Clinically relevant *in vitro* biofilm models: A need to mimic and recapitulate the host environment

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A B S T R A C T

Biofilm-associated infections are difficult to treat and eradicate because of their increased antimicrobial tolerance. *In vitro* biofilm models have enabled the high throughput testing of an array of differing novel antimicrobials and treatment strategies. However, biofilms formed in these oftentimes basic *in vitro* systems do not resemble biofilms seen *in vivo*. As a result, translatability from the lab to the clinic is poor or limited. To improve translatability, *in vitro* models must better recapitulate the host environment. This review describes and critically evaluates new and innovative *in vitro* models that better mimic the environments of a variety of clinically important, biofilm-associated infections of the skin, oropharynx, lungs, and infections related to indwelling implants and medical devices. This review highlights that many of these models represent considerable advances in the field of biofilm research and help to translate laboratory findings into the clinical practice.

1. Introduction

Microbial biofilms exist and persist in a variety of niche environments of the human host. Biofilms cause an array of infections and diseases that range in their severity and chronicity. This is inextricably underpinned by their ability to evade host immune action and notable tolerance towards antimicrobials [1,2,3]. A plethora of novel and effective antimicrobials and treatment strategies have been explored. However, translation of their anti-biofilm activity from results obtained in the lab to treatment success under a clinical setting is hindered by currently available biofilm models. Consequently, in the past five to ten years alone there has been much discussion of model appropriateness, and a gap identified toward models most closely representing the host.

To date, most studies within the biofilm field have been performed using *in vitro* systems or animal models that do not mimic or accurately represent the human host environment. Undeniably, there is much merit to these studies, providing valuable and fundamental insight into biofilms and a means to assess antimicrobial efficacy. Animal models have aimed to shed light on host immune responses and represent human pathogenesis, whilst many of the currently available *in vitro* models enable us to collect information in a manner that is high throughput, easy to use, flexible, controllable, and cost effective. However, both can be limited or restricted in their translatability to biofilms present *in vivo*. Animal models present considerable ethical barriers, are complicated to

run, require expertise, and cannot always accurately reflect human physiology and disease [4]. And as our understanding of the biofilms present at various sites of infection increases, the more we uncover of the stark and vast differences to biofilms generated in many of the simpler and most commonly used *in vitro* models that fail to consider the host environment [5,6]. Of note, basic models lacking appropriate host factors (relevant substratum, flow/non-flow, host fluids etc) have been noted to profoundly impact the biofilms formed, including their architecture/structure, gene expression, quorum sensing, virulence, and antimicrobial tolerance profiles - to name a few [5,6,7]. This has prompted the generation of several innovative, and more advanced *in vitro* models that aim to better recreate a variety of biofilm infected sites such as a chronic wound via artificial dermis biofilm model, acneic skin via artificial sebum pellet, fungal nail models, and so forth [8,9,10]. While these newer models include host factors, it is important to recognise that none of them can completely represent an *in vivo* biofilm environment and additional host interaction will play an important role.

In this review, we discuss newer *in vitro* models that aim to replicate the environments of a variety of biofilm-associated infections ranging from those of the skin, oropharynx, lungs, and indwelling implants/ medical devices. We provide the details of each of these models (their set up, components being mimicked/encompassed etc.), advantages and limitations, their utility and findings. Overall, this review aims to evaluate such models which offer more relevant conditions towards

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specific infection scenarios compared to general *in vitro* models.

2. Skin infections

The skin is the largest organ of the human body and forms a protective barrier from potential mechanical/chemical harm of the outside world and foreign invading microbes and pathogens. Damage to the skin can facilitate the introduction of microbes to the site. The aetiology of the diseases that result is attributed to initial microbial accumulation followed by biofilm formation. Non-healing chronic wounds (burns, pressure ulcers, diabetic foot ulcers etc.) are a prominent example of biofilm-associated skin infections that are particularly challenging to treat [1]. Biofilms have also been implicated in several other persistent, and oftentimes chronic skin infections such as acne, atopic dermatitis, eczema, dermatophytosis, and onychomycosis which all present their

own set of treatment challenges and burdens to both the patient and healthcare system [11,12,9].

2.1. Chronic wounds

A wound occurs when there is injury or damage to the skin's integrity and impairment of physiological function. Unlike acute wounds (abrasions, blisters, lacerations, surgical incisions), chronic wounds fail to proceed through the normal healing stages in a timely manner, a process necessary for the restoration of anatomical and functional integrity [13, 14]. Consequently, chronic wounds represent an ideal environment for microbial colonisation [15,16]. Specifically, a wound provides a moist, nutrient-rich environment. The wound bed offers a biochemically and physically complex environment of varying nutrient gradients and gas levels, pH, commensals, local biomechanical forces, and unique

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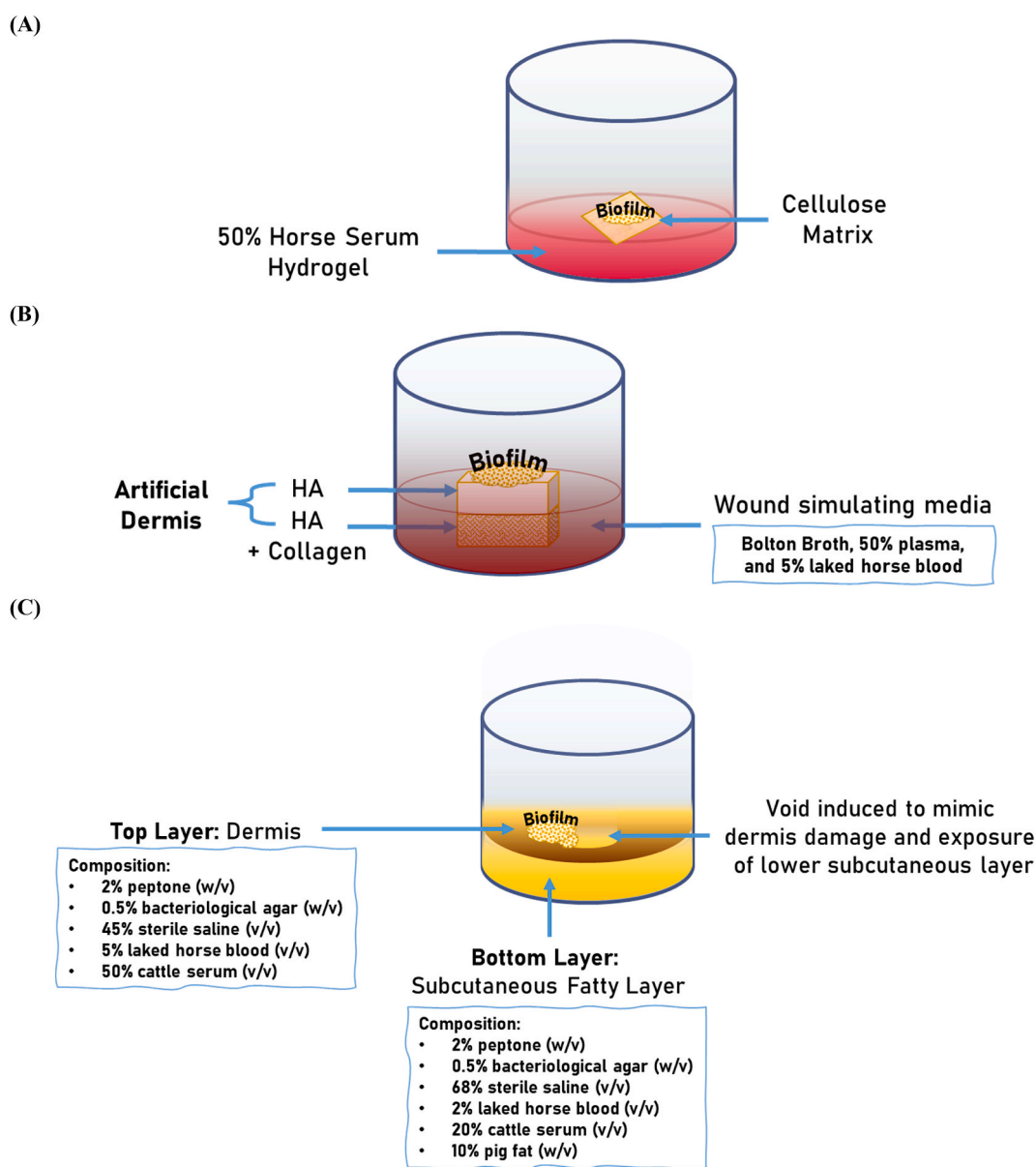
Fig. 1. Schematic representations of three *in vitro* wound biofilm models. (A) Hydrogel wound biofilm model developed by Townsend et al. (2016) [18]. The hydrogel model comprises a 50% horse serum hydrogel topped with a cellulose matrix where the biofilm is formed. Models **(B)** and **(C)** encompass the multi-layered nature of the skin. **(B)** Brackman et al. (2016) [8] model is composed of a spongy hyaluronic acid (HA) top layer and a bottom hyaluronic acid-collagen blend layer which is initially infused in the wound simulating media (50% plasma-5% laked horse blood) and then kept partially immersed in this medium. **(C)** The complexity of each layer's composition, together with the uniqueness of the intentional void mimicking dermal damage distinguishes the Chen et al. (2021) [19] model from the two-layer Brackman et al. (2016) [8] wound model seen in **(B)**.

architectural scaffolds/topographical landscapes. Collectively, these factors facilitate and shape microbial interactions occurring with the host towards initial adherence, colonisation, and subsequent biofilm formation and establishment [17]. It is widely recognised that the healing of chronic wounds is often complicated due to the biofilm formation by pathogens [1]. Here, we present some of the latest developments in *in vitro* chronic wound models that encompass components of the wound microenvironment.

Townsend et al. (2016) [18] developed an *in vitro* hydrogel-based cellulose substratum wound model (Fig. 1A) for the study of commonly used topical wound treatments povidone-iodine (PVP–I) and chlorhexidine (CHX) against inter-kingdom polymicrobial biofilms of *Candida albicans*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. The model is contained in a 12-well plate and is composed of a 50% horse serum hydrogel topped with a cellulose matrix. The semi-solid hydrogel material serves as a nutrient source, whilst the cellulose matrix provides a three-dimensional structure facilitating polymicrobial biofilm formation. The polymicrobial biofilms formed via the *in vitro* hydrogel model displayed significantly reduced antimicrobial susceptibility to both PVP-I and CHX when compared to biofilms formed in a traditional polystyrene plate setup. This aligns with clinical findings, whereby both treatments have been ineffective for the management of chronic wounds. Biofilms formed in the hydrogel model were also noticeably different in architecture/structure when compared to biofilms formed in polystyrene plates. Specifically, *C. albicans* individual biofilm cell morphology was altered, with yeast cells found in biofilms grown via the hydrogel model whilst hyphae predominated biofilms formed in the polystyrene plate setup. A limitation of this model is that cellulose is not naturally present in a wound and may impact the biofilms formed. Future adaptions of this model may wish to use more host representative growth matrices whereby fibroblasts or keratinocytes serve as the main matrix component [17].

Other *in vitro* models exist that mimic the multilayering of the skin. Encompassing aspects such as the epidermis, dermis, and subcutaneous tissue layers as well as the respective features unique to each layer; collagen, blood to represent vascularisation, and adipose distinctly present in the subcutaneous tissue layer [20,21]. Brackman et al. (2016) [8] developed a two-layered *in vitro* artificial dermis (AD) wound biofilm model (Fig. 1B) comprising of an upper spongy layer of chemically cross-linked hyaluronic acid (HA) and a spongy lower layer of HA mixed with collagen. The AD is soaked and partially immersed in a wound simulating media (WSM) of microbial growth media supplemented with 50% plasma and 5% laked horse blood. This *in vitro* model was utilised to assess the antimicrobial efficacy of hydroxypropyl-β-cyclodextrin-functionalised gauzes loaded with hamamelitannin and vancomycin. The gauzes impacted quorum sensing and biofilm formation of *P. aeruginosa* and *S. aureus*. This model advantageously provides a three-dimensional structure for single- and mixed-species biofilm growth, a nutrient-rich environment, relevant host matrix components, and WSM which can be easily refreshed or replenished in the wells of the plate.

Recently, Chen et al. (2021) [19], developed a more complex two-layered *in vitro* model of differential compositional blends of peptone, bacteriological agar, laked horse blood, cattle serum, and sterile saline (Fig. 1C). The subcutaneous layer contained pig fat to mimic the high adipose content and to further distinguish it from the dermis layer. Moreover, the model is comprised of an intentional void/breach of the dermis layer to represent a breach in skin barrier function, with the model exposing the lower subcutaneous layer. This uniquely enables the bacteria to spread both over and into the subcutaneous layer. Biofilms of *P. aeruginosa* and *S. aureus* were inspected for their ability to co-exist using this model as often seen *in vivo*. Biofilms were also subjected to antimicrobial testing to i) assess the model's practical utility (i.e., ease of applying antimicrobial solutions and dressings) and ii) translatability of antimicrobial susceptibility findings in accordance with clinical susceptibility. The study found that

two-species biofilms were achievable, co-existing for up to 96 h. Moreover, the biofilms formed existed as non-surfaced attached microcolonies, which the authors stated resembled those seen in clinical samples. A 0.1% polyhexamethylene biguanide antimicrobial rinsing solution and a variety of wound dressings (silver and honey-based) were easily appliable to the biofilms. In both instances, the log reduction in biofilm reflected clinical findings, with antimicrobial resilience much higher than other similar published *in vitro* biofilm studies that fail to recapitulate the wound environment. Overall, this model offers much promise towards replicating a chronic wound.

Here, three *in vitro* chronic wound models of varying complexities were explored. It is evident that models which aim to better recapitulate a wound, yield biofilms phenotypically similar to those present *in vivo*, with antimicrobial susceptibility closely reflecting clinical findings. Of course, there are other limitations that all three models could further consider such as wound bed temperature, gas diffusion, antimicrobial diffusion, and the inclusion of all three major skin layers.

In vitro wound models also exist exploring and focussing on improving the wound fluid component. Many models utilise plasma and serum to recreate wound fluid, intending to capture both the nutrient richness and biochemical complexities. However, the wound milieu, which plays a crucial role in wound healing, is far more complex and comprises a variety of host factors [22]. Kadam et al. (2021) [23] present an *in vitro* wound milieu (IVWM) to further facilitate a shift from models that solely rely upon laboratory refined media (e.g., Luria-Bertani, LB) and serum/plasma. This wound milieu incorporates serum, matrix elements (collagen, fibrinogen, and fibronectin), and other host factors (lactoferrin and lactic acid). Utilising the IVWM, biofilms of *P. aeruginosa* and *S. aureus* were assessed and compared to biofilms formed with LB and fetal bovine serum. It was found that biofilms formed with the IVWM were distinctly different to biofilms formed utilising both lab media and serum. More importantly, biofilms formed via the IVWM more closely represented those seen *in vivo* in their metabolic activity, biomass, structure, and formation. Antibiotic tolerance profiles aligned more closely to those established under clinical settings. Further work needs to be done in recapitulating wound fluids, and consideration made on their complex/heterogeneous nature with variability dependent on; wound type (foot ulcer, burn etc), chronic vs acute wound, and phase of wound healing [24,25]. Nonetheless, these findings highlight that the physical landscapes provided in *in vitro* models are only a part of recapitulating the chronic wound environment. It is also the fluids that comprise the wound environment that further shape and direct biofilm formation, and thus much consideration is needed into replicating them and understanding the role they play in wound-associated biofilms.

2.2. Acne

In the U.S alone, acne affects ~85% of individuals between the ages of 12–24 and accounts for 20% of dermatologist visits [26]. Acne is a chronic cutaneous inflammatory disorder and its pathogenesis is characterised by hyperproliferation, altered follicular epithelium differentiation, and excess sebum production [27]. *Cutibacterium acnes*, a gram-positive anaerobe is a naturally residing member of the skin flora implicated in acne. *C. acnes* biofilm formation has been attributed to the chronicity of acne [28]. Moreover, *C. acnes* biofilms reduce antimicrobial treatment success [29].

Spittaels and Coenye (2018) [9] developed an *in vitro* artificial sebum model comprising of an artificial sebum pellet which is inoculated with clinically relevant *C. acnes* strains (Fig. 2). In acneic skin, the pilosebaceous unit is blocked by overproduced sebum, which creates an anaerobic environment rich in lipids. This presents an ideal environment for *C. acnes* biofilm growth. Hence, this model aimed to recreate the lipid-rich environment of the pilosebaceous unit via the sebum pellet. The artificial sebum is composed of a variety of physicochemically related components typically present in human sebum. This *in vitro*
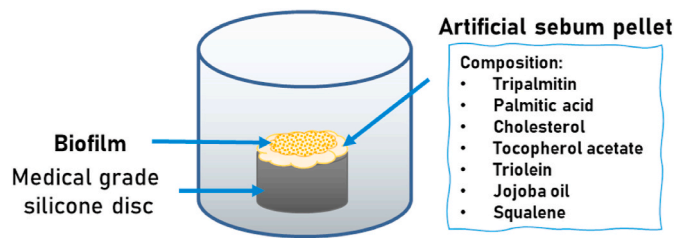
Fig. 2. Schematic representation of *in vitro* artificial sebum model developed by Spittaels and Coenye (2018) [9]**.** The model comprises of sebum pellets made of components chosen to physicochemically represent those seen in human sebum (e.g., free fatty acids are represented by palmitic acid). The sebum pellets sit on top of sterile medical-grade silicone discs which are loaded into 48-well plates. *C. acnes* inoculum is added into each well and incubated for 15 min at 37 °C, unattached *C. acnes* is aspirated off, and biofilms are formed from the sebum pellet attached bacteria.

model successfully generated *C. acnes* biofilms for a variety of clinically relevant strains obtained directly from acneic skin. Biofilms formed resemble those found *in vivo*, demonstrating virulence factor production (e.g., viable lipase and protease function). This is an important find, as exogenous lipase is abundantly produced by *C. acnes* which triggers a variety of host inflammatory responses that contribute to an exacerbated and chronic acneic state [30]. The authors suggest that this model is most beneficial and relevant over other currently available *in vitro* models used in the biofilm field which are reliant on microtitre plates, flow cells, or glass beads as it closely mimics the nutrient-deficient environment of the pilosebaceous unit. The authors also highlight its ease of use and cost-effectiveness. Taken together it is an attractive *in vitro* model that should be explored and utilised to characterise *C. acnes* biofilms and their antimicrobial susceptibility to further ascertain its clinical relevance and translatability.

2.3. Other skin soft tissue infections

Necrotising fasciitis (NF) and related necrotising soft tissue infections (NSTIs) are invasive forms of skin soft tissue infections (SSTIs). They are severe and life-threatening, with necrosis rapidly progressing through all layers of the skin, and into the muscle. NF and NSTIs pose a high mortality rate, especially in the absence of surgical intervention (roughly 86% mortality rate) [31]. *Streptococcus pyogenes* (Group A Streptococcus; GAS), is a gram-positive human pathogen involved in NF/NSTIs. Until recently, biofilms were not implicated as a mechanism for GAS virulence in NF/NSTIs.

Siemens et al. (2016) [32] developed a three-dimensional *in vitro* human skin tissue organotypic model to investigate biofilms formed by three NSTI GAS strains. The air-exposed skin model was generated from human keratinocytes (N/TERT-1 cells) and non-human dermal fibroblasts. To mimic key anatomical features and functions of human skin, a stratified dermis and fibroblast dermal layer was created, with structural framework proteins incorporated. All three GAS strains readily infected the tissue of the organotypic model. Throughout the model tissue, GAS appeared as aggregates when visualised via confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). To confirm if GAS biofilms form *in vivo* for NSTI infections, clinical NSTI patient biopsies (derived from patient fascia, soft tissue, and muscle) were investigated. As per the organotypic model, both CLSM and SEM revealed aggregates of GAS biofilms throughout the biopsy tissue. This study was the first to determine GAS biofilm formation as a complicating component in GAS NSTIs. Moreover, the *in vitro* three-dimensional human skin tissue organotypic model sufficiently recapitulated the NSTI environment, with GAS biofilms phenotypically resembling those present in patient NSTI biopsy samples. Future studies should consider exploring the antimicrobial susceptibility of GAS biofilms generated via the *in vitro* organotypic model to further assess its clinical translatability

and relevance for GAS NF/NSTIs.

Another group, Wu et al. (2021) [33]; described a generalised *in vitro* N/TERT keratinocyte epidermal skin organoid model which they claim can be used for a variety of biofilm-associated skin infections and SSTIs in the context of anti-biofilm drug screening. Of note, this model generates distinguishable stratified skin layers; stratum corneum, stratum granulosum, stratum spinosum, and a basal cell layer. 24 h biofilms of methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* were found to form on the skin surface as aggregates. CLSM revealed that whilst MRSA was able to penetrate through the epidermis, *P. aeruginosa* remained atop the skin surface as seen via SEM. These biofilms were also challenged for one or three days with DJK-5, a novel anti-biofilm peptide. Biofilm burden was reduced significantly for both bacteria. MRSA was eradicated by DJK-5, as well as other peptides (DJK-6 and 1018). However, mupirocin was ineffective against MRSA biofilms formed via the organoid model. This coincides with clinical findings whereby antimicrobial treatment failure is common against biofilm-associated SSTIs. Lastly, Wu et al. (2021) [73] contextualised this *in vitro* skin epidermal organoid model to recreate a thermal burn wound. Specifically, 100 °C heat was applied to the skin epidermal organoid model and MRSA biofilms treated with a topical preparation of 0.4% DJK-5. MRSA biofilms displayed a significant reduction post-treatment. Interestingly, pro-inflammatory cytokine levels were also monitored, which saw an increase in IL-1β and IL-8. This model does enable successful screening of novel anti-biofilm drugs under clinically representative conditions whilst enabling the monitoring of immune responses. Taken together Wu et al. (2021) [33], have demonstrated an efficacious model which has much promise in replacing the limited and basic *in vitro* models currently in circulation, whilst overcoming the ethical implications and challenges of animal models.

2.4. Nail onychomycosis

Onychomycosis is a chronic fungal infection of the nail. It is the most common nail pathology, underpinning 50% of nail diseases worldwide. Clinically, the condition is presented by discolouration of the nail, nail brittleness, nail thickening, and hyperkeratosis [34]. Dermatophytes (e. g., *Trichophyton rubrum* and *Trichophyton mentagrophytes*), non-dermatophyte moulds (e.g., *Fusarium oxysporum* and *Scopulariopsis brevicaulis*) and yeasts (e.g., *C. albicans* and *Candida parapsilosis*) have been implicated in nail disease [35,10]. Treatment typically comprises of topical and systemic antifungals (e.g., terbinafine, itraconazole, or fluconazole), pulse therapy, and/or in some instances the nail plate is completely removed. Treatment success is limited as recurrence rates are high, resulting in extended treatment regimens [36]. Thick fungal biofilm biomasses have been described both within and under the nail plate, which have been linked to treatment failure and recurrence [12].

Given the complexity of the nail environment, the necessity of the three-layered nail plate, and the residing polymicrobial flora, an *in vitro* substitute (e.g., a simple multi-well plate system) to model nail onychomycosis is not feasible. Specifically, biofilms form on nails with varying levels of trauma (e.g., indentations, splitting, and breakages) and dryness/brittleness [12]. As such, studies in this space oftentimes utilise models comprising of human nail clippings and removed animal hooves (e.g., bovine or porcine) which aim to preserve the three-dimensional nail structure, whilst cultivating and modelling biofilm growth in culture [37,10].

Vila et al. (2015) [10] developed a novel *in vitro* non-dermatophyte and yeast biofilm model (Fig. 3A) of *F. oxysporum* and *C. albicans*, typically implicated in nail onychomycosis. Healthy human fingernail fragments collected from donors were autoclave sterilised, and biofilms (24 h *C. albicans* and 48 h *F. oxysporum*) formed on the inner nail surface of nail fragments placed in polystyrene wells of a 96-well plate. SEM revealed *F. oxysporum* filamentous biofilms, and notably dense *C. albicans* biofilms arranged in a complex network of hyphae/buds. The authors acknowledged their alikeness to those seen *in vivo*. Moreover,

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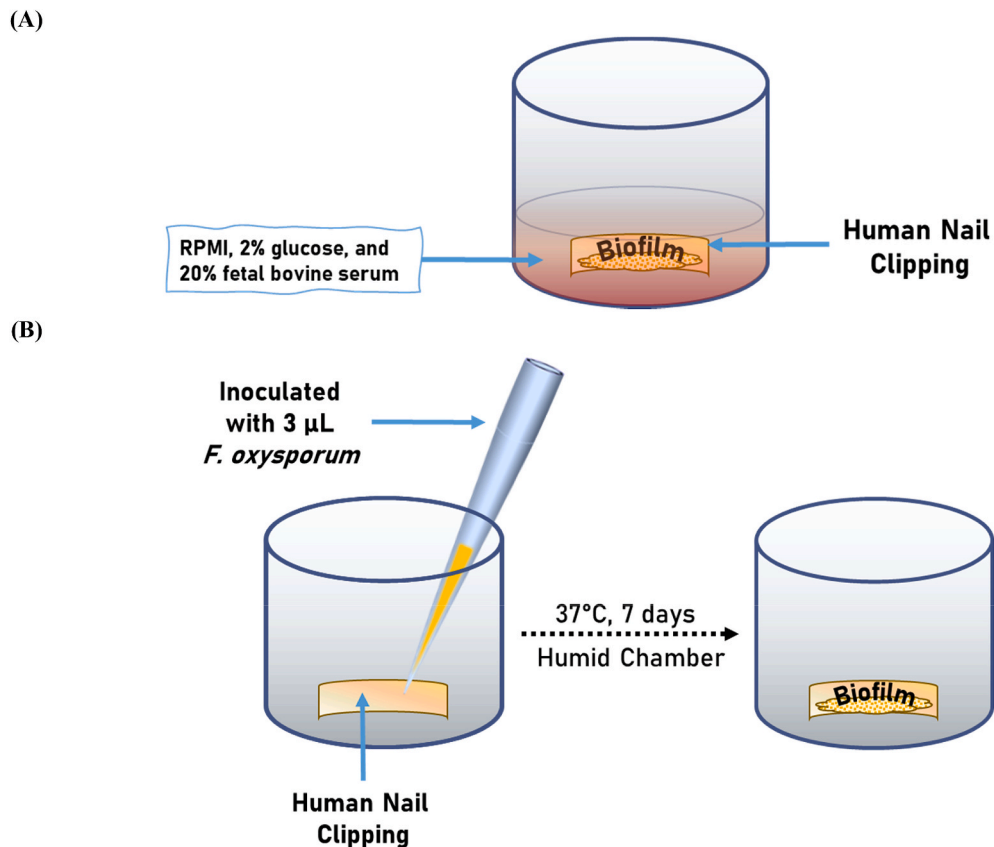
Fig. 3. Schematic representation of *in vitro* fungal nail biofilm models. (A) Non-dermatophyte and yeast biofilm model developed by Vila et al. (2015) [10]. The model comprises of pre-sterilised human fingernail clippings placed in polystyrene wells of a 96-well plate. Biofilms of *C. albicans* (24 h) and *F. oxysporum* (48 h) are grown on the nail at 36 °C in Roswell Park Memorial Institute (RPMI) 1640 growth medium supplemented with 2% glucose and 20% fetal bovine serum. **(B)** Veiga et al. (2021) [38] *in vitro* model comprises of a human nail clipping that is inoculated with *F. oxysporum*. Unlike the model presented in **(A)**, this model is not submerged in liquid media to support biofilm growth. Instead, the human nail is kept dry, and is the only nutrient source.

this model enabled efficacy testing for laser and light therapies, which are a relatively newer treatment modality over topical/systemic antifungals. Biofilms formed via the *in vitro* model were susceptible to laser therapy alone and in combination with intense pulsed light, as well as near-infrared treatment.

Veiga et al. (2021) [38] *in vitro* model also uses sterile healthy human fingernail clippings to form the three-dimensional structure and substratum for biofilm growth (Fig. 3B). However, in this model, the nail is not submerged in growth media. Instead, the nail is inoculated with a 3 μL suspension of a clinical strain of *F. oxysporum* incubated at 37 °C for seven days in a humid chamber. Here, the nail served as the only nutrient source for biofilm growth, recapitulating infection *in vivo*. This study observed the fungal-nail relationship over a seven-day period, noting that the first, fourth, and seventh days were critical in biofilm formation (morphology, mechanistic relationship to the nail, biochemical responses, and metabolism).

3. Oropharyngeal infections

The oral cavity and contiguous regions of the tonsils, pharynx, and upper oesophagus comprise the oropharynx. Several infections may result in this vast landscape: at the oral cavity dental plaque and root carries predominate, whilst sore throats, tonsilitis, and pharyngitis affect the throat/pharynx. Oftentimes, these infections are persistent and chronic. The interplay between naturally residing microflora, pathogenic microorganisms, and the host tissue at the oropharynx has garnered considerable attention [39]. Moreover, biofilm formation has been investigated as a complicating component for many of these infections that span the breadth of the oropharynx.

3.1. Dental plaque and root carries

Dental plaque is a prime example of a biofilm. These biofilms are a microorganism rich reservoir, with some dental plaque biofilms teeming with over 700 bacterial species. Among the plethora of bacterial species identified, 40 species have been distinctly linked to caries [40]. Although fermented sugars and several host factors (e.g., salivary flow, teeth, immune factors, pH) contribute to dental caries, it is the dental plaque biofilms that underpin much of the localised destruction of dental enamel, resulting in caries [40,41]. Recently, there has been an exploration into reproducible biofilm model systems based on batch culture, continuous culture, and flow cells. These systems enable the study of caries biofilm formation as well as the testing of potential antimicrobial treatments and identification of preventative actions or strategies.

Periasamy and Kolenbrander (2010) [42] established a saliva-fed flow cell model to investigate *Veillonella*-based mixed-species communities on biofilm formation, metabolic changes, and physiological function of the community. The two-chamber flow cells (Fig. 4A) were incubated with 25% sterile human saliva (v/v, in dH₂O) to coat the glass substratum with salivary components. Single-species and mixed-species biofilms containing appropriate combinations of six species (*Veillonella* sp., *Streptococcus oralis*, *Actinomyces oris*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Aggregatibacter actinomycetemcomitans*) linked to initial, early, mid, and late-colonisers of enamel were grown in the pre-conditioned flow cells at 37 °C in an anaerobic chamber. 25% sterile human saliva served as the sole nutrient source for biofilm formation. This was consistently supplied at a flow rate of 0.2 mL/min to mimic unstimulated salivary flow at the oral cavity. Utilising this model, high specificity was found among the six species towards community interactions and partnerships. These distinct interactions further enhanced biofilm growth. Moreover, the commensal veillonellae were able to metabolically communicate with species representative of each stage of colonisation via lactic acid for the facilitation of multispecies biofilm formation. However, this model could benefit from incorporating a host epithelial substratum for biofilm growth (e.g., OKF4, a

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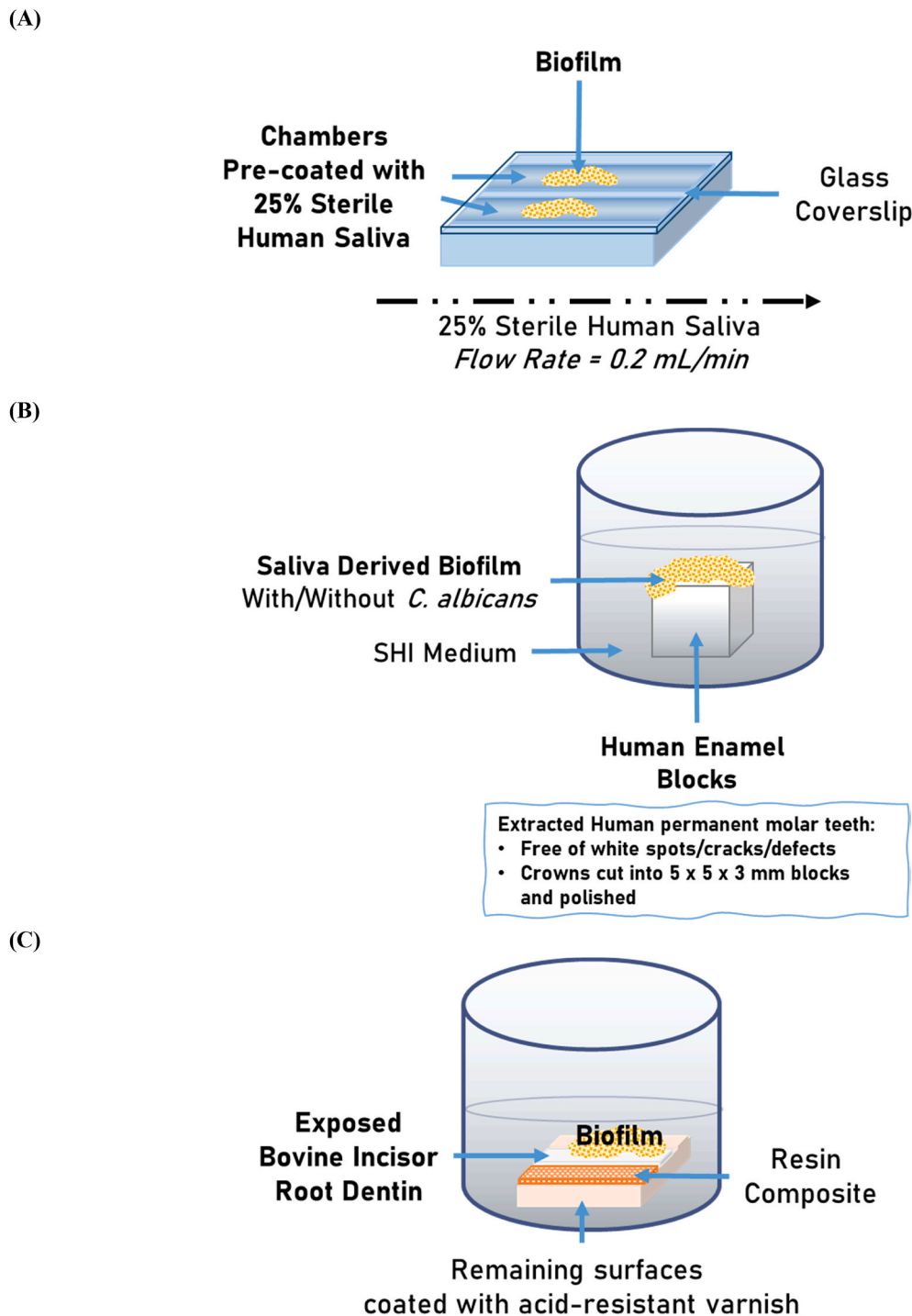
Fig. 4. Schematic representation of *in vitro* dental plaque and root carries biofilm models. (A) Periasamy and Kolenbrander (2010) [42] saliva-fed flow cell model utilises a two-chamber flow cell setup. Each chamber is pre-conditioned with 25% sterile saliva. *Veillonella*-based mixed-species biofilms are grown under flow conditions (0.2 mL/min flow rate) with 25% sterile saliva as the only nutrient source. **(B) Du et al. (2021)** [43] *in vitro* artificial caries model utilises extracted human molar teeth. The crowns are cut into enamel blocks and seeded with saliva derived biofilms (with/-without *C. albicans*). Biofilms are grown for five days in SHI medium under anaerobic conditions at 37 °C. **(C)** Zhou et al. (2020) [44] *in vitro* recurrent root caries model comprises of bovine incisor root dentin as the substratum for biofilm growth. *S. mutans*, *L. acidophilus*, and *C. albicans* biofilms were chosen to study the efficacy of nanoparticles that reduce demineralisation.

human oral epithelial cell line) to cover the glass surface prior to salivary coating to better recreate the host environment.

Fungi have also been associated with asymptomatic oral carriage and disease. Up to 80% of the healthy human population can asymptomatically carry *C. albicans* [45]. Although several bacterial species have been considered key players in dental plaque, there has been a growing appreciation of *C. albicans* as a mediator of dental disease. Oftentimes, *C. albicans* is needed to facilitate and support the physical and/or metabolic processes of other oral bacteria (e.g., *Streptococcus mutans*, *S. oralis*, *Streptococcus gordonii* etc) towards colonisation, efficient sugar metabolism, and biofilm formation [45]. A more recent study by Du et al. (2021) [43] evaluated the demineralisation capacity of *C. albicans*

biofilms in the context of root caries, a subtype of dental caries prominent amongst the aging population [46]. In brief, the *in vitro* artificial caries model (Fig. 4B) comprised of human enamel blocks seeded with human saliva-derived biofilm either supplemented with or without *C. albicans*. SHI medium, an enriched growth medium capable of supporting a diverse inoculum was utilised and refreshed every 24 h over five days. Biofilms were cultivated under anaerobic conditions at 37 °C, with SHI medium pH and ammonia levels monitored. *C. albicans* alone was not acidogenic, however, when present in the polymicrobial biofilms, a lower pH was noted alongside decreased ammonia levels. Moreover, demineralised lesion depth was significantly increased, and notable mineral loss of hard tissue was observed for polymicrobial

biofilms containing *C. albicans*.

Another study by Zhou et al. (2020) [44] developed a biofilm-based *in vitro* recurrent root caries model using root caries pathogens *S. mutans*, *Lactobacillus acidophilus*, and *C. albicans* for the study of anti-biofilm bioactive nanoparticles. The nanoparticles were hypothesised to release calcium and phosphate that supersaturates the site, reducing biofilm-induced demineralisation whilst aiding in remineralisation. In brief, the model comprised of bovine teeth which formed the substratum for polymicrobial biofilm growth (Fig. 4C). Interestingly, the McBain artificial saliva medium utilised was also dosed with 0.2% sucrose and cultured for 4 h to mimic oral food cycling in a 24 h period. Moreover, the concentration of sucrose in this artificial saliva can alter the pH towards an acidic pH due to the polymicrobial biofilm. Lastly, the buffering effect of the McBain medium is meant to capture the bicarbonate and phosphate buffering capabilities of whole saliva. Utilising this *in vitro* model, it was found that the bioactive nanoparticles had a protective effect, reducing root dentin demineralisation and preserving dentin hardness. Moreover, the novel bioactive nanocomposites significantly inhibited the formation of root biofilms of all three root caries pathogens. Overall, this *in vitro* caries model simulated the environment of the oral cavity in a relatively simple and easy to re-create set up which offers clinical relevance. The model enabled the evaluation of novel bioactive nanoparticle composites as promising anti-biofilm agents against root caries, that also offer protection of tooth structures.

3.2. Pharyngitis and tonsillitis

Pharyngitis and tonsilitis are common infections of the throat, primarily caused by viruses and bacteria. GAS is the most common bacterial causative agent of pharyngitis and tonsillitis diagnosed in 20–40% of children, and 5–15% of adults [47]. Globally, GAS causes 600 million tonsillopharyngeal infections per year [31]. Alarmingly, an antibiotic treatment failure rate of 20–40% has been documented, despite GAS remaining susceptible to penicillin, the antibiotic of choice. GAS biofilm formation has been explored as a complicating factor in the antibiotic treatment of recurrent GAS tonsillopharyngitis [48]. To date, much of the biofilm research undertaken in the GAS field is performed in simple microtiter plate setups. Oftentimes, *in vitro* GAS biofilm studies have been reliant on abiotic surfaces as the substratum for biofilm growth.

Some studies have extended upon this, recognising the benefits and importance of incorporating host factors [49,50,51,52]. Specifically, microtiter plate well surfaces have been coated with host extracellular matrix components such as collagen, fibronectin, fibrinogen, and laminin (reviewed in Ref. [52]). However, the human host is the only natural reservoir for GAS, and the complex consortia of interactions occurring within the host environment are inextricably necessary for shaping initial GAS adherence and subsequent GAS biofilm formation.

Recently, this has seen the development of an *in vitro* GAS-pharyngeal cell biofilm model by Vyas et al. (2021) [52]. The model comprises of a fixed pharyngeal cell monolayer of Detroit 562 pharyngeal cells obtained directly from the human pharynx as the substratum for biofilm growth. It was found that GAS biofilm biomass significantly increased when grown on fixed Detroit 562 pharyngeal cell monolayers compared to the plastic well surface [52]. Highlighting the importance of incorporating host factors, i.e. relevant epithelial substratums for GAS biofilm growth. The authors also assessed differing biofilm growth periods (48, 72, and 96 h) to determine the most optimal time period that yielded robust GAS biofilms. Across all five GAS strains, 72 h was determined to be the most optimal growth period [52]. This model has further been utilised to assess the role of pharyngeal cell surface glycans in mediating GAS biofilm formation in the context of recurrent GAS pharyngitis and antibiotic treatment failure (Fig. 5) [53]. In brief, Vyas et al. (2020) [53] found that the removal of terminal mannose and sialic acid residues from the Detroit 562 pharyngeal monolayer surface resulted in a significant increase in GAS biofilm biomass for M12 GAS, an M-type frequently associated with GAS pharyngitis [54,31]. These biofilms demonstrated significant tolerance to penicillin, with a minimum biofilm eradication concentration value of 125 μg/mL. Alarmingly, these biofilms displayed a 5000-fold greater penicillin tolerance when compared to the MIC generated for planktonic M12 GAS. Overall, this model displayed great utility, offering a starting point for the *in vitro* modelling of GAS pharyngeal biofilms for an otherwise relatively new and emerging field of biofilm study amongst the GAS research community. As highlighted by the authors, adaptation of this model is encouraged (e.g., utilising unfixed monolayers, alternate pharyngeal cell lines, and primary oropharyngeal tissue). The model could further benefit from incorporating saliva and flow conditions to mimic the constant salivary movement and bathing of the pharynx.
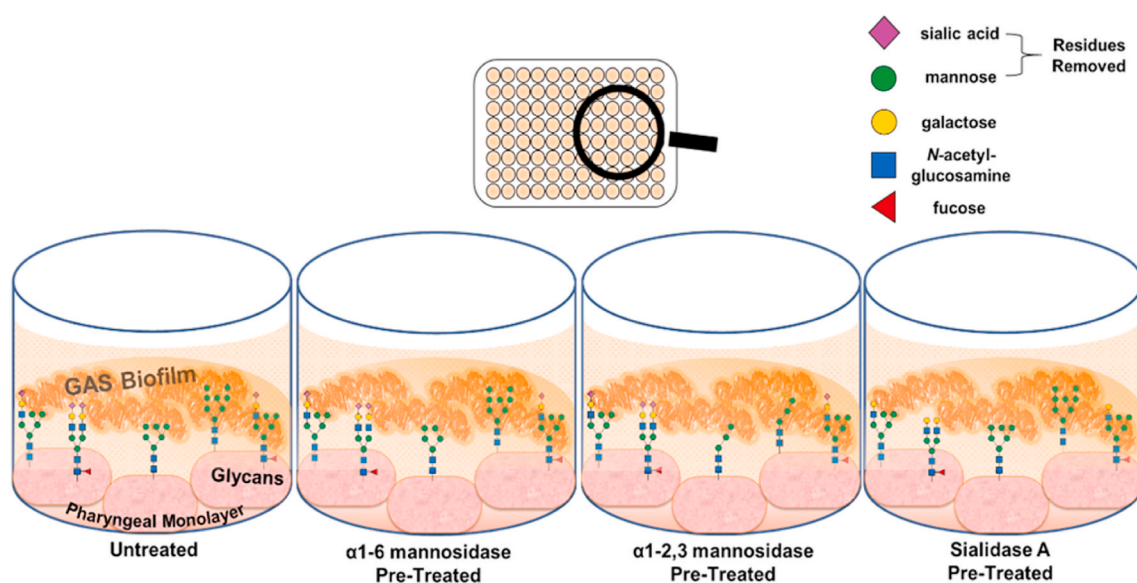

Fig. 5. *In vitro* GAS pharyngeal cell biofilm model developed by Vyas et al. (2021) [52] for assessing the role of pharyngeal cell surface glycans in GAS biofilm formation [53]. The model comprises of fixed Detroit 562 pharyngeal cell monolayers which have been pre-treated with exoglycosidases (which remove distinct terminal mannose and sialic acid glycan residues) or untreated control (glycans intact). 72 h M12 GAS biofilms were formed from planktonic GAS that had initially adhered to the Detroit 562 pharyngeal cell monolayers after 2 h incubation. Figure reproduced from Vyas et al. (2020) [53].

4. Cystic fibrosis

Biofilm formation is oftentimes a hallmark of lung infections caused by *Mycobacterium* or *P. aeruginosa* [55] and the associated increased antimicrobial resistance is of great concern [56]. One highly studied example is the genetic disease cystic fibrosis (CF) which is marked by an associated biofilm lung infection involving the opportunistic pathogen *P. aeruginosa*. CF is the most common hereditary disease in Caucasians affecting approximately 1 in 3500 newborns [57]. To achieve better therapeutic outcomes, it is extremely important to study this pathogen in a relevant model and investigate the effectiveness of appropriate therapies as it was shown that antimicrobial testing in simple *in vitro* modelling is a poor predictor of successful antibiotic treatment.

An understanding of the microenvironment provided by the host CF lung and factors such as iron, oxygen availability, and the presence of mucus is important for meaningful antibiotic testing and prediction [58]. Specific growth conditions that are relevant to the lung have been investigated, including mucin-containing artificial growth medium [59] and the use of artificial sputum medium (ASM) that mimics CF lung habitats [60,61,62]. Iglesias et al. (2019) [60] established an *in vitro* biofilm ASM model to evaluate the pharmacodynamics of common antibiotics (meropenem, vancomycin, azithromycin, linezolid, rifampin, ciprofloxacin, tobramycin) used to treat CF patients with active staphylococci. Firstly, the rheology of their ASM was investigated, finding it to be more elastic than viscous. This result matched the rheology of their CF patient-derived sputa. Then, 24 h *S. aureus* biofilms formed in ASM were assessed for their biofilm biomass and metabolic activity. Biofilms formed in ASM had reduced biomass and lower metabolic activity compared to biofilms formed in trypticase soy broth supplemented with 1% glucose and 2% NaCl (TGN). Moreover, biofilms formed in ASM displayed drastically reduced susceptibility to all antibiotics tested compared to biofilms formed in TGN. Generally, all antibiotics tested were deemed less potent and efficient in the ASM compared to TGN. This study underscores the importance of culture medium in influencing biofilm responsiveness to antibiotics. Particularly, the need to use ASM to better determine translatable and efficacious drug concentrations and therapeutic strategies for CF patients.

The importance of lung epithelial cells as a component of the microenvironment that shapes initial host-pathogen interactions and subsequent infection and should not be ignored. Despite this much remains unknown of the influence of the lung epithelium on biofilm antibiotic susceptibility.

Crabbé et al. (2017) [63] utilised a previously developed rotating wall vessel-derived three-dimensional *in vitro* A549 epithelial cell lung model to assess *P. aeruginosa* biofilm antimicrobial sensitivity. Firstly, *P. aeruginosa* biofilms formed at the *in vitro* model's lung epithelial cell interface displayed *in vivo* bacterial biofilm phenotype, whereby the biofilms could not be completely eradicated despite being challenged with high concentrations of antibiotics. Moreover, differential biofilm-inhibitory activity was noted for biofilms formed on three-dimensional epithelial cells compared to an abiotic plastic substratum. Specifically, the biofilm-inhibitory activity of all three aminoglycosides tested (tobramycin, amikacin, and gentamicin) was most effective for biofilms formed on the epithelial substratum. This study highlights the value of mimicking the host lung microenvironment, particularly the influence of the lung epithelium in biofilm antibiotic susceptibility. Although, a notable caveat of this model is that it does not consider that chronic CF infection is characterised by *P. aeruginosa* aggregating within the mucus plugs found in the airways of CF patients, as opposed to directly adhering to CF lung epithelia [64].

5. Implant and device related infections

The insertions of implants and medical devices are very common medical procedures, including dental and hearing implants, joint replacements, and catheter insertions. While these procedures undoubtedly improve or even save lives, they are also a leading cause of infections. The devices can be contaminated with microorganisms, and when inserted they can break epithelial and mucosal barriers leading to chronic infections and tissue necrosis [65]. For intravascular devices and prosthetic joints, the most common colonising pathogens are *Staphylococcus epidermidis* and *S. aureus*. Whereas for urinary catheters, it is *E. coli*, Candida, and Enterococcus [66]. Microbial contamination on implants and devices can render them non-useable or cause the device to fail. More importantly, contamination can lead to infections that are chronic and/or life-threatening. High dose antibiotic treatment regimens are implemented, and in some instances, the implant or device has to be removed and replaced. This can be a costly and extremely risky procedure, with antibiotic treatment not always successful. Antibiotic treatment failure can be attributed to antibiotic resistance, however, oftentimes the bacteria are found existing as biofilms increasing the resistance further. Taken together, there is an unmet need for other alternative antimicrobial treatment strategies that can target biofilm-infected implants and devices.

Dental implants have been researched considerably, as dental implants comprise a major portion (~50%) of biomedical implants. In Europe alone, 1.2 million dental implants are inserted annually. Worryingly, dental implants rank amongst the highest for implant-associated infection, with patients developing chronic inflammation, gingival bleeding, swelling, and bone loss within five to ten years of implant insertion [67,68,69]. Several *in vitro* implant biofilm models have been developed to understand the biofilms that form. Such insight can mitigate implant failure, enabling the optimisation or design of more sophisticated dental implants, and the development of novel antimicrobials and treatment strategies. When investigating device-related biofilms, their formation and antimicrobial susceptibility, the properties of the implant (e.g., implant material) and the insertion environment (e.g., saliva, flow, teeth, appropriate microorganisms of the mouth) must be considered.

Sánchez et al. (2014) [70] utilised a previously developed and optimised *in vitro* tooth (hydroxyapatite disc) biofilm model [71] to study the biofilm-forming abilities of six relevant oral strains (*S. oralis*, *Actinomyces naeslundii*, *Veillonella parvula*, *F. nucleatum*, *P. gingivalis*, and *A. actinomycetemcomitans*) on titanium and zirconium implant surfaces. In brief, the model comprised of sterile un-stimulated saliva pre-coated discs of calcium hydroxyapatite (mimicking the tooth surface) and discs of titanium and zirconium, representative of common dental implants (Fig. 6). Discs were placed in a 24-well plate and inoculated with mixed bacterial suspensions at anaerobic conditions (10% H_2, 10% CO_2, and N_2) at 37 °C for up to 120 h. The authors noted that resultant mixed-species biofilms formed via this *in vitro* model cultivated sub-gingival dental plaque/peri-implant plaque similar to those found orally *in vivo*. Biofilms adhered and matured to all three surfaces with similar dynamics. However, the thickness and accumulation of the EPS, alongside bacterial cell organisation differed noticeably dependent on the disc surface. This aligns with other studies, whereby microbiota are influenced by the implant surface/material and the environment of the peri-implant [72]. It would be interesting to investigate how differing levels of surface roughness may impact microbial adherence and biofilm formation for titanium and zirconium implant materials, as implants with rougher surfaces have been shown to enhance initial microbial adhesion [73,68]. For improved physiological relevance, this oral mixed-species implant biofilm model could benefit from incorporating flow conditions and assessing sequential addition/distinct combinations of oral species as this will further shape the biofilms formed [67,74]. Nonetheless, from their findings, the authors suggest that clinically, antimicrobial treatment strategy and susceptibility of such biofilms may subsequently vary dependent on the implant material.

6. Conclusions

In vitro models have contributed considerably to our understanding
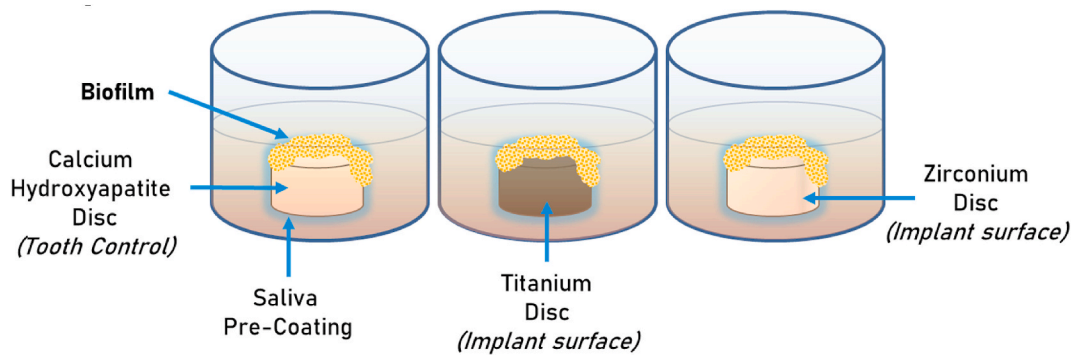
Fig. 6. Schematic representation of *in vitro* tooth biofilm model developed by Sánchez et al. (2011) [71] to study implant materials and their impact on mixed-species biofilms [70]. The model comprises of sterile un-stimulated saliva pre-coated discs of calcium hydroxyapatite (tooth surface control), titanium, and zirconium placed in wells of a 24-well plate. Six relevant oral strains (*S. oralis*, *A. naeslundii*, *V. parvula*, *F. nucleatum*, *P. gingivalis*, and *A. actinomycetemcomitans*) were inoculated into each well and grown under anaerobic conditions (10% H_2, 10% CO_2, and N_2) at 37 °C. Biofilms were grown to 1, 12, 24, 48, 72, 96, and 120 h, and their structure and viability were assessed via CLSM and SEM.

of biofilms. They have enabled high-throughput testing of novel antimicrobials and treatment strategies in a manner that is cost-effective, simple, scalable, and adaptable. However, many *in vitro* models are far too reductionistic, resulting in oversimplified models. These basic models typically rely on abiotic substratum for biofilm growth. Biofilms that form on such surfaces may not represent those seen *in vivo*. Consequently, prediction of antimicrobial efficacy and success under clinical settings is poor. This underscores the importance of host physiology and the local microenvironment in mediating biofilms present in infection and disease.

Here, an array of *in vitro* biofilm models have been explored that aim to recreate various *in vivo* infection scenarios and microenvironments of the skin, oropharynx, lungs, and indwelling medical implants/devices to varying degrees. Notably, most *in vitro* models presented here facilitated the formation of biofilms that are phenotypically similar to those seen *in vivo*. Moreover, in contrast to simpler *in vitro* biofilm models that use abiotic surfaces and standard growth media, biofilms formed under conditions representative of the host displayed decreased antimicrobial susceptibility. This is more realistic, representative, and consistent with antimicrobial treatment under clinical settings. As such, these models should replace reductionist *in vitro* systems where possible to enable the design and development of efficacious antimicrobials and anti-biofilm treatment strategies. Additionally, some *in vitro* models of greater complexity (e.g., three-dimensional organoid model by Wu et al. (2021) [33]) can reconcile host immune responses to biofilms that are present and appropriate antimicrobial treatments. However, increasing model complexity doesn't necessarily always translate to improved microenvironment/infection scenario recapitulation. For example Cornforth *et al.*, 2020 [75], surveyed a variety of *P. aeruginosa* CF models for their accuracy via RNA sequencing data. The infection transcriptome of *P. aeruginosa* in expectorated human sputum was more closely replicated via a defined synthetic CF sputum medium model and an *in vitro* CF epithelial cell model over a common mouse CF lung infection model [75]. As such, caution is needed when selecting and applying such *in vitro* models. Consideration is needed surrounding the research questions being asked and the various factors and conditions of both the host microenvironment and pathogen/s being studied (e.g., human infection transcriptome, pathogen genotype being used, physicochemical environment being replicated etc). And perhaps model selection does require a framework grounded in an evidence-based approach [75]. Together, these will underpin the relevance and/or accuracy of the model in question [75,17]. Nonetheless, *in vitro* models that recreate the host environment are beneficial, overcoming many of the limitations presented by simpler *in vitro* models and the ethical barriers posed by animal models and *in vivo* human studies.

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