was inoculated into 2 ml of LB broth contained within a sterile borosilicate vial. The vial was then incubated overnight at 37°C in a JSR shaking incubator, set at 120 rpm. Working within a Haier biomedical biosafety cabinet, 850 µL of the resultant bacterial culture was combined with 150 µL of sterile 15% glycerol solution, and this mixture was transferred to a cryovial. For preservation, the cryovial was subsequently stored in a -20°C freezer.

3.2 Culture media preparation

3.2.1 Luria Bertani broth (LB)

Luria Bertani broth (LB) (HiMedia, USA) was used as a general medium to grow most bacterial cultures. LB was supplied in the form of dehydrated powdered medium and prepared using distilled water (dH2O). The required amount of dry medium was added to the deionized water, mixed thoroughly in a conical flask, and sterilised at 121°C for 15 min as per the manufacturer's instructions. The sterilised medium was cooled and maintained sterile before use.

3.2.2 Tryptone Soy Broth (TSB)

Trypticase Soy Broth (TSB) (Oxoid, UK) was used as a general medium to grow most bacterial cultures. TSB was supplied in the form of dehydrated powdered medium and prepared using distilled water (dH2O). The required amount of dry medium was added to the distilled water, mixed thoroughly in a conical flask, and sterilised at 121°C for 15 min as per the manufacturer's instructions. The sterilised medium was cooled and maintained sterile before use.

3.2.3 Alkaline Peptone Water (APW)

Alkaline Peptone Water (APW) (HiMedia, USA) was used as an enrichment medium to grow *Vibrio* cultures. The required amount of dry peptone and sodium chloride powder was added to the distilled water, and mixed thoroughly in a conical flask. pH was adjusted to between 8.4 and

8.6 using NaOH and sterilised at 121°C for 15 min as per the manufacturer's instructions. The sterilised medium was cooled and maintained sterile before use.

3.2.4 Luria Bertani agar (LA)

Luria Bertani agar (LA) (HiMedia, USA) was used as a general medium for bacterial enumeration to determine cell concentration. The required amount of LA dehydrated powdered medium was added to the distilled water as per the manufacturer's instructions, mixed thoroughly, and sterilised at 121°C for 15 min. The sterilised agar media was kept warm at 50°C in a water bath until the next step of pouring into Petri plates. A sterile pipette was used to dispense agar into sterile Petri plates and left until solidified before the lid was placed and kept in storage prior to use.

3.2.5 Tryptone Soya Agar (TSA)

Tryptone Soya agar (TSA) (Oxoid, UK) was used as a general medium for bacterial enumeration to determine cell concentration. The required amount of TSA dehydrated powdered medium was added to the distilled water as per the manufacturer's instructions, mixed thoroughly, and sterilised at 121°C for 15 min. The sterilised agar media was kept warm at 50°C in a water bath until the next step of pouring into Petri plates. A sterile pipette was used to dispense agar into sterile Petri plates and left until solidified before the lid was placed and kept in storage prior to use.

3.2.6 Mannitol Salt Agar (MSA)

Mannitol Salt Agar (MSA) (Oxoid, UK) was used as a differential medium for Streptococcal enumeration to determine cell concentration. The required amount of MSA dehydrated powdered medium was added to the distilled water as per the manufacturer's instructions, mixed thoroughly, and sterilised at 121°C for 15 min. The sterilised agar media was kept warm at 50°C in a water bath until the next step of pouring into Petri plates. A sterile pipette was used to

dispense agar into sterile Petri plates and left until solidified before the lid was placed and kept in storage prior to use.

3.2.7 MacConkey Agar (MAC)

MacConkey Agar (HiMedia, USA) was used as a differential medium for Escherichia enumeration to determine cell concentration. The required amount of MacConkey agar dehydrated powdered medium was added to the distilled water as per the manufacturer's instructions, mixed thoroughly, and sterilised at 121°C for 15 min. The sterilised agar media was kept warm at 50°C in a water bath until the next step of pouring into Petri plates. A sterile pipette was used to dispense agar into sterile Petri plates and left until solidified before the lid was placed and kept in storage prior to use.

3.2.8 Thiosulfate-Citrate-Bile Salts-Sucrose Agar (TCBS)

Thiosulfate-Citrate-Bile Salts-Sucrose Agar (HiMedia, USA) was used as a differential medium for *Vibrio* enumeration to determine cell concentration. The required amount of TCBS dehydrated powdered medium was added to the distilled water as per the manufacturer's instructions, mixed thoroughly, and sterilised at 121°C for 15 min. The sterilised agar media was kept warm at 50°C in a water bath until the next step of pouring into Petri plates. A sterile pipette was used to dispense agar into sterile Petri plates and left until solidified before the lid was placed and kept in storage prior to use.

3.2.9 Soft Agar (0.7%)

Begin the preparation by weighing out the required amount of LB (Luria Bertani) powder as recommended by the manufacturer to prepare 1 litre of LB broth. To this, add 7 grams of bacteriological agar for every litre of medium you're preparing, resulting in a 0.7% agar concentration. Combine the LB powder and bacteriological agar with distilled in a flask.

Swirl the mixture to ensure that the LB and agar are distributed throughout the water and then autoclave at 121°C for 15 min. Pour the soft agar into microcentrifuge tubes under sterile conditions. For storage of liquid soft agar, ensure the container is well-sealed to prevent contamination. Store in a cool place, and always sterilise the medium again before use if stored in liquid form.

3.3 Reagent Preparation

3.3.1 Sodium Dodecyl Sulfate, SDS (10%)

On a balance, weigh out 10 grams of SDS powder. Transfer the SDS powder to a conical flask or beaker. Add approximately 90 mL of distilled or deionized water. This brings the total volume close to, but not exactly, 100 mL, allowing for the additional volume of the SDS powder. Place a stirring bead in the flask and place it on a magnetic stirrer. Allow the solution to stir until the SDS is fully dissolved. Once the SDS is fully dissolved, adjust the total volume to 100 mL with distilled water. Transfer the 10% SDS solution to a sterile conical flask, label it with the concentration and date of preparation, and store it at room temperature. Ensure the container is well-sealed to prevent contamination.

3.3.2 Phenol:chloroform:isoamyl alcohol (25:24:1)

First, measure out 25 mL of phenol. Next, measure 24 mL of chloroform and 1 mL of isoamyl alcohol. Pour the measured amounts of phenol, chloroform, and isoamyl alcohol into a glass bottle. Using a glass stirring rod, mix the components thoroughly. After mixing, tightly cap the glass bottle and invert it several times to ensure the components are well-blended. For storage, place the mixture in a cool, dark location. It's important to note that over time, a white precipitate might form at the bottom of the bottle. Before using the mixture, be sure to invert the bottle several times to re-suspend any settled precipitate. Always handle the chemicals with care and employ appropriate safety equipment and procedures.

3.3.3 Chloroform:isoamyl alcohol (24:1)

Begin by measuring out 24 mL of chloroform. Following that, measure 1 mL of isoamyl alcohol. Pour both the chloroform and isoamyl alcohol into a glass bottle. Using a glass stirring rod, thoroughly mix the components together. After achieving a homogeneous mixture, tightly seal the glass bottle with its cap. For storage, it's recommended to keep the mixture in a cool, dark location. Before each use, ensure to shake or invert the bottle several times to maintain the mixture's uniformity. As always, when working with these chemicals, it's essential to handle them with care and to use appropriate safety equipment and procedures.

3.3.4 Sodium Acetate (3M, pH 5.2)

Begin by weighing out 24.6 grams of sodium acetate trihydrate. Dissolve the sodium acetate in approximately 80 mL of distilled or deionized water. Once dissolved, adjust the pH to 5.2 using glacial acetic acid. It's essential to add the acid dropwise and check the pH intermittently to avoid overshooting the desired pH. After reaching the target pH, adjust the final volume to 100 mL using distilled or deionized water. Transfer the prepared sodium acetate solution into a suitable storage bottle, ensuring it's properly labelled. For long-term storage, it's advisable to keep the solution at room temperature and away from direct sunlight.

3.3.5 TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)

Initiate the process by solubilizing 1.21 grams of Tris base in approximately 800 mL of distilled water. Subsequently, incorporate 0.186 grams of EDTA into the mixture, ensuring thorough dissolution. For pH adjustment, incrementally introduce hydrochloric acid (HCl) to achieve a pH of 8.0. It's paramount to administer the acid in a controlled manner, intermittently gauging the pH to prevent surpassing the intended value. Upon reaching the stipulated pH, augment the solution to a final volume of 1 litre using deionized or distilled water. Decant the formulated TE buffer into an aptly labelled storage container. It is advisable to preserve the buffer at ambient temperature, shielded from direct sunlight. In the context of chemical solution preparation,

meticulous handling of reagents and adherence to safety protocols, complemented by the use of appropriate protective equipment, is essential.

3.3.6 Saline (0.9%)

To formulate the saline solution, solubilize 9 grams of sodium chloride in 1 litre of distilled water. Upon complete dissolution of the sodium chloride, decant the solution into an appropriate storage vessel. Prior to securely capping the vessel, affix a label delineating both the concentration and the preparation date. It's advisable to sterilise the solution by autoclaving at 121°C for 15 minutes if it's intended for biological applications. Store the saline solution at room temperature, and always ensure the storage area is free from direct sunlight. Remember to handle all chemicals and solutions with care, using the necessary safety equipment and observing best laboratory practices.

3.3.7 Crystal Violet (0.1%)

To prepare a 0.1% solution of crystal violet, dissolve 0.1 grams of crystal violet in 100 mL of distilled water. Thoroughly mix the solution until the crystal violet is fully dissolved, which will result in a clear, deep purple solution. Once prepared, transfer the solution to an appropriate storage container. It's essential to label the container with the concentration, date of preparation, and any safety warnings associated with crystal violet. If intended for specific biological applications, it might be necessary to filter sterilise the solution using a 0.22-micron filter. For storage, keep the solution in a cool, dark place away from direct sunlight to maintain its stability. Always handle chemicals with care and ensure that safety protocols are strictly followed, particularly since crystal violet is a dye and can stain easily.

3.3.8 Glacial Acetic Acid (33%)

Begin the preparation by carefully measuring out 33 mL of glacial acetic acid. To this, add 67 mL of distilled or deionized water in a suitable glass container. Gently stir the mixture to ensure homogeneity. Due to the exothermic nature of this dilution, it's crucial to add the acid to the

water and not vice versa to prevent excessive heat generation and potential splashing. Once the solution is well mixed, transfer it to an appropriate storage bottle. Label the bottle with the concentration, date of preparation, and any safety warnings associated with glacial acetic acid. For safety reasons, always use protective equipment such as gloves and safety goggles when handling concentrated acids. Store the 33% glacial acetic acid solution in a cool place, ensuring it's well-sealed to prevent any evaporation or contamination.

3.4 Genomic DNA Extraction

3.4.1 Whole Genome DNA Extraction

Chemicals & Reagents

1. Bacterial culture
2. 10% SDS (sodium dodecyl sulphate)
3. Phenol:chloroform:isoamyl alcohol (25:24:1)
4. Chloroform:isoamyl alcohol (24:1)
5. 3M Sodium acetate, pH 5.2
6. Ice-cold 70% ethanol
7. Ice-cold Absolute ethanol
8. TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)

Equipments

1. Sterile microcentrifuge tubes
2. Microcentrifuge
3. Water bath
4. Heat block

Procedures

1. Cell Harvesting
    a. Inoculate bacteria in the LB medium and incubate overnight at 37°C in a JSR shaking incubator, set at 120 rpm.
    b. Transfer 1 mL of the culture into a microcentrifuge tube.
    c. Harvest the bacterial cells by centrifugation at 8,000 rpm for 10 minutes
    d. Discard the supernatant and resuspend the pellet in 100 µL of TE buffer.

2. Lysis
    a. Place the tube in a heat block set to 100°C and boil for 15 minutes.
    b. Add 10% SDS (about 50 µL) and mix gently by inversion.
    c. Incubate at 55°C for 1 hour in a water bath.
    d. Immediately cool the tube on ice for 10 minutes to ensure the DNA remains intact.

3. Phenol:Chloroform Extraction
    a. Add an equal volume of phenol:chloroform:isoamyl alcohol to the sample. Vortex vigorously for 20 seconds.
    b. Centrifuge at 14,000 for 10 minutes to separate the phases.
    c. Carefully transfer the aqueous (upper) phase to a new sterile tube.

4. Chloroform Extraction
    a. Add an equal volume of chloroform:isoamyl alcohol to the aqueous phase. Vortex vigorously for 20 seconds.
    b. Centrifuge at 14,000 for 10 minutes to separate the phases.
    c. Carefully transfer the aqueous (upper) phase to a new sterile tube.

5. DNA Precipitation
    a. Add 0.1 volumes of 3M sodium acetate and 2.5 volumes of ice-cold absolute ethanol to the aqueous phase. Mix well.
    b. Incubate at -20°C overnight.
    c. Centrifuge at 15,000 rpm for 30 minutes to pellet the DNA.

d. Carefully remove the supernatant.

6. DNA Washing
    a. Add 1 mL of ice-cold 70% ethanol to the DNA pellet.
    b. Centrifuge at 15,000 rpm for 15 minutes.
    c. Remove the supernatant and air-dry the DNA pellet for 10 minutes.

7. DNA Resuspension
    a. Resuspend the DNA pellet in 200 µL of TE buffer
    b. Store the DNA at -20°C.

8. DNA Dilution
    a. Begin by adding 100 µL of DNA to an Eppendorf tube containing 900 µL of TE buffer. Vortex the mixture thoroughly to achieve a 10^-1 dilution.
    b. From this 10^-1 dilution, transfer 100 µL into a new Eppendorf tube containing 900 µL of TE buffer. Vortex to mix, resulting in a 10^-2 dilution.
    c. Repeat the above step using the 10^-2 dilution to obtain a 10^-3 dilution.

3.4.2 Fragmented Genome DNA Extraction

Chemicals & Reagents

1. Bacterial culture
2. 10% SDS (sodium dodecyl sulphate)
3. Phenol:chloroform:isoamyl alcohol (25:24:1)
4. Chloroform:isoamyl alcohol (24:1)
5. 3M Sodium acetate, pH 5.2
6. Ice-cold 70% ethanol
7. Ice-cold Absolute ethanol
8. TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)

Equipments

1. Sterile microcentrifuge tubes
2. Microcentrifuge
3. Water bath
4. Heat block
5. Sonicator

Procedures

1. Cell Harvesting
    a. Inoculate bacteria in the LB medium and incubate overnight at 37°C in a JSR shaking incubator, set at 120 rpm.
    b. Transfer 1 mL of the culture into a microcentrifuge tube.
    c. Harvest the bacterial cells by centrifugation at 8,000 rpm for 10 minutes
    d. Discard the supernatant and resuspend the pellet in 100 µL of TE buffer.

2. Lysis
    a. Place the tube in a heat block set to 100°C and boil for 15 minutes.
    b. Add 10% SDS (about 50 µL) and mix gently by inversion.
    c. Incubate at 55°C for 1 hour in a water bath.
    d. Immediately cool the tube on ice for 10 minutes to ensure the DNA remains intact.

3. Phenol:Chloroform Extraction
    a. Add an equal volume of phenol:chloroform:isoamyl alcohol to the sample. Vortex vigorously for 20 seconds.
    b. Centrifuge at 14,000 for 10 minutes to separate the phases.
    c. Carefully transfer the aqueous (upper) phase to a new sterile tube.

4. Chloroform Extraction
    a. Add an equal volume of chloroform:isoamyl alcohol to the aqueous phase. Vortex vigorously for 20 seconds.
    b. Centrifuge at 14,000 for 10 minutes to separate the phases.
    c. Carefully transfer the aqueous (upper) phase to a new sterile tube.

5. DNA Precipitation
    a. Add 0.1 volumes of 3M sodium acetate and 2.5 volumes of ice-cold absolute ethanol to the aqueous phase. Mix well.
    b. Incubate at -20°C overnight.
    c. Centrifuge at 15,000 rpm for 30 minutes to pellet the DNA.
    d. Carefully remove the supernatant.

6. DNA Washing
    a. Add 1 mL of ice-cold 70% ethanol to the DNA pellet.
    b. Centrifuge at 15,000 rpm for 15 minutes.
    c. Remove the supernatant and air-dry the DNA pellet for 10 minutes.

7. DNA Resuspension
    a. Resuspend the DNA pellet in 200 µL of TE buffer.

8. DNA Fragmentation
    a. Place the sample microcentrifuge tube containing DNA on an ice block.
    b. Set the sonicator at 10 Watts and clean the microtip.
    c. Pulse the sonication for 20 seconds and turn it off for 60 seconds.
    d. Repeat for three cycles.
    e. Store the sonicated DNA at -20°C

9. DNA Dilution
    a. Begin by adding 100 µL of DNA to an Eppendorf tube containing 900 µL of TE buffer. Vortex the mixture thoroughly to achieve a 10^-1 dilution.

b. From this 10^-1 dilution, transfer 100 µL into a new Eppendorf tube containing 900 µL of TE buffer. Vortex to mix, resulting in a 10^-2 dilution.

c. Repeat the above step using the 10^-2 dilution to obtain a 10^-3 dilution.

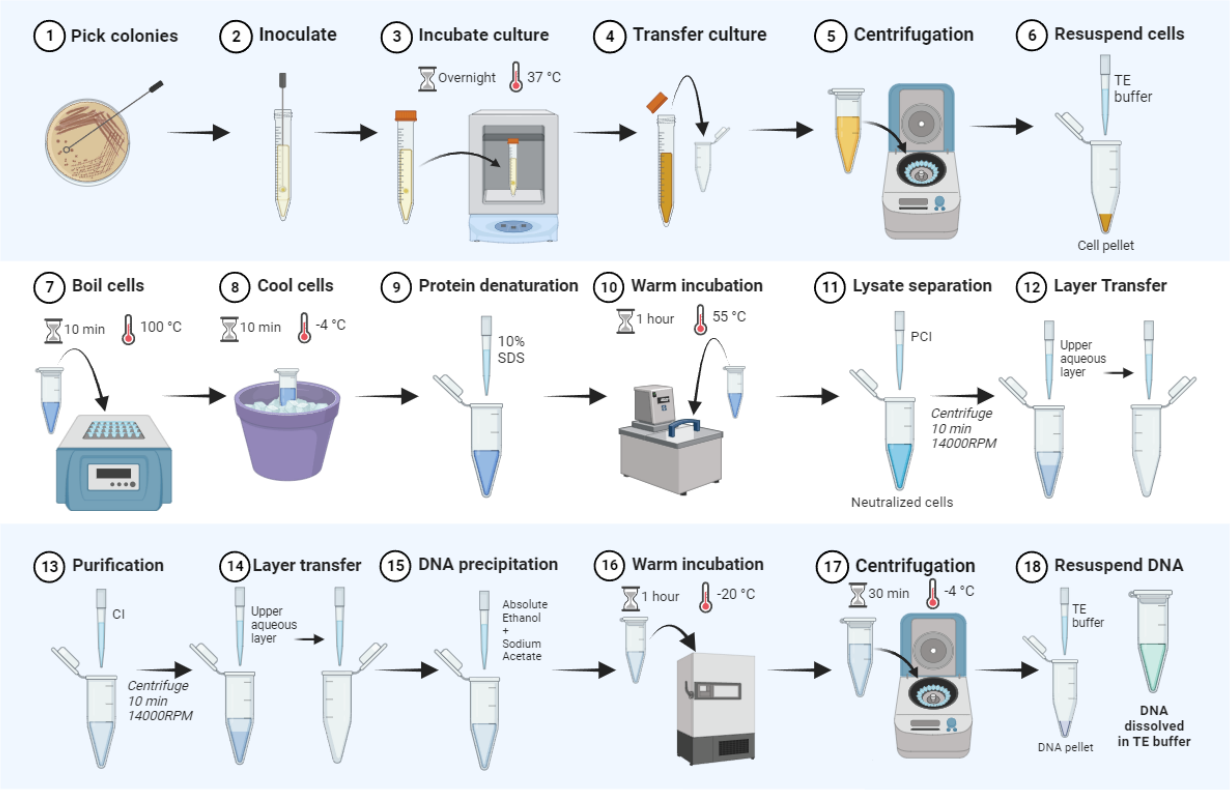


Figure 5: Illustrated summary of the DNA isolation protocol.

3.5 Crystal Violet Biofilm Formation Assay

3.5.1 Inocula Preparation

The bacterial inoculum was prepared using a protocol tailored to each species, beginning with the growth of a 3-hour culture initiated from a single colony taken from a fresh agar plate. Depending on the bacterial species, various nutrient broths were used:

Vibrio: Inoculum was prepared using Alkaline Peptone Water (APW).

Escheria: Inoculum was prepared using Luria Bertani (LB) Broth.

Streptococcal: Inoculum was prepared with Tryptic Soy Broth (TSB).

To further process the young culture:

Prepare a 1:1 dilution by combining 750 µL of the bacterial culture with 750 µL of fresh medium in a sterile borosilicate vial. Depending on the experimental condition, either:
Add no DNA to the vial, or introduce 200 µL of DNA (this can be either Whole DNA or Fragmented DNA) to the vial. Incubate the vial at room temperature for 36 hours, ensuring it remains undisturbed and stationary, without any shaking.

3.5.2 Washing the biofilm

After the incubation period, carefully decant the medium from each vial, ensuring the biofilm at the bottom remains undisturbed. To remove any planktonic or loosely attached cells, wash the vials three times with a sterile 0.9% saline solution.

3.5.3 Staining

To each vial, add 2 mL of a 0.1% crystal violet solution. Let the vials stand at room temperature for 15 minutes. Following this, gently rinse the vials with water to eliminate any excess crystal violet, ensuring that the stained biofilm remains undisturbed.

3.5.4 Dissolution

Once the wells are dry, introduce 2 mL of 33% acetic acid into each vial to dissolve the bound crystal violet. Let the vials stand at room temperature for 45 minutes, occasionally giving them a gentle shake to ensure complete solubilization of the crystal violet.

3.5.5 Quantification

Transfer 200 µL of the solubilized crystal violet solution into each well of a 96-well flat-bottomed microtiter plate. Using a microplate reader, measure the absorbance of each well at 620 nm. To determine the net biofilm formation, subtract the absorbance value of the control wells (those containing only the medium) from the other readings.

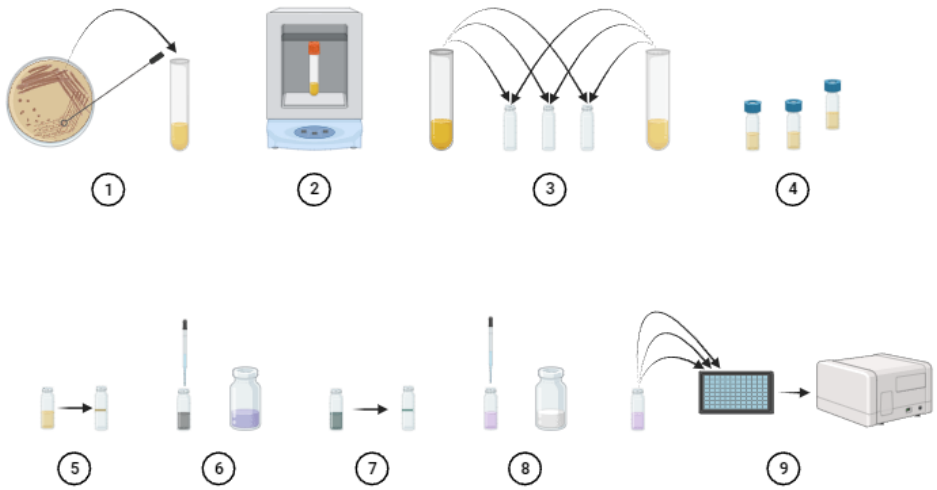


Figure 6: Biofilm formation assay process.

(1) Inoculate fresh medium using a single colony from a fresh agar plate. (2) Incubate the test tube for 3 hours in a shaker incubator. (3) Transfer 750 ul of the bacterial inocula along with 750 ul of fresh medium to each of 3 sterile vials. (4) Incubate the vials at room temperature for 36 hours with no shaking. (5) Discard the inocula carefully. Wash each vial three times using 0.9% saline. (6) Add 2 ml crystal violet and let stand for 15 minutes. (7) Discard the crystal violet. Wash the vials twice using 0.9% saline. (8) Add 2 ml acetic acid and let stand for 45 minutes. (9) Transfer 200 ul of the dissolved biofilm ring into 3 different wells on the microtiter plate. Use the ELISA machine to measure the OD at 620 nm.

CHAPTER 4 RESULT

Multiple strains of *V. cholerae*, *E. coli*, and *S. aureus* were procured from the laboratory repository at BRAC University and subsequently subjected to examinations to determine their biofilm-forming potentials. Following standard protocols, the assessment progressed until the staining of the biofilm on borosilicate vials. Through a comparative analysis of biofilm thickness across the various strains, four predominant strains were identified: *V. cholerae* WT346, *V. cholerae* 1877, *E. coli* 0157:H7, and *S. aureus* ATCC 25923. These strains exhibited pronounced biofilm development, discernible to the unaided eye, and manifested superior thickness relative to their counterparts.

Genomic DNA was meticulously extracted from these quartet strains. This extracted DNA, both in its whole and fragmented forms, was utilised to assess the impact of its addition on the biofilm development of the corresponding strains under both inter and intra-species conditions. The parameters, including incubation temperature, duration of incubation, and volume of inoculum, were meticulously maintained constant throughout the experiment.

Subsequently, the resultant biofilms were solubilized in glacial acetic acid, facilitating the measurement of the optical density (OD) of the solution, thereby ensuring quantifiable results. Both the visual evaluation of stained biofilms and the derived OD values indicated an augmented biofilm formation for a majority of the strains when supplemented with DNA, in contrast to the control (biofilm formation in the absence of DNA). To elucidate the observed variations in biofilm development, optical density values were employed to compute the average biofilm yield for both the control and DNA-added vials. These quantified values were then systematically represented in bar charts, showcasing the relative enhancement or attenuation in biofilm formation.

4.1 Growth in Nutrient and Selective Media

For the empirical investigation, the quartet of strains was initially cultured on Luria Agar (LA) plates from their respective glycerol stock collections, followed by incubation at 37°C for a

24-hour period to facilitate colony formation. Subsequently, individual colonies from each strain were cultured onto fresh, selective media plates, undergoing another 24-hour incubation period. These validated colonies were once again cultivated on pristine LA plates, which post-incubation, served as the working plates designated for inoculating the requisite liquid cultures tailored for biofilm development. It is imperative to note that this cultivation procedure was systematically repeated at seven-day intervals, ensuring the consistent availability of refreshed culture plates for the ongoing experimental requirements.

4.1.1 Growth of *Vibrio cholerae* WT346

The *V. cholerae* WT346 strain was meticulously cultured on pristine LA plates. Post incubation, the emergence of white, medium-sized individual colonies was discernibly observed within the second and third quadrants of the methodically streaked plates. To further validate the strain, an isolated colony from these LA plates was subsequently cultivated onto a fresh TCBS plate. Following another incubation period, this procedure yielded bright yellow, distinct colonies predominantly in the third and fourth quadrants. Colonies characterised by their considerable size, vivid yellow hue, combined with a distinguishably opaque central region and translucent peripheries, were conclusively identified as the singular colonies of *V. cholerae* WT346.

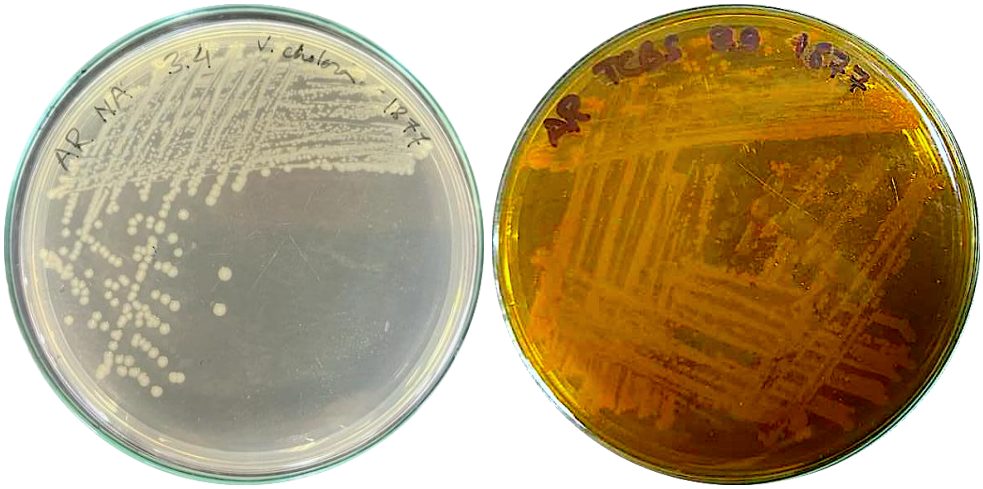
(a) (b)

Figure 7: Growth of Vibrio cholerae WT346

(a) WT346 cultured on the Luria Agar (LA) plate manifested as discrete white colonies, indicative of bacterial proliferation. (b) A representative colony from the LA medium was subsequently transferred to the Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar, resulting in the emergence of characteristic yellow colonies, corroborating the identity of the isolate as WT346.

4.1.2 Growth of *Vibrio cholerae* 1877

V. cholerae 1877 was streaked on fresh LA plates and after incubation, white medium-sized single colonies were observed in the 2nd and 3rd quadrants of the streaked plates. A single colony was taken from LA plates and streaked on the fresh TCBS plate which, after incubation, formed bright yellow single colonies in the 4th quadrant. The large and yellow colonies with opaque centres and translucent edges were taken as confirmed single colonies of *V. cholerae* 1877.



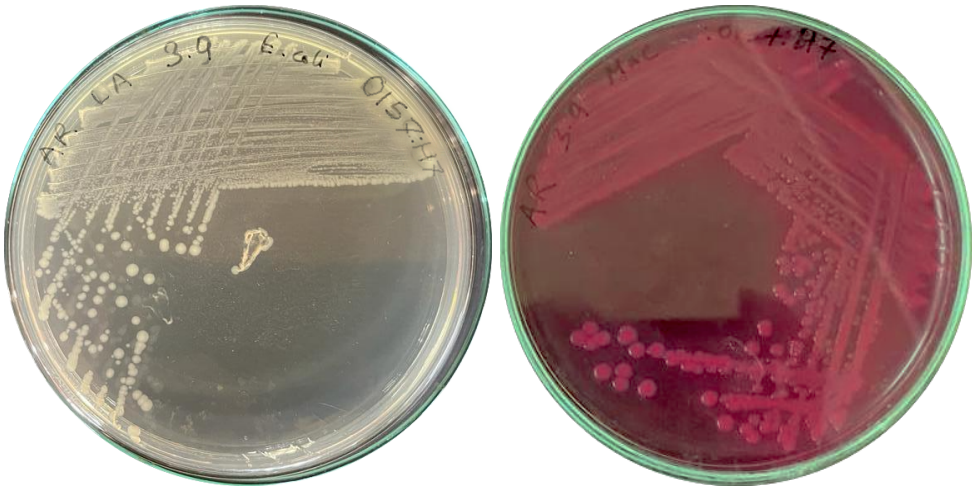
(a) (b)

Figure 8: Growth of Vibrio cholerae 1877

(a) 1877 streaked onto the LA plate, with white colonies showing bacterial growth. (b) A single colony from the LA plate streaked onto the TCBS plate which gave yellow colonies confirming that the bacteria was 1877.

4.1.3 Growth of *Escherichia coli* 0157:H7

The strain *E. coli* 0157:H7 was diligently propagated on pristine LA plates. Following the incubation period, one could discern the appearance of white, medium-sized solitary colonies predominantly within the second quadrant of the methodically streaked plates. For further validation, a distinct colony from these LA plates was subsequently cultivated onto a freshly prepared MAC plate. After the ensuing incubation, this procedure yielded expansive, matte pink individual colonies, primarily evident in the third and fourth quadrants. Colonies manifesting a pink to deep pink hue, coupled with a dry texture and a more intensified pink halo, were unequivocally identified as the singular colonies of *E. coli* 0157:H7.



(a) (b)

Figure 9: Growth of Escherichia coli 0157:H7

(a) The 0157:H7 isolate, when cultured on Luria Agar (LA) medium, exhibited discernible white colonies, denoting bacterial proliferation. (b) Subsequent sub-culturing of a distinct colony from the LA medium onto MacConkey (MAC) agar resulted in the formation of characteristic pink colonies, unequivocally validating the bacterium's identity as 0157:H7.

4.1.4 Growth of *Staphylococcus aureus* ATCC 25923

The *S. aureus* ATCC 25923 strain was cultured on pristine TSA plates. Post-incubation, there was a discernible presence of white, medium-sized, individualised colonies, predominantly

localised within the second quadrant of the systematically streaked plate. For further validation, a representative colony from the TSA plate was cultivated onto a novel MSA plate. After the subsequent incubation period, this procedure yielded diminutive yellow singular colonies, principally within the third and fourth quadrants. It's noteworthy to mention that these yellow colonies, set against the light pink agar backdrop, were conclusively recognized as the solitary colonies of *S. aureus* ATCC 25923.

(a) (b)

Figure 10: Growth of Staphylococcus aureus ATCC 25923

(a) The isolate ATCC 25923, when cultured on Tryptic Soy Agar (TSA) medium, manifested distinct white colonies indicative of bacterial growth. (b) A subsequent transfer of a specific colony from the TSA medium to Mannitol Salt Agar (MSA) resulted in the emergence of characteristic yellow colonies, conclusively affirming the bacterial identity as ATCC 25923.

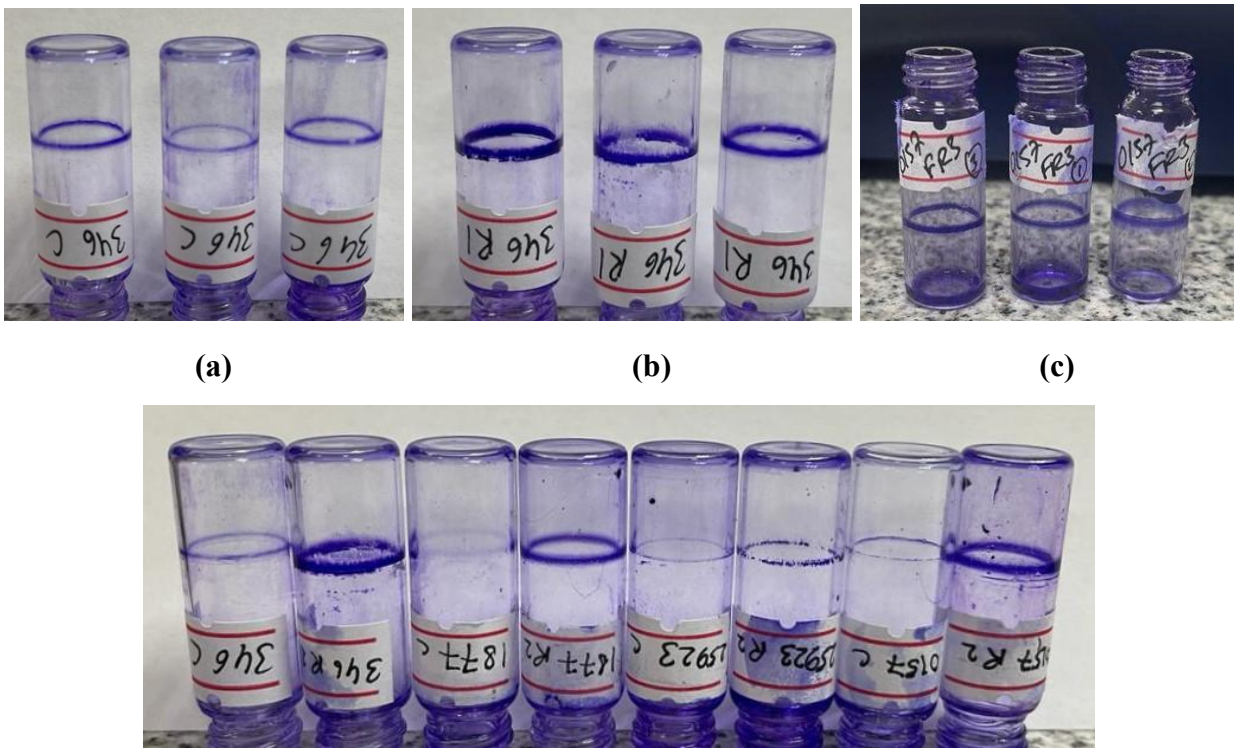
4.2 Biofilm formation on borosilicate glass vials

Upon concluding a 36-hour incubation period of the liquid inoculum in a quiescent state, manifestations of biofilm became discernibly evident on the inoculum's surface. Subsequent to the disposal of the inoculum and its rinsing with saline solution, only the biofilm remained affixed to the borosilicate glass vial's surface. Employing a 0.1% crystal violet solution for staining yielded lucidly distinguishable biofilms with quantifiable thickness. Adhering to standard protocols, these stained biofilms were solubilized using a 33% glacial acetic acid

solution. Solutions derived from this dissolution process were aliquoted into microtiter plates, followed by obtaining optical density readings at 620 nm. Notably, these optical density values exhibited a direct proportionality to the biofilm thickness originally presented in the vials.

4.2.1 Visual Comparison of the Thickness of Biofilm Developed with and without DNA

Upon employing crystal violet staining, biofilms exhibiting varied thicknesses were discernibly identified. Subsequent observations illuminated a pronounced augmentation in biofilm thickness when cultivated in the presence of both whole and fragmented DNA, relative to the control which remained devoid of any exogenous DNA introduction. A discernible trend in biofilm thickness was evident upon utilising varied DNA concentrations. Furthermore, a nuanced differential was observed between the biofilms fostered in the presence of whole DNA and those cultivated with fragmented DNA.



(a) **(b)** **(c)**

(d)

Figure 11: Comparison of stained biofilm for whole, fragmented, and no DNA

Stained biofilms cultivated in vials with diverse DNA conditions provide revealing insights: (a) The vials labelled '346 C' host biofilms engendered by *V. cholerae* WT346 strain devoid of any DNA exposure. The notation '346' corresponds to the WT346 strain, while 'C' denotes the control, establishing a baseline for biofilm evaluation. (b) Vials marked '346 R1' exhibit biofilms developed by the *V. cholerae* WT346 strain in the presence of whole DNA. In this nomenclature, '346' is indicative of the WT346 strain, 'R' symbolises whole DNA, and '1' designates the first trial iteration. A noticeable increase in biofilm density relative to the control group is evident. (c) The '0157 FR3' vials contain biofilms engendered by the *E. coli* 0157:H7 strain, where fragmented DNA was introduced. Here, '0157' signifies the 0157:H7 strain, 'F' indicates fragmented DNA, and '3' points to the third trial. These biofilms demonstrate a thickness comparable to their counterparts formed in the presence of whole DNA. (d) A comparative assessment between the control vials and those enriched with whole DNA, encompassing all four bacterial strains. The labels '346', '1877', '25923', and '0157' represent the strains *V. cholerae* WT346, *V. cholerae* 1877, *S. aureus* ATCC25923, and *E. coli* 0157:H7, respectively. The ensuing data reveals a consistent enhancement in biofilm robustness across all strains when whole DNA is incorporated, as juxtaposed with the foundational biofilms.

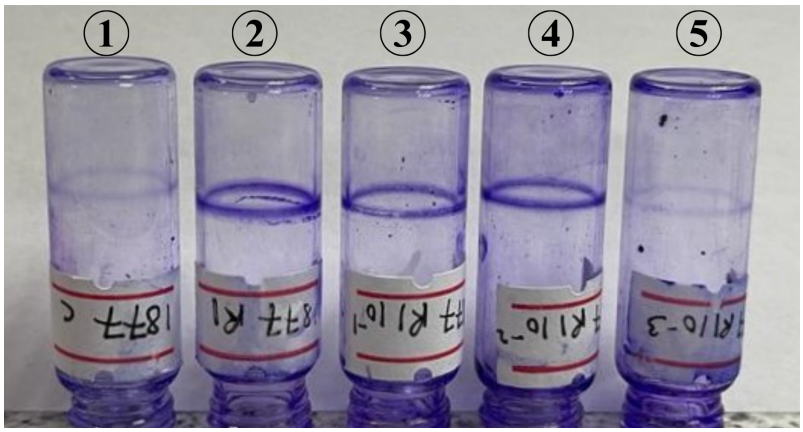


Figure 12: Vials with stained biofilms cultivated under varying DNA concentrations

Vials showcasing stained biofilms cultivated under varying DNA concentrations for the *V. cholerae* 1877 strain: (1) Commencing from the leftmost vial, the biofilm represents a control condition devoid of any DNA addition. This is labelled as '1877 C', where '1877' specifies the strain and 'C' represents 'Control'. Subsequent to this are vials that have biofilms fostered in the milieu of progressively diluted DNA concentrations. (2) The '1877 R1' vial contains a biofilm nurtured in the presence of undiluted or raw DNA. (3, 4 & 5) Vials tagged as '1877 R1 10^-1', '1877 R1 10^-2', and '1877 R1 10^-3' represent biofilms grown with DNA concentrations diluted 10-fold, 100-fold, and 1000-fold, respectively. A discernible trend emerges from this array: as the DNA concentration dwindles, so does the biofilm's thickness. The most substantial biofilm is attributed to the raw DNA ('R1'), while the most diluted concentration ('R1 10^-3') results in the leanest biofilm formation.

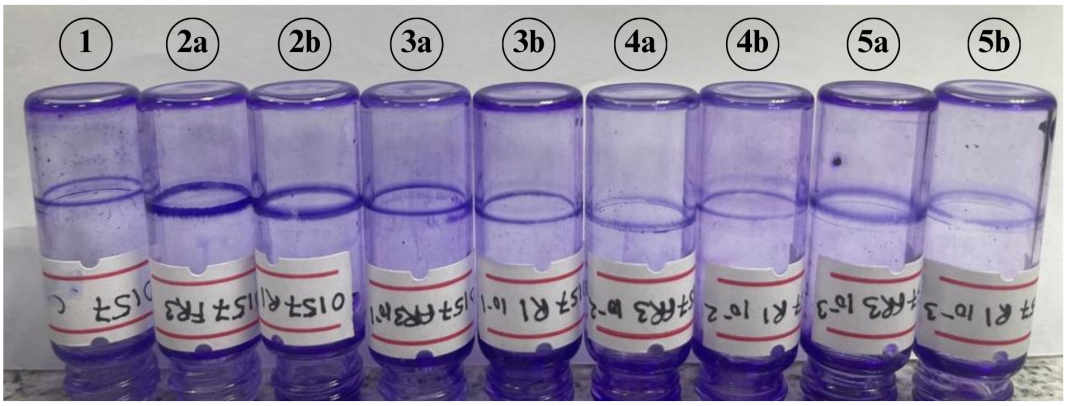


Figure 13: Comparative Analysis of Biofilm Formation under Various DNA Conditions

Comparative Analysis of Biofilm Formation under Various DNA Conditions: (1) Control vial without DNA infusion. (2a & 2b) Biofilms developed in the presence of fragmented and whole DNA. (3a & 3b) Biofilms in 10-fold diluted fragmented and whole DNA environments. (4a & 4b) Those exposed to 100-fold diluted fragmented and whole DNA. (5a & 5b) Biofilms cultivated with 1000-fold diluted fragmented and whole DNA, respectively.

4.3 Biofilm formation in the presence of gDNA

Upon the successful identification of bacterial strains *V. cholerae* WT346, *V. cholerae* 1877, *E. coli* 0157:H7, and *S. aureus* ATCC25923 via selective media plate cultivation, genomic DNA (gDNA) was systematically extracted in accordance with established protocols. The extracted gDNA was incorporated into the experimental framework in two primary forms: undisturbed whole genomic DNA and segmented DNA. These forms functioned as primary experimental variables, while parameters such as incubation durations, temperatures, DNA extraction volume, and inoculum volume were standardised throughout the study.

Post an initial 3-hour incubation, DNA was incorporated into cultures housed in borosilicate vials, and subsequent incubation extended for an additional 36 hours at a consistent temperature range of 25°C to 30°C.

For ensuring experiment reliability, a control mechanism was instituted: one vial contained an inoculum devoid of any DNA (Control, C) while another vial housed only the nutritional medium, serving as the Negative Control (NC). Biofilm generation was analysed under varying

DNA concentrations, specifically: 1 x 10^-0 (Raw), 1 x 10^-1, 1 x 10^-2, and 1 x 10^-3. Each DNA concentration was tested in triplicate to ensure consistency.

For precision, biofilms were dissolved in glacial acetic acid, with the resulting solutions allocated across three microtiter plate wells to facilitate accurate optical density (OD) readings. These readings were crucial in ascertaining the average biofilm quantity, ensuring an accurate representation of biofilm generation.

4.3.1 Effect of Whole DNA on Biofilm Formation

In the designed experiment, one of the primary variables introduced was the addition of whole genomic DNA extracted from each of the four distinct bacterial strains. Specifically, two key scenarios were investigated. The first, termed "Intraspecies Addition," involved adding the genomic DNA from a specific strain to its own culture. An example of this would be supplementing WT346 DNA to a WT346 culture, with the intention of discerning the impact of a bacterium's inherent genomic DNA on its biofilm formation capabilities. The second scenario, known as "Interspecies Addition," contrastingly added genomic DNA from one bacterial strain to the culture of a different strain. For instance, introducing WT346 DNA into the 1877 culture. The objective here was to uncover any potential variations in biofilm formation when a culture is exposed to the genomic DNA from an entirely different bacterial species.

Intraspecies: Effect of isogenic Whole DNA on Biofilm development

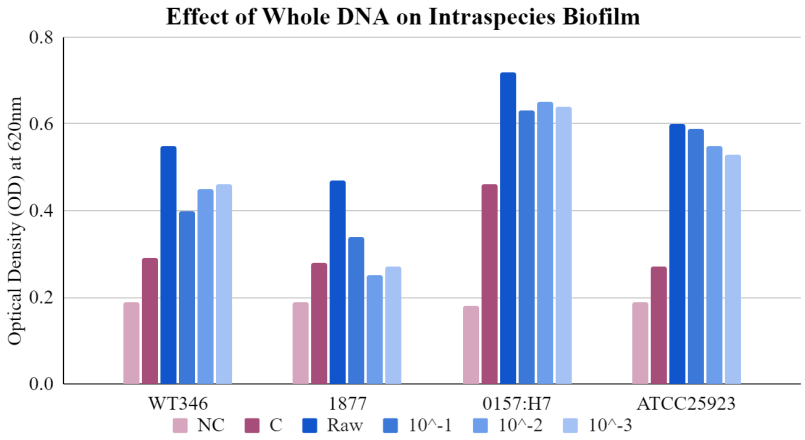


Figure 14: Effect of whole DNA on intraspecies biofilm formation.

The chart illustrates the Optical Density (OD) values, which serve as a quantitative measure of biofilm production, for the bacterial strains *V. cholerae* WT346, *V. cholerae* 1877, *E. coli* 0157:H7, and *S. aureus* ATCC25923. The OD values presented are associated with the introduction of Isogenic whole DNA into each strain.

Interspecies: Effect of heterogenic Whole DNA.

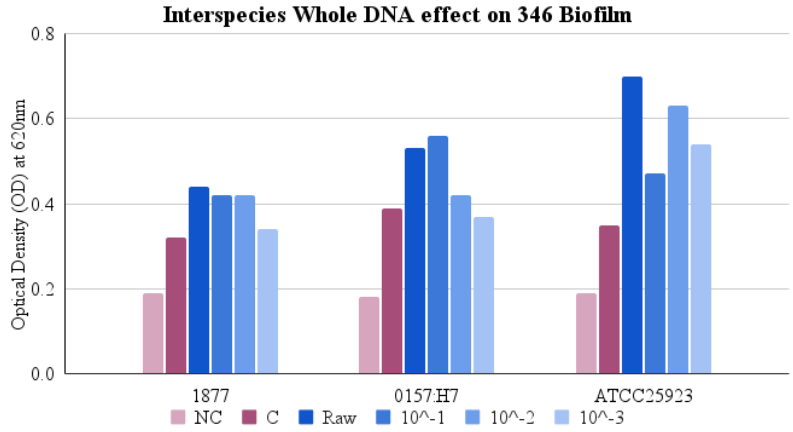


Figure 15a: Effect of heterogenic whole DNA on WT346 biofilm formation.

We investigated the impact of introducing whole DNA from V. cholerae 1877, E. coli 0157:H7, and S. aureus ATCC25923 on the biofilm synthesis of V. cholerae WT346.

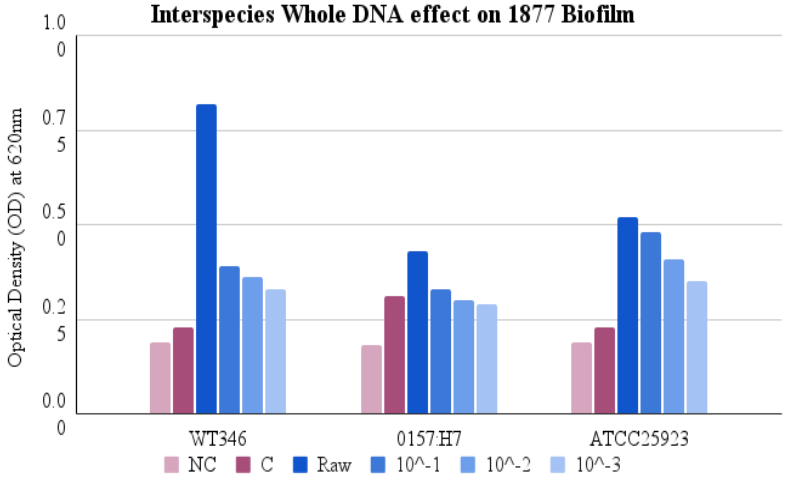


***Figure 15b*: Effect of heterogenic whole DNA on 1877 biofilm formation.**

Similarly, the role of whole DNA from V. cholerae WT346, E. coli 0157:H7, and S. aureus ATCC25923 was assessed in relation to the biofilm production of V. cholerae 1877.

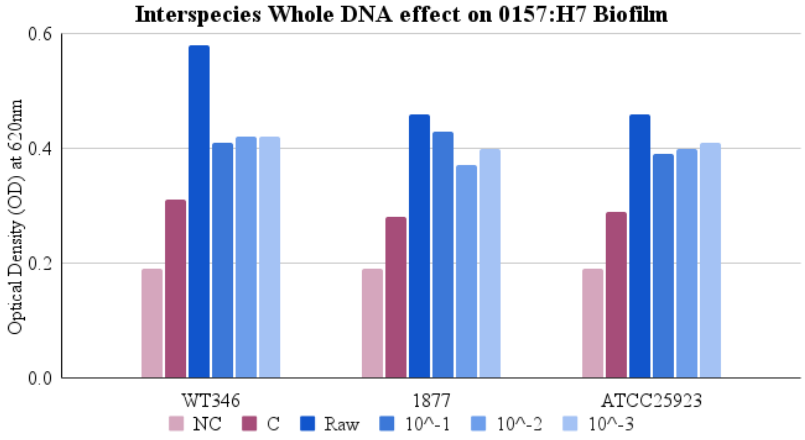


Figure 15c: Effect of heterogenic whole DNA on 0157:H7 biofilm formation.

For E. coli 0157:H7, the effect on biofilm formation was gauged by introducing whole DNA from V. cholerae WT346, V. cholerae 1877, and S. aureus ATCC25923.

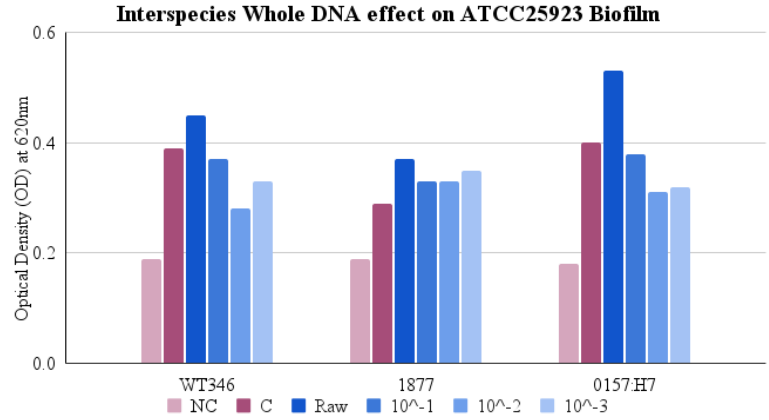


Figure 15d: Effect of heterogenic whole DNA on ATCC 25923 biofilm formation.

Lastly, for S. aureus ATCC25923, we examined the influence of whole DNA from V. cholerae WT346, V. cholerae 1877, and E. coli 0157:H7 on its biofilm development.

4.3.2 Effect of Fragmented DNA on Biofilm formation

The subsequent variable explored in this study pertains to the introduction of fragmented DNA sourced from the quartet of strains under examination. This fragmented DNA was derived from the initially extracted whole DNA, which underwent sonication to yield fragmentary DNA components. Analogous to the methodology applied with whole DNA, this fragmented version was incorporated in both an isogenic and heterogenic fashion. Specifically, isogenic inclusion implied the integration of fragmented DNA from a specific strain into the culture of that identical strain. Conversely, the heterogenic approach involved the amalgamation of fragmented DNA from one strain into the culture medium of a disparate strain. This experimental design facilitates an in-depth understanding of how fragmented DNA influences biofilm formation, both in homologous and non-homologous settings.

Intraspecies: Effect of isogenic Fragmented DNA on Biofilm development

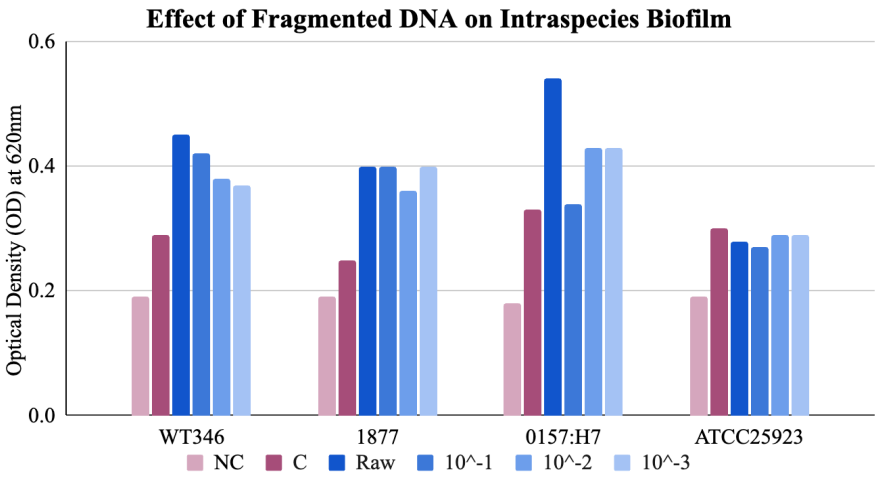


Figure 16: Effect of fragmented DNA on intraspecies biofilm development

The chart presents the optical density (OD) values, indicative of biofilm production, for the bacterial strains *V. cholerae* WT346, *V. cholerae* 1877, *E. coli* 0157:H7, and *S. aureus* ATCC25923. These values were derived under conditions wherein each bacterial culture was exposed to fragmented DNA of the same strain (isogenic).

Interspecies: Effect of heterogenic Fragmented DNA

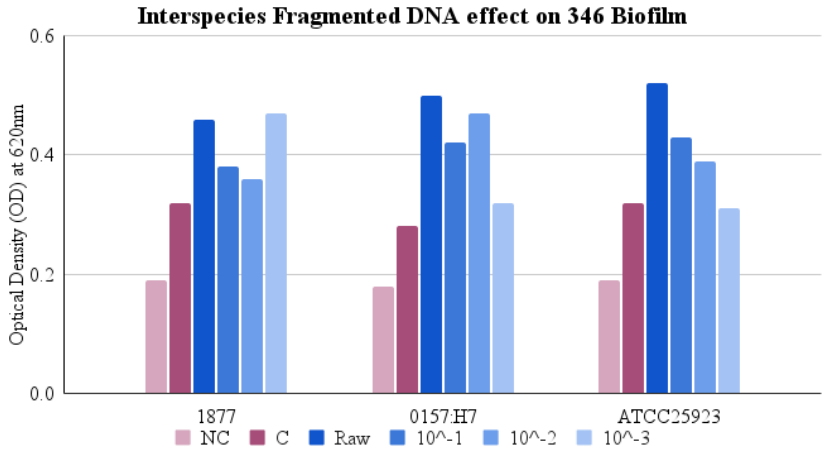


Figure 17a: Effect of heterogenic fragmented DNA on WT346 biofilm development. Illustrates the influence of fragmented DNA from V. cholerae 1877, E. coli 0157:H7, and S. aureus ATCC25923 on the biofilm yield of V. cholerae WT346.

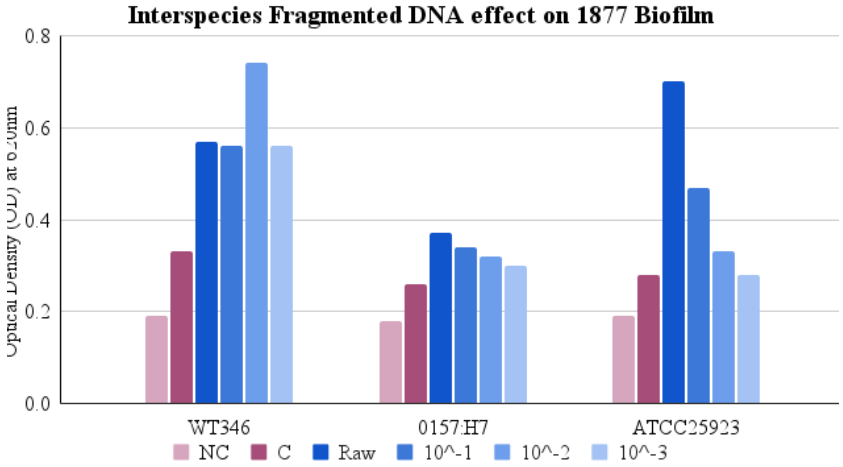


Figure 17b: Effect of heterogenic fragmented DNA on 1877 biofilm development.

Depicts the alteration in biofilm production of V. cholerae 1877 when exposed to fragmented DNA from V. cholerae WT346, E. coli 0157:H7, and S. aureus ATCC25923.

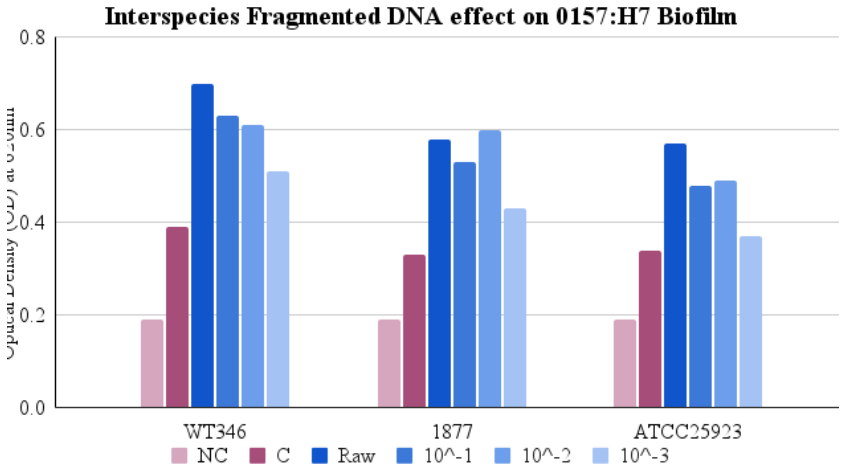


Figure 17c: Effect of heterogenic fragmented DNA on 0157:H7 biofilm development.

Reveals the biofilm-forming capacity of E. coli 0157:H7 in the presence of fragmented DNA obtained from V. cholerae WT346, V. cholerae 1877, and S. aureus ATCC25923.

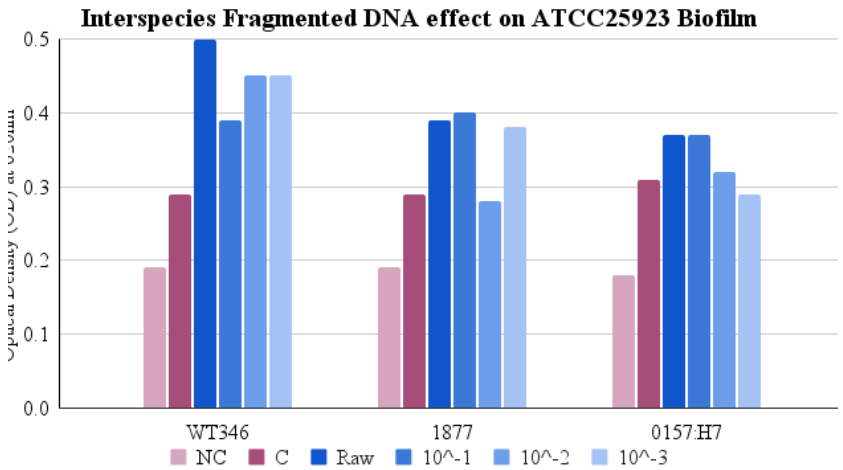


Figure 17d: Effect of heterogenic fragmented DNA on ATCC 25923 biofilm development.

Showcases the modulation in biofilm formation by S. aureus ATCC25923 under the influence of fragmented DNA from V. cholerae WT346, V. cholerae 1877, and E. coli 0157:H7.

4.4 Compilation of data

Table 2: Percentage Variation in Biofilm Formation due to Whole and Fragmented DNA Induction

Strain	DNA Source	Control Without DNA	DNA (Whole)	% Change	Control Without DNA	DNA (Fragmented)	% Change
***Vibrio cholerae* WT346**	WT346	0.285	0.542	90.2%	0.283	0.440	55.5%
	1877	0.315	0.430	36.5%	0.309	0.457	47.9%
	0157:H7	0.380	0.522	37.4%	0.272	0.492	80.9%
	ATCC 25923	0.349	0.689	97.4%	0.318	0.510	60.4%
***Vibrio cholerae* 1877**	WT346	0.223	0.810	263%	0.322	0.563	74.8%
	1877	0.269	0.464	72.5%	0.240	0.394	64.2%
	0157:H7	0.298	0.422	41.6%	0.248	0.358	44.4%
	ATCC 25923	0.227	0.510	125%	0.274	0.686	150%
***Eshcherichia coli* 0157:H7**	WT346	0.303	0.574	89.4%	0.382	0.697	82.5%
	1877	0.271	0.453	67.2%	0.323	0.568	75.9%
	0157:H7	0.453	0.711	56.9%	0.326	0.528	61.9%
	ATCC 25923	0.289	0.452	56.4%	0.331	0.565	70.7%
***Staphylococcus aureus* ATCC 25923**	WT346	0.390	0.436	11.8%	0.282	0.495	75.5%
	1877	0.286	0.361	26.2%	0.286	0.376	31.5%
	0157:H7	0.390	0.529	35.6%	0.301	0.393	30.6%
	ATCC 25923	0.269	0.589	119%	0.303	0.273	-9.90%

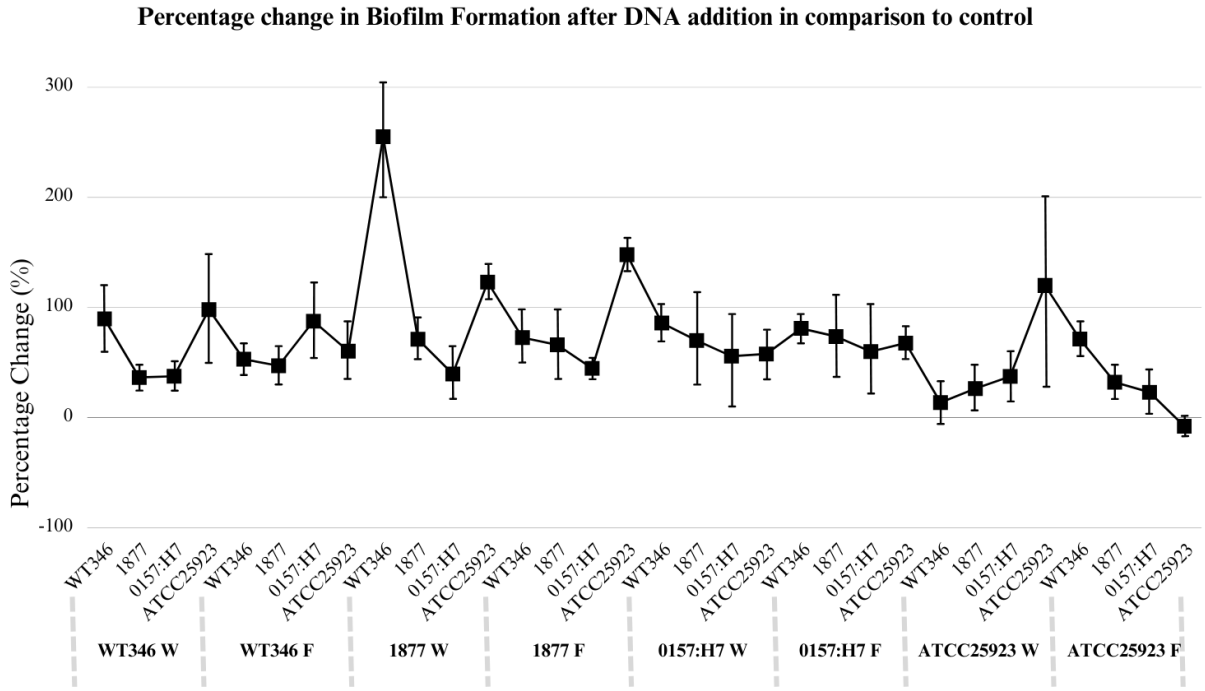


Figure 18. Percentage change in Biofilm Formation after DNA Addition in Comparison to Control

CHAPTER 5 DISCUSSION AND ANALYSIS

The intricate dance between microbial communities and their surrounding environments continues to be an unfolding narrative, marked by breakthroughs that shed light on the adaptive strategies of these microorganisms. Central to this saga is the biofilm – an assembly of microbial cells recognised not merely as a passive congregation but as a dynamic, responsive entity. This is especially pertinent when considering *Vibrio cholerae*, where biofilm formation emerges as a critical defence mechanism against threats, such as phage predation.

While biofilms have been recognized for their adaptability to various environmental factors, the role of genomic DNA (gDNA) in influencing their behaviour has remained in the shadows. Much of the biofilm research has highlighted its interaction with extracellular DNA (eDNA), but the unique influence of gDNA, especially its varied configurations, is still enigmatic. This is a knowledge gap that cannot be ignored, given the ubiquity and potential roles of gDNA in microbial communication, defence, and evolution within biofilm matrices. Furthermore, distinctions in responses between Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Vibrio cholerae* & *Escherichia coli*) bacteria to gDNA require closer scrutiny.

This research, a fragment of a broader endeavour, targets the multitudinous factors that promote biofilm production in *Vibrio cholerae*. Our discussion will not only explore the nuances of gDNA's influence on biofilm formation dynamics but also juxtapose these findings with known interactions of eDNA and biofilm morphogenesis. In doing so, we venture deeper into the intricate interplay of gDNA with Gram-positive and Gram-negative biofilms and the mysteries surrounding intra and inter-species gDNA modulations. As we weave our observations into the expansive tapestry of biofilm research, our overarching aspiration is to offer invaluable insights and, in the process, carve out new avenues for future exploration.

5.1 Recapitulation of the findings from the preliminary characteristics

Our overarching aim was to optimise the biofilm formation process, ensuring consistency across three distinct bacterial strains. Understanding that biofilm formation can be inherently

unpredictable, our methodology prioritised reproducibility and reliability over achieving maximal biofilm yield for any individual strain. Here's a recapitulation of the key findings and methodological adjustments made during our preliminary studies:

Given the stochastic nature of biofilm formation, we employed a triplicate vial approach for each bacterial strain. This allowed us to ensure that biofilm formation was consistent across all replicates, minimising the variability often encountered in such processes. Similarly, every exposure to fragmented, whole, isogenic, or heterogenic gDNA was repeated thrice across three vials. For precise quantification, samples from each vial were then allocated to three distinct microtiter plate wells for ELISA readings. This multi-tiered replication strategy ensured our results were both robust and reproducible, despite the stochastic nature of biofilm development.

A significant breakthrough was realised with *Vibrio* strains. Instead of the commonly used culture mediums, we transitioned to using Alkaline Peptone Water (APW). This adjustment was pivotal in achieving consistent biofilm formation, underscoring the importance of medium selection in biofilm consistency. APW is a culture medium that contains alkaline peptone, which provides a rich source of nutrients for bacterial growth. The alkaline pH of APW creates an environment for the enrichment of *Vibrio* strains (Nhu et al., 2021).

It's worth reiterating that our methodological approach was geared towards achieving consistent biofilm formation across the board, rather than optimising the yield for any single bacterial strain. This approach ensures that our subsequent studies on gDNA's influence on biofilm dynamics are grounded in consistent and reproducible biofilm samples.

Across the board, our adjusted methodology yielded uniform biofilm thickness. We quantified thickness using Optical Density (OD) measurements at 620 nm. Our refined methodology consistently produced uniform biofilm thickness across diverse bacterial strains, quantified using Optical Density (OD) measurements at 620 nm. Specifically, *V. cholerae* WT346 registered an average OD of 0.296 with a minimal variability of 0.34%. *V. cholerae* 1877, on the other hand, clocked an average OD of 0.294, with its 1.41% standard deviation underscoring its consistent biofilm formation. *E. coli* 0157:H7 exhibited a marginally thicker biofilm at an average OD of

0.344, albeit with a variability of 0.94%. Lastly, *S. aureus* ATCC25923 showed an average OD of 0.327 with a standard deviation of 3.9%, further attesting to the reliability of our adjusted methodology.

In essence, our preliminary characterizations highlighted the crucial balance between optimization and consistency. The findings and adjustments from this stage provided a robust foundation, setting the stage for our in-depth exploration of gDNA's interactions with biofilms.

5.2 Interactions and Implications of gDNA on Biofilm Dynamics

The interaction between biofilms and genomic DNA (gDNA) forms a crucial juncture in our research. By exposing biofilms to varying configurations of gDNA, we sought to uncover patterns, nuances, and implications that could provide fresh insights into biofilm dynamics. Furthermore, genomic DNA (gDNA), in its various forms, offers a unique lens through which to study biofilm formation and modulation. The introduction of isogenic (from the same species) and heterogenic (from a different species) gDNA provides an opportunity to explore the intricacies of bacterial behaviour, identity recognition, and adaptability. Here, we present a comparative study of biofilm responses to these.

5.2.1 Comparison of biofilm responses to Isogenic Whole gDNA

In a comprehensive study across three trials involving nine vials, we assessed the baseline biofilm accrual, quantified via optical density. The results indicated average biofilm formations for *V. cholerae* WT346 at 0.285, *V. cholerae* 1877 at 0.269, *E. coli* 0157:H7 at 0.453, and *S. aureus* ATCC 25923 at 0.269. These baseline measurements, especially the notably higher accrual of *E. coli*, provide crucial reference points for evaluating the subsequent impacts of gDNA on each strain's biofilm dynamics.

Upon introducing 20 µl of isogenic whole gDNA configurations to the biofilms, a pronounced escalation in biofilm thickness was evident across all strains. Specifically, *V. cholera* WT346 exhibited a substantial rise, reaching an average optical density (OD) of 0.542. Similarly, *V.*

cholera 1877's biofilm thickness increased to an OD of 0.464. Notably, *E. coli* 0157:H7, already demonstrating a higher baseline, further surged to an impressive OD of 0.711. Finally, *S. aureus* ATCC 25923 showed a heightened biofilm density with an OD of 0.589. These findings underscore the significant role isogenic gDNA plays in enhancing biofilm accrual across diverse bacterial strains.

The discernible elevation in biofilm thickness upon exposure to whole gDNA carries significant implications. This heightened response suggests a potential role of intact genomic sequences in enhancing biofilm resilience or promoting structural integrity. Such marked enhancements, particularly in response to isogenic gDNA, allude to an intrinsic recognition mechanism within bacterial communities. The biofilm's augmented response to its native genetic material posits an intriguing theory: bacteria could potentially leverage gDNA as a signalling or regulatory molecule. This might serve dual purposes, facilitating nuanced intra-community communication and bolstering the biofilm's structural robustness, thereby providing an evolved defence against environmental challenges and perturbations.

5.2.2 Comparison of biofilm responses to Heterogenic Whole gDNA

In our study, we assessed biofilm formation across three different types of heterogenic whole gDNA. Each gDNA type underwent three individual trials, and within each trial, the experiment was replicated across three vials to ensure consistency. This brought us to a total of nine trials involving 27 vials. Additionally, for each of these DNA types, we established a baseline by measuring the biofilm accrual in three separate vials, prior to exposing the bacterial strains to the heterogenic whole DNA. *V. cholerae* WT346 demonstrated an average biofilm accrual of 0.348, whereas its counterpart *V. cholerae* 1877 exhibited a slightly lower accrual at 0.249. *E. coli* 0157:H7 had an average accrual of 0.288, and *S. aureus* ATCC 25923 showed the highest baseline biofilm accrual of 0.355.

For *V. cholerae* WT346, the response was particularly intriguing. Exposed to the DNA of *V. cholerae* 1877, the biofilm thickness increased to 0.430 from its baseline of 0.348. An even more significant jump was observed upon introducing DNA from *E. coli* 0157:H7, with the accrual

reaching 0.522. The most dramatic augmentation was witnessed when exposed to DNA from *S. aureus* ATCC 25923, escalating the thickness to 0.689. This escalating trend may signify *V. cholerae* WT346's varied adaptive or defensive reactions to foreign genetic material.

Conversely, *V. cholerae* 1877 displayed a diversified pattern. The strain's biofilm saw a pronounced spike to 0.810 upon exposure to DNA from *V. cholerae* WT346. However, the values dropped significantly in the presence of *E. coli* 0157:H7 and *S. aureus* ATCC 25923 DNAs, registering at 0.422 and 0.510 respectively. This variation hints at potential genetic factors influencing the strain's ability to recognize and react differently to closely related versus distantly related gDNAs.

For *E. coli* 0157:H7, there was a moderate biofilm response to the heterogenic DNAs. Post-exposure to *V. cholerae* WT346's DNA, the accrual rose slightly to 0.574. The presence of *V. cholerae* 1877 DNA resulted in a decrease to 0.469, while the introduction of *S. aureus* ATCC 25923 DNA brought it near this value at 0.487. This consistency, albeit with mild fluctuations, suggests *E. coli* 0157:H7 might possess a more stable or generalised biofilm reaction to foreign gDNAs.

Lastly, *S. aureus* ATCC 25923 demonstrated variable responses. Its biofilm thickness peaked at 0.529 with *E. coli* 0157:H7 DNA, but the accruals were more subdued with the *V. cholerae* strains' DNAs, settling at 0.436 and 0.361 respectively. This could imply a differential recognition or adaptive mechanism inherent in *S. aureus* when confronted with diverse genetic inputs.

5.2.3 Comparison of biofilm responses to Isogenic Fragmented gDNA

In a meticulously structured study over three trials using nine vials, we established control biofilm accrual measurements for several strains alongside the introduction of isogenic fragmented gDNA. Quantifying via optical density, the controls indicated average biofilm formations for *V. cholerae* WT346 at 0.283, *V. cholerae* 1877 at 0.240, *E. coli* 0157:H7 at 0.326, and *S. aureus* ATCC 25923 at 0.302. These control values serve as essential benchmarks, aiding

in the evaluation of any perturbations or influence the fragmented gDNA might have on each strain's biofilm dynamics.

Upon exposure to fragmented gDNA, biofilms showcased a more tempered reaction in comparison to their whole gDNA counterparts. Specifically, *V. cholerae* WT346 reflected an optical density (OD) of 0.440, a subdued increase from its control. *V. cholerae* 1877 presented an OD of 0.394, *E. coli* 0157:H7 recorded an OD of 0.528, showing a modest escalation, while *S. aureus* ATCC 25923 interestingly displayed a decrease with an OD of 0.273. These observations indicate the varied influence fragmented gDNA can exert across different bacterial strains, with some enhancing their biofilm accrual and others displaying a contrary behaviour.

Upon the introduction of isogenic gDNA, biofilms manifested distinct characteristics, including enhanced structural integrity with increased microbial density. These reactions possibly stem from a recognition mechanism of self-DNA, which could lead to coordinated communal responses or heightened protective mechanisms within the biofilm matrix. Conversely, the more muted response to fragmented gDNA might be attributed to the biofilm's selective interaction with particular gDNA sequences or a reduced ability to recognize and assimilate fragmented genetic material. This differential behaviour, especially the pronounced positive reaction to native genetic material, underscores the hypothesis that bacteria may deploy gDNA as a regulatory tool. Such utilisation could serve as a signalling molecule, fostering intra-community communication, or as a structural enhancer, bolstering the biofilm's resilience against environmental challenges.

5.2.4 Comparison of biofilm responses to Heterogenic Fragmented gDNA

In our meticulously designed study on biofilm formation influenced by heterogenic DNA, we utilised three distinct DNA types, each subjected to three trials and replicated across three vials—resulting in 27 trials in total. Prior to the exposure to heterogenic fragmented gDNA, baseline biofilm measurements were established: *V. cholerae* WT346 had an accrual of 0.299, *V. cholerae* 1877 at 0.281, *E. coli* 0157:H7 at 0.345, and *S. aureus* ATCC 25923 at 0.290. This

baseline data, especially with *E. coli* 0157:H7 displaying the highest accrual, sets the stage for assessing the nuanced effects of heterogenic DNA on biofilm dynamics.

Firstly, *V. cholerae* WT346 was observed to have a baseline biofilm accrual of 0.299. When introduced to fragmented DNA from different strains, the dynamics changed significantly. The addition of DNA from *V. cholerae* 1877 resulted in a biofilm thickness of 0.457. However, it was the exposure to *E. coli* 0157:H7 and *S. aureus* ATCC 25923 DNAs that led to more pronounced increases, recording values of 0.492 and 0.509 respectively. This gradient increase in biofilm thickness might reflect WT346's differential recognition or adaptive mechanisms when faced with genetic material from varying bacterial sources.

Secondly, *V. cholerae* 1877 started with a baseline biofilm accrual of 0.281. Post-exposure dynamics exhibited a pronounced elevation with *S. aureus* ATCC 25923 DNA, skyrocketing the thickness to 0.685. This was in sharp contrast to the subdued reaction observed with *E. coli* 0157:H7 DNA, which settled at 0.358. The moderate response to *V. cholerae* WT346 DNA, resulting in a biofilm accrual of 0.563, might indicate a potential recognition of closer genetic elements or the presence of specific pathways that respond to related strains.

Beginning with the highest baseline biofilm accrual of 0.345 among the tested strains, *E. coli* 0157:H7 showcased a distinctive reaction to fragmented gDNA exposure. The biofilm thickness augmented most significantly, reaching 0.697, when exposed to *V. cholerae* WT346 DNA. Responses to *V. cholerae* 1877 and *S. aureus* ATCC 25923 DNAs were comparatively similar, registering at 0.568 and 0.565, respectively. The strain's heightened baseline and subsequent increases might allude to inherent genetic or metabolic factors that predispose *E. coli* 0157:H7 to more pronounced biofilm formations under genetic perturbations.

Lastly, *S. aureus* ATCC 25923 started with a baseline of 0.290 in biofilm accrual. Following exposure to fragmented DNAs, the most substantial biofilm thickness was observed with *V. cholerae* WT346 DNA, settling at 0.495. In contrast, *V. cholerae* 1877 and *E. coli* 0157:H7 DNAs resulted in more tempered responses, with biofilm accruals of 0.376 and 0.363 respectively. These variations might highlight ATCC 25923's differential mechanisms of

recognizing or adapting to foreign genetic inputs, further emphasising the intricacies of bacterial-bacterial interactions in biofilm settings.

5.2.5 Deciphering the Biofilm-gDNA Nexus: Implications for Microbial Dynamics and Interventions

Biofilms, representing sophisticated microbial architectures, have long intrigued researchers due to their complex dynamics and interactions. Our recent exploration into the relationship between biofilms and genomic DNA (gDNA) has yielded insights of paramount importance.

The structural configuration of gDNA, rather than merely being a passive entity within the biofilm matrix, exerts a pronounced influence on biofilm behaviour. Particularly, the whole gDNA has emerged as a formidable modulator, potentially amplifying biofilm resilience and architectural integrity. This observation prompts an academic curiosity into the cellular and molecular mechanisms that are invoked upon gDNA interaction, pointing towards potential targets for biotechnological or therapeutic endeavours.

The study further delineates a distinctive dichotomy in biofilm responsiveness. Isogenic gDNA appears to fortify the communal cohesion within the biofilm, acting as a cohesive agent. Conversely, the introduction of heterogenic gDNA instigates a range of adaptive and structural modifications within the biofilm matrix. Such differential responsiveness not only underscores the inherent adaptability of biofilms but also hints at a dynamic interplay of gene exchange, intra-bacterial communication, and possible competitive phenomena.

This nuanced behaviour of biofilms, especially in the context of gDNA interactions, accentuates their evolutionary sophistication. It paints a picture of biofilms as not merely microbial aggregates but as discerning, responsive assemblies attuned to their genetic milieu.

In conclusion, this research has unravelled a new dimension in the understanding of biofilm-gDNA interactions. The unveiled insights provide a solid foundation for subsequent inquiries and have far-reaching implications. As the scientific community delves deeper into this

nexus, there lies the promise of novel therapeutic strategies, enhanced understanding of microbial ecology, and potential biotechnological innovations.

5.3 Effects of gDNA in Gram-Positive vs. Gram-Negative Biofilms

5.3.1 Comparative analysis of biofilm formation between gram-positive and gram-negative strains before and after gDNA introduction.

To truly understand the nuanced effects of gDNA on biofilm formation, it's paramount to dissect its interactions with different bacterial classifications. This comparative analysis draws upon observations from both gram-positive strains which included *S. aureus* and gram-negative strains which included *V. cholerae* and *E. coli*, before and after gDNA exposure:

The gram-positive strain, *S. aureus* ATCC25923, has evolved a dense, layered biofilm with an average thickness of 0.313. This structure suggests a fortified defence mechanism, potentially honed over time to combat varied environmental challenges.

In contrast, gram-negative strains like *V. cholerae* WT346, *V. cholera* 1877, and *E. coli* 0157:H7 showcase a more porous biofilm morphology. Their respective thicknesses - 0.314, 0.262, and 0.335 - reflect a spectrum of adaptabilities. *V. cholera* WT346's biofilm is comparably robust to *S. aureus*, indicating similar protective goals despite structural differences. *V. cholera* 1877 leans towards a thinner formation, perhaps to optimise other biological functions. Meanwhile, *E. coli* 0157:H7's slightly thicker biofilm might signify its versatility across different habitats.

Gram-Positive Strains: *S. aureus* ATCC25923

Upon the introduction of isogenic gDNA to *S. aureus* ATCC25923, a substantial shift in biofilm formation became evident. The integration of whole gDNA caused a pronounced 119% increase in biofilm thickness, pegging it at 0.589. In contrast, the assimilation of fragmented gDNA resulted in a modest dip, curbing thickness by 9.9% to a value of 0.273.

When we delved into the effects of heterogenic gDNA, the strain exhibited diverse responses. DNA from *V. cholera* WT346 caused an 11.8% augmentation in thickness when whole (0.436) and a 75.5% increase when fragmented (0.495). *V. cholera* 1877's DNA accounted for a 26.2% rise when whole (0.361) and 31.5% when fragmented (0.376). Lastly, *E. coli* 0157:H7's whole DNA marked a 35.6% increase (0.529), while its fragmented counterpart registered a 30.6% elevation (0.393).

Gram-Negative Strains

V. cholera WT346:

For this strain, the introduction of isogenic whole gDNA led to a significant 90.2% increase in biofilm thickness, measuring at 0.542. However, its fragmented variant exhibited a more moderate effect, recording a 55.5% increase, reaching 0.44.

In the context of heterogenic gDNA, *V. cholera* WT346's biofilm structure revealed nuanced responses. *V. cholera* 1877's DNA caused a 36.5% enhancement in thickness when whole (0.430) and surged by 47.9% when fragmented (0.457). *E. coli* 0157:H7's DNA, in its whole form, instigated a 37.4% ascent (0.522), whereas its fragmented version saw an 80.9% rise (0.492). Notably, *S. aureus* ATCC25923's whole DNA contributed to a stark 97.4% growth (0.689), and its fragmented variant marked a 60.4% increase (0.510).

V. cholera 1877:

When isogenic gDNA was introduced, the whole form caused a 72.5% upsurge in thickness (0.464), whereas its fragmented counterpart reported a 64.2% elevation (0.394).

Regarding heterogenic gDNA, *V. cholera* 1877 responded with noticeable variances. *V. cholera* WT346's DNA, in its whole form, registered an immense 263% boost (0.810) and rose by 74.8% in its fragmented state (0.563). *E. coli* 0157:H7's DNA showcased a 41.6% increment when whole (0.422) and increased by 44.4% when fragmented (0.358). Meanwhile, *S. aureus*

ATCC25923's DNA highlighted a 125% rise when whole (0.510) and leapt by 150% when fragmented (0.686).

E. coli 0157:H7:

For this strain, the isogenic whole gDNA introduction marked a 56.9% growth in thickness (0.711). In contrast, fragmented gDNA revealed a 62% rise, settling at 0.528.

Upon heterogenic gDNA introduction, *E. coli* 0157:H7 manifested differential reactions. *V. cholera* WT346's whole DNA led to an 89.4% enhancement (0.574), while its fragmented version was responsible for an 82.5% rise (0.697). *V. cholera* 1877's DNA, when whole, achieved a 67.2% boost (0.453) and ascended by 75.9% when fragmented (0.568). *S. aureus* ATCC25923's DNA, in its whole configuration, reported a 56.4% growth (0.452), while its fragmented type induced a 70.7% surge (0.565).

Concluding Implications and Interpretations

The differential responses of gram-positive and gram-negative bacteria to gDNA introduction offer a compelling narrative on the adaptability and complexity of microbial communities. Gram-positive *S. aureus* ATCC25923 demonstrated marked resilience, with notably enhanced biofilm thickness upon whole gDNA integration. Its capacity to discern and subsequently integrate genetic material is apparent, indicating an evolutionary adaptation that might facilitate its persistence in diverse environments. Conversely, gram-negative strains, encompassing *V. cholera* WT346, *V. cholera* 1877, and *E. coli* 0157:H7, presented a more nuanced and varied response to gDNA. Their biofilm adaptations, while evident, were more heterogeneous and reflective of potential strain-specific genetic interaction dynamics. Their structural and physiological distinctions from gram-positive bacteria, such as the presence of an outer membrane, could influence their interactions with introduced gDNA. Ultimately, this study underscores the inherent genetic and adaptive disparities between gram-positive and gram-negative bacteria. Their differential responses to gDNA augmentations might be rooted in their distinct evolutionary trajectories, cellular architectures, and survival strategies. Such

insights not only deepen our understanding of bacterial adaptability but also provide avenues for targeted microbial interventions in health and environmental contexts.

5.3.2 Delineation of gDNA's Interaction with Cellular Walls of Gram-Positive and Gram-Negative Bacteria: A Comparative Study

Bacterial cell walls, paramount for survival and adaptability, vary between Gram-positive and Gram-negative strains. These differences influence how each reacts to external elements, such as genomic DNA (gDNA). Through this comparative analysis, we aim to illuminate the nuanced dynamics of gDNA interactions within these distinct cellular architectures.

Gram-Positive Bacteria:

Characterised by a dense peptidoglycan layer with interspersed teichoic acids, Gram-positive bacteria present a relatively porous barrier. This thick yet permeable wall interacts efficiently with gDNA, a phenomenon possibly stemming from the gDNA's negative charge finding affinity with the wall's positive regions. Our study aligns with prior literature indicating the importance of extracellular DNA (eDNA) in these bacteria. eDNA facilitates initial adhesion and microcolony formation (Das et al., 2010). Moreover, it not only provides a scaffold to maintain biofilm integrity but also enhances biofilm resistance to antimicrobial agents (Das et al., 2017).

Gram-Negative Bacteria:

In contrast, Gram-negative bacteria possess a dual-layered wall: an inner peptidoglycan layer encapsulated by an outer lipid membrane. This outer membrane, rich in lipopolysaccharides, poses a more complex interface for gDNA interaction. In our observations, gDNA exhibited limited permeation through the outer membrane, suggesting restricted access. Yet, similar to their Gram-positive counterparts, Gram-negative bacteria utilise eDNA for matrix stabilisation (Barnes et al., 2012). eDNA augments biofilm resilience against shear forces and facilitates bacterial persistence on surfaces.

Both bacterial types exploit eDNA for biofilm dynamics, from cellular aggregation to the secretion of an extracellular polymeric substance (EPS) (Laverty et al., 2014). eDNA's presence amplifies biofilm robustness and serves as a crucial scaffold. Further, its role in enabling horizontal gene transfer within biofilms emphasises its importance in genetic exchange (Das et al., 2010).

In summary, the disparities in gDNA interactions between Gram-positive and Gram-negative bacteria derive mainly from their inherent structural variations. Gram-positive bacteria, with their thicker peptidoglycan layers, seem more receptive to gDNA. In contrast, the intricate dual-membrane configuration in Gram-negative bacteria modulates gDNA accessibility. This analysis accentuates the significance of bacterial structural disparities in influencing gDNA dynamics. Such insights not only provide a deeper understanding of bacterial behaviour but also pave the way for innovations in microbial ecology, biotechnology, and disease control.

5.4 Comparative Framework of eDNA and gDNA Influences: Synthesis and Speculation on DNA-induced Biofilm Transformations

The symbiotic relationship between biofilm structures and their interactions with extracellular DNA (eDNA) and genomic DNA (gDNA) extracted via the PCI method deepens our understanding of biofilm evolution and functionality. Marrying our recent findings with established literature provides a holistic perspective on the transformative powers DNA components exert on biofilm dynamics and hypothesises potential underlying molecular mechanisms.

A. eDNA Dynamics and Its Role in Biofilm Lifecycle

Existing literature, such as that by Steinberger & Holden (2005), has illuminated the differential capabilities of bacterial species to produce eDNA. Pseudomonas strains, for example, emerge as prominent eDNA producers. Whitchurch et al. (2002) highlighted the foundational role of eDNA in facilitating bacterial cell surface attachment. On the other hand, our findings accentuated the

enhanced biofilm resilience with isogenic gDNA introduction, indicating a multifaceted interplay between eDNA and gDNA in biofilm dynamics.

B. Diverse Impacts of eDNA and gDNA on Biofilm Maturation

Biofilm-genetic interactions are multi-dimensional. Works like those by Allesen-Holm et al. (2006) differentiate DNA release patterns in biofilms versus planktonic cultures. This resonates with our findings, which further align with Harmsen et al. (2010) and Chiang et al. (2013), accentuating the combined contributions of eDNA and gDNA in enhancing biofilm resilience. Thus, emphasising eDNA's role in augmenting biofilm robustness.

C. Genetic Interchange and Evolutionary Paradigms

Merging our insights with Ibáñez de Aldecoa et al. (2017), we discern the pivotal role of eDNA in horizontal gene transfer. The synergistic production of eDNA and the commencement of natural competence mark eDNA's evolutionary utility. Lorenz and Wackernagel (1994) further fortify this by spotlighting eDNA's role in various environmental genetic exchanges. Similarly, the structural and functional roles of eDNA in biofilm formation and gene transfer, as detailed by Whitchurch et al. (2002), resonate with our observations, highlighting its evolutionary significance. Additionally, the primary sequence resemblance of eDNA to cellular DNA in some bacterial species, as observed by Steinberger & Holden (2005), warrants further exploration. Nevertheless, The genetic composition of eDNA in these biofilms was found to be unique from both cellular and total biofilm DNA, emphasising eDNA's distinct origin and role.

D. Influences on Microbial Community Characterization

Steinberger & Holden's (2005) insights into eDNA's influence on microbial community assessments highlight its intricate roles. Delving deeper, contributions from researchers like Alm et al. (1996) and Kjelleberg & Molin (2002) expound on the intersections between eDNA, microbial detection, and biochemical biofilm interactions. Given the disproportionate accumulation of eDNA in biofilms relative to initial species distribution, understanding its

multifaceted influence on both the analysis and intrinsic microbial interactions becomes paramount.

E. Diverse Yet Intersecting Functions of eDNA and gDNA

Whitchurch et al. (2002) and Steinberger & Holden's studies give insights into the differential roles and production patterns of eDNA across species. In contrast, Flemming & Wingender (2010) focus on gDNA's contribution to biofilm stability, a facet our study further explored. Research from Decho & Gutierrez (2017) further contextualises the distinct yet intersecting roles of eDNA and gDNA in diverse environments, like marine settings. The unpredictability observed in the eDNA content of dual-species biofilms indicates intricate microbial interactions which could be influenced by the environment.

F. Speculative Molecular or Cytological Pathways Underpinning Observations

Considering our novel findings and the available literature, it's plausible that isogenic gDNA acts as a molecular signalling agent, promoting communal cohesion within biofilms. This internal signalling could amplify biofilm structural integrity through heightened gene expression or activation of pathways promoting adherence and matrix synthesis. The differential biofilm response to heterogenic gDNA also indicates a sensing capability, possibly through molecular sensors that detect DNA sequence differences. Furthermore, eDNA's role is not confined to structural integrity alone. As highlighted by Mulcahy et al. (2010), eDNA in biofilms also acts as a chelator, inducing antibiotic resistance, and underscoring its defensive capability. Okshevsky & Meyer (2015) further reiterate the significance of eDNA in biofilm formation, stabilisation, and maintenance. Together, these studies illuminate the multifaceted roles of eDNA in biofilms. Whether it's facilitating genetic exchange, aiding in biofilm stabilisation, or acting as a nutrient reservoir, the ecological and functional significance of eDNA is undeniable.

The interactions between eDNA and gDNA, whether naturally occurring or experimentally introduced, offer rich insights into biofilm research. Our comparative analysis, enriched by integrating novel findings with established research, underscores the crucial roles DNA

components play in biofilm formations. As our understanding deepens, we move closer to unlocking the mysteries of microbial interactions and their potential applications across diverse fields.

5.5 Implications and Significance: Evolutionary Perspective

5.5.1 Biofilm Dynamics and the Evolutionary Context

Intrinsic Recognition Mechanism and Genomic Memory: The pronounced elevation in biofilm thickness in response to isogenic whole gDNA suggests that bacteria might possess an evolutionary conserved intrinsic mechanism for recognizing their genomic DNA. This implies that over time, bacteria have evolved to reinforce their biofilm structures when their genomic integrity is perceived to be at risk.

gDNA as a Signalling or Regulatory Molecule: The possibility that bacteria leverage gDNA as a signalling or regulatory molecule implies the evolution of complex communication systems. In an evolutionary context, the ability to sense, decode, and respond to genomic information could confer significant advantages in terms of survival, adaptation, and competition.

Adaptive Responses to Heterogenic gDNA: The varied reactions of bacterial strains to heterogenic whole gDNA suggest that biofilm organisms have evolved adaptive or defensive mechanisms against foreign genetic material. This kind of response can be seen as an evolved strategy to mitigate potential threats from closely or distantly related organisms.

Fragmented gDNA Responses and Selective Evolution: The more subdued response of biofilms to fragmented gDNA, when compared to whole gDNA, alludes to the evolutionary honing of bacteria to react more prominently to entire genomic sequences rather than fragments. This might be a result of the fragmented sequences not being perceived as an immediate or significant threat, or it could be indicative of an evolved selectivity in genomic recognition.

5.5.2 Strain-specific Implications

V. cholerae WT346's Evolutionary Strategy: The escalating biofilm response to heterogenic DNA, especially from *S. aureus* ATCC 25923, could point towards a heightened defence mechanism. This might represent an evolutionary advantage where the bacteria amplifies its defence against what it perceives to be a more distantly related or potentially harmful organism.

V. cholerae 1877's Genomic Intelligence: The pronounced spike in response to DNA from its counterpart WT346, followed by a decrease in response to other DNAs, might suggest an evolved pathway that prioritises or is more sensitive to closely related strains.

E. coli 0157:H7's Stable Biofilm Reaction: The modest and consistent biofilm response, regardless of the heterogenic DNA source, could indicate a more stable evolutionary trait, where the bacteria does not drastically alter its biofilm dynamics in response to external genetic cues. This could offer robustness to its survival strategy.

S. aureus ATCC 25923's Differential Recognition: The variable biofilm responses might suggest that this strain has developed a nuanced evolutionary mechanism to differentiate and adapt to diverse genetic inputs. This could enhance its survival in mixed microbial communities.
Especially the evolutionary Perspective.

5.5.3 Interplay of Bacteriophages, Genomic DNA, and *V. cholerae* Biofilms in cholera Epidemiology

Cholera, primarily triggered by the toxigenic *Vibrio cholerae*, remains a pressing global health predicament. This bacterium's intricate life cycle, combined with its nuanced interactions with the environment and particularly with bacteriophages, provides a detailed narrative about the multifarious nature of cholera's epidemiology. Intriguingly, the dual manifestation of *V. cholerae* in biofilm and planktonic forms underscores the profound influence of environmental bacteriophages on their behaviour and survival (Gallego-Hernández et al., 2020).

Biofilms, acting as protective bacterial conglomerates, offer *V. cholerae* dual advantages. They not only ensure the bacterium's amplified persistence in varied environmental conditions but also accentuate its infectivity potential. Furthermore, these fortified structures, acting as microbial bastions, provide a resilient shield against environmental challenges, including but not limited to threats from protozoa and bacteriophages (Gallego-Hernández et al., 2020). Their significance in cholera's epidemiology is magnified when considering endemic regions, where dismantling these biofilms can translate into a pronounced decrease in disease incidence. This complexity is heightened when analysing patient stool samples, which, apart from revealing the free-floating planktonic form of *V. cholerae*, often spotlight the more structured and notably more infectious biofilm aggregates.

Within this ecological tapestry, the pivotal role of bacteriophages is undeniable. Typically, their action culminates in the release of eDNA, thereby influencing microbial community dynamics (Carrolo et al., 2010). However, our study introduces a novel dimension to this interplay by focusing on the deliberate incorporation of in vitro extracted gDNA into *V. cholerae's* milieu. This strategic introduction mimics the genetic upheaval synonymous with bacteriophage activity but under more controlled conditions. From an evolutionary standpoint, such conditions can be leveraged by bacteria for opportunistic adaptation. The eDNA, typically resultant of bacteriophage-mediated bacterial lysis, serves as a genetic reservoir. Still, the gDNA, by its sheer concentration and controlled nature, might offer a denser cache of genetic information. This influx might not only be a trigger for dormant *V. cholerae* cells to resuscitate but might also arm them with augmented genetic traits essential for environmental adaptability (Carrolo et al., 2010).

Biofilms, with their strategic structural adaptations, act as bastions against various environmental challenges, including phage attacks. In our observations, *V. cholerae,* when exposed to gDNA, appeared to reinforce its biofilm dynamics, possibly as a preemptive defence mechanism. Such a phenomenon can be viewed as an evolved bacterial strategy to bolster communal defences in an environment perceived as rich in phages or other potential threats (Carrolo et al., 2010) The intricate interplay between *V. cholerae's* biofilm dynamics, bacteriophages, and introduced gDNA unveils the sophisticated adaptive strategies that bacterial communities might deploy

when exposed to concentrated genetic material. As shown in both the S. pneumoniae study (Carrolo et al., 2010) and your *V. cholerae* observations, the microbial world is replete with mechanisms that counterbalance external challenges, ensuring survival and evolutionary advantage.

5.6 Limitations and Future Directions

5.6.1 Limitations of the Study

Our research centred on specific bacterial strains, potentially limiting the generalizability of our findings to the broader microbial world. Each biofilm exhibited unique characteristics, suggesting that extrapolating results from one biofilm to another, even within related strains, might be over simplistic. The distinct responses of *V. cholera* WT346 and *V. cholera* 1877 further highlighted this inter-strain variability. In essence, while our findings are significant for the studied strains, they may not encompass the diverse reactions of all bacterial species.

In this study, we relied on the crystal violet staining technique to quantify biofilm biomass, a method that might miss intricate structural details. Alternative techniques, such as optical microscopy, might provide more detailed insights. While our primary genome extraction protocol involved the PCI method, the absence of specific reagents (Proteinase K, RNase, and lysozyme) necessitated protocol modifications. We incorporated a boiling method, aiming to disrupt cells and inhibit proteins, especially nucleases, before the traditional PCI steps. Additionally, our ELISA device didn't support the optimal 590 nm wavelength for crystal violet assay readings, and we occasionally noted discrepancies when measurements were repeated. These limitations could influence the interpretation of our results.

Our study's intricacies extend to the genomic DNA (gDNA) extraction process. While the DNA is typically dissolved in the TE buffer, the effect of this buffer on biofilm remains undetermined. Given the known influence of pH on biofilm formation, the TE buffer’s composition could potentially introduce variations. Additionally, traces of impurities, like sodium acetate from the extraction process, may remain. These factors, combined with potential variations due to

extraction efficiency, introduce another layer of complexity that might subtly affect the interpretation of our results.

Furthermore, while we employed a specific procedure for DNA fragmentation, the lack of a quantifying method to ascertain the average size of these fragments posed a limitation. The variability in biofilm response to fragmented DNA might, in part, stem from inconsistencies in fragment sizes, underscoring the need for more precise characterization in future investigations.

In addition to the aforementioned considerations, a significant technical challenge encountered was the use of acetic acid dissolution in conjunction with the crystal violet staining. The balance between ensuring complete removal of crystal violet from the vials, while preserving the integrity of the biofilm ring, was particularly delicate. This delicate equilibrium invariably introduced a margin of error in our measurements, underscoring the need for developing or adopting more precise methodologies.

5.6.2 Future Research Avenues Stemming from Our Findings

Our current methodology centred on the use of crystal violet staining—a tried and tested approach, but one that inherently captures only a macroscopic view of biofilm accrual. The future holds promise for more sophisticated characterization methods. Tools such as confocal laser scanning microscopy (CLSM) or atomic force microscopy (AFM) stand out as exemplary techniques, providing a more granular view of biofilm formations down to the microscale. Not only do they grant insights into the intricate architecture and three-dimensional organisation of biofilms, but they also have the added advantage of being largely automated. This automation diminishes the potential for human error, ensuring more consistent, replicable, and reliable data outputs. Leveraging such technologies in subsequent studies can deepen our understanding of biofilm behaviour and its underlying molecular mechanisms, bridging gaps left by traditional staining techniques.

Our initial observations have underscored some overlap between eDNA and gDNA's impact on biofilm development and resilience. Yet, the depth and breadth of their mutual interactions

remain to be fully charted. A study delineating the unique and shared roles of these DNA types in biofilm progression could shed profound light on their distinct and collective impacts on microbial community structures. An exciting extension to this inquiry would involve phage-lysed DNA. Given its origins — emerging from bacteriophage-triggered bacterial dissolution — its inclusion in studies could yield seminal insights. Unravelling the nuances of biofilm responses to this type of DNA can illuminate the broader bacterial-bacteriophage evolutionary dance and the resulting microbial ecosystem dynamics.

The varied biofilm reactions in the presence of heterogenic versus isogenic DNA also beckon a deeper analysis. Leveraging state-of-the-art tools, like RT-PCR, can be instrumental in teasing apart the genetic mechanisms driving these differential responses. Such an inquiry will not only deepen our understanding of DNA-mediated microbial interactions but also provide a holistic view of the microbial ecosystem. Another dimension brought to light in our research pertains to the varied biofilm reactions to DNA fragments of different sizes. This prompts the question: How pivotal is fragment size in influencing biofilm dynamics? A focused study, employing sophisticated techniques for precise DNA fragmentation analysis, could unravel this mystery. Although prior studies have differentiated between Cellular DNA, Total DNA, and eDNA, our findings hint at a more multifaceted role of eDNA in the biofilm matrix. A comparative sequencing of these DNA forms, side by side with gDNA fragments, would provide a panoramic view of DNA's roles in bolstering biofilm stability and structure.

CHAPTER 6 CONCLUSION

Biofilms, with their inherent structural complexity and adaptability, have consistently emerged as a formidable force in microbial ecology. Our investigation into the intricate dance between biofilm dynamics and the incorporation of eDNA and gDNA has illuminated multifaceted interactions, revealing not just the adaptability of bacterial communities but also the evolutionary strategies underpinning their survival and propagation. The pronounced influence of both isogenic and heterogenic DNA on biofilm integrity and resilience underscores the nuanced adaptability of bacterial strains, suggesting an intricate interplay of intrinsic recognition, genomic signalling, and defensive mechanisms.

The contrasting yet complementary roles of eDNA and gDNA have been brought to the fore, prompting a reconsideration of traditional perspectives on biofilm development. These DNA entities, beyond their genetic coding capabilities, emerge as potent modulators of biofilm structure and function, with ramifications spanning from public health to environmental sustainability. Furthermore, the observed disparities in biofilm responsiveness to isogenic versus heterogenic DNA sources present an evolutionary narrative, hinting at bacteria's refined ability to distinguish between self and non-self genetic cues. Such discernment likely confers survival advantages, enhancing adaptability and competitiveness within diverse microbial ecosystems. The case of *V. cholerae*, with its intricate relationship to both biofilm structures and bacteriophages, encapsulates the broader theme of this investigation—demonstrating how microbial interactions, when seen through the lens of genomic interplay, provide profound insights into the complex tapestry of microbial ecology.

As we advance, it is evident that the future of biofilm research lies in embracing more sophisticated methodologies, deepening our understanding and potentially opening avenues for targeted interventions in clinical, environmental, and industrial contexts. From public health challenges like cholera to the broader evolutionary dynamics of bacteria in the face of genetic perturbations, our exploration has reinforced the importance of understanding biofilms in all their complexity. As we move forward, it is with anticipation that continued research will not

only unravel deeper mysteries of microbial world interactions but also pave the way for innovations that can harness or mitigate the powers of these microbial communities.

REFERENCE LIST

1. Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M., & Tolker-Nielsen, T. (2006). A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Molecular Microbiology, 59(4), 1114-1128.
2. Alm, E. W., Oerther, D. B., Larsen, N., Stahl, D. A., & Raskin, L. (1996). The oligonucleotide probe database. Applied and Environmental Microbiology, 62(10), 3557-3559.
3. Awuor, S. O., Omwenga, E. O., Mariita, R. M., & Daud, I. I. (2022). Cholera outbreak: antibiofilm activity, profiling of antibiotic-resistant genes and virulence factors of toxigenic Vibrio cholerae isolates reveals concerning traits. Access Microbiology, 4(3), 000324.
4. Baliarda, A., Winkler, M., Tournier, L., Tinsley, C. R., & Aymerich, S. (2021). Dynamic interspecies interactions and robustness in a four-species model biofilm. MicrobiologyOpen, 10, e1254.
5. Ballén, V.; Cepas, V.; Ratia, C.; Gabasa, Y.; Soto, S.M. (2022). Clinical Escherichia coli: From Biofilm Formation to New Antibiofilm Strategies. Microorganisms, 10(6), 1103.
6. Banin, E., Vasil, M. L., & Greenberg, E. P. (2005). From The Cover: Iron and Pseudomonas aeruginosa biofilm formation. Proceedings of the National Academy of Sciences, 102(31), 11076–11081.
7. Barnes, R. J., Bandi, R. R., Wong, M. L., & Hernday, A. D. (2012). Exploring the role of extracellular DNA in the evolution, maintenance, and spread of bacterial biofilms. Journal of bacteriology, 194(21), 3779-3787.
8. Barraud, N., Moscoso, J. A., Ghigo, J.-M., & Filloux, A. (2014). Methods for Studying Biofilm Dispersal in Pseudomonas aeruginosa. Pseudomonas Methods and Protocols, 643–651.
9. Berk, V., Fong, J. C. N., Dempsey, G. T., Develioglu, O. N., Zhuang, X., Liphardt, J., Yildiz, F. H., & Chu, S. (2012). Molecular architecture and assembly principles of Vibrio cholerae biofilms. Science, 337(6091), 236–239.

10. Berne, C., Ellison, C. K., Ducret, A., & Brun, Y. V. (2018). Bacterial adhesion at the single-cell level. Nature Reviews Microbiology, 16, 616–627.
11. Beyenal, H., Lewandowski, Z., & Harkin, G. (2004). Quantifying Biofilm Structure: Facts and Fiction. Biofouling, 20(1), 1–23.
12. Boles, B. R., Thoendel, M., & Singh, P. K. (2005). Rhamnolipids mediate detachment of Pseudomonas aeruginosa from biofilms. Molecular Microbiology, 57(5), 1210–1223.
13. Bridier, A., Piard, J.-C., Pandin, C., Labarthe, S., Dubois-Brissonnet, F., & Briandet, R. (2017). Spatial Organization Plasticity as an Adaptive Driver of Surface Microbial Communities. Frontiers in Microbiology, 8.
14. Brown, H. L., Hanman, K., Reuter, M., Betts, R. P., & van Vliet, A. H. M. (2015). Campylobacter jejuni biofilms contain extracellular DNA and are sensitive to DNase I treatment. Frontiers in Microbiology, 6.
15. Brucker, D.K., Tan, Y., Vints, K., Cremer, D.K., Braem, A., Verstraeten, N., Michiels, J., Vleugels, J., Cammue, B. P. A., & Thevissen, K. (2015). Fungal β-1,3-Glucan Increases Ofloxacin Tolerance of Escherichia coli in a Polymicrobial E. coli/Candida albicans Biofilm. American Society for Microbiology, 3052-3058.
16. Carrolo, M., Frias, M. J., Pinto, F. R., Melo-Cristino, J., & Ramirez, M. (2010). Prophage Spontaneous Activation Promotes DNA Release Enhancing Biofilm Formation in Streptococcus pneumoniae. PLoS ONE, 5(12), e15678.
17. Chaali, M., Naghdi, M., Brar, S. K., & Avalos-Ramirez, A. (2018). A review on the advances in nitrifying biofilm reactors and their removal rates in wastewater treatment. Journal of Chemical Technology & Biotechnology.
18. Chao, Y., Marks, L. R., Pettigrew, M. M., & Hakansson, A. P. (2015). Streptococcus pneumoniae biofilm formation and dispersion during colonization and disease. Frontiers in Cellular and Infection Microbiology, 4.
19. Chen, X., Xiong, X., Jiang, X., Shi, H., & Wu, C. (2019). Sinking of floating plastic debris caused by biofilm development in a freshwater lake. Chemosphere, 222, 856–864.
20. Chiang, W. C., Nilsson, M., Jensen, P. Ø., Høiby, N., Nielsen, T. E., Givskov, M., & Tolker-Nielsen, T. (2013). Extracellular DNA shields against aminoglycosides in Pseudomonas aeruginosa biofilms. Antimicrobial agents and chemotherapy, 57(5), 2352-2361.

21. Christner, M., Franke, G.C., Schommer, N.N., Wendt, U., Wegert, K., Pehle, P., Kroll, G., Schulze, C., Buck, F., Mack, D., Aepfelbacher, M. and Rohde, H. (2010), The giant extracellular matrix-binding protein of Staphylococcus epidermidis mediates biofilm accumulation and attachment to fibronectin. Molecular Microbiology, 75(1), 187–207.
22. Chua, S. L., Liu, Y., Yam, J. K. H., Chen, Y., Vejborg, R. M., Tan, B. G. C., Kjelleberg, S., Tolker-Nielsen, T., Givskov, M., & Yang, L. (2014). Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. Nature Communications, 5, 4462.
23. Conover, M. S., Mishra, M., & Deora, R. (2011). Extracellular DNA Is Essential for Maintaining Bordetella Biofilm Integrity on Abiotic Surfaces and in the Upper Respiratory Tract of Mice. PLoS ONE, 6(2), e16861.
24. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin-Scott, H. M. (1995). Microbial biofilms. Annual review of microbiology, 49(1), 711-745.
25. Coughlan, L. M., Cotter, P. D., Hill, C., & Alvarez-Ordóñez, A. (2016). New Weapons to Fight Old Enemies: Novel Strategies for the (Bio)control of Bacterial Biofilms in the Food Industry. Frontiers in Microbiology, 7.
26. Culotti, A., & Packman, A. I. (2014). Pseudomonas aeruginosa Promotes Escherichia coli Biofilm Formation in Nutrient-Limited Medium. PLoS ONE, 9(9), e107186.
27. Dale, J. L., Nilson, J. L., Barnes, A. M. T., & Dunny, G. M. (2017). Restructuring of Enterococcus faecalis biofilm architecture in response to antibiotic-induced stress. Npj Biofilms and Microbiomes, 3(1).
28. Das, T., Sharma, P. K., Busscher, H. J., van der Mei, H. C., & Krom, B. P. (2010). Role of Extracellular DNA in Initial Bacterial Adhesion and Surface Aggregation. Applied and Environmental Microbiology, 76(10), 3405–3408.
29. Das, T., Simone, M., Ibugo, A. I., Witting, P. K., Manefield, M., & Manos, J. (2017). Glutathione enhances antibiotic efficiency and effectiveness of DNase I in disrupting Pseudomonas aeruginosa biofilms while also inhibiting pyocyanin activity, thus facilitating restoration of cell enzymatic activity, confluence, and viability. Frontiers in Microbiology, 8.

30. Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W., & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science, 280(5361), 295-298.
31. Decho, A. W., & Gutierrez, T. (2017). Microbial Extracellular Polymeric Substances (EPSs) in Ocean Systems. Frontiers in Microbiology, 8, 922.
32. Domenico, E.G.D.; Oliva, A.; Guembe, M. The Current Knowledge on the Pathogenesis of Tissue and Medical Device-Related Biofilm Infections (2022). Microorganisms 2022, 10(7), 1259.
33. Donlan, R. M. (2001). Biofilm Formation: A Clinically Relevant Microbiological Process. Clinical Infectious Diseases, 33(8), 1387–1392.
34. Donlan, R. M. (2002). Biofilms: Microbial Life on Surfaces. Emerging Infectious Diseases, 8(9), 881–890.
35. Drago, L., De Vecchi, E., Torretta, S., Mattina, R., Marchisio, P., & Pignataro, L. (2011). Biofilm formation by bacteria isolated from upper respiratory tract before and after adenotonsillectomy. APMIS, 120(5), 410–416.
36. Dranguet, P., Le Faucheur, S. and Slaveykova, V.I. (2017), Mercury bioavailability, transformations, and effects on freshwater biofilms. Environ Toxicol Chem, 36: 3194-3205.
37. Espeland, E. M., & Wetzel, R. G. (2001). Complexation, Stabilization, and UV Photolysis of Extracellular and Surface-Bound Glucosidase and Alkaline Phosphatase: Implications for Biofilm Microbiota. Microbial Ecology, 42(4), 572–585.
38. Faruque, S. M., & Mekalanos, J. J. (2012). Phage-bacterial interactions in the evolution of toxigenicVibrio cholerae. Virulence, 3(7), 556–565.
39. Faruque, S. M., Naser, I. B., Islam, M. J., Faruque, A. S. G., Ghosh, A. N., Nair, G. B., & Sack, D. A. (2005). Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages. Proceedings of the National Academy of Sciences, 102(5), 1702-1707.
40. Fazli, M., Almblad, H., Rybtke, M. L., Givskov, M., Eberl, L., & Tolker-Nielsen, T. (2014). Regulation of biofilm formation in Pseudomonas and Burkholderia species. Environmental Microbiology, 16(7), 1961-1981.

41. Fleming, D., & Rumbaugh, K. (2017). Approaches to Dispersing Medical Biofilms. Microorganisms, 5(2), 15.
42. Flemming, H.C., & Wingender, J. (2010). The biofilm matrix. Nature Reviews Microbiology, 8(9), 623–633.
43. Fong, J. C. N., & Yildiz, F. H. (2008). Interplay between Cyclic AMP-Cyclic AMP Receptor Protein and Cyclic di-GMP Signaling in Vibrio cholerae Biofilm Formation. Journal of Bacteriology, 190(20), 6646–6659.
44. Foreman, A., Holtappels, G., Psaltis, A. J., Jervis-Bardy, J., Field, J., Wormald, P.-J., & Bachert, C. (2011). Adaptive immune responses in Staphylococcus aureus biofilm-associated chronic rhinosinusitis. Allergy, 66(11), 1449–1456.
45. Frantz, C., Stewart, K. M., & Weaver, V. M. (2010). The extracellular matrix at a glance. Journal of Cell Science, 123(24), 4195–4200.
46. Funari, R., & Shen, A. Q. (2022). Detection and characterization of bacterial biofilms and biofilm-based sensors. ACS Sensors, 7(2), 347-357.
47. Gallego-Hernandez, A. L., DePas, W. H., Park, J. H., Teschler, J. K., Hartmann, R., Jeckel, H., Drescher, K., Beyhan, S., Newman, D. K., & Yildiz, F. H. (2020). Upregulation of virulence genes promotes Vibrio cholerae biofilm hyperinfectivity. Proceedings of the National Academy of Sciences, 117(20), 11010-11017.
48. Gao, P., Ho, P. L., Yan, B., Sze, K. H., Davies, J., & Kao, R. Y. T. (2018). Suppression of Staphylococcus aureus virulence by a small-molecule compound. Proceedings of the National Academy of Sciences, 115(31), 8003–8008.
49. Ghanbari, A., Dehghany, J., Schwebs, T., Müsken, M., Häussler, S., & Meyer-Hermann, M. (2016). Inoculation density and nutrient level determine the formation of mushroom-shaped structures in Pseudomonas aeruginosa biofilms. Scientific Reports, 6(1).
50. Gilbert, P., Allison, D., & McBain, A. J. (2002). Biofilms in vitro and in vivo : do singular mechanisms imply cross-resistance? Journal of Applied Microbiology, 92, 98S-110S.
51. Gomes, L. C., Carvalho, D., Briandet, R., & Mergulhão, F. J. (2016). Temporal variation of recombinant protein expression in Escherichia coli biofilms analysed at single-cell level. Process Biochemistry, 51(9), 1155–1161.

52. Guzmán-Soto, I., McTiernan, C. D., Gonzalez-Gomez, M., Ross, A., Gupta, K., Suuronen, E. J., Mah, T., Griffith, M., & Alarcon, E. I. (2021b). Mimicking biofilm formation and development: Recent progress in in vitro and in vivo biofilm models. iScience, 24(5), 102443. https://doi.org/10.1016/j.isci.2021.102443
53. Hall-Stoodley, L., Costerton, J. W., & Stoodley, P. (2004). Bacterial biofilms: from the Natural environment to infectious diseases. Nature Reviews Microbiology, 2(2), 95–108.
54. Harmsen, M., Yang, L., Pamp, S. J., & Tolker-Nielsen, T. (2010). An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal. FEMS Immunology & Medical Microbiology, 59(3), 253-268.
55. Harriott, M. M., & Noverr, M. C. (2009). Candida albicans and Staphylococcus aureus Form Polymicrobial Biofilms: Effects on Antimicrobial Resistance. Antimicrobial Agents and Chemotherapy, 53(9), 3914–3922.
56. Hinsa, S.M., Espinosa-Urgel, M., Ramos, J.L. and O'Toole, G.A. (2003), Transition from reversible to irreversible attachment during biofilm formation by Pseudomonas fluorescens WCS365 requires an ABC transporter and a large secreted protein. Molecular Microbiology, 49(4), 905-918.
57. Hollenbeck, E. C., Fong, J. C. N., Lim, J. Y., Yildiz, F. H., Fuller, G. G., & Cegelski, L. (2014). Molecular Determinants of Mechanical Properties of V. cholerae Biofilms at the Air-Liquid Interface. Biophysical Journal, 107(10), 2245–2252.
58. Hoque, M. M., Naser, I. B., Bari, S. M. N., Zhu, J., Mekalanos, J. J., & Faruque, S. M. (2016). Quorum Regulated Resistance of Vibrio cholerae against Environmental Bacteriophages. Scientific Reports, 6(1).
59. Hori, K., & Matsumoto, S. (2010). Bacterial adhesion: From mechanism to control. Biochemical Engineering Journal, 48(3), 424–434.
60. Huseby, M. J., Kruse, A. C., Digre, J., Kohler, P. L., Vocke, J. A., Mann, E. E., Bayles, K. W., Bohach, G. A., Schlievert, P. M., Ohlendorf, D. H., Earhart, C. A., & Novick, R. P. (2010). Beta toxin catalyzes formation of nucleoprotein matrix in staphylococcal biofilms. Proceedings of the National Academy of Sciences of the United States of America, 107(32), 14407-14412.

61. Ibáñez de Aldecoa, A. L., Zafra, O., & González-Pastor, J. E. (2017). Mechanisms and Regulation of Extracellular DNA Release and Its Biological Roles in Microbial Communities. Frontiers in Microbiology, 8, 1390.
62. Izano, E. A., Amarante, M. A., Kher, W. B., & Kaplan, J. B. (2007). Differential Roles of Poly-N-Acetylglucosamine Surface Polysaccharide and Extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis Biofilms. Applied and Environmental Microbiology, 74(2), 470–476.
63. Jiang, Y., Geng, M., & Bai, L. (2020). Targeting Biofilms Therapy: Current Research Strategies and Development Hurdles. Microorganisms, 8(8), 1222.
64. Kamruzzaman, M., Udden, S. M. N., Cameron, D. E., Calderwood, S. B., Nair, G. B., Mekalanos, J. J., & Faruque, S. M. (2010). Quorum-Regulated Biofilms Enhance the Development of Conditionally Viable, Environmental Vibrio cholerae. Proceedings of the National Academy of Sciences of the United States of America, 107(4), 1588-1593.
65. Kanematsu, H., Kudara, H., Kanesaki, S., Kogo, T., Ikegai, H., Ogawa, A., & Hirai, N. (2016). Application of a Loop-Type Laboratory Biofilm Reactor to the Evaluation of Biofilm for Some Metallic Materials and Polymers such as Urinary Stents and Catheters. Materials, 9(10), 824.
66. Kaplan, J. B. (2010). Biofilm Dispersal: Mechanisms, Clinical Implications, and Potential Therapeutic Uses. Journal of Dental Research, 89(3), 205–218.
67. Karatan, E., & Watnick, P. (2009). Signals, Regulatory Networks, and Materials That Build and Break Bacterial Biofilms. Microbiology and Molecular Biology Reviews, 73(2), 310–347.
68. Karygianni, L., Ren, Z., Koo, H., & Thurnheer, T. (2020). Biofilm Matrixome: Extracellular Components in Structured Microbial Communities. Trends in Microbiology.
69. Katharios-Lanwermeyer, S., Whitfield, G. B., Howell, P. L., & O'Toole, G. A. (2021). Pseudomonas aeruginosa Uses c-di-GMP Phosphodiesterases RmcA and MorA To Regulate Biofilm Maintenance. American Society for Microbiology, 12(1), 2161-2129.
70. Kaur, S., Sharma, P., Kalia, N., Singh, J., & Kaur, S. (2018). Anti-biofilm Properties of the Fecal Probiotic Lactobacilli Against Vibrio spp. Frontiers in Cellular and Infection Microbiology, 8.

71. Kierek, K., & Watnick, P. I. (2003). Environmental Determinants of Vibrio cholerae Biofilm Development. Applied and Environmental Microbiology, 69(9), 5079–5088.
72. Kitts, G., Giglio, K. M., Zamorano-Sánchez, D., Park, J. H., Townsley, L., Cooley, R. B., Wucher, B. R., Klose, K. E., Nadell, C. D., Yildiz, F. H., & Sondermann, H. (2019). A Conserved Regulatory Circuit Controls Large Adhesins in Vibrio cholerae. mBio, 10(6).
73. Kjelleberg, S., & Molin, S. (2002). Is there a role for quorum sensing signals in bacterial biofilms? Current Opinion in Microbiology, 5(3), 254-258.
74. Landini, P., & Zehnder, A. J. B. (2002). The Global Regulatory hns Gene Negatively Affects Adhesion to Solid Surfaces by Anaerobically Grown Escherichia coli by Modulating Expression of Flagellar Genes and Lipopolysaccharide Production. Journal of Bacteriology, 184(6), 1522–1529.
75. Laverty, G., Gorman, S. P., & Gilmore, B. F. (2014). Biomolecular mechanisms of Pseudomonas aeruginosa and Escherichia coli biofilm formation. Pathogens and disease, 70(3), 336-347.
76. Lawrence, J. R., Korber, D. R., Hoyle, B. D., Costerton, J. W., & Caldwell, D. E. (1991). Optical sectioning of microbial biofilms. Journal of Bacteriology, 173(20), 6558–6567.
77. Leid, J. G., Shirtliff, M. E., Costerton, J. W., & Stoodley, P. (2002). Human Leukocytes Adhere to, Penetrate, and Respond to Staphylococcus aureus Biofilms. Infection and Immunity, 70(11), 6339–6345.
78. Lewandowski, Z., & Beyenal, H. (2013). Fundamentals of Biofilm Research. In CRC Press eBooks. https://doi.org/10.1201/b16291
79. Li, G., Park, S., Kang, D.-W., Krajmalnik-Brown, R., & Rittmann, B. E. (2011). 2,4,5-Trichlorophenol degradation using a novel TiO2-coated biofilm carrier: Roles of adsorption, photocatalysis, and biodegradation. Environmental Science & Technology, 45(19), 8359-8367.
80. Li, Y., Hao, G., Galvani, C. D., Meng, Y., Fuente, L. D. L., Hoch, H. C., & Burr, T. J. (2007). Type I and type IV pili of Xylella fastidiosa affect twitching motility, biofilm formation and cell-cell aggregation. Microbiology, 153(3), 719–726.
81. Liu, C., Sun, D., Zhu, J., Liu, J., & Liu, W. (2020). The Regulation of Bacterial Biofilm Formation by cAMP-CRP: A Mini-Review. Frontiers in Microbiology, 11.

82. Liu, X., Yao, H., Zhao, X., & Ge, C. (2023). Biofilm Formation and Control of Foodborne Pathogenic Bacteria. Molecules, 28(6), 2432.
83. Lorenz, M. G., & Wackernagel, W. (1994). Bacterial gene transfer by natural genetic transformation in the environment. Microbiological reviews, 58(3), 563-602.
84. Ma, L., Conover, M., Lu, H., Parsek, M. R., Bayles, K., & Wozniak, D. J. (2009). Assembly and Development of the Pseudomonas aeruginosa Biofilm Matrix. PLoS Pathogens, 5(3), e1000354.
85. Madsen, J. S., Røder, H. L., Russel, J., Sørensen, H., Burmølle, M., & Sørensen, S. J. (2016). Coexistence facilitates interspecific biofilm formation in complex microbial communities. Environmental Microbiology, 18(8), 2565–2574.
86. Maes, S., Vackier, T., Nguyen Huu, S., Heyndrickx, M., Steenackers, H., Sampers, I., … De Reu, K. (2019). Occurrence and characterisation of biofilms in drinking water systems of broiler houses. BMC Microbiology, 19(1).
87. Magrex-Debar, É. L., Lemoine, J., Gellé, M., Jacquelin, L. F., & Choisy, C. (2000). Evaluation of biohazards in dehydrated biofilms on foodstuff packaging. International Journal of Food Microbiology, 55(1–3), 239–243.
88. Mah, T. F. C., & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology, 9(1), 34–39.
89. Mathlouthi, A., Pennacchietti, E., & De Biase, D. (2018). Effect of temperature, pH and plasmids on in vitro biofilm formation in Escherichia coli. Acta Naturae, 10(4), 129–132.
90. McDougald, D., Rice, S. A., Barraud, N., Steinberg, P. D., & Kjelleberg, S. (2011). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nature Reviews Microbiology, 10(1), 39–50.
91. McNeill, K. D., & Hamilton, I. R. (2003). Acid tolerance response of biofilm cells ofStreptococcus mutans. Fems Microbiology Letters, 221(1), 25–30.
92. Monir, M.M., Islam, M.T., Mazumder, R. et al. Genomic attributes of Vibrio cholerae O1 responsible for 2022 massive cholera outbreak in Bangladesh. Nat Commun 14, 1154 (2023).
93. Morinaga, K., Yoshida, K., Takahashi, K., Nomura, N., & Toyofuku, M. (2020). Peculiarities of biofilm formation by Paracoccus denitrificans. Applied Microbiology and Biotechnology, 104, 2427–2433.

94. Muhammad, MH., Idris. A.L., Fan, X., Guo, Y., Yu, Y., Jin, X., Qiu, J., Guan, X., & Huang, T. (2020). Beyond Risk: Bacterial Biofilms and Their Regulating Approaches. Frontiers in Microbiology, 11.
95. Mulcahy, H., Charron-Mazenod, L., & Lewenza, S. (2008). Extracellular DNA chelates cations and induces antibiotic resistance in Pseudomonas aeruginosa biofilms. PLoS Pathog, 4(11), e1000213.
96. Nair, D., Memmi, G., Hernandez, D., Bard, J., Beaume, M., Gill, S., Francois, P., & Cheung, A. L. (2011). Whole-genome sequencing of Staphylococcus aureus strain RN4220, a key laboratory strain used in virulence research, identifies mutations that affect not only virulence factors but also the fitness of the strain. American Society for Microbiology, 193(9), 2332-2335.
97. Naser, I. B., Hoque, M. M., Nahid, M. A., Tareq, T. M., Rocky, M. K., & Faruque, S. M. (2017). Analysis of the CRISPR-Cas system in bacteriophages active on epidemic strains of Vibrio cholerae in Bangladesh. Scientific Reports, 7(1).
98. Naser IB, Hoque MM, Faruque SN, Kamruzzaman M, Yamasaki S, Faruque SM (2019) Vibrio cholerae strains with inactivated cqsS gene overproduce autoinducer-2 which enhances resuscitation of dormant environmental V. cholerae. PLoS ONE 14(10): e0223226
99. Naser IB, Shishir TA, Faruque SN, Hoque MM, Hasan A, et al. (2021) Environmental prevalence of toxigenic Vibrio cholerae O1 in Bangladesh coincides with V. cholerae non-O1 non-O139 genetic variants which overproduce autoinducer-2. PLOS ONE 16(7): e0254068.
100. Nhu, N. T. Q., Lee, J. S., Wang, H. J., & Dufour, Y. S. (2021). Alkaline pH Increases Swimming Speed and Facilitates Mucus Penetration for Vibrio cholerae. Journal of Bacteriology, 203(7).
101. Okshevsky, M., & Meyer, R. L. (2015). The role of extracellular DNA in the establishment, maintenance, and perpetuation of bacterial biofilms. Critical reviews in microbiology, 41(3), 341-352.
102. Okshevsky, M., Regina, V. R., & Meyer, R. L. (2015). Extracellular DNA as a target for biofilm control. Current Opinion in Biotechnology, 33, 73–80. https://doi.org/10.1016/j.copbio.2014.12.002

103. O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). Biofilm Formation as Microbial Development. Annual Review of Microbiology, 54(1), 49–79.

104. O'Toole, G. A., & Kolter, R. (1998). Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Molecular Microbiology, 30(2), 295–304.

105. Pakkulnan, R., Anutrakunchai, C., Kanthawong, S., Taweechaisupapong, S., Chareonsudjai, P., Chareonsudjai, S. (2019). Extracellular DNA facilitates bacterial adhesion during Burkholderia pseudomallei biofilm formation. PLoS ONE 14(3): e0213288

106. Pang L, Lin H, Yang F and Deng D (2023) Editorial: Mechanisms of biofilm development and antibiofilm strategies. Front. Microbiol. 14:1190611.

107. Petersen, F. C., Tao, L., & Scheie, A. A. (2005). DNA Binding-Uptake System: a Link between Cell-to-Cell Communication and Biofilm Formation. Journal of Bacteriology, 187(13), 4392–4400.

108. Peterson, B. W., He, Y., Ren, Y., Zerdoum, A., Libera, M. R., Sharma, P. K., & van Winkelhoff, A. J. (2015). Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges. FEMS Microbiology Reviews, 39(2), 234-245.

109. Petrova, O. E., & Sauer, K. (2009). A Novel Signaling Network Essential for Regulating Pseudomonas aeruginosa Biofilm Development. PLoS Pathogens, 5(11), e1000668.

110. Petrova, O. E., & Sauer, K. (2012). Sticky Situations: Key Components That Control Bacterial Surface Attachment. Journal of Bacteriology, 194(10), 2413–2425.

111. Purdy, A. E., & Watnick, P. I. (2011). Spatially selective colonization of the arthropod intestine through activation of Vibrio cholerae biofilm formation. Proceedings of the National Academy of Sciences, 108(49), 19737–19742.

112. Razatos, A., Ong, Y. L., Sharma, M. M., & Georgiou, G. (1998). Molecular determinants of bacterial adhesion monitored by atomic force microscopy. Proceedings of the National Academy of Sciences, 95(19), 11059-11064.

113. Reisner, A., Haagensen, J. A. J., Schembri, M. A., Zechner, E. L., & Molin, S. (2003). Development and maturation of Escherichia coli K-12 biofilms. Molecular Microbiology, 48(4), 933–946.

114. Reisner, A., Krogfelt, K. A., Klein, B. M., Zechner, E. L., & Molin, S. (2006). In Vitro Biofilm Formation of Commensal and Pathogenic Escherichia coli Strains: Impact of Environmental and Genetic Factors. Journal of Bacteriology, 188(10), 3572–3581.

115. Research On Microbial Biofilms: PA-03-047. (2003). Journal of Investigative Medicine, 51(3), 162–163.

116. Rittmann, B. E., & McCarty, P. L. (2001). Environmental biotechnology: Principles and applications. McGraw-Hill.

117. Saini, R., Saini, S., & Sharma, S. (2011). Biofilm: A dental microbial infection. Journal of Natural Science, Biology, and Medicine, 2(1), 71-75.

118. Sauer, K., Stoodley, P., Goeres, D. M., Hall-Stoodley, L., Burmølle, M., Stewart, P. S., & Bjarnsholt, T. (2022). The biofilm life cycle: expanding the conceptual model of biofilm formation. Nature Reviews Microbiology, 20, 608–620.

119. Senpuku, H., Nakamura, T., Iwabuchi, Y., Hirayama, S., Nakao, R., & Ohnishi, M. (2019). Effects of Complex DNA and MVs with GTF Extracted from Streptococcus mutans on the Oral Biofilm. Molecules, 24(17), 3131.

120. Seper, A., Fengler, V. H. I., Roier, S., Wolinski, H., Kohlwein, S. D., Bishop, A. L., … Schild, S. (2011). Extracellular nucleases and extracellular DNA play important roles in Vibrio cholerae biofilm formation. Molecular Microbiology, 82(4), 1015–1037.

121. Steinberger, R. E., & Holden, P. A. (2005). Extracellular DNA in Single- and Multiple-Species Unsaturated Biofilms. Applied and Environmental Microbiology, 71(9), 5404-5410.

122. Stewart, P. S., & Costerton, J. W. (2001). Antibiotic resistance of bacteria in biofilms. The Lancet, 358(9276), 135–138.

123. Stewart, P. S., & Franklin, M. J. (2008). Physiological heterogeneity in biofilms. Nature Reviews Microbiology, 6(3), 199–210

124. Stoodley, P., Lewandowski, Z., Boyle, J. D., & Lappin-Scott, H. M. (1999). Structural deformation of bacterial biofilms caused by short-term fluctuations in fluid shear: An in situ investigation of biofilm rheology. Biotechnology and Bioengineering, 65(1), 83–92.

125. Stoodley, P., Wilson, S., Hall-Stoodley, L., Boyle, J. D., Lappin-Scott, H. M., & Costerton, J. W. (2001). Growth and Detachment of Cell Clusters from Mature Mixed-Species Biofilms. Applied and Environmental Microbiology, 67(12), 5608–5613.

126. Teitzel, G. M., & Parsek, M. R. (2003). Heavy Metal Resistance of Biofilm and Planktonic Pseudomonas aeruginosa. Applied and Environmental Microbiology, 69(4), 2313–2320.

127. Tetz, G. V., Artemenko, N. K., & Tetz, V. V. (2008). Effect of DNase and Antibiotics on Biofilm Characteristics. Antimicrobial Agents and Chemotherapy, 53(3), 1204–1209.

128. Tolker-Nielsen, T., Brinch, U. C., Ragas, P. C., Andersen, J. B., Jacobsen, C. S., & Molin, S. (2000). Development and Dynamics of Pseudomonas sp. Biofilms. Journal of Bacteriology, 182(22), 6482–6489.

129. Tolker-Nielsen, T. (2015). Biofilm Development. Microbiology Spectrum, 3(2).

130. Velmourougane, K., Prasanna, R., & Saxena, A. K. (2017). Agriculturally important microbial biofilms: Present status and future prospects. Journal of Basic Microbiology, 57(7), 548–573.

131. Verma-Gaur, J., Qu, Y., Harrison, P. F., Lo, T. L., Quenault, T., Dagley, M. J., Bellousoff, M., Powell, D. R., Beilharz, T. H., Traven, A., & Butler, G. (2015). Integration of posttranscriptional gene networks into metabolic adaptation and biofilm maturation in Candida albicans. PLoS Genetics, 11(10), e1005590.

132. Vilain, S., Pretorius, J. M., Theron, J., & Brozel, V. S. (2009). DNA as an Adhesin: Bacillus cereus Requires Extracellular DNA To Form Biofilms. Applied and Environmental Microbiology, 75(9), 2861–2868.

133. Wang, H., Li, Y., Yang, H., Lin, K.-A., Shao, T., & Hope, J. (2023). Biofilms controlling in industrial cooling water systems: A mini-review of strategies and best practices. ACS Applied Bio Materials, 6(8), 3213-3220.

134. Waters, C. M., & Bassler, B. L. (2005). QUORUM SENSING: Cell-to-Cell Communication in Bacteria. Annual Review of Cell and Developmental Biology, 21(1), 319–346.

135. Watnick, P., & Kolter, R. (2000). Biofilm, City of Microbes. Journal of Bacteriology, 182(10), 2675–2679.

136. Wei, G., & Yang, J. Q. (2023). Impacts of hydrodynamic conditions and microscale surface roughness on the critical shear stress to develop and thickness of early-stage Pseudomonas putida biofilms. Biotechnology and Bioengineering, 120, 1797–1808.

137. Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C., & Mattick, J. S. (2002). Extracellular DNA required for bacterial biofilm formation. Science, 295(5559), 1487.

138. Wimpenny, J. W. T., & Colasanti, R. (2006). A unifying hypothesis for the structure of microbial biofilms based on cellular automaton models. FEMS Microbiology Ecology, 22(1), 1–16.

139. Wu, Y., & Outten, F. W. (2008). IscR Controls Iron-Dependent Biofilm Formation in Escherichia coli by Regulating Type I Fimbria Expression. Journal of Bacteriology, 191(4), 1248–1257.

140. Xavier, J. B., Picioreanu, C., & van Loosdrecht, M. C. M. (2004). A modelling study of the activity and structure of biofilms in biological reactors. Biofilms, 1(4), 377–391.

141. Xia, W., Li, N., Shan, H., Lin, Y., Yin, F., Yu, X., & Zhou, Z. (2021). Gallium Porphyrin and Gallium Nitrate Reduce the High Vancomycin Tolerance of MRSA Biofilms by Promoting Extracellular DNA-Dependent Biofilm Dispersion. ACS Infectious Diseases, 7(8), 2565-2582.

142. Yan, J., Sharo, A. G., Stone, H. A., Wingreen, N. S., & Bassler, B. L. (2016). Vibrio cholerae biofilm growth program and architecture revealed by single-cell live imaging. Proceedings of the National Academy of Sciences, 113(36), E5337–E5343.

143. Yin, W., Wang, Y., Liu, L., & He, J. (2019). Biofilms: The Microbial “Protective Clothing” in Extreme Environments. International Journal of Molecular Sciences, 20(14), 3423.