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REVIEW

Innocent until proven guilty: mechanisms and roles of *Streptococcus–Candida* interactions in oral health and disease

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SUMMARY

***Candida albicans* and streptococci of the mitis group colonize the oral cavities of the majority of healthy humans. While *C. albicans* is considered an opportunistic pathogen, streptococci of this group are broadly considered avirulent or even beneficial organisms. However, recent evidence suggests that multi-species biofilms with these organisms may play detrimental roles in host homeostasis and may promote infection. In this review we summarize the literature on molecular interactions between members of this streptococcal group and *C. albicans*, with emphasis on their potential role in the pathogenesis of opportunistic oral mucosal infections.**

INTRODUCTION

The bacterial genus *Streptococcus* consists of gram-positive organisms that are typically classified into α-, β-, and γ-hemolytic groups, based on the type or presence of blood agar hemolysis that they trigger (incomplete, complete or absent, respectively). The streptococci most frequently isolated from the oral cavity are either α-hemolytic (also known as viridans) or non-hemolytic and, with the exception of *Streptococcus mutans*, are broadly considered avirulent or even beneficial organisms. For example, certain oral streptococcal species can hinder the development of a cariogenic *S. mutans* biofilm (Kuramitsu *et al.*, 2007).

Recent attention has been focused on a subgroup of these streptococci, known as the mitis group (MGS), since members of this group have been implicated in driving a pathogenic multi-species biofilm phenotype when they coaggregate with other bacterial or fungal species (Ramsey *et al.*, 2011; Whitmore & Lamont, 2011; Xu *et al.*, 2013b). The MGS are principally represented by the species *Streptococcus gordonii*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus parasanguinis* and *Streptococcus sanguinis* and comprise approximately 5% (Moore *et al.*, 1982) to over 60% (Syed & Loesche, 1978) of the recoverable human oral microbiota, depending on the method of analysis. MGS colonize the majority of healthy humans (Alam *et al.*, 2000) and are also credited with initiating community assembly in oral biofilms (reviewed in Whitmore & Lamont, 2011).

The ability of any microorganism to form an adhering biofilm on oral surfaces is essential for stable colonization of the oral cavity (Jakubovics & Kolenbrander, 2010). During biofilm growth, coaggregation of MGS with other organisms frequently

confers a mutual advantage in biofilm formation. For example, other colonizers such as *Actinomyces* spp., *Capnocytophaga* spp., *Eikenella* spp., *Haemophilus* spp., *Prevotella* spp., *Propionibacterium* spp. and *Veillonella* spp., appear to benefit from the presence of MGS (Kolenbrander *et al*., 2002). Inter-Kingdom co-aggregation interactions between MGS members and *Candida albicans* have also been extensively studied *in vitro* (Jenkinson *et al*., 1990) and there is now evidence for *Candida*–streptococcal biofilm interactions *in vivo*, both in humans and animal models (Zijnge *et al*., 2010; Xu *et al*., 2013b). Like MGS, *C. albicans* colonizes the oral cavity of the majority of healthy individuals (Odds, 1988; Wilkieson *et al*., 1991), so interactions between these organisms are likely to play a role not only during commensal colonization of oral surfaces, but also during the course of opportunistic infections.

Multi-species biofilm communities may play both beneficial and detrimental roles in host homeostasis. Within these communities, microorganisms use quorum-sensing molecules and other metabolites to communicate with each other, adjust their population density, change gene expression patterns, and continuously adapt to the biofilm microenvironment, or release planktonic cells and colonize new, distant sites. During these processes, cell–cell interactions in the community result in antagonism, synergy or mutualism. These events not only affect the behavior of microorganisms but also modulate host responses and the ability to trigger infection (Wright *et al*., 2013). As a consequence of the shared ecological niche and high frequency of oral carriage of oral streptococci and *C. albicans*, it is important to understand how these organisms modulate each other's capacity to interact with the host. This review summarizes recent evidence on this topic.

COMMON COLONIZATION SITES IN ORAL HEALTH AND DISEASE

The human oral cavity contains a number of different potential habitats for oral microorganisms such as the keratinized and non-keratinized oral mucosa, dorsal and lateral tongue surfaces, subgingival crevices and teeth. The commensal microbiota at these sites have been traditionally regarded as important in maintaining oral health. Oral streptococci colonize both teeth and oral mucosal surfaces in healthy individuals (Frandsen *et al*., 1991). *Streptococcus oralis* and its closely related species *S. sanguinis* and *S. mitis* comprise 60–80% of the primary colonizers of clean tooth surfaces, depending on the method of analysis used (16S rRNA sequencing vs. culture) (Gibbons, 1989; Diaz *et al*., 2012a). Importantly, although streptococci had been traditionally regarded as early or late (as in the case of *S. gordonii*) tooth colonizers, recent 454 microbiome pyrosequencing analysis has revealed that MGS (particularly *S. mitis*) dominate in the buccal mucosa of healthy individuals (Diaz *et al*., 2012a). Furthermore, when *C. albicans* is present, MGS colonization and biofilm efficiency on mucosal surfaces increase, as shown in three-dimensional models of the human oral and esophageal mucosae, under conditions of salivary flow (Diaz *et al*., 2012b). These results were confirmed in a mouse model of *Candida*–streptococcal oral co-inoculation, where it was shown that *S. oralis* colonization of the oral and gastrointestinal tract is significantly augmented by the presence of *C. albicans* (Xu *et al*., 2013b). Similarly, in the healthy vaginal mucosa, group B streptococci have a greater probability of isolation when *C. albicans* is present (Monif & Carson, 1998).

Although *C. albicans* appears to favor streptococcal colonization of mucosal surfaces, the pioneer binding of MGS to oral surfaces has been proposed to directly reduce the opportunities of pathogens such as *Candida* species and *Staphylococcus aureus* to colonize (Wade, 2013). Indeed some oral streptococci produce small molecules with antibiotic-like activity that can inhibit growth of other organisms. For example, *S. mutans*, *S. mitis*, *S. oralis, and S. sanguinis* produce diffusible signal factors (*Streptococcus* diffusible signal factors), which can repress the yeast-to-hypha transition of *C. albicans* that is essential for mucosal colonization and virulence (Lo *et al*., 1997; Vilchez *et al*., 2010). In other instances, streptococci can serve as probiotics, as in the case of *S. salivarius* K12, which has probiotic properties that protect mice from severe candidiasis (Ishijima *et al*., 2012).

Similar to MGS*, Candida* species (principally represented by *C. albicans*) also colonize both hard (teeth, dentures) and soft (mucosal) surfaces (Campos *et al*., 2008; Zijnge *et al*., 2010). Interestingly, *C. albicans* isolates from dental plaque and tracheobronchial sites from the same mechanically ventilated intensive care patients are genetically identical (Heo *et al*., 2011), underscoring the potential for systemic fungal spread

from this oral habitat. Although coaggregation of *Candida* and streptococci has been observed on human dental plaque (Zijnge *et al.*, 2010), and on denture biofilms (Campos *et al.*, 2008) *in vivo* the clinical or biological significance of these interactions is unknown. *Candida albicans* is frequently isolated in dental caries of children with high oral *Candida* carriage, but again whether it plays a direct pathogenic role or merely co-exists in these lesions with cariogenic viridans streptococci is not clear (Raja *et al.*, 2010).

On oral mucosal surfaces *C. albicans* can trigger an inflammatory response associated with the development of candidiasis, a common oropharyngeal infection primarily afflicting immunosuppressed individuals (reviewed in Villar & Dongari-Bagtzoglou, 2008). Although the presence of *C. albicans* is required for the development of this infection, it is increasingly appreciated that oral candidiasis is a mixed fungal–bacterial mucosal biofilm-induced or denture biofilm-induced disease (Dongari-Bagtzoglou *et al.*, 2009; Nett *et al.*, 2010; Johnson *et al.*, 2012; Nobile *et al.*, 2012; Xu *et al.*, 2013b). In fact two recently reported rodent models of denture stomatitis identified endogenous bacteria forming biofilm communities with *C. albicans* on denture surfaces (Nett *et al.*, 2010; Johnson *et al.*, 2012). *Candida albicans* biofilm formation is controlled by a complex transcriptional network essential for *C. albicans* adaptation to different host habitats, resistance to immune system, survival and growth (Dwivedi *et al.*, 2011; Nobile *et al.*, 2012).

In an experimental oropharyngeal candidiasis mouse model *C. albicans* formed complex polymicrobial biofilms on the dorsal surface of the tongue with indigenous bacterial cocci (Dongari-Bagtzoglou *et al.*, 2009). The potential role of streptococci in the development of this lesion was recently explored experimentally in a mouse model where oral co-inoculation of *C. albicans* with *S. oralis* led to increased frequency and severity of biofilm lesions (Xu *et al.*, 2013b). In humans, *S. oralis* and *C. albicans* are frequently co-isolated from the sputum of antibiotic-treated cystic fibrosis patients (Maeda *et al.*, 2011). Furthermore, co-isolation of *C. albicans* and *Streptococcus pneumoniae*, which has a high degree of genetic relatedness to *S. oralis* (Johnston *et al.*, 2010), was implicated as the cause of pulmonary infection (Yokoyama *et al.*, 2011). However, MGS have not yet been implicated in the pathogenesis of oropharyngeal candidiasis in humans.

MOLECULAR MECHANISMS OF STREPTOCOCCAL–*CANDIDA* CELL INTERACTIONS

Adhesive and coaggregation interactions

Streptococci bind directly to salivary proteins that are abundant in the pellicle covering teeth and mucosal surfaces, and this binding facilitates their initial colonization under salivary flow conditions. MGS members *S. sanguinis*, *S. gordonii* and *S. oralis* have the ability to coaggregate with many microorganisms on salivary pellicles (Kolenbrander *et al.*, 2002), including *C. albicans* (Hsu *et al.*, 1990; Jenkinson *et al.*, 1990). Coaggregation of *Candida* with oral streptococci was suggested to be important for *C. albicans* colonization of oral surfaces (Jenkinson *et al.*, 1990) but more recent evidence suggests that *Candida* facilitates oral biofilm accretion of MGS species that lack the ability to form robust mucosal biofilms, such as *S. oralis* (Diaz *et al.*, 2012b; Xu *et al.*, 2013b).

The dual binding ability to host surfaces and to other microorganisms promotes streptococcal multispecies communities in the oral cavity. Binding to the pleiomorphic fungus *C. albicans* can be both streptococcal species-specific and fungal morphotype-specific. *Streptococcus sanguinis* and *S. gordonii* adhere to starved *C. albicans* yeast cells and *S. salivarius* also shows some coaggregating ability to yeast, but *S. mutans* and *Enterococcus faecalis* are deficient (Jenkinson *et al.*, 1990). *In vitro*, in the absence of nutrients, *S. oralis* cells bind to *Candida* germ tubes as visualized with multi-color fluorescence microscopy (Diaz *et al.*, 2012b) and in the presence of nutrients, both *S. oralis* and *S. gordonii* cells form clusters along hyphae within a few hours (Bamford *et al.*, 2009; Diaz *et al.*, 2012b).

Streptococcus–*Candida* cell adhesion is physically mediated by a number of well-characterized cell wall surface proteins/receptors on both organisms. Streptococci have abundant surface cell wall-anchored protein adhesins discussed in detail elsewhere (Nobbs *et al.*, 2009). Untill now, two families of proteins on the streptococcal cell surface have been found to be involved in *C. albicans* binding. Both protein families contain an LPXTG motif recognized by sortase A

enzymes, tethering the related proteins to the peptidoglycan layer. These proteins have multiple functions, including binding to both human proteins and microorganisms. One family is the CshA protein family first identified in *S. gordonii* DL1 (McNab & Jenkinson, 1992). The CshA protein comprises 2508 amino acid residues containing three domains, leader sequence, non-repetitive region (NR), and amino acid repeat block region(R), and it forms fibrillar structures on the cell surface with adhesive and hydrophobic properties (McNab *et al.*, 1999). *Streptococcus gordonii* DL1 *cshA* mutants are deficient in binding to immobilized human fibronectin and other oral bacterial and *Candida* cells and both NR and R domains of CshA protein are responsible for binding to *Candida* cells (Holmes *et al.*, 1996). CshA-like proteins have also been found in *S. oralis* and *S. sanguinis* (Elliott *et al.*, 2003). However, the binding mechanism of CshA is still not clear and no ligands of CshA on the *Candida* cell surface have been identified to date.

The second streptococcal adhesin family implicated in binding to *C. albicans* consists of the SspA and SspB proteins that belong to the antigen I/II polypeptide family. SspA and SspB precursors comprise 1575 and 1499 amino acid residues respectively and contain seven structural regions: signal peptide, N-terminal region, Ala-rich repeats, divergent region, Pro-rich repeats, C-terminal region, and cell-wall anchoring sequence (Jenkinson & Demuth, 1997). Interestingly, the divergent region shows sequence similarity with streptococcal glucan-binding protein C, which is involved in dextran-dependent aggregation of streptococci (Okamoto-Shibayama *et al.*, 2006). The *S. gordonii* strain DL1 has both SspA and SspB proteins and the two genes are in one operon, but *S. oralis* only has an *sspA* gene (Vickerman *et al.*, 2007; Reichmann *et al.*, 2011). Both SspA/B proteins participate in S. *gordonii* binding to fungi, whereas binding of SspB protein to *C. albicans* requires *C. albicans* cell surface expression of protein ALS3 (agglutinin-like sequence 3), because *S. gordonii* cells fail to attach to Δ*als3*/Δ*als3 Candida* cells (Silverman *et al.*, 2010). Heterologous expression of SspB and ALS3 proteins in *Lactobacillus lactis* and *Streptococcus cerevisiae*, respectively, confers coaggregation properties between the two organisms, which supports the idea that SspB and ALS3 proteins interact directly (Silverman *et al.*, 2010).

The cell wall of the trimorphic fungus *C. albicans* contains proteins and carbohydrates that differ in yeast, pseudohyphal and hyphal forms and are also tightly regulated by the type and/or duration of the environmental stimuli that drive morphogenesis (Gopal *et al.*, 1984; Ebanks *et al.*, 2006). Different environmental stimuli may therefore lead to hyphae expressing different surface proteins or having different amounts or types of skeletal polysaccharides, depending on the specific environmental triggers (Gow & Hube, 2012). The best studied adhesins in *C. albicans*, belong to one of three families, the ALS, Hwp1 (Hyphal Wall Protein 1), and Iff/Hyr (Hyphally Regulated Protein 1) families, which are reviewed in detail elsewhere (de Groot *et al.*, 2013). Some proteins of the ALS and Hwp1 families are involved in adhesion to both host epithelial cells and bacteria. The ALS family consists of eight large, cell wall surface GPI-anchored glycoproteins that show differential expression profiles in yeast and hyphae. ALS3, ALS1 and ALS5 proteins mediate recognition of *S. gordonii* cells, whereas ALS3 also mediates *Staphylococcus aureus* adhesion to *C. albicans* (Klotz *et al.*, 2007; Silverman *et al.*, 2010). Hwp1 and Eap1, which belong to the Hwp1 family, confer on yeast the ability to bind to *S. gordonii* when the proteins are heterologously expressed in *S. cerevisiae*, and the interactions do not depend on the *S. gordonii* CshA protein (Nobbs *et al.*, 2010).

In addition to protein–protein types of adhesive interactions between *C. albicans* and oral streptococci, carbohydrate-mediated or extracellular polysaccharide (EPS)-mediated interactions may also play important roles in multi-species community assembly. The EPS is a major component of the biofilm matrix. Both oral streptococci and *C. albicans* produce EPS in the biofilm state, albeit with a different composition. The EPS of viridans streptococci is synthesized by multiple glucosyl-transferases (Gtfs) that exist widely in the streptococcal genus. GtfB is secreted by *S. mutans* and can bind to candidal yeast cells in an enzymatically active form, depositing α-glucans on the yeast cell surface (Gregoire *et al.*, 2011). The newly synthesized α-glucans promote *S. mutans* binding to yeast cells and enhance *S. mutans–C. albicans* coaggregation (Gregoire *et al.*, 2011). Similarly, GtfG-derived α-glucans play a role in promoting biofilm interactions between *S. gordonii* and *C. albicans* (Ricker *et al.*, 2014).

Other streptococcal molecules that play a role in inter-species and inter-generic aggregation interactions are the coaggregation receptor polysaccharides (RPS). These are six, structurally distinct receptors for lectin-like adhesins mediating inter-species and intra-species streptococcal aggregation, or inter-generic interactions of streptococci with *Actinomyces*, and *Veilonellae* (Yoshida *et al*., 2006). For example, Yoshida *et al.* (2006) have shown that an RPS-negative *S. oralis* mutant fails to form mixed biofilms with an adhesin-expressing *Actinomyces*. Sequence analysis of human dental plaque organisms showed that RPS-positive streptococci clustered with *S. oralis* and RPS-negative clustered with *S. gordonii* (Hoshino *et al*., 2005), indicating species heterogeneity in the expression of these receptors within the MGS group. There is no information on the potential role of these receptors in inter-Kingdom interactions.

The major carbohydrate component of *C. albicans* EPS is β-glucans (Al-Fattani & Douglas, 2006). In *C. albicans* biofilms β-glucans are immunodetectable not only within the matrix but also on the hyphal surface (Dongari-Bagtzoglou *et al*., 2009). Since streptococci form 'Corncob–like' structures on hyphae in mixed biofilms both *in vitro* and *in vivo* (Bamford *et al*., 2009; Zijnge *et al*., 2010; Diaz *et al*., 2012b), this suggests that streptococci may benefit from the β-glucan-rich surface provided by hyphae in the biofilm growth state. It is therefore conceivable that glucan binding proteins of certain streptococci have both α- and β-glucan-binding properties that mediate such interactions, since these proteins are known to have multiple functions [for example glucan-binding protein D also has lipase activity (Shah & Russell, 2004)].

Inter-Kingdom signaling

During formation of polymicrobial biofilm communities streptococcal and *Candida* cells communicate by using their own 'languages', consisting of chemical signal molecules or metabolites that alter microbial behavior. Quorum-sensing systems are well-known communication systems in bacteria and fungi. Bacteria produce and secrete chemical signal molecules termed autoinducers to modulate cell behavior within communities (Miller & Bassler, 2001). There are at least two types of autoinducers, autoinducer 1 (AI-1) mediating intra-species communication, and autoinducer 2 (AI-2) serving as universal language involved in inter-species communication (Miller & Bassler, 2001; Vendeville *et al*., 2005). AI-2 is a byproduct of the activated methyl cycle and the *luxS* gene encodes the protein precursor responsible for its production (Vendeville *et al*., 2005). Streptococcal species possess a homologue of the *luxS* gene and a *S. gordonii luxS* mutant is unaffected in planktonic growth or monospecies biofilm formation (McNab *et al*., 2003). However, *luxS* mutants of *S. gordonii* fail to form mixed biofilms with *Porphyromonas gingivalis* (McNab *et al*., 2003). Along the same lines, the biofilm mass of *C. albicans* mixed with a *S. gordonii luxS* mutant is reduced, and interestingly *C. albicans* produces shorter hyphae, suggesting a role of this bacterial gene product in inter-Kingdom communication (Bamford *et al*., 2009).

Farnesol, the first quorum-sensing molecule identified in eukaryotes, accumulates abundantly in *Candida* biofilms to inhibit hyphal growth (Hornby *et al*., 2001). Farnesol also blocks yeast to hyphae transition induced by a variety of environmental cues such as serum, *N*-acetylglucosamine, and glucanase (Biswas *et al*., 2007; Sudbery, 2011; Xu *et al*., 2013a). As a consequence, farnesol downregulates a number of hyphal genes, including ALS3 and heat-shock protein 90 (Uppuluri *et al*., 2007). AI-2 of *S. gordonii* relieves the repression of *Candida* hyphae and biofilm formation triggered by farnesol, further supporting the idea that *C. albicans* responds to this signal (Bamford *et al*., 2009). Hence by relieving farnesol's hyphal repression, AI-2 may indirectly increase hyphal virulence gene expression and increase fungal pathogenicity. On the other hand, farnesol can decrease the accumulation of EPS in *S. mutans* biofilms, without affecting bacterial cell viability, so potentially reducing the cariogenic potential of this organism (Koo *et al*., 2003).

Fatty acid diffusible signal factors (DSF) were recently identified as interspecies small signaling molecules that are structurally homologous to *cis*-11-methyl-2 dodecanoic acid (*cis*-DA). DSF were first identified in *Xanthomonas campestris* and were implicated in biofilm dispersal and full virulence of this organism (Barber *et al*., 1997; Dow *et al*., 2003). The synthesis of DSF is dependent on the RpfF protein while a two-component system (sensor kinase) RpfC/(regulator) RpfG is implicated in DSF perception. DSF of *Stenotrophomonas maltophilia* can be detected by *Pseudomonas aeruginosa* PA sensor kinase and

influences dual-species biofilm structure, supporting a role in communication between the two species (Ryan *et al.*, 2008). Similarly, these molecules play a role in inter-Kingdom communication since DSF purified from *S. mutans* cell culture supernatants inhibit yeast-to-hyphal transition of *C. albicans* and suppress HWP1 gene expression. *Streptococcus mitis*, *S. oralis* and *S. sanguinis* also produce DSF-type activity (Vilchez *et al.*, 2010).

Streptococcus mutans produces another quorum-sensing molecule known as competence-stimulating peptide (CSP). CSP is encoded by the *comC* gene, which is under the regulation of ComD and a two-component regulatory system (Pestova *et al.*, 1996). CSP not only inhibits *Candida* germ tube formation but also stimulates the hyphae-to-yeast transition (Jarosz *et al.*, 2009). In conclusion, the net result of signaling between *Candida* and streptococci in mixed biofilms is not always the same but bound to be dictated by environmental conditions, population densities and the streptococcal species participating in these communities.

Metabolic interactions

Oral streptococci produce lactic acid as an end product of their carbohydrate fermentation process, which can contribute to a rapid decline in the environmental pH. Extracellular pH can modulate *Candida* hyphal growth, and in turn, biofilm formation. *Candida albicans* grows in the yeast form at acidic pH and in the hyphal form at alkaline pH (Calderone, 2002). PHR1 and PHR2 (pH-Response) are two pH-regulated genes in *C. albicans* that encode homologues of *S. cerevisiae* GAS1, predicted to be anchored to the plasma membrane (Fonzi, 1999). *PHR1* expression is repressed at pH value <5.5 whereas *PHR2* is repressed at pH $>$ 6. Both *PHR1* and *PHR2* mutants exhibit a pH-conditional morphological defect (Saporito-Irwin *et al.*, 1995; Muhlschlegel & Fonzi, 1997). Hence, in theory, by decreasing the environmental pH as a result of the fermentative process, streptococci may repress *PHR1* gene expression and increase *PHR2* expression leading to inhibition of the yeast-to-hyphae transition. However, under glucose-limiting conditions, *C. albicans* has the ability to neutralize the environmental pH and proceed with hyphal morphogenesis by excreting ammonia into the environment (Vylkova *et al.*, 2011).

Viridans group streptococci generate hydrogen peroxide under standard laboratory growth conditions. In fact *S. gordonii* spent culture medium contains up to 0.15 mM H_2O_2. Genotoxic and oxidative stress mediated by streptococcal H_2O_2 can stimulate filamentous growth in *C. albicans* (Barnard & Stinson, 1996; Nasution *et al.*, 2008). The H_2O_2 released by MGS may also increase catalase gene expression in *C. albicans*, which in turn may protect the organism from neutrophil oxidative killing, in a model similar to that proposed to exist in the pathogenic synergy between *S. gordonii* and *Aggregatibacter actinomycetemcomitans* (Ramsey *et al.*, 2011).

As in any well-regulated community system with checks and balances, metabolic signals in polymicrobial communities can promote or repress activities of its member species, control population growth, and manipulate the community hierarchical structure with the establishment of 'leader' and 'follower' or 'accessory' species. The end result of these metabolic interactions for the microorganism community is a homeostatic state that extends the life of the community as a whole. For the host the end point is a beneficial, symbiotic or pathogenic relationship with the microbial community. The remainder of this review will concentrate on *Candida*–streptococcal interactions contributing to a pathogenic relationship with the host.

VIRULENCE MODELS OF *CANDIDA* AND STREPTOCOCCAL POLYMICROBIAL INFECTION

There is an abundance of *in vivo* models to study virulence and pathogenic mechanisms of *C. albicans* mono-infection. These include insects, fruit flies, nematodes, zebrafish, mice and rats (Cotter *et al.*, 2000; Alarco *et al.*, 2004; Hamamoto *et al.*, 2004; Hanaoka *et al.*, 2008; Meeker & Trede, 2008; Pukkila-Worley *et al.*, 2009; Johnson *et al.*, 2012; Solis & Filler, 2012). Larger mammals such as macaques, piglets, rabbits and guinea pigs have also been used (Fransen *et al.*, 1984; Filler *et al.*, 1991; Steele *et al.*, 1999; Andrutis *et al.*, 2000). Mice have been useful in the development of mucosal, intravenous and gastrointestinal dissemination models (MacCallum & Odds, 2005; Clemons *et al.*, 2006; Koh *et al.*, 2008; Dongari-Bagtzoglou *et al.*, 2009) (also see review by MacCallum, 2012). Similarly, the virulence and pathogenetic mechanisms of an α-hemolytic *Strepto-*

coccus, phylogenetically very closely related to some MGS (*S. pneumoniae*) have been studied in mouse, rat and rabbit models (Chiavolini *et al.*, 2008). Mixed infections with this pathogen have also been modeled *in vivo*. For example, mouse models of pneumonia and otitis media have been used to study the synergistic effect of *S. pneumoniae* with non-typeable *Haemophilus influenzae* (Ratner *et al.*, 2005; Ramsey & Whiteley, 2009). Rodent models have also been used to study the cariogenicity of *S. mutans* (Bowen *et al.*, 1988, 1991), or the oral colonization capabilities of *S. gordonii* (Tanzer *et al.*, 2001). However, with the exception of *S. mutans*, no other oral streptococcal species has shown virulence properties in a rodent model of single oral infection, even when animals were immunocompromised and inoculated with a high number of organisms (Xu *et al.*, 2013b).

With the possible exception of virally triggered diseases, most oral infectious diseases are polymicrobial in nature or require 'cooperation' by multiple microbes, because they develop in oral habitats that harbor a vast number of different bacterial and fungal species. Despite this central axiom, animal models of oral polymicrobial diseases are scarce, and models of mixed infection have only recently begun to emerge. Because of the lack of significant virulence properties when inoculated on their own, the role of MGS as 'accessories' to established pathogens was recently tested in two mixed infection models. Ramsey and colleagues used a mouse abscess model to study co-infection with *A. actinomycetemcomitans* and *S. gordonii* (Ramsey *et al.*, 2011). An oral co-infection mouse model has also been used to study alveolar bone loss caused by *P. gingivalis* in the presence of *S. gordonii* (Daep *et al.*, 2011). In both studies this MGS species was implicated in enhanced pathogenicity of the primary oral pathogens (*A. actinomycetemcomitans* and *P. gingivalis*) and so the term 'accessory pathogen' was introduced to describe this organism (Whitmore & Lamont, 2011).

The first mixed *Candida*–bacterial (*Escherichia coli*) co-infection model was described in the 1950s (Gale & Sandoval, 1957) and has been followed by very few studies ever since. Recently, Peters & Noverr (2013) developed a murine model of peritonitis by co-infecting mice with *C. albicans* and *Staphylococcus aureus*. Using this model, the investigators found that co-infection of mice can lead to 40% mortality while single infection with either organism is non-lethal (Peters & Noverr, 2013). A murine oral *Candida*–streptococcal co-infection model was recently described by Dongari-Bagtzoglou and colleagues. In this model, it was shown that the MGS species *S. oralis* can significantly enhance *C. albicans* mucosal pathogenicity in mice immunocompromised with cortisone (Xu *et al.*, 2013b). This resembles the significant enhancement in *P. aeruginosa* pathogenicity in a rodent lung co-infection model where MGS on their own show no virulence attributes (Duan *et al.*, 2003). Finally, in a fruit fly model of polymicrobial gastrointestinal infection oral streptococci, including *S. oralis* and its close phylogenetic relative *S. mitis*, were grouped into three distinct pathogenicity groups: virulent, avirulent and synergistic, i.e. streptococci that alone are not pathogenic but in combination with a pathogen can significantly enhance pathogenicity (Sibley *et al.*, 2008b). Collectively most animal models of mixed infection have so far consistently demonstrated that, although MGS lack significant virulence properties on their own, they can promote the virulence of established bacterial and fungal oral opportunistic pathogens.

MECHANISMS OF SYNERGISTIC VIRULENCE IN *CANDIDA* AND STREPTOCOCCAL POLYMICROBIAL INFECTIONS

Microorganisms can interact to increase each other's pathogenicity in four principal ways: (i) modulation of host responses; (ii) increased virulence; (iii) environmental alterations; and (iv) metabolic interactions. Below, we present a comprehensive discussion of evidence of each of these mechanisms as they pertain to *C. albicans*-streptococcal interactions.

Mixed biofilm growth and virulence gene expression

The mechanisms of synergistic virulence of *Streptococcus* and *Candida* are still not fully understood. One early mechanism of synergy may be at the level of colonization, whereby one species helps others to colonize certain oral surfaces more efficiently. Although most MGS streptococci preferentially colonize tooth surfaces, when a perturbation of the oral microbial flora is present they may colonize mucosal sites in higher numbers. For example, in lactobacilli-free and streptococci-free mice, when orally inoculated,

S. gordonii colonizes the palatal and tongue surfaces in higher numbers compared with in mice with intact oral microbiota (Loach *et al.*, 1994). Similarly the introduction of *C. albicans* to the oral cavity of mice facilitates colonization of mucosal surfaces by *S. oralis* (Xu *et al.*, 2013b). Hence, *C. albicans* creates favorable conditions for mucosal colonization and biofilm growth of these bacteria, in line with recent evidence showing that introduction of *C. albicans* in the gastrointestinal tract of antibiotics-treated mice leads to a preferential re-growth of enterococci (Mason *et al.*, 2012). Because increased *S. oralis* mucosal colonization in the presence of *C. albicans* led to an increased size and frequency of oral lesions (Xu *et al.*, 2013b), it is conceivable that like many opportunistic pathogens, a critical mass of this species, reached in the presence of fungal organisms, is needed to induce pathology. These results dispute the long-held belief that these members of the commensal bacterial microbiota protect the host against mucosal candidiasis (Liljemark & Gibbons, 1973). Similarly, *C. albicans* promoted *Staphylococcus aureus* burdens and pathology in a peritoneal co-infection model (Peters & Noverr, 2013).

A second mechanism of synergy at the biofilm accretion level is when one species modulates the structure of a polymicrobial biofilm to form certain architectural patterns, which may promote biofilm pathogenicity. One such example is the filamentous architecture of *P. aeruginosa* within biofilms mixed with *S. maltophilia*, which is not found in *P. aeruginosa* mono-species biofilms. This structural change was linked to an increased tolerance of *P. aeruginosa* to polymyxins (Ryan *et al.*, 2008). In *C. albicans–S. oralis* mixed biofilms, *C. albicans* hyphae obtain a more uniform vertical orientation against the biofilm substratum surface, both *in vitro* and *in vivo* (Fig. 1). Based on the well-accepted role of hyphal organisms in mucosal invasion (Gow & Hube, 2012), it is tempting to speculate that such an orientation against the oral mucosal surface may be more conducive to tissue pathology and systemic dissemination, as seen in this co-infection model (Xu *et al.*, 2013b).

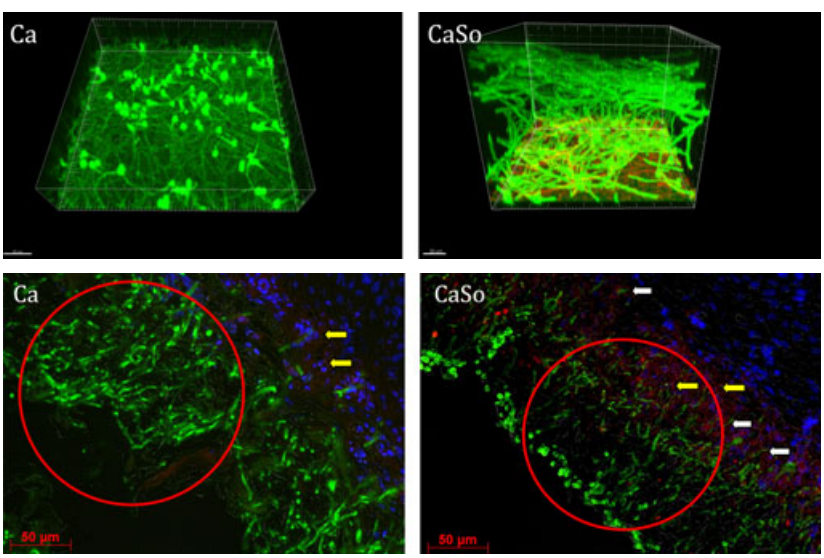

Figure 1 *Streptococcus oralis* modifies *Candida albicans* biofilm structure. Upper panel: *C. albicans* SC5314 biofilms were grown with (CaSo) or without (Ca) *S. oralis* 34 on a glass surface under static conditions at 37°C. After 20 h, *C. albicans* and *S. oralis* 34 were labeled with a fluorescein isothiocyanate-conjugated anti-*C. albicans* polyclonal antibody (shown in green) and a *S. oralis*-specific fluorescence *in situ* hybridization probe labeled with Alexa 546 (shown in red), then observed by confocal scanning laser microscopy. Note the orientation of the hyphae toward the substratum of the dual biofilm. Lower panel: Overlay images of mouse tissue sections showing *C. albicans* biofilms (green) growing on the oral mucosal surface of animals infected with *C. albicans* SC5314 alone (Ca), or *C. albicans* SC5314 plus *S. oralis* So34 (CaSo). Stained in red are neutrophils infiltrating the oral mucosa. Mucosal cells are counterstained with the nucleic acid stain Hoechst 33258 (blue). Sections were stained as described elsewhere (Xu *et al.*, 2013b). Note the difference in hyphal orientation toward the mucosal surface in single (Ca) and mixed (CaSo) infection, associated with more localized mucosal invasion (white arrows), as well as more pronounced neutrophil infiltration (yellow arrows) in the latter.

Finally, in polymicrobial biofilms gene expression patterns of organisms can be mutually modulated, which can have an important impact on the virulence of opportunistic pathogens. For example, proteomic analysis of *in vitro Candida–Staphylococcus* co-culture biofilms showed that 27 proteins of *C. albicans* and *Staphylococcus aureus* are significantly upregulated (Peters *et al.*, 2010). Similarly, genome-wide transcriptional analysis shows that about 4% of genes in the *P. aeruginosa* genome are modulated by the presence of MGS strains isolated from cystic fibrosis patients, and that a large portion of upregulated genes are involved in *P. aeruginosa* pathogenesis (Duan *et al.*, 2003). Using *Drosophila* as a surrogate host for polymicrobial infection it was shown that nearly half of the MGS strains isolated from human sputum have pathogenic synergy with *P. aeruginosa*, by altering *Pseudomonas* gene expression without affecting its growth (Sibley *et al.*, 2008a). Since MGS species have been shown to promote *Candida* yeast to hyphae transition under certain *in vitro* conditions (Bamford *et al.*, 2009) (Fig. 2), it is plausible that several virulence genes involved in mucosal invasion are upregulated during these interactions.

Influence on host response through pattern recognition receptors

One principal way by which microorganisms can interact to increase the pathogenicity each of the other is by modulation of host responses. Even with the introduction of more pathogenic species, multiple bacterial species from the commensal microbiota can contribute to mucosal inflammation (Jergens *et al.*, 2007; Hajishengallis *et al.*, 2011) and there are several examples of co-infections where infection with one organism influences the innate host response to the other (Jamieson *et al.*, 2010; Vylkova *et al.*, 2011). One possible molecular mechanism of such interactions is a unilateral or reciprocal upregulation of pattern recognition receptors in the oral mucosa, which drive inflammatory responses to otherwise commensal or opportunistic organisms. It has been postulated that commensal oral microorganisms alone do not trigger host inflammatory responses because a critical density of such receptors is not present on oral epithelia to transmit inflammatory signals. This is supported by the fact that, even when given in high infectious doses, *S. oralis* did not induce an inflammatory response in the oral mucosa (Xu *et al.*, 2013b).

When *S. oralis* is orally co-inoculated with *C. albicans* an exaggerated mucosal inflammatory response ensues (Xu *et al.*, 2013b). The majority of the immune regulatory genes upregulated in the co-infected animals involve genes in the general categories of chemotaxis response, neutrophilic response, cytokine activity and phagocytosis. Interestingly, strong induction of multiple neutrophil-activating cytokines (interleukin-17C, Chemokine (C-X-C motif) ligand 1/CXCL1, macrophage inflammatory protein-2/MIP-2, tumor necrosis factor, interleukin-1α, interleu-

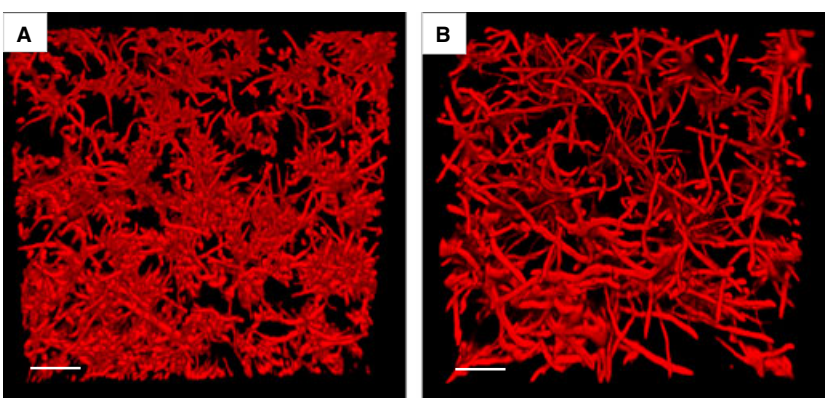

Figure 2 *Streptococcus gordonii* stimulates yeast to hypha transition in *Candida albicans*. The *C. albicans* biofilms were grown with or without *S. gordonii* DL1 expressing green fluorescent protein under flow conditions at 37°C. After 6 h the biofilms were visualized by confocal scanning laser microscopy. The growth medium contained Calcofluor white (1 μg ml^{-1}) to fluorescently label *C. albicans* (shown in red). (A) An underneath image of a *C. albicans* biofilm grown without *S. gordonii*, and (B) an underneath image of a dual species biofilm of *C. albicans* with *S. gordonii*. In panel B the *S. gordonii* component has been subtracted using Volocity® computer software. (Scale bars, 50 μm.)

kin-1β) with concomitant increased neutrophilic infiltration was observed in this model (Fig. 1). Synergistic virulence mediated by an exaggerated host inflammatory response was also observed in mice co-infected peritoneally with *Staphylococcus aureus* and *C. albicans*, with increased expression levels of proinflammatory cytokines that augment a neutrophilic response, including interleukin-6, granulocyte colony-stimulating factor, keratinocyte-derived chemokine, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1. In addition, the cyclooxygenase pathway was implicated in synergistic virulence in this model (Peters & Noverr, 2013).

Gram-positive bacteria-triggered epithelial inflammation is principally regulated by Toll-like receptor 2 (TLR2) (Aderem & Ulevitch, 2000), however, TLR2 plays a secondary role in bacterial clearance, at least in chronic models of infection (Knapp *et al.*, 2004; Burns *et al.*, 2006). In an oral infection model, it was shown that the TLR2 oral mucosal expression levels increased in mice infected with *C. albicans* and that *S. oralis* can signal directly via this receptor to activate a neutrophilic response (Xu *et al.*, 2013b). Oral mucosal epithelial cells normally express low levels of TLR2, but can be stimulated to express higher levels by *C. albicans* (Zhang *et al.*, 2004). Hence, a model of synergy was suggested whereby infection with *C. albicans* increases both the biomass of *S. oralis* and the TLR2 expression to critical levels required for mucosal proinflammatory signaling by this otherwise commensal organism. This was supported by the fact that in $TLR2^{-/-}$ co-infected mouse proinflammatory gene expression and polymorphonuclear cell infiltration were partly attenuated (Xu *et al.*, 2013b). TLR2-dependent epithelial innate immune response genes also augmented the neutrophilic influx in an alveolar mucosa co-infection model with oral streptococci (Sibley *et al.*, 2008a).

Similarly, respiratory co-infection with *H. influenzae* and *S. pneumoniae* in mice leads to increased production of nuclear factor-κB in mouse lung tissue and this synergistic effect is dependent on TLR2 because nuclear factor-κB is not increased in $TLR2^{-/-}$ mice (Lim *et al.*, 2008). The TLR2 expression in the alveolar mucosa is upregulated by *S. pneumoniae*, but can be amplified further by co-infection with *S. pneumoniae* with *H. influenzae*, as seen in the oral co-infection model with *C. albicans* and *S. oralis* (Xu *et al.*, 2013b). Interestingly, TLR2 induction by *S. pneumoniae* and/or *H. influenzae* in murine lung tissues is dependent on the TLR4 receptor (Lim *et al.*, 2008).

Not all oral streptococcal species are involved in increased virulence of primary pathogens or in exaggerated epithelial inflammatory responses. In fact, *S. salivarius* strain K12 antagonizes *P. aeruginosa*-induced interleukin-8 secretion from human bronchial epithelial cells, suggesting a role for commensal streptococci in down-regulating epithelial cell inflammatory responses in the nasopharynx (Cosseau *et al.*, 2008). Hence, there is a great variability in the involvement of oral streptococcal species in oral health and disease, with some species having probiotic properties and others acting as accessories in infectious disease pathogenesis.

Influence on epithelial barrier integrity and invasion

Invasion of epithelial surfaces allows organisms to reach the bloodstream, with potential subsequent escape into deep organs. Gram-positive commensals exert the opposite effects on mucosal epithelial barriers depending on their population size, ranging from promotion of homeostasis in low numbers (Rakoff-Nahoum *et al.*, 2004), to deleterious effects in high numbers (Clarke *et al.*, 2011). Although the resident bacterial biota can play an important protective role against the invasion of pathogens in the lower gastrointestinal tract (Falk *et al.*, 1998), certain members of the MGS promote *C. albicans* breaching of the mucosal barrier in organotypic constructs *in vitro* (Diaz *et al.*, 2012b) and facilitate systemic dissemination of fungal cells *in vivo* (Xu *et al.*, 2013b).

E-cadherin plays a crucial role in epithelial cell tight junctions and in the maintenance of the stratified oral mucosal tissue architecture. Although barrier function regulation in oral epithelium is not well defined, E-cadherin protein integrity is important in different oral models of infection (Katz *et al.*, 2002; Dongari-Bagtzoglou *et al.*, 2009). In addition to causing extensive epithelial cell damage via cytoplasmic invasion (Zhu & Filler, 2010), *C. albicans* uses a paracellular pathway of mucosal invasion via protease-mediated E-cadherin degradation (Villar *et al.*, 2007), in agreement with the reduced E-cadherin protein levels in human oral candidiasis biopsies (Leigh *et al.*, 2004). During the invasion process, hyphae bind to E–cadh-

erin on epithelial cells via the hyphal-specific protein ALS3. Subsequently, hyphae secrete aspartic proteinases that, apart from hydrolyzing tight junction proteins, bind to integrins on the epithelial surface and induce apoptosis, further expediting the breach of the mucosal barrier (Villar *et al.*, 2007; Parnanen *et al.*, 2010; Liu & Filler, 2011; Wu *et al.*, 2013). This process may be further enhanced or expedited by the synergistic effect of MGS members that can promote yeast to hyphal transition (Bamford *et al.*, 2009).

Epithelial cells are the direct targets of bacterial cytolysins, including Pneumolysin (Ply). Ply is a major toxin of *S. pneumoniae* that belongs to cholesterol-dependent cytolysins that attack cholesterol-rich membranes to form large oligomeric transmembrane pores on cell membranes and kill target cells (Morgan *et al.*, 1994). Increased colonization of the mouse upper respiratory tract of *S. pneumoniae* and *H. influenzae* is dependent on Ply (Ratner *et al.*, 2005). It has been suggested that peptidoglycan of *H. influenzae*, a key mediator of inflammation in this model, can access the epithelial cytoplasm via Ply-induced pores to trigger cytoplasmic inflammatory signaling (Ratner *et al.*, 2005). Sequence analysis suggests that the *S. mitis* genome contains putative virulence factor genes encoding pneumolysin and autolysin that are also found in virulent *S. pneumoniae* strains (Whatmore *et al.*, 2000). Also, an *S. oralis* clinical isolate from endocarditis produces sialidase (Byers *et al.*, 2000), an enzyme that is thought to facilitate breach of mucosal barriers and provide nutritional support to this organism. Hence it is not entirely surprising that these streptococci can disseminate systemically and cause sepsis, especially when the mucosa is injured or disrupted by cytotoxic chemotherapy (Khan & Wingard, 2001). Surprisingly, although the biomass of *S. oralis* 34 on the oral mucosa increases in the presence of *C. albicans*, bacterial breach of the mucosa and systemic dissemination is not promoted (Xu *et al.*, 2013b). This is probably attributed to the large strain to strain and species to species genetic variability in MGS.

Lethal co-infection of mice with the pandemic H1N1 virus (Mex09) and *S. pneumoniae* is associated with severe lung epithelial cell damage. Co-infection with Mex09 and *S. pneumoniae* decreases the expression of a number of genes associated with tissue repair or remodeling and cell differentiation, including KGF and Sftpa1 (Kash *et al.*, 2011). Along similar lines, in *S. oralis* 34 and *C. albicans* co-infected mice more severe oral lesions were accompanied by downregulation of a large number of epithelial cell structure genes, including several cytokeratins, (Xu *et al.*, 2013b). Hence streptococcal synergy in tissue pathology and invasion may also be mediated by a compromise in host structural repair pathways.

CONCLUSIONS AND FUTURE DIRECTIONS

Studies of synergistic or antagonistic roles of the oral microbiota in oral health and disease are still in their infancy, despite decades of pioneering, productive

Table 1 Summary of molecular interactions between oral streptococci and *Candida albicans*

Streptococcal molecules	Functional interaction with *Candida*	References
CshA	Binds to unidentified ligand(s) on *C. albicans* to promote coaggregation	Holmes *et al.*, 1996; McNab *et al.*, 1999
SspA/SspB	Bind to hyphae-specific ALS3 cell wall protein of *C. albicans* to promote coaggregation	Jenkinson & Demuth, 1997; Silverman *et al.*, 2010
α-glucans	Synthesized by GtfB or GtfG to promote mixed biofilm accretion with *C. albicans*	Gregoire *et al.*, 2011; Ricker *et al.*, 2014
Autoinducer-2	Relieves farnesol repression of *C. albicans* hyphal growth	Bamford *et al.*, 2009
Competence-stimulating peptide	Inhibits germ tube formation and stimulates hyphae-to-yeast transition	Jarosz *et al.*, 2009
Streptococcus diffusible signal factors	Inhibit yeast-to-hyphae transition	Vilchez *et al.*, 2010
Lactic acid	Decreases pH potentially affects pH- response gene expression	Calderone, 2002; Vylkova *et al.*, 2011
H_2O_2	Oxidative and genotoxic stress, promotes filamentous growth	Barnard & Stinson, 1996; Nasution *et al.*, 2008

research by oral microbiologists. To begin with, animal or complex organotypic *in vitro* models of oral polymicrobial infections are scarce and new ones are urgently needed. One intriguing aspect of microbial commensalism is defining the conditions under which a commensal relationship with the host may be transformed to pathogenic. Dysbiotic changes in the commensal microbiota quantity or composition, accompanied by a distorted mucosal immunological or inflammatory response, appear to play central roles in the development of several polymicrobial infections in the upper and lower gastrointestinal tract. It is possible that a two-step process is needed by 'ordinary commensals' to trigger disease either as accessories to primary pathogens, or as primary opportunistic pathogens themselves: (i) increase in colonization efficiency at oral sites not abundantly colonized in health; and (ii) local mucosal alterations that favor initiation of inflammatory signaling cascades that are not conducive to microbial clearance.

Due to their abundance and plethora of oral habitats, oral streptococci of the MGS are bound to play important roles in both health and disease (Table 1). We propose that the interactions of *C. albicans* with members of this group serve as a prototype of the interactions between opportunistic pathogens with commensals and their influence on oral mucosal homeostasis. A wide range in disease manifestations of oral candidiasis (pseudomembranous, erythematous, hyperplastic) has not been adequately explained by differences in *Candida* virulence or host factors. We therefore further propose that differences in clinical manifestations of this infection may be explained by interactions of this organism with the commensal streptococcal biota that occupies each site. *Streptococcus pneumoniae*, a non-oral member of the MGS, shares an extremely high degree of genetic homology with both *S. mitis* and *S. oralis* (Kawamura *et al.*, 1995). Despite the close genetic relationships between these species, while *S. pneumoniae* can be a severe respiratory pathogen, *S. oralis* and other MGS members have only been implicated in opportunistic bloodstream infections. This major phenotypic difference is intriguing because a whole genome microarray analysis showed that *S. oralis* hybridized to 83% of pneumococcal virulence genes (Johnston *et al.*, 2010). Based on existing evidence we propose that *C. albicans* can trigger a dysbiotic change in *S. oralis* by promoting colonization and growth at mucosal sites that would normally not be a primary habitat for this organism in health. Whether this dysbiotic change is merely a result of favorable coaggregation interactions between these microbes or is secondary to epithelial changes induced by the primary pathogen, *C. albicans*, is still incompletely understood. Inter-Kingdom signaling, metabolic interactions and local environmental changes are all likely to play a role in the development of this polymicrobial mucosal biofilm infection. Modulation of host responses by oral streptococci and increased *Candida* virulence may lead to a pathogenic synergy of these microorganisms in the oral mucosa. As the infection progresses there is a possible fluctuation of the role and contribution of each organism in the infectious process, with one or the other playing central or accessory roles in host responses or tissue pathology, at different disease stages. More investigations are needed into the inter-Kingdom communication systems that have a bearing on virulence gene expression patterns when these organisms co-inhabit new sites. In addition to partaking in dysbiosis and inflammatory changes, MGS and *C. albicans* may regulate pattern recognition receptor expression to multiple other microorganisms that also occupy these sites. Future studies should focus on defining the molecular mechanisms of these complex microbial interactions with the host.

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