Biofilm Formation on Implants and Prosthetic Dental Materials

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Abstract

The human body is subdivided into niches containing a wide variety of commensal microorganisms with essential functions for the host's health. When the balance of the resident microflora changes, pathological conditions may occur. Based on this premise, this chapter first describes the composition of one of these niches, the oral cavity: its oral microbiome and the most frequent biofilm-related medical device infections promoted by multidrug-resistant strains, the so-called super bacteria or super bugs. In this context, the discussion focuses on the key events that unbalance the microbiome homeostasis and induce commensal bacteria to biofilm formation and describes how metabolites can influence the prevalence of bacterial species within the microbial community, thus promoting the onset of infectious diseases. As implantable devices are increasingly being used in dentistry, as in other medical fields, there is a pressing need for control strategies, able to counteract the events involved in biofilm formation, especially the adhesion phase, in order to reduce the occurrence of infection-associated implant failures. In this connection, the second part of this chapter briefly examines currently available strategies and the role of chemistry in biofilm prevention: the development of materials with intrinsic antibacterial properties, bioactive coatings with bactericide agents or materials delivering antibiotics, and nanostructured anti-adhesion surfaces or anti-biofilm bioactive molecules. Emerging and future approaches to fight biomaterial-associated infections are still to be clarified.

Keywords

Dental materials • Dental plaque • Oral biofilm • Oral diseases • Oral implants • Oral microbiome

Introduction

The human body contains numerous different tissues and cell types, as well as a huge variety of different microorganisms. The commensal human microbiome is now estimated to outnumber human cells ($\sim 10^{13}$) about tenfold ($\sim 10^{14}$); they include viruses, protozoa, fungi, archaea, and bacteria. Members of this complex microbial community are normal residents of the skin, the oral cavity, and the vaginal and intestinal mucosa and exercise a broad range of functions indispensable for the host's well-being; these include providing energy for our metabolism, making essential vitamins, and acting as first-line defense against potential pathogens. In only a few

cases are they harmful and a potential source of disease; this occurs when, for any reason, the balance between microbiota and host is lost: weakened host defense mechanism, increased bacterial proliferation, and prevalence of some species over others.

The oral cavity is a very interesting example of the interaction between the human body and commensal microbiome; the latter has now been studied minutely, and phylogenetic data on all oral bacteria have been collected into a huge database, known as the human oral microbiome database (HOMD). As an example, studies on the salivary microbiome report that this biological fluid can hold about 10^8 colony-forming units (CFU)/ml of bacteria, an enormous number of microorganisms. The oral cavity appears to possess one of the most complex microbiomes of the organism, second only to the colon, and of the various biological niches, such as the gastroenteric tract or the skin; it has the largest core of commonly shared microorganisms among unrelated individuals. The HOMD is thus a comprehensive repository of oral bacteria taxa, obtained using 16 rRNA identification tools, and of oral bacterial genome sequences, and it may also play a pivotal role in understanding oral health and diseases.

Moreover, intriguingly and unlike other sites of the human body, an imbalance between host and normal oral microbiome underlies the development of most oral diseases, including caries, prosthetic stomatitis, periodontal disease, peri-implant mucositis, and peri-implantitis. However, this microbiome-related etiopathogenesis does not correlate with the concept of "infection": it distinctively indicates an impaired balance among commensal microbiota, host susceptibility, and environmental factors, such as dietary and smoking habits. Among others, one crucial element that can contribute in disturbing this equilibrium is the presence of dental biomaterials within the oral cavity. Microorganisms, adhering to and proliferating on these "exogenous" substrates, show a range of capacities to form their peculiar biofilm structure, known as "dental plaque." Particularly in the presence of dental biomaterials, such as titanium implants, or the ceramics, composites, and metals used in restorative and prosthetic dentistry, dental plaque can acquire a distinctive composition, producing local inflammation and promoting the in situ adhesion of cariogenic, periodontal, and peri-implant pathogens. Nonetheless, evidence shows that the oral biofilm forming on the resin surfaces of removable dentures chiefly comprises fungal species, such as *Candida albicans*, which can produce mucosal inflammation under the prosthetic resin base, for the most part on the palate; this condition is called prosthetic stomatitis and is one of the most common diseases of the elderly.

Mechanical oral hygiene comprises tooth brushing, with the adjunctive use of toothpaste and, in selected cases, of antiseptic mouthwashes, is able to remove dental biofilm and maintain a healthy dental status. Disruption of the biofilm is pivotal for preserving the correct balance of the oral microbiome, which, in turn, is directly related to oral health. Oral hygiene procedures enable local plaque accumulation to be controlled and successfully counteract the preponderance of one or more pathogen species over the others. In particular, they limit both their overgrowth and the increase of sugars on dental surfaces, which are their major sources of energy.

Table 1 Genera and species of bacteria frequently found in the oral cavity

Genus	Species	Genus	Species
Actinomyces	*israelii*	*Haemophilus*	*influenzae*
Aggregatibacter	*naeslundii*	*Lactobacillus*	*haemolyticus*
Arachnia	*viscosus*	*Leptotrichia*	*acidophilus*
Bacteroides	*odontolyticus*	*Neisseria*	*casei*
Campylobacter	*actinomycetemcomitans*	*Rothia*	*salivarium*
Capnocytophaga	*propionica*	*Selenomonas*	*buccalis*
Clostridium	*gingivalis*	*Staphylococcus*	*dentium*
Eikenella	*intermedius*	*Streptococcus*	*pharyngis*
Fusobacterium	*melaninogenicus*	*Treponema*	*catarrhalis*
	loescheii	*Veillonella*	*meningitidis*
	denticola	*Wolinella*	*dentocariosa*
	sputorum		*sputigena*
	ochracea		*aureus*
	sputigena		*epidermidis*
	gingivalis		*mutans*
	tetani		*sanguinis*
	botulinum		*salivarius*
	corrodens		*mitis*
	nucleatum		*denticola*
	polymorphum		*oralis*
			vincentii
			parvula
			recta

For these reasons, a full understanding of the mechanisms regulating the oral microbiome in health and in disease, as well as its interaction with oral tissues and dental materials, is a key factor in the diagnosis, prevention, and successful treatment of these multifactorial, biofilm-related dental diseases.

The oral cavity has from the early days of microbiology provided an extraordinary opportunity for microbiological investigation. The description of bacteria as "living animalcules" was first given by van Leeuwenhoek (1632–1723), who observed materials taken from his gums, using his primitive microscopes. The microbes sketched in his notebook are graphical representations of some of the most abundant bacteria in the oral cavity, including cocci, fusiform bacteria, and spirochetes. W. D. Miller was the next important scientist to make a significant contribution to our understanding of oral microbiology. In 1890, he published a book entitled *Microorganisms of the Human Mouth* in which he postulated a correlation among dental caries, microorganisms, and fermentable carbohydrates. He hypothesized that the microorganisms found in "dental plaque" were responsible for carbohydrate fermentation, which led to acid production and the dissolution of mineralized dental tissues. "Dental plaque," the three-dimensional biofilm growing on oral surfaces, is still the term commonly used to identify the oral microbiome. It is now known that dental plaque comprises a multitude of microorganisms, mostly identified (Table 1) by culture-based methods. It is highly probable that even more microbes will be found, since the number of classified species is increasing, more or

less in parallel with the development of new the technologies becoming available for their taxonomic classification, as discussed in the following paragraphs.

Innovative Methods for Oral Microbiome Analyses

The innovative methods that have recently been made available by molecular biology include the use of culture-independent methods to identify the composition of the oral microbiome, along with the new molecular techniques for DNA sequencing. The latter have greatly increased the resolution of detection and can be applied both to identify the genetic heterogeneity of bacterial species and to investigate the effect of the environment on each microbial phenotype. Thus, over time, the number of sequences obtained per sample has risen very markedly, with a corresponding significant reduction in both time and economic costs of the determination.

Typically, analyses on microbiota include three main fields: (i) the composition of microorganisms; (ii) the environmental conditions in which the microbial communities grow, basically consisting of host nutritional status, salivary pH variations, and reduction-oxidation potential; and (iii) functions and metabolic activities of the microbiota, in turn pivotal outputs for pathogenesis. Investigations in each of these fields may entail several steps, ranging from genetics to metabolomics, following a closely interconnected biological hierarchy. Briefly, the multistage path from gene to metabolite analyses can be described as follows. The genome comprehends the whole set of genes belonging to the human body, thus the genetic information that explains which gene is used for what function or activity; the same group of genes can then be transcribed to the corresponding ensemble of mRNAs, namely, the transcriptome. Subsequently, the transcriptome is translated into its corresponding set of proteins, known as the proteome, comprising the potential effectors of a specific function. When these concepts are related specifically to the oral microbiome, the groups of expressed genes and proteins produced by the dental plaque are called metatranscriptome and metaproteome, respectively. The final step of this biological hierarchy corresponds in determining the resulting assortment of metabolites, the so-called metabolome, which, in the case of the oral biofilm, intuitively involves the metabolism of the microbial community [1].

Genetics – Through the operational taxonomic unit (OTU), the operational definition of a species or group of species can be defined; OTUs identify a specific genus or family and correspond to 16S rRNA gene variable v3–v5 region sequences, clustered at 97 % similarity. Indeed, 16S rRNA genes, via the pyrosequencing of 16S rRNA polymerase chain reaction (PCR), have been used to define the identity or closest relatives of the species in the oral microbiome, in order to obtain a comprehensive description of the oral microbiota. However, the PCR method may lead to some bias: although accurate identification of the taxonomic composition of oral samples is of great importance in terms of scientific knowledge, it only provides scant information about the specific functional activity of each microbial community within the oral cavity.

Metagenomics – Moving beyond genetics, metagenomics provides a new tool to understand the genetic information relating to the oral microbiome and ideally to obtain details on the function of each member of this structure. Without the need for traditional culturing and/or PCR techniques, metagenomics consists of the direct analysis of the total DNA content belonging to bacterial communities. This is achieved using the DNA extracted from oral samples and then analyzed by the following two methods:

(i) *Direct DNA sequencing* of the total DNA belonging to a bacterial community. This group of methods has been widely used to investigate dental plaque from donors. Interestingly, functional assignment via currently available databases indicated the putative function in about half of the sequences. This finding confirms that a large portion of oral bacteria genes remains functionally unknown. A wide interindividual difference among samples has also emerged, and subjects who have never experienced carious lesions during their lives displayed an overexpression of genes encoding antimicrobial peptides and quorum-sensing genes, compared to the oral specimens from subjects having experienced caries. The latter specimens also showed an increased frequency of genes deputized to iron scavenging and oxidative and osmotic stress [1].

(ii) *DNA cloning methods*, which include a first step of DNA fragmentation, a second stage of fragment cloning into a vector within a "bacterial host," and a third phase in which the cloning process is repeated, leading to a "metagenomic library" with multiple clones. This library can be defined as the ensemble of all different fragments of DNA coming from the bacterial community under investigation. Metagenomic vectors, which in most cases correspond to the bacteria *Escherichia coli*, are microorganisms able to accommodate large DNA inserts; these artificial bacterial chromosomes possess the advantage of cloning the entire operons and enhancing the probability of detecting their functions. In addition, the possibility of freezing libraries for future experiments makes it possible to further sequence the inserted DNA by traditional methods and to revise the genetic information in consequence. Regarding the oral microbiome, four metagenomic libraries have been produced by the DNA cloning technique and then screened for antibiotic resistance: all libraries contained clones resistant to tetracycline and amoxicillin, while only three of them included clones resistant to gentamicin [1].

Metatranscriptomics – Although the above metagenomics approach reveals the total genetic potential of a microbial community, it should be taken into account that the "functionally active" bacterial pool may be modified under the pressure of several environmental conditions. As an example, changes in the salivary flow rate, which varies with the time of day or since the last meal, can significantly influence bacterial activities.

Environmental effects can, at different stages, alter the oral microbiome composition and also biofilm formation. In the light of these alterations, metatranscriptomics aims to determine which microorganisms are active and which genes

are expressed under particular conditions, by analyzing the RNA extracted from the samples. First applied to human gut specimens and in vitro oral biofilm models, metatranscriptomics entails extracting total RNA and then reversely transcribing it to cDNA and sequencing by ad hoc technologies. A recent metatranscriptomics study on oral microbiota showed that each combination of disease plus its associated bacterial community displayed a distinct metabolic profile and that this did not differ among patients [2]. The most important limitation of this approach is the high percentage of rRNA in bacterial samples, which normally accounts for over 90 % of total RNA and can confuse findings.

Metagenomic and metatranscriptomic methods are to a great extent complementary, successfully contributing in investigating the taxonomic composition of oral microorganisms, their functional outputs, and the actively expressed genes.

Metabolomics – The term "metabolome" was first used in the 1960s to identify and quantify metabolites in certain biological systems. A Human Metabolome Database is now available. It was initially used to recognize the intermediates of the Embden-Meyerhof-Parnas (EMP) pathway in human red blood cells, after which it was successfully customized and implemented for detecting oral bacteria metabolites, among others *Streptococcus* spp. and *Actinomyces* spp. In the last two decades, metabolomics has rapidly grown on the wave of technological advances in molecular biology and chemical analyses. It has now become a highly reliable tool, able to accurately identify biological molecules. In terms of the instruments employed, capillary electrophoresis (CE) associated to mass spectrometry (CE-MS) has been proposed as one of the most reliable approaches to separate and compute metabolites from the different metabolic pathways, i.e., the central carbon metabolism, the pentose-phosphate pathway, and the tricarboxylic acid (TCA) cycle. The results appear to be reliable even using the very small samples coming from supragingival plaque. Using these techniques, the human metabolome profiles of supragingival plaque have been obtained, before and after a glucose rinse; the changes in these profiles mirrored those occurring individual bacterial strains of *Streptococcus sanguinis*, *Streptococcus mutans*, *Actinomyces oris*, and *Actinomyces naeslundii*. These findings support the recent idea of a unique "bacterial superorganism," since a microbial community consists of an enormously large number of different bacteria but expresses its functions, from the metabolomics perspective, as one single organism, i.e., the "superorganism" [1].

Considering that the presence of dental plaque is not univocally related to dental diseases, the oral biofilm can be associated to either a healthy or a pathological status and is affected by wide intra- and interindividual variability, mainly due to specific environmental conditions. Metabolome analyses could contribute in investigating the composition of the microbiome, possibly helping to explain the fundamental concept of "dental plaque homeostasis." The supragingival plaque contains bacteria that utilize endogenous energy sources, mainly from the salivary substrate. Saliva contains a plethora of proteins, glycoproteins, and urea which can, respectively, be degraded by bacterial enzymes to peptides and amino acids, to sugars, or to ammonia and carbon dioxide. Supragingival bacteria thus appear able to produce alkalis as well as acids, ensuring the stability of the supragingival plaque pH. The pH is also

associated to the correct balance between demineralization and remineralization processes at the tooth surface, ensuring the healthy condition of enamel and dentine. When sugars are supplied to the oral cavity, bacterial acid production rapidly decreases the plaque pH; the pH then slowly returns to the original level, mainly through the salivary buffer effect and bacterial alkali production. In the case of carious lesions, acid production exceeds alkali production, leading to tooth decay. In this connection, complete analyses of the metabolic pathways involved in alkali/acid production are likely to add new information about oral microbiome homeostasis during health and disease [1].

Metaproteomics – Metaproteomics lies between metagenomics/metatranscriptomics and metabolomics. The application of proteomics to the oral biofilm, generally using differential proteomic analysis such as 2D electrophoresis, may provide pivotal information on the synthesis of proteins and on their posttranslational modifications. The main limitation of this approach is the difficulty of recognizing microbial proteins among the wealth of host proteins: the former are highly variable and cannot be identified univocally. Future research will focus on overcoming this drawback, also considering that preliminary proteomic findings already enable the appropriate protocols for sample treatment and data analysis to be set up successfully.

Taken together, the above *–omics* techniques will contribute in clarifying changes occurring to the oral microbiota during pathogenesis and in response to therapies, acquiring even more importance in the presence of dental biomaterials.

The Normal Oral Microbiome

The human oral cavity is heavily colonized by a wide range of microorganisms which, including bacteria, fungi, archaea, viruses, and protozoa, form the oral microbiome. Currently, most studies investigating the "normal" microbiome limit their findings to the so-called bacteriome, often generically named "microbiome," and only a small number of reports specifically refer to the mycobiome (fungal-related microbiota).

Recently, the American organization "National Institute of Health" (NIH) conducted the "Human Microbiome Project" (HMP), one of the most important scientific missions of the twentieth century. The HMP discovered that (i) the oral microbiome is highly defined at the species level, with certain geographical differences, and (ii) it faces daily mechanical and chemical modifications, due to the intake of nutritional substances and personal oral hygiene practices (for instance, the number of tooth brushings or the number of meals during the day). A number of external agents and mechanical forces can change the temperature and pH of the oral cavity and influence the composition of its microbiota; these include the use of antiseptic compounds, diet, smoking, as well as oral hygiene procedures.

Besides viruses, the most frequent and important species detectable within the oral cavity are bacteria, fungi, and mycoplasmas. Briefly, the features of these three classes will be described:

(i) *Bacteria*. The HMP initially analyzed the bacterial composition of oral microbiome, from 200 subjects of both genders, and identified 185–355 genera, belonging to 13–19 bacterial phyla. Nine intraoral sites were considered: buccal mucosa, hard palate, keratinized gingiva, palatine tonsils, saliva, sub- and supragingival plaque, throat, and tongue dorsum. Although depending upon the specific oral site considered, the high-abundance core genera (defined as genera present at >10 % abundance and at >75 % ubiquity) can be summarized in two groups: *Streptococcus* (OTU, 2, 6) and unclassified *Pasteurellaceae* (OTU, 19, −, 16). Further major core genera (>1 % abundance at >80 % ubiquity) include *Gemella* (OTU, 11), *Veillonella* (OTU, 4), *Prevotella* (OTU, 10), *Fusobacterium* (OTU, 9), *Porphyromonas* (OTU, 7), *Neisseria* (OTU, −, 8), *Capnocytophaga* (OTU, −), *Corynebacterium* (OTU, −, 15), unclassified *Neisseriaceae* (OTU, 21), *Actinomyces* (OTU, 14), and unclassified *Lactobacillales* (OTU, 13) [3]. A single OTU dominated nearly all oral mucosal sites of this large cohort: *Streptococcus* (OTU, 2).

Thus, about half of the total cultivable flora comprises oral streptococci, which can be detected on almost all surfaces of the oral cavity; these are dominated by *S. mutans*, i.e., the pathogen primarily responsible for dental caries. Other Gram-positive cocci, such as enterococci and staphylococci, are usually in less abundance, as are *Actinomyces* and lactobacilli, in turn the most frequently detectable Gram-positive rods. The Gram-negative cocci species *Neisseria*, seldom implicated in dental diseases, are also a common finding, with an abundance equal to that of *Actinomyces* spp. The most frequent Gram-negative oral rods are *Haemophilus* spp. and *Aggregatibacter* spp. Of note, *Aggregatibacter actinomycetemcomitans* has been associated with aggressive forms of periodontal diseases, and the relative abundance in the oral microbiome of healthy subjects appears negligible. Considering other bacteria involved in the pathogenesis of periodontal diseases, i.e., *Porphyromonas* species, *Treponema denticola*, and *Fusobacterium nucleatum*, evidence exists to support their drastic increase in dental plaque when the appropriate mechanical oral hygiene procedures are not performed.

(ii) *Fungi*. *Candida* is the main fungal component of the oral environment, also being found in healthy people because of its commensal feature, together with further genera, such as *Aspergillus* and *Saccharomyces*, detectable as a minor component. *Candida albicans* is the most commonly isolated yeast species, followed by other clinically relevant "non-*Candida albicans* species," which include *Candida tropicalis*, *Candida krusei*, and *Candida glabrata. Candida* is a usual component of the oral biofilm, and its relative abundance in dental plaque is particularly high in patients with oral candidiasis: as mentioned above, one of the most common clinical pictures is prosthetic stomatitis, occurring in areas beneath the resin base of removable dentures.

(iii) *Mycoplasmas*. These pleomorphic microorganisms differ from other oral bacteria in that they lack an outer membrane. They have been isolated from the oral cavity, the most typical species being *Mycoplasma pneumoniae*, considered a surface parasite; it may be an etiological factor in infections of the upper respiratory tract, mainly in immune-compromised patients.

Development of the oral microbiome – The oral microbiome as it exists today can be seen as the product of microorganisms' long adaptation in cohabiting within the human body. From this fascinating perspective, microorganisms have been tailored to live in human organisms under a mutually beneficial symbiosis between microorganisms and human tissues [3]. This coevolution of the oral microbiome with the human host has resulted in a process known as "colonization resistance": this term describes the ensemble of host-associated microbial communities, fully equipped with mechanisms enabling them to prevent colonization by and establishment of foreign microbes. Five principal types of interactions among oral bacteria have been identified, namely, competition for nutrients, synergy, antagonism, neutralization of virulence factors, and interference in signaling mechanisms. Bacterial interspecies communication is a cornerstone of colonization resistance, together with a broader inter-kingdom communication, both processes being crucial in oral microbial ecosystem homeostasis. For instance, biofilm formation by *C. albicans* appears to be partially regulated by certain bacteria that produce a range of selective signaling molecules; *C. albicans*'s metabolites are, in turn, compounds known to be able to influence bacterial growth.

The acquisition of a normal, beneficial oral microbiome, including the process of colonization resistance, is an essential step in the growth of newborns. The oral microbiome in infants is closely connected to that of the gastroenteric tract, but after 2 weeks of life, it already differs as the oral cavity is rapidly colonized by bacteria originating from the environment where the newborn lives. Bacterial transfer from the mother, or from other external sources, including other people sharing the same environment, greatly affects dental biofilm morphogenesis.

As recently reviewed by Zaura et al. [3], the key aspects pivotal for the physiological acquisition of a normal microbiota during development are:

(i) *Vertical transmission of the microbiome* from mother to child starts with delivery, whether vaginal or through Caesarian section, which to a large extent determines which microorganisms initially colonize the infant's oral cavity (vagina- or skin-derived). Infants born by Caesarian section acquire *Streptococcus mutans* earlier than vaginally born infants, while vaginal birth enables newborns to acquire a greater bacterial taxonomic diversity by the third month of life. Similarly, breast-feeding versus infant formula feeding appears to influence acquisition of the oral microbiome; breastfeeding gives the infant "beneficial" oral lactobacilli that are not detectable in formula-fed infants.

(ii) *Preservation of the oral microbiome*, after acquisition during the first stage of life, involves bidirectional interactions between the microbiome and the host; thus, the human immune system develops in a continuing dialogue with the commensal populations of microbiota. This communication exploits the following three main ways: the first includes the host pattern recognition receptors (PRRs), especially the toll-like receptor (TLR) family, expressed by oral mucosa cells, i.e., keratinocytes, macrophages, mucosal dendritic cells (DCs, which belong to the Langerhans cell subtype), polymorphonuclear leukocytes, and natural killer cells. Altered expression patterns of TLRs have been found in

several dental and oral diseases, suggesting their specific role in pathogenesis, while it has been suggested that mucosal DCs are peculiar intermediaries able to avoid infectivity of the oral cavity by commensal microbiota. A second tool to stimulate antigenic tolerance, and thus avoid the risk of local infectious disease, is the expression of lipopolysaccharide (LPS) receptors CD14, TLR2, and TLR4 by DCs, at the level of the non-inflamed oral epithelium. Finally, chemical sensing is the third pivotal tool that the host can exploit to monitor microbial activity. In recent decades, studies have suggested there may be a direct link between secreted bacterial products and chemosensory activation mechanisms for mucosal clearance.

The fundamental role of the immune system in preserving oral health becomes increasingly evident when examining the impaired situation due to the patient's pathological status; typical examples are those of patients receiving hematopoietic stem cell transplant and who require immunosuppressive therapy or of patients affected by head and neck carcinoma and treated with local radiotherapy. One of the most severe and painful adverse effects is the mucosal damage known as "severe mucositis," which is potentially associated to life-threatening viral and fungal supra-infections.

(iii) *The secretory immunoglobulin A* (S-IgA), usually delivered via the saliva and gingival crevicular fluid, limits and controls microbial adhesion and colonization. Conversely, bacterial ability to evade S-IgA guarantees their survival within the oral cavity, again highlighting their ongoing symbiotic coevolution with the human body host. S-IgA elusion is mainly achieved through bacterial IgA proteases, which neutralize the immunoglobulin. These proteases are known virulence factors of several human pathogens, such as *Neisseria meningitidis* and *Streptococcus pneumoniae*, and of other commensal streptococci (*Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus sanguinis*). The latter have been defined as "primary colonizers" and are also the foremost species in infants.

(iv) *Salivary flow rate and saliva composition* also play key roles in maintaining the healthy oral microbiome. Focusing on protein composition of the saliva, microbial homeostasis is strongly affected by the presence of salivary glycoproteins, because they contain glycans that may act as traps to prevent pathogens from adhering to epithelial cells. Other salivary proteins that influence the oral microbiome include lysozyme, peroxidase, mucins, lactoferrin, defensins, and agglutinins.

The oral cavity as a biological niche – From the topographical standpoint, two main subniches are described in the oral cavity: the supragingival niche and the subgingival niche. The supragingival niche includes the teeth or implants and the mucosal tissue outside the gingival sulcus. The plaque recovered from this area in healthy subjects generally comprises aerobic Gram-positive bacteria, mostly *Streptococcus* spp. (*Streptococcus sanguinis*, *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus salivarius*) and lactobacilli. In contrast, the subgingival niche (i.e., the gingival sulcus) is characterized by the presence of some Gram-negative microaerophilic bacteria, in addition to Gram-positive and aerobic species. Many

Table 2 Bacterial species recoverable subgingivally in healthy subjects

Gram-positive	Morphotypes	Gram-negative	Morphotypes
Actinomyces	Rod	*Bacteroides*	Rod
Clostridium	Rod	*Fusobacterium*	Rod
Lactobacillus	Rod	*Neisseria*	Coccus
Staphylococcus	Coccus	*Prevotella*	Rod
Streptococcus	Coccus	*Treponema*	Motile rod
		Veillonella	Coccus
		Wolinella	Rod
		Eikenella	Coccus
		Aggregatibacter	Coccus
		Porphyromonas	Rod/coccus
		Tannerella	Rod
		Campylobacter	Long rod
		Capnocytophaga	Rod

of these are rods, with some motile bacteria and facultative intracellular bacteria (e.g., *Porphyromonas gingivalis*) [4].

Table 2 lists the most frequent genera and species recoverable from the sulci of healthy subjects.

These microorganisms are natural commensals of the oral cavity, where they are found either in their planktonic form or within structured and complex 3D biofilm communities. The formation of the biofilm community is a key factor in the transition of bacteria from commensals to putative pathogens. When bacteria grow in the biofilm, they may accumulate high concentrations of bacterial metabolites (e.g., fatty acid end products, ammonia, hydrogen peroxide, oxidants, and carbon dioxide) in their local environment, which influence the prevalence of species both within the microbial community and in the host. For instance, as already mentioned, carious lesions are closely related to certain biofilm-forming bacteria, mainly *Streptococcus mutans*, which is able to adhere to the teeth, proliferate, and produce lactic acid, which in turn can dissolve the mineralized components of enamel and dentine. In the presence of sugar, *S. mutans* overwhelms the other non-acid-producing *Streptococcus* spp. that make up the supragingival plaque. *Actinomyces* spp. are among the dominant taxa in both the supra- and the subgingival plaque, from both healthy subjects and periodontitis patients. *Porphyromonas gingivalis*, *Bacteroides forsythus*, and *Treponema denticola* have been detected in supragingival and subgingival plaque samples of both healthy subjects and individuals affected by periodontitis, although they are significantly more prevalent in both supra- and subgingival plaque samples from the latter.

The Oral Microbiome in Oral Diseases: A Focus on Implant and Prosthetic Dental Materials

In the light of the concepts described above, biological properties that confer stability to the microbiome are important for the prevention of disease-related

"dysbiosis," producing the microbial shift toward periodontitis or carious lesions. Although the processes underlying the healthy equilibrium of a normal microbiome remain poorly understood, the mechanisms that underlie oral diseases have been investigated in depth; in particular, research has focused on oral microbiome changes that occur on the surface of implants and prosthetic dental materials. In addition, the surface adhesion of microbes, such as bacteria and fungi, and the subsequent formation of biofilms contribute to multidrug-resistant infections in humans and, consequently, to the failure of medical devices.

Peri-implant microbiome – Having been the object of numerous high-quality studies, osseointegrated dental implants are today a therapeutically successful option in prosthetic dentistry, for the rehabilitation of complete, partial, and single edentulism. Oral implantology is based on technologically advanced devices, highly customized to replace missing teeth, satisfying both functional and esthetic requirements.

Implant rehabilitation may be considered one of the foremost discoveries of the twentieth century; however, from the oral microbiome perspective, dental implants also represent new artificial surfaces within the oral cavity, which appear more prone than natural tooth surfaces to form bacterial biofilms. Dental plaque, similar to what occurs on natural teeth, can easily accumulate. Biofilm formation on the implant surface is a trigger factor for the further inflammatory process of peri-implant tissues, namely, peri-implant mucositis (when the inflammation only involves the peri-implant mucosa) or peri-implantitis (when the inflammation progresses toward the surrounding alveolar bone).

The oral microbiome in peri-implant infections has been studied by conventional, molecular, and metagenomic analyses. Using the 16S rRNA-based PCR detection method on crevicular fluid samples, the biofilm adhering to abutments showed the presence of both *A. actinomycetemcomitans* and *P. gingivalis* [5]. Moreover, the oral microbiota growing on dental and implant surfaces has recently been investigated in partially edentulous patients, in a large, 10-year retrospective clinical trial, on 504 implants and 493 adjacent teeth [6]. The microbiota analyses of dental plaque specimens, collected after the placement of sandblasted and acid-etched implants, revealed the presence of some bacterial species associated with periodontitis, such as aerobic Gram-negative rods and staphylococci, although abundances were very wide ranging (from 6.2 % to 78.4 % of implants). The study authors reported a higher abundance of *Tannerella forsythia*, *Parvimonas micra*, *Fusobacterium nucleatum/necrophorum*, and *Campylobacter rectus* at implant sites than on dental surfaces. Based on these data, the prevalence of *Prevotella intermedia*, *Treponema denticola*, *Campylobacter rectus*, and *Staphylococcus warneri* has been suggested to be associated with peri-implantitis. In addition, comparing smokers versus nonsmokers, the latter showed higher counts of periodontopathogenic species; similar to the comparison between periodontal versus non-periodontal patients. These latter findings again support the role of the two major risk factors, i.e., smoking and periodontal disease, in the pathogenesis of peri-implant inflammation.

Considering the composition of the oral microbiome, although some evidence suggests that the miscellaneous microbial flora of peri-implant infections may bear a

resemblance to that of periodontal infections, some recent studies suggest there may be certain differences. It is likely that future breakthroughs will occur with the increasing application of metagenomics and metatranscriptomics. These innovative technologies have recently been applied to evaluate the microbiota associated with osseointegrated implants and to investigate peri-implant disease pathogenesis. The current state of the art was reviewed by Charalampakis and colleagues, who analyzed the existing knowledge on peri-implant microbiology and the diversity of the microbial communities associated with peri-implantitis [7].

The peri-implant microbiome in healthy individuals includes a preponderance of Gram-positive cocci and nonmotile bacilli, with a small number Gram-negative anaerobic species, similar to what occurs in the normal gingival tissue. The switch to the first step of inflammation around implants, i.e., peri-implant mucositis, correlates with the increased presence of cocci, motile bacilli, and spirochetes, to an extent equivalent to that of gingivitis. Conversely, the further shift to peri-implantitis is mainly related to the appearance of Gram-negative, motile, and anaerobic species, which are frequently detected in periodontitis. Through molecular biology, *A. actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and several *Fusobacterium* spp. have been detected in dental implant plaque specimens.

It has been suggested that, in general, the bacterial profile of peri-implantitis derives from periodontitis, since most peri-implant lesions shares common features with periodontal disease. In particular, the so-called "red complex" group of periodontopathogens (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) was found to be more abundant at sites affected by peri-implant disease than at healthy ones. Conversely, the count of *S. aureus*, markedly higher at implant sites than at others, supports the possibility of detecting unique and distinctive microbiological features related to peri-implantitis. Indeed, although the metagenomics approach has yet to provide robust data, owing to the paucity of investigations, emerging data support the view that the peri-implant microbiome is a specific entity, different from the periodontal microbiome. Interestingly, further evidence suggests that implant sites and adjacent teeth appear to share similar microbiota, probably because they are spatially close and comparable ecological niches. Indeed, in the case of fully edentulous patients, healthy implants displayed similar bacterial colonizers as do healthy periodontal sites. However, in the case of partially edentulous patients, the implant surface was colonized by the same species as the adjacent teeth and oral mucosa. In addition, *A. actinomycetemcomitans* and *P. gingivalis*, usually detectable only in the presence of teeth, were detected in peri-implantitis in fully edentulous patients, indicating that the bacterial species might originate from niches in the oral cavity other than the subgingival sites, such as the soft tissues or saliva; alternatively, these bacteria might remain in place after tooth extraction and subsequently colonize the oral surfaces, including dental implants [8].

Dental caries and the oral microbiome – Dental caries have been investigated microbiologically, at the molecular level, in a number of studies, and the principal findings have been summarized in a recent review by Nyvad and colleagues [1]. In order to extend scientific knowledge of this common disease, cariology

progressively exploits the new and complementary approaches, including metagenomics, metatranscriptomics, metaproteomics, and metabolomics analyses of dental biofilms, along with refined microbial sampling techniques. One of the priorities for caries microbiologists in the near future will be to verify the performance, and not just the composition, of the entire microbial community. In particular, the metabolism resulting from the activities of oral microbiota greatly affects the dynamic processes of caries. Chiefly for this reason, metabolomics is expected to acquire a decisive role in this field, to facilitate research in assessing bacterial functions. Integrated approaches will make it possible to assess which genes are expressed and which phenotypic characteristics of the biofilms are detectable at specific dental sites, since caries is a localized disease. Taking into account that one of the major difficulties is sample collection, it is essential that biofilm specimens be taken from specific and specified tooth sites. Indeed, the use of pooled samples has been found not to be appropriate, since the bacterial inoculum collected from salivary samples of patients with different caries experiences would be unable to provide insight into the cariogenic potential of site-specific biofilms. Unfortunately, most molecular studies on caries have used saliva samples or pooled plaque samples.

In a recent study, the 16S rRNA gene was cloned and sequenced, in order to characterize the microbial composition of the oral biofilm in the presence of carious lesions. Custom-made arrays, specifically targeted to individual patient groups, detected a microbial diversity in patients' subgingival plaque. The main methodological limitation of this technique is that only those microorganisms specifically targeted by the probes can be detected. Samples collected from healthy and carious root surfaces of older patients were analyzed for their taxonomic microarray, showing that great bacterial diversity and the presence of *Actinomyces* spp. were more frequent at healthy sites, whereas several species of lactobacilli and *Pseudoramibacter alactolyticus* were associated with root caries [1]. A further study on the transcriptome determined a functional core microbiota, consisting of about 60 species; it identified numerous functional networks and provided support for the hypothesis that interindividual environmental differences affect the selection of microbial groups. Dominant functions of bacteria, such as the capacity of dental plaque microbes to metabolize diverse sugars and to handle the acid production and oxidative stress that result from sugar fermentation, were expressed by the oral microbiota [9].

Metagenomics and metatranscriptomics, via pyrosequencing analyses, can retrieve millions of partial 16S rRNA gene sequences in one sequencing run; they have been used in a cross-sectional study to analyze the oral microbiota of Chinese children with and without dental caries. The findings supported the hypothesis that the presence in the plaque of the genera *Streptococcus*, *Veillonella*, and *Actinomyces* is significantly associated with dental caries. Focusing on adulthood, the comparison between "healthy" and "cariogenic" salivary microbiome revealed that the latter was significantly more variable in terms of community structure. This outstanding result, i.e., that "healthy" microbiomes are more preserved than caries microbiomes, was consistent with other evidence from a study applying microarrays to analyze the microbial composition of saliva in children in relation to their caries status [1].

Removable denture oral microbiome – Changes of the oral microbiota before and after wearing removable dentures (RD) appear possibly related to a local imbalance of the microbial community, leading to oral candidiasis; however, there is as yet no certainty. Possible variations in the human oral bacterial community related to wearing partial RD have been analyzed in the four main kinds of biological oral specimens: saliva, supra- and subgingival plaque, and oral mucosal surfaces. A recent study collected these four types of plaque samples from RD wearers ($n = 10$) at three different times, i.e., before and after 1 and 6 months of wearing RD; a further ten healthy adults were selected as control group [10]. After cloning and sequencing, the health-associated genera, such as *Streptococcus*, *Neisseria*, *Corynebacterium*, *Gemella*, *Veillonella*, *Selenomonas*, and *Actinomyces*, showed a decreasing trend in RD wearers, while species associated to disease, mainly *Streptococcus mutans*, appeared to increase.

Considering that *Candida*-related prosthetic stomatitis is correlated to a marked elevation in the number of *Candida* species cells present on the acrylic base of dentures, an interesting recent trial investigated the relationship between the *Candida* load and the bacterial diversity, in the saliva of older patients [11]. Patients were partially edentulous, with or without partial RD, or edentulous, with total upper and lower RD: almost all subjects were positive for *Candida*, with a negative correlation between *Candida* load and bacterial profiles of the saliva. When the *Candida* load increased, the diversity of the salivary microbiome decreased, and its composition shifted toward dominance by streptococci and lactobacilli, while genera within the *Fusobacteria* and *Bacteroidia* classes disappeared. Decreased bacterial variety was associated with a lack of equilibrium among the microbiome communities.

Definition, Structure, and Composition of the Biofilm

The oral microbiome, adhering to hard substrates, can assemble into three-dimensional structures, called “dental biofilm” or “dental plaque”: the soft white material that may be observed on the surfaces of both teeth and dental materials.

The term “biofilm” indicates a community of microorganisms adhering to a surface, glued into an extracellular polymer matrix, also known as “slime,” within which there are water channels. These channels generally consist of glycoproteins, proteins, and polysaccharides, which are secreted by the microorganisms themselves. Thanks to this complex and dynamic structure, the microorganisms acquire multiple properties, including improved protection against host defenses and against new invading microbes. Salivary proteins, adhering onto tooth surfaces and forming the dental pellicle, help microorganisms to bind to the surface, which is the first step of biofilm arrangement. Biofilms can form on both body tissues and material surfaces. Although mixed-species biofilms predominate in most environments, including the oral cavity, single-species biofilms exist in a variety of infections and on the surface of implantable medical implants such as orthopedic implants or

catheters [12]. Indeed, the dental biofilm (corresponding to the oral microbiome) is composed of all the components of the oral microbiota and may thus comprise a single or multiple microbial species, mainly bacteria, fungi, and mycoplasmas. The saliva can also contain certain types of protozoa, such as *Trichomonas* species, but mainly in immunocompromised subjects.

In the process of biofilm development, Gram-positive bacteria, such as *Streptococcus* spp. and *Actinomyces* spp., are called "pioneer species," since they are usually the first to adsorb onto the dental pellicle and start to proliferate. They play an important role in producing conditions suitable for other microbes to further colonize the substrate; indeed, their respiration process reduces the oxygen tension and increases the level of carbon dioxide, resulting in hypoxic conditions that are suitable for anaerobic species. A number of oral microorganisms easily proliferate in this environmental setting: they are facultative anaerobes and account for most oral cavity bacteria, for example, oral streptococci, which survive deep within the dental biofilm.

Biofilm Metabolism

Bacterial biofilm metabolism chiefly relies upon carbohydrates as principal source of energy, in order to produce ATP. In particular, glucose is converted to pyruvate via the glycolysis metabolic pathway; pyruvate then follows diverse pathways depending on the oxygen tension and the type of microorganism. For example, glucose is degraded to pyruvate via the central carbon metabolism, following the classic glycolysis reaction; however, under anaerobic conditions, it is further degraded into lactate and acetate, by bacteria including *Streptococcus*, *Actinomyces*, and *Lactobacillus* spp. Conversely, in the presence of oxygen, pyruvate is converted to acetate by *Streptococcus* and *Lactobacillus*, and lactate is converted to acetate by *Actinomyces*. When bicarbonate is also present, as often occurs in the saliva, phosphoenolpyruvate is converted to succinate with bicarbonate assimilation; for instance, *Actinomyces* follows this metabolic pathway. Lastly, in *C. albicans* pyruvate is directly metabolized into acetyl CoA by the pyruvate dehydrogenase complex in aerobic conditions, but under hypoxia the anaerobic route is activated, and small amounts of acetaldehyde may be produced. Since ethanol is toxic to microorganisms at high concentrations, the preferential metabolism is aerophilic and avoids ethanol accumulation.

As has been said, biofilm metabolism is crucial for several dental diseases, and microorganisms' metabolic pathways have been elucidated, using single bacterial strains in preclinical studies; they have not yet been confirmed by in vivo analyses on supragingival plaque. The main limitation on these studies is that the amount of supragingival plaque that can be sampled from the oral cavity is insufficient for a conventional metabolic study: metabolome analysis could be an excellent alternative to overcome this difficulty.

Biofilm-Related Medical Device Infections

The most common biofilm-related medical device infections are due to the Gram-negative *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, or *Escherichia coli* or to the Gram-positive *Staphylococcus epidermidis*, *Staphylococcus aureus*, or enterococci.

Hospital and health-care facilities are peculiar environments in which dangerous antibiotic-resistant pathogens can live and evolve. Hospital-based pathogens show continuous dynamic change, and this influences their distribution through the body over time and their pathogenicity [13]. To fight the multidrug resistance (MDR) of several bacteria is still the major global challenge. Table 3 summarizes the strains correlated to hospital-based infections; to date, the MDR strains identified are the species *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., collectively known under the acronym of "ESKAPE." Hospital-based pathogens may infect the oral cavity and intraoral devices.

At the beginning of the antibiotic era, hospital-acquired infections were mainly due to S*taphylococcus* spp., initially kept under close control by penicillin. Then, as *Staphylococci* started producing beta-lactamase, beta-lactamase-resistant compounds were synthesized in order to counteract these pathogens. Subsequently, methicillin-resistant *S. aureus* (*MRSA*) and Gram-negative bacilli emerged and became the chief bacteria responsible for hospital-acquired infections (HI); however, the use-abuse of antibiotics has favored the selection of bacteria with methicillin resistance combined with resistance to other types of antibiotics. In the late 1960s, *Enterobacteriaceae*, such as *Escherichia* spp., became increasingly involved in hospital-based infections, finally leading to the emergence of multidrug-resistant (MDR) Gram-negative *Pseudomonas aeruginosa* and *Acinetobacter* spp., causing very difficult therapeutic problems and a frustrating and never-ending search for a solution.

The World Health Organization (WHO) has now recognized MDR as one of the three most important problems facing human health [14]. MDR is often due to the presence of specific resistance gene "islands" that, under the pressure of antibacterial agents, can be rapidly switched, developing a dynamic and always novel mechanism of antibiotic counteraction. In most cases, MDR strains attain these "islands" from bacteria of unrelated genera, as confirmed by sequence similarity and phylogenetic analyses [15]. This gives rise to the so-called super bacteria or super bugs, resistant to most, if not all, antibiotic regimes. However, the mechanisms underlying MDR vary in different pathogens, often reflecting the cellular structure of the bacterium.

Biofilm Formation and Propagation

Oral biofilm formation is part of a biological cycle that includes four main stages: initiation, maturation, maintenance, and dissolution (Fig. 1).

Table 3 Principal hospital-based infections, microorganisms, and human body sites involve

Hospital-based infection (HI)	Infective agents at different body sites Microorganism
Surgical skin (SSI) and soft tissue infections (SSTI)	*Staphylococcus* (*S.*) *aureus and epidermidis*; *Acinetobacter* (*A.*) *baumannii*; *Escherichia* (*E.*) *coli*; *Pseudomonas* (*P.*) *aeruginosa*; *Enterococcus* (*E.*) *faecalis*; coagulase-negative *Staphylococci*; *Candida* (*C.*) *albicans*
Bloodstream infections (BSI)	*S. aureus*; *E. coli*; *Enterococcus* spp.; *Streptococcus* (*S.*) spp.; *Proteus* spp.; *Staphylococcus* (*S.*) spp.; *P. aeruginosa*; *Candida* (*C.*) spp.; hepatitis B and C virus; *Cytomegalovirus*
Meningitis (MI)	*Enterovirus*; herpes simplex type II; varicella-zoster virus; *Adenovirus*; parotitis virus; HIV; *Flavivirus*, *Arbovirus*; *Neisseria* (*N.*) *meningitidis*; *Streptococcus* (*S.*) *pneumoniae*; *Haemophilus* (*H.*) *influenzae*; *S. aureus*; *P. aeruginosa*; *E. coli*; *Listeria monocytogenes*; *Cryptococcus neoformans*; *Histoplasma capsulatum*; *Coccidioides immitis*; *Blastomyces dermatitidis*; *Candida* spp.
Respiratory infections (RI) in intensive care units (ICU)	*Streptococcus* (*S.*) *pneumoniae*; *Haemophilus* (*H.*) *influenzae*; *Moraxella* (*M.*) *catarrhalis*; *S. aureus*; *P. aeruginosa*; *A. baumannii*; *E. coli*; *Legionella*; *Aspergillus* (*A.*) *fumigatus*; *Pneumocystis* (*P.*) *jirovecii*; *Mycobacterium* (*M.*) *tuberculosis*, *Klebsiella* (*K.*) *pneumoniae*; *Serratia* (*S.*) *marcescens*
Endocarditis (EC)	*S. aureus*; *Streptococcus* (*S*) *pyogenes* and *pneumoniae*; *E. faecalis*; *P. aeruginosa*; *Candida* (*C.*) *albicans*
Gastroenteritis (GI)	*Rotavirus*; *Campylobacter*; *S. aureus*; *Pseudomonas* (*P.*) *aeruginosa*; *E. coli* O157:H7; *Salmonella* spp.; *Giardia* (*G.*) *lamblia* and *intestinalis*; *E. faecalis* and *faecium*; Norwalk virus, *Adenovirus*, *Astrovirus*; *Calicivirus*; *Cryptosporidium parvum*
Urinary infections (UI)	*E. coli*; *Pseudomonas* (*P.*) *aeruginosa*; *Klebsiella* spp.; coagulase-negative *Staphylococci*; *E. faecalis* and *faecium*; *S. aureus; Proteus* (*P.*) spp.*; S. marcescens; Citrobacter* (*C.*) spp.
Genital/pelvic infections (GI)	Human papillomavirus; *Trichomonas vaginalis*; *E. faecalis* and *faecium*; *C. albicans*; *Proteus* (*P.*) spp.; *Klebsiella* spp.; *E. coli*; group B hemolytic *Streptococcus*; *Gonococci*; *Chlamydia*; *Herpes Simplex Virus; Mycoplasma*

Bacteria appear to initiate biofilm development in response to specific environmental cues, such as nutrient availability: microorganisms undergo a transition from free-living, planktonic cells to sessile, surface-attached cells in response to a nutrient-rich medium. Biofilms continue to develop as long as fresh nutrients are provided, but when bacteria are nutrient deprived, they detach from the surface and return to a planktonic mode of growth. This starvation response is thought to allow the cells to search for a fresh source of nutrients and is driven by well-known adaptations that bacteria actuate when nutrients become scarce.

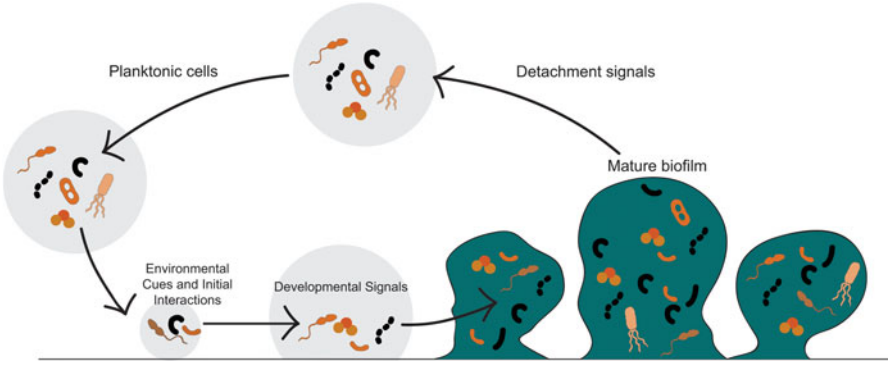

Fig. 1 Biological cycle of bacteria, including initiation, maturation, maintenance, and dissolution of the biofilm (Artgraph by Eng. Ettore Varoni and Dr. Silvia Bovo)

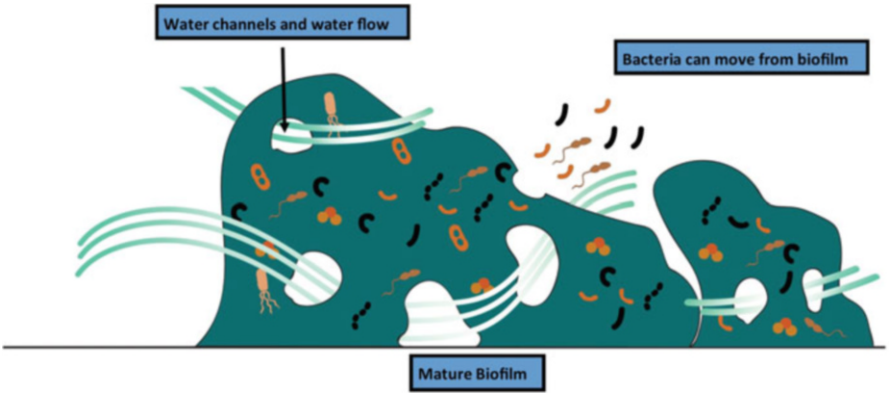

Fig. 2 Mature biofilm water channels. Channels can be used for microbial network signaling and to dilute drugs (Artgraph by Eng. Ettore Varoni and Dr. Silvia Bovo)

Conversely, the biofilm is also a complex protected arrangement, self-developed by the bacteria to enable them to survive in a hostile environment more easily than when they are in planktonic form. In particular, it enables them to optimize nutrient uptake, shelters them from removal forces, and protects them from desiccation, from host defense mechanisms, and from potential toxic or harmful agents, including antimicrobial agents. Interestingly, it is easy for the biofilm to develop antibiotic resistance, and it very frequently occurs, because the microbial community can regulate the opening and closing of the water channels biochemically (Fig. 2) and can consequently control the concentration of metabolites within the structure and/or stop the entrance of drugs.

Bacteria cells in the biofilm community coordinate efforts with their neighbors, to accomplish cooperative activities such as bioluminescence production, biofilm

development, and exoenzyme secretion. Coordination occurs through a mechanism of cell-to-cell communication called quorum sensing. This mechanism gives bacteria the capacity to recognize the population density by measuring the accumulation of a specific signaling molecule secreted by members of the community. When this density reaches a certain level, accumulation of the signal in the extracellular environment is sufficient to promptly activate the biofilm response to maintain its correct balance [16]. Moreover, "quiescent cells" have been also found inside the biofilm. These cells cannot be killed by antibiotics because they are at a low metabolic stage, assuring the protection of the structure and sustaining the drug's ineffectiveness [17–19].

Biofilm formation stages – It is crucial to clarify and understand in depth the events involved in biofilm formation on material surfaces, in order to develop effective control strategies. Adhesion is the first step in colonization and is a cornerstone for starting biofilm formation, since it allows bacteria to grow on certain surfaces and then invade host tissues. The sequence of the interaction, between floating bacteria and a surface, may be summarized as follows [20]:

1. Convective transport of fluids and active bacterial chemotaxis.
2. Van der Waals attractive forces, which operate at separation distances greater than 50 nm.
3. At distances of 10–20 nm, the interaction of van der Waals attractive forces and electrostatic repulsion produces a weak area of attraction, which maintains reversible adhesion.
4. At the same distance and even closer, adhesion between bacterial adhesin and ligands adsorbed onto the biomaterial surface from biological fluids, when the material was installed, begins to operate.

After surface colonization by pioneer bacteria, co-aggregation of other bacteria to cells that are already attached can occur. Multiplication of the attached organisms produces confluent growth of microorganisms, and a biofilm starts to form.

Figure 3 shows the sequential steps of supragingival biofilm formation on a root cementum surface, through scansion electronic microscope (SEM) images.

Biofilm/Substratum/Environment Interaction

Materials science and tissue engineering offer a unique opportunity to investigate biofilm formation. The availability of a stable surface is a prerequisite for the bacteria cells to attach and for consequent biofilm formation, and the properties of the surface can affect the outcome and bacteria/surface interactions.

Several aspects can affect biofilm formation and growth; the most important effects related to substrate and environment will be described in the next sections.

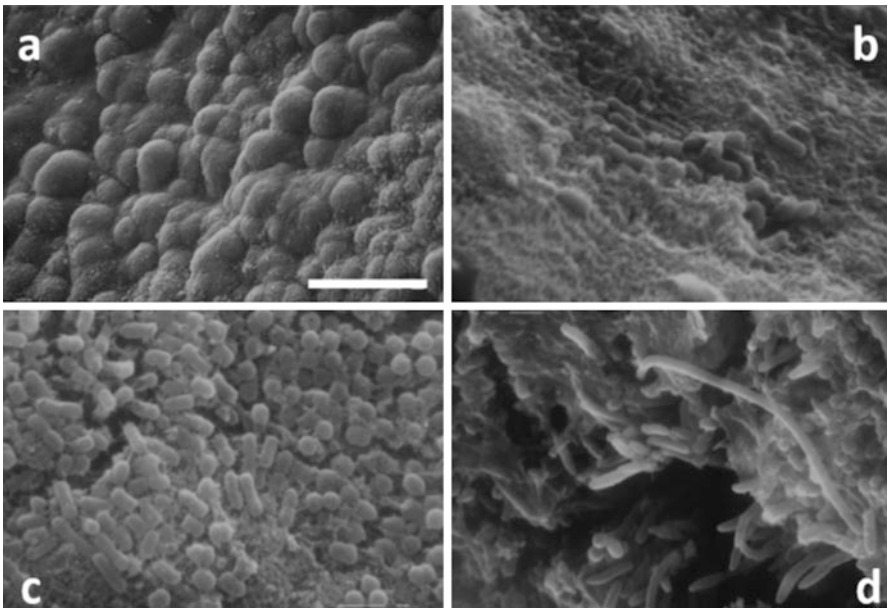

Fig. 3 Sequential steps of bacterial colonization in the oral cavity (SEM images). Clean and sterile cementum of the dental root surface, which is suddenly covered by a salivary pellicle (Adapted from Carrassi [21]): after 2 h, the cementum surface is colonized by a few cocci; after 12 h, the surface is completely covered by cocci and short rods; after 24 h, the biofilm has developed. Many bacteria, cocci, and short and long rods can be observed on the root surface, adhering to the slime layer

The Substrate Effect: Surface Energy and Hydrophilic/Hydrophobic Properties

The initial interactions between the bacterial cell wall and a surface (including those of other cell walls) are primarily influenced by interfacial electrostatic forces (repulsion or attraction) and van der Waals forces. However, many different nonspecific interactions and interfacial forces also influence cell attachment, including hydration forces, hydrophobic interactions, and steric forces [22]. Hydrophobic (low surface energy) and electrostatic (charge) interactions are the most widely investigated phenomena.

In general, bacteria may be modeled as colloidal particles approaching surfaces with a Brownian motion [48]. The interaction may be described by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory focused on long-range interactions between particles and substrate. This interaction includes the Lifshitz-van der Waals interaction and the interaction resulting from the overlapping of two layers of interactions. The forces are additive, and the energy of adhesion is a function of the distance between the particle and the substrate. In the case of bacteria, the DLVO

is not fully descriptive, and short-range Lewis acid-base interaction and hydration must also be taken into account (XDLVO).

The charge upon the bacteria wall is generally measured as electrophoretic mobility. It is usually electronegative, especially in the case of Gram-negative bacteria, as is that of many material surfaces. Thus, from the theoretical standpoint, bacteria do not adhere closely except to strongly electropositive surfaces. However, in practice they may show paradoxical behavior, because of the ability of the cell wall to dynamically alter its charge in response to environmental conditions, such as pH or ionic strength in the medium. In addition, fibrils, fimbriae, and flagella may expose different charges at their tips. The walls may also be penetrated by solvents, causing dynamic rearrangement of the wall polymers and consequently altering surface charge. These phenomena explain why the bacteria/substrate interaction is not fully described by the DLVO or XDLVO theories, and bacterial behavior in regard to the electrostatic properties of the substrate is not fully predictable (Table 4) [23].

In addition to electrostatic attraction, chemotaxis and possibly haptotaxis also contribute to the initial attachment [24]; this occurs in response to chemoattractants in the environment or adsorbed onto the surfaces, such as amino acids, peptides, and glucides.

The interactions between bacteria and surface, as described above, are generally reversible, but they evolve rapidly toward irreversible bonds characterized by molecular-specific reactions between bacterial surface structures and the substratum. The interactions are mediated by bacterial surface polymeric structures, called adhesins, included in the capsules, fimbriae, or pili and in the slime. For instance, *S. aureus* binds fibronectin, while *S. epidermidis* has several polysaccharide adhesins that mediate the adhesion of this bacterium to various material surfaces and protein tissues. Of the adhesins, the most important are (i) capsular polysaccharide/adhesion (PS/A), (ii) a biosurfactant known as "surface-active agent" (SAA), (iii) polysaccharide intracellular adhesion (PIA), (iv) a polysaccharide composed of β-1,6-linked *N*-acetylglucosamines with partly deacetylated residues, and (v) peptidoglycan, an accumulation-associated protein (AAP). PS/A and SAA take part in bacteria-material interactions, whereas PIA and AAP are implicated in cell-cell interactions [25] (Fig. 4).

The Environment Effect

Temperature, exposure time, bacterial concentration, and the presence of antibiotics or other antibacterial molecules affect bacteria adhesion and biofilm development. In addition, physical stresses, including flow, scraping, or epithelial detachment, have a great influence on biofilm formation. In general, high mechanical stresses inhibit biofilm formation and its maturation.

All these phenomena are evident in the oral cavity, where environmental conditions change frequently. It is a common observation that, in subjects who

Table 4 Representative examples of deviations from the DLVO or XDLVO theory observed in bacterial adhesion studies (Adapted from Poortinga et al. [23])

Strain	Experiment	Findings
Arthrobacter, *Corynebacterium*, *Rhodococcus*, *Pseudomonas*, *Gordona*	Adhesion to glass and Teflon	Experimentally obtained energy barriers against adhesion are some orders of magnitude smaller than DLVO predictions at low ionic strength
E. coli	Adhesion to sludge flocs	Adhesion does not correlate with bacterial zeta potential but with a fraction of the positive charge present on the bacterial cell surface
Vibrio alginolyticus	Adhesion to hydroxyapatite	Bacterial adhesion increases at increasing ionic strength, in accordance with the DLVO theory, but decreases when ionic strength exceeds 0.1 M
Corynebacterium	Accumulation of bacteria at air-water interface	In contrast to DLVO predictions, under repulsive conditions, accumulation decreases for increasing ionic strength
S. salivarius	Adhesion to glass	Despite small differences in DLVO interaction energies, adhesion rates of a fibrillated and non-fibrillated strain differ greatly
Marine strains	Adhesion to hydrophobic and hydrophilic polystyrene	No correlation found between adhesion and ionic strength
Sphingomonas paucimobilis	Adhesion to bare glass and EPS-coated glass	The XDLVO theory can explain adhesion to glass but cannot explain adhesion to glass coated with bacterial EPS
Pseudomonas	Adhesion to sand	A fraction of the bacteria adheres faster than the rest, while DLVO calculations predict no difference
E. coli	Direct measurement of bacterial interaction force with glass, mica, and hydrophobic polymers	Force measurements do not correlate with XDLVO calculations for a lipopolysaccharide covered strain but do correlate for a strain with truncated lipopolysaccharide chain
Pseudomonas and *Burkholderia*	Measurement of bacterial interaction with silicon nitride AFM tip	A repulsive force extending over longer distances (>100 nm) than predicted by the DLVO theory is measured

do not brush their teeth efficiently, plaque accumulation is abundant. In xerostomic patients, who are deficient in saliva amount and flow, plaque accumulates very

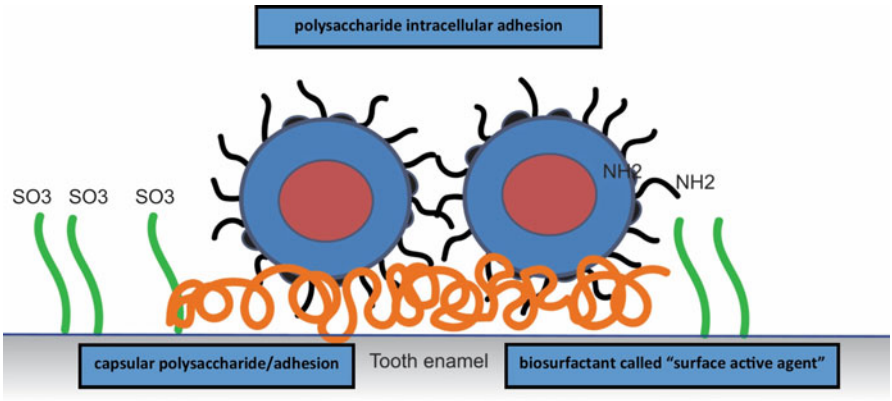

Fig. 4 Molecular interaction between bacteria and substrate (Adapted from Katsikogianni and Missirlis [25])

rapidly, and the clinical consequences consist of a prevalence of caries and periodontal disease.

The Effect of Surface Roughness at Micro- and Nanoscales

Certain physical parameters, such as surface roughness and morphology, are thought to closely affect biofilm formation. It is well known that rough restorative materials accumulate more plaque and expose patients to the risk of developing caries and gum diseases at neighboring sites. This is a key aspect in implantology, because most implants available on the market are designed to be rough and grooved, in order to improve primary stability, healing of mineralized and soft tissues, and maintenance of tissue integration around the implants over time, whether in healthy or diseased subjects. However, when rough surfaces are exposed to the oral environment, biofilm formation is swift, mainly because of the roughness and grooving shelter bacteria from physical removal, hindering cleaning procedures. Biofilm formation around implants is an etiological factor for peri-implantitis and implant failure or loss [26].

It has been observed that, although roughness and wettability are related, the roughness parameter is often predominant [22]. The clinical roughness threshold for biofilm formation in the oral cavity has been shown to be Ra = 0.2 μm: below this threshold, for Ra values within the microscale, there is no significant improvement in inhibiting bacterial adhesion [21, 27]. In contrast, at the nanoscale, rough and geometrically determined surface morphology has been shown to produce antifouling properties. At this scale, interaction of the bacteria with the surface remains limited to the surface of physical protrusions, like drops of dew on the leaves of a lotus flower (*Nelumbo* spp.), and bacteria are repulsed [28].

The Effect of Protein Absorption

As described earlier in this chapter, the first step in the pathogenesis of foreign body-related infections is bacterial adhesion. The mechanisms involved in adhesion lead to passive adsorption of the bacterial cells on the solid material, through physico-chemical surface interactions with bacterial structures termed bacterial adhesins. Thus, bacterial behavior varies as a function of material hydrophobicity and electrostatic charge. Chemo-physical properties and functional groups exhibited by the biomaterial surface interact with those of the bacterial cells, determining the kinetics of microbial adhesion.

However, in many cases of implanted or invasive medical devices, materials first come into contact with body fluids. This is particularly true in the oral cavity, where installed materials are immediately wetted by the saliva, crevicular fluid, or blood, depending on the anatomic site of application.

The components of body fluids, mainly proteins, are rapidly adsorbed onto the material surface. The protein film that quickly forms on the biomaterial surface during the initial exposure to physiologic fluids may thus be considered as the true interface with the bacteria. Nonspecific effects have been described, such as those derived from albumin surface adsorption, thought to alter the physicochemical characteristics of the surface and to increase the degree of hydrophobicity, while competing for the surface with other pro-adhesive host proteins. In addition, various host proteins mediate bacterial adhesion by interacting with bacterial adhesins; these are frequently receptor proteins known as "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs). The bacteria-binding host proteins include collagen, fibrinogen, fibronectin, laminin, vitronectin, clumping factors A and B, bone sialoprotein, elastin, and IgG. Charged surfaces can also interact electrostatically with other extracellular polymeric components. In addition to polysaccharides, other extracellular polymeric substances are produced by biofilm-forming bacteria; these include extracellular DNA, teichoic acids, and amphiphilic molecules, whose production or proportion may depend on the specific growth phase. Effective low-adhesion surfaces are thus hydrophilic, highly hydrated, and non-charged. These types of surface appear to prevent or limit contact between a bacterium and the potential attachment points of the material surface [28].

The adsorption of proteins on a surface can be reduced, either by altering the interaction potential or by slowing down the rate of adsorption through high-potential barriers to interaction. This latter method of controlling the kinetics of adsorption can be achieved by polymer grafting, resulting in the introduction of long-range repulsive forces. Other strategies to achieve lower bacterial adhesion to biomaterials exposed to protein solutions rely on conditioning the surface by pre-adsorption of molecules claimed to increase apolar hydrophilicity and hydrophobicity or to compete with host adhesion adsorption [29]. In addition, the possibility of controlling tissue integration while contrasting bacterial adhesion, simply by acting on the topographical features of the biomaterial surface, is certainly very

attractive. Specifically patterned surfaces can direct the alignment and spatial distribution of bacterial cells. At the same time, customized superficial nanostructures can reduce the areas of contact where eukaryotic and bacterial cells can anchor. Topographies can achieve a degree of complexity that confers entirely new properties on the material surface [30].

Biofilm Formation on Dental Implants and Prosthetic Dental Materials

Biofilm Formation on Dental Implants

Biofilm formation on dental implants is the crucial step toward the inflammation of peri-implant tissues, jeopardizing the long-term success of osseointegrated implants. In general, the assessment of the microbiological and immunopathological aspects of peri-implant diseases has shown a microbiological diversity of peri-implantitis biofilms and a specific local immune response of the host [8].

Bacterial colonization and adhesion at the implant surface starts already 30 min after placing the device and lasts for several months. For instance, the presence of *S. aureus* has been confirmed as long as 1 year later. As pointed out in the paragraph "Peri-implant microbiome," the bacterial composition of the newly formed implant biofilm closely resembles that of the nearest teeth, suggesting that the microbial flora on dental substrates can act as a "reservoir" for the bacteria that compose the biofilm around implants. Importantly, bacteria of subgingival biofilms, collected from peri-implantitis patients, displayed multiple antibiotic resistances in vitro, for example, in the case of *Prevotella intermedia*, *Prevotella nigrescens*, or *Streptococcus constellatus*.

Although the qualitative composition of the biofilm in peri-implantitis shows similarities to that of periodontitis, supporting the hypothesis that patients with active periodontal disease are at higher risk for developing peri-implantitis, several further microorganisms, very uncommon in periodontitis, have been recognized in peri-implantitis; these include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Peptostreptococcus micros*, and *Pseudomonas* spp. [8]. A further peculiar element in peri-implant mucositis, and subsequently in peri-implantitis, is that inflammation acquires typical features defined as the "specialized innate response." Peri-implantitis displays larger numbers of immune cells, mainly interstitial dendritic cells and related inflammatory mediators. The progression from mucositis to peri-implantitis is characterized by a drastic increase in neutrophils, osteoclasts, macrophages, and lymphocytes, in findings supported by transcriptome analyses. Compared to the inflammatory tissue from periodontitis sites, the peri-implant granulation tissue displayed a specific innate response, with greater mRNA expression of pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, and IL-8. Moreover, resident primary fibroblasts showed increased production of

vascularization factors, matrix metalloproteases, and complement receptor C1q, with decreased production of metalloprotease inhibitors and growth factors for collagen synthesis [8].

Recent studies have analyzed samples of crevicular fluid collected from the sulcus around abutments and report a significant difference between supra- and subgingival plaque: these findings supported the hypothesis that the cellular adherence of peri-implant tissue to titanium implant, via hemidesmosome, actin filaments, and microvilli, greatly reduces the risk of formation of anaerobic subgingival pockets. Indeed, the biofilm coating observable on supragingival abutment surfaces appeared significantly thicker than that on subgingival sites.

Together with surface localization (supra- and subgingival) of oral biofilm, surface modification of biomaterial also appeared to significantly affect the health status of tissues around implant abutments. Two main aspects are particularly involved, i.e., the local immune response to biomaterial and the biofilm adhesion and proliferation on it. With regard to the former, particularly in the case of mucositis, the physicochemical treatment of the implant surface during manufacturing appears to affect the inflammatory response of the adjacent mucosal tissue, in terms of different microvessel density and amount of inflammatory infiltrate. Regarding biofilm adhesion and growth, the surface chemistry and the design features of the implant-abutment configuration can affect biofilm formation. As mentioned, increased surface roughness and surface free energy appear to promote dental plaque formation on implant and abutment surfaces, although this conclusion derives chiefly from descriptive literature, rather than from high-quality meta-analyses. A considerable debate still surrounds the issue, and in particular the precise role played by physicochemical and textural properties of the implant surface on microbial composition is still unknown. It is hypothesized that greater roughness and higher free energy at the implant surface might promote biofilm formation, so that peri-implantitis might occur and progress more quickly. However, and conversely, some evidence also supports the hypothesis that abutments with different surface characteristics do not greatly influence either biofilm formation on the implant surface or the extent and composition of the inflammatory response. No implant system or surface type has been found superior over any other in terms of marginal bone preservation, the main reason for this probably being related to the presence of salivary proteins at the interface between the host tissue and biomaterial. The latter adheres first at the implant surface and can mediate bacterial adhesion: any differences in bacterial adhesion due to surface microstructures may partially be "counteracted" or masked by this salivary pellicle, which mediates the mucosa-implant interconnection [8].

Taken together, the results of these studies suggest that the diversity of the microbial community and the subsequent immunity response of peri-implantitis versus periodontitis might not be as close as has been believed: further investigations targeting the multiplicity of peri-implant-specific microbiota will be needed to identify the best approach for peri-implantitis management, still an important clinical challenge.

Biofilm Formation on Restorative and Prosthetic Materials

As was said in the paragraph "Dental caries and the oral microbiome," dental caries is chiefly the result of an imbalance in metabolic activity within the oral biofilm, which becomes skewed toward a strong acidification of the milieu at the tooth surface, leading to the dissolution of hard dental tissues (enamel and dentine). From a metabolomics perspective, the cariogenic potential of the microbial community must be described in terms of activities relevant to acid production. Recent studies have shown that metabolomics may explain caries pathogenesis better than a focus solely on microbiome composition; unsurprisingly, sound evidence exists to confirm that carbohydrate metabolism is a cornerstone in caries development, because of its capacity to acidify the environment and dissolve dental tissues, leading to tooth decay.

For dental applications, antimicrobial coatings killing bacteria upon contact are more promising than antimicrobial-releasing coatings. Moreover, certain natural polymers, used as biomaterials with intrinsic antibacterial properties, such as chitosan or pectins, could be useful tools, in that they would contextually exert antimicrobial activity during tissue regeneration [31, 49].

Biofilms appear to form in different ways, depending on the different types of biomaterials used in restorative and prosthetic dentistry. On gold and amalgam, the in vivo growth of dental plaque appears thick and can almost completely coat the substrate, but it is also barely viable. Conversely, on ceramics oral biofilms are thin but highly viable. Dental plaque on composites and glass ionomer cements has been reported to produce surface decay, which appears to further enhance biofilm proliferation. In particular, residual monomers released from composites affect plaque development in vitro, but the corresponding in vivo effects are less striking, probably due to the greater dilution of these compounds, which become dissolved in a huge volume of saliva, which is continuously replaced by the flow rate.

Dental plaque grows readily on the acrylic bases of dentures, mainly because of their porous structure. The composition of oral biofilms on the mucosal and prosthetic surfaces has been investigated, to determine any differences. A recent study analyzed 61 edentulous subjects with complete maxillary and mandibular dentures [32]: "supragingival" plaque samples were collected from the acrylic base; from the dorsal, lateral, and ventral surfaces of the tongue; from the floor of the mouth, the buccal mucosa, the hard palate, the vestibule/lip, and the attached gingiva; and from the saliva. The microbial profiles of plaque from the soft tissues differed with the site considered, but the main periodontal pathogens, i.e., *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, were detectable in all specimens. In particular, samples from the dorsum of the tongue showed the highest bacterial counts, followed by the adherent gingiva and the lingual margins; the lowest counts were recorded for samples from the buccal mucosa and the labial vestibular mucosa. The patterns of microbial colonization versus harvesting site showed three clusters: the first cluster included the saliva, the supragingival plaque, and the lateral and dorsal surfaces of the tongue; the second cluster comprised the six remaining soft tissues; and the third cluster comprised all species on the denture palate.

The Role of Chemistry in Dental Biofilm Limitation

Current Strategies

Numerous strategies are currently available to hinder the formation of "pathogenic" oral biofilm on dental biomaterials and the development of related dental disease. These include strategies relating to the materials themselves, substances used to dope materials, and different types of surface coating including bioactive coatings, micro- and nano-particles, etc.

Materials with intrinsic antibacterial properties – Bulk materials that exert antibacterial action without requiring any modification are generally described as intrinsically antibacterial. Numerous metals, such as silver, zinc, and copper, are known to be intrinsically bactericidal. However, their activity is not usually highly specific and is not solely oriented against prokaryotic cells: there is generally a certain degree of cytotoxicity against host cells in peri-prosthetic tissues, reducing their viability. This is often due to the metals becoming corroded in the physiological environment or to its inexorable leaching that leads to the release of high concentrations of active ions, causing local toxicity and, in some cases, accumulation in distant target organs. Silver is certainly the most widely used for biomedical applications; its bactericidal activity is related to the inactivation of critical enzymes of the respiratory chain (e.g., succinate dehydrogenase) by binding to thiol groups and induction of hydroxyl radicals. Recently, the utilization of silver as thin nano coatings, in doped solid or hydrogel materials, in the formulation of bioactive alloys and glasses and its use in the form of micro- or nanoparticles, has progressively advanced, although the possible inactivation of silver-mediated antibacterial activity in physiological fluids and the low biocompatibility index are still debated.

Gallium-based treatments provide promising titanium anti-biofilm coatings to develop new bone-implantable devices for oral, maxillofacial, and orthopedic applications [33]. Recent evidence shows that the biological functions of Fe^{3+} are impaired by replacing iron with gallium; gallium inhibits Fe^{3+} biological functions by what is known as a "Trojan horse" strategy [33].

Chitosan is another substance known to possess intrinsic antibacterial and antifungal activities [34]. However, chitosan is a polycationic polymer derived from chitin, and it only has bland bactericidal activity, usually enhanced at low pH.

Bioactive coatings with bactericidal agents – Bioactive antibacterial coatings have been developed with the purpose of achieving desirable new anti-infective properties at the biomaterial-tissue interface, without compromising the characteristics of the bulk material. In the so-called contact biocides, anti-infective surfaces involve the use of non-leachable substances, such as some antimicrobial peptides, quaternary amines, and *N*-halamines. These bioactive surfaces only kill bacteria on contact, as the bactericidal substances are not released, and are activated following direct interaction with the bacterial cells. Direct contact-killing is based on extremely high electrostatic forces on the surface that can disrupt bacterial cell membranes by removing anionic lipids [35]. The limit of this strategy is that surfaces can potentially

be masked and inactivated when filmed by the host proteins present in protein-rich physiologic fluids.

Nitrogen monoxide (NO), a natural molecule with pleiotropic functions, usually produced by leukocytes as host defense against microbial pathogens, plays an important role as a bioactive bactericide [36]. However, NO can interact with superoxide in the tissues, in conditions of oxidative stress, generating the highly cytotoxic peroxynitrite ($ONOO^-$); this makes it very important to fine-tune the beneficial and toxic effects of NO, by carefully controlling the release kinetics.

Great interest is directed toward substrates that become antimicrobial following a process of photoactivation; these include titanium oxide (TiO_2). TiO_2 surfaces undergo photoactivation upon irradiation, with an adsorption wavelength of 385 nm; this irradiation excites the anatase allomorph, which is one of the three main TiO_2 polymorphs. The bactericidal action of irradiated titanium surfaces is due to reactions of photooxidation, which involve O_2 and H_2O, with the formation of hydroxyl radicals (HOO-) and the direct and indirect oxidation of organic substances. These radicals are highly effective at disrupting bacterial membranes. In particular, AgeTiO_2 appears to be a very promising coating, combining the known oligodynamic bactericidal properties of silver ions with an enhanced photocatalytic activity, conferred by facilitating electron-hole separation and/or increasing the surface area for adsorption [24].

Materials delivering antibiotics – An obvious step to produce biomaterials with anti-infective properties is to incorporate antibiotics within the biomaterials. Antibiotics can be incorporated variously into the bulk or coating of a biomaterial, and the incorporation can be either in molecular or in particle form. The release can consequently occur by different modalities, including diffusion to the aqueous phase, erosion/degradation of resorbable loaded matrices, and hydrolysis of covalent bonds. Thus, delivery kinetics depends on the stability of the molecular bonds or on the rate of biodegradation/bioerosion of the matrices entrapping the antimicrobial agent. However, these delivery mechanisms have been widely debated, especially regarding their efficacy over the long term (>3 weeks) [37].

Urinary and central venous catheters provide a significant example of the use of materials delivering antibiotics: a study comparing different types of antibiotic- and metal/antibiotic-doped urinary catheters found no difference in bacteria reduction at 3 weeks between doped and non-doped catheters; however, during the first week, the bactericidal efficacy of the doped catheters was clearly superior to that of their non-doped counterparts. A study examining the bactericidal efficacy of central venous catheters found efficacy to be closely related to the implant site: the infection rate was reduced in the femoral and jugular veins but remained unchanged in the subclavian vein [50].

There is general concern that the routine use of antibiotic-loaded biomaterials will increase the spread of antibiotic resistance: after an initial burst, antibiotic release diminishes and becomes subinhibitory. A number of studies have reported that subinhibitory concentrations of certain antibiotics enhance, rather than inhibit, biofilm formation by bacteria. This leads to the need for new therapeutic agents.

Antimicrobial peptides (AMPs) are a very interesting emerging class of molecules that occur naturally in the mechanisms of innate immune defenses in multicellular organisms. AMPs show broad-spectrum activity against a large class of pathogens, and their microbicidal action is related to their ability to determine transmembrane pores. Thus, AMPs are considered to be a very promising class of bactericidal agents, and they have been studied in depth and tested in several clinical trials in order to clarify their biocompatibility.

Nanostructured anti-adhesion surfaces – Certain nanostructural features of material surfaces have been shown capable of altering the 3D conformation of adsorbed proteins, and this might have an effect on host adhesins that film the biomaterial surfaces [38]. In this connection, one of the most rapidly expanding strategies in the field of nanotechnologies is the exploitation of the antibacterial properties of nanoparticles (NPs). The bactericidal activity clearly depends on the NPs' characteristics in terms of material, charge, and size. In the case of gold NPs, the bactericidal action has been found to be determined by inhibition of ATP synthase activity associated to the change in membrane potential and by inhibition of the subunit of ribosome for tRNA binding. Silver NPs (AgNPs) appear to interact with the bacterial cell wall, disturbing its permeability, inactivating essential proteins such as thiol-containing enzymes, causing DNA condensation, and leading to Reactive Oxygen Species (ROS) generation [39]. However, together with these positive bactericidal effects, it must be stressed that NPs can sometimes have toxic effects: the induction of apoptosis and genotoxic effects related to NPs' translocation to distant tissues/organs have been reported [40]. Thus, the chemical composition, size, shape, concentration, rate of dissolution/degradation, and surface properties of nanoparticles must be clearly understood and fine-tuned to achieve the best performance in terms of the benefits/drawbacks ratio.

Anti-biofilm bioactive molecules – Recent progress in understanding the molecular mechanisms implicated in the physiology of biofilm formation has opened new vistas concerning how to contrast the colonization of bacteria on biomaterial surfaces [41, 42]. This has led to the development of numerous different active substances, including molecules with different mechanisms of action: enzymes capable of selectively degrading extracellular polymeric substances of the biofilm (e.g., dispersin B, rhDNase I), bactericidal molecules capable of killing metabolically quiescent bacterial cells (e.g., lysostaphin, certain AMPs), molecules and other microorganisms interfering with the quorum sensing system and inducing biofilm dispersion (e.g., furanones), and molecules downregulating the expression of biofilm extracellular polymeric substances (e.g., *N*-acetylcysteine) [43]. All these molecules have a serious defect in common: their efficacy is limited to a single species or at best to a small number of species; this greatly restricts their effectiveness against bacterial communities. Exceptions are the proteolytic enzymes, such as trypsin and proteinase K, which can degrade even host extracellular matrix proteins, and whose internal use in an in vivo physiological environment could obviously have adverse effects on the wound healing process. The most promising therapies now being studied comprise combinations of anti-biofilm molecules and conventional wide-spectrum antibiotics, as, for example, was shown in a study [44] combining

dispersin B and cefamandole for the treatment of staphylococcal biofilm growth on polyurethanes.

Further Strategies

With regard to pathogenesis, the combination of the different "*–omics*" and related innovative technologies will provide an increasingly comprehensive view of the role that the oral microbiota can play in health and biomaterial-related dental diseases, from peri-implantitis to prosthetic candidiasis. Among others, metabolome analysis is probably the most promising method to monitor these dynamic metabolic activities, helping to clarify pathogenesis. Nonetheless, it may also be applied in examining the effectiveness of both conventional drug therapies and novel compounds and might even provide useful insights for the identification of pioneering biomarkers relevant for the development and progression of biomaterial-related diseases.

While ongoing preclinical and clinical studies hope to accumulate more data on the disease pathogenesis, as well as on the efficacy of current anti-infective strategies, new possibilities to counteract biomaterial-associated infections are advancing. Pre-inoculating urinary catheters with nonpathogenic *E. coli* were found to significantly impede catheter colonization by *E. faecalis*. However, some practical difficulties surround the introduction of this approach into clinical trials, as it would entail applying non-sterile catheters.

The use of phages as "biological weapons" has been attempted, with controversial results: whereas a high inhibition ratio >4 log has been shown in in vitro experiments, no significant result emerged from in vivo studies [45]. Moreover, the use of phages as therapeutic agents is severely limited by (i) their high specificity, (ii) bacterial resistance, (iii) pre-inactivation by the immune system, (iv) poor resistance in the surface immobilization step, and (v) high risk of unpredicted virus expansion using phages as vector.

A possible future approach to combating biomaterial-associated infections, while avoiding the use of today's antibiotics, might be provided by antisense peptide nucleic acids (PNAs). These can interfere with the expression of critical bacterial genes that are involved in antibiotic resistance, biofilm formation, and bacterial reproduction/survival. Gram-positive bacteria are less susceptible to cell-penetrating peptides conjugated with PNAs; however, studies have shown positive results on Gram-negative bacteria by targeting the rpoD gene, which encodes an RNA polymerase primary σ (70) that is essential for bacterial growth [46]. However, a number of critical concerns surrounding the safety of PNAs must be addressed before this technology will be able to enter clinical trials on human patients; in particular, these concern possible mutagenic effects deriving from the complexation of PNAs and their degradation products, which might match DNA and knockdown, or even knockoff, sequences of the human genome.

An alternative strategy has been presented, which is based on contrasting bacterial infections by modulating the host's local immune response, rather than by

counteracting bacterial colonization directly [47]. Two active cytokines, namely, monocyte chemoattractant protein-1 (MPC-1) and interleukin 12 p70 (IL-12), were tested. The former is a powerful macrophage-recruiting cytokine, while the latter, IL-12, can induce T-helper cells to secrete Th1 cytokines, such as interferon-g (IFN-g), which in turn stimulate the bactericidal activity of macrophages. The results are promising, but no synergic activity between the cytokines was observed.

Finally, autologous platelet-rich plasma (PRP) was also found to be bactericidal when used as surface coating: in vitro experiments have shown that PRP can cause a reduction in colony-forming units of two logs.

The increasing use, in dentistry as well as in other medical fields, of implantable devices and the apparently unstoppable advance of drug-resistant bacteria are combining to make it imperative that we understand and combat the development of bacterial biofilm on non-biological surfaces. Several interesting approaches are being developed, in the hope that further research will lead to eradicating infection-associated implant failures.

Summary

- The human body contains complex microbial communities with essential functions for the host's health.
- The oral cavity is an example of a dynamic microbial niche.
- The increasing use of implantable devices has led to the emergence of biofilm-related device infections on the part of apparently unstoppable multidrug-resistant bacteria.
- New prevention strategies are being developed in order to reduce the frequency of infection-related implant failures.
- Emerging approaches are still a matter of debate.

References

1. Nyvad B, Crielaard W, Mira A et al (2013) Dental caries from a molecular microbiological perspective. Caries Res 47:89–102. doi:10.1159/000345367
2. Jorth P, Turner KH, Gumus P et al (2014) Metatranscriptomics of the human oral microbiome during health and disease. MBio 5:e01012–e01014. doi:10.1128/mBio.01012-14
3. Zaura E, Nicu EA, Krom BP, Keijser BJF (2014) Acquiring and maintaining a normal oral microbiome: current perspective. Front Cell Infect Microbiol 4:85. doi:10.3389/fcimb.2014.00085
4. Takeuchi H, Furuta N, Amano A (2011) Cell entry and exit by periodontal pathogen via recycling pathway. Commun Integr Biol 4:587–589. doi:10.4161/cib.4.5.16549
5. Heuer W, Elter C, Demling A et al (2007) Analysis of early biofilm formation on oral implants in man. J Oral Rehabil 34:377–382. doi:10.1111/j.1365-2842.2007.01725.x
6. Eick S, Ramseier CA, Rothenberger K et al (2015) Microbiota at teeth and implants in partially edentulous patients. A 10-year retrospective study. Clin Oral Implants Res. doi:10.1111/clr.12588

7. Charalampakis G, Belibasakis GN (2015) Microbiome of peri-implant infections: lessons from conventional, molecular and metagenomic analyses. Virulence 6:183–187. doi:10.4161/21505594.2014.980661
8. Belibasakis GN (2014) Microbiological and immuno-pathological aspects of peri-implant diseases. Arch Oral Biol 59:66–72. doi:10.1016/j.archoralbio.2013.09.013
9. Peterson SN, Meissner T, Su AI et al (2014) Functional expression of dental plaque microbiota. Front Cell Infect Microbiol 4:108. doi:10.3389/fcimb.2014.00108
10. Zhu X, Wang S, Gu Y et al (2012) Possible variation of the human oral bacterial community after wearing removable partial dentures by DGGE. World J Microbiol Biotechnol 28:2229–2236. doi:10.1007/s11274-012-1030-5
11. Kraneveld EA, Buijs MJ, Bonder MJ et al (2012) The relation between oral Candida load and bacterial microbiome profiles in Dutch older adults. PLoS One 7:e42770. doi:10.1371/journal.pone.0042770
12. Shah SR, Tatara AM, D'Souza RN et al (2013) Evolving strategies for preventing biofilm on implantable materials. Mater Today 16:177–182. doi:10.1016/j.mattod.2013.05.003
13. Bereket W, Hemalatha K, Getenet B et al (2012) Update on bacterial nosocomial infections. Eur Rev Med Pharmacol Sci 16:1039–1044
14. Bassetti M, Ginocchio F, Mikulska M (2011) New treatment options against Gram-negative organisms. Crit Care 15:215. doi:10.1186/cc9997
15. Perez F, Hujer AM, Hujer KM et al (2007) Global challenge of multidrug-resistant *acinetobacter baumannii*. Antimicrob Agents Chemother 51:3471–3484. doi:10.1128/AAC.01464-06
16. Solano C, Echeverz M, Lasa I (2014) Biofilm dispersion and quorum sensing. Curr Opin Microbiol 18:96–104. doi:10.1016/j.mib.2014.02.008
17. Gilbert P, Maira-Litran T, McBain AJ et al (2002) The physiology and collective recalcitrance of microbial biofilm communities. Adv Microb Physiol 46:202–256
18. Kamruzzaman M, Udden SMN, Cameron DE et al (2010) Quorum-regulated biofilms enhance the development of conditionally viable, environmental *vibrio cholerae*. Proc Natl Acad Sci U S A 107:1588–1593. doi:10.1073/pnas.0913404107
19. Wu M-Y, Sendamangalam V, Xue Z, Seo Y (2012) The influence of biofilm structure and total interaction energy on *Escherichia coli* retention by *Pseudomonas aeruginosa* biofilm. Biofouling 28:1119–1128. doi:10.1080/08927014.2012.732070
20. Yang L, Liu Y, Wu H et al (2011) Current understanding of multi-species biofilms. Int J Oral Sci 3:74–81. doi:10.4248/IJOS11027
21. Rimondini L, Farè S, Brambilla E et al (1997) The effect of surface roughness on early in vivo plaque colonization on titanium. J Periodontol 68:556–562. doi:10.1902/jop.1997.68.6.556
22. Cochis A, Fini M, Carrassi A et al (2013) Effect of air polishing with glycine powder on titanium abutment surfaces. Clin Oral Implants Res 24:904–909. doi:10.1111/j.1600-0501.2012.02490.x
23. Poortinga AT, Bos R, Norde W, Busscher HJ (2002) Electric double layer interactions in bacterial adhesion to surfaces. Surf Sci Rep 47:1–32. doi:10.1016/S0167-5729(02)00032-8
24. Foster HA, Ditta IB, Varghese S, Steele A (2011) Photocatalytic disinfection using titanium dioxide: spectrum and mechanism of antimicrobial activity. Appl Microbiol Biotechnol 90:1847–1868. doi:10.1007/s00253-011-3213-7
25. Katsikogianni M, Missirlis YF (2004) Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. Eur Cell Mater 8:37–57
26. Lang NP, Berglundh T, Working Group 4 of Seventh European Workshop on Periodontology (2011) Periimplant diseases: where are we now? – consensus of the seventh European workshop on periodontology. J Clin Periodontol 38(Suppl 11):178–181. doi:10.1111/j.1600-051X.2010.01674.x

27. Teughels W, Van Assche N, Sliepen I, Quirynen M (2006) Effect of material characteristics and/or surface topography on biofilm development. Clin Oral Implants Res 17(Suppl 2):68–81. doi:10.1111/j.1600-0501.2006.01353.x
28. Crick CR, Ismail S, Pratten J, Parkin IP (2011) An investigation into bacterial attachment to an elastomeric superhydrophobic surface prepared via aerosol assisted deposition. Thin Solid Films 519:3722–3727. doi:10.1016/j.tsf.2011.01.282
29. Ruggieri MR, Hanno PM, Levin RM (1987) Reduction of bacterial adherence to catheter surface with heparin. J Urol 138:423–426
30. Mitik-Dineva N, Wang J, Truong VK et al (2008) *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* attachment patterns on glass surfaces with nanoscale roughness. Curr Microbiol 58:268–273. doi:10.1007/s00284-008-9320-8
31. Varoni EM, Iriti M, Rimondini L (2012) Plant products for innovative biomaterials in dentistry. Coatings 2:179–194. doi:10.3390/coatings2030179
32. Sachdeo A, Haffajee AD, Socransky SS (2008) Biofilms in the edentulous oral cavity. J Prosthodont 17:348–356. doi:10.1111/j.1532-849X.2008.00301.x
33. Cochis A, Azzimonti B, Della Valle C et al (2015) Biofilm formation on titanium implants counteracted by grafting gallium and silver ions. J Biomed Mater Res A 103:1176–1187. doi:10.1002/jbm.a.35270
34. Muzzarelli R, Tarsi R, Filippini O et al (1990) Antimicrobial properties of *N*-carboxybutyl chitosan. Antimicrob Agents Chemother 34:2019–2023
35. Bieser AM, Tiller JC (2011) Mechanistic considerations on contact-active antimicrobial surfaces with controlled functional group densities. Macromol Biosci 11:526–534. doi:10.1002/mabi.201000398
36. Friedman A, Friedman J (2009) New biomaterials for the sustained release of nitric oxide: past, present and future. Expert Opin Drug Deliv 6:1113–1122. doi:10.1517/17425240903196743
37. Brambilla E, Ionescu A, Gagliani M et al (2012) Biofilm formation on composite resins for dental restorations: an in situ study on the effect of chlorhexidine mouthrinses. Int J Artif Organs 35:792–799. doi:10.5301/ijao.5000165
38. Ferraris S, Venturello A, Miola M et al (2014) Antibacterial and bioactive nanostructured titanium surfaces for bone integration. Appl Surf Sci 311:279–291. doi:10.1016/j.apsusc.2014.05.056
39. Hajipour MJ, Fromm KM, Ashkarran AA et al (2012) Antibacterial properties of nanoparticles. Trends Biotechnol 30:499–511. doi:10.1016/j.tibtech.2012.06.004
40. Albers CE, Hofstetter W, Siebenrock KA et al (2013) In vitro cytotoxicity of silver nanoparticles on osteoblasts and osteoclasts at antibacterial concentrations. Nanotoxicology 7:30–36. doi:10.3109/17435390.2011.626538
41. Sungurtekin-Ekci E, Ozdemir-Ozenen D, Duman S et al (2014) Antibacterial surface properties of various fluoride-releasing restorative materials in vitro. J Appl Biomater Funct Mater. doi:10.5301/jabfm.5000212
42. Van Staden AD, Dicks LMT (2012) Calcium orthophosphate-based bone cements (CPCs): applications, antibiotic release and alternatives to antibiotics. J Appl Biomater Funct Mater 10:2–11. doi:10.5301/JABFM.2012.9279
43. Artini M, Papa R, Scoarughi GL et al (2013) Comparison of the action of different proteases on virulence properties related to the staphylococcal surface. J Appl Microbiol 114:266–277. doi:10.1111/jam.12038
44. Donelli G, Francolini I, Romoli D et al (2007) Synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth on polyurethanes. Antimicrob Agents Chemother 51:2733–2740. doi:10.1128/AAC.01249-06
45. Phee A, Bondy-Denomy J, Kishen A et al (2013) Efficacy of bacteriophage treatment on *Pseudomonas aeruginosa* biofilms. J Endod 39:364–369. doi:10.1016/j.joen.2012.10.023
46. Bai H, You Y, Yan H et al (2012) Antisense inhibition of gene expression and growth in gram-negative bacteria by cell-penetrating peptide conjugates of peptide nucleic acids targeted to rpoD gene. Biomaterials 33:659–667. doi:10.1016/j.biomaterials.2011.09.075

47. Li B, Jiang B, Boyce BM, Lindsey BA (2009) Multilayer polypeptide nanoscale coatings incorporating IL-12 for the prevention of biomedical device-associated infections. Biomaterials 30:2552–2558. doi:10.1016/j.biomaterials.2009.01.042
48. Israelachvili JN (2011) Intermolecular and surface forces, rev 3rd edn. Academic Press, Elsevier, Waltham, Massachusetts, United States
49. Cochis A, Fracchia L, Martinotti MG, Rimondini L (2012) Biosurfactants prevent in vitro *Candida albicans* biofilm formation on resins and silicon materials for prosthetic devices. Oral Surg Oral Med Oral Pathol Oral Radiol 113:755–761. doi:10.1016/j.oooo.2011.11.004
50. Jahn P, Beutner K, Langer G (2012) Types of indwelling urinary catheters for long-term bladder drainage in adults. Cochrane Database Syst Rev 10:CD004997. doi:10.1002/14651858.CD004997.pub3