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ORIGINAL ARTICLE

The positive impact of *Streptococcus mutans* on the growth of *Candida albicans* within mixed-species biofilms and implications to dental health

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Abstract

Introduction: Candida albicans and *Streptococcus mutans* co-exist in biofilms in the oral cavity. In this study, the impact of *S. mutans* on the growth of *C. albicans* within a mixed-species biofilm was examined. *Materials and Methods:* Single species *C. albicans* biofilms and mixed species biofilms containing *C. albicans* and *S. mutans* at 1:3 and 1:10 ratios were constructed in 6-well microtiter plates. After 24 hours of incubation, the density of resuspended biofilm cells was determined as CFU/ml and used to compare the growth of *C. albicans* in single species and mixed species biofilms. *Results:* The CFU/ml of *C. albicans* in mixed-species biofilms was found to be higher than that in single-species biofilms. *Conclusion: S. mutans* promotes the growth of *C. albicans* in a co-inhabited biofilm.

Keywords: impact; *S. mutans*; growth; *C. albicans*; mixed-species biofilms

INTRODUCTION

Candida albicans typically exists as a commensal fungus in the human body. However, certain conditions, such as disruptions in the microbiota or immune dysfunction, can trigger its transformation into a pathogenic state, leading to infections.[1] These infections, localized in different areas including the skin, the mucosal surfaces of the gastrointestinal tract and the female reproductive tract, can sometimes result in severe invasive diseases associated with high mortality.[2] One important factor contributing to *Candida* infections is the formation of biofilms. These biofilms are commonly found on host cells or on the surfaces of medical devices.[3] *Candida* biofilms are characterised by slow growth and exhibit greater resistance to the environment compared to the free-floating form of the pathogen.[4] These features enable *C. albicans* to thrive within the host, providing it with enhanced opportunities for growth and survival.

Fungal pathogenesis is influenced by a range of activities and factors, with polymorphism playing a significant role.[5] Polymorphism refers to the ability of fungi to exist in different forms, such as yeast or hyphae, and their capacity to switch between these forms. Yeast cells aid in dissemination, while hyphae enable invasion during infections.[6] Another crucial factor is the adaptability of fungi to changing environmental conditions,[7] such as variations in pH levels and the immune status of the host. The fungus possesses the capability to adjust its behavior and morphology in response to these environmental cues. Fungal morphogenesis is also influenced by microbial communication, known as quorum sensing. Through quorum sensing, fungal cells cooperate and coordinate their activities to gain advantages and optimize their survival strategies.[8] This behavior allows them to divide tasks effectively. For instance, yeast cells are responsible for adherence, while hyphae contribute to the establishment of biofilms and invasion, ultimately leading to the success of fungal infections.

Streptococcus mutans is a gram-positive bacterium commonly found in the human oral cavity where it is a key player in the development of dental caries. It possesses the ability to adhere to the tooth surface and produce extracellular polysaccharides that form the matrix of the dental plaque.[9] On the colonized tooth surface,

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particularly in plaque biofilms, it thrives in the presence of fermentable carbohydrates from the diet and causes demineralization of the tooth enamel that leads to the development of cavities. *S. mutans* is often found in association with other oral microorganisms including *C. albicans*, particularly in the context of biofilm formation.

Many studies have shown synergistic interactions between *Candida* species and oral streptococci at mucosal sites, in plaque communities and within biofilms. The co-existence between *S. mutans* and *C. albicans* can lead to an increased ability to adhere to tooth surfaces, enhanced production of virulence factors, and increased resistance to antimicrobial agents, making mixed-species biofilms more difficult to control.[10] Severe lesions in early childhood caries have been attributed to metabolic and glucan-dependent synergism between *C. albicans* and *S. mutans*.[11] Co-infection with *S. mutans* has been reported to promote deep organ dissemination of *C. albicans*.[12]

On the other hand, *C. albicans* has also been found to reduce the cariogenic potential of *S. mutans* [13] and *S. mutans* was shown to modulate biofilm formation as well as attenuate the virulence of *C. albicans*.[14] Santos *et al*.[15] reported *S. mutans*'s inhibition of candidiasis in a murine model.

In this study, we examined the impact of *S. mutans* on the growth of *C. albicans* within a mixed-species biofilm by comparing the colony-forming units (CFU) of *C. albicans* in single-species and mixed-species biofilms containing different ratios of *C. albicans* and *S. mutans*.

MATERIAL AND METHODS

Biofilms were formed using *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. *C. albicans* was cultured on Sabouraud dextrose agar (SDA) (OXOID, UK) and incubated at 37ºC for 24 hours. *S. mutans* was cultured on brain heart infusion agar (BHIA) (Himedia, India) and incubated at 37ºC with 5% CO_2 for 48 hours. After the incubation period, single colonies from both agar plates were inoculated into separate 1:1 mixtures of brain heart infusion broth (BHIB) (Himedia, India) and Sabouraud dextrose broth (SDB) (OXOID, UK) and placed in a shaking incubator set at 37ºC and 250 rpm for 18 hours. The effectiveness of this mixture of BHI broth and SDB in developing biofilm growth was confirmed, while the use of BHI broth with 5% fetal bovine serum was found to be ineffective in cultivating single-species and mixed-species biofilms. This determination was made by observing the formation of biofilms in a 6-well plate using microscopy. The turbidity of the cultures was then measured using a McFarland (McF) densitometer and the colony forming units/ml (CFU/ml) was determined by the Miles and Misra method.[16]

Two sets of mixed species biofilms with cell number ratios of 1:10 and 1:3 for *C. albicans* to *S. mutans* were prepared. These ratios were arbitrarily chosen to represent high and low concentrations of *S. mutans* that could affect *C. albicans* growth. To prepare the mixed-species biofilms of 1:10 cell number ratio, 2 ml each of 5 McF *C. albicans* suspension (1.5×10^7/ml) and 3.5 McF of *S. mutans* suspension (1.5×10^8/ml) were used. For the mixed-species biofilms of 1:3 cell number ratio, the 3.5 McF suspension of *S. mutans* was first diluted three-fold, then 2 ml of the diluted suspension was added to 2 ml of 5 McF *C. albicans* suspension. Four ml of 5 McF *C. albicans* suspension was used to make the single-species biofilms. In all cases, 4 ml of the respective cell suspensions were added to each well of a 6-well plate (Orange Scientific, USA). The biofilms were incubated at 37ºC for 90 minutes, then washed three times with 4 ml sterile phosphate buffered saline (PBS) before 4ml fresh broth was added. Incubation of the biofilms continued at 37ºC for 24 hours.

After the incubation period, the biofilms were washed twice with 4 ml sterile PBS. One ml of PBS was then added into each well containing a biofilm and the biofilms were detached using a cell scraper to facilitate the preparation of the cell suspension in PBS. Biofilms in the 6-well-plates were used to determine the CFU/ml of *C. albicans*. Statistical analysis was conducted using GraphPad software.

RESULTS

The results showed the CFU/ml of *C. albicans* in the mixed-species biofilms to be higher than that in single-species biofilms, particularly at the *C. albicans*: *S. mutans* ratio of 1:3 (Figure 1). An ANOVA test, followed by a Tukey post-hoc test revealed a statistically significant difference between the CFU/ml of *C. albicans* in single-species biofilms and mixed-species biofilms at a ratio of 1:3 ($p<0.05$).

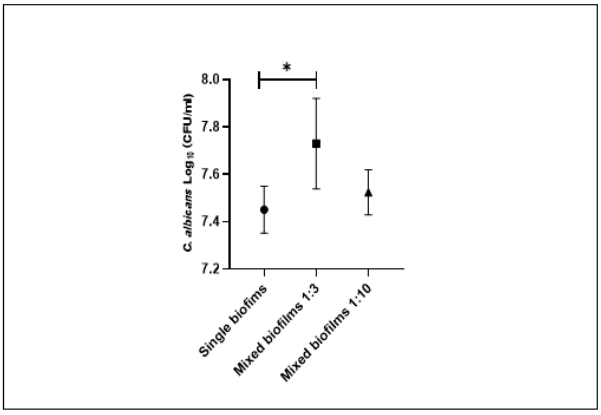

Figure 1. Comparison of *C. albicans* viable counts in single and mixed species biofilms. *p<0.05

DISCUSSION

In a healthy oral cavity, fungi are much less abundant than bacteria but they maintain complex and dynamic relationships with their bacterial counterparts via physical binding, quorum sensing and metabolic exchanges. *C. albicans* is the fungus found most frequently in the oral cavity during dysbiotic disease, whereas *S. mutans* is the dominant bacterial species in dental plaque. The *C. albicans* : *S. mutans* ratio varies in different individuals and under different conditions of health and disease.[17]

The outcome of *C. albicans* and *S. mutans* interaction has been variously described as synergistic or antagonistic in different in vitro and in vivo investigations. In this study, we compared the growth of *S. mutans* and *C. albicans* in single-species and mixed-species biofilms. Both types of biofilms were developed under identical conditions, including media composition, *C. albicans* concentration, and growth conditions. Our hypothesis was that the presence of *S. mutans* in a mixed-species biofilm would impact the growth of *C. albicans* due to alterations in growth conditions. The results showed a significant increase in the CFU/ml of *C. albicans* in biofilms constructed with a *C. albicans* : *S. mutans* ratio of 1:3, suggesting a positive population growth effect of *S. mutans* on *C. albicans* in the mixed-species biofilm. The difference in the amount of enhanced *C. albicans* growth observed for the 1:3 and 1:10 mixtures could be due to nutrients availability for *C. albicans* which would be more in the biofilms made with *C. albicans* and *S. mutans* at 1:3 ratio compared to the biofilms with *C. albicans* and *S. mutans* at 1:10 ratio. Another possible explanation is quorum sensing, a phenomenon known to be influenced by cell numbers and cell density. Different concentrations of cells in the mixed biofilms could trigger distinct signaling pathways to lead to variations in the results. This highlights the possible influence of cell density on communication and associated downstream effects in mixed-species biofilms. Xiao *et al*.[18] conducted a study on the transcriptomes of *C. albicans* and *S. mutans* in cross-kingdom interactions in biofilms and found that virulence genes such as *HWP1*, *ERG4* and *CHT2* exhibited both upregulation and downregulation under different conditions and at different times during biofilm formation. The results from our in vitro model of a dual-species biofilm showed *S. mutans* promoting the growth of *C. albicans*, with the extent of growth promotion being influenced by the *C albicans* : *S.mutans* ratio.

The observations made in this study are compatible with the concept of multifaceted interactions among microbial inhabitants in a biofilm, influenced by the variety and relative quantity of microbial species. The clinical relevance of our *in vitro* observations is not yet clear. The oral cavity is heavily colonized by diverse microbes whose complex interactions and their contributions to disease pathogenicity are still largely unknown. Although our results suggest that decreasing the population of *S. mutans* might prevent the overgrowth of *C. albicans,* this manoeuvre might result in microbiome dysbiosis and other yet unknown adverse sequelae. Further investigations are required to identify the specific mechanisms of microbial synergy or antagonism and to see whether such interactions can be utilized for the development of new strategies for the treatment of oral infections.

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Author contributions: Wang XL performed all experiments; YF Ngeow and SF Yap conceptualized and supervised the study. All authors contributed to the manuscript writing.

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