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Pathogenesis of mucosal biofilm infections: challenges and progress

Anna Dongari-Bagtzoglou

Department of Oral Health Sciences, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA, Tel.: +1 860 679 4543, Fax: +1 860 679 1027, adongari@uchc.edu

Abstract

Living-tissue biofilms remained unrecognized until very recently, mainly as a result of traditional microbial sampling techniques or histologic processing, which disrupt the spatial organization of the tissue microorganisms. Thus, the biofilm nature of certain mucosal infections was frequently unintentionally missed or disregarded. To a large extent, the study of human tissue biofilms is still in its infancy. However, with the advent of newer methodologies, such as fluorescent *in situ* hybridization and endoscopic confocal laser scanning microscopy, which combine the identification of microbes with *in situ*, direct visualization of their relationships with each other and with their substratum, mucosal tissue biofilms are becoming easier to study and, thus, their role in human infections is becoming more apparent. This review summarizes the challenges in the study of tissue biofilms, proposes two inflammation-centered – albeit opposite – pathogenetic models of mucosal tissue biofilm infections and suggests directions for future research and novel therapeutic approaches.

Keywords

biofilm; extracellular matrix; inflammation; mucosal tissue

Mucosal biofilms are implicated in a wide variety of human infections

The term biofilm has been used to describe a well-organized microbial community enmeshed in a polymeric, carbohydrate-rich extracellular matrix (ECM) and adhering to an inanimate or living surface [1]. Most bacteria and fungi exist predominantly in such organized communities in nature and, according to a recent public announcement from the US NIH, trigger more than 80% of human soft- and hard-tissue infections [101]. Biofilm development proceeds in two main steps: first, microbial adhesion to a surface and, second, cell-to-cell adhesion with multicellular structure formation. These processes are, at least partly, controlled by quorum-sensing molecules, which are intercellular communication molecules produced by bacteria and fungi and are analogous to cytokines produced by mammalian cells [1]. The term ‘mucosal biofilm’ was coined by Garth Ehrlich [2,3] and denotes biofilms that grow on mucosal surfaces. Proposed clinical criteria for mucosal biofilm infections include: signs and symptoms of infection in otherwise culture-negative patients, chronicity or recurrence with periodic exacerbations and remissions, and minimal or no response to antimicrobials [4].

Pulmonary infection by *Pseudomonas aeruginosa*, which afflicts people with cystic fibrosis, is the prototype of a mucosal biofilm [5]. Once this biofilm develops, *P. aeruginosa* colonizes the airway indefinitely. Mucosal biofilms have also been identified in the gut mucosa and, in one study, were found in practically all inflammatory bowel disease (IBD) patients [6]. In contrast to the well-characterized high degree of diversity in the gut flora, the diversity of mucosal biofilm bacteria was astonishingly low, with *Bacteroides* species making up more

than two-thirds of the biofilm mass [6]. Recently, the mucosal biofilm hypothesis gained significant ground in the pathogenesis of chronic otitis media, with the demonstration of biofilms forming directly on the mucosal surface of the middle ear in animals and humans [7,8]. This finding can explain the paradox of bacterial mRNA and DNA found in otherwise sterile middle-ear effusions in children with this infection [3]. In addition to the alveolar, gut and ear mucosa, oropharyngeal, urinary tract, nasal, sinus and vaginal mucosae, as well as skin, can be covered by soft-tissue biofilms, exhibiting a tenacious and intimate association with the epithelium and triggering chronic or recurrent inflammatory diseases. Examples of such inflammatory diseases are tonsillitis, stomatitis, rhinitis, sinusitis, otitis, urethritis, cystitis, vaginitis and dermatitis [2,9–14].

Mucosal biofilms are difficult to study

There is universal agreement among microbiologists that the study of biofilm organisms is far more difficult than that of planktonic (freely suspended) cells. As a rule, biofilms are harder to grow *in vitro*, harder to quantify using colony-forming unit (CFU) counts or metabolic assays and more difficult (if not impossible) to examine with traditional transmission light microscopy. For example, without the use of specialized cell-disruptive techniques, CFU-based quantitation severely underestimates the number of biofilm microorganisms as it results in one colony per aggregate as opposed to one colony per microbial cell. Furthermore, the use of metabolic assays, such as the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)2H-tetrazolium-5-carboxanilide (XTT) assay that is commonly used to extrapolate cell density in fungal biofilms, is fraught with difficulty since cell metabolic activity and cell numbers are only linearly related in the early stages of biofilm growth [15].

The difficulties in the study of biofilms listed previously pertain to both tissue and abiotic surface biofilms. However, the study of tissue biofilms is further complicated by two factors. First, human tissue samples, faithful animal models of infection or *in vitro*-engineered mucosal tissue models that provide substrates for biofilm growth, are not always available. Poor accessibility to human tissue samples may explain the fact that, compared with human mucosal biofilms, mucosal biofilms in animals have been better characterized (for review see [16]). Second, when tissues can be sampled for study, tissue preservation techniques have to be such that biofilm distortion is avoided. Since the mucopolysaccharide-containing ECM generally collapses following aldehyde-based fixation and dehydration, specimens may have to be stored frozen or transported in a physiologic saline solution immediately for imaging [17,18]. It has also been shown that, although intestinal biofilms are sufficiently visible in Carnoy solution-fixed biopsy specimens, no biofilm can be observed in formalin-fixed specimens from the same locations, regardless of the visualization technique employed [6]. These investigators further suggested that tissue fixation in nonaqueous Carnoy solution preserves the mucopolysaccharide layer [6]. However, owing to the fact that ECM hydrophobicity and solubility can vary in biofilms depending on the substratum, level of maturation or type of microorganism [19], standard aldehyde fixation can be used successfully with some biofilms prior to imaging [20].

Fluorescent *in situ* hybridization (FISH) is a well-established molecular diagnostic assay, commonly used in pathology laboratories to detect and differentiate between microorganisms in histological sections. FISH can distinguish organisms at the species level or higher (i.e., genus, phylum or domain) and allows simultaneous identification of up to seven different microbial species in one sample (for review, see [21]). One of the limitations of FISH is that it employs ribosomal (r)RNA-targeted probes and, thus, its sensitivity is limited by the metabolic state of the microorganisms within the mucosal biofilm, since metabolically inactive organisms are generally considered to have a lower cellular ribosomal content. This limitation may explain the relative ‘inaccessibility’ of FISH probes in metabolically silent biofilm areas,

which otherwise stain very well with nuclear stains, such as 4'-6-diamidino-2-phenyl-indole (DAPI) [6]. However, metabolic activity does not correlate well with ribosomal content in all microbial species, since some slow growing bacteria retain high cellular ribosomal content, even in conditions of low metabolic activity [21]. To detect metabolically active cells more accurately, FISH oligo-nucleotide probes can be synthesized that bind to intergenic spacer regions in rRNA genes, which are quickly degraded during ribosome maturation in bacteria. Thus, these probes would detect only cells that are producing new rRNA (i.e., metabolically active cells) at the time of sampling [21]. Since traditional histologic processing of tissue specimens for FISH requires fixation and a number of washing steps that undoubtedly remove or distort a significant portion of biofilms, the combination of FISH with confocal laser-scanning microscopy of fresh or frozen specimens may be more appropriate in the study of mucosal biofilms. In fact, the combination of FISH with confocal imaging, followed by image analysis with stereologic or spatial statistics softwares, constitute state-of-the-art technologies in the description of spatial localization patterns of microorganisms growing in environmental biofilms, and may find similar applications in tissue biofilms [21].

Another problem in studying mucosal biofilms *in situ* is the poor accessibility of certain mucosal locations, such as the lower GI tract, the middle ear and the urinary tract. Newer technologies, such as laser-scanning confocal endoscopy, which allow microscopic evaluation of living tissues without the need for biopsy or histologic processing [18], may be exploited to address this problem. Although conventional one-photon excitation laser confocal microscopy is generally sufficient for biofilm image analysis; two photon excitation laser-scanning microscopy may allow a deeper penetration of tissue biofilms and enhanced resolution [20,21]. Finally, atomic force microscopy, an essentially nondestructive sample-imaging technique with nanoscale resolution, has been used to study ultrastructural and nano-mechanical properties of abiotic biofilms and may, ultimately, find some applications in the study of tissue biofilms [22].

'Hunt' for the ECM

The presence of an ECM, or glycocalyx, is considered to be the hallmark of a biofilm [19]. In most biofilms studied to date, the composition and biosynthetic regulation of the ECM remain elusive research goals and represent the 'darker' side of biofilm biology. Even the mere visualization of the ECM has been problematic and some studies have considered the microscopic observation of 'blebs' under phase-contrast microscopy and staining with carbohydrate-specific dyes, such as Alcian blue, as indirect evidence for the presence of ECM [10].

Part of the problem in identifying tissue biofilm ECM *in situ* is its potential sensitivity to standard histologic processing, as described previously [6,17,18] and, in part, is due to the fact that its quantity and composition can vary in different mucosal sites depending on environmental cues, ranging from nutrient availability to mechanical stimuli [19,23]. For example, it is well established that under static conditions, matrix synthesis is minimal by certain mucosal pathogens [24,25]. Therefore, overall, it is not surprising that biofilm formation is favored in mucosal sites with fluid flow (e.g., oral mucosa and urethra) or tissue motility (e.g., alveoli and gut). In addition, the composition of mucosal biofilm ECM is inherently more complex than abiotic surface biofilms, owing to the fact that host glycoproteins, nucleic acids and cells may participate in its development [16,26–28]. For example, neutrophils contribute to *Pseudomonas*-induced lung biofilms by providing a source of the DNA and actin polymers found in the ECM [26,27]. Similarly, in the GI tract mucosa, it is reasonable to suggest that host mucus and sloughing epithelial cells participate in the ECM structure [16].

For some bacterial and fungal microorganisms, such as *Streptococcus aureus*, *P. aeruginosa* or *Cryptococcus neoformans* the composition of the ECM has been well characterized [28–31]. For example, in staphylococci, the ECM consists mainly of a β -1,6-linked *N*-acetylglucosamine polymer, whose synthesis is regulated by the *ica* operon [31]. Some Gram-negative bacteria are known to secrete lipid biosurfactants as part of the matrix, which enable biofilms to solubilize and utilize certain substrates that would otherwise be inaccessible [28]. For other pathogens, indirect evidence exists that favors a certain mix of polysaccharides in the ECM. For example, glucose accounts for almost 20% of extra-cellular polysaccharide dry weight in *Candida albicans* biofilms, whereas mannose is found in lower quantities [25]. In addition, *Candida* biofilms are easily destroyed by treatment with a β -1,3-glucanase [24], suggesting that glucose forms an extracellular glucan polymer. In support of this, a glucan cross-linked protein has been shown to be critical in biofilm development and recent data suggest that it may be ‘shed’ from the cell wall during ECM synthesis [32]. We were able to confirm the presence of β -glucan in the ECM and characterize its distribution within an oral mucosal biofilm *in situ*, using a double immunolabeling technique, followed by confocal microscopy (Figure 1).

Abiotic-surface biofilms can give some clues for mucosal biofilms

Biofilm formation on abiotic and tissue surfaces appears to be regulated by similar processes in certain mucosal pathogens. For example, the ability to autoaggregate *in vitro* is positively associated with the ability of mucosal pathogens, such as *Streptococcus*, *Proteus* and *Lactobacillus*, to adhere to the host epithelium [9,33]. The formation of a urinary tract mucosal biofilm by *Proteus* species is dictated by the expression of fimbriae leading to bacterial aggregation, which plays a similar role in biofilm formation on cover glass or plastic surfaces [9]. Similarly, specific surface proteins of uropathogenic *Escherichia coli* allow this microorganism to form biofilms on both abiotic and epithelial surfaces [34]. Recently, *Candida* alcohol dehydrogenase was shown to play a role in biofilm formation, acting as a negative regulator both in oral mucosa tissue and abiotic models [35]. It is also remarkable that *Streptococcus pneumoniae* gene-expression patterns are almost identical in tissue and abiotic biofilms, considering the enormous differences in the microbial growth conditions of these two locations [10]. Based on this information, it is reasonable to expect that, at least for some pathogens, there may be common regulatory pathways controlling biofilm growth on both abiotic and tissue surfaces. Therefore, knowledge on regulatory pathways of biomaterial biofilms can provide important clues about mucosal tissue biofilms.

Host response in soft-tissue–biofilm infections

A prominent feature of skin and alveolar infections characterized by tissue biofilms is an inflammatory response with many of the cellular, molecular and histologic features of an acute response to infection [12,26]. Histologic hallmarks of these superficial lesions include hydrolytic enzyme-mediated destruction of the cell junctions, premature keratinocyte detachment from each other and shedding (acantholysis), intercellular edema (spongiosis) and, finally, a large number of neutrophils traversing the epithelial layers and reaching into the biofilm pockets [12,26,27]. Other histopathologic features include superficial necrosis and ulceration pockets packed with microbial communities which, in general, do not penetrate deeply into the tissues [12,26,36].

Neutrophils are abundant within catheter biofilms, as well as within human tissue biofilms *in vivo* [26,37,38]. Whether they become actively recruited by host or microbial proteins is currently unknown. In addition, their functional activation and phagocytic status within biofilms seems to vary, with active phagocytosis being detected within biofilms of certain bacterial species [25,26] but not others [38]. With the exception of the alveolar mucosa, the

host response to biofilms forming on non-keratinizing epithelia, such as the urinary tract and lower GI tracts, is largely unknown.

Pathogenesis of mucosal biofilm infections

Although biofilms have been detected in several mucosal locations, their ability to trigger human disease is still a matter of active investigation. For example, it has been hypothesized that IBD results from an aberrant host inflammatory response to intestinal bacteria [6]. However, the fact that the gut epithelium that lies in contact with these biofilms is intact and not invaded by the microorganisms [6] clouds their direct role in pathogenesis. Thus, at least in the case of IBD, the possibility still exists that mucosal biofilm formation is secondary to an aberrant immune response to self-antigens. Indeed, it is plausible that the release of nutrients from immunologically driven epithelial cell lysis may promote growth of biofilm microorganisms. Furthermore, locally released proinflammatory cytokines can act as biofilm-specific growth factors [39]. Along these lines, it has been suggested that the first step in the pathogenesis of chronic rhinosinusitis is inflammation of the nasopharynx, triggered by allergy, viral disease or gastroesophageal reflux, which is followed by biofilm formation by known rhinosinusitis pathogens, such as *Haemophilus influenzae*, *S. pneumoniae* or *S. aureus* [14]. Thus, it is possible that tissue biofilms form as a consequence of inflammation, triggered initially by biofilm-unrelated stimuli.

However, other microscopic studies have shown that colonic biofilms are usually associated with histologically abnormal mucosal architecture [40] and, thus, may initiate local tissue damage. Therefore, it is possible that mucosal biofilms provide a protective environment for microorganisms from innate defense mechanisms, which may promote their persistence and, ultimately, invasion into host tissues, triggering direct tissue damage. In fact, the ability of certain pathogens to form a soft-tissue biofilm may dictate their ability for soft-tissue invasion and destruction. Along these lines, it has been proposed that the ability to form a biofilm in the digestive tract may allow *Candida* to invade across the intestinal epithelium into the submucosa and disseminate systemically [35]. This suggestion was prompted by evidence showing that thicker biofilms formed by an aldehyde dehydrogenase mutant were accompanied by increased tissue invasion and damage in a 3D model of the human oral mucosa [35]. A close association between biofilm formation and invasion has also been strongly suggested for this pathogen by data showing that invasive growth and biofilm development are regulated by common signaling pathways [41].

Another complicating factor in dissecting the pathogenesis of mucosal biofilm infections is the fact that most human mucosal surfaces are constantly colonized by multiple commensal microorganisms with variable potential to contribute to opportunistic infections. As a result, mucosal biofilms are frequently polymicrobial in nature and may be readily influenced by synergistic or inhibitory interactions of bacteria, viruses and fungi. Therefore, understanding the cellular and molecular interactions of pathogens with the normal flora, as well as the interactions among pathogens themselves, is of paramount importance in understanding the pathogenesis of mucosal biofilm infections. For example, the ability of various bacteria in the oral microflora to express molecules (e.g., bacteriocins) that interact with other bacterial species and host cells has been suggested to be a critical component in dental plaque development, which constitutes perhaps the most classic example of a pathogenic biofilm in humans [42].

Proposed inflammation-centered pathogenesis of mucosal biofilm infections

Two inflammation-centered pathogenesis models of mucosal biofilm infections are proposed in the following sections, which may help to explain the potentially divergent roles of tissue biofilms in human disease. The preponderance of one of the two pathogenetic schemes may

depend on mucosal site characteristics, such as epithelial type and specialized function, on virulence characteristics of the microorganisms or, more likely, on both.

Mucosal biofilms may trigger an excessive proinflammatory response leading to indirect host damage

It was hypothesized recently that damage to mucosal tissues from biofilms results mainly from an aberrant immune and inflammatory response to the microbial aggregates, rather than direct damage from microbial hydrolytic enzymes [6]. In fact, it has been shown that neutrophils contribute to *Pseudomonas*-induced lung pathology by excessive use of their toxic secretory arsenal such as oxidative agents that, although targeted against the biofilm bacteria, are instead destroying host tissues owing to the bacteria-shielding action of the ECM [26,27]. It has also been proposed that the engine that drives the chronic inflammatory exudate (otorrhea) in chronic otitis media is the bacterial biofilm in the mid-ear mucosa (primarily consisting of *H. influenzae* and *S. pneumoniae*) [8]. However, neither the mechanisms by which soft-tissue biofilms affect the development of inflammatory events, nor the disease-specific changes in biofilm communities, have been determined in these mucosal biofilm infections.

Mucosal biofilms may block the initial host inflammatory response leading to innate immune cell paralysis & promoting biofilm growth & tissue invasion

The critical role of proinflammatory cytokines in the host defense against many mucosal pathogens has been unequivocally demonstrated in numerous *in vitro* and animal studies (for review, see [43]). Proinflammatory cytokines are necessary for the upregulation of numerous microbial pattern-recognition receptors in innate immune cells, as well as for the activation of several microbicidal functions [44–46]. According to this pathogenesis model, biofilm components may attenuate the mucosal inflammatory response to infection and dysregulate phagocytic cell function, providing an early growth advantage to biofilm organisms. Recently, an animal model of otitis media provided evidence in support of this model [47]. It was reported that *H. influenza* biofilms are characterized by high levels of cell-surface-associated phosphorylcholine-containing lipooligosaccharides, with decreased potency as inflammatory agonists during the early stages of infection, thus allowing the development of stable biofilm communities. Similarly, *C. albicans* biofilms were shown to downregulate secretion of most proinflammatory cytokines by peripheral blood mononuclear cells, and the addition of these cells to biofilms had a stimulatory, rather than inhibitory, effect on biofilm growth *in vitro* [48]. Finally, it is proposed that although proinflammatory cytokines may not be affected by biofilm components, anti-inflammatory cytokines, such as IL-10 and TGF- β , may be substantially stimulated. For example, such cytokines are inducible by *C. neoformans* capsular polysaccharides [49]. Potentially, these polysaccharides are very important in the pathogenesis of biofilm infections, since their secretion promotes biofilm development [30].

Therapeutic challenges in mucosal biofilm infections

Antimicrobial susceptibility testing of *in vitro* biofilm models has shown bacterial survival after treatment with concentrations 100–1000-times the MIC for bacteria in a suspension culture (for review, see [50]). Multiple mechanisms of antimicrobial resistance have been shown to play a role in this biofilm phenotype in several microorganisms. These mechanisms include the inactivation of the antimicrobial by the ECM via reactivity or adsorption, increased production of antimicrobial-degrading enzymes, high-density-induced stress responses, slow growth rates, facilitation of plasmid gene transfer and induction of multidrug efflux-pump genes. All of these mechanisms are considered to be part of a biofilm-specific phenotype, which is induced in a subpopulation of the multicellular community by biofilm-related factors, such as nutrient limitation and density-dependent environmental stresses [51]. Numerous studies

have also suggested that the number of resistant organisms within a biofilm may be small during initial exposure to antibiotics, but that repeated exposure to the same class of antimicrobials rapidly drives the resistant cells to repopulate the biofilm. The end result is the development of second- or third-generation biofilms, which are much more resistant than the original biofilm [52–54]. Clinically, this implies that traditional antimicrobial treatments that target single microbial cells within biofilms will never be able to eliminate them. This may be true also for non-traditional therapeutic approaches, such as antibody-delivered cytotoxic radiation, since even these approaches are not completely capable of eradicating biofilms [55] and may antagonize the effects of traditional antifungal drugs [56].

Therefore, it has been suggested that antibiofilm therapies that target the entire biofilm as a complex multicellular organism or prevent unique, biofilm-specific processes, are needed to fight biofilm infections [57]. Future directions in therapeutics may include chemical synthesis and the use of quorum-sensing molecules to prevent microbial cell-to-host and microbial cell-to-microbial cell attachment, or promote cell detachment from mucosal surfaces or biofilms. Since some quorum-sensing molecules (autoinducers) may play a role in priming cells for antimicrobial resistance [58], inhibitors of synthesis or activity of these molecules may be used to render the biofilm more resistant to conventional antimicrobials. Other potential approaches include the discovery of inhibitors of ECM biosynthesis or developing strategies for probiotic biofilm promotion that precludes the development of pathogenic biofilms. In addition, the combination of locally delivered proinflammatory cytokine-based therapeutics with traditional antimicrobial drugs may be a reasonable future approach to treatment. For example, agents that promote invasion of phagocytic cells into the biofilm, in combination with glycocalyx-reducing drugs, may increase the effectiveness of treatment in biofilm-associated skin infections [57]. Along these lines, the beneficial effects of a granulocyte–macrophage colony-stimulating factor-based mouthrinse was shown recently in radiation-treated patients with pseudomembranous candidiasis [59].

Expert commentary

Recently, newer *in situ* visualization technologies have led to the identification of well-structured tissue biofilms associated with soft tissues *in vivo* [6–8,12] or in 3D-engineered mucosal tissue equivalents *in vitro* [35,60,61]. Although biofilms have been detected in several mucosal locations, their ability to trigger human disease is still a matter of active investigation. It is currently hypothesized that the indolent, long-term persistence of a mucosal biofilm provides conditions conducive to recurrent infections, to periods of exacerbations and remissions in chronic infections, or to sloughing or release of planktonic organisms and chronic seeding of distant locations [6,7,12]. There are two major characteristics of mucosal biofilms that distinguish them from abiotic surface biofilms. First, since most mucosal surfaces are not sterile, there is a dynamic interplay between the local commensal microflora and the pathogenic microorganisms that colonize these sites. The second characteristic is a dynamic, two-way relationship between mucosal biofilms and the host, as proposed in this review, whereby biofilms both modulate and become modulated by the host inflammatory response, as well as by cellular and molecular host components, which may contribute to biofilm development. An inflammation-centered epithelial type-specific pathogenesis model, modulated by the virulence characteristics of each pathogen, is also suggested to prevail at different mucosal sites.

Five-year view

In the next few years, it is critical to examine the mucosal biofilm processes independently of abiotic surface biofilms and dissect the virulence factors uniquely important in pathogenesis within the tissue environment. This is because host cell participation and the potential

interactions between microorganisms and host proteins are more complex in tissues compared with abiotic substrata. Multispecies and even multigenus biofilms are more likely to trigger disease in nonsterile mucosal sites, such as the alimentary tract and, thus, experimental modeling of such mucosal biofilm infections will have to account for this fact. Finally, since traditional single-cell-targeting antimicrobial treatments will probably not be able to eradicate multicellular biofilm structures, novel therapeutic avenues should target beneficial biofilm relationships between microorganisms and the host, rather than biologic processes that take place in microbial cells upon colonization of abiotic surfaces.

Key issues

- Mucosal biofilms were recently shown to be involved in a plethora of human infections, and the mechanisms involved in the pathogenesis of these infections are under active investigation.
- Mucosal biofilms are more complex than abiotic-surface biofilms owing to the host cell participation in their development and structure.
- A dynamic, two-way relationship between mucosal biofilms and the host is proposed, whereby biofilms both modulate and become modulated by the host response to infection.
- Mucosal biofilms may directly or indirectly contribute to tissue damage, via an enhanced host proinflammatory response.
- Since traditional antimicrobial treatments targeting single cells will probably not be able to eradicate multicellular biofilm structures, novel therapeutic avenues are needed that target biofilm-beneficial relationships between microorganisms and the host.

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Figure 1.

Confocal image of an oral mucosal biofilm formed by *Candida albicans* (strain SC5314) on a 3D *in vitro*-engineered model of the human oral mucosa.

The microorganisms (green) were stained with a polyclonal rabbit fluorescein-conjugated antibody specific for *C. albicans* (Biodesign International, ME, USA). The biofilm extracellular matrix (red) was stained with BFDiv, a mouse monoclonal antibody specific for β -glucan (kindly provided by Biothera), followed by incubation with Cy3-conjugated F(ab')₂ fragment goat antimouse IgM antibody (Jackson ImmunoResearch, PA, USA). Appropriate isotype controls were used, which stained negative (not shown). Biofilms were examined in a confocal-scanning laser microscope (Zeiss LSM510/Confocor2, PlanNeofluar objective [20 $\times$,

NA0.5]). Organisms in contact with the oral mucosa (left side of image) appear to be associated with more abundant quantities of β -glucan.