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Simulated Media for Mimicking the Human Environment In Vitro

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ABSTRACT

The phrase 'All models are wrong but some are useful' spoken by George Box in 1976 is as relevant today as ever. Modern research relies heavily on models and the use of in vitro models is the cornerstone of developing novel treatments for various infectious diseases. Simple growth media have been, and still are, heavily used when performing research involving biofilms and infectious pathogens. However, using modern technologies, large discrepancies are now being revealed between bacteria grown in simple media versus those grown in more authentic media. These discrepancies can lead to significant differences in bacterial tolerances, growth patterns, biofilm formation abilities, etc. Hence, if the aim is to replicate the in vivo situation in a laboratory setting, the creation of realistic simulated bodily fluids should be prioritised. This paper presents a range of simulated human fluids from various body sites where infections often occur. Bacterial behaviour has been evaluated in all these media and is often compared to a simple growth medium counterpart. In all instances, significant differences are observed which might lead to important discrepancies, particularly in potential treatment efficiency. We hope this may serve as inspiration for any researcher doing in vitro work, attempting to mimic reality.

1 | Introduction

Biofilms are often implicated in the contamination of medical implants and chronic infections [1]. Microorganisms within the biofilm phenotype display emergent properties, behaving differently than when in a planktonic mode of growth. This includes increased tolerance and resistance to antibiotics, habitat diversity and changes in resource capture and enzyme retention [2]. Consequently, research into biofilms is essential for developing effective prevention and treatment strategies for a range of chronic infections. As a result, biofilm models and simulated media have a crucial role in advancing knowledge and building on the progression of anti-biofilm therapeutics. The scientific community has developed a range of in vitro biofilm models varying in complexity, some of which have become the 'gold standard' in biofilm research [3]. To ensure reflective and comparative results to in vivo infection, accuracy in model composition and critical evaluation of experimental setup is paramount.

The basis of all in vitro models includes microorganisms attached to a preferred substrate surrounded by a representative liquid media, either with or without flow and shear forces. Preferred substrates include synthetic materials like agar, microtitre plates, petri dishes, polycarbonate discs and glass slides, none of which are usually present in in vivo infections [3]. Most simulated liquid media consist of a nutrient source, salts, buffers and appropriate enzymes, which depend on the function of the mimicked fluid. Even though many of these elements are present within in vivo infection, nutrient availability and environmental conditions like pH and oxygen concentration can be unrealistic, and many important fluid components are neglected to control for cost and

experimental ease. This includes, but is not limited to, immune cells, signalling molecules like hormones, cell contents and normal microflora. As a result of the simplified media, observed effects may not be accurate to the in vivo setting, and bacterial protein expression and secretion can be influenced. For example, in a study from 2020, the transcriptome of *P. aeruginosa* was compared in in vitro LB medium and synthetic cystic fibrosis sputum media (SCFM2) to gene expression in an in vivo infection, and accuracy scores were calculated [4]. The results showed a gene expression accuracy of 86% in SCFM2 media when compared to the in vivo infection. However, LB media only produced an accuracy score of 80%, highlighting the importance of accurate simulated media on bacterial gene expression and thus potential treatment results [4]. Therefore, specialist simulated media have been developed and enhanced for a variety of different body areas to study the common infections found at each site (Figure 1). However, it is worth noting that complex, specialist media are not always required. When studying bacterial gene expression in *Porphyromonas gingivalis* in both liquid midlogarithmic growth cultures and a murine model, the results showed that 96% of genes were expressed similarly in in vivo infection when compared to in vitro liquid midlogarithmic growth cultures [5]. In comparison, only 85% of genes were similarly expressed in the murine model compared to in vivo infection [5]. Thus, media selection should be specific to each experimental question.

In this TechNote, we aim to examine a range of different simulated media for different infection sites and compare their composition to the actual in vivo milieu. The effect these media have on bacterial behaviour, tolerances and resistances, and the biofilm environment will also be discussed, emphasising the significance and awareness of using realistic media in infection research.

2 | Simulated In Vitro Media

2.1 | Saliva

The oral cavity contains a rich microbiome with the identification of more than 700 bacterial species found in human saliva [6]. Many of these species are part of the natural flora and aid in digestion; however, a shift in the oral microbiome can cause serious health consequences. This includes the development of dental caries, gingivitis and periodontal disease [7]. Human saliva composition is 98% water, with the remaining percentage made up of amylase, proteins, mineral salts and mucus, with a pH of around 6.5–7 [8]. However, despite being mainly water, the development of an artificial saliva medium has been difficult owing to the complex rheological characteristics of natural saliva. Glenister et al. [9] developed a standard simulated oral fluid

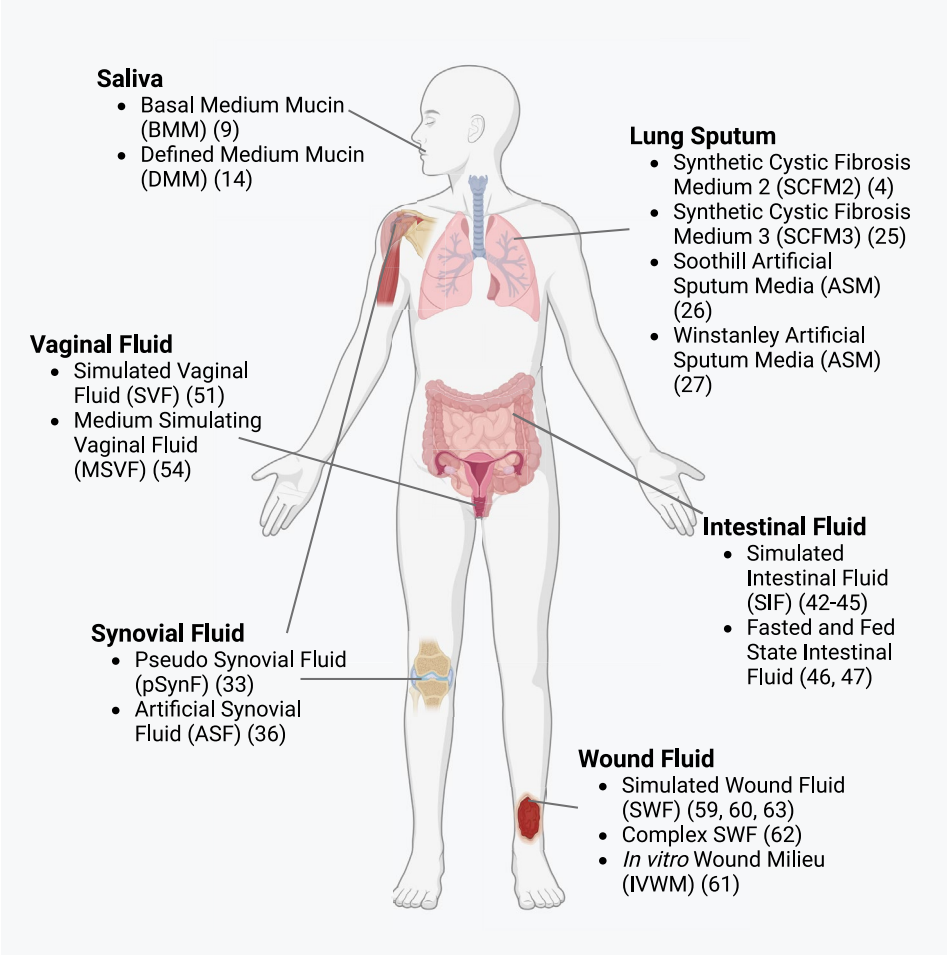

FIGURE 1 | Schematic of six body sites where infections commonly occur. Synthetic mediums mimicking these sites are listed, including their references. *Figure made using BioRender.*

called basal medium mucin (BMM) which contains yeast extract, proteose and trypticase peptones and mucin at a pH of 7.4 [9]. Dental biofilms grown in this media exhibited pH changes characteristic of in vivo Stephan curves and growth rates similar to that seen in in vivo dental biofilms [10, 11]. It was also shown that synergy between microbial species arose in biofilms formed in BMM, demonstrated by higher metabolic activity of each individual species in the mixed biofilms compared to the single species biofilms [12]. Microscopy of biofilms grown in BMM also showed a similar cell distribution to that seen in natural dental biofilms, with the microbes seeding together in all conditions tested, suggesting the presence of a mutualistic interaction between the microbial species [12]. This could be linked to the increase in resistance profiles seen in biofilms grown in BMM compared to traditional microbiology media, mimicking the resistance trend seen in healthcare.

Although BMM has been demonstrated as a good natural saliva alternative, it contains a significantly higher protein percentage than human saliva and is chemically undefined [13]. Wong and Sissions [11] developed a defined medium mucin (DMM) simulated oral fluid which contains commercially available components that mimic relevant constituents of saliva at an applicable concentration [14]. DMM contains ions, mucin, a comparable mixture of amino acids to natural saliva, vitamins and protein/peptide equivalent amino acids to model salivary proteins, all dissolved in water and adjusted to a pH of 6.8 [14]. Oral biofilms grown in DMM displayed growth patterns similar to those seen in BMM and natural saliva; however, greater biphasic growth patterns than BMM were detected, implying changes in metabolism and interspecies organisation. The development of a chemically defined artificial salivary medium provides the opportunity for modification, allowing further research into bacterial resistance and the structure of dental biofilms.

2.2 | Lung Sputum

Normal lung sputum is comprised of small amounts of mucins, nonmucin proteins, salts, lipids and cellular debris in water, which makes up about 97% of the medium [15]. However, in patients with cystic fibrosis (CF) the cystic fibrosis transmembrane conductance regulator (CFTR) gene has a mutation which leads to reduced levels of water and increased levels of mucin in the lung sputum [16]. As a result, the mucus produced is thicker and harder to clear, leading to a vicious cycle of infection and inflammation, with bacteria forming difficult-to-treat biofilms. To simulate the CF lung environment allowing research into these biofilms, a variety of different artificial sputum media (ASM) have been developed, which can be split into Soothill-derived ASM or synthetic CF medium series (SCFM) depending on the foundational formulations [17]. These have a basic composition of mucin, DNA, lipids and amino acids at varying compositions and pH. Bacterial responses and biofilm formation have been characterised predominantly in synthetic cystic fibrosis sputum media iteration 2 (SCFM2) out of the nine currently developed ASMs [18].

SCFM2, chemically defined from average concentrations in CF sputum samples, has been shown to support biofilm formation in a range of bacterial species [19–21]. Transposon sequencing revealed that the SCFM2 exhibits selective pressures on *P. aeruginosa* very similar to that seen with natural CF sputum, which was further corroborated by similar bacterial gene expression to in vivo infection [4, 18, 22]. However, as with all current simulated media, not all bacterial genes are identically expressed in SCFM2 when compared to human infection, meaning media selection should be specific for each experimental question to ensure similar expression levels. Fluorescent microscopy confirmed the successful formation of both mono-species and dual-species biofilms in SCFM2, with biovolume densities demonstrating the nutritional availability of the media sufficient to support a range of bacterial growths [23]. Minimum inhibitory concentrations (MICs) and minimum biofilm eradication concentration (MBEC) of colistin, a common antibiotic used to treat bacterial infections in patients with CF, were quantified for laboratory and clinical strains of *P. aeruginosa* in SCFM2. The results showed higher MICs and MBECs for most strains when grown in SCFM2 compared to standard broth, leading to a change in classification from sensitive to resistant for many of the strains tested [24]. This demonstrates the importance of testing antimicrobials in relevant media. A further iteration of SCFM has been developed, termed SCFM3, which includes more amino acids and metabolites to address the abundant bioavailability in CF sputum [25]. However, biofilm formation and bacterial behaviour have yet to be fully studied in this medium.

Unlike SCFM media, Soothill ASM was formulated from literature concentrations of mucin, DNA, ions and lipids identified in CF sputum [17]. *P. aeruginosa* biofilms were shown to have increased resistance to ceftazidime and gentamicin when grown in Soothill ASM compared to conventional testing, a developing trend seen in patients with CF [26]. This ASM was modified with the addition of amino acids and manipulation of its buffering capacity to better simulate in vivo lung sputum by Winstanley et al. (2012) [27]. Winstanley ASM was used to test *P. aeruginosa* biofilm susceptibility to tobramycin under microaerophilic conditions mimicking the reduced oxygen concentration seen in CF mucus. The results showed increased resistance greater than 128-fold in Winstanley ASM grown in reduced oxygen conditions compared to aerobic conditions [27]. This highlights the importance of environmental conditions on bacterial resistance even with appropriate simulated media. Further variations of Soothill ASM have been developed, varying in mucin concentration, DNA levels, bovine serum albumin (BSA) and amino acids, to study *P. aeruginosa* in CF infection [28–30].

2.3 | Synovial Fluid

Synovial fluid, found in shoulder, hip and knee joints, lubricates joints to reduce friction and provides nourishment to the cartilage. However, free-floating biofilm aggregates may form within this fluid, especially in the case of prosthetic joint surgeries, leading to difficult-to-treat prosthetic joint infections (PJIs) and surgery failures [31]. Human synovial fluid is comprised of 85% water, and as it is derived from the dialysate of blood plasma, it also contains glucose, dissolved proteins like albumin and globulins, mineral ions and hyaluronic acid [32]. Substitutes of human synovial fluid have been developed for studying the formation and treatment/prevention of PJIs. Pseudo synovial fluid (pSynF) developed by Knott et al. [33]

contains hyaluronic acid, fibrinogen and albumin dissolved in tryptic soy broth (TSB), a highly nutritious liquid growth medium [33]. Aggregate formation, virulence factor expression and antimicrobial activity of *Staphylococcus aureus* were investigated in pSynF to see if the fluid could model the bacterial behaviour seen in human synovial fluid. The results showed that the proteins sequestered in the *S. aureus* aggregates when grown in pSynF were similar to those proteins found in aggregates grown in human synovial fluid; however, pSynF aggregates were smaller in size [33]. Antibiotic responsiveness was also investigated using amikacin, which is commonly used to treat PJIs, in TSB compared to pSynF. In standard TSB media, complete sensitivity of *S. aureus* to amikacin was seen at 30 μg/mL [33]. However, a dose-dependent response on *S. aureus* CFUs in pSynF was discovered, with bacteria recoverable at up to 200 μg/mL amikacin. Virulence expression patterns under the control of the phenol-soluble modulin (PSM) alpha and beta were also monitored in *S. aureus* grown in the different media [33]. The luciferase expression patterns of *psmα* and *psmβ* were similar in pSynF to that seen in serum and synovial fluid [34]. However, the viscosity of pSynF is markedly lower than that found in human synovial fluid, potentially due to the absence of proteoglycans like lubricin produced in the joint capsule [35]. This suggests further modifications of the simulated medium could be needed.

To bypass the issues that can be created by the absence of trace proteins, other simulated synovial fluids are based on blood plasma from donors. Artificial synovial fluid (ASF) is created from plasma purified from blood, which is then supplemented with glucose and electrolytes [36]. Biofilm formation, cell aggregation and gene expression profiles of *Staphylococcus epidermidis* were investigated in ASF and TSB media. Phenotypic analysis showed that ASF-induced cell cluster and biofilm formation resembling microscopic findings from native synovial fluid. Expression of genes related to biofilm formation and regulation in *S. epidermidis* was also conducted, showing increased expression of *icaA*, *embp*, *aap* and *altE* after 24 h growth in ASF compared to TSB [36]. Differential gene expression of *argR*, a master regulator of virulence, was also seen, resembling that seen in in vivo infection [37, 38]. Both simulated synovial fluids could be further adapted for advanced models of PJIs, which could include the addition of host immune cells and hormones found in natural synovial fluid.

2.4 | Intestinal Fluid

The gastrointestinal (GI) tract contains a rich community of microorganisms, with the colon containing 10^{13} bacteria [39]. The gut microbiota is not only involved in food digestion and nutrient intake but also plays key roles in immune and nervous system maturation, along with colonisation resistance against pathogens [40]. However, dysregulation and imbalances of the microbiome can occur, leading to biofilm formation and infection by pathogenic bacteria like *Clostridium difficile*. Human GI fluid is a complex medium that can vary immensely between subjects. It contains bile salts, electrolytes, lipids, cholesterol, proteins and enzymes at a pH between 3.4 and 8.3 [41]. To study the effects of intestinal dysregulation, simulated intestinal fluids (SIFs) have been developed with physiologically relevant physiochemical conditions (e.g., pH and osmolality) and concentrations of human intestinal fluid components. Most simulated media contain trypsin and bile salts at varying concentrations adjusted to a pH of 8.0 [42–44]; however, occasionally the trypsin is substituted with pancreatin [45].

Survival and morphological changes of *Vibrio parahaemolyticus* were investigated in SIFs as a liquid medium and a component of an in vitro continuous digestion model. The results demonstrated an optimal growth environment for *V. parahaemolyticus* with upregulation of virulence factor genes coding for T3SS2 effector proteins, toxin genes and transcriptional regulatory proteins in the intestinal fluid [44]. Similarly, SIF has also been used to monitor *C. difficile* survival and biofilm formation as well as planktonic and biofilm growth of *Salmonella typhimurium* and *Lactiplantibacillus plantarum* [42, 43, 45]. Further work into the development and refinement of SIFs is ongoing with a particular focus on fasted vs. fed state intestinal fluids due to the differences in pH and composition [41, 46, 47]. This will affect not only biofilm formation by different bacterial species but also the solubility and sensitivity of antimicrobial therapies. However, as this is a new area of research, these intestinal fluid mimics have not been used within microbiology, and so bacterial behaviour data within the simulated fluids are limited.

2.5 | Vaginal Fluid

The vaginal canal is home to many microorganisms which, when the normal microbiome is dysregulated, can cause infections like bacterial vaginosis (BV) and listeriosis in both women and in neonates when the microflora is transferred during birth [48, 49]. The vaginal fluid contains lactic acid, acetic acid, urea, glucose, glycerol and varying concentrations of proteins dependent on the menstrual cycle phase [50]. The pH also varies throughout the cycle from 3.5 to 7, but a pH of 4.2 was proposed for fluid mimics, as this represents the typical value for healthy, nonmenstruating, premenopausal women [50]. A simulated vaginal fluid (SVF) containing all the components mentioned above at a pH of 4.2 was first developed by Owen and Katz [50]. The formulation was initially used to test contraceptive and prophylactic drug delivery but has also been used to study biofilm formation of different microbes [51]. Survival and biofilm formation of *Listeria monocytogenes* was investigated in SVF, revealing that at a normal vaginal pH, pathogen growth is inhibited [52]. However, at a higher pH of 6.5, the bacteria proliferated and formed biofilms, suggesting that women who have increased vaginal pH values during pregnancy may be at higher risk of listeriosis [52].

Biofilm formation of *Candida* species and alternative treatment strategies were tested in a modified version of SVF containing yeast nitrogen base and fine-tuned concentrations of ions [53]. The vaginal *Candida* species demonstrated both planktonic growth and biofilm formation in the modified SVF, drawing the conclusion that SVF is a good medium to isolate and culture vaginal *Candida* species. Bacterial interactions have also been studied in vaginal fluid mimics. Medium simulating vaginal fluid (MSVF) was developed by Juárez Tomás and Nader [54] and contains the same components as SVF at different concentrations, along with the addition of Tween 80 and mucin [54]. The

production of extracellular factors by *Lactobacillus jensenii* and *Gardnerella piotii* and the influence they have on each other's viability were explored in MSVF. *L. jensenii* eradicated multiple different strains of *G. piotii* but was ineffective against other species of lactobacilli, suggesting the production of a bacteriocin-like inhibitory substance that targets *Gardnerella* species [55]. The study into an effective vaginal fluid mimic has provided a platform for developing a potential treatment for *Gardnerella*-related BV [55].

2.6 | Wound Fluid

With an ageing community, chronic wounds affect 1%–2% of the population, with the common types including diabetic ulcers, vascular ulcers and pressure ulcers [56]. A number of microbial species are isolated from chronic wounds, including *P. aeruginosa*, *S. aureus*, *C. albicans*, coagulase-negative staphylococci, *Enterococcus faecalis* and *Proteus* species [57]. Wound exudate, which is mainly produced in the inflammatory phase, is derived from blood and contains fibrin, glucose, proteins including albumin, proteases and immune cells, all in water [58]. Many versions of a simulated wound fluid (SWF) have been developed, with the majority containing foetal bovine serum (FBS) as a main component [59–63]. This closely mimics in vivo wound exudate, as FBS is also derived from blood [64].

Simple SWFs often include the addition of peptone water, which contains amino acids and sodium chloride, to FBS in a 1:1 ratio [63]. SWFs have been used in chronic wound biofilm models supporting the growth and production of biofilms for different bacterial species, along with testing antibiofilm therapies against chronic wound infections [65, 66]. Most commonly, the growth of *P. aeruginosa* and *S. aureus* in biofilm models containing SWF has been studied, along with the effect of different treatments including hydrogels and antibiotics on the bacteria in both mono- and dual-species biofilms [63, 67]. More complex SWFs have been developed, containing FBS, sodium hydrogen carbonate and a mixture of salt chlorides in water, which allows the modification of more realistic chloride concentrations not offered by using peptone water [62]. This medium has been used to test the antimicrobial activity and efficacy of various therapies against different wound microorganisms including *P. aeruginosa*, *C. albicans* and *E. faecalis* [62, 68]. The activity and efficacy of an Alginate/CMC Silver Dressing (ACSP) were determined against planktonic bacteria in complex SWF, showing antimicrobial efficacy against *S. epidermis*, *E. coli*, *C. albicans* and *P. aeruginosa* [62]. An advanced SWF, called in vitro wound milieu (IVWM), has been prepared to recapitulate the key features of mixed species biofilms [61]. As well as containing FBS, host matrix proteins, collagen, fibronectin and fibrinogen are included along with lactoferrin and lactic acid in a base of water to accurately mimic host conditions. IVWM encourages the formation of metabolically active *P. aeruginosa* and *S. aureus* mono-species biofilms and supports the coexistence of both species [61] with an observed increased tolerance to antimicrobials when compared to LB, recapitulating the increased tolerance seen in in vivo infections. A selection of SWFs with varying compositions is available, which support bacterial and biofilm growth, allowing the selection of the appropriate medium for chronic wound research.

3 | Conclusions

In this TechNote we have introduced a range of different simulated media available and strived to emphasise how the chosen media of an experiment can affect the outcome. Switching from a simple media to a more complex one can promote more multifaceted bacterial responses in terms of interactions, tolerances, biofilm formations, growth patterns and transcribed virulence factors. This demonstrates the pivotal role media can have when comparing in vitro results to in vivo observations; the more similar the environments, the more translatable the results will be to clinical infections. However, it is worth noting that complex is not always better or needed, and as such research should be undertaken on the simulated media available when deciding which is most appropriate for each experiment. While it is important to consider and mimic the composition of bodily fluids where infection occurs, some simple media and models can also give relevant experimental outcomes, and as such the role of simple media in microbiology should not be negated.

Infection research is an ever-growing area, challenged by the rising threat of resistant microbes, emerging outbreaks and an ageing population. The need to understand infections in detail is as prominent as ever to allow the development of novel treatments that can withstand the spread of resistance across all diseases. To this end, realistic simulated media are crucial for model development and our understanding of biofilm biology.

4 | Articles for Further Reading

For further reading, we have made a list of comprehensive reviews covering in vitro models of each body part:

Oral biofilm models: Luo et al., 2021. DOI: 10.1111/jam.15200

Lung models: Grassi and Crabbe, 2024. DOI: 10.1183/16000617.0062-2024

Implant associated models: Cometta et al., 2024. DOI: 10.1016/j.biomaterials.2024.122578

Intestinal models: Anjum et al., 2022. DOI: 10.3389/fmicb.2022.831455

Vaginal models: Shapiro et al., 2022. DOI: 10.1016/j.addr.2022.114543

Wound models: Brackman and Coenye, 2015. DOI: 10.1007/5584_2015_5002

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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