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Review

Pharmaceutical Contamination by Biofilms Formed of the *Burkholderia cepacia* Complex: Public Health Risks

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Abstract: Biofilms formation by the *Burkholderia cepacia* complex (Bcc) poses a considerable risk to hospital environments, particularly for immunocompromised individuals. These bacteria exhibit notable resistance to disinfectants and antibiotics, mainly due to their ability to adhere to biotic and abiotic surfaces, forming highly persistent biofilms, contamination, and pharmaceutical solutions. These microbial structures function as protective shields, impeding the effective action of antimicrobial compounds and facilitating the occurrence of chronic infections and outbreaks in healthcare settings. The high genetic plasticity of the Bcc, evidenced by the presence of multiple chromosomes and the ease of horizontal gene transfer, further enhances its capacity for adaptation and treatment resistance. Moreover, the ability of the Bcc to survive in aquatic environments and withstand unfavorable conditions heightens concerns regarding the contamination of pharmaceutical products. This study examines the molecular mechanisms underlying Bcc biofilm formation, its impact on hospital infections, and the challenges associated with its eradication. It also discusses the current detection techniques available and innovative approaches to mitigating contamination in pharmaceutical products. In summary, a thorough understanding of the mechanisms underlying Bcc biofilm formation and maintenance is crucial for implementing more effective preventive measures and minimizing the risks associated with hospital infections.

Keywords: *Burkholderia cepacia* complex; antibiotics; biocides; biofilm; chronic granulomatous disease; cystic fibrosis; melioidosis; nosocomial outbreaks; pharmaceutical contamination; quorum sensing; resistance

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1. Introduction

The genus *Burkholderia*, which includes species from the *Burkholderia cepacia* complex (Bcc), is found in diverse environments, including soils, plants, medical devices, and pharmaceutical formulations. While some species have beneficial applications in agriculture and bioremediation, Bcc members are particularly concerning due to their pathogenic potential, being associated with chronic respiratory infections, septicemia, and hospital outbreaks [1,2]. The presence of multiple chromosomes and the high genetic plasticity of these bacteria provide a remarkable capacity for adaptation, enabling their survival under adverse conditions and facilitating the acquisition of genes associated with antimicrobial resistance [3]. These characteristics make the Bcc a significant challenge for healthcare systems, as infections caused by these bacteria frequently result in therapeutic failures and increased morbidity and mortality among hospitalized patients [4,5].

Contamination of pharmaceutical products by microorganisms poses a significant threat to public health, particularly in hospital settings. Among the most concerning pathogens in this context, bacteria from the Bcc are particularly notable due to their intrinsic antimicrobial resistance and their capacity to form biofilms on both biotic and abiotic surfaces [1]. These biofilm structures serve as protective barriers, shielding the bacteria from the effects of disinfectants and antibiotics, as well as increasing the likelihood of infectious outbreaks, especially among immunocompromised patients [2]. Consequently, a thorough understanding of the mechanisms underlying the formation and persistence of Bcc biofilms on pharmaceutical products and hospital surfaces is essential for the development of effective strategies to control and prevent such infections [6,7].

In this context, this study reviews the main aspects related to the contamination of pharmaceutical products by biofilms formed by the Bcc, highlighting their relevance in the dissemination of nosocomial infections. The molecular mechanisms involved in the formation of these biofilms, the challenges associated with their detection and eradication, and innovative strategies to mitigate their impact, will be explored. In light of the growing concern over the microbiological safety of pharmaceutical products, it becomes essential to deepen our understanding of the persistence of the Bcc in these environments and propose effective measures for its control, aiming at protecting public health and reducing the risks associated with hospital infections.

2. The Genus *Burkholderia*

The genus *Burkholderia*, belonging to the class *β-Proteobacteria*, order *Burkholderiales*, and family *Burkholderiaceae*, is a fascinating and complex group of Gram-negative bacilli, obligate aerobic, and motile bacteria, distinguished by their complexity and genetic diversity. These microorganisms are widely distributed across various ecosystems, such as soils, aquatic environments, and plant rhizospheres, as well as in hospitals and on medical devices [2,8]. The isolation from pharmaceutical facilities is common, mainly from pharmaceutical water samples [9]. Their interactions with hosts range from beneficial mutualistic relationships to opportunistic infections, highlighting their relevance in both medical and environmental microbiology [3,10]. Furthermore, *Burkholderia* establishes symbiotic relationships with insects, such as ants and termites, acting as gut symbionts that assist in cellulose digestion and protection against pathogens, which reinforces their ecological versatility [11,12].

The type species of the genus, *Burkholderia cepacia*, was originally classified as *Pseudomonas cepacia*. It was later reclassified based on taxonomic analyses, including 16S rRNA sequencing, DNA-DNA hybridization, and studies of cell membrane lipid and fatty acid composition [13,14]. With the expansion of the genus, a taxonomic reorganization became necessary to differentiate pathogenic lineages from those with biotechnological and environmental potential. As a result, mutualistic and environmental species were reclassified into other genera, such as *Paraburkholderia* and *Caballeronia*, due to their distinct genetic and functional characteristics, including associations with plants and mycorrhizal fungi and lower virulence [15,16]. This division was essential to separate clinically relevant lineages, such as those of the Bcc, from others with lower pathogenic risk and greater biotechnological applications [15,17].

The Bcc is composed of approximately 24 genetically distinct species, characterized by high phenotypic variability [18,19]. Initially categorized as genomovars due to differences in DNA-DNA hybridization levels, these species were later recognized as independent [20,21]. Bcc species are important agents of chronic respiratory infections in immunocompromised patients, especially those with cystic fibrosis (CF) and chronic granu-

lomatous disease (CGD), and they are also associated with nosocomial outbreaks due to their high antimicrobial resistance [19,22].

The genome of *Burkholderia* is marked by remarkable genetic plasticity, often organized into multiple chromosomes, with sizes ranging between 6 and 9 Mb and a high proportion of G + C base pairs [23]. This genomic complexity allows for rapid adaptation to diverse environments and the acquisition of resistance to physical and chemical stresses, contributing to its widespread ecological distribution and evolutionary success [1,24]. The presence of mobile genetic elements, such as transposons and integrons, facilitates horizontal gene transfer, enabling *Burkholderia* to quickly acquire new adaptive traits in response to environmental pressures [25,26].

Antimicrobial resistance in the genus *Burkholderia* is multifactorial, involving mechanisms such as Resistance–Nodulation–Division (RND) efflux pumps, enzymatic modification of antibiotics, and low outer membrane permeability, making the treatment of infections caused by the Bcc particularly challenging [1]. Additionally, these bacteria employ the type VI secretion system (T6SS), a crucial mechanism for virulence and interbacterial competition, allowing the injection of toxins into competing cells and the host, facilitating their persistence and evasion of the immune response [27]. Other secretion systems, such as type III (T3SS), are also used by pathogenic species, like *B. mallei* and *B. pseudomallei*, to secrete protein effectors into host cells, promoting biofilm formation, modulating the immune response, and facilitating infection [28].

The ability to form biofilms is another critical aspect of *Burkholderia's* pathogenicity, conferring resistance to antimicrobials and complicating the eradication of infections, especially in hospital environments and the respiratory tract of CF patients [22,29]. Gene expression regulation in these bacteria is mediated by quorum sensing (QS) systems based on N-acyl-homoserine lactones (AHLs) and small regulatory RNAs, influencing biofilm formation, oxidative stress resistance, and adaptation to different environmental conditions [30].

In addition to their clinical impact, the genus *Burkholderia* has various biotechnological and environmental applications. Some species promote plant growth through biological nitrogen fixation and the production of phytohormones, while others synthesize antimicrobial compounds effective against phytopathogens. These characteristics make the genus promising for the biological control of agricultural diseases [31]. Additionally, their ability to degrade environmental pollutants, such as hydrocarbons and halogenated solvents, has sparked interest in bioremediation [32]. Studies have also explored their potential in producing bioplastics, such as polyhydroxyalkanoates (PHA), and enzymes like lipases and proteases, with applications across various industrial sectors, including food and pharmaceuticals [33].

However, the application of *Burkholderia* in agriculture and industrial processes requires caution, as several species exhibit high virulence and antimicrobial resistance, posing risks to public health. In particular, *B. mallei* and *B. pseudomallei* are classified by the Centers for Disease Control and Prevention (CDC) as Tier 1 bioterrorism agents, alongside *Bacillus anthracis* and *Yersinia pestis*, due to their high lethality and antibiotic resistance [34,35]. These bacteria are responsible for glanders and melioidosis, respectively, serious infections that affect equines and humans, particularly in tropical and subtropical regions [36,37]. Melioidosis, in particular, is a neglected disease that primarily affects rural populations in endemic areas such as Southeast Asia and Northern Australia [38]. The lack of rapid and accurate diagnosis, combined with antimicrobial resistance, makes this disease a significant threat [37,38].

Given the functional and clinical diversity of the *Burkholderia* genus, a deeper understanding of its pathogenicity mechanisms, antimicrobial resistance, and ecological

interactions is essential for developing effective diagnostic, treatment, and control strategies [1,38]. Advances in genomics and bioinformatics have provided new insights into these bacteria, with significant implications for medical microbiology, environmental biotechnology, and sustainable agriculture [23,31]. Additionally, climate change may influence the geographic distribution of *Burkholderia*, particularly pathogenic species, as rising temperatures and shifting precipitation patterns expand endemic areas into previously unaffected regions [37,39].

3. Biofilms: Resistance Strategy

In the environment, bacteria often organize into polymicrobial biofilms, which consist of microbial communities adhering to biotic surfaces (such as plant or animal tissues) or abiotic surfaces (such as plastics, metals, or rocks). The formation of these biofilms is a dynamic, multicellular process, beginning with planktonic cells (free and motile) that attach to surfaces through adhesins, proteins, or polysaccharides present on the cell surface, or through extracellular secretions such as exopolysaccharides. Initially, this attachment is reversible, but over time, it becomes irreversible as the cells start to produce a complex extracellular matrix [6,40].

The extracellular matrix is composed of a mixture of polysaccharides, proteins, extracellular DNA (eDNA), and lipids, forming a three-dimensional structure that keeps the cells together and protected [40]. This matrix not only provides structural support but also acts as a physical and chemical barrier against external agents, such as antimicrobials and protozoa [41]. Additionally, the matrix facilitates intercellular communication through signaling molecules, such as AHLs, which regulate processes like biofilm formation and the expression of virulence genes in a mechanism known as QS [30,42].

Biofilms play a crucial role in microbial survival in various environments, providing protection against environmental stresses such as dehydration, nutrient scarcity, ultraviolet (UV) radiation, and predation by protozoa or other microorganisms [40,43]. The biofilm structure creates microniches where cells can organize into different layers, allowing for the division of metabolic tasks and the optimization of resource usage [44]. For example, cells on the surface of the biofilm may be more exposed to nutrients and oxygen, while cells inside may be in a metabolically inactive state but protected from adverse conditions [6,44].

A notable characteristic of biofilms is their high resistance to the penetration of antimicrobial agents. The extracellular polymeric matrix hinders the diffusion of antimicrobial molecules, such as antibiotics and disinfectants, and neutralizes enzymes and other toxic compounds [40]. Additionally, bacteria in biofilms exhibit reduced growth rates, making them less susceptible to antibiotics that rely on the metabolic activity of cells to be effective [45,46]. Studies indicate that bacteria in biofilms can be up to 1.000 times more resistant to antimicrobials than in their planktonic state, posing a significant challenge for the treatment of biofilm-associated infections [40,47].

In addition to antimicrobial resistance, biofilms also provide protection against the host's immune system. The extracellular matrix can inhibit phagocytosis by immune cells, such as macrophages and neutrophils, preventing these cells from recognizing and eliminating the bacteria [40,48]. Furthermore, biofilms can modulate the host's immune responses by inducing the production of pro-inflammatory cytokines, which, instead of eliminating the infection, cause tissue damage and chronic inflammation [49]. This ability to evade the immune system and persist in the host contributes to the establishment of chronic infections, such as those observed in CF patients; chronic wound infections; and infections associated with medical devices, such as catheters and prosthetics [50,51].

The formation of biofilms represents a serious threat to human health, especially in hospital settings. Biofilm-associated infections are often chronic and difficult to treat due

to the intrinsic resistance of the bacteria and the challenge of completely eradicating the biofilm [6,7].

Additionally, biofilms can act as reservoirs for pathogenic bacteria, which can spread to other parts of the body or to other individuals, especially in hospital environments [52]. The presence of biofilms on medical devices, such as urinary catheters and heart valves, is a common cause of nosocomial infections, which can lead to severe complications and increased mortality [53,54].

4. Adaptation System: From Susceptible Planktonic to Resistant Sessile

The transition of bacteria from a planktonic state (free-moving) to a sessile state (adhered to surfaces) is a complex, multi-phase process regulated by a combination of environmental and genetic factors [40,55]. This process is fundamental to both microbial ecology and human health, as biofilms are often associated with persistent infections and antimicrobial resistance [53,56]. Below, we detail each phase of this process, emphasizing the molecular mechanisms as well as their ecological and clinical implications [6,52].

4.1. Phase 1: Initial Adhesion

Initially, planktonic bacterial cells recognize and adhere to biotic surfaces (such as plant or animal tissues) or abiotic surfaces (such as plastics, metals, or rocks). This process is often triggered by adverse environmental conditions, including nutrient scarcity, fluctuations in pH or temperature, or mechanical stress [57,58]. Initial adhesion is mediated by specialized cellular structures, such as fimbriae, pili, and flagella, which enable bacteria to anchor to the surface [56,59]. Additionally, cell surface proteins, such as adhesins, play a crucial role by interacting with specific surface molecules through receptor–ligand binding [60]. These interactions can be either specific, depending on molecular compatibility, or non-specific, based on electrostatic or hydrophobic forces [61]. At this early stage, cells begin secreting small amounts of EPS, but a well-structured matrix has not yet formed. Initial adhesion is reversible, allowing cells to detach if conditions are unfavorable [62] (Figure 1A).

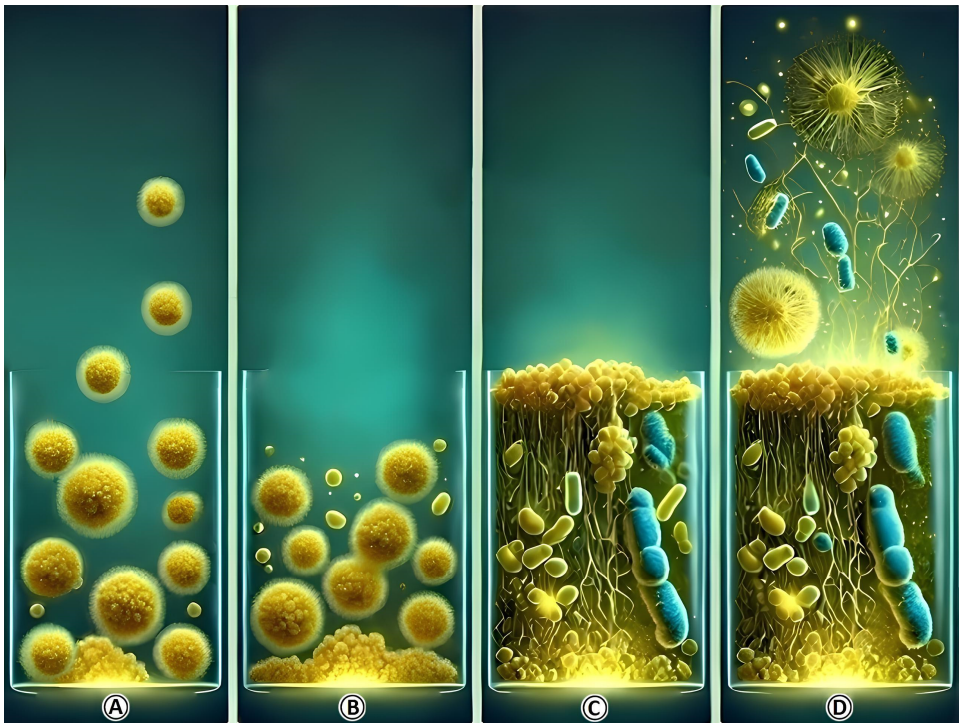


Figure 1. Polymicrobial biofilms. Structurally complex microbial communities composed of multiple interacting species, occasionally identified in storage and distribution systems of raw materials within

the pharmaceutical industry. (**A**) Phase 1: Initial Adhesion. This phase represents the initial stage of biofilm development, during which microbial aggregates in aqueous suspension sediment and transiently adhere to biotic or abiotic surfaces. Adhesion is mediated by physicochemical forces, including hydrophobic and electrostatic interactions, and is initially reversible, allowing cells to migrate or detach from the surface. (**B**) Phase 2: Growth and Intercellular Communication. After irreversible adhesion, microorganisms actively proliferate, forming organized microcolonies. Concurrently, the production of extracellular polymeric substances (EPS) increases, contributing to the development of the biofilm matrix, a complex three-dimensional network that offers mechanical protection, nutrient retention, and structural cohesion. Intercellular communication is mediated by quorum sensing (QS) systems, through which signaling molecules regulate the expression of genes involved in virulence, antimicrobial resistance, and EPS biosynthesis. (**C**) Phase 3: Maturation. During this stage, the biofilm acquires a highly organized and functional three-dimensional architecture. Biochemical and physical interactions between cells intensify, promoting metabolic cooperation, horizontal gene transfer, and enhancement of resistance mechanisms against antimicrobial agents and environmental stressors. Marked physiological heterogeneity is observed, with cells in diverse metabolic states, including viable but non-culturable (VBNC) forms, persister cells, and actively dividing populations. In industrial polymicrobial biofilms, this phase exemplifies a dynamic equilibrium between cooperative and competitive interactions, enhancing biofilm stability and resilience. (**D**) Phase 4: Dispersion and Recycling. This final phase is characterized by the release of individual cells or small aggregates from the mature biofilm. Dispersion is triggered by environmental signals and internal regulatory pathways, enabling colonization of new ecological niches and initiating a new cycle of biofilm formation. This dispersal mechanism plays a crucial role in microbial adaptation and dissemination across clinical, industrial, and natural settings [63].

4.2. Phase 2: Growth and Intercellular Communication

Following the initial adhesion phase, bacteria begin to multiply and establish intercellular communication through a process known as QS. This signaling mechanism enables microorganisms to synchronize the expression of specific genes based on population density [58]. QS plays a crucial role in activating genes related to EPS synthesis and biofilm development [59]. As EPS production increases, the extracellular matrix acquires a more structured organization, providing resistance and cohesion to the developing biofilm [60]. The progressive increase in bacterial population and the continuous accumulation of EPS lead to the formation of intricate three-dimensional arrangements, which can take on various shapes, such as mushroom-like structures or other organized conformations [61]. These arrangements consist of interconnected microcolonies that facilitate nutrient circulation and the transmission of signaling molecules within the environment [60] (Figure 1B).

4.3. Phase 3: Maturation

When the biofilm reaches its "maturity" stage, the extracellular matrix becomes the predominant component, consisting of a complex combination of polysaccharides, lipids, proteins, and eDNA [40]. This matrix not only provides structural support but also acts as a protective barrier, shielding bacteria from external threats such as antimicrobial agents and host defense mechanisms [64]. However, the high density of the matrix can hinder metabolite exchange, leading to the accumulation of potentially toxic substances within the biofilm [60]. To counteract this issue, some bacteria activate enzymatic mechanisms to degrade EPS components, triggering cell dispersion [59]. This stage is crucial for the propagation of microorganisms to new niches, enabling the colonization of other surfaces and the reinitiation of the biofilm formation cycle [65] (Figure 1C).

4.4. Phase 4: Dispersion and Recycling

In the final stage, the partial degradation of the extracellular matrix enables the release of previously attached cells, which return to the planktonic state [40]. This dispersion

mechanism is essential for biofilm propagation across diverse ecosystems and is directly linked to the persistence of chronic and recurrent infections [64]. The ability of bacteria to transition between planktonic and sessile lifestyles provides a significant evolutionary advantage, allowing these microorganisms to adapt to variable environmental conditions and develop resistance to antimicrobial therapies [59] (Figure 1D).

4.5. Temperature Variation

Temperature is a critical environmental factor that significantly influences biofilm formation, particularly in species belonging to the *Burkholderia* genus. While the optimal temperature range for the growth of these microorganisms is between 30 and 35 °C, research shows that more robust biofilm production tends to occur under thermal conditions that reflect the natural environment of the strains [14,66]. Silva [67] analyzed the biofilm-forming ability of *B. cepacia* isolates at 20 °C, the average water temperature in Botucatu, Brazil, and at 35 °C, considered optimal for bacterial growth. The results revealed that at 20 °C, 93.3% of the isolates were classified as biofilm producers, while at 35 °C, only 51.7% exhibited this characteristic. These data suggest that lower temperatures, closer to the bacterium's natural habitat, may stimulate biofilm formation, possibly providing an adaptive advantage in natural environments.

It is important to highlight that the ideal temperature for biofilm formation may vary among different *Burkholderia* species and strains, influenced by factors such as the availability of carbon sources and specific conditions of the culture medium. Therefore, when studying biofilm formation in this genus, it is essential to consider the environmental and physiological particularities of each species [14].

5. Extracellular Matrix: Complex Structure

The extracellular matrix (ECM) of biofilms is a dynamic and multifunctional structure primarily composed of EPS, which play crucial roles in adhesion, cohesion, protection, and functionality of microbial communities [40]. The ECM is not merely a passive byproduct of bacterial activity, but rather an active and indispensable component for the stability and resilience of biofilms [41]. The majority of biofilm biomass is made up of highly hydrated EPS, representing between 85 and 98% of its total composition [40]. This high level of hydration gives the matrix a gel-like consistency, which facilitates the diffusion of nutrients, chemical signaling molecules, and metabolites, while also acting as a physical barrier against external agents such as antimicrobial substances and immune system cells [64,68].

The composition of the ECM is highly diverse, including a variety of macromolecules such as lipids, proteins, eDNA, and EPS [40]. EPS are the most abundant and structurally significant components of the ECM, functioning as the "skeleton" of the biofilm [41]. They consist of long-chain polymers, such as cellulose, alginate, Pel, and Psl, which vary in structure and function depending on the bacterial species and environmental conditions [56].

In addition to EPS, ECM proteins play crucial roles in stabilizing the matrix and mediating cell–surface and cell–cell interactions. Adhesive proteins, such as lectins, facilitate binding to both biotic and abiotic surfaces, while extracellular enzymes, such as proteases and lipases, assist in the degradation of complex nutrients and the remodeling of the matrix [40]. eDNA is another critical component of the ECM, acting as a "glue" that holds the cells together and contributes to resistance against mechanical and chemical stresses. eDNA is primarily released through cell lysis or extracellular vesicles and can interact with other matrix macromolecules, such as polysaccharides and proteins, to form cohesive structural networks [69,70].

The macromolecular composition of the ECM is not static and varies significantly in response to environmental factors such as nutrient availability, pH, temperature, oxygen

presence, and mechanical stresses. For instance, in environments with low carbon availability, bacteria may prioritize the synthesis of reserve polysaccharides, like glycogen [71], while under oxidative stress conditions, the production of eDNA and heat shock proteins may increase [68,72]. This plasticity in ECM composition reflects the adaptive capacity of bacteria to form biofilms across a wide range of environments, from industrial surfaces to human tissues [40,73].

The variability of the ECM is also influenced by the polymicrobial nature of many biofilms. In mixed communities, different bacterial species can contribute distinct types of EPS, creating a more complex and functional matrix. For example, in biofilms associated with chronic infections, such as those found in diabetic wounds or lung infections in cystic fibrosis patients, the interaction between *Burkholderia* and other species such as *Pseudomonas* and *Staphylococcus*, results in a highly heterogeneous matrix that confers resistance to multiple antimicrobial treatments [40,42]. This interspecies synergy can lead to the production of complementary EPS, such acidic polysaccharides by *Burkholderia*, which together enhance the robustness of the biofilm [74,75].

The complexity of the ECM presents significant challenges for research and the development of biofilm control strategies. The variability in matrix composition complicates the identification of universal therapeutic targets, as the components of the ECM can differ between species and even between strains of the same species [6,40]. Additionally, the ability of bacteria to modulate EPS production in response to environmental stresses makes biofilms highly adaptable and resistant to external interventions [76,77].

6. Molecular Mechanisms Associated with Substrate Attachment

6.1. Flagellum: Motility and Immunomodulation

Flagellin, the main protein component of bacterial flagella, plays a crucial role in biofilm formation in *Burkholderia*, particularly during the initial phase of adhesion to surfaces. The motility provided by the flagella facilitates the movement of bacterial cells, promoting more efficient adhesion and resulting in the formation of more highly persistent biofilms. In *B. pseudomallei*, for example, strains with intact flagella exhibit a significantly higher capacity to form highly persistent biofilms compared to flagellin-deficient strains [78,79]. Mutants in the *fliC* gene, responsible for encoding flagellin, show a marked reduction in biofilm formation compared to wild-type strains, highlighting the importance of this component not only in motility but also in adhesion and biofilm establishment [80,81].

In addition to its structural and motility functions, flagellin from *Burkholderia* exhibits immunogenic properties that play a key role in modulating the host's inflammatory response. It is recognized by the innate immune system through Toll-like receptors (TLR5), which are responsible for detecting pathogen-associated molecular patterns [78,82]. This interaction triggers an inflammatory response, promoting the production of pro-inflammatory cytokines, such as interleukins and tumor necrosis factor (TNF) [83,84]. While this response is crucial for host defense, excessive inflammation can damage tissues, contributing to pathogenesis [81,85].

In addition to recognition by TLR5, flagellin can be internalized by host cells and detected in the cytoplasm by intracellular sensors, such as NOD-like receptors (NLRs). Specifically, it is recognized by NLRs NAIP5 and NLRC4, which form the inflammasome complex [86,87]. Activation of this inflammasome triggers caspase-1 activation, resulting in the cleavage of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and interleukin-18 (IL-18), as well as inducing a programmed cell death mechanism known as pyroptosis [88,89]. This process is crucial for host defense against bacterial infections,

including those caused by *Burkholderia*. Pyroptosis helps to limit the spread of the infection but can also contribute to tissue destruction and disease progression [38,90].

The expression of flagellin in *Burkholderia* is regulated by various environmental factors and cellular signals. Variables such as nutrient availability, temperature, and the presence of specific chemical signals directly influence the expression of genes related to flagella. For example, under conditions of nutritional stress or lower temperatures, flagellin expression can be increased to facilitate motility and the search for new ecological niches [78,80]. This regulation allows bacteria to adjust their motility and adhesion capacity according to environmental conditions, optimizing biofilm formation and colonization of different habitats [79,81].

6.2. Pili: Connecting Motility and Adhesion

Structures such as fimbriae and pili in *Burkholderia* play crucial roles in the initial adhesion to surfaces, being essential for biofilm formation and maturation. Pili, in particular, is a filamentous structure located on the bacterial surface that facilitate adhesion to a variety of surfaces, both biotic and abiotic [79,80]. In addition to their primary role in adhesion, these structures also modulate the host's immune responses, influencing processes such as phagocytosis and interaction with immune system cells [78,81]. This ability to interact with the host promotes bacterial survival and persistence, contributing to its virulence and pathogenicity [85,91].

Studies indicate that species of the Bcc possess various types of pili, including network, filamentous, spiny, and cable-type forms. Cable pili is known for its role in adhesion to human epithelial cells, being crucial for biofilm formation on biotic surfaces [92,93]. However, the genes responsible for encoding this pili are not uniformly distributed among the different Bcc species. For example, the *cblA* gene, associated with the production of cable pili, is not present in all species, and even among those that possess the gene, not all express these pili in a functional manner [42]. This genetic variability suggests that the presence and function of cable pili may vary significantly among different species and strains of Bcc, which may influence biofilm formation and antibiotic resistance [94].

Although cable pili has been implicated in the initial adhesion to host cells, its specific role in biofilm formation is not yet fully understood. Some studies suggest that other types of pili or adhesion mechanisms may compensate for the absence of cable pili in certain strains, allowing for the formation of highly persistent biofilms [92,93]. Additionally, flagellar motility also contributes to initial adhesion and biofilm formation by facilitating the movement of bacterial cells toward surfaces and promoting cell-to-cell interactions that are essential for biofilm development [42,95]. The variability in the expression of these mechanisms among *Burkholderia* species and strains may have implications for the bacterium's resistance to different treatments and its ability to persist in clinical environments [94].

In *B. cenocepacia* H111, the *fimA* gene encodes a type I pilin protein regulated by the CepIR quorum sensing (QS) system. However, a *fimA* mutant did not show significant defects in biofilm biomass on abiotic surfaces, suggesting that other pili genes may compensate for its absence [92]. Additionally, other factors, such as the large adhesion *bapA* gene, are also crucial for biofilm structure and architecture [42]. The presence of these additional genes highlights the complexity of biofilm formation, as well as the diversity of adhesion mechanisms present in different strains [93,94].

Additionally, type IV pili (T4P) plays a crucial role in twitching motility, a movement characterized by abrupt contractions that allow the bacterium to move along solid surfaces. This form of motility is distinct from that provided by flagella and is associated with biofilm formation and pathogenicity in various bacteria [96,97]. While twitching motility mediated by T4P is well documented in other bacterial species, such as *P. aeruginosa*, its occurrence

and specific function in *Burkholderia* still require further investigation [42,93]. Modulation of these structures, as well as post-translational modifications in T4P, such as glycosylation, may provide insights into the bacterium's interaction with host tissues and environmental surfaces, influencing both virulence and infection persistence [96].

6.3. Type III Secretion System: Pathogen–Host Interaction

The T3SS (Type III Secretion System) is a highly specialized virulence factor present in several *Burkholderia* species, including *B. mallei*, *B. pseudomallei*, and *B. cenocepacia*. This system functions as a "molecular syringe", injecting effector proteins directly into the host cell's cytoplasm, thereby subverting cellular processes in favor of the bacterium [98]. The presence and diversity of the T3SS in *Burkholderia* suggest a crucial role in pathogenesis, immune evasion, and adaptation to different ecological niches. The T3SS consists of over 20 structural proteins that form a multiprotein complex, including a needle that traverses the host cell membrane and a pore that allows the passage of effectors [99,100].

In *B. pseudomallei*, the T3SS is essential for intracellular survival and the pathogenesis of melioidosis, a severe disease affecting humans and animals. The bacterium uses this system to escape from the phagosome following phagocytosis by macrophages and other immune cells, allowing it to replicate in the host cell's cytoplasm [101]. One of the key effectors secreted by *B. pseudomallei* is the protein BopA, which contributes to the evasion of autophagy, a cellular defense mechanism aimed at eliminating intracellular pathogens. Additionally, the T3SS is also associated with biofilm formation, conferring antibiotic resistance and promoting infection persistence. Studies have shown that T3SS-deficient mutants have a reduced capacity to form biofilms and are less virulent in animal models [99,100,102].

In *B. cenocepacia*, the T3SS plays a crucial role in interaction with epithelial cells and immune evasion, being a determining factor in the colonization of CF patients. Studies suggest that this system contributes to the remodeling of the host cell cytoskeleton, facilitating bacterial adhesion and invasion [103,104]. For example, the effector BopE, secreted by *B. cenocepacia*, acts as a GTPase-activating protein that induces cytoskeletal rearrangements, promoting bacterial internalization. However, the presence and expression of genes related to the T3SS vary across different Bcc species, influencing their ability to cause disease and form biofilms. This genetic variability presents a challenge in understanding the exact mechanisms by which the T3SS contributes to the virulence and adaptation of different strains in both hospital and community environments [42,93,105].

Comparative genomic studies have been conducted to understand the distribution and evolution of T3SS in *Burkholderia*. Evidence suggests that different lineages within the genus possess distinct T3SS subtypes, indicating that this system has evolved to perform specific functions in different ecological contexts [106,107]. For example, some *Burkholderia* sensu stricto species harbor T3SS variants associated with pathogenicity in mammals, while *Paraburkholderia* species exhibit homologous systems that may play a role in interactions with plants and other environmental organisms [15,108]. These findings suggest that the T3SS may not only be involved in infecting animal hosts but also play a role in symbiosis and microbial competition in natural environments [42,92].

The integration of genomic and transcriptomic approaches has provided new insights into the regulation of the T3SS and its relationship with biofilm formation. Evidence suggests that the activation of the T3SS may be coordinated with the production of EPS and other virulence factors, enhancing the bacterium's ability to colonize different surfaces and persist in chronic infections [99,102]. Additionally, gene expression profiling has shown that T3SS regulation can be modulated by environmental signals, such as the presence of specific nutrients and interactions with host cells. For instance, the expression of the T3SS

in *B. pseudomallei* is regulated by factors like iron availability and cell density, indicating an adaptive response to environmental conditions [42,92].

Another relevant aspect is the interaction of the T3SS with components of the host's immune system. Studies suggest that certain effectors secreted by *Burkholderia* can interfere with the activation of the inflammasome, a protein complex crucial for the innate immune response [92,109]. This modulation may influence the inflammatory response and determine the progression of the infection, making the T3SS a potential target for the development of new therapeutic strategies. Selective inhibition of T3SS components, through small molecules or specific antibodies, could represent a promising approach to reduce the virulence of *Burkholderia* without compromising the patient's natural microbiota [42,102].

Furthermore, research indicates that the T3SS may be involved in complex interactions with other microorganisms, including fungi. This function suggests a possible role in microbial competition and colonization of specific niches, highlighting the versatility of this secretion system [42,106]. In environmental contexts, the T3SS could provide *Burkholderia* with a competitive advantage by allowing it to exclude competing microorganisms and adapt to new environments. For example, in interactions with fungi, the T3SS may be used to inhibit the growth of competitors or to establish symbiotic relationships [92,107].

6.4. Lipopolysaccharides: Adhesion and Immune Evasion

Infections caused by *B. pseudomallei* are often associated with recurrences, especially in immunocompromised patients, such as individuals with diabetes. One of the factors contributing to the persistence of these infections is the bacterium's ability to form biofilms, structures that confer resistance to conventional antimicrobials and hinder the complete eradication of the pathogen. Research indicates that biofilm formation by *B. pseudomallei* is directly related to an increase in relapse rates in patients with melioidosis [42,78,92].

Moreover, the lipopolysaccharide (LPS) produced by *B. pseudomallei* plays a central role in the pathogenesis of infections. The LPS of this species has unique structural characteristics that distinguish it from other Gram-negative bacilli, reducing its immunogenicity and facilitating evasion of the host's immune response [37,110]. Studies show that the LPS of *B. pseudomallei* has a distinct molecular structure, with a low capacity to activate phagocytic cells, such as macrophages and neutrophils, resulting in a milder immune response and reduced production of pro-inflammatory cytokines [111]. This ability to modulate the immune response is particularly relevant in immunocompromised patients, such as diabetics, in whom the immune response is already compromised, promoting the persistence and recurrence of the infection [112].

The LPS of *B. pseudomallei* is composed of three main regions: lipid A, the oligosaccharide core, and the O antigen. Lipid A, in particular, exhibits structural modifications that reduce its immunological activation capacity. It is penta-acylated and contains a modified bifosphorylated group with 4-amino-4-deoxy-arabinose (Ara4N) on both phosphates, as well as long-chain fatty acids such as C(14:0)(3-OH), C(16:0)(3-OH), and C(14:0)(2-OH). The presence of the latter fatty acid is exclusive to *B. pseudomallei* and is not found in related species. These modifications in lipid A are crucial for immune evasion, as they reduce the binding of LPS to TLR4-type receptors, which are responsible for detecting pathogen-associated molecular patterns and triggering an acute inflammatory response [37,110,112] (Figure 2).

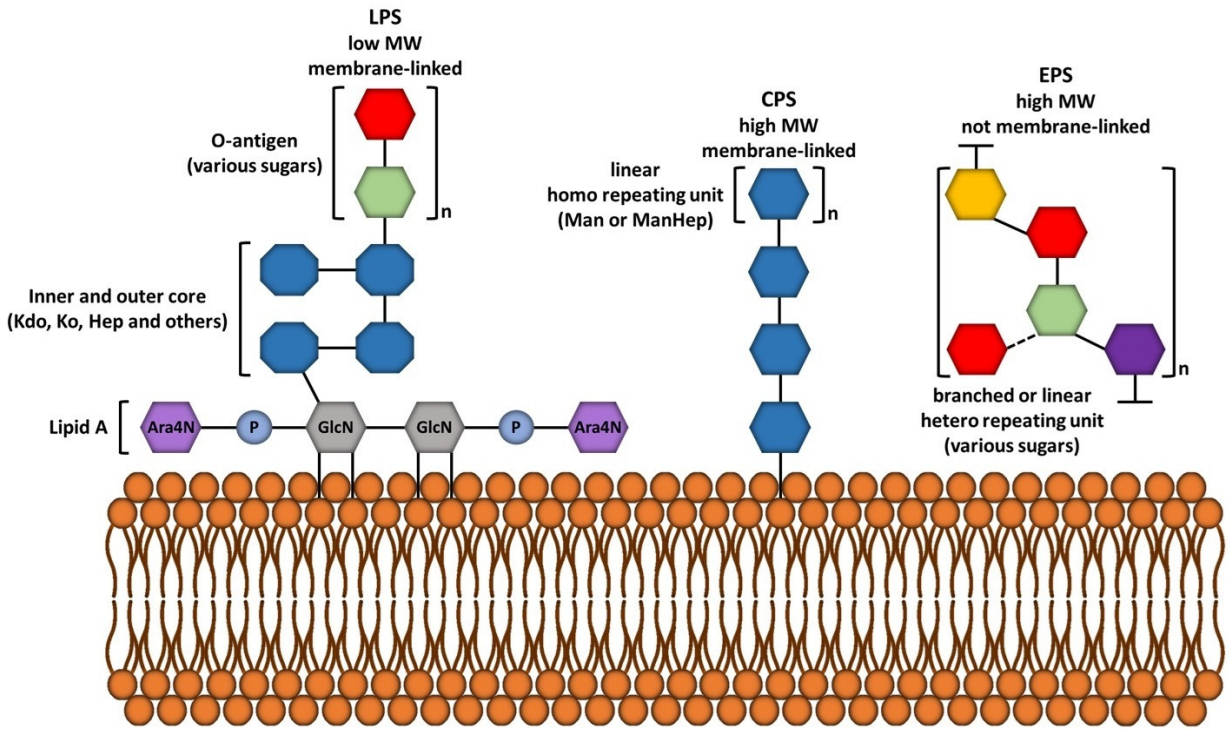


Figure 2. Schematic overview of the main surface polysaccharides produced by *Burkholderia* spp. LPS, lipopolysaccharide: Lipid A, 2-amino-2-deoxy-D-glucose (GlcN) is penta-acylated with a bifosphorylated group and with 4-amino-4-deoxy-L-arabinose (Ara4N), in addition to long-chain fatty acids such as C(14:0)(3-OH), C(16:0)(3-OH), and C(14:0)(2-OH); the O-antigen is diversified with several sugars, with two main serotypes standing out: type A and type B. CPS, capsular polysaccharide: composed of an unbranched homopolymer featuring residues of 2-O-acetyl-6-deoxy-β-D-manno-heptopyranose linked by (1→3) linkages. EPS, exopolysaccharide: includes EPS-I, cepacian, cocepacian, KdoEPS, levan, and poly-β-(1/6)-N-acetyl-2-deoxy-glucosamine (PNAG). Abbreviations: low molecular weight (low MW); high molecular weight (high MW); 2-amino-2-deoxy-D-glucose (GlcN); 3-deoxy-D-manno-oct 2-ulosonic acid (Kdo); D-glycero-D-talo-oct-2-ulosonic acid (Ko); D-mannose (Man); 6-deoxy-D-manno-heptose (ManHep); heptose (Hep) [113].

The O antigen of the LPS is highly diversified, with two main serotypes described: type A and type B. Type A is predominant in strains from Southeast Asia and is associated with more severe infections, while type B is more common in Australian strains and appears to be related to lower virulence [114]. The O antigen of type A consists of repeating disaccharides of 6-deoxy-α-L-talopyranose and β-D-glucopyranose, frequently modified by acetylation and methylation. Type B has a more complex structure, with additional substitutions of β-D-xylopyranose in some rhamnose residues [115]. This structural diversity has significant implications for the development of diagnostics and vaccines against *B. pseudomallei*, as antigenic variability can hinder the creation of universal immunogens [110,116].

The identification of specific antibodies against the O antigen has been associated with protection in experimental models of melioidosis [114]. However, the genetic variability of the LPS and its low immunogenicity present challenges for the development of effective vaccines [116]. Additionally, the modified LPS may influence the bacterium's ability to persist in the host by preventing the effective activation of the innate immune system. For example, the low activation of TLR4 by *B. pseudomallei* LPS results in reduced production of pro-inflammatory cytokines, such as TNF-α and IL-6, which may contribute to the persistence of the bacterium in host tissues [117].

In addition to its role in immune evasion, *B. pseudomallei* LPS also plays a role in biofilm formation. Studies suggest that LPS may interact with components of the ECM, such as EPS

and proteins, to stabilize the biofilm structure and increase its resistance to environmental stresses. This interaction may be particularly important in hospital settings, where *B. pseudomallei* biofilms are often associated with chronic and recurrent infections [42,78,118].

6.5. Exopolysaccharides: Structural Diversity and Implications in Pathogenesis

The production of EPS by Bcc has been extensively studied, revealing the diversity and complexity of these polysaccharides. Cepacian (PS-I and PS-II) is the most identified EPS, but other forms of EPS also play significant roles in pathogenesis and biofilm formation. In addition to providing structural protection and resistance to adverse environmental factors, EPS modulate the interaction between *Burkholderia* and the host, contributing to immune evasion and the persistence of the infection [119,120] (Figure 2).

The genes in the *bceABCDEFGHIJKL* cluster encode the enzymes required for the formation of sugar precursors, the glycosyltransferases, and the proteins involved in the polymerization and export of cepacian. These genes are highly conserved within Bcc and are regulated by environmental signals, such as nutrient availability, the presence of oxidative stress, and QS [121]. The QS system plays a crucial role in regulating EPS production, allowing bacteria to coordinate gene expression in response to population density [42]. Interestingly, the *B. cenocepacia* J2315 strain does not produce cepacian due to a frameshift mutation in *bceB* (BCAM0856), which encodes a putative glycosyltransferase essential for the biosynthesis of the polysaccharide [120]. This mutation indicates that some Bcc lineages may rely on other mechanisms for biofilm formation and antimicrobial resistance, such as the production of other EPS or the expression of adhesins and surface proteins [120,121].

Cepacian is frequently acetylated, and although the role of acetylation remains unknown, it is speculated that this modification may protect bacteria against opsonization and phagocytosis, similar to alginate in *P. aeruginosa*. Studies suggest that, in CF patients infected with *P. aeruginosa*, mucoid isolates producing alginate are associated with progressive deterioration of lung function and increased resistance to both antimicrobials and the immune system. These factors may also be attributed to cepacian in *Burkholderia*, although further studies are needed to confirm this hypothesis. Acetylation may alter the physicochemical properties of the EPS, increasing its hydrophobicity and consequently its ability to adhere to surfaces and form more stable biofilms [120].

Cunha et al. [122] reported that cepacian is not essential for biofilm formation, although it may enhance their formation, making them more robust and mature. The transition from a mucoid to a non-mucoid phenotype through variation in cepacian production was observed in Bcc isolates from patients with CF. This observation suggests that EPS production is involved in the persistence of infections, while the loss of EPS production may increase the invasiveness of these strains, facilitating their spread within the host. This phenotypic plasticity may be an adaptive strategy to survive in different environments, such as the respiratory tract of CF patients, where conditions can vary widely [121,122].

The EPS of Bcc are not only essential for the architecture of biofilms but also act as a physical barrier that protects encapsulated bacterial cells against environmental insults, such as antibiotics and UV light, in addition to capturing reactive oxygen species (ROS) and interfering with neutrophil chemotaxis. For example, the EPS barrier slows the diffusion of small molecules, such as hydrogen peroxide, preventing them from efficiently reaching the bacterial cells within the biofilm. Furthermore, it decreases the rate of drug penetration, providing crucial time for other resistance mechanisms, such as the activation of β-lactamase expression and antibiotic efflux, to act. This biofilm-mediated resistance represents a significant challenge in the treatment of Bcc infections [120,123].

Negatively charged polysaccharides, through their linkages in the EPS, can effectively block the penetration of positively charged antibiotics, such as aminoglycosides. Additionally, the ECM of biofilms can act as a reservoir for virulence factors, such as degradative enzymes and siderophores, which contribute to the adaptation and survival of Bcc in hostile environments. This biofilm-mediated resistance represents a significant challenge in the treatment of Bcc infections, especially in immunocompromised patients [120,121].

In addition to cepacian, other EPSs have been identified in Bcc species, such as galactan and mannan, which may play distinct roles in host interaction and adaptation to different ecological niches. The identification and characterization of these molecules are crucial for the development of effective therapeutic strategies, including the use of EPS synthesis inhibitors, degradative enzymes, and combination therapies aimed at biofilm dispersion. For example, dispersin B, an enzyme that degrades ECM polysaccharides, has been explored as a strategy to enhance the effectiveness of antibiotics against biofilms [120,123].

Studies suggest that EPS production may be regulated by environmental factors, such as iron availability and the presence of other microorganisms in the pulmonary microbiota. This complex regulation highlights the need for integrated approaches that combine genomic, transcriptomic, and proteomic analyses to better understand the biosynthesis mechanisms and functions of EPS in Bcc species. The integration of these approaches may reveal new therapeutic targets and strategies to combat persistent infections caused by these opportunistic bacteria [120,121].

6.6. Extracellular DNA: Structural Component and Resistance

eDNA is a fundamental component of the ECM in bacterial biofilms, playing essential structural and regulatory roles [70]. It facilitates the initial adhesion of bacterial cells to surfaces, promotes cellular cohesion, and contributes to resistance to antimicrobial agents [124]. In biofilms formed by *Burkholderia* species, such as *B. cepacia*, *B. pseudomallei*, and *B. thailandensis*, eDNA is involved in multiple physiological and structural processes, influencing biofilm formation, maturation, and dispersion [125]. eDNA can be released through various mechanisms, including controlled cell lysis, active secretion, and stress-induced release. Programmed cell lysis of a subpopulation of cells can release eDNA as a structural and virulence factor [126]. Furthermore, some bacteria secrete eDNA through outer membrane vesicles, a mechanism that may be relevant for *Burkholderia* [125]. Environmental factors, such as oxidative stress, exposure to antibiotics, and microbial competition, can also trigger eDNA release, aiding bacterial adaptation and biofilm stability [41].

Studies indicate that eDNA primarily concentrates at the base of the biofilm matrix of *Burkholderia*, suggesting its importance in initial adhesion and structural organization. In a mouse stomach tissue model, Austin et al. [127] observed significant accumulation of eDNA from *B. pseudomallei* K96243 during colonization, without cell lysis, which resulted in an increase in the number of cells adhering to the stomach tissue. This eDNA accumulation was associated with the formation of a structural network that facilitated adhesion and cellular cohesion. On the other hand, Garcia et al. [128] demonstrated that in *B. thailandensis*, eDNA is not required for initial attachment, but becomes essential for the cell–cell interaction in biofilm formation after 16 h. These data suggest that different *Burkholderia* species may utilize distinct mechanisms for eDNA during biofilm formation, reflecting the adaptive diversity of the genus.

Studies have shown that eDNA accumulation in the biofilm matrix of *B. pseudomallei* alters the microenvironment, making it more acidic and favoring resistance to aminoglycosides, a mechanism that may contribute to the prolonged survival of the pathogen in adverse environments. The acidification of the biofilm microenvironment reduces the effectiveness of antibiotics such as gentamicin, which are less active in acidic pH [125].

Additionally, eDNA can sequester divalent cations, such as calcium and magnesium, which are essential for bacterial membrane stability and the activity of certain antibiotics. The degradation of eDNA by DNase I has been shown to significantly impact the structure and stability of the biofilm [70]. The application of DNase I reduced biofilm adhesion, suggesting its therapeutic potential in preventing biofilms in CF patients, where *Burkholderia* poses a significant risk for chronic infection [124,129].

Confocal laser scanning microscopy analyses and biofilm quantification by COMSTAT have reinforced the importance of eDNA. Studies have shown that treatment with DNase I significantly reduced biofilm formation, depending on the stage at which the enzyme was added. When DNase was introduced early in biofilm development, between 0 and 24 h, there was a significant reduction in biofilm structure formation, with a noticeable effect up to 48 h of growth [129,130]. This suggests that eDNA acts as an essential intercellular connector for the initial attachment and early development of the biofilm. However, the degradation of eDNA in mature biofilms had a variable effect, reducing formation in only two out of three clinical isolates of *B. pseudomallei*, suggesting that other ECM components may compensate for the loss of eDNA in later biofilm stages. These components include EPS, proteins, and lipids, which may assume structural functions similar to eDNA [129,130].

7. Population Density Sensing Mechanism

7.1. Quorum Sensing

The QS system is an essential mechanism for cellular communication that coordinates population-level behaviors in bacteria, regulating processes such as virulence, antimicrobial resistance, and biofilm formation [131–133]. This system was first described in the 1960s and 1970s in the bioluminescent marine bacteria *Vibrio fischeri* and *Vibrio harveyi* [134], and since then, it has been extensively studied in various microorganisms, including species of the Bcc [135]. QS operates through the production, release, and detection of signaling molecules called autoinducers (AIs), whose concentration increases proportionally to bacterial population density [131]. When these molecules reach a critical threshold, they activate transcriptional regulators that modulate collective gene expression, enabling the bacterial population to respond in a coordinated manner to different environmental conditions [132,136].

QS is primarily mediated by signaling molecules from the AHL family in Gram-negative bacteria, including *Burkholderia*. The classic AHL-based QS system consists of two main genes: one encodes the LuxI enzyme, responsible for synthesizing the signaling molecules, and the other encodes a LuxR family receptor, which detects the signal and regulates gene expression [137,138]. A classic example occurs in *V. fischeri*, where the AHL N-3-oxo-hexanoyl-homoserine lactone ($3OC_6$-HSL) diffuses through the cell membrane and binds to the transcriptional regulator LuxR, activating the expression of the *luxCDABEG* operon, responsible for bioluminescence [138,139].

In the Bcc, the first evidence of the presence of QS was obtained through cross-feeding experiments, in which spent culture supernatants from *P. aeruginosa* containing AHLs stimulated the production of siderophores, proteases, and lipases in *B. cepacia* 10661. These results suggested the existence of a functional QS system in *Burkholderia*, capable of responding to signals produced by other bacteria [140]. Subsequent studies identified multiple QS systems within the *Burkholderia* genus, with variations in the structure and function of the AHLs involved [141,142].

In *B. thailandensis*, a widely used model for studying pathogens of the *Burkholderia* genus, three distinct QS circuits have been identified: QS-1, QS-2, and QS-3 [138]. QS-1 consists of the BtaI1-BtaR1 pair and uses N-octanoyl-homoserine lactone (C_8-HSL) as a signaling molecule. QS-2 includes BtaI2-BtaR2, which regulates the production of N-3-

hydroxy-decanoyl-homoserine lactone ($3OHC_{10}$-HSL), while QS-3 consists of the BtaI3-BtaR3 pair and utilizes N-3-hydroxy-octanoyl-homoserine lactone ($3OHC_8$-HSL). These systems work in an integrated manner to regulate various aspects of bacterial physiology, including cell aggregation, EPS production, and biofilm formation [139,143].

B. thailandensis QS-deficient mutants exhibit defects in cell aggregation during the planktonic phase, resulting in less highly persistent biofilms. This occurs because QS regulates the production of EPS, such as capsular polysaccharides I (CPSI), CPSII, and CPSIII (also known as cepacian), which are essential for the structural cohesion of the biofilm. Deficiency in these polysaccharides compromises biofilm stability, making the bacteria more susceptible to environmental factors and antimicrobial agents [144–146].

QS plays a key role in biofilm formation by regulating bacterial motility, surface adhesion, microcolony formation, maturation, and dispersion [147]. In addition, it influences the production of virulence factors, such as proteases, lipases, and siderophores, contributing to the pathogenicity of opportunistic bacteria like *B. cepacia* and *B. pseudomallei* [148]. In *B. pseudomallei*, QS regulates the production of toxins and degradative enzymes, as well as contributing to the bacteria's survival inside macrophages [143]. QS is also involved in antimicrobial resistance by modulating the expression of efflux pumps and antibiotic-degrading enzymes [139,149].

QS is modulated by environmental factors such as nutrient availability, pH, presence of antibiotics, and ion concentration, allowing bacteria to adapt their physiology to the environmental conditions [147]. Additionally, QS can mediate complex interactions with other microorganisms, including fungi and competing bacteria [149]. In natural environments, QS can provide a competitive advantage for *Burkholderia* by enabling the exclusion of competing microorganisms and the colonization of specific niches [143]. For example, in interactions with fungi, QS can be used to inhibit the growth of competitors or to establish symbiotic relationships [139,148].

7.2. Quorum Sensing Through Acyl-Homoserine Lactone

All members of the Bcc encode at least one QS system composed of homologs of the LuxR and LuxI proteins, which are responsible for the synthesis and recognition of signaling molecules known as AHLs. The production of these molecules varies among different *Burkholderia* species and even among different strains, with their concentration and type directly depending on bacterial cell density and the interaction between these molecules [135,150].

The first genes involved in QS in Bcc were identified through a transposon insertion mutant screening, which resulted in the overproduction of siderophores in Chrome Azurol S agar medium. This research revealed that the QS system of *B. cenocepacia* K56-2 is mediated by the AHL synthase CepI, which primarily produces C_8-HSL and, to a lesser extent, N-hexanoyl-homoserine lactone (C_6-HSL). The transcriptional regulator CepR, when activated by these AHLs, modulates the expression of various virulence genes, including extracellular proteases (ZmpA and ZmpB), chitinases, lipopeptide toxins, and other pathogenicity-related factors [137,151] (Figure 3A).

The QS system of Bcc consists of two main gene sets: *cepIR* and *cciIR*. These systems are widely distributed among different *Burkholderia* species and are considered essential for regulating the expression of virulence factors. The *cepIR* system, present in all Bcc species, positively regulates the expression of several virulence factors, including protease production and biofilm formation. The *cciIR* system, in turn, is also involved in virulence regulation and interspecies interactions among *Burkholderia* [132,150].

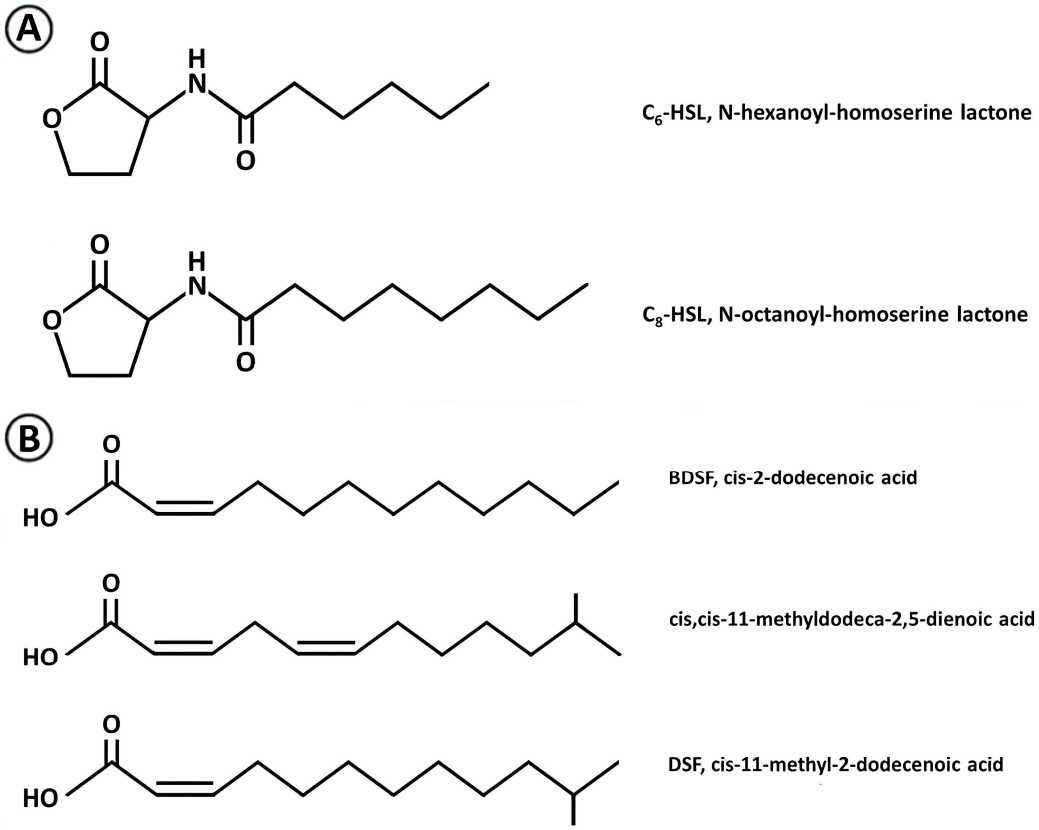


Figure 3. Different signaling molecules in quorum-sensing produced by Bcc species. (**A**) Signaling molecules in AHL-based; (**B**) signaling molecules in the DSF family-based [152].

The mechanism of action of both systems is similar: at low cell densities, there is a basal production of AHLs by the CepI synthase, which accumulates as the cells multiply. When the concentration of AHLs reaches a critical threshold, they bind to the CepR regulator, activating the expression of genes related to virulence and biofilm formation [132]. This mechanism allows bacteria to coordinate population-wide behaviors in response to changes in cell density and environmental conditions, as previously mentioned [132,150].

The interaction between the CepIR system and biofilm formation is crucial. In *B. cenocepacia* H111, mutants with deletions in the *cepI* or *cepR* genes exhibit a significant reduction in biofilm maturation, forming thinner and less structured biofilms [135]. Interestingly, in *B. cenocepacia* K56-2, which possesses both the CepIR and CciIR systems, the inactivation of either system also impairs biofilm formation. However, mutations in *cciI* or *cepI*/*cciI* do not produce the same effect, suggesting that the CciIR system plays a secondary role compared to CepIR in biofilm formation [150,153] (Figure 4).

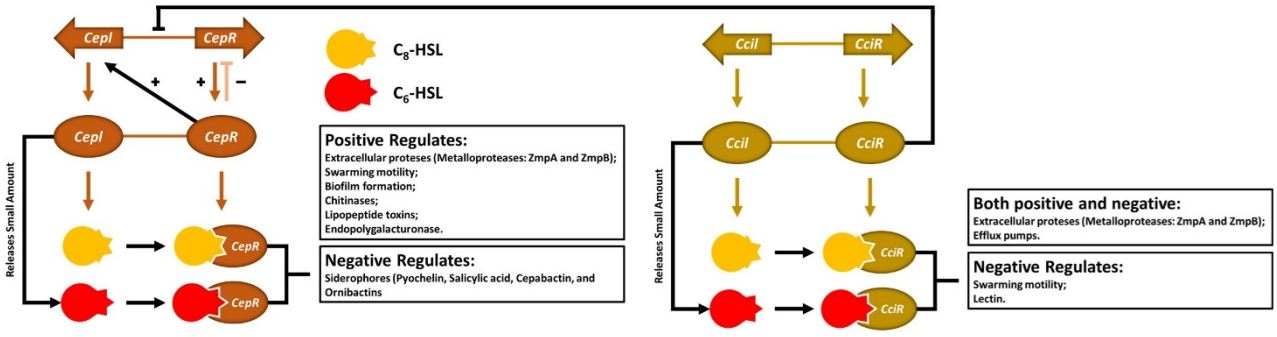


Figure 4. Quorum-sensing scheme in *Burkholderia* mediated by C_8-HSL and C_6-HSL [154].

The diversity in AHL production within the Bcc is remarkable. While some species, such as *B. multivorans* and certain strains of *B. cenocepacia*, produce very low levels of C_8-HSL (less than 1 nM), others can generate significantly higher concentrations, ranging from 10 nM to 1 mM, depending on the species and strain [155,156]. This variation suggests that the regulation of the QS system in *Burkholderia* is complex and that the expression of *cepI* may be influenced by additional regulatory factors that are not yet fully understood. For instance, in *B. multivorans* ATCC 17616, mutants that produce high levels of AHLs do not exhibit mutations in the CepIR QS locus, indicating the presence of external regulatory mechanisms that affect AHL production [156,157].

The CepIR system can interact with other QS systems present in the Bcc, such as the BviIR system of *B. vietnamiensis*, which utilizes N-decanoyl-homoserine lactone (C_{10}-HSL) as a signaling molecule [158]. The interaction between these different QS systems may contribute to a more refined regulation of virulence and bacterial adaptation in competitive environments, such as those found in hosts or under adverse environmental conditions [153,159].

7.3. Quorum Sensing Through Cis-2-Unsaturated Fatty Acids

The signaling system mediated by cis-2-dodecenoic acid, known as the *Burkholderia* diffusible signal factor (BDSF), represents a distinct and essential cell-to-cell communication mechanism for regulating physiological and pathogenic processes in *Burkholderia*. Identified by Ryan et al. [149], BDSF is structurally similar to the diffusible signal factor (DSF) previously described in *Xanthomonas campestris* pv. *campestris*. However, the BDSF signaling system in *B. cenocepacia* exhibits unique characteristics, particularly regarding its regulation and interaction with other signaling pathways, such as the CepIR system and the cyclic diguanylate monophosphate (c-di-GMP) molecule [160,161].

BDSF is synthesized by the enzyme RpfF, encoded by the *rpfF* gene, which converts fatty acid precursors into cis-2-dodecenoic acid [161]. Unlike the CepIR system, which is regulated by positive feedback, the production of BDSF does not rely on this mechanism [147]. This suggests that the BDSF system operates independently and complements the AHL-based QS system. The expression of *rpfF* is modulated by environmental conditions such as nutrient availability, pH, and stress, allowing *B. cenocepacia* to adjust its physiology in response to changes in the environment [160,161] (Figure 3B).

BDSF plays a central role in regulating various physiological processes in *B. cenocepacia* [161]. In biofilm formation, BDSF regulates the production of c-di-GMP, a crucial second messenger for biofilm formation and stabilization [162]. In *B. cenocepacia* H111, biofilm formation is mediated by the interaction between c-di-GMP, the protein BerB, and the alternative sigma factor RpoN (σ54) [147]. This interaction activates the transcription of the *berA* gene, which encodes a transcriptional regulator responsible for the expression of a gene cluster (*bepA–L*) involved in the production of the exopolysaccharide Bep. Bep is essential for biofilm stability, providing resistance to antimicrobial agents and host defenses [42,163].

In virulence factor production, BDSF regulates the expression of genes related to the production of extracellular proteases and lipases, which are important for host tissue degradation and nutrient acquisition [164,165]. Additionally, BDSF influences swarming motility, a behavior associated with surface colonization and bacterial dissemination [147]. In antimicrobial resistance, BDSF is involved in regulating mechanisms of antibiotic resistance, such as the expression of efflux pumps and antibiotic-degrading enzymes. This ability allows *B. cenocepacia* to survive in hostile environments, such as those encountered in chronic infections [31]. In environmental adaptation, BDSF facilitates *B. cenocepacia*'s adaptation to adverse environmental conditions, such as pH variations, osmotic stress, and

nutrient limitation. This flexibility is particularly crucial for the survival of the bacterium in both hospital and natural environments [165,166].

The c-di-GMP is a central second messenger in the regulation of biofilm formation and other virulence processes in *B. cenocepacia*. BDSF modulates the levels of c-di-GMP, which, in turn, activate the BerB and RpoN-dependent signaling pathway. Activation of this pathway leads to the production of the exopolysaccharide Bep, an essential component for biofilm stability. Bep protects bacterial cells from antimicrobial agents and host defenses, contributing to infection persistence [42,163] (Figure 4).

Recent studies have investigated the composition and function of EPS produced by *Burkholderia*. In *B. cenocepacia* H111, an insoluble water-extractable exopolysaccharide was identified, composed of a repetitive tetrasaccharide unit with the structure [3)-α-D-Galp-(1→3)-α-D-Glcp-(1→3)-α-D-Galp-(1→3)-α-D-Manp-(1→]n. This polysaccharide is likely the Bep, which plays a crucial role in biofilm stabilization. Additionally, a soluble water-extractable exopolysaccharide was isolated, whose structure and function are still under investigation [167,168]. These studies are expanding our knowledge of biofilm formation mechanisms and composition in *B. cenocepacia*, revealing the complexity and dynamics of molecular interactions that ensure biofilm stability and resistance [168,169].

7.4. Quorum Sensing in Burkholderia mallei and Burkholderia pseudomallei

The detection of quorum sensing in *B. mallei* and *B. pseudomallei*, primary pathogens responsible for glanders and melioidosis, respectively, presents particularly complex characteristics compared to other *Burkholderia* species. These pathogens have sophisticated QS networks, utilizing multiple homologs of the LuxIR systems, involving various AHL signaling molecules. These signaling molecules play a crucial role in the regulation of physiological and pathogenic processes, such as biofilm formation, virulence factor production, and adaptation to adverse environmental conditions [166,170,171].

In *B. pseudomallei*, three homologs of the *luxI* gene and five homologs of the *luxR* gene have been identified, which are part of the AHL-based QS system [172]. These signaling systems are involved in the regulation of gene expression for various physiological and pathogenic traits [81]. Studies using mass spectrometry to analyze the supernatants of *B. pseudomallei* cultures and *luxI*-defective mutants revealed the production of several AHL signaling molecules, including C_8-HSL and C_{10}-HSL, which are also produced by other species within the Bcc [173]. Additionally, *B. pseudomallei* synthesizes additional AHL molecules, such as N-(3-hydroxyoctanoyl)-homoserine lactone, N-(3-hydroxydecanoyl)-homoserine lactone, and N-(3-oxotetradecanoyl)-homoserine lactone [174]. These molecules have unique characteristics that may be associated with specific physiological functions and the environmental adaptation of *B. pseudomallei* [175,176].

Mutagenesis studies of key components of the QS circuit, such as the *luxI* and *luxR* genes, have indicated that disruption of this system can significantly affect the pathogenicity of *B. pseudomallei*. Ulrich et al. [172] demonstrated that mutating components of the QS system increased the survival time of BALB/c mice exposed to the pathogen via aerosol, in addition to reducing organ colonization. Although virulence was attenuated, the production of extracellular protease, lipase, and phospholipase C was not significantly affected by the disruption of the QS system. This finding suggests that QS in *B. pseudomallei* specifically regulates pathogenicity without completely compromising the virulence functions mediated by these enzymes.

Additionally, inactivation of one of the *luxI* genes of *B. pseudomallei*, such as *pmlI*, has been associated with a reduction in virulence in murine models of intraperitoneal, subcutaneous, and intranasal infection [172]. Interestingly, the *pmlI* mutant exhibited overproduction of the metalloprotease MprA, an enzyme associated with *B. pseudomallei*

virulence [81]. This suggests that the QS system may modulate the production of proteins associated with virulence in a complex and specific manner, possibly to optimize pathogenicity in different host niches [137,174].

In *B. mallei*, the QS system appears to be very similar to that of *B. pseudomallei*, but with some notable differences. In particular, *B. mallei* lack a pair of *luxIR* genes identified in *B. pseudomallei*, called *bpmIR2* [172]. This *luxIR* pair, present in *B. pseudomallei*, shares the highest degree of homology with the BviIR system of *B. vietnamiensis* [174]. A genomic comparison between *B. pseudomallei* and *B. mallei* revealed that the region encoding *bpmIR2* was likely deleted during the evolution of *B. mallei*, resulting in the loss of this specific QS system [177,178]. This difference may have significant implications for the regulation of physiological and pathogenic processes, as the *bpmIR2* system could play a role in adapting to specific environments or modulating interactions with the host [30,172,177].

7.5. Quorum sensing Between Pseudomonas aeruginosa and Burkholderia cenocepacia

The chronic co-infection in the lungs of CF patients caused by *P. aeruginosa* and *B. cenocepacia* represents a significant clinical challenge due to the formation of highly resistant polymicrobial biofilms and the complex dynamics of bacterial interactions mediated by signaling molecules, such as AHLs [179]. These interactions not only influence the formation and stability of the biofilms but also modulate the virulence and adaptation of the bacteria to the hostile lung environment, making these infections particularly difficult to treat [29,180].

Studies using green fluorescent protein (GFP)-based biosensors have revealed that *B. cenocepacia* is capable of sensing and responding to AHL signals produced by *P. aeruginosa*, demonstrating cross-species communication [181]. However, *P. aeruginosa* does not respond to AHL molecules produced by *B. cenocepacia*, indicating that the communication is unidirectional [182]. This asymmetry in communication could have significant implications for the virulence of polymicrobial biofilms, where *B. cenocepacia* may be more influential in the collective behavior of the bacterial community [173,181].

To study these interactions, experimental models simulating the biofilm conditions found in the lungs of CF patients were used. These models include artificial flow chambers and alginate spheres implanted in mouse lung tissue [50,181]. These systems allow direct observation of AHL-mediated communication between *P. aeruginosa* and *B. cenocepacia* in a more natural and relevant environment for studying chronic lung infections. The results of these studies confirmed that the unidirectional communication between the two species can modulate biofilm formation and stability, as well as the expression of virulence factors [181].

The formation of polymicrobial biofilms by *P. aeruginosa* and *B. cenocepacia* significantly increases resistance to antimicrobials and the host immune system. Biofilms are protective structures that hinder the penetration of antibiotics and immune cells, creating an environment conducive to bacterial persistence [6]. AHL-mediated communication between the two species may result in a more stable biofilm that is harder to eradicate, exacerbating the severity of lung infections in CF patients [180,181,183].

The ability of *B. cenocepacia* to perceive AHL signals from *P. aeruginosa* but not respond to signals produced by *B. cenocepacia* suggests an adaptive mechanism that allows *B. cenocepacia* to manipulate the biofilm environment to its advantage, without reciprocation from *P. aeruginosa*. This unidirectional interaction may be beneficial to *B. cenocepacia* as it enables the regulation of its own virulence in a polymicrobial environment without submitting to the QS control of *P. aeruginosa*. This behavior may contribute to the persistence and pathogenicity of *B. cenocepacia* in chronic infections [181,184].

The cross-species communication mediated by AHLs between *P. aeruginosa* and *B. cenocepacia* has significant clinical implications, particularly in the context of pulmonary infections in CF patients. Modulating this interaction could provide new therapeutic strategies to reduce virulence and biofilm formation. For example, the inhibition of QS (quorum quenching) may be a promising approach to disrupt communication between the two species, destabilizing the biofilm and enhancing the effectiveness of conventional antimicrobial treatments [181,184]. This could be achieved through molecules that interfere with the synthesis, release, or detection of AHLs, or that block the bacterial response to these molecules [180,181].

8. Biofilms Associated with the Healthcare Environment

Microbial biofilms, formed by bacteria and fungi, constitute one of the major obstacles in controlling healthcare-associated infections, accounting for 65% to 80% of these occurrences [185]. These complex structures can develop on various surfaces, including medical devices such as catheters, prostheses, and heart valves, and are frequently associated with urinary, respiratory, and bloodstream infections [6,186]. Patients with compromised immune systems, such as those with CF or CGD, are particularly susceptible to these infections due to the prolonged use of medical devices, which facilitate biofilm formation [50,187,188].

The Bcc, composed of highly resistant opportunistic bacteria, is notable for its ability to form persistent biofilms in clinical and industrial environments [189]. Patients with CF are particularly vulnerable to Bcc infections, as these bacteria colonize the lungs and produce an ECM that protects them from both antimicrobial agents and the host's immune system. This matrix is one of the main factors that hinder the eradication of these infections [188].

The organization of Bcc biofilms is mediated by QS systems that regulate the expression of virulence factors. The CepIR system, for example, plays a crucial role in the production of exoenzymes and adhesion factors, which are essential for maintaining the infection [190,191]. Studies conducted with sputum samples from patients with CF revealed the presence of QS signaling molecules, such as AHLs, in infections caused by *B. cepacia* and *P. aeruginosa*. In contrast, infections caused by *S. aureus* rarely exhibit these markers. Furthermore, it was observed that functional QS systems are maintained during chronic infections, suggesting that natural selection favors strains with active cell-to-cell communication mechanisms in CF patients [192,193].

The environmental and hospital distribution of the Bcc also contributes to its pathogenicity. Most species within the complex, except for *B. latens* and *B. metallica*, have been isolated from both natural environments (such as soil and water) and hospital infections, demonstrating their ecological versatility. This adaptability facilitates their dissemination and biofilm formation on hospital surfaces and medical devices, creating infection reservoirs that are difficult to decontaminate [121,192].

The resistance conferred by Bcc biofilms is multifactorial. The ECM acts as a physical barrier, preventing the penetration of antibiotics and immune cells. Additionally, bacterial cells within the biofilm exhibit a tolerant phenotype, characterized by a reduced metabolic rate and slower cell division, making them less susceptible to antimicrobial agents that rely on metabolic activity for their effectiveness [6,50].

The clinical implications of Bcc biofilm formation are severe, especially in immunocompromised patients. Persistent infections can lead to complications such as chronic pneumonia, septicemia, and pulmonary fibrosis. Antimicrobial resistance often necessitates the removal of infected medical devices and prolonged use of combination therapies, further increasing the challenges in managing these cases [121,194].

Strategies for controlling Bcc biofilms include the development of antimicrobial materials for medical devices, which can inhibit bacterial adhesion and biofilm formation [195].

Additionally, the implementation of strict decontamination protocols in hospital environments is essential to reduce the spread of these pathogens. The combination of preventive and therapeutic approaches is crucial to mitigating the impact of these infections in high-risk patients [195,196].

8.1. Infections Associated with Biofilms in Cystic Fibrosis

CF is an autosomal recessive genetic disease that primarily affects populations of Caucasian descent, with an estimated incidence of 1 in every 2000 to 3500 live births in Europe, the United States, and Canada. In recent decades, advances in medical treatment have increased the life expectancy of patients in developed countries to between 40 and 50 years [197]. The disease is caused by mutations in the *CFTR* gene (CF transmembrane conductance regulator), located on chromosome 7. This gene encodes a protein essential for chloride ion transport and regulation of the epithelial sodium channel (ENaC). The most common mutation, ΔF508, is characterized by the deletion of three nucleotides, resulting in the loss of a phenylalanine residue. This alteration leads to the production of a protein with an abnormal conformation, which is retained in the endoplasmic reticulum and fails to reach its functional form [198,199].

The pathogenic mechanism of CF is related to the dysfunction of the CFTR protein, which compromises ionic balance in epithelial cells [200]. Excessive absorption of sodium and chloride results in dehydration of the epithelial surface and the production of thick, sticky mucus [201]. These changes lead to systemic clinical manifestations, including exocrine pancreatic insufficiency; pulmonary damage; and liver, kidney, and gastrointestinal dysfunctions. The primary cause of mortality in CF is airway obstruction by viscous mucus, which impairs mucociliary clearance and promotes colonization by pathogens such as the Bcc [202,203].

Patients with CF, who are often immunocompromised, experience initial pulmonary colonization by pathogens such as *S. aureus* and *Haemophilus influenzae* [202]. As the disease progresses, coinfection by more aggressive bacteria, such as *B. cepacia* and *P. aeruginosa*, occurs, leading to severe respiratory complications [204,205]. The clinical outcome of this coinfection can range from asymptomatic carriers to fatal necrotizing pneumonia, known as cepacia syndrome (CS) [1,192,206].

The CS affects more than 20% of CF patients and is characterized by a rapid decline in lung function, accompanied by high fever, bacteremia, respiratory failure, and sepsis, often with a fatal outcome [207]. Studies suggest that CS is associated with the inability of ROS-deficient neutrophils to eliminate *B. cenocepacia* after phagocytosis. This results in necrotic cell death, release of toxic contents, and worsening of tissue inflammation [121,208].

The transmission of Bcc in CF patients occurs through environmental sources, such as water, and by patient-to-patient spread, including direct transmission [209]. Outbreaks of Bcc have been documented in specialized CF treatment centers, such as the Johns Hopkins Cystic Fibrosis Center (USA), the Royal Brompton Hospital (UK), and the Hôpital Necker-Enfants Malades (France), highlighting the need for strict infection control measures [209]. The most isolated species include *B. cenocepacia* and *B. multivorans*, recognized for their high virulence and transmissibility [1,192]. Other species, such as *B. cepacia* and *B. dolosa*, have also been identified, demonstrating the diversity within the Bcc [204,210].

The incidence of Bcc infections in CF patients varies geographically and over time. In 2021, the Cystic Fibrosis Foundation (USA) reported that 2.5% of patients were infected, while the Cystic Fibrosis Registry (UK) indicated a rate of 3.5%. These values represent a slight decrease compared to data from 2012 and 2013, which showed infection rates of 2.6 and 3.8% in the USA and UK, respectively, possibly due to improvements in infection control practices [211,212]. However, eradicating these infections remains a significant

challenge, primarily due to the intrinsic antimicrobial resistance of Bcc, which limits available therapeutic options [197,213].

8.2. Infections Associated with Biofilms in Chronic Granulomatous Disease

CGD is a rare hereditary disorder with an estimated incidence of 1 in 200,000 to 250,000 individuals. This condition is characterized by mutations in genes responsible for the production of ROS, which are essential components of immune defense. One of the main genes involved is CYBB, located on the X chromosome, which encodes the NOX2 subunit (gp91ˆphox) of the NADPH oxidase (NOX) enzyme. This enzyme plays a crucial role in generating the superoxide radical (O_{2-}), which is necessary for the elimination of microorganisms phagocytosed by neutrophils and macrophages [214,215]. A deficiency in ROS production compromises the immune system's ability to fight infections, making CGD patients highly susceptible to opportunistic pathogens [215].

Among the microorganisms frequently associated with CGD, species of the Bcc, such as *B. cepacia*, *B. cenocepacia*, and *B. multivorans*, are particularly notable. These pathogens are known to cause severe infections, including necrotizing pneumonia, bacteremia, and deep abscesses, especially in immunocompromised patients. The formation of granulomas, a common feature in CGD, reflects the inability of phagocytes to eliminate these microorganisms, perpetuating local inflammation [216,217]. The failure to produce ROS in CGD patients hampers the eradication of *Burkholderia*, promoting infection persistence and increasing the risk of systemic dissemination [216].

Infections caused by *B. cenocepacia* and *B. multivorans* are often fatal due to the high virulence of these species and their intrinsic resistance to antimicrobials. Treating these infections is particularly challenging, requiring advanced therapeutic approaches. Hematopoietic stem cell transplantation has emerged as a promising option for patients with recurrent and severe infections, aiming to restore NOX function [218]. Additionally, prophylactic regimens with antimicrobials, such as trimethoprim-sulfamethoxazole, and antifungals, such as itraconazole, are widely used to prevent infections in CGD patients [217].

The life expectancy of CGD patients has increased, with the current average survival being approximately 40 years. However, it remains lower than that of the general population due to chronic and difficult-to-eradicate infections. Additionally, patients face challenges associated with the prolonged use of antimicrobial and immunosuppressive treatments [219]. Effective management of CGD requires rigorous monitoring, with an emphasis on early infection detection and the implementation of appropriate therapies, such as the use of interferon-gamma (IFN-γ) to enhance phagocyte function. Hematopoietic stem cell transplantation is a relevant therapeutic option in severe cases, aiming to restore NOX function and reduce disease-associated morbidity. Gene therapy, still in the experimental stage, presents a promising approach to improving NOX function and reducing susceptibility to pathogens such as *Burkholderia* [218,220].

8.3. Infections Associated with Biofilms in Melioidosis

The glanders and melioidosis are infectious diseases caused by *B. mallei* and *B. pseudomallei*, respectively. Both were first identified in 1911 and are commonly found in soil and water. Transmission to humans primarily occurs through the inhalation of aerosols; ingestion of contaminated water or food; or inoculation through skin wounds, often associated with everyday activities. Since its discovery, melioidosis has been recognized as a growing global threat, with high mortality rates and significant public health impacts, particularly in endemic regions [35,221].

Melioidosis is one of the leading causes of death from infectious diseases in several regions, such as northeastern Thailand. In 2006, the minimum population mortality rate

was estimated at 8.63 per 100,000 inhabitants, positioning the disease as the third leading cause of infectious death in the region, behind only HIV/AIDS and tuberculosis. During the same period, the mortality rate among patients was 42.6%, with a decrease observed from 49% in 1997 to 40.5% in 2006 [222]. Globally, it is estimated that melioidosis causes up to 165,000 annual cases, resulting in approximately 89,000 deaths, including 2800 in Thailand. Mortality rates vary significantly between endemic regions: 23% in Australia, 39% in Thailand, and up to 61% in Cambodia [37]. The disease is often underreported in areas such as South America and West Africa, where public knowledge of preventive practices, such as wearing rubber boots and boiling water, is limited, particularly in rural areas [223].

Melioidosis presents a wide spectrum of clinical manifestations, ranging from asymptomatic infections to severe cases of pneumonia, abscesses in multiple organs, and septic shock. Recurrence of the infection occurs in about 10% of patients, often associated with the formation of biofilms by *B. pseudomallei*, a virulence factor that complicates bacterial eradication [224]. Early diagnosis and the use of effective antimicrobial therapies, such as ceftazidime in the acute phase and trimethoprim-sulfamethoxazole in the maintenance phase, can reduce mortality rates to less than 10% [36,225].

The severity of melioidosis depends on factors such as the route of infection, the host's immune status, the bacterial strain, and the microbial load [35,221]. Patients with comorbidities, such as diabetes and chronic kidney failure, are particularly susceptible to severe complications, such as acute septic shock, which can reach mortality rates of 70 to 80% in endemic regions of Thailand. Additionally, the infection can remain latent for years, manifesting later under conditions of immunosuppression [225].

Genetic factors play a crucial role in susceptibility to melioidosis. Mutations in genes involved in the IFN-γ pathway, essential for macrophage activation and the elimination of intracellular pathogens, increase the risk of infection. For example, mutations in the IFN-γ receptor (IFNGR) gene impair the immune response, while variations in the major histocompatibility complex (MHC), particularly in class II molecules, affect the coordination of adaptive immunity [38]. Polymorphisms in genes related to the inflammatory response, such as IL-12 and TNF-α, also influence the host's ability to control the infection. The production of IFN-γ, mediated by NK and T cells, is essential for initial resistance to infection, highlighting the importance of these cytokines in immune defense [116,226].

Furthermore, polymorphisms in genes encoding Toll-like receptors (TLRs), responsible for recognizing pathogen-associated molecular patterns (PAMPs), may impair the initial detection of *B. pseudomallei* and activation of innate immunity. Epigenetic changes, such as DNA methylation in immune-related genes, also modulate susceptibility to infection, although the exact mechanisms still require further investigation [38,227].

Currently, there are no vaccines available to prevent melioidosis or glanders, although intensive research is ongoing. Clinical management mainly relies on the use of antibiotics, with ceftazidime as the first-line treatment during the acute phase and trimethoprim-sulfamethoxazole during the maintenance phase. These strategies have shown efficacy in reducing the mortality associated with the disease [36,225].

9. Biofilms Associated with the Pharmaceutical Industry

Members of the Bcc stand out for their remarkable resistance and ability to contaminate non-sterile pharmaceutical products, representing a significant risk to public health, especially for immunocompromised patients, such as those with CF or cancer, or individuals undergoing transplants [22]. The presence of these bacteria in medications can result in severe infections, product recalls, and economic losses for the pharmaceutical industry. Additionally, their ability to degrade organic compounds present in medications

can compromise the stability and efficacy of products, generating toxic substances and reducing their therapeutic potency [228,229].

Historically, the identification of *Burkholderia* in pharmaceutical products was a challenge due to the limitations of traditional taxonomic identification methods. Many contamination episodes in the past may have been caused by members of the Bcc but were not properly reported [230]. With the advancement of molecular techniques, such as 16S rRNA gene sequencing and real-time PCR, it has become possible to detect and identify these bacteria in various sources of contamination, including raw materials, water, and surfaces of equipment used in the manufacture of pharmaceutical products [9,231]. These techniques have allowed for a better understanding of the distribution and impact of Bcc in the pharmaceutical industry [14,228].

Data from the U.S. Food and Drug Administration (FDA) highlight the significance of the Bcc as a frequent contaminant in non-sterile pharmaceutical products [232]. Between 1998 and 2006, *B. cepacia* was responsible for 22% of recalls of non-sterile products [233]. This percentage increased to 34% between 2004 and 2011, reflecting greater surveillance and identification of the presence of *Burkholderia* [234]. Between January and July of 2012, *B. cepacia* was the most common microbial contaminant found in non-sterile products, accounting for 39% of the bacterial species isolated. Other genera, such as *Pseudomonas*, *Staphylococcus*, and *Enterobacter*, were identified less frequently, emphasizing the predominance of the Bcc in this context [235].

Species of the Bcc have been isolated from a wide variety of pharmaceutical and personal care products, including nasal sprays, lotions, oils, mouthwashes, cleaning towels, wet wipes, pre-operative skin solutions, hand sanitizers, and liquid drops for intestinal gas relief [232]. While sterile products, such as those administered intravenously or ocularly, require extremely controlled environments to prevent severe infections, non-sterile products, applied topically or orally, pose a lower risk of infection. However, the contamination of these products should not be underestimated, especially considering the ability of the Bcc to cause opportunistic infections in vulnerable patients [236].

One of the most concerning characteristics of the Bcc is its ability to metabolize a variety of organic compounds as carbon and energy sources. This capability allows *Burkholderia* to proliferate in different types of medications, compromising their stability and effectiveness. The bacterium can metabolize nitroaromatic and halogenated compounds, commonly found in medications, leading to the degradation of both active ingredients and excipients. This degradation can result in toxic substances and reduce the potency of the pharmaceutical product, as well as affect its physical, chemical, and organoleptic properties, such as color, taste, and odor. In some cases, the degradation can generate new compounds with toxic potential, posing an additional risk to consumers, especially those already facing other pathological conditions [22].

The natural resistance of *Burkholderia* to various antimicrobials is another factor contributing to its persistence in environments prone to contamination [1,237]. Many species of the Bcc possess intrinsic mechanisms of antibiotic resistance, such as the production of efflux pumps and antibiotic-degrading enzymes [121,237]. Additionally, the ability to form biofilms on equipment surfaces and storage tanks makes the eradication of these bacteria even more challenging [1]. These biofilms protect bacterial cells from antimicrobial agents and adverse environmental conditions, allowing *Burkholderia* to persist for extended periods in pharmaceutical manufacturing and storage environments [121].

Rigorous surveillance and quality controls are essential to prevent *Burkholderia* contamination in pharmaceutical and cosmetic products. Implementing manufacturing and storage protocols that minimize contamination risks is crucial to ensure consumer safety and protect the integrity of the pharmaceutical industry [238]. Recommended measures

include validating cleaning and disinfection processes, monitoring water quality, using advanced detection techniques, controlling raw materials, and educating and training pharmaceutical industry staff about the risks associated with *Burkholderia* contamination. These are the best practices for preventing it [239].

10. Resistance to Antibiotics

10.1. Resistance of Planktonic Cell

The intrinsic resistance of bacteria belonging to the Bcc to various antimicrobial agents, including antibiotics, disinfectants, and preservatives, poses a significant challenge in managing infections associated with these microorganisms. This multifactorial resistance results from a combination of biological mechanisms that enable Bcc species to survive and proliferate in adverse environments, such as non-sterile pharmaceutical products. Elucidating these mechanisms is crucial for the development of effective therapeutic approaches and for the prevention of infections caused by these pathogens [240].

One of the main factors contributing to the resistance of Bcc is the unique structure of the outer membrane of these bacteria, characterized by reduced permeability. This physical barrier prevents the effective penetration of various antimicrobial agents, compromising their therapeutic action [241]. The outer membrane of *Burkholderia* is composed of LPS that undergo structural modifications, such as the substitution of phosphate groups with aminoarabinose and the addition of galactose residues. These alterations decrease the affinity of cationic antibiotics, such as ceftazidime and clavulanic acid, for bacterial cells, reducing their effectiveness [24]. Additionally, the presence of porins with low permeability restricts the diffusion of antimicrobial molecules into the cell, further enhancing resistance [241,242].

Another critical resistance mechanism in the Bcc involves the activity of multidrug efflux pumps belonging to the RND family. These specialized proteins are responsible for the active expulsion of a variety of toxic substances, including antibiotics. In the genome of *B. cenocepacia* J2315, several efflux pumps have been identified, each playing specific roles in resistance to different classes of antimicrobials. For instance, one of these pumps is highly regulated in the presence of chloramphenicol, while another is associated with resistance to fluoroquinolones and streptomycin [243]. Additionally, a homolog of the MexAB-OprM pump from *P. aeruginosa* was identified in *B. cenocepacia*, mediating the efflux of chloramphenicol, trimethoprim, and ciprofloxacin [244]. The function of other putative RND pumps, whose genes have been identified in the *B. cenocepacia* J2315 genome, has not yet been fully elucidated, but it is suggested that they may contribute to resistance to a wide range of antimicrobials [23,242].

Inducible β-lactamases also play a central role in Bcc resistance. These enzymes are capable of hydrolyzing β-lactam antibiotics, such as penicillins and cephalosporins, inactivating their therapeutic action. In the genome of *B. cenocepacia* J2315, genes encoding β-lactamases from classes A, C, and D have been identified, which confer resistance to a broad range of β-lactam antibiotics [23]. Although the presence of aminoglycoside-inactivating enzymes has not been experimentally confirmed, genes encoding a putative aminoglycoside O-phosphotransferase and an aminoglycoside O-adenylyltransferase have been identified, suggesting a potential mechanism of resistance to these antibiotics. These enzymes can chemically modify aminoglycosides, preventing their binding to the bacterial ribosome and, consequently, neutralizing their antibacterial activity [240].

In addition to the mechanisms mentioned, Bcc also employs other resistance strategies, such as the production of antibiotic-modifying enzymes and the alteration of molecular targets. For example, mutations in genes encoding DNA gyrases and topoisomerase IV can confer resistance to fluoroquinolones, while modifications in ribosomes can reduce

the efficacy of aminoglycosides and tetracyclines [242,245]. These combined mechanisms highlight the complexity of Bcc's intrinsic resistance and the need for innovative therapeutic approaches to overcome these challenges [242].

10.2. Resistance of Sessile Cells

Biofilms formed by Bcc bacteria are complex and highly organized structures that provide these microorganisms with remarkable resistance to antibiotics and disinfectants [230]. This characteristic complicates both the treatment of infections and control measures in hospital environments [22]. These biofilms consist of an ECM that surrounds the bacterial cells, creating a protected and highly resistant microenvironment. Biofilm formation is a multifactorial process involving various resistance mechanisms, making infections associated with Bcc particularly challenging to treat [24,246].

The ECM plays a central role in the increased resistance of bacteria in biofilms. This matrix acts as a physical barrier that hinders the penetration of antimicrobial agents, providing time for bacteria to develop defensive responses [71]. The EPS is composed of a variety of components, including polysaccharides, proteins, eDNA, and other macromolecules, which form a complex three-dimensional network [41]. This structure not only prevents the diffusion of antibiotics but also protects bacterial cells from adverse environmental conditions, such as desiccation and attacks from the host's immune system [247]. Furthermore, the EPS can sequester metal ions and other potentially toxic molecules, further contributing to bacterial survival [40,248].

Heterogeneity within biofilms is another factor contributing to the increased resistance. Different regions of the biofilm exhibit varying growth rates, including areas of slow or inactive growth [247]. This heterogeneity results from gradients of nutrients, oxygen, and other environmental factors that vary throughout the biofilm structure [6]. In areas with slow or inactive growth, bacterial cells show a reduction in metabolic rate, which decreases the effectiveness of antibiotics that target active metabolic processes, such as protein synthesis or DNA replication [46]. Additionally, the presence of subpopulations of persistent cells, which are highly resistant to antibiotics, further complicates treatment. These persistent cells can survive lethal doses of antibiotics and, once the treatment is interrupted, can repopulate the biofilm, leading to recurrence of the infection [46,249,250].

The rate of antimicrobial agent transport into biofilms is a critical factor influencing treatment efficacy. Unlike planktonic cells, where exposure to the antimicrobial agent is immediate, biofilms can delay this exposure, allowing bacteria to develop defensive responses [6]. The penetration of antimicrobial agents into biofilms is a widely discussed topic in scientific literature [46]. Although physicochemical calculations suggest that diffusion limitations should not be significant for most compounds, experimental data indicate that the penetration rate varies depending on the compound and the specific biofilm [251].

In the case of biofilms formed by *B. pseudomallei*, studies have shown that antibiotic penetration is significantly limited, resulting in resistance to conventional antimicrobials such as doxycycline, ceftazidime, imipenem, and trimethoprim-sulfamethoxazole. This resistance is attributed not only to the physical barrier provided by the EPS but also to the ability of bacterial cells to modify their molecular targets and express enzymes that degrade or inactivate antibiotics. Additionally, the presence of efflux pumps, which actively expel antibiotics from bacterial cells, contributes to the increased resistance [38].

11. Resistance to Biocides

The term "biocide" refers to chemical agents capable of destroying or inhibiting the growth of living organisms. Within this category, antiseptics are applied to living tissues

to reduce the microbiota, while disinfectants are used on inanimate surfaces to eliminate pathogenic microorganisms. Preservatives, on the other hand, are added to products such as medicines, food, and cosmetics to prevent microbial growth. The concentration of these agents varies depending on the application, whether for disinfection or preservation. For example, chlorhexidine, a biguanide, is used in concentrations ranging from 0.5 to 4% for surface disinfection, 0.02 to 4% for antisepsis, and 0.0025 to 0.01% for preservation [22].

A concerning characteristic of Bcc species is their ability to survive and proliferate in commercially available biocide formulations, showing low susceptibility to these compounds [1]. This resistance makes the Bcc a significant contaminant in the pharmaceutical industry, where it can colonize and form biofilms on equipment, pipes, and raw materials in the presence of moisture [252]. This ability to persist in hostile environments and in the presence of biocides is one of the main reasons why the Bcc is considered a major opportunistic pathogen of concern in hospital and industrial settings [252,253].

Among the most used biocides in healthcare environments and personal care products are benzalkonium chloride (BZK), chlorhexidine (CHX), cetylpyridinium chloride, triclosan, povidone, and octenidine [254]. These compounds, classified as low-level antiseptics, have a broad spectrum of activity against Bcc bacteria [255]. However, the intrinsic resistance of the Bcc to these biocides has been widely documented, highlighting the need for a deeper understanding of resistance mechanisms and strategies to overcome them [256].

BZK is one of the most widely used biocides as an antimicrobial preservative in pharmaceutical products, particularly in multidose aqueous formulations such as nasal sprays, eye drops, and otic solutions [255]. As a quaternary ammonium compound, BZK contains a positively charged nitrogen atom linked to three alkyl groups and a benzyl group [256]. Its mechanism of action primarily occurs by destabilizing the bacterial membrane, leading to the leakage of cellular components and cell death [254]. Despite its widespread use, BZK solutions contaminated with Bcc bacteria have been associated with numerous nosocomial outbreaks, more so than any other biocide available on the market. This is due to the remarkable ability of the Bcc to survive and proliferate in BZK solutions [253,255].

CHX, another widely used biocide, is primarily employed for hand hygiene and as a component of mouthwashes. As a biguanide, CHX works by compromising the cell membrane structure, breaking divalent cation and LPS bonds, which results in the loss of cytoplasmic components and periplasmic enzymes [255]. Despite its effectiveness against a broad range of microorganisms, CHX has also been associated with nosocomial outbreaks related to the Bcc, particularly due to contamination of CHX solutions [254]. These outbreaks highlight the ability of the Bcc to resist biocides that are considered effective against most bacteria [256].

Initial studies identified a strain of *B. cepacia* that remained viable for 14 years in a saline solution supplemented with 0.05% BZK, demonstrating the remarkable ability of the Bcc to survive in adverse conditions [254]. In a study, the susceptibility of 36 species within the Bcc to BZK and CHX, following incubation in distilled water for 40 days, was assessed using minimum inhibitory concentration (MIC) assays [255]. Although the incubation period increased susceptibility to BZK and CHX, six strains of *B. cenocepacia* maintained a high level of resistance. This result suggests that, even under adverse conditions, some strains of the Bcc are capable of maintaining their resistance to biocides, contributing to their persistence in hospital and industrial environments [254].

In the same study, clinical strains of *B. contaminans*, *B. multivorans*, *B. vietnamiensis*, and *B. ambifaria* were recovered after 14 days in the presence of BZK, compared to the first 24 h [255]. These observations suggest that BZK was inactivated by the bacteria and even used as a carbon and energy source for bacterial growth and metabolism. This ability to utilize biocides as a nutrient source is particularly concerning, as it indicates that the Bcc not

only resists these compounds but may also benefit from them under certain conditions [254]. Additionally, long-term incubations (18 months) with BZK at 53 µg/mL and 500 µg/mL induced the formation of cell aggregates resembling biofilms, visible both macroscopically and microscopically. These aggregates may represent a survival strategy for the Bcc in hostile environments, allowing the bacteria to withstand biocides and persist for extended periods [255].

The resistance of the Bcc to biocides such as BZK and CHX is multifactorial and involves mechanisms such as modification of the cell membrane, production of degradative enzymes, and biofilm formation. The ability of the Bcc to form biofilms is particularly relevant, as the ECM of biofilms acts as a physical barrier that prevents biocides from penetrating and protects bacterial cells from adverse conditions [254]. Additionally, the heterogeneity within the biofilm, with areas of slow or inactive growth, contributes to increased resistance, as cells in these zones are less susceptible to biocides that rely on metabolic activity for their effectiveness [255].

12. Frequent Resistance Mechanisms

12.1. Heterogeneity

The heterogeneity within biofilms is a critical factor contributing to antimicrobial resistance, making biofilm-associated infections particularly challenging to treat [257]. This heterogeneity arises from variable conditions within the biofilm, such as nutrient, oxygen, and pH gradients, which create distinct microenvironments within its structure [6]. These variations lead to differences in bacterial growth rates and metabolic activities, directly influencing their susceptibility to antimicrobial agents [45].

In the deeper regions of the biofilm, nutrient and oxygen availability is significantly reduced due to the limited diffusion through the ECM. This resource limitation causes bacterial cells to enter a state of slow metabolism or even dormancy [258]. Under conditions of low metabolic activity, cells become less susceptible to antibiotics that rely on active metabolic processes to be effective [259]. For example, antibiotics such as β-lactams and aminoglycosides require bacteria to be actively replicating or synthesizing proteins to exert their effects [77]. In dormant cells, these antibiotics are ineffective, allowing the bacteria to survive even after antimicrobial treatments [256]. Consequently, these subpopulations of low-metabolic-activity cells can persist and, once treatment is discontinued, repopulate the biofilm, perpetuating the infection [46,260].

The presence of "persister" cells within the biofilm is another resistance mechanism that contributes to the difficulty of eradicating these infections [261]. Persister cells are a subpopulation that enters a temporary dormant state, becoming highly resistant to antibiotics [262]. Although they represent only a small fraction of the total biofilm population, their ability to survive antimicrobial treatments is crucial for the persistence of infection. These cells can remain viable for extended periods, even in the presence of lethal antibiotic concentrations, and once environmental conditions become favorable, they can resume growth and repopulate the biofilm [263]. This phenomenon is particularly concerning in chronic infections, where the presence of persister cells can lead to infection recurrence after the completion of therapy [46,264].

The differential expression of proteins in biofilms also plays a fundamental role in antimicrobial resistance. Biofilm-associated cells can express specific proteins that are not produced in planktonic cells, providing adaptive advantages in hostile environments. For instance, the production of enzymes that degrade or modify antibiotics is a common strategy used by biofilm-forming bacteria to resist treatments [45]. Enzymes such as β-lactamases can hydrolyze β-lactam antibiotics, including penicillins and cephalosporins, rendering them ineffective [265]. Additionally, biofilm cells can express efflux systems that

actively expel antimicrobials from the cell, reducing their intracellular concentration and, consequently, their effectiveness [40,266].

12.2. Efflux Pumps

Efflux pumps are active transport systems that play a fundamental role in antimicrobial resistance by expelling toxic compounds, including antibiotics, out of bacterial cells [267]. These systems are particularly important in biofilms, where the overexpression of efflux pumps significantly contributes to antibiotic tolerance and infection persistence [268]. Understanding the mechanisms of action and regulation of these pumps is essential for the development of therapeutic strategies aimed at overcoming biofilm-mediated resistance [269,270].

A notable example of efflux pumps involved in biofilm resistance is the PA1874-1877 operon in *P. aeruginosa*. This operon encodes an ABC (ATP-binding cassette) efflux system, which utilizes the energy derived from ATP hydrolysis to transport toxic substances out of the cell. Studies have shown that the genes of this operon are overexpressed in biofilms compared to planktonic cells, indicating their involvement in biofilm-specific resistance to antibiotics. Deletion of this operon resulted in increased susceptibility to antibiotics, suggesting that it plays a crucial role in biofilm-mediated resistance. These findings highlight the importance of ABC-type efflux pumps in protecting bacterial cells in biofilms against the action of antimicrobial agents [269,271].

In addition to ABC-type efflux systems, efflux pumps from the resistance-nodulation-division (RND) family in *P. aeruginosa* are also closely implicated in multidrug resistance. These pumps are protein complexes that span both the inner and outer membranes of the bacterium, allowing the active expulsion of a wide range of toxic compounds. Among the most studied RND systems in *P. aeruginosa* are MexAB-OprM and MexCD-OprJ, which are associated with resistance to various antibiotics, including β-lactams, fluoroquinolones, and macrolides [272]. The overexpression of these pumps is directly related to the increase in multidrug resistance, and their inhibition can restore bacterial susceptibility [273]. For example, the overexpression of the MexCD-OprJ pump has been correlated with resistance to antibiotics and the emergence of multidrug-resistant strains, highlighting the critical role of these systems in antimicrobial resistance [274–276].

The BrlR regulator, of the MerR type, is another important component in antibiotic tolerance in biofilms. BrlR acts as a transcriptional regulator that positively regulates the expression of efflux pumps, such as MexAB-OprM and MexEF-OprN, increasing the bacterium's ability to expel antibiotics. Inactivation of BrlR resulted in decreased expression of these efflux pumps and an increase in antibiotic susceptibility, highlighting its importance in biofilm-mediated resistance. These findings suggest that BrlR is a potential target for the development of therapies aimed at reducing antimicrobial resistance in biofilms [277,278].

In addition to expelling antibiotics, efflux pumps can also influence bacterial communication systems, such as QS. QS is a genetic regulation mechanism that allows bacteria to coordinate population behaviors in response to changes in cell density. The overexpression of efflux pumps, such as MexCD-OprJ, can affect the production of QS signaling molecules, such as AHLs, modulating the expression of virulence factors and contributing to resistance in biofilms. For example, changes in AHL production can affect biofilm formation, exoenzyme production, and the expression of other virulence factors, making bacteria better adapted to hostile environments and less susceptible to antimicrobial treatments [275,276].

The inhibition of efflux pumps has been explored as a promising strategy to overcome antimicrobial resistance in biofilms. Molecules that block the activity of efflux pumps, known as efflux inhibitors, can enhance the efficacy of antibiotics, making bacterial cells more susceptible to treatment [279]. For example, the combination of antibiotics with efflux

inhibitors, such as phenylalanylalanylalanylarginyl-β-naphthylamide (PAβN), has been shown to increase the susceptibility of *P. aeruginosa* to antibiotics like fluoroquinolones and β-lactams [280]. This approach may be particularly useful in treating biofilm-associated infections, where efflux pump-mediated resistance is a critical factor [274,281].

13. Therapeutic Methods Against Biofilm-Induced Infections

Due to the intrinsic resistance to multiple antimicrobials and the ability to form biofilms, infections caused by the Bcc are difficult to eradicate, even with prolonged treatments. Innovative and combined strategies are being investigated to overcome these limitations and improve clinical management [282].

13.1. Antibiotic Therapy: Antimicrobial Synergy

The treatment of infections caused by *Burkholderia* is notoriously challenging due to its intrinsic resistance to multiple antibiotics. To overcome this resistance, the use of combination therapies has proven to be an effective approach, aiming to circumvent bacterial resistance mechanisms and improve antibiotic penetration in biofilms. These combinations typically involve antibiotics with different mechanisms of action, which act synergistically to enhance the effectiveness of the treatment [240,283].

One of the most commonly used combinations in the treatment of *Burkholderia* infections includes ceftazidime, a broad-spectrum β-lactam, and meropenem, a potent carbapenem. Ceftazidime is effective against many Gram-negative bacteria, but its activity may be compromised by the production of β-lactamases. The addition of meropenem, which is more stable against these enzymes, can help overcome this resistance. Additionally, the combination of β-lactams with β-lactamase inhibitors, such as clavulanic acid, has been explored to increase the effectiveness of treatment. This approach aims to block the activity of β-lactamases, allowing β-lactams to act more effectively [284,285].

Another frequently used combination is sulfamethoxazole-trimethoprim (SMX-TMP), a therapeutic regimen widely employed in bacterial infections. SMX-TMP works by inhibiting folic acid synthesis, which is essential for bacterial replication [286]. This combination has shown efficacy against some *Burkholderia* strains, especially in chronic pulmonary infections in CF patients. However, resistance to SMX-TMP has also been documented, highlighting the need for constant monitoring of antimicrobial susceptibility [240,287].

The combination of β-lactams with aminoglycosides, such as tobramycin, has also been investigated as a strategy to overcome *Burkholderia* resistance [288]. Aminoglycosides, such as tobramycin, work by inhibiting bacterial protein synthesis, while β-lactams interfere with cell wall synthesis [289]. This combination can result in a synergistic effect, increasing the treatment's effectiveness [290]. Tobramycin has been used in inhalation formulations for the treatment of chronic pulmonary infections in CF patients, where antibiotic penetration into the biofilm is a significant challenge. Inhalation administration allows high concentrations of the antibiotic to be directly delivered to the site of infection, enhancing its efficacy [291,292].

In addition to these combinations, other therapeutic strategies have been explored to improve the treatment of *Burkholderia* infections. For example, the combination of fluoroquinolones, such as ciprofloxacin, with β-lactams or aminoglycosides has shown synergistic effects in some cases. Fluoroquinolones work by inhibiting the enzymes DNA gyrase and topoisomerase IV, which are essential for bacterial DNA replication [293,294]. The combination with other antibiotics can enhance the effectiveness of the treatment, especially in multidrug-resistant strains [294].

Combination therapy may also include the use of agents that increase membrane permeability, such as polymyxin B. Polymyxin B is a polypeptide antibiotic that destabilizes

the outer membrane of Gram-negative bacteria, increasing permeability and facilitating the entry of other antibiotics. The combination of polymyxin B with β-lactams or carbapenems has been investigated as a strategy to overcome resistance mediated by efflux pumps and the low membrane permeability in *Burkholderia* [240,295].

Another promising approach is the inhibition of efflux pumps, which actively expel antibiotics from the bacterial cell. Molecules such as PAβN have been tested as efflux pump inhibitors in combination with antibiotics, increasing the intracellular concentration of the drug and restoring its efficacy. This strategy may be particularly useful in the treatment of *Burkholderia* infections, where the overexpression of efflux pumps is a common resistance mechanism [296,297].

Combination therapy can also be enhanced with the use of agents that degrade the ECM of the biofilm, such as DNase or dispersin B. These agents can destabilize the biofilm, facilitating antibiotic penetration and increasing their effectiveness [40].

13.2. Association Between Mechanical Force and Antibiotic Therapy

The combination of surgical debridement followed by antibiotic treatment has been established as a highly effective strategy for controlling and preventing biofilm formation in wounds, particularly in chronic wound cases. Studies have shown that bacteria can begin biofilm formation in wounds as early as 10 h after colonization. Once established, these biofilms can persist for weeks or even months, contributing to wound chronicity and the failure of conventional treatments. Surgical debridement, which involves the mechanical removal of necrotic tissue, debris, and biofilms from the wound, is a fundamental approach to disrupting this bacterial structure. However, clinical research indicates that even after effective debridement, biofilms can rapidly reform, reaching a mature state within 72 h. This phenomenon occurs because some bacterial cells may remain at the site or in the surrounding wound area, serving as a nucleus for biofilm reformation [298,299].

At this point, the combination with antibiotics becomes crucial. After debridement, the non-adherent (planktonic) bacteria that remain in the wound are significantly more susceptible to antibiotic action than when encapsulated in biofilms. Therefore, the administration of antibiotics immediately after debridement is a practical and effective strategy to eliminate these remaining bacteria and prevent biofilm recurrence. This combined approach has proven particularly useful in overcoming the intrinsic resistance of biofilms to antibiotics, which is one of the greatest challenges in the treatment of chronic infections [51,300].

Moreover, the use of ultrasound-targeted microbubble destruction (UTMD) in combination with antibiotics has been shown to enhance the efficacy of these drugs. UTMD has emerged as an innovative and highly promising approach for treating biofilm-associated infections, particularly when used alongside antibiotics. This technology leverages the physical properties of microbubbles and the energy of ultrasound waves to improve the delivery and effectiveness of antimicrobial agents, overcoming the barriers imposed by the biofilm's ECM [301,302].

UTMD employs small gas-filled spheres coated with a layer of lipids, proteins, or polymers. These microbubbles are loaded with therapeutic agents, such as antibiotics, and injected into the wound or infection site. When exposed to high-frequency ultrasound waves, the microbubbles oscillate and eventually implode, a phenomenon known as cavitation. This implosion generates localized mechanical forces, including microjets and shock waves, capable of disrupting the biofilm ECM, increasing tissue permeability, and releasing therapeutic agents directly at the infection site [301,302].

The ECM acts as a physical barrier that protects bacteria from antibiotics and the immune system. UTMD disrupts this matrix, exposing the bacteria and facilitating the action of antimicrobial agents. Additionally, the mechanical forces generated by UTMD

create micropores in the surrounding tissues, allowing antibiotics to penetrate more deeply and reach the inner layers of the biofilm. The implosion of the microbubbles also enables the controlled and targeted release of surface-loaded antibiotics, increasing local drug concentration while reducing systemic side effects. A notable example of UTMD's efficacy is its combination with vancomycin, an antibiotic commonly used to treat infections caused by Gram-positive bacteria, such as *S. aureus* [303,304].

The implosion of the microbubbles not only disrupts the ECM of the biofilm but also enhances the penetration of vancomycin into the deeper layers of the bacterial structure. As a result, the local concentration of the antibiotic is amplified, and its bactericidal action is potentiated. Compared to the use of vancomycin alone, the combination with UTMD has shown a dramatic reduction in bacterial viability and a higher biofilm eradication rate [301,304]. UTMD offers several advantages in biofilm treatment, including improved antibiotic efficacy, reduced antibiotic dosage requirements, complementary physical action that destroys the biofilm structure, and targeted application focusing specifically on the area of infection without affecting surrounding healthy tissues [302,303].

Despite promising results, UTMD still faces challenges that need to be overcome for its widespread clinical adoption. Among these are the optimization of ultrasound parameters (frequency, intensity, and duration) for different types of biofilms and tissues, the standardization of microbubbles, and ensuring safety in sensitive tissues [305,306]. Furthermore, more large-scale clinical studies are needed to validate the efficacy and safety of this technique in humans [304]. In the future, UTMD could be combined with other advanced therapies, such as the use of nanoparticles loaded with antibiotics or enzymes that degrade the ECM, to create even more effective multidisciplinary approaches for combating biofilm-associated infections. This technology also has the potential to be applied in other areas of medicine, such as cancer treatment (for targeted delivery of chemotherapeutics) and gene therapy [307,308].

13.3. Synthetic and Natural Peptides

Antimicrobial peptides (AMPs), whether synthetic or derived from natural sources, have shown significant potential in combating bacterial infections, especially in light of the rise of strains resistant to conventional antibiotics [309]. These peptides can act in various ways, including destabilizing the bacterial membrane and interfering with essential intracellular processes [310]. Their ability to target multiple bacterial sites and their rapid action make them a promising alternative in the current scenario of antimicrobial resistance [311,312].

Polymyxins, such as colistin, are examples of AMPs that interact with the outer membrane of Gram-negative bacteria by binding to LPS and phospholipids, leading to membrane disorganization and subsequent cell death [295]. Colistin has been used as a last-resort treatment for infections caused by multidrug-resistant strains due to its effectiveness against these bacteria. However, its use is limited by concerns over toxicity, such as nephrotoxicity and neurotoxicity, which has driven the search for safer and more effective analogs [313,314].

Cathelicidins and defensins are classes of AMPs that play key roles in the innate immunity of mammals. Cathelicidins, such as LL-37, have an amphipathic structure that allows them to interact with bacterial membranes, leading to pore formation and cell lysis [315]. In addition, LL-37 can modulate immune responses, including the chemotaxis of immune cells and the neutralization of endotoxins [316]. This dual function, antimicrobial and immunomodulatory, makes cathelicidins versatile molecules in combating infections and promoting tissue healing [317].

Defensins are subdivided into α-defensins and β-defensins, both characterized by structures rich in β-sheets stabilized by disulfide bridges [318]. They primarily act by inserting into bacterial membranes, forming pores that increase permeability and result in cell death. In addition to their direct antimicrobial activity, defensins also participate in modulating the immune system, influencing processes such as dendritic cell activation and cytokine release [319]. This ability to interact with the host's immune system enhances their therapeutic potential, especially in chronic or persistent infections [318,320].

The efficacy of AMPs is related to factors such as their amino acid sequence, three-dimensional structure, and electric charge. Most of these peptides are cationic, which facilitates electrostatic attraction to bacterial membranes, which are generally anionic [321]. After the initial binding, AMPs can insert into the membrane, forming structures that lead to destabilization and permeabilization, ultimately resulting in bacterial cell death [322]. In addition to their action on membranes, some AMPs can penetrate the bacterial cytoplasm and interfere with vital processes, such as DNA, RNA, and protein synthesis, or inhibit the activity of essential enzymes [323]. This versatility of mechanisms reduces the likelihood of resistance development by bacteria, making AMPs promising candidates for the development of new antimicrobial therapies [321,324].

However, challenges such as in vivo stability, potential toxicity to host cells, and production costs still need to be overcome for the widespread clinical application of AMPs [311]. The engineering of synthetic peptides and chemical modifications have been explored to optimize these molecules, aiming to increase their antimicrobial efficacy and reduce adverse effects [325]. For example, the incorporation of non-natural amino acids or conjugation with nanoparticles can improve the stability and bioavailability of AMPs. With the advancement of research, it is expected that AMPs will play an increasingly important role in the therapeutic arsenal against resistant infections, offering an effective and sustainable alternative to combat one of the greatest challenges in global health [326,327].

13.4. Extracellular Polymeric Substances as a Therapeutic Target

The ECM is a key component in the formation and maintenance of bacterial biofilms, playing a central role in protecting bacterial communities against antimicrobial treatments and the host immune system [41]. This matrix not only provides a physical barrier that prevents the penetration of antibiotics and immune cells but also facilitates intercellular communication and nutrient exchange between bacteria, promoting biofilm survival and persistence in hostile environments [50]. Strategies targeting the EPS have been widely explored as a promising approach to prevent biofilm formation or promote its dispersion [328]. These strategies can be divided into three main categories: inhibition of EPS production or secretion, blocking the adhesion of EPS to surfaces, and direct degradation of matrix components. Each of these approaches aims to disrupt the structural integrity of the biofilm, making bacteria more vulnerable to conventional treatments [40,329].

The inhibition of EPS synthesis and secretion has been investigated as a way to prevent the initial formation of biofilms. One of the key targets in this approach is the modulation of cyclic nucleotides, such as cyclic diguanylate (c-di-GMP) and cyclic diadenylate (c-di-AMP), which act as secondary messengers regulating EPS production and biofilm formation. c-di-GMP, in particular, is known to promote the synthesis of matrix polysaccharides and bacterial adhesion. Small molecule screenings have identified compounds capable of inhibiting enzymes involved in the synthesis or degradation of c-di-GMP, thereby reducing EPS production [330]. Additionally, inhibitors of glucosyltransferases, enzymes responsible for polysaccharide synthesis, and the formation of pili, protein structures essential for bacterial adhesion and matrix construction, have shown potential in preventing biofilm formation [331,332].

Enzymatic degradation of EPS is another promising strategy, especially for established biofilms. Enzymes such as glucanohydrolases and dispersin B have been used to digest specific matrix components, such as polysaccharides, weakening the biofilm structure and facilitating bacterial removal. For example, dispersin B, an enzyme that degrades polysaccharides, has shown effectiveness against biofilms of *S. aureus* and *P. aeruginosa* in experimental models [333]. Additionally, glycoside hydrolases, which break glycosidic bonds in polysaccharides, have been effective in degrading biofilms in murine models of chronic wounds, paving the way for clinical applications [334].

Another interesting approach involves the use of substances naturally secreted by commensal bacteria, which can inhibit biofilm formation by pathogenic species. A notable example is the serine protease Esp, secreted by *S. epidermidis*, a commensal bacterium of the human skin. Esp is capable of reducing biofilm formation by *S. aureus* and even destroying pre-existing biofilms of this species in in vitro studies. Additionally, *S. epidermidis* that secretes Esp has been shown to eliminate *S. aureus* nasal colonization in experimental models, suggesting a therapeutic potential for preventing biofilm-associated infections [335].

eDNA is another critical component of the EPS matrix, acting as a "glue" that holds the bacteria together and contributing to the structural stability of the biofilm [41]. Enzymes that degrade DNA, such as DNase I, have been extensively studied for their ability to disrupt biofilms [336]. DNase I breaks down eDNA, destabilizing the matrix and making the bacteria more susceptible to antimicrobial treatments. In vitro and in vivo studies have shown that the application of DNase I can significantly reduce biofilm biomass and enhance the effectiveness of antibiotics, particularly in chronic and persistent infections [69,337].

These approaches targeting the biofilm ECM offer promising avenues for the development of more effective therapies against biofilm-associated infections [41]. However, the clinical application of these strategies requires a deep understanding of the specific composition of the matrix in different bacterial species and environments. EPS can vary significantly between species and even between strains of the same species, which necessitates the customization of therapies based on the context of the infection [337]. Furthermore, the safety and efficacy of these agents must be rigorously evaluated in clinical models, considering potential side effects and interactions with the host's immune system [51,336].

13.5. Immunological Modulation in Enhancing Defense

Immunotherapy has emerged as a promising approach in the fight against infections caused by *Burkholderia*. Immunotherapy aims to modulate and strengthen the host's immune response, offering an alternative or complement to conventional antimicrobial treatments, which often prove inadequate due to bacterial resistance [307].

One of the strategies under investigation involves the use of cytokines, signaling proteins that play crucial roles in communication between immune system cells. Cytokines such as interferons (IFNs) and colony-stimulating factors (CSFs) have been explored for their ability to modulate both innate and adaptive immune responses. IFNs, for instance, are well known for their antiviral and immunomodulatory properties. They activate immune cells such as macrophages and natural killer (NK) cells and promote the expression of major histocompatibility complex (MHC) molecules, which are essential for antigen presentation and T lymphocyte activation. In the context of bacterial infections, IFNs can enhance the ability of immune cells to recognize and eliminate pathogens, including *Burkholderia* [338,339].

The CSFs, such as granulocyte-colony-stimulating factor (G-CSF) and granulocyte-macrophage-colony-stimulating factor (GM-CSF), are cytokines that promote the production, differentiation, and activation of leukocytes, including neutrophils and macrophages, which are essential for defense against bacterial pathogens [340]. In *Burkholderia* infections, the use of CSFs has been investigated as a means to enhance the quantity and functionality

of these immune cells, thereby improving the host's ability to combat the infection [341]. While the use of cytokines has been more extensively studied in oncological contexts and certain viral infections, their application in bacterial infections, such as those caused by *Burkholderia*, is being explored as a strategy to potentiate the immune response and overcome bacterial resistance [340,342].

In parallel, the development of vaccines targeting *Burkholderia* has been a focus of research, aiming to prevent infections and control the spread of these bacteria [343]. A promising approach involves designing vaccines based on adhesion proteins and biofilm matrix components. The biofilm ECM is a strategic target, as it plays a crucial role in shielding bacteria from the immune system and antibiotics. By targeting specific EPS components, such as polysaccharides or adhesion proteins, these vaccines seek to prevent biofilm formation and, consequently, reduce the virulence and persistence of infections [40,344].

Protein subunit vaccines, which use purified fragments of bacterial proteins to stimulate an immune response without introducing the entire pathogen, have shown potential in this field [344]. For instance, adhesion proteins from *Burkholderia* type 1 fimbriae or EPS components such as the polysaccharide alginate have been investigated as vaccine antigens. This approach offers the advantage of faster and more targeted production while reducing the risk of side effects associated with live or attenuated pathogens [345]. Furthermore, subunit-based vaccines can be combined with adjuvants—substances that enhance the immune response—to improve their efficacy [346].

Another strategy under development is the use of DNA or RNA vaccines, which introduce genes encoding bacterial antigens into the host organism, inducing the production of these antigens and, consequently, a specific immune response. This approach has been explored for various bacterial and viral infections and can be adapted for *Burkholderia*, targeting specific antigens associated with biofilm formation or bacterial virulence [347,348].

Although these strategies are still in experimental phases, they represent significant advances in the search for therapeutic and preventive alternatives against *Burkholderia* infections, particularly given the challenges posed by antimicrobial resistance and the ability of these bacteria to form persistent biofilms. The combination of cytokine-based immunotherapy and the development of vaccines targeting biofilm components and adhesion proteins may provide new tools for controlling these infections, reducing dependence on antibiotics and improving clinical outcomes for patients. As research progresses and these approaches are validated in clinical trials, they are expected to become an integral part of the therapeutic arsenal against *Burkholderia* infections and other resistant pathogens [307,345].

13.6. Recombinant Bacteriophages: A Promising Alternative

Bacteriophages, or simply phages, were discovered in the early 20th century as viruses that specifically infect and lyse bacteria. Félix d'Hérelle, a pioneer in this field, was the first to use phages therapeutic in humans, demonstrating their ability to eliminate pathogens such as *Shigella* and *Vibrio cholerae*. Although phage therapy was largely replaced by broad-spectrum antibiotics in Western countries from the 1940s onward, it remained a common practice in several Eastern Bloc nations, including Georgia, Russia, and Poland. Institutions such as the Eliava Institute in Georgia have preserved the tradition of phage therapy, which continues to be used today, particularly in cases of antibiotic-resistant infections [349].

With the decline in the discovery of new antibiotics and the alarming rise in bacterial resistance, phage therapy research has experienced a resurgence since the 1980s. Currently, phages are being tested in various therapeutic strategies against a growing list of multidrug-resistant and increasingly dangerous bacterial pathogens, including species from the ESKAPE group (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter*

baumannii, *P. aeruginosa*, and *Enterobacter* spp.) [350]. Phage therapy has been explored not only for acute infections but also for chronic infections associated with biofilms, which are particularly difficult to treat with conventional antibiotics [351,352].

Bacteriophages are extremely abundant in the environment, outnumbering bacteria by approximately ten to one [353]. It is estimated that they destroy about 50% of the global bacterial population every 48 h, playing a crucial role in regulating microbial ecosystems. Many phages possess enzymes called depolymerases, which degrade the ECM of bacterial biofilms. This capability makes phages ideal for targeting biofilms. Phage depolymerases break down these components, exposing the bacteria and allowing the phages to infect and destroy them [354,355].

To maximize the effect of phages against biofilms, multiple phages can be combined to formulate a "phage cocktail". This approach increases the likelihood of success, as different phages can target specific subpopulations within a heterogeneous bacterial community [356]. For example, bacteriophage K, a polyvalent phage of *Staphylococcus*, can lyse ten different strains of *S. epidermidis* and nine different *Staphylococcus* species. This "super phage" is also effective against vancomycin-resistant *S. aureus* (VRSA) and some methicillin-resistant *S. aureus* (MRSA) strains, highlighting its potential in combating multidrug-resistant pathogens [47,357].

In addition to their potent antibacterial properties, phages offer several advantages over traditional antibiotics. They exhibit extraordinary target specificity, attacking only specific bacteria without affecting the host's beneficial microbiota [358]. This specificity reduces the risk of dysbiosis and the side effects associated with broad-spectrum antibiotics [359]. Phages are abundant in the environment, making their acquisition easier from sources such as water, soil, and sewage. Their pharmacokinetics are advantageous, allowing efficient distribution throughout the body, including tissues and biofilms where antibiotics often struggle to penetrate. Notably, the mechanism of action of bacteriophages is entirely distinct from that of antibiotics. While antibiotics inhibit cellular processes such as cell wall or protein synthesis, phages infect bacteria, replicate within them, and destroy them through cell lysis. This means that antibiotic-resistant bacteria remain susceptible to bacteriophages, making phage therapy a valuable alternative in the current landscape of antimicrobial resistance [358,360].

With the rise of bacterial resistance and the decline in the development of new antibiotics, phage therapy is reemerging as a promising alternative for combating bacterial infections, particularly those caused by multidrug-resistant pathogens and biofilm-associated bacteria [358]. Ongoing research aims to optimize the use of phages, either alone or in combination with antibiotics, to develop effective and safe therapeutic approaches for bacterial infection control [359]. The combination of phages and antibiotics, for instance, has shown synergistic effects, where phages weaken bacteria, allowing antibiotics to complete their elimination. Additionally, genetic engineering of phages is being explored to create phages with enhanced properties, such as a broader host range or the ability to produce enzymes that degrade biofilms [361].

Despite its promising potential, phage therapy still faces regulatory and technical challenges [362]. Standardizing phage production, ensuring safety in humans, and gaining acceptance within the medical community are issues that need to be addressed [363]. However, with advances in research and the implementation of rigorous clinical trials, phage therapy is expected to become an essential tool in the therapeutic arsenal against bacterial infections, providing a sustainable and effective solution to one of the greatest challenges in global health [364,365].

13.7. Inhibition of Quorum Sensing: Innovative Therapy

QS is an intercellular communication system based on the production, release, and detection of signaling molecules known as AIs. This mechanism plays a crucial role in

regulating coordinated gene expression in bacterial populations, being essential for processes such as biofilm formation and maturation, virulence factor production, horizontal gene transfer, and antibiotic resistance [147]. The presence of biofilms grants bacteria increased resistance to antibiotics and the host immune response, making infections more difficult to treat [366]. In this context, disrupting QS signals through anti-QS agents emerges as a promising approach to prevent biofilm formation and mitigate antimicrobial resistance [131].

Based on the QS signaling mechanism, interference with this system can be achieved through various strategies, including blocking the synthesis of signaling molecules; degrading or sequestering these molecules using chemicals, antibodies, or specific enzymes; blocking QS receptors; and inhibiting the signal transduction pathway [367]. This phenomenon of QS deactivation is generally referred to as quorum quenching (QQ), and its application has been extensively studied as an innovative alternative for combating biofilm-associated bacterial infections [368]. QQ aims to disrupt bacterial communication, preventing the coordination of density-dependent behaviors such as biofilm formation and the expression of virulence genes [369].

The QQ methods can be classified into four main categories: (i) inhibition of AI synthesis, which can be achieved by directly interfering with the genes responsible for the biosynthesis of these molecules [367]; (ii) degradation or modification of AIs through enzymes such as lactonases and acylases, which break down or alter the structure of QS signals, preventing their detection by target cells [368]; (iii) QS receptor blockade using specific antagonists that compete with AIs for binding sites on the receptors, thereby inhibiting signal pathway activation [182]; and (iv) interference with QS signal transduction, which prevents the activation of QS-regulated genes, thereby disrupting virulence factor expression and biofilm formation. Each of these strategies aims to destabilize bacterial communication, making bacteria more susceptible to conventional treatments [370,371].

Significant efforts have been dedicated to developing QS inhibitors, and many new agents have been reported in the literature. Among the promising compounds, peptide-based QS modulators stand out, as they mimic natural signaling molecules and interfere with QS pathway activation [372]. These synthetic peptides have shown particular efficacy against Gram-positive bacteria, such as *S. aureus*, where they block AI binding to receptors or inhibit toxin production and biofilm formation. Additionally, phytocompounds and plant-derived byproducts have been widely investigated as sources of anti-QS agents due to their bioavailability and low toxicity. Natural compounds such as flavonoids, alkaloids, and terpenoids have demonstrated anti-QS activity in various studies [373].

Examples of natural compounds with anti-QS properties include baicalin, a flavonoid extracted from *Scutellaria baicalensis*, and cinnamaldehyde, a phenolic compound found in cinnamon. Baicalin has been shown to increase the susceptibility of biofilms formed by *B. multivorans* and *B. cenocepacia* to antibiotics, suggesting potential as both an antibacterial and antibiofilm agent [374]. Cinnamaldehyde, in turn, has demonstrated efficacy in inhibiting biofilm formation and reducing the production of virulence factors in various bacterial species [375]. Furthermore, studies indicate that pyrazole- and pyridazine-derived compounds are effective in reducing biofilm formation and virulence factor production in biofilms formed by the Bcc [376,377].

Interference with QS not only reduces biofilm formation but also decreases the expression of virulence factors, making bacteria more vulnerable to the host's immune system and antibiotics [378]. Moreover, unlike traditional antibiotics, which kill or inhibit bacterial growth, anti-QS agents do not exert direct selective pressure for the development of resistance, making them an attractive alternative in the current antimicrobial resistance scenario [379,380].

However, challenges such as bioavailability, chemical stability, and potential cytotoxic effects still need to be overcome before these molecules can be applied in clinical practice and industry. A deeper understanding of the mechanisms of QS and QQ could open pathways for the discovery of new therapeutic agents aimed at regulating bacterial communication and inhibiting biofilm formation. Future research should focus on optimizing these compounds, evaluating their safety and efficacy in animal models and human use, and exploring synergistic combinations with antibiotics and other therapies. As these studies advance, it is expected that anti-QS agents will become a valuable tool in combating resistant bacterial infections and biofilm-associated infections, contributing to improved clinical outcomes and reducing the global burden of infectious diseases [32,381].

13.8. Innovative Approaches: Nanoparticles and Electric Currents

In addition to the strategies previously discussed, other innovative approaches are being explored to prevent or inhibit biofilm formation, especially in light of the growing concern over infections caused by multidrug-resistant microorganisms. Among these approaches, the use of nanoparticles with anti-biofilm properties and physical methods based on low-intensity electric currents stand out [382].

Nanoparticles have been extensively investigated due to their antimicrobial properties and their ability to interfere with the mechanisms of biofilm formation and maturation. Titanium dioxide (TiO_2) nanoparticles, for example, have shown effectiveness in preventing biofilms formed by clinically relevant pathogens, including *S. aureus*, *S. epidermidis*, *P. aeruginosa*, *E. coli*, and Candida albicans. Their mechanism of action involves the generation of ROS under light irradiation, which leads to the destruction of the biofilm ECM and cell death due to oxidative stress. ROS, such as hydroxyl radicals and superoxide, damage essential cellular components like DNA, proteins, and lipids, as well as degrade the EPS matrix that protects the bacteria [383,384].

In addition to TiO_2, silver and chitosan nanoparticles also exhibit promising anti-biofilm activity. Silver, due to its well-known bactericidal properties, works by inhibiting bacterial adhesion to surfaces and altering cell membrane integrity [385]. Silver ions (Ag+) interact with thiol (-SH) groups in bacterial proteins and enzymes, inhibiting their function and leading to cell death [386]. When combined with chitosan, a natural biopolymer derived from chitin, these nanoparticles have shown significant effectiveness in preventing biofilm formation by Bcc species, a group of bacteria notoriously associated with colonization of medical devices and respiratory infections in immunocompromised patients [387]. Chitosan, in turn, has intrinsic antimicrobial properties, in addition to being biocompatible and biodegradable, making it an attractive option for biomedical applications [385,388].

An even more advanced strategy involves the development of functionalized nanoparticles for the controlled release of antimicrobial agents directly at the site of infection. These particles can be designed to release antibiotics or other antimicrobial substances in response to specific stimuli, such as changes in pH or temperature, which improves the treatment's effectiveness and reduces the side effects associated with the systemic use of antibiotics [389]. For example, pH-sensitive nanoparticles can release their contents in acidic environments, such as those found in biofilms or sites of inflammatory infection. Some of these nanoparticles can also be coated with targeting molecules, such as antibodies or specific peptides, increasing their specificity for bacterial cells and biofilms without affecting healthy human cells. This targeting approach allows for more precise and efficient delivery of therapeutic agents, minimizing damage to surrounding tissues [390,391].

In addition to strategies based on nanotechnology, physical methods such as the application of low-intensity electric currents have been studied as an alternative to enhance the action of antibiotics and destabilize already established biofilms. This phenomenon,

known as the "bioelectric effect", occurs because direct electric current can interfere with the polarization of the bacterial membrane, making the cells more susceptible to the action of antimicrobials. The electric current affects the integrity of the cell membrane, increasing its permeability and facilitating the entry of antibiotics and other bactericidal substances [6,392].

Studies have shown that the application of low-intensity direct electric currents can significantly reduce bacterial load in biofilms. Caubet et al. [393] observed that this type of electrical stimulation impairs the growth of biofilms formed by *E. coli*. Niepa et al. [394] demonstrated that cathodic electrochemical currents are highly effective in eliminating persistent cells in *P. aeruginosa* biofilms, one of the most challenging pathogens in the context of hospital infections. They showed that the application of direct electric currents not only compromises the cellular integrity of *P. aeruginosa*, but also destabilizes the biofilm ECM, facilitating its removal. The electric current can induce the formation of ROS at the infection site, contributing to the destruction of the bacteria and the EPS matrix.

These innovative approaches represent promising advances in the fight against biofilm-associated infections, especially in the context of growing antimicrobial resistance. The continuous development of new technologies, combining nanotechnology, bioelectrochemistry, and controlled drug release strategies, could offer effective alternatives for biofilm control in clinical and industrial settings [382].

14. Nosocomial Outbreaks Caused by Contaminated Pharmaceutical Products

Nosocomial outbreaks related to contamination by Bcc bacteria represent a critical challenge for global public health, particularly in hospital settings [1]. These microorganisms, known for their resistance and ability to form biofilms, are frequently associated with infections in hospitalized patients, especially those with weakened immune systems [121]. Contamination by the Bcc is commonly linked to pharmaceutical products, non-sterile cosmetics, and medical devices, especially those containing water in their composition, such as aqueous solutions and gels [22]. The persistence of the Bcc in water purification systems and medical equipment, combined with its resistance to conventional disinfectants, makes these outbreaks particularly difficult to control and eradicate [22,395].

The main transmission route of the Bcc in hospital environments is contaminated water used in the production of pharmaceutical products and cosmetics, as well as in medical devices. Products such as cleaning solutions, mouthwashes, eye drops, and gels are especially susceptible to contamination due to their high-water content, which serves as an ideal vehicle for the spread of the pathogen [22]. Additionally, poorly maintained or contaminated water purification systems are frequently identified as sources of outbreaks [396]. The formation of biofilms in pipes, water reservoirs, and medical equipment is a crucial factor contributing to the persistence of the Bcc. These biofilms not only protect the bacteria from disinfectants but also act as continuous reservoirs of contamination, even after cleaning and disinfecting surfaces [121].

Several studies have documented outbreaks associated with the Bcc in different regions around the world, highlighting the global nature of the problem (Table 1). In Morocco, a study conducted at a hemodialysis center in Fez revealed that 17% of water samples collected were contaminated with *B. cepacia*, representing a significant risk to patients undergoing renal treatment [397]. In Brazil, a study conducted in São Luís, Maranhão, analyzed 18 water samples collected from pre- and post-treatment points at three hospital units, identifying the presence of *B. cepacia* in two of them, reinforcing the need for stricter filtration and disinfection protocols for water used in healthcare services [398].

Table 1. Pharmaceutical contaminations and outbreaks caused by the *Burkholderia cepacia* complex.

Period	Country	Location	Product	Species	N° of Infected	Clinical Data	Underlying Conditions	References
ND	Portugal	CF Center	ND	*B. cepacia* (62.5%) *B. multivorans* (25.0%) *B. cenocepacia* (12.5%)	32	CRTI and DPF	CF	[122]
1995–2009	Portugal	CF Center	ND	*B. cepacia* (56.5%) *B. contaminans* (18.5%) *B. multivorans* (14.8%) *B. cenocepacia* (7.4%)	81	CRTI and DPF	CF	[399]
1999–2015	USA	VHA	CVC	Bcc	248	CVC (41%), PNM (20%), D at 14 days (16%), at 30 days (25%), and at 90 days (36%)	NCDs	[400]
ND	Brazil	HDU	Dialysis solution	*B. cepacia*	ND	ND	CKD, IMS, CVC, and IMDU	[398]
2007–2008	USA	UH	Nasal spray	Bcc	36	ARTC, ARTI, and PNM	IMS and CRIs	[401]
2008–2009	Germany	TH	Wet wipes	*B. contaminans*	29	ARTI and SW	NCDs, IMS, IMDU, and SW	[396]
2011	India	OC	Anesthetic eye drops	Bcc	12	RDN, Ed, OP, PD, BV, AE, and VL	OS, CAT, and CT	[402]
2012	USA	TH	Intravenous fentanyl	*B. contaminans*	6	FV, LKC, TCH, and HPT	NCDs and IMDU	[403]
2013	Ecuador	ICU	Mouthwash	*B. cepacia*	10	ARTC and ARTI	MV and SMC	[404]
2013	China	TH	Ultrasound coupling gel	*B. cepacia*	8	RDN, Ed, ISP, PD, and FV	PCS	[405]
2013	South Korea	BMC	CHX 0.5%	Bcc	21	SIS	VMC	[406]
2013	Argentina	ICU, NICU	Ultrasound coupling gel	Bcc	6	FV, LKC, and BCT	NCDs and IMDU	[407]
2013	India	ACU	Antiemetic (granisetron)	Bcc	6	FV, Ch, and BCT	IMS and CAN	[408]
2013–2014	Paraguay	HDU	Heparin solutions	*B. cepacia*	12	FV, Ch, and SIS	HTN and DM,	[409]
2013–2018	Turkey	TH	Medical devices	*B. cepacia*	45	UTI, ARTI, and BCT	NCDs, IMS, and IMDU	[410]

Table 1. *Cont.*

Period	Country	Location	Product	Species	N° of Infected	Clinical Data	Underlying Conditions	References
2014	Brazil	ND	Mouthwash	*B. cepacia*	ND	MI, LP, OI, and TI	ND	[411]
2014	China	NPD Ward	Saline solution	*B. stabilis*	15	AS	CNDs	[412]
2014	India	NICU	Dextrose 5.0%, NS, and CPAP	*B. cepacia*	10	FV, LKC, TCH, HPT, and Seps	PT and SMC	[413]
2014	Spain	HDU	Heparin solutions	*B. cepacia*	10	FV, Ch, and SIS	HTN, DM, and CKD	[414]
2015	India	NICU	Liquid soap	*B. cepacia*	6	FV, LKC, TCH, HPT, and NS	PT and SMC	[415]
2015	India	TH, PEDU	Injection vials (AMK)	Bcc	15	ARTI, FV, LKC, and Seps	NCDs, IMS, and IMDU	[416]
2015	China	SICU	Ventilators	*B. cepacia*	10	CPD, FV, LKC, PI, and VAP	NCDs, IMS, and IMDU	[417]
6 months	Egypt	UH	ND	Bcc	35	PD and SB	ND	[397]
2015–2016	Switzerland	9HCs	Washing gloves	*B. stabilis*	46	BCT and OIs	ND	[418]
2015–2016	Australia	4HCs	Gel packed in sachets (SUPC)	*B. cenocepacia*	15	AC, ARTI, and BCT	NCDs, IMS, and IMDU	[419]
2016	Australia	H and HC	Mouthwash (CHX)	*B. lata*	12	ARTC and ARTI	CLDs, IMS, and IMDU	[420]
ND	India	TH, PICU	Distilled water	Bcc	3	VSD, PNM, PTX, and RMS	IC/BSAT	[421]
2016	USA	TH, PEDU	Liquid sodium docusate	Bcc	12	AC, ARTI, and BCT	NCDs, IMS, and IMDU	[422]
2016	USA	16HCs	Liquid sodium docusate	Bcc	63	AC, ARTI, and BCT	NCDs, IMS, and IMDU	[5]
2016	Saudi Arabia	TH	Ultrasound coupling gel	*B. cepacia*	12	FV, Ch, and SIS	NCDs, IMS, and IMDU	[423]
2016	South Korea	NICUs	CHX 0.5%	*B. cepacia*	10	RF	PT and SMC	[424]
2016	USA	TH, ICU, PEDU	Liquid sodium docusate	Bcc	15	UTIs, ARTI, and BCT	ND	[425]
2016–2017	USA	59HCs	Syringes with saline solution	Bcc	162	AC, ARTI, and BCT	NCDs, IMS, and IMDU	[426]
ND	Jordan	UCs	ND	Bcc	2	PLR, Dys, and FV	ND	[427]

Table 1. *Cont.*

Period	Country	Location	Product	Species	N° of Infected	Clinical Data	Underlying Conditions	References
2017	USA	VH	CHX 2%	*B. cenocepacia*	5	Ed, LP, FV, and CLL	VMC	[428]
2017	India	NTICU	CVC	*B. cepacia*	12	AC, ARTI, and BCT	NCDs, IMS, CST, and IMDU	[429]
2017	Canada	PDU	Dialysis solution	*B. cepacia*	6	AC, BCT, and PRT	HTN, DM, and CKD	[430]
2017–2018	USA	Multiple HCs	No-rinse cleansing foam	*B. cenocepacia* *B. vietnamiensis*	59	AC, ARTI, and BCT	NCDs, IMS, and IMDU	[395]
2018	Germany	CTICU	Mouthwash (Oct)	Bcc	12	AC, ARTI, and BCT	NCDs, IMS, and IMDU	[431]
2018	China	TH	Anesthetic gel, and injectables	*B. cepacia*	12	UTI and BCT	NCDs, IMS, and IMDU	[432]
2019	China	TH	CHX 0.5%	*B. cepacia*	10	AC, BCT, and PRT	HTN, DM, and CKD	[433]
2020	China	TH	Anesthetic gel (cystoscopy)	*B. cepacia*	8	Dys, UTI, and FV	VMC	[229]
ND	Argentina	PhI	IS, PP, and MM	*B. aenigmatica* *B. arboris* *B. cenocepacia* *B. cepacia* *B. contaminans* *B. multivorans* *B. vietnamiensis*	135 isolates	NA	NA	[228]

Abbreviations: 16HCs, Sixteen Healthcare Centers; 4HCs, Four Healthcare Centers; 59HCs, Fifty-Nine Healthcare Centers; 9HCs, Nine Healthcare Centers; AC, Asymptomatic Colonization; ACU, Ambulatory Chemotherapy Unit; AE, Acute Endophthalmitis; ARTC, Asymptomatic Respiratory Tract Colonization; ARTI, Acute Respiratory Tract Infection; AS, Asymptomatic; BCT, Bacteremia; BMC, Boramae Medical Center; BV, Blurred Vision; CAN, Cancer; CAT, Cataract; CF Center, Cystic Fibrosis Treatment Center; CF, Cystic Fibrosis; Ch, Chills; CHX, Chlorhexidine; CKD, Chronic Kidney Disease; CLDs, Chronic Lung Diseases; CLL, Cellulitis; CNDs, Chronic Nasal Diseases; CPD, Cough with Purulent Discharge; CRIs, Chronic Respiratory Infections; CRTI, Chronic Respiratory Tract Infection; CST, Craniospinal Trauma; CT, Corneal Transplant; CTICU, Cardiothoracic Intensive Care Unit; CVC, Central Venous Catheter; D, Death; DM, Diabetes Mellitus; DPF, Decline in Pulmonary Function; Dys, Dysuria; Ed, Edema; FV, Fever; H and HC, Hospitals and Healthcare Centers; HDU, Hemodialysis Unit; HPT, Hypotension; HTN, Hypertension; IC/BSAT, Immunocompromised and/or Broad-Spectrum Antibiotic Therapy; ICU, Intensive Care Unit; IMDU, Invasive Medical Device Use; IMS, Immunosuppression; IS, Industrial Solutions; ISP, Incision Site Pain; LKC, Leukocytosis; LP, Localized Pain; MI, Mouth Inflammation; MM, Miscellaneous Materials; Multiple HCs, Multiple Healthcare Centers; MV, Mechanical Ventilation; NA, Not Applicable; NCDs, Non-Communicable Diseases; ND, Not Declared; NICU, Neonatal Intensive Care Unit; NPD, Nasal Disease Patients; NS, Neonatal Sepsis; NTICU, Neurotrauma Intensive Care Unit; OC, Ophthalmology Center; Oct, Octenidine; OI, Oral Infection; OIs, Other Infections; OP, Ocular Pain; OS, Ophthalmic Surgery; PCS, Post-Cesarean Section; PD, Purulent Discharge; PDU, Peritoneal Dialysis Unit; PEDU, Pediatric Unit; PhI, Pharmaceutical Industry; PI, Pulmonary Infiltrates; PICU, Pediatric Intensive Care Unit; PLR, Polyuria; PNM, Pneumonia; PP, Pharmaceutical Products; PRT, Peritonitis; PT, Preterm; PTX, Pneumothorax; RDN, Redness; RF, Respiratory Failure; RMS, Rhabdomyosarcoma; SB, Skin Burns; Seps, Sepsis; SICU, Surgical Intensive Care Unit; SIS, Systemic Infection Symptoms; SMC, Severe Medical Conditions; SW, Surgical Wounds; TCH, Tachycardia; TH, Tertiary Hospital; TI, Throat Infection; UCs, Urology Clinics; UH, University Hospital; UTIs, Urinary Tract Infections; VAP; Ventilator-Associated Pneumonia; VH, Veterinary Hospital; VHA, Veterans Health Administration; VL, Vision Loss; VMC, Various Medical Conditions; VSD, Ventricular Septal Defect.

Contamination cases in hospital products are also frequent. In 2014, in Brazil, seven batches of a mouthwash were recalled in Minas Gerais due to contamination by *Burkholderia* bacteria. This incident highlighted how failures in quality control can pose significant risks to patients [411]. In Paraguay, an outbreak in a Hemodialysis Unit (UH) was linked to the contamination of 75% of the dialysis water samples by *B. cepacia*, indicating widespread dissemination within the hospital system [409].

Outbreaks of *B. cepacia* have also been reported in surgical contexts. A notable example occurred in 2014, when thirteen patients undergoing cataract surgery with intraocular lens insertion developed acute endophthalmitis due to the use of contaminated anesthetic eye drop. This case reinforced the need for rigorous microbiological testing in ophthalmic products to prevent serious postoperative complications [402]. In 2016, in the United States, an epidemiological investigation analyzed 15 cases of Bcc infections in pediatric intensive care unit patients in Texas and 4 similar cases in Illinois. Molecular analyses revealed that the isolated strains were indistinguishable, indicating a common source of contamination. The investigation traced the origin of the infections to contaminated products from a single supplier, highlighting the importance of epidemiological tracking to identify and contain the clonal spread of pathogens in hospital products [425].

In Germany, an outbreak caused by *B. cepacia* was identified in a cardiothoracic Intensive Care Unit (ICU) due to contamination of a batch of octenidine-based mouthwash. The manufacturer was notified, and the product was immediately withdrawn from the market, leading to the interruption of the outbreak [396]. In Argentina, a study analyzed 135 cultures from different pharmaceutical companies, with 46 samples being from water used in the production of medications. *B. cepacia* and other species from the complex were detected in these samples, highlighting the need for strict water quality control in industrial settings [228].

Another relevant example occurred in a Chinese hospital, where four cases of *B. cepacia* infection were linked to the use of a 0.05% CHX solution. The investigation revealed that the contamination originated at the supplier's bottling facility, where biofilms had formed, allowing the pathogen to spread to the final product [433]. Additionally, recalls were issued on several occasions due to contamination of essential hospital products. A notable case occurred in the USA, where a no-rinse cleaning foam used for patient hygiene in hospitals across six different states was recalled after reports of associated infections. The infections included severe cases, such as respiratory and urinary infections, wound infections, sepsis, contamination of peritoneal fluid, and genital infections in at least 60 patients, highlighting the risks associated with *B. cepacia* contamination in medical and cosmetic products [395].

These outbreaks demonstrate the ability of the Bcc to survive and persist in hospital environments, especially in water-based products, which act as ideal reservoirs for biofilm development. The intrinsic resistance of the Bcc to common disinfectants and its ability to form biofilms make it essential to adopt stringent microbiological monitoring measures in water purification systems, medical equipment, and pharmaceutical product manufacturing processes [22]. More rigorous hygiene protocols, the implementation of new technologies for early detection of contamination, and innovative strategies to inhibit biofilms are crucial to mitigate the risk of outbreaks and protect vulnerable patients in hospital settings [121]. The prevention and control of these outbreaks require a multifaceted approach, involving collaboration between healthcare professionals, medical product manufacturers, and regulatory authorities to ensure patient safety and the quality of products used in healthcare environments [1].

The Bcc is recognized for its ability to survive and persist in hospital environments, especially in water-based products, which serve as ideal reservoirs for biofilm development [22]. The intrinsic resistance of the Bcc to common disinfectants and its ability to form

biofilms make it essential to adopt stringent microbiological monitoring measures in water purification systems, medical equipment, and pharmaceutical product manufacturing processes [1,237]. More rigorous hygiene protocols, the implementation of new technologies for early detection of contamination, and innovative strategies to inhibit biofilms are crucial to mitigate the risk of outbreaks and protect vulnerable patients in hospital settings [121]. The prevention and control of these outbreaks require a multifaceted approach, involving collaboration between healthcare professionals, medical product manufacturers, and regulatory authorities to ensure patient safety and the quality of products used in healthcare environments [4].

15. Control and Prevention of Contamination

The persistence of Bcc bacteria in pharmaceutical products is a multifactorial issue, often linked to failures in good manufacturing practices (GMP) implementation [22]. Pharmaceutical industries are responsible for ensuring the quality control of their products and monitoring the processing stages, including the selection and control of raw materials used. However, several factors contribute to microbiological contamination in pharmaceutical environments, such as improper cleaning procedures; the use of unsuitable water grades in inadequate purification and distribution systems; prolonged use of the same disinfectants without proper rotation [232]; insufficient microbiological controls [434]; and a lack of robust guidelines for testing, specifications, and process validation [236,435].

According to Technical Report No. 67 from the Parenteral Drug Association (PDA), titled "Exclusion of Objectionable Microorganisms from Nonsterile Pharmaceuticals, Over-the-Counter (OTC) Products, Medical Devices, and Cosmetics", which addresses the exclusion of objectionable microorganisms from nonsterile products, an objectionable microorganism is defined as one capable of proliferating in a given pharmaceutical product and compromising its chemical, physical, functional, or therapeutic properties. It is important to highlight that this concept is not limited to classical pathogens but includes any microorganism that—depending on the type of product, route of administration, target population, and level of contamination—may compromise the product's safety, efficacy, or quality. The definition emphasizes that pathogenic microorganisms, particularly when present at elevated concentrations, may pose an infection risk to patients, thus justifying their classification as objectionable [436]. The high rate of product recalls due to contamination by Bcc bacteria, along with the numerous hospital outbreaks associated with these microorganisms, further reinforces their classification as objectionable [232]. Furthermore, their opportunistic nature, affecting both CF patients and individuals without the disease; their resistance to antimicrobial agents and preservatives; and their remarkable ability to persist in hospital and industrial environments collectively make their control a considerable challenge [437].

In Brazil, the National Health Surveillance Agency (Anvisa) has published several Collegiate Board Resolutions (RDC), such as RDC No. 658 of 2022, RDC No. 48 of 2013, and RDC No. 481 of 1999, which, along with the Brazilian Pharmacopeia, establish guidelines for GMP for pharmaceuticals and cosmetics. These regulations define the permissible microbiological limits for raw materials and products, specifying the organisms that must be absent. For cosmetic products, the bacterial limits are 5×10^3 colony-forming unit (CFU)/g or mL for products of risk grade 1 and 5×10^2 CFU/g or mL for products of risk grade 2, with the requirement for the absence of *P. aeruginosa*, *S. aureus*, *E. coli*, coliforms, and clostridia. The microbiological limits for non-sterile pharmaceutical raw materials, as well as the non-permitted pathogenic organisms, vary according to the pharmaceutical formulation, extraction process, origin, and route of administration of each product. However, studies on contamination by *Burkholderia* in cosmetics in Brazil are recent, and

Bcc species are not yet specifically addressed in these regulations; moreover, the Brazilian Pharmacopeia does not include specific tests for their identification [230,438].

Unlike the Brazilian scenario, in 2019, the United States Pharmacopeia (USP) included guidelines for microorganism detection in non-sterile products, such as USP 61 ("Microbial Enumeration Tests") and USP 62 ("Tests for Specified Microorganisms") [439–441]. However, despite recognizing the significance of Bcc bacteria, the USP does not yet provide specific methods for their detection in non-sterile pharmaceutical products, such as inhalers or aqueous preparations for oral, cutaneous, or nasal use [439,440]. This gap in regulations and control methods contributes to the oversight in detecting these bacteria, increasing the risk of releasing contaminated products into the market [442–444]. However, standards from the American Society for Testing and Materials (ASTM) and the Personal Care Products Council (CTFA) include identification tests for *Burkholderia* in water-miscible cosmetic products [442,443,445,446].

The concern regarding the undetected presence of Bcc in pharmaceutical products is not a new topic and has been discussed by the FDA since 1981, when the regulatory agency identified irregularities in the validation and control processes of a water deionization system, leading to the contamination of a pharmaceutical product with *B. cepacia* [253,447,448]. In 1993, a recall was issued for a metaproterenol sulfate inhalation solution due to *B. cepacia* contamination, even though the USP did not require microbiological testing for this product [238]. A more recent case involved the contamination of a chemically preserved fexofenadine oral suspension by *B. cepacia* six months after its manufacture. The objectionable microorganism was not previously detected because it was not included in the routine tests described in USP 62 and was not considered a threat, even though its presence had once been detected in the manufacturer's water system [22].

Studies conducted by the FDA showed that 34% of recalls for non-sterile pharmaceutical products between 2004 and 2011 were due to *B. cepacia* contamination [233,234]. An update of this study revealed that between 2012 and 2019, there was an average of 30 recalls per year due to the presence of this pathogen, with 47.8% of these recalls involving non-sterile raw materials [235].

Given this scenario, the implementation of strict safety measures is essential to mitigate the risk of *B. cepacia* contamination in the pharmaceutical industry. This includes rigorous monitoring of water sources, especially pharmaceutical water systems, which are the primary vectors of contamination [232]; the adoption of enhanced cleaning and disinfection protocols throughout all stages of the production process; and the application of more sensitive and specific microbiological tests for the detection of objectionable organisms, including Bcc bacteria [449]. Investigating the presence of these bacteria as contaminants in the pharmaceutical industry is an increasing necessity and a crucial aspect of sanitary regulation, aiming to reduce nosocomial outbreaks and adverse public health impacts [22].

16. Conclusions

The contamination by biofilms formed by the Bcc presents a significant challenge both for the pharmaceutical industry and for hospital environments. These microorganisms, known for their high adaptability and resistance, stand out as pathogens that are difficult to control. The analysis of the molecular mechanisms involved in biofilm formation—such as adhesion, maturation, dispersion, regulation by QS, and the remarkable genetic plasticity of the Bcc—reveals the complexity that hampers its eradication. This survival capacity not only contributes to the persistence of nosocomial infections but also limits the effectiveness of conventional disinfection and microbiological control methods.

In this context, the development of integrated strategies that combine innovative diagnostic techniques, advanced decontamination technologies, and combined therapies

is essential to address biofilm formation. Ongoing research in this area, with a focus on early detection methods and specific interventions, is crucial to improve microbiological control protocols, ensuring the safety of pharmaceutical products and patient protection. In summary, a detailed understanding of the mechanisms behind Bcc biofilm formation and maintenance is critical for the implementation of more effective preventive measures and for reducing the risks associated with hospital infections.

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