Trends in Microbiology

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Review

Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments

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Biofilms are microbial communities embedded within an extracellular matrix, forming a highly organized structure that causes many human infections. Dental caries (tooth decay) is a polymicrobial biofilm disease driven by the diet and microbiota–matrix interactions that occur on a solid surface. Sugars fuel the emergence of pathogens, the assembly of the matrix, and the acidification of the biofilm microenvironment, promoting ecological changes and concerted multispecies efforts that are conducive to acid damage of the mineralized tooth tissue. Here, we discuss recent advances in the role of the biofilm matrix and interactions between opportunistic pathogens and commensals in the pathogenesis of dental caries. In addition, we highlight the importance of matrix-producing organisms in fostering a pathogenic habitat where interspecies competition and synergies occur to drive the disease process, which could have implications to other infections associated with polymicrobial biofilms.

Oral Biofilms: More than Just the Sum of Their Bacterial Parts

Many infectious diseases are caused or exacerbated by biofilms [1,2]. Oral infectious diseases are prime examples of the consequences of dynamic interactions between microorganisms, their host, and the host's diet, leading to microbial colonization of oral surfaces and the establishment of pathogenic biofilms (or dental plaque) [3]. Biofilms are defined as structured communities of microorganisms that are attached to a surface and enmeshed in an extracellular polymeric matrix [1,4]. Advances in DNA- and RNA-sequencing technologies are revealing important information about the diversity in composition, genome content, and behaviors of the biofilm microbiota at different oral sites (Box 1). In parallel, the knowledge about the biological impacts of the extracellular matrix in governing cell–cell interactions, creating microenvironments, and modifying the virulence of the biofilms continues to augment our understanding of the pathogenesis of infectious diseases [1,5].

There are many factors that affect the composition of the microbiota found on various surfaces of the mouth, especially when teeth begin to erupt, providing novel, nonshedding surfaces for colonization by commensals and opportunistic **pathogens** (see Glossary). These include, but are not limited to, age, diet, oral hygiene, systemic and immune conditions, and the use of certain medications that induce, for example, hyposalivation. The critical role that diet plays in microbial colonization is well illustrated in patients or experimental animals [6,7]. When hosts are

Trends

New knowledge on biofilm matrix biology and polymicrobial composition has indicated the importance of the local microenvironment where pathogens and commensals interact.

Opportunistic oral pathogens evolved in intimate association with not only the human host and resident microbiota, but also with a constantly changing diet to enhance virulence potential of the biofilm.

Although the oral microbiota and its function should be viewed as a whole, the matrix which provides the spatial, physical, and chemical environment is equally important.

In polymicrobial communities, matrix-producing pathogens can be considered 'biofilm environment conditioners' that help to build up a pathological habitat on biotic and abiotic surfaces.

Understanding of functional interactions between matrix components and the local microbiome could lead to new approaches to prevent polymicrobial biofilm-associated infections.

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Box 1. The Microbiomes and Oral Diseases: The Need for Mechanistic Studies

Defining the 'microbiome' in the oral cavity may be far more complex and challenging than initially envisioned, which, in addition to many bacterial species (bacteriome), also harbors fungi (mycobiome), viruses (virome), and even ultrasmall organisms (candidate phyla radiation group) [69]. The total surface of the adult mouth is ~215 cm^2 of which 20% is comprised of tooth surfaces, 50% keratinized, and 30% non-keratinized epithelium. The surface topography, including different areas of teeth, fissures in the tongue, and gingival crevices, provide unique retention sites for microbes, which are continually bathed by saliva. Microbiome-profiling has revealed a remarkable variety of species in different oral biofilm communities, allowing researchers to make associations between microbial composition and health status. Yet, it remains uncertain whether these 'microbiome snap-shots' demonstrate 'causation or correlation' with disease or health. Sometimes, a particular bacterial group or distinctive species may merely be bystanders or opportunistic colonizers with little or no contribution to the onset or severity of disease. In-depth mechanistic studies are required to precisely define the role of microbial components in the pathological process by omitting specific taxa from reconstituted communities, or by assessing the pathogenic potential of specific interacting species using *in vivo* models. For example, robust molecular and *in vivo* studies have helped to establish periodontitis as an exemplar of polymicrobial synergy and dysbiosis (PSD) on the basis of animal model mechanistic studies and human periodontal metagenomics/metatranscriptomic data [16]. A similar PSD concept, sometimes overlapping with ecological models, has been proposed to explain the etiopathogenesis of dental caries, where microbial community members might play roles comparable to those determined in periodontitis (keystones, accessory pathogens/pathobionts). This hypothesis certainly merits rigorous investigation as research in this direction has thus far been, at best, descriptive lacking mechanistic understanding. However, there are indeed fundamental differences. The diet (sugars) is a major driving force for the development of cariogenic biofilm communities. The biofilm also harbors abundant extracellular polysaccharides that enmesh the microorganisms, forming a diffusion-modifying matrix that profoundly changes the chemical and physical microenvironment on a nonshedding surface (instead of soft tissues with localized immune responses). These distinct factors raise the question whether caries can be characterized using the PSD/keystone model. Nevertheless, the available data point towards the assembly of a localized 'pathogenic habitat' via matrix production, where polymicrobial interactions between pathogens and commensals occur within highly structured biofilms to modulate the disease process. As much as departing from reductionism to holism created great strides to understand the complexity of oral microbiomes, a return to a reductionist approach will be essential to understand the pathogenic mechanisms, as well as to validate the emerging concepts in longitudinal clinical studies.

overexposed to dietary sugars, the structure and composition of biofilms formed on teeth changes significantly and the residing microbial communities become highly fit to metabolize carbohydrates and produce acids leading to **dental caries** [8,9]. Sequencing of ancient dental plaque provides additional evidence that shifts in oral microbiota are associated with dietary changes [10]. For example, *Streptococcus mutans* (a cariogenic bacterium) was not detected in plaque samples from Neolithic or Mesolithic fossils [10]. However, only when humans adopted agriculture, with a sugar-rich diet, did *S. mutans* begin to appear in the fossil record and became more prevalent as refined sugars in the diet continually increased, as did the incidence of dental caries [10].

Although early studies focused on microbial composition of biofilms, it is now clear that microorganisms residing within biofilms are embedded in a matrix containing **extracellular polymeric substances (EPS)**. The importance of the matrix in the collective microbial behavior and virulence, as well as for tolerance of antimicrobials, is being increasingly recognized and considered integral to the biofilm lifestyle [1,2,4]. EPS production directly mediates microbial adherence to a surface and cell-to-cell adhesion, while forming a polymeric matrix that enhances mechanical stability of biofilms. Furthermore, the diffusion-modifying properties of EPS matrix cause chemical/nutrient gradients to form, thereby creating microenvironments within biofilms that can vary widely from other sites in key environmental inputs known to affect microbial behaviors, including pH, redox, and nutrient availability. Thus, the matrix allows the cells to organize into cohesive multicellular ecosystems where cooperative and antagonistic interactions occur within a heterogeneous chemical and physical milieu [1], helping to create localized niches with differing pathogenic potentials.

As the science of microbiomics and matrix biology evolved, it became clear that polymicrobial interactions and the local biofilm microenvironment play instrumental roles in modulating health and disease conditions [11–13]. Conversely, decades of research have clearly demonstrated

that pathogens have developed an arsenal of mechanisms to enhance the virulence potential of the biofilm [14–16]. Changes experienced by the host (e.g., increased consumption of sugars or alterations in immune responses) can trigger pathogens to reshape the local microenvironment and the microbial community. Still, little is known about how pathogens modify the spatial–temporal organization and communal behavior to create localized pathological niches. Furthermore, how commensals and pathogens coexist and battle with one another within a biofilm matrix to mediate the disease process remains poorly defined. Here, we review and provide new perspectives about the role of pathogens based on current knowledge of matrix biology and the data accumulated from microbiome studies using dental caries as an exemplar. Recognizing that opportunistic pathogens evolved in intimate association with not only the human host and resident microbiota, but also with a constantly changing diet, we focus on their collective impact on the plaque microbiota and its virulence potential. We hope that this article will stimulate new dialogue, hypotheses, and approaches that establish a more robust framework to understand and resolve a persistent and costly oral disease, while concurrently providing new insights to other polymicrobial biofilm-associated infections.

The Pathogens and Dietary Sugars: Modulating Biofilm Virulence

Tooth surfaces are coated with a proteinaceous film known as acquired enamel pellicle, which is derived from host and microbial sources, such as salivary proteins and bacterial exoenzymes. Within the oral microbiome, a small group of bacteria (such as streptococci and *Actinomyces* spp.) are known to adhere to pellicle-coated surfaces via combinations of highly specific adhesin–receptor interactions augmented by hydrophobic or electrostatic forces, followed by coadhesion events that drive microbial colonization and biofilm initiation on teeth [17,18]. During this process the various bacterial species interact physically and metabolically to shape the initial biofilm community structure. Certain microbial interactions are beneficial as they interfere with caries pathogens, whereas others can function in synergy with cariogenic bacteria to accelerate the disease progression. The dynamic balance between commensals and opportunistic pathogens can be disrupted by frequent sugar consumption and poor oral care, which promotes the development of virulent biofilms in close proximity to the tooth surface.

Dental caries is a classic biofilm-induced disease that causes the destruction of the mineralized tooth tissue [8,9,13]. The microorganisms in the oral cavity are required, but not sufficient, to cause dental caries because the formation of cariogenic biofilms is dependent on the host diet [8,9]. A diet rich in sugar promotes the assembly of EPS matrix and enhances accumulation of acidogenic and acid-tolerant microbiota, which can explain microscopic images of plaque-biofilms collected from caries-active sites, revealing bacteria enmeshed in EPS (Figure 1). *S. mutans,* a member of the mutans streptococci (MS) group, has long been implicated with dental caries in humans. Extensive clinical, epidemiological, and experimental animal studies have shown, conclusively, though not exclusively, that MS are strongly associated with the disease, especially in **early childhood caries (ECC)** [19]. One of the primary adaptations that allow *S. mutans* to become such an efficient opportunistic pathogen within the oral microbiota resides with its exceptional capacity to utilize a wide variety of carbohydrates to produce EPS and acids, and to live a biofilm lifestyle, including stress resistance and **bacterial competence** mechanisms (Box 2). One sugar in particular, sucrose, the ordinary table sugar and the historical sweetener in cooking, is most cariogenic as the component hexoses (glucose and fructose) provide the building blocks of EPS and are efficiently fermented to produce acids. Sucrose is essential to the organism's success as a pathogen because of the unique ability of *S. mutans* to convert sucrose to extracellular insoluble glucans that enhance bacterial adhesion–cohesion, and form the core of the EPS matrix [20]. *S. mutans* clearly does not act alone to cause dental caries [21]. Rather, it interacts with other organisms in a dynamic and concerted polymicrobial effort to assemble a cariogenic biofilm.

Glossary

Bacterial competence: the ability of bacterial cells to take up foreign genetic material (DNA) from the environment.

Dental caries: a polymicrobial- and diet-dependent disease that is characterized by the development of acidogenic biofilms (or dental plaque) that cause demineralization of the enamel surface over time, eventually leading to the clinical onset of cavitation or tooth decay.

Early childhood caries (ECC): a virulent type of dental caries that disproportionately afflicts underprivileged preschool children. The onset and progression of ECC is aggressive, leading to rampant tooth destruction that are painful and recurring, which may require total oral rehabilitation under general anesthesia if left untreated.

Ecological plaque hypothesis: a model that encompasses microbiological, biochemical, and ecological properties of biofilms associated with oral diseases. Host-derived changes (e.g., frequent dietary sugar exposure) trigger biofilm environmental acidification and selection of an acidogenic-aciduric microbiota, resulting in net mineral loss and the onset of dental caries.

eDNA: extracellular DNA material that can be either actively secreted or result from cell lysis. eDNA can bind to components of other extracellular polymeric substances, contributing to the biofilm matrix structural organization while also serving as a nutrient source for resident microbes.

Emergent properties: novel structures, activities, patterns, and properties that arise during the process of self-organization in complex systems, which in the biofilm context include surface adhesion–cohesion, spatial organization, physical and social interactions, chemical heterogeneity, and increased tolerance to antimicrobials.

Extracellular polymeric substances (EPS): extracellular biomolecules, often termed EPS, that form the biofilm matrix. EPS can be exopolysaccharides, fibrous and globular proteins (including extracellular enzymes), lipids, and nucleic acids/eDNA, which can be microbial surface-associated,

Over the years, the **ecological plaque hypothesis**, whereby microbial community shifts occur due to environmental acidification caused by sugar metabolism, has provided a logical and a tractable model for studying polymicrobial communities inhabiting the oral cavity [3]. However, an often forgotten question arises as to how localized acidification within biofilms and resultant caries on tooth surface occurs in the presence of powerful buffering saliva and the slightly alkaline environment of the human mouth. One explanation is that saliva cannot gain access to the acid produced in the depths of plaque formed on teeth, while 'the fuel' (dietary sugars) can readily diffuse throughout the biofilm [13,22,23]. The presence of an EPS matrix with diffusion-modifying properties plays an important role in creating the pathological (acidic) microenvironment adjacent to the tooth surface. Thus, dental caries, though undoubtedly a result of polymicrobial acidogenesis, can be better understood conceptually as a pathological process that relies not only on microbial composition and metabolic activity but also on the milieu within which the organisms interact and acids accumulate. In this regard, *S. mutans* can play a key role in biofilm matrix assembly as the main producer of insoluble glucans among oral bacteria (Box 2), 'resetting' the microenvironment for other aciduric-cariogenic bacteria to thrive and become established, possibly at the expense of *S. mutans* itself, consistent with the variable levels of the bacterium in human plaque (<1–30%) as the disease progresses [21,24,25].

The EPS Matrix: Assembling a Complex Biochemical Microenvironment

Recent advances in the understanding of the EPS matrix biology have revealed a 'multifunctional scaffold' essential for the biofilm lifestyle [1,13]. The structural and biochemical properties of the matrix provide the **emergent properties** of biofilms, including surface adhesion, spatial and chemical heterogeneities, synergistic/competitive interactions, and increased tolerance to antimicrobials [1] (Figure 2). The formation of EPS matrix depends on substrate availability, the synthesis and secretion of extracellular materials, shear and other stresses. The major matrix components in oral biofilms associated with dental caries are polysaccharides, particularly *S. mutans*-derived glucans [20]. Furthermore, soluble glucans and fructans produced by other

Box 2. *Streptococcus mutans* Biofilm Lifestyle: An Avid Sugar Consumer and EPS/Acid Producer

Streptococcus mutans and dental caries became more prevalent after dietary shifts of the Neolithic and Industrial revolutions, which were characterized by the introduction of sugar-rich diets [10]. Coevolution of *S. mutans* with the increased sugar consumption largely explains how well adapted *S. mutans* is to colonize and thrive on teeth when its human host ingests sugars. *S. mutans* has at least seven enzymes that hydrolyze sucrose, some yielding polymers and free glucose or fructose, and one that cleaves the sucrose-6-phosphate generated by sucrose transporters. *S. mutans* secretes multiple exoenzymes, particularly glucosyltransferases (Gtfs), which are able to cleave sucrose to produce extracellular glucans (homopolymers of glucose) and free fructose. The glucans are key constitutes of the matrix in cariogenic biofilms, and are associated with enhanced virulence in experimental animals and in humans. The Gtfs make a polymer that is adhesive, relatively water-insoluble, and rich in (α1,3) linkages, with (α1,6) branching, which, in turn, are important for biofilm scaffolding and stability. While there are numerous oral microbial species, most of them are incapable of producing insoluble glucans until they are coated by secreted Gtfs. Interestingly, the ratio of 1,3-to-1,6 linkages increases by the action of secreted dextranases attacking the (α1,6) chains as glucans are produced [20]. However, no oral bacteria have yet been found that can hydrolyze the (α1,3) bonds, including *S. mutans*. Expression of Gtfs and the production of a glucan-matrix are dependent on environmental cues, including pH and carbohydrate source, involving signaling, such as the second messenger c-di-AMP and possibly unique small noncoding RNAs called microRNAs [70–72]. The discovery of the latter may provide new understanding of the matrix biology and regulation of biofilm formation. How *S. mutans* modulates this integrative regulatory network to respond to environmental challenges and coordinate expression of genes required for matrix synthesis remains poorly defined. Furthermore, all isolates of *S. mutans* harbor high-affinity, high-capacity transport systems for a variety of carbohydrates, which are primarily mediated by the sugar:phosphotransferase system (PTS). The PTS can rapidly internalize mono- and disaccharides, including glucose, mannose, and fructose among others, which can be further metabolized into acids. When sugar is present in excess, the cells produce mostly lactic acid via a lactate dehydrogenase. In low-carbohydrate conditions, a pyruvate-formate lyase funnels the pyruvate to acetate and formate, gaining an additional ATP, compared to the lactate pathway [73]. By efficiently utilizing sucrose, *S. mutans* can orchestrate EPS production to help bacteria accumulate and form a matrix. In turn, the adherent bacteria rapidly transport and efficiently metabolize different sugars to produce acids via concerted actions of transporters and catabolic enzymes. These properties (with acid-tolerance) allow *S. mutans* to build up, interact with, and live in an acidified habitat that becomes a hallmark of cariogenic biofilms.

secreted extracellularly, or deposited on abiotic and biotic surfaces.
F_1F_0-ATPase: a membrane-associated proton-translocating enzyme that is key for bacterial acid tolerance, particularly in *Streptococcus mutans*. During glycolysis, protons are pumped out by F-ATPase to help maintain ΔpH across the cell membrane, preventing acidification of the cytoplasm, which would typically inhibit intracellular enzymes.
Pathogen: any organism that supports or enhances the virulence potential of the biofilm to cause infectious diseases.

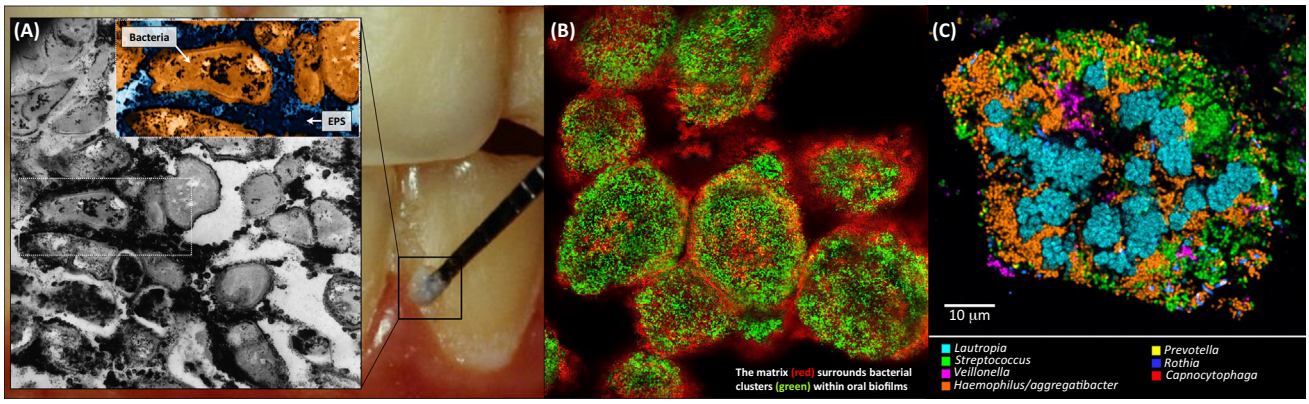

Figure 1. Dental Plaque Architecture: The EPS Matrix, Spatial Organization, and Polymicrobial Composition. (A) Plaque biofilm from a caries-active subject (photo courtesy of Dr Jaime A. Cury): microscopic image (inset) of plaque-biofilm showing a selected area containing bacterial cells (highlighted in orange) enmeshed in EPS (in dark blue); the image was pseudo-colored using Adobe Photoshop software for visualization purposes (adapted from [19]). (B) Bacterial clusters (green) surrounded by EPS matrix (red) detected in mature mixed-species oral biofilms formed in sucrose (adapted from [5]). (C) Spatial organization of human dental plaque showing multiple clusters of varying sizes containing different microbial species (adapted from [31]). Abbreviation: EPS, extracellular polymeric substances.

species (e.g., *Actinomyces, Streptococcus salivarius*, and *Streptococcus gordonii*) and hybrid starch-glucans are also present. Like other matrices, cariogenic biofilms also appear to contain **eDNA** [26], bacterially-derived proteins that possess amyloid-like properties [27], as well as host proteins and glycoproteins, that can contribute to the matrix scaffold, often in association with glucans (e.g., eDNA–glucan complexes) [28]. However, the function and structural organization of these other exopolymers in the matrix remain poorly understood and further studies are required to elucidate their role in the microbial composition and cariogenic potential of the biofilm. Glucans are comprised of glucose moieties linked primarily by α1,3 and α1,6 glycosidic bonds produced by a concerted action of streptococcal exoenzymes termed glucosyltransferases (Gtfs) [20,29]. Intriguingly, Gtf enzymes released extracellularly can bind to the tooth surface in active form, producing glucans *in situ* that provide novel bacterial binding sites. Furthermore, secreted Gtfs also bind to other oral microbes (commensal streptococci, actinomyces, lactobacilli, and even *Candida albicans*), thereby converting them into glucan producers. The EPS glucans formed on surrogate surfaces enhance microbial accumulation on teeth, while forming new interspecies interactions and increasing cell–cell cohesion. Using direct incorporation of fluorescent probes during the synthesis of glucans by Gtfs, a detailed portrait of the spatio–temporal order of EPS-matrix assembly and its spatial arrangement with bacterial cells in mixed-species biofilms has emerged [30]. These extracellular polymers accumulate at a different location on the apatitic surface and on the cell membrane, each with complementary roles to form a nascent EPS matrix and coordinate biofilm development, including surface adherence, cell–cell adhesion, and formation of cell clusters similar to micro-colonies found in other biofilm systems [4]. As the biofilm matures, the continued EPS production *in situ* expands the matrix tri-dimensionally, encasing cells clusters, and creating a bridge from one to another forming a highly compartmentalized yet cohesive structure within a 3D matrix scaffold. Such spatial organization and heterogeneities shaped by EPS synthesis could explain the detection of different microbial clusters varying in size and composition found in human oral biofilms [31,32] (Figure 1).

EPS deposition on surfaces and development into a polymeric matrix also affect the mechanical properties of biofilms, such as increasing adhesive strength to surfaces and cohesiveness [33]. Well established biofilms are mechanically difficult to remove from tooth surfaces and often

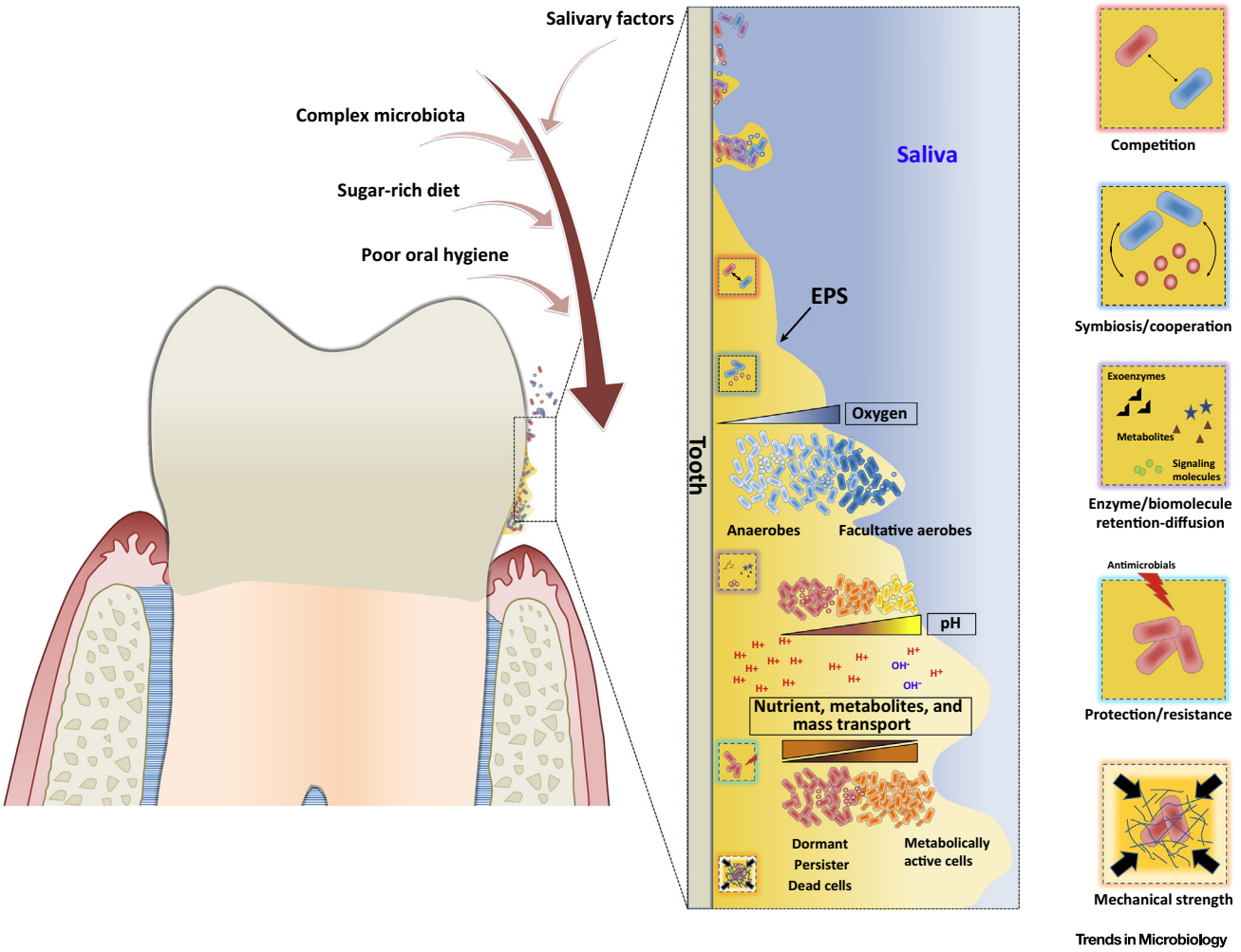

Figure 2. The Biofilm Properties: Assembling a Complex Microenvironment. In the oral cavity, a diet rich in sugar, particularly sucrose, provide a substrate for the production of extracellular polysaccharides, which form the core of the extracellular matrix in cariogenic biofilms. The matrix drastically changes the physical and biological properties of the biofilm. The exopolysaccharides enhance microbial adhesion–cohesion and accumulation on the tooth surface, while forming a polymeric matrix that embeds the cells. The matrix provides a multifunctional scaffold for structured organization and stability of the biofilm's microbial community, where microorganisms coexist and compete with each other. The diffusion-modifying properties of the matrix, combined with the metabolic activities of embedded organisms, help to create a variety of chemical microenvironments, including localized gradients of oxygen and pH. Furthermore, the matrix can trap or sequester a diverse range of substances, including nutrients, metabolites, and quorum-sensing molecules. Similarly, enzymes can be retained and stabilized, transforming the matrix into a *de facto* external digestive system [1]. These properties provide the distinctive characteristics of the biofilm lifestyle, including mechanical stability, spatial and chemical heterogeneity, and drug tolerance [1].

display enhanced viscoelasticity, which can help them persist by partially yielding rather than detaching when subjected to fluid shear stresses. However, the EPS matrix can be constantly remodeled locally through the actions of dextranases, DNAses, and proteolytic enzymes dynamically altering the mechanical properties and/or exposing new binding sites to additional microorganisms that were unavailable during biofilm initiation [20]. Indeed, matrix stiffness appears to increase as the biofilm matures. At later stages, mature biofilms can release small aggregates or even individual cells (a process termed dispersal), often through matrix degradation, to seed uncolonized sites and reinitiate the biofilm life cycle [34]. The physicochemical properties of the biofilm matrix can also provide protection to embedded bacteria by reducing drug access and triggering antimicrobial tolerance. For example, the EPS can bind cationic antimicrobials, such as chlorhexidine and antimicrobial peptides, preventing penetration into the deeper layers of the biofilm, and thereby reducing killing efficacy [30,35].

The formation of chemical and nutritional 'gradients' within most biofilms involves a balance between the matrix acting as a physical barrier (sequestering molecules or affecting diffusion of substances in and out of the biofilms) and local microbial consumption/metabolism (Figure 2). These processes can create numerous tailored biological niches with varying concentrations of pH, O_2, inorganic ions, signaling molecules, metabolites, and other solutes [1,2,4]. Thus, the positioning of microorganisms and their stress response mechanisms may correlate with their susceptibility to, or affinity for, low pH or hypoxic environments and availability of specific ligands, nutrients, or biomolecules. Such a heterogeneous milieu can modulate gene expression locally and influence the metabolic exchange and intercellular signaling among different species or between different clusters of cells distributed within the biofilm structure, orchestrating their communal 'social behavior', spatial organization, and/or physiological heterogeneity [1,2,4,31,32] to develop pathogenic niches. Recent studies have shown that bacterial aggregates can create pH or O_2 microgradients and induce transcriptomic changes in a subpopulation of cells within the biofilm [22,36,37].

In the context of dental caries, how and where acidic microenvironments are formed, maintained, and protected within the 3D biofilm architecture may be the key determinant because the buffering saliva surrounding the tooth surfaces is capable of neutralizing acids produced in the mouth. The heterogeneous spatial distribution of pH across oral biofilm structure has been long appreciated [13]. A fluorescent pH probe, directly immobilized in the biofilm matrix, revealed a fascinating 3D pH distribution within intact biofilms despite exposure to neutral pH buffer [30]. Localized regions of low pH values (4.5–5.5) throughout the biofilm structure and at the biofilm–apatite interface suggest that the acids accumulated and confined in these specific areas are not readily neutralized. The EPS matrix has been shown to limit diffusion of charged ions in buffers, whereas uncharged solutes, such as sucrose, can diffuse into biofilms and can be readily metabolized into acids by the embedded bacteria [22]. Furthermore, extracellular glucans appear to directly trap protons to help retain and accumulate acids within biofilms [23]. The biofilm matrix can also act as an external digestion system by immobilizing exoenzymes, allowing them to metabolize substrates in close proximity to cells while also participating in matrix remodeling. For instance, soluble fructans and glucans present in the matrix can be degraded by fructanase and dextranase, providing readily fermentable carbohydrates on-site and thereby extending the duration of the acid challenge [20]. Thus, EPS can modulate persistent acidification at the tooth interface by helping biofilms to adhere, spatially localize metabolites, and possibly restrict access to buffering saliva, which helps to create a cariogenic microenvironment.

Biofilm Microbiota: Pathogens, Commensals, and Interspecies Interactions

Commensals have a significant advantage over cariogenic pathogens when the host's diet is not rich in fermentable carbohydrates, particularly sucrose. Many commensal bacteria associated with oral health can adhere more avidly to saliva-coated tooth surfaces, can grow substantially better than *S. mutans* and many other aciduric species, and have multiple mechanisms to interfere with their establishment and growth. However, the 'microbial battlefield' can change dramatically if sugar is supplied frequently, promoting EPS matrix synthesis, acid production, and the creation of localized acidic microenvironments, where *S. mutans* can work synergistically with other aciduric species and cause ecological changes to shape the biofilm community, structure, and metabolism conducive to caries development (Figure 3).

Competition/Antagonism

The inverse association between the abundance of commensals with mutans streptococci has been well documented, indicating that these organisms can control the growth of cariogenic bacteria. The simplest mechanism involves the production of basic (alkaline) compounds that

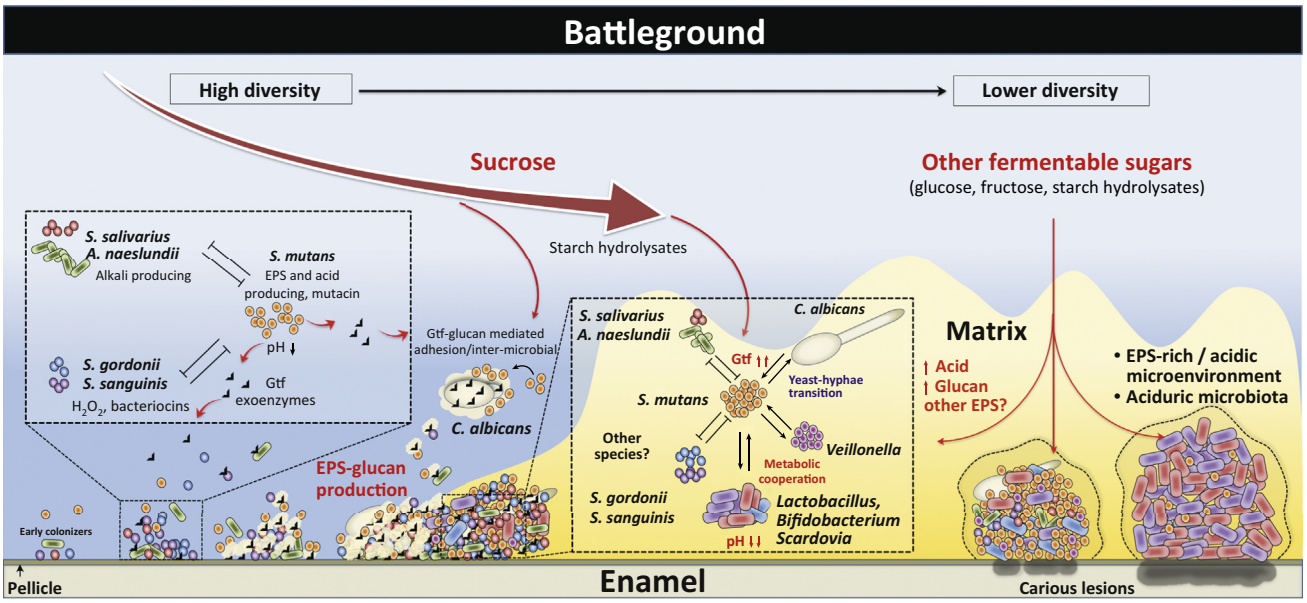

Figure 3. The Biofilm Battleground: Antagonistic, Synergistic, and Mutualistic Interactions to Build a Pathogenic Habitat. The social interactions of the oral microbial community start with early colonizers that can rapidly adhere to the tooth surface, and then coadhere with other microorganisms. During this process the various species interact physically and metabolically to shape the initial biofilm community. The interactions can be both antagonistic and cooperative, which can dynamically change depending on the host. Certain interactions are beneficial as *Streptococcus gordonii/Streptococcus salivarius* and *Actinomyces naeslundii* interfere with caries pathogens such as *Streptococcus mutans* by secreting bacteriocins and hydrogen peroxide as chemical weapons or counter the deleterious effects of acidification by producing alkali. However, the balance between commensals and pathogens can be disrupted by frequent sugar consumption and poor oral care. When sucrose is available, EPS-producing exoenzymes such as Gtfs, present in the pellicle and also bound to different microbes (including *Candida albicans*), produce copious amounts of glucans. Furthermore, the Gtfs can also use starch hydrolysates (from α-amylase activity on pellicle and bacterial surfaces) to produce hybrid glucan polymers. The surface-formed glucans provide novel binding sites for adhesion and coadhesion, which mediate new interspecies interactions and microbial clustering on the tooth surface, while assembling a polymeric matrix that provides protection and mechanical stability, making the biofilm recalcitrant to antimicrobials and difficult to remove. Competitive and synergistic interactions continue to develop between the microbes embedded in the biofilm structure. If the biofilm remains on teeth, and the consumption of carbohydrate-rich diets persists, the amount of EPS and the extent of acidification of the matrix increases. The diffusion-modifying properties of the matrix, combined with microbial metabolic activities, help to create a highly acidic and increasingly anaerobic (hypoxic) niche. Such conditions elicit biochemical, ecological, and structural changes that favor the survival and dominance of highly acid-stress-tolerant organisms that can synergize with each other. The microbial diversity can further reduce in favor of an aciduric microbiota, helping to maintain an acidified microenvironment. The low-pH condition at the tooth–biofilm interface promotes demineralization of the enamel, leading to the onset and progression of dental caries. This model may explain the rapid accumulation of cariogenic plaque in the presence of sucrose (and other fermentable sugars) in the diet, even if the initial population of pathogens, such as *S. mutans*, is numerically low. Abbreviations: EPS, extracellular polymeric substances; *S. sanguinis, Streptococcus sanguinis*.

maintain pH values near neutrality, allowing commensals to outcompete *S. mutans* and acid-tolerant organisms that are able to grow and dominate at lower pH conditions. The two prevailing pathways for alkali production are urea metabolism by bacterial ureases, mainly *S. salivarius*, certain *Actinomyces* species, and a few oral haemophili [38]. Urea hydrolysis yields ammonia and CO_2. While the CO_2 can provide some modest buffer capacity, ammonia can rapidly equilibrate with a proton to yield NH_4^+, raising the pH and providing the bacteria with a source of nitrogen. Arginine can be metabolized by numerous oral bacteria via various pathways. A dominant route for arginine catabolism by oral commensals is the arginine deiminase system (ADS), which metabolizes internalized arginine yielding ornithine, ammonia, CO_2, and ATP. Urea and arginine metabolism not only results in alkalinization of oral biofilms, preventing demineralization and promoting remineralization, it also provides bioenergetic advantages to the organisms that harbor ureases and the ADS. This can provide ecological advantages to commensals and deter the growth of caries pathogens, promoting the development of healthy oral biofilms.

In addition to adhesion and alkali generation, many commensals have more 'active measures' to interfere with the growth and biological processes of caries pathogens, and *S. mutans* in particular. The production of H_2O_2 via pyruvate oxidase and other enzymes, and the secretion of bacteriocins and other antimicrobial compounds by commensals, are important 'chemical weapons' that are inhibitory to the growth of *S. mutans* [39]. Interestingly, *S. mutans* can 'counter-attack' by releasing mutacins (lantibiotic and non-lantibiotic peptide antibiotics), which are effective against a variety of commensals. However, certain commensal streptococci (e.g., *S. gordonii*) have the ability to interfere with intercellular signaling systems required for the production of mutacin [40]. In particular, a recent clinical isolate, designated as *Streptococcus* A12, produces a protease (similar to *S. gordonii* challisin) that breaks down competence-stimulating peptide (CSP), which activates mutacin production by *S. mutans* via a two-component signal transduction systems [41]. Alkali-generating or bacteriocin/toxin-producing bacteria within biofilms can influence interspecies interactions, local pH, and microenvironment dynamics [42].

The development of dental caries is also intertwined intimately with stress tolerance by caries pathogens. In addition to the aforementioned competitive interactions with commensals, coping with a large influx of carbohydrates in the diet by rapidly shifting metabolism can induce a variety of stress pathways. Perhaps, though, the most significant stress under cariogenic conditions is acid. The *in situ* measurements of plaque pH following a carbohydrate challenge reveal that the pH can drop within 2–3 min to values as extreme as 4.0 and below [43]. This is a true 'acid shock' that would be intolerable to most health-associated commensals as they cease growth below 6 or 5.5, and are rapidly killed at pH 4.0 [44,45]. By contrast, *S. mutans*, lactobacilli and other aciduric bacteria can cope with such suddenly dropped pH by using various adaptive strategies. A primary determinant of acid tolerance in *S. mutans* is the proton-extruding **F_1F_0-ATPase** pump, which is highly active, has a much lower optimal pH than the same enzyme in commensal streptococci, and is produced in greater quantity when the organisms are challenged with low pH [45]. However, acid tolerance is a complex trait with many factors contributing to constitutional and adaptive acid resistance, including restructuring the bacterial membrane structure–composition. These mechanisms have been reviewed elsewhere [43,46].

Cooperation/Synergism

If the environmental acidic stress persists, other acidogenic and aciduric bacteria such as non-mutans streptococci, actinomyces, lactobacilli, bifidobacteria, and *Scardovia* species are detected, which can synergize to enhance acidification of the biofilm milieu as detailed in excellent in-depth review articles [8,11,47]. Cariogenic biofilms are acidic, hypoxic, but rich in carbohydrates, which creates an ideal microenvironment for opportunistic organisms like lactobacilli to grow and accelerate caries progression. Concomitantly, EPS production by *S. mutans* Gtfs is induced under acidic pH. *Lactobacillus casei*, frequently isolated from the cariogenic plaque with *S. mutans*, is known for its high capacity to produce and tolerate acid, although it has poor ability to colonize teeth. The presence of *S. mutans* and sugar exposure promotes colonization by certain lactobacilli [48] through Gtf binding and a glucan-mediated adhesion mechanism, increasing accumulation of both organisms within biofilms. Further, some *Lactobacillus reuteri* strains possess two different enzymes: 4,6-α-glucanotransferase that uses starches to synthesize α-glucan-type polysaccharides, and a typical glucansucrase that synthesizes α-glucans from sucrose [49]. In addition, the presence of amylase bound on bacterial (e.g., *S. gordonii, Streptococcus parasanguinis*) and tooth surfaces produce a variety of starch hydrolysates *in situ* that can be metabolized into acids and incorporated into glucans synthesized by Gtfs forming hybrid polymers [50–52]. Additional EPS produced by other oral bacteria may also contribute to biofilm matrix assembly, even if the proportion of *S. mutans* is declining as the biofilm matures and the caries lesion worsens. Thus, we speculate that

co-colonization with a diverse group of EPS-producing or EPS-modifying organisms can expand the substrate repertoire that allows oral bacteria to build complex EPS matrix with properties that can enhance caries development that glucans alone cannot achieve.

The creation of acidic microenvironments benefits not only acid producers and strongly acid-tolerant species [53] but also those organisms that use lactate as a carbon source, whereas other species that are acid sensitive, or that cannot metabolize the acids present in their surroundings, may perish. *Veillonella*, an obligate anaerobic Gram-negative bacterium, is considered a bridge organism in the oral biofilm [54]. *Veillonella* does not utilize carbohydrates as a source of energy; instead, it utilizes lactate. Such nutritional preference renders this organism dependent on streptococci that produce copious amounts of lactic acid. In fact, *Veillonella* often coaggregates with oral streptococci and, not surprisingly, it is often identified as a signature organism in caries-active subjects [55]. It has been long perceived that the presence of this bacterium in plaque neutralizes the acidic pH of the oral biofilm, thereby preventing enamel demineralization. However, evidence from microbiome studies demonstrates that both *Veillonella* and *S. mutans* are highly associated with caries lesions [56]. Acetate produced from *Veillonella* lactate catabolism can be damaging to enamel, while *Veillonella* promotes *S. mutans* growth despite the presence of antagonistic *S. gordonii in vitro* [57]. Conversely, some bacteria found in cariogenic biofilms do not fit the classical profile of being acidogenic–aciduric, such as *Prevotella and Atopobium* [58]. Whether they are just bystanders or play an active role in caries pathogenesis remains to be elucidated.

Intriguingly, results from several clinical studies reveal that the fungus *C. albicans* is frequently detected in higher numbers in plaque-biofilms from toddlers with ECC, as reviewed in [19]. In the mouth, *C. albicans* is known to form mixed microbial communities on soft-tissue and prosthetic surfaces, causing mucosal infections. However, *C. albicans* can coadhere with *S. mutans* and colonize tooth surfaces in the presence of sucrose [59,60]. Specifically, this cross-kingdom interaction appears to be largely mediated by *S. mutans*-derived Gtfs that bind avidly onto the *Candida* surface and produce large amounts of glucans on the fungal surface, boosting the ability of both microbes to form biofilm together while increasing the amount of EPS-matrix. Once together within biofilms, these organisms can cooperate by providing substrates/metabolites and growth-stimulating factors [61,62], while enhancing Gtfs production [60,62,63]. Using a rodent model, a synergistic enhancement of biofilm virulence was observed when *S. mutans* was coinfected with *C. albicans* and exposed to a sucrose-rich diet, leading to rampant caries on teeth similar to those found clinically in ECC [60]. Further investigation into how other metabolic pathways contribute to the symbiotic interactions and matrix production may offer additional insights into the disease process.

Dental caries is a highly dynamic pathological process where the host's diet fuels the assembly of virulent biofilms by promoting EPS matrix assembly and polymicrobial interactions that cause profound changes in the local environment. EPS-producing pathogens such as *S. mutans* appear to battle with commensal organisms and, when conditions are conducive, build-up a 'habitat' to work synergistically with other aciduric species to acidify the biofilm milieu and cause demineralization of the tooth surface. Yet, it is indeed difficult to fully assess the absolute contribution of many species to the caries process because of the spatial/chemical heterogeneity, the continually changing microenvironments, and the fact that organisms could be beneficial or detrimental depending on the conditions and degree to which a lesion had progressed (e.g., proteolytic bacteria could accelerate the caries process in advanced lesions where dentin is exposed). Nevertheless, this evolving view of ecological battles and polymicrobial synergies within a heterogeneous yet structured environment has direct implications in

Box 3. Challenges and Therapeutic Opportunities for Polymicrobial Biofilm Oral Infections

Oral biofilms are one of the most complex polymicrobial communities found in nature, where ecological and dysbiosis principles have been applied to explain their ability to cause diseases. Multiple biofilm types are formed on mucosal, abiotic (e.g., implants and restorative materials), or tooth surfaces in the oral cavity, where interspecies and even cross-kingdom associations occur while interacting with host saliva, diet, and immunity. This unique milieu provides challenges for design and delivery of therapeutics, but also offers fertile ground for the development of innovative anti-biofilm strategies targeting host–microbe–diet interactions that could have broader implications. Saliva and the diet of the host play key roles in modulating biofilm structure, spatial organization, microenvironments, and the development of microbial communities. Sugar-rich diets fuel opportunistic pathogens such as *Streptococcus mutans* to create an acidic microenvironment protected by an EPS-rich matrix. Conversely, keystone pathogens such as *Porphyromonas gingivalis* inhabiting the subgingival environment modulate the host immune response and polymicrobial balance, resulting in a dysbiotic biofilm community that causes periodontitis. Conventional antimicrobial elimination of the pathogen has been proven difficult. The complexity of biofilm biology highlights the need for new therapeutic strategies to effectively control oral biofilms. Emerging technologies can modulate polymicrobial or host–microbe interactions. Community manipulation through depleting pathogens and favoring the growth of antagonizing commensal organisms could reduce the overall biofilm virulence. For example, L-arginine can be used for alkali production by arginolytic bacteria (e.g., *Streptococcus gordonii*), which can neutralize acids and modulate pH homeostasis within oral biofilms *in vivo* [74]. Similarly, probiotic approaches that increase the proportions of beneficial bacteria at the expense of pathogens may be another viable strategy to modulate the pathogenic potential of oral biofilms. Furthermore, species-specific targeting antimicrobial peptides or small molecules can selectively inhibit *S. mutans* from multispecies biofilms to promote a healthy microbiome *in vitro* [75,76]. Likewise, modulation of the immune response can limit destructive inflammation and deplete the source for dysbiosis in periodontitis [77]. However, multitargeted approaches may be needed to disrupt both the microbes and the surrounding matrix. New pH-sensitive multifunctional nanoparticles are capable of simultaneous EPS degradation and bacterial killing once activated by acidic pH values found within the cariogenic biofilm microenvironment [78,79]. Antimicrobial peptides combined with anti-EPS strategies may further increase the access and permeabilizing properties of the peptides once in the biofilm [35]. These targeted therapies may lead to more effective and precise oral biofilm control, while providing important therapeutic insights against other polymicrobial infections.

Outstanding Questions

In polymicrobial biofilm communities, how do diffusion-modifying matrix and compartmentalization modulate intra- and intercellular responses among pathogens, commensals, and other organisms to trigger the development of a pathogenic niche?

How do chemical and mechanical signals, and the microenvironment heterogeneity, impact spatial-temporally the ecological changes or polymicrobial synergies that enhance biofilm virulence potential?

Considering that oral bacteria display tremendous genomic and phenotypic heterogeneity, can a focus on core genome factors further advance our mechanistic understanding of the microbial etiology in polymicrobial diseases?

Whether the new organisms (and other matrix components) detected in the biofilm are just bystanders or play an active role in the disease process needs to be explored using relevant human microbiota and *in vivo* models.

Can a better understanding of the matrix biology and polymicrobial interactions aid the development of improved risk assessment and targeted therapeutic strategies?

understanding the pathogenic mechanisms of dental caries and in developing effective therapeutics (Box 3).

Concluding Remarks and Future Perspectives

The diet–microbe interactions in the oral cavity play a key role in determining the fate of the colonizing microbiota from birth. The available evidence clearly indicates that the pathogenesis of dental caries involves the assembly of an EPS matrix and synergistic multispecies efforts triggered by the host's dietary sugars that promote cariogenic biofilm development. Although the complex microbiota and its function should be viewed as a whole, the 'battleground' where the ecological battles and polymicrobial synergy ensue to drive the disease process is equally important (Figures 2 and 3). EPS enhances bacterial adhesion–cohesion and interspecies binding interactions, while forming a matrix that embeds the cells into spatially organized microbial clusters. The EPS matrix acts as a diffusion-controlling barrier by modulating the access of solutes to the interior and by trapping produced acids inside the biofilm, while also serving as an endogenous source for acid production in addition to creating oxygen gradients. The embedded microbes must then cope with a wide range of stresses (acidic, hypoxia) and large fluctuations in nutrient availability to persist in oral biofilms, a prerequisite for contributing to the onset of caries. Thus, the matrix helps cariogenic biofilms to 'cling' onto teeth, despite exposure to shear forces, and create an acidic pH microenvironment in spite of being 'bathed' by buffering saliva.

In this context, the primary role of *S. mutans* as a pathogen may reside with its exceptional ability to alter the local physicochemical environment by utilizing dietary sugar to assemble an insoluble polymeric matrix, thereby providing mechanical stability and protecting the acid milieus within which commensals may perish and more potent acidogenic/aciduric organisms flourish to become dominant, making the biofilm difficult to treat. We propose that EPS-producing pathogens can be considered 'biofilm environment conditioners' that help to build

up a pathological niche (habitat) within a complex microbial community (Figure 3). This concept can be integrated with current understanding about the biofilm matrix biology and microbiome-based data, which may have import to other polymicrobial infections.

Future studies need to elucidate the molecular and functional diversity of extracellular matrix and their relationship with spatial–temporal changes of the polymicrobial interactions during the transition between health and disease (see Outstanding Questions). Furthermore, it remains unclear how the EPS matrix provides structural scaffolds, and governs microbial clustering, positioning, and activity either directly or through generation of local microenvironments (niches). Matrix-mediated changes can modify cell–cell interactions, specifically between different microbial species. In turn, dynamic reciprocity between cells and matrix may provide a complex, interconnected molecular network governing cellular functionality at both single-cell and multicellular levels. Technological advances in microscopy and spectroscopy-based methods have provided impressive details about EPS secretion and interactions at single-polymer/protein precision or biofilm formation *in vivo* [5,64–66]. *In vivo*-like environments afforded by microfluidic devices/3D printing (e.g., organ-on-chip) may help to assess mechanisms of biofilm formation in conditions mimicking clinical situations. Finally, how host factors/genetics associated with saliva composition, tooth defects (e.g., hypoplasia) [67], immune response among others influence pathogenic biofilm development remain to be fully elucidated [68]. In-depth analysis of the structural and functional interaction between the many components of the biofilm matrix, the local microbiome, and host factors shall advance our understanding of the pathogenic mechanisms of oral and other polymicrobial diseases, and lead to precise targeted therapies to prevent or treat them.

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