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Dental implant surfaces and their interaction with the oral microbiome

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ABSTRACT

Objective: This review aims to collate the current knowledge in the field of antimicrobial surfaces on dental implant materials, focusing on microbial population and functional responses, predominantly from omics-based studies.

Design: Extensive searching of Scopus and Pubmed databases informed a narrative review on the antimicrobial impact of implant surfaces on the complex oral microbiome.

Results: The awareness of this issue has led to considerable research resources being directed towards the augmentation of implant surfaces to counteract microbial colonization. Whilst the implant material itself has a direct influence on bacterial adhesion and viability, the surface finish and putative antimicrobial coatings are critical to countering early biofilm formation. Multiple modes of surface modification have been developed to counteract early colonization, including direct physical contact effect, such as anti-adhesion strategies and extract effects, through antimicrobial release chemistry or material leaching. These concepts deploy different techniques, including nano-texturing, surface chemistry alteration and controlled release, each with a diverse set of benefits and drawbacks. Novel surface finishes and coatings require investigation with regards to their influence on oral biofilms, whether on individual bacterial species or against mature biofilms.

Conclusion: The search for optimal implant surfaces is necessary for the reduction of the peri-implantitis burden and the longevity of dental implants. To date, next generation sequencing methodologies, enabling a greater depth of understanding of the complex interactions between oral microorganisms, host response, and implant surface coatings are under used in this area of research.

Introduction

Implants are becoming increasingly common in today's populace, with reports in some groups of ~1,000% increase in prevalence since the turn of the century [41]. Projections based on a US cohort indicate the potential for up to 23% of the partially edentulous population to opt for dental implants to improve oral aesthetics [10,57], mastication ability [51,70] and overall quality of life [57,65]. Ensuring longevity of these implants is crucial to positive outcomes, fulfilling patients' expectations of a life-long solution [58] and minimizing the risks and disruption associated with secondary surgeries [68,105]. Dental implant failure generally refers to a lack of fulfilment of the desired function of an implant [146], clinically defined to include any of the following: pain on use, mobility, bone loss > half the length of the implant, uncontrolled exudate or complete loss [104]. Failure rates are reported in the range of 5-10%, dependent on a multitude of factors, including implant position, age, oral healthcare and lifestyle choices [1,27,54,60,84,117,129,133,146]. Smoking and co-morbidities such as diabetes mellitus are also often associated with an increased risk of implant failure [6,37,152]. Failures can occur due to a lack of osseointegration or peri-implant infection resulting in a host immune response leading to bone resorption and destabilization of the implant.

An influential review of peri-implantitis risk factors by a panel of international experts indicated potential alternative, non-microbiological aetiologies for peri-implantitis, including host foreign body responses, where cellular invasion of implant materials is considered a defensive mechanism to protect surrounding tissues, titanium particle release inducing osteoclastic host responses, overloading with occlusal forces, and even genetic predisposition, with cytokine polymorphisms associated with the disease [20]. Furthermore, they concluded that implant material, shape and surface characteristics could all be associated with increased risk of peri-implantitis. These ethiopathologies have been

Abbreviations: PiRC, peri-implantitis-related complex; SFE, surface free energy; CFU, colony forming units.

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comprehensively reviewed elsewhere [20,48] and may contribute extensively to the peri-implantitis burden. However, here we focus on the complex microbial influence on implant environment and the interactions associated with different surface configurations.

Infections are often polymicrobial in nature, caused by the invasion of bacteria into surrounding tissues. In the case of the oral cavity, the accumulation of microorganisms on the surfaces of teeth and soft tissues results in the formation of plaque or biofilm, creating anoxic niches required by many dental pathogens. In periodontitis and peri-implantitis, studies have suggested that Gram-negative, red complex pathogens such as *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* proliferate in these anaerobic pockets [13,77,78,80,97]. Through a plethora of virulence factors *P. gingivalis* has been shown to both increase and evade the host immune response [2,56], as well as promote osteoclastogenesis through lipopolysaccharide activation of toll-like receptors, stimulating bone resorption [72,95]. Along with caries, alveolar bone loss associated with periodontal disease is one of the primary reasons for native tooth loss [112,118]. Similarly, peri-implantitis can be described as inflammation of the tissues surrounding an implant and the associated loss of periodontal bone (Renvert et al., 2018).

The prevalence of peri-implant disease has been widely reported [22,96,103], with a comprehensive systematic review and meta-analysis by Rakic et al. determining high levels of heterogeneity amongst study protocols and definitions confounding results [128]. The study, including 29 clinical studies, determined patient prevalence of 18.5%, whilst occurrence at implant sites averaged 12.8%. Nonetheless, the ranges varied extensively, between 1-46% and 0.2-63% for patient and implant levels, respectively. This level of inconsistency was attributed to a lack of consensus in the definition and application of strict clinical parameters. Therefore, adherence to the definition refined by a clinical working group into peri-implant disease research and its clinical and radiological parameters (e.g., bleeding on probing and marginal bone loss >2mm), is essential for translation of future studies [136]. Interestingly, this meta-analysis highlighted the implant surface type as crucial to disease status, reporting significant reductions in peri-implantitis associated with moderately rough surfaces compared to others. This is likely due to improved osseointegration over smoother finishes.

As one of the primary aetiologies of peri-implant disease, a strategy to counteract bacterial accumulation and infection must be considered. There is an array of antimicrobial mechanisms attributed to the prevention of microorganism proliferation, whether through physical or chemical disruption of cell integrity, or through inhibition of nucleic acid and protein synthesis, cell adherence or metabolic pathways [71,76,142,156]. Although dental hygiene compliance remains paramount post-implant, if the surfaces themselves can offer a barrier to biofilm formation by harnessing these antimicrobial properties, then their long-term success rate can be improved. Therefore, the material and surface coating of an implant is crucial to its integration with the bone and soft tissues, as well as its antimicrobial capabilities to repel infection, ultimately supporting longstanding retention of the implant.

The current breadth of material and surface modifications employ a series of antimicrobial mechanisms, which can be divided into four main categories (Fig. 1). These include the intrinsic properties of the implant material, surface contact effect, inhibition of surface adhesion and release of antimicrobial agents. Testing of these material properties has often been reported through antibacterial effects on individual organisms [21,38,93,151,157]. However, to closely imitate the complex interactions between microbial plaque and implant material *in vivo*, we must consider the polymicrobial nature of dental biofilms.

While the presence and abundance of organisms is important, the contributions each make to metabolic pathways and environmental conditions is crucial to microbiome homeostasis or dysbiosis. The term microbiome was originally coined by Joshua Lederberg and was defined as "*the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body*" [82]. Since the emergence of next generation sequencing and the development of more cost-effective technologies, the molecular analysis of the microorganisms and their genetic material present in our body has expanded exponentially [66]. By enabling detailed access to the complex interactions between microorganism and host, there is now a greater understanding of the synergistic or antagonistic effects on the system in both health and disease. Furthermore, distinctions can be made between the microbiomes of specific sites of the body, particularly divergent in the gut, skin, vagina

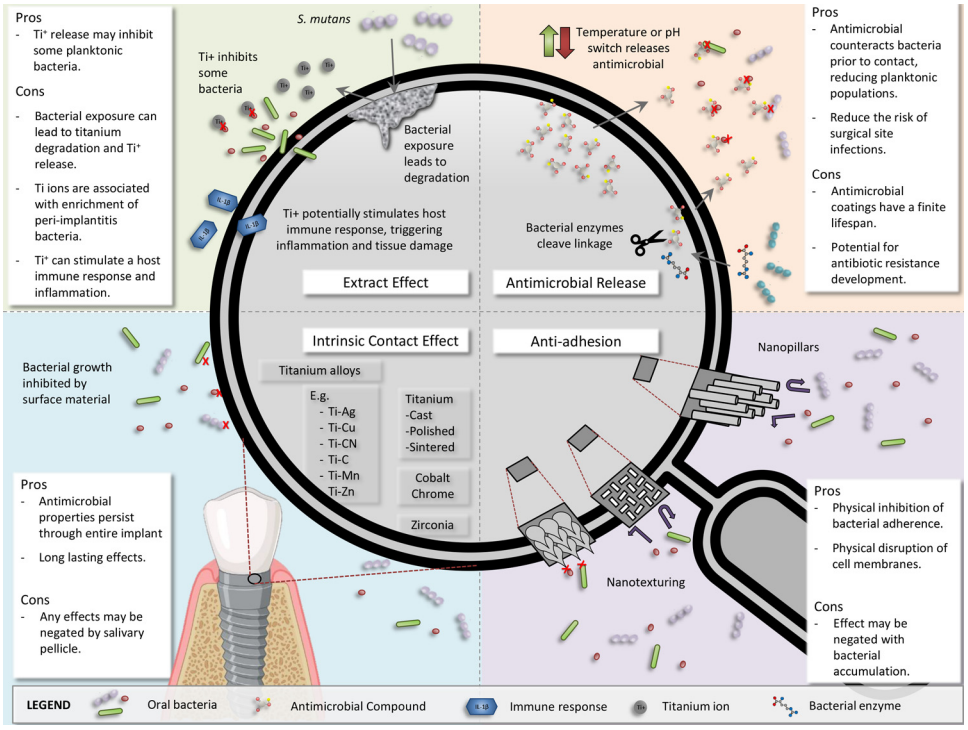

Fig. 1. Diagrammatic representation of dental implant material and surface effects on bacterial challenge in the oral cavity and their advantages and disadvantages. Bottom left: Intrinsic contact effects of pure materials and their alloys can persist throughout the entire material for the implant's lifetime. Top left: Extract effect of implant material can impact the local environment through leaching of potentially bactericidal and immune response stimulating components. Bacterial exposure can result in surface degradation and ion release. Titanium ions have also demonstrated correlations with enriched peri-implantitis-associated pathogens. Bottom right: Differing nanostructures can impact both the adhesion of bacteria and the cell wall through direct disruption and lysis. Top right: Antimicrobial surface coatings can act upon contact with bacteria or release compound into the local environment through gradual degradation of bonds or active release through enzymatic cleavage or temperature/pH control. Ti – titanium, Ag – silver, CN – carbon nitride, N – nitride, Mn – manganese, Zn - zinc.

and oral cavity, with each exhibiting discrete microbiota and metabolic pathways for health maintenance and immunological homeostasis [26]. Therefore, the location of an implant within the human body, even the specific position in the oral cavity, is of high importance when considering its colonization (or absence of), with the multitude of symbiotic bacteria, fungi, viruses, archaea and protozoa inhabiting the host pan-microbiome. This notion is further complicated by the intra-host diversity and the variation in microbiota between diseased and healthy states. For instance, in the oral cavity a healthy microbiome is generally composed of the following genera: *Streptococci, Actinomyces, Lactobacillus, Rothia, Neisseria, Veillonella, Fusobacterium*, to name a few [31]. However, when the environment becomes dysbiotic, due to dietary, oral hygiene or systemic host factors, a shift occurs in the microbe diversity and abundance. For example, in periodontal disease, keystone pathogens including the red complex bacteria *P. gingivalis, T. forsythia* and *T. denticola* contribute to an altered microbial population [4,59,144]. Due to this wide variation in organisms, implant surfaces with broad antimicrobial effects are likely to offer greater translational potential. Nonetheless, the paradoxical nature of treating dysbiosis, often caused by antibiotics in the first place, with further antimicrobial effects, must not be overlooked.

In this review we discuss the available literature reporting the microbiological response to the array of available implant materials and surface characteristics, with a particular focus on oral biofilms and the complex network of interactions of the microbiome (Table 1).

Microbiological differences between periodontitis and peri-implantitis

If we are to consider the potential microbial populations and antimicrobial properties of new surface types, understanding the complexity of *in vivo* oral biofilms is essential. Therefore, we must first delineate the target organisms relevant to implant disease. As a widely studied disease, periodontitis has well defined aetiological associations with oral pathogens [4,24,67,91,143,144] and is often used as a disease model for peri-implant infection. However, the differences between the microbiota associated with periodontal disease and peri-implantitis is an area for debate. There does not appear to be a consensus into whether the periodontal disease-associated organisms are the same as those in implant infections, with some studies reporting distinct taxa, [13,77,78,80,97] whilst others report no significant differences [132,138,162]. Furthermore, the high failure rates of periodontal disease-based treatments in peri-implantitis cases suggests that there are major differences in their responses to treatment regimen. During a five-year follow up study, Leonhardt et al. identified only 6/26 subjects demonstrated bone mass increases, post-targeted antibiotic therapy. However, therapeutic targets were periodontal disease-associated pathogens, which may be less crucial in peri-implantitis, emphasizing the necessity for the use of different targets with this disease [83]. Further exhibiting the potential differences between the two diseases, this highlighted that for *in vitro* disease modelling, careful selection of peri-implantitis specific organisms is necessary, as opposed to assuming existing periodontal disease models/organisms will suffice.

A seminal systematic review by Rakic et al. reported notable separation between the microorganisms associated with periodontal and peri-implant diseases, identifying 21 eligible studies describing the microbiological content of supra- and subgingival plaque samples in healthy, periodontitis and peri-implant disease cohorts [127]. Of these studies, 15 targeted specific pathogens through culture, PCR, fluorescent probing, and DNA hybridization checkerboard methodologies. Whilst these are valid approaches, they rely on pre-existing expectations of microbial content, potentially missing crucial elements of the microbiome relevant to disease. The six remaining studies utilised sequencing techniques to harness a greater understanding of the incumbent microorganisms. However, only two featured >10 peri-implantitis subjects, indicative of an understudied area. Furthermore, extensive inter-study heterogeneity prevented meta-analysis, whilst only one study indicated the implant type and material. Nonetheless, the review highlighted a distinction between periodontal disease and peri-implantitis, indicating the presence of more anaerobic Gram-negative pathogens, such as *P. gingivalis*, and a more complex population of bacterial species in implant associated infections. Whilst many studies of the peri-implant microbiome rely on 16S rRNA sequencing [5,7,43,79,149], this technique cannot outline the detailed functional properties of the biofilms. Recently, Ghensi et al. reported an extensive clinical study where shotgun metagenomics of 113 plaque microbiomes from healthy, mucositis and peri-implantitis sites was performed, with contralateral controls [49]. Here they identified significant differences between peri-implantitis and healthy cohorts both in taxonomic composition and functionality. Seventy-one species were differentially abundant between the groups, with 10 of these contributing to >73% of peri-implantitis plaque composition. From these data the authors defined a signature set of species markers for disease versus health, termed the peri-implantitis-related complex (PiRC). The red complex pathogens, *P. gingivalis, T. forsythia* and *T. denticola*, along with common oral bacteria *Prevotella intermedia, Fusobacterium nucleatum, Porphyromonas endodontalis* and *Fretibacterium fastidiosum* encompassed the seven species PiRC. Through machine learning techniques this study was able to identify that the functional contribution of the PiRC to the peri-implantitis biofilms was up to 64% of the total pathways identified.

One direct comparison of periodontitis and peri-implantitis microbiomes using pyrosequencing highlighted significant decreases in species richness in implant-associated populations, with principal component analysis demonstrating clear distinctions between groups [80]. Interestingly, peri-implantitis microbiomes were dominated by three genera, *Treponema, Streptococcus* and *Butyrivibrio*, contributing 75% of populations. Conversely, periodontal disease communities consisted of an average of ten genera for the same proportion. These different abundances potentially reflect the smaller proportion of bacteria suited to culture on (or in the presence of) inorganic surfaces. Furthermore, the importance of the higher proportions of *Streptococcus mutans* in peri-implant versus periodontitis samples reported by Kumar et al., [80] is supported by the findings of reductions in this bacterium post-treatment [122]. Additionally, the authors identified butyrate fermenting organisms; *Butyrivibrio, Burkholderia, Anaerococcus* and *Anaerovorax*, solely in peri-implant communities, suggesting this as a future area of research as a potential therapeutic target. Nonetheless, substantial inter-subject variability was apparent, highlighting the heterogenous nature of microbiome analyses.

If we accept that there are likely differences between peri-implantitis and periodontal disease, we next must consider the reasoning behind this. Since the origin of biofilms in both circumstances is likely to be from the same source as in health or periodontitis, the obvious differential factor is the composition of material surfaces, whether mineral, organic or inorganic in nature.

Implant material interactions

The major differences to consider between implant materials and native teeth are their composition and structural influence on the topography of the external surface. Human teeth are primarily composed of the biomineral hydroxyapatite and organic collagen [14]. The surface is comprised of a network of misaligned crystals that produce hard, smooth enamel surfaces, accommodating to microbial plaque formation.

The inherent constituents of an implant material can be biologically inert, in the case of titanium, or exhibit fundamental antimicrobial properties. Therefore, the selection of primary implant material may be crucial to the prevention of biofilm accumulation and subsequent peri-implant disease, promoting longevity. Whilst inert substances may benefit from an absence of cytotoxic effect on host cells, neither will they impact on exogeneous cells, such as bacteria.

Table 1
List of studies using molecular and omics methods for the comparison of dental implant materials. Publications were identified via extensive searching of Scopus and Pubmed databases, with studies included that compared complex microbiome data between multiple implant surface types/finishes

Authors	Material/Surfaces Tested	Methodologies	Research/Clinical Observations	Molecular Microbiological Observations	Citation
Liu et al. [92]	Ti vs Ti-Cu implants	Canine ligature model and sucrose supplementation (3 month follow up). 16S rRNA gene sequencing (V4 region), Illumina HiSeq 2500 platform. Sequences aligned to KEGG database by Diamond.	Histopathological and clinical observations indicated healthy tissues in Ti-Cu subjects vs peri-implantitis associated with Ti implants.	Ti-Cu displayed enriched health-associated genera: *Prevotella, Filifactor, Catonella* and *Bergeyella*. Ti-Cu also harboured increased proportions of *Proteobacteria* and *Verrucomicrobia*, capable of methane removal. Ti demonstrated greater abundance of *Sphaerochaeta* and *Synergistaceae*, able to utilize sucrose and galactose.	[92]
Shokeen et al. [141]	Ti-CN vs Ti-N disks	*In vitro* disk model, human saliva +/-0.5% sucrose or mannose (Days 1, 3 & 7). Crystal violet assay. 16S rRNA gene sequencing (V4 region). QIIME2 analysis.	Biofilm biomass on Ti-CN was significantly reduced vs Ti-N	Comparable species-level abundance observed between surfaces. Carbohydrate supplementation reduced diversity in both surfaces, *Streptococci* and *Lactobacillus fermentum* proliferated. Ti-CN demonstrated greater biofilm biomass with increased hydrophilicity or surface free energy suggested as mechanism.	[141]
Sun et al. [147]	Mechanically polished Ti vs TiO_2 nanotubes	Canine model (8 week follow up). Supra- and sub-mucosal plaque collected. Multi-omics approach. 16S rRNA sequencing (V3-V4 region), Illumina MiSeq with Mothur and Qiime OTU analysis. Comparative metatransciptomics: RNA sequencing, Illumina HiSeq2000. GO and KEGG analysis.	No significant differences in clinical evaluation.	*In vitro* testing revealed reduced bacterial numbers. However, genomics approaches indicated no significant differences in microbial composition at phylum or genus level between surfaces. Gene expression showed down regulation of pathogen invasion and bacterial migration pathways with TiO_2 nanotubes.	[147]
Daubert et al. [29]	Human Ti implants	Ten-year clinical follow up study of 36 patients with 61 implants. 16S rRNA sequencing analysis. Dissolved Ti analysis via ICP-MS.	High Ti levels in plaque are associated with peri-implantitis.	Significant correlation between the presence of dissolved Ti in the oral cavity and peri-implantitis, observed in 40% of peri-implantitis sites. Ti enriches peri-implantitis taxa. *Veillonella* spp. prevalence directly correlated with Ti quantity.	[29]
de Avila [30]	Machined pure Ti vs zirconia abutments	*In vitro* biofilm disk model. 48 hr incubation of a cultivable microbial saliva community. Denaturing gradient gel electrophoresis of 16S DNA.	Ti demonstrated a 6.1-fold reduction in bacteria vs zirconia.	Similar distributions of microbes were observed between materials, both in early and mature biofilms. Authors suggest that comparable low surface polarities between Ti and zirconia might explain the findings.	[30]
Nascimento et al. [109]	Zirconia vs machined Ti vs cast Ti abutments	Randomized clinical cross-over with test materials in intra-oral splints for 24 hrs. 38 species DNA checkerboard hybridization	Cast Ti (2.21×10^5) demonstrated significantly greater bacterial numbers than mechanically polished Ti (1.13×10^5) or zirconia (0.74×10^5). Cast Ti showed greater incidence of species vs polished Ti or zirconia.	Only *Fusobacterium nucleatum, Tannerella forsythia, Staphylococcus aureus, Streptococcus gordonii, Streptococcus parasanguinis, Neisseria mucosa, Pseudomonas aeruginosa* and *Peptostreptococcus anaerobios* demonstrated non-significant differences between zirconia and both machined and cast Ti. Dominant species differed: *Streptococcus mitis* - zirconia, *Porphyromonas endodontalis* - machined-Ti and *Actinomyces actinomycetemcomitans* for cast-Ti.	[109]
Van Brakel [154]	Zirconia vs Ti abutments	Two-week and 3-month follow up of 20 patients with 2 dental implants each. Real time PCR of 16S rDNA for 7 oral pathogens.	Healthy tissue observed with both abutment types. Mean probing depth slightly higher for Ti (2.2 mm) vs zirconia (1.7 mm).	Minimal difference observed between materials regarding the abundance of key periodontal pathogens *P. gingivalis, A. actinomycetemcomitans, Prevotella intermedia, Treponema denticola* and *T. forsythia*.	[154]

Abbreviations: Ti - titanium, Cu - copper, N - nitride, CN - carbon nitride, KEGG - Kyoto Encyclopedia of Genes and Genomes, OTU - operational taxonomic units, GO - Gene Ontology, ICP-MS - Inductively coupled plasma mass spectrometry.

Titanium alloys

Titanium is one of the most widely used medical implant materials, [94,164] with the grade V alloy Ti-6Al-4V most commonly used in dentistry [148]. Due to favourable mechanical properties, such as high tensile strength and a corrosion-resistant layer of inert titanium oxide, titanium offers strong biocompatibility and a robust structure. Furthermore, titanium alloy assembly can be altered due to the ability to shift from α or β-phase dependent on the manufacturing conditions [153]. By changing the structural arrangement, the properties of the alloy can be optimized for a given purpose.

Identifiable differences between titanium dioxide layers and organic tooth surfaces suggest that compositional variance between peri-implantitis and periodontal disease biofilms likely originates from adhesion disparities, despite both being coated in a salivary pellicle [78].

Much like cytotoxic effects, antimicrobial properties can be delineated into the direct impact on microorganisms upon contact and the influence on the surrounding environment (extract effect).

Titanium alloy contact effect

When considering the propensity for microbial adhesion and growth on titanium implants, the focus is the direct contact effect. Particularly in dental implants where they will be constantly challenged by the presence of bacteria from the oral cavity. Therefore, substantial research efforts have focused on this effect in titanium and its alloys, although often restricted to selected bacteria.

Supplementation of titanium with additional metals demonstrating antibacterial properties, such as Ag, [25,69] Cu, [11,93,120,130,159,165] manganese [18] and Zn, [90] add persistent antimicrobial potential to the core implant material. Compared with surface release chemistry, which suffer from a limited life span, antimicrobial alloys have no loss of efficacy through degradation as the effect can be imparted through the entire material.

There is a plethora of research focusing on the antimicrobial effects of Cu supplementation into titanium implants [11,93,120,130,159,165]. Zhaung and colleagues developed a copper Ti-6Al-4V alloy demonstrating a sustained release of Cu^{2+} ions [165]. Here they focused on medical implants, inserting rods into rat femurs in an *in vivo* implant-associated infection model. Although not representing a dental setting, the alloy demonstrated significant reductions in biofilm biomass and down regulation of biofilm-associated genes. Liu et al. investigated the antimicrobial properties of a combination of Cu and pure titanium against known oral pathogens, *S. mutans* and *P. gingivalis*, observing only 43.37% and 26.9% of glucosyltransferase gene expression versus Ti, responsible for extracellular polysaccharide contribution to biofilm formation [93]. The aforementioned study utilized transmission electron microscopy to highlight morphological changes in both pathogens, with observations of compromised cell walls, suggesting a mechanism where Cu^{2+} effects the membrane, resulting in leakage and DNA synthesis inhibition.

A recent study from Liu and colleagues reported 16S rRNA gene sequencing data for comparisons of titanium and Ti-Cu, implanted into a dog ligature model [92]. The study determined that Ti-Cu reduced bacterial populations in polymicrobial infections versus Ti, with significant enrichment of health-associated genera; *Prevotella, Filifactor, Catonella* and *Bergeyella*. Furthermore, detailed metabolic pathway analysis revealed significant differences in carbohydrate metabolism between the two implant materials. The authors proposed that homeostasis was maintained through carbohydrate adherence to the implant surface, providing metabolic targets for the tricarboxylic acid cycle, in turn reducing acidification of the local milieu. The findings also revealed that Ti-Cu harboured increased proportions of Proteobacteria and Verrucomicrobia, capable of methane removal, reducing its availability to anaerobic pathogens, suggesting that these organisms were enriched due to copper ion release from the implant surface. Conversely, titanium implants had greater abundance of acidogenic and anaerobic genera, such as *Sphaerochaeta* and *Synergistaceae*, capable of utilizing sucrose and galactose, whilst further contributing to oral acidification and pathogen enrichment.

Whilst there is a consensus for the antibacterial properties of Cu, as well as an observed lower toxicity compared to Ag, [130] both the concentration and manufacturing process can alter the efficacy. Tao et al. reported 100% killing of *S. aureus* and *Escherichia coli* with Ti-3Cu, [151] whilst others have reported optimum antibacterial effect at 10% Cu [11,47]. However, the desired mechanical properties start to deteriorate at these concentrations, so Fowler and colleagues have suggested the use of Ti-3Cu for dental implants [47]. Post-manufacturing treatments can also influence the material properties, with Peng et al. observing the effects of annealing temperature on microstructure, mechanical properties and antimicrobial ability [120]. They determined that Ti_2Cu alloys treated at higher temperatures (860-910°C) exhibited minimal antibacterial effects against *S. aureus*. However, those treated at 740°C displayed *in vitro* biofilms only 40-55% the thicknesses of Ti-6Al-4V comparators. Manufacturing conditions can contribute extensively to material characteristics, with the predominance of α-phase Ti_2Cu generated by lower annealing temperatures potentially leading to preferential bacterial adherence.

Enhanced tribological surface coatings, such as titanium nitride (Ti-N) or titanium carbon nitride (Ti-CN) [139] have demonstrated the potential for biomass reductions on implant materials [52,141]. Furthermore, Shokeen et al. identified significant decreases in biofilm biomass on Ti-CN disks versus Ti-N via crystal violet assay [141]. However, more detailed investigations using 16S rRNA analysis showed comparable species-level abundance for both materials, increasing in diversity over time. Interestingly, the addition of the carbohydrates, glucose and mannose reduced diversity indices and allowed Gram-positive organisms, such as *Streptococci* and *Lactobacillus fermentum* to proliferate. Nonetheless, whilst the biomasses differed, the microbial compositions between the two materials remained very similar, suggesting that the hydrophilic nature of the rougher Ti-CN surface, or even the surface free energy (SFE) may have impacted the strength of bacterial adherence [141].

Whilst there are copious studies investigating the anti-adhesive abilities of nano-texturing implant surfaces with various structures, such as nanotubes, [42,61,88,108,121] pillars [21,64,160] and grooves, [44] these tend to report findings for individual bacteria as opposed to a more clinically reflective complex community. It is not completely understood why nanostructures produce anti-adhesive properties, but it is potentially related to the hydrophilicity and negative charge associated with the surface [88]. Cao et al. reported interesting findings where spear-like structures were more effective at reduced *Staphylococcus epidermidis* biofilms than pocket-based architecture, likely due to the physical disruption caused to bacterial cells [21]. However, they discovered that killing efficacy was not sustained, as dead cells began to block the nanostructures and allow substantial biofilm accumulation, highlighting that no single modification may suffice.

One recent study used a canine model to investigate the differential gene expression in plaque samples from mechanically polished titanium implants and a TiO_2 nanotube coated surface [147]. Interestingly, whilst *in vitro* testing demonstrated a promising antimicrobial effect associated with the nanotube surface, the multi-omics approach revealed no significant impact on microbial composition. However, gene expression analysis identified inhibition of bacterial migration and pathogen invasion pathways, whilst a mechanical impact on cell membranes were observed with the TiO_2 nanotubes. Although only three replicates were tested, this further highlights the requirement for implant material testing against complex, polymicrobial biofilms to observe a more accurate assessment of novel surfaces. Unfortunately, notwithstanding the aforementioned study, there is a distinct absence of reports assessing nanostructure influence on complex bacterial communities, as found in the oral cavity. Whilst there are endless parameters associated with nanotexturing, such as nanotube diameter, spacing and groove depth, that can impact adhesion and biofilm formation on implants, [42,108,121] detailed microbial population studies would provide great insight into these types of surface enhancements.

Titanium extract effect

Implant materials subjected to continually shifting environmental conditions and microbial exposures are at risk of degradation and the undesirable detrimental release of particles, unfortunately titanium is no different.

Titanium surfaces may be considered to leach ions into solution, [19,74,75,119,155] even though the formation of a titanium oxide layer offers some protection against this. Sridhar et al. demonstrated the capability of *S. mutans* to corrode titanium surfaces, identifying a potential

source of particle release [145]. A recent ten-year follow up study of titanium implants focused on the relevance of dissolved titanium in the oral milieu and indicated a significant association with peri-implantitis groups and its presence in 40% of peri-implantitis sites [29]. Furthermore, direct correlations were observed between titanium quantities and microbiome composition, with *Veillonella* spp. demonstrating an elevated prevalence in its presence. Moreover, studies have shown that titanium particles can elicit proinflammatory responses [123,155]. Petterson et al. revealed IL-1β activation in an *in vitro* macrophage model stimulated by titanium particles, but interestingly, not from cobalt-chrome. These findings reveal potential differences in the host immune response to non-biological implants and teeth, with dysbiosis proving to exacerbate this. The combination of this divergent immunological environment and the impact of implant material's properties on the early colonizer's ecosystem serves to shape the peri-implantitis microbiome differently to one of periodontal disease.

Zirconia

Zirconium dioxide (zirconia) is a bioceramic alternative to titanium, used widely in dental implants due to its non-cytotoxicity, strong biocompatibility, and provision of good aesthetics [28,140]. Modern implants are often composed of aluminium oxide toughened or yttria-stabilized zirconia, combining the temperature resistant properties of ceramic and fracture-proofing of metal [53].

Titanium vs zirconia antimicrobial comparisons

The research is not clear as to whether zirconia exhibits comparable, or even greater antibacterial effects than titanium, with contrasting findings reported in the literature. [30,109,134,154] Here we outline several studies investigating the microbial population differences on these surfaces, although complex microbiome comparison studies are lacking.

In a complex human saliva-seeded model, de Avila and colleagues observed 6.1-fold reductions in bacterial adherence on pure titanium versus zirconia disks, as well as decreases in biofilm mass and density via confocal laser scanning microscopy and crystal violet assay. [30] Interestingly, they reported no differences in taxonomic profiles, only reduced numbers with Ti. Whilst roughness characteristics were matched between variables, the authors suggested that increased hydrophobicity and surface tension due to differing chemical compositions led to an improved electrical conductivity and greater bacterial adherence with zirconia. The importance of surface characteristics, such as SFE should be considered, particularly as pre-incubation with saliva to form a pellicle may impact on SFE and any differences between implant materials could be masked due to comparable cell wall interactions with the pellicle. [81,131] Nonetheless, to replicate *in vivo* scenarios *in vitro*, this pellicle is essential, so the pellicle-implant interaction should always be considered.

Conversely, Roehling et al. determined significant reductions in both 3-species and complex human plaque-derived biofilm thicknesses on zirconia compared with Ti, 8.41 μm vs 13.12 μm and 9.04 μm vs 13.42 μm; respectively. [134] Unfortunately, no species analysis was performed, and the short incubation period of 72 hours may not be sufficient to establish the differences generated by the slow growing anaerobic pathogens, such as *P. gingivalis*. Nascimento et al. implemented a randomized cross-over clinical investigation using abutment material disks on splints, inserted into six participants' mouths. [109] Notwithstanding the limited number of participants, the findings indicated wide varying effects between zirconia and both machined and cast Ti, with only *F. nucleatum, T. forsythia, S. aureus, Streptococcus gordonii, Streptococcus parasanguinis, Neisseria mucosa, Pseudomonas aeruginosa* and *Peptostreptococcus anaerobios* demonstrating non-significant differences between materials out of 38 species tested by DNA checkerboard hybridization. While zirconia exhibited the lowest total colony forming units (CFU) 0.74×10^5, machine titanium performed better than cast titanium (1.13×10^5 vs 2.21×10^5 CFU). Interestingly, each of the materials harboured a different dominant species, reported as *Streptococcus mitis, P. endodontalis* and *Actinomyces actinomycetemcomitans* for zirconia, machined-Ti and cast-Ti respectively. Nonetheless, only selected organisms were tested, and the methodology was limited to a detection level of greater than 10^4 CFU, indicating that some key contributors may have been neglected.

However, one study reporting two week and three month follow-ups of 20 patients with either zirconia or titanium implants identified very little difference between materials regarding the abundance of key periodontal pathogens *P. gingivalis, A. actinomycetemcomitans, P. intermedia, T. denticola* and *T. forsythia* [154]. Nevertheless, mean probing depths were higher with titanium implants (2.2 mm vs 1.7 mm for zirconia), suggesting alternative contributors to gingival inflammation. Furthermore, a study incubating materials in intra-oral splint devices for 60 hours reported reduced bacterial counts on Ti-N (1×10^9 CFU) and Zr-N (1.2×10^7 CFU) versus titanium alone (9.8×10^{10} CFU) [52].

Whilst investigating pellicle formation using radio-labelled *S. mutans* and *A. naeslundii*, Lima et al. indicated differences in the early colonizer proportions between hydroxyapatite and titanium or zirconia surfaces [89]. They demonstrated that hydroxyapatite surfaces followed the well-defined early colonization progression of *Streptococci* and *Actinomyces* co-aggregation, however, inorganic implant surfaces showed less reliance on the former and more of a co-relationship with *Veillonella* spp.

Antimicrobial agent release

Developing implants with antimicrobial release functionality offers protection from planktonic microbes before they can adhere. The delivery system of this antimicrobial compound is crucial to the longevity of effect. By functionalizing a surface with a degradable, rapid release chemistry, an implant can secrete a considerable antimicrobial dose in the early stages, post implantation, thus providing protection against surgical site infection. However, since peri-implantitis is associated with late-failure, these methods may result in early depletion of the active compound, exposing an implant to unimpeded biofilm growth and subsequent infection. Whilst a burst-release system has its advantages, combining this with other antimicrobial coating methods for longitudinal implant protection is preferable. Unfortunately, there is a paucity of research studies utilizing metagenomic approaches for the investigation of antimicrobial agents from dental implant surfaces. This is an area of research requiring further attention to report the efficacy of these coatings against complex biofilms.

Nonetheless, research using less complex microbial models remains informative to a certain extent. Wu and colleagues assessed an N-halamine polymeric coating of titanium implants with antimicrobial efficacy in both release into the proximal milieu and direct contact effect [157]. This coating demonstrated reductions of planktonic *S. aureus* and *P. gingivalis* populations by 64% and 42%, respectively. By depleting populations prior to contact, the potential for adhesion to implant surfaces is reduced before contact is made. Indicating a surface persistence of up to 16 weeks, the N-halamine coating eliminated a further 96% and 91% of *S. aureus* and *P. gingivalis*, respectively. Two-pronged antimicrobial approaches, such as this, show promise for the control of oral biofilms, however, since peri-implantitis is often responsible for late implant failure, a lifespan of beyond 16 weeks would be preferable.

If prolonging the lifespan of implant coatings is the ultimate objective, then utilizing response release chemistry to coordinate antimicrobial dosing offers value. By harnessing alterations in the local milieu, such as temperature [86] and pH changes, [38] to trigger compound release, the antimicrobial properties of a surface may be preserved until required. Dong and colleagues developed a silver nanoparticle release system, connected with a pH-sensitive acetal linker molecule, which cleaved upon environmental reductions to pH 5.5, akin to bacterial acidification in caries-associated organisms [38]. Whilst the authors' have

demonstrated effective killing of both *S. aureus* and *E. coli*, evidence of the antimicrobial effect of this coating against dental caries-specific organisms would be invaluable in its evaluation for use in oral implants. Whilst this concept demonstrates promise, when considering potential dental applications, it is conceivable that unintentional release may occur due to ingestion of acidic foods or those with high temperature, which could reduce the longevity of coating. Therefore, more specific release strategies have been proposed. Yuan et al. linked vancomycin to implant surfaces using a combination of chitosan and hyaluronic acid [163]. By exploiting the release of bacterial-specific hyaluronidase enzymes to free the antimicrobial, this study demonstrated substantial reductions in *S. aureus* both *in vitro* and through *in vivo* rat models. Again, there is minimal data reporting the efficacy of these coatings for complex biofilms, where detailed metagenomic data could enlighten the mechanisms further.

Clinical trial evidence

Several clinical trials have investigated the accumulation of plaque on different implant surfaces. However, there is no clear consensus as to the influence of the biomass on bone resorption and implant failure. The findings of Quirynen et al. support the *in vitro* correlations of roughness and bacterial load, reporting 25 times more microorganisms on roughened surfaces compared to standard abutments, with a reduced coccoid population, indicative of a more mature biofilm [125]. Nonetheless, surface roughness does not always correlate with clinical impact, as outlined by two longitudinal follow ups of randomized clinical trial cohorts [32,98]. Higher plaque indices were reported for dual acid-etched surfaces, but these did not correlate with deleterious impact on oral tissue health, and even demonstrated reduced bone resorption in some instances. Alternatively, a five-year randomized clinical trial reporting on 48 minimally or moderately rough implants in 18 patients identified no significant differences in bacterial populations determined by qPCR [126]. Interestingly, they described greater bone loss in rougher implants in the partially edentulous group, indicating the complex influence of a combination of factors in the onset of peri-implant disease.

Importantly, Bollen et al. tested the hypothesis that a surface roughness threshold of 0.2 μm was sufficient to minimise bacterial adhesion [17]. Here, the authors observed minimal differences in bacterial load between machine titanium (Ra = 0.2 μm) and a highly polished ceramic (Ra = 0.06 μm). They reported slightly elevated populations of Gram-negative organisms, but none of these were the common oral pathogens. Therefore, ultra-smoothness of surfaces may not be a necessary characteristic for anti-biofilm properties.

Additional factors for consideration

Microbiological factors are not the only key elements to implant success. There is a recent trend identifying the role of epigenetic mechanisms, such as methylation and microRNA influence on osseointegration, with evidence of titanium surface functionalization with microRNAs increasing osteogenic gene expression [34,99].

Also, whilst we might naturally consider the degree of inflammatory response induced by the contrasting bacterial accumulations on different materials, the potential pro-inflammatory responses associated with the actual implant surface may be vital to implant survival. Menini and colleagues highlighted the differing macrophage responses associated with four titanium and one zirconia implant [100]. Although minimal pro-inflammatory responses were observed, one titanium implant induced significantly increased IL-1β and IL-6 expression, potentially due to different surface microstructures on this implant type. A recent study also demonstrated divergent pro-inflammatory responses between eleven titanium implant variants, both *in vitro* and *in vivo*, highlighting the multifaceted influence of not just hydrophilicity, but surface topography and composition [3]. These differences could be compounded by bacterial activation of immune responses. Furthermore, Morra et al. reported varying bacterial endotoxin (e.g. lipopolysaccharide, lipoteichoic acid, peptidoglycan) adherence across machined, sandblasted and acid-etched titanium surfaces [106]. Machined surfaces stimulated increases in macrophage expression of pro-inflammatory cytokines, IL-6 and IL-8, by up to 30- and 100-fold, respectively. These material differences must be considered, alongside the direct bacterial stimulation as to the suitability of an implant.

Peri-implantitis modelling

Whilst *in vitro* modelling will never reflect the entire complexity of the *in vivo* situation, the movement away from the ethical issues of animal studies, and the requirement for extensive medical device testing prior to implantation, indicates the necessity for laboratory simulations. To offer insight into how different surface finishes interact with both host and microbial cells, *in vitro* modelling of responses has substantial value in both health and disease. Here we outline the models reported in the literature.

By facilitating reproducibility, control of variables and environment, multiple hypotheses can be tested in the absence of any potential bias from cohort characteristics. Whilst clinical trial samples directly from the human oral cavity are invaluable in the investigations of oral microbiota and the interactions with the host, there are many instances where this is not achievable, whether due to safety, ethics or logistics. Furthermore, whilst animal models may proffer an alternative, they too are hindered by ethical issues, the lack of direct translatability with human response, high-expense, intensity of labour and the specialist skills and facilities required [114]. Thus, the value of *in vitro* models is further highlighted. To ensure that modelling reflects the environment *in vivo*, complex multi-species biofilms must be used.

The multiple cell types and immunological response of the oral mucosa, as well as the wide heterogeneity in microorganism populations across individuals [87,110,111] ensures that *in vitro* modelling of oral disease is extremely challenging [50]. Selection of the right model for the experimental requirement is essential, with simulations varying in complexity from simple two-dimensional oral biofilm models, [46,102,161] to complex three-dimensional organotypic co-culture replicas, [39,63,101,113] often more reflective of true mucosal response [158]. Simple, single species co-culture models will not represent the complexity of interactions *in vivo*, however, can be a useful screening test to assess the interactions between individual bacteria and epithelial cells, for instance. Three-dimensional models have been used to investigate oral disease through fibroblast and oral keratinocyte cell lines at the liquid surface interface [15,16,39,137]. Whichever the model of choice, the use of saliva is essential to reflect the environment *in vivo*, either through provision of a cocktail of proteins, mucins, enzymes and immunoglobulins to allow for early colonizer adherence, or to enable the establishment of complex communities representative of the oral microbiota at the time of sampling [62].

Bodet and colleagues used both macrophages and epithelial cells to measure inflammatory responses to periodontal pathogens *P. gingivalis, T. denticola* and *T. forsythia* [15,16]. This work highlighted the importance of balancing of the eukaryotic cell lines, as differing ratios produced different strengths of immune response. Differing responses to model types have also been observed when testing similar variables. Pinnock et al. embedded fibroblasts in a collagen matrix, adding a surface layer of epithelial cells to form an organotypic model to test response to *P. gingivalis* exposure [124]. Here, they demonstrated three-times cellular survival rates and reduced cytokine degradation compared to monolayer culture. Furthermore, Gursoy et al. observed divergent responses of a similar epithelial model to *F. nucleatum* influence versus a two-dimensional setup [55]. A further consideration for these oral co-culture models is the balancing of the cell line growth conditions and the anaerobic environment, essential for many periodontal pathogens. Although once part of a complex biofilm, anoxic niches will form, the oxygen re-

quired for Eukaryotic cell line growth will impact on the behavior of organisms such as *P. gingivalis*, which has been demonstrated to alter its gene expression in the presence of low levels of oxygen [35,85].

Choice of cell line will also impact on the results from *in vitro* modelling experiments. Primary cells are closely reflective of *in vivo* responses; however, it can be difficult to maintain their consistency and can quickly alter phenotype after several passages [8,116]. Therefore, immortalized cell lines can offer easily culturable alternatives, delivering consistent behaviours. However, there have been demonstrations of morphological and genetic differentiation from primary lines [36,73,116]. Nonetheless, others have demonstrated expected *in vivo* behavior in immortalized lines of Human gingival fibroblasts and Human gingival keratinocytes, [12] suggesting a promising compromise.

Co-culture models for oral disease have recently been comprehensively reviewed, [107] so these are not covered in detail here. Instead, we focus on the literature featuring models specifically of peri-implant disease, adding further complexities to assess. Roffel and colleagues developed a three-dimensional oral mucosa model with a fibroblast-containing hydrogel covered with gingival epithelial cells, which they inserted two types of abutment in a space created with a tissue punch [135]. Soft tissue attachment was assessed through immunohistochemical characterization, via observations of gingival attachment length, sulcus depth and gingival expansion at the abutment surface. With additional protein expression analysis, the reconstructed gingiva enabled the authors to demonstrate no discernible differences between two titanium alloy abutment surfaces, one machined and one anodized. Unfortunately, the microbiological impact was not considered in this study and is critical to modelling *in vivo* host response with greater accuracy. By modelling host, implant and biofilm simultaneously, using a collagen-based hydrogel laden with human gingival fibroblasts and a layer of oral keratinocytes, Ingendoh-Tsakmakidis and colleagues reported upregulation of initial epithelial stress responses and a down regulation of IL6 and CXCL8 by *Streptococcus oralis* [63]. This was indicative of a balanced, protective immune response, incited by this commensal organism. The addition of *A. actinomycetemcomitans*, an opportunistic oral pathogen, led to further reductions in host immune response, potentially offering a colonization advantage prior to pathogenic effect. Although the biofilm was not cultured *in situ*, it was grown prior to inverting on top of the mucosal model, by introducing the microbial element this model, it provides findings more reflective of true physiological behaviours. Building upon this model, Mikolia et al. were able to observe the early immune responses to initial bacterial colonisers, *S. oralis, A. naeslundii, Veillonella dispar* and *P. gingivalis* [101]. Through biofilm analysis and immunological ELISA targets they demonstrated reductions in biofilm mass associated with the first 24 hours contact with the mucosal model and a weak early inflammatory response, indicative of the constant, primed immunological state, protecting against pathogen invasion. Live/Dead staining showed increased bacterial viability by 48 hours, whilst cytokine secretion also increased, potentially reflecting the impact of a *P. gingivalis*-induced reaction. By having all the necessary elements in one model, histological analysis was able to highlight visible mucosal detachment beyond 48 hours and offer confidence in the findings.

Just as next generation sequencing technologies have progressed microbiome analyses, advancements in bioprinting methodologies can be used to harness magnetic nanoparticle labelling of cells for 3-D structure assembly, [23,45] or scaffold-based structures created through printing in hydrogels encapsulating specific cell lines [9,33,40,115,150]. This technology could be adapted for the generation of oral mucosa replicas, implanted with abutment materials to simultaneously assess bacterial response to the foreign material and the eukaryotic response to both.

Concluding remarks

Here we have discussed the breadth of literature examining implants materials, surface alterations, antimicrobial coating and the availability of *in vitro* models for assessing oral bacterial colonization, biofilm formation and the host immune response. Whilst there are many promising techniques for adapting implant surfaces, employing a combination of methods may be the answer. Detailed analyses of the microbiota and its interactions with oral tissues through next generation sequencing will continue to provide a greater depth of knowledge to inform the material science. Unfortunately, information on the complexity of biofilm interactions with multiple implant surface types is limited in the current literature, highlighting the need for further metagenomic studies in this field.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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