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Antigen I/II mediates interactions between *Streptococcus mutans* and *Candida albicans*

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SUMMARY

Streptococcus mutans and *Candida albicans* are frequently co-isolated from dental plaque of children with early childhood caries (ECC), and only rarely found in non-ECC children, suggesting that these species interact in a manner that contribute to the pathogenesis of ECC. Previous studies have demonstrated that glucans produced by *S. mutans* are crucial for promoting biofilm formation and cariogenicity with *C. albicans*; however, it is unclear how non-glucan *S. mutans* biofilm factors contribute to increased biofilm formation in the presence of *C. albicans*. In this study, we examined the role of *S. mutans* antigen I/II in two-species biofilms with *C. albicans,* and determined that antigen I/II is important for the incorporation of *C. albicans* into the two-species biofilm*,* and is also required for increased acid production. The interaction is independent of Als1 and Als3 proteins, which are known streptococcal receptors of *C. albicans*. Moreover, antigen I/II is required for the colonization of both *S. mutans* and *C. albicans* during co-infection of *Drosophila melanogaster in vivo*. Taken together, these results demonstrate that antigen I/II mediates the increase of *C. albicans* numbers and acid production in the two-species biofilm, which represents new activities associated with this known *S. mutans* adhesin.

Keywords

Streptococcus mutans; *Candida albicans*; Antigen I/II; Early childhood caries

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INTRODUCTION

Early childhood caries (ECC) remains one of the most common childhood diseases affecting underprivileged children in developed countries [1,2]. *Streptococcus mutans* is one of the most important etiologic agents for ECC [3,4]. *S. mutans* thrives in a cariogenic biofilm with multiple species of bacteria and fungi. The commensal fungus *Candida albicans* is frequently detected in high numbers with *S. mutans* recovered from ECC-affected children, but only sporadically from ECC-free children[5–7]. *C. albicans* is a common colonizer of the oral cavity and an opportunistic pathogen. Previous studies demonstrate that cross-kingdom interactions between *C. albicans* and *S. mutans* enhance co-species biofilms that promote caries development [8,9].

Streptococcus-derived glucosyltransferases (Gtfs) bind to *C. albicans* in an enzymatically active form, thereby facilitating the promotion of the two-species biofilm[10,11] in a sucrose-dependent manner[10,11]. Although much is known about the contribution of exopolysaccharides from *S. mutans* and other streptococci in two-species biofilm development with *C. albicans*, the role of other *S. mutans* biofilm related factors is poorly understood.

The antigen I/II family of adhesins are cell wall-associated polypeptides that are widely distributed on the cell surface of many streptococci[12]. Antigen I/II is not only important for initial streptococcal adhesion to the host, but also for inter-bacterial adhesion and "secondary" colonization [12]. Antigen I/II of *S. gordonii* has been shown to mediate co-aggregation with Agglutinin-like family (Als1 and Als3) proteins on *C. albicans* [13]. However, the role of antigen I/II in the interaction between *C. albicans* and *S. mutans* under sucrose-dependent conditions is unknown. Given the fact that high sugar consumption is an important risk factor for the development of ECC, our goal was to determine how antigen I/II modulates the interaction between *C. albicans* and *S. mutans* in the presence of sucrose.

In this study, we demonstrate that *S. mutans* antigen I/II facilitates the attachment of *C. albicans* to *S. mutans* in the two-species biofilm and concurrently promotes acid production. This interaction is independent of known antigen I/II receptors, *C. albicans* Als1 and Als3 proteins. Moreover, antigen I/II is required for colonization of *C. albicans* during co-infection with *S. mutans* in a *Drosophila melanogaster* sucrose-dependent feeding model. Taken together, these results suggest that antigen I/II is critical for the interaction of *S. mutans* with *C. albicans* under sucrose conditions, which may contribute to heightened pathogenicity during co-infection with these two microbes.

METHODS

Bacterial strains and culture conditions

S. mutans UA159 and *C. albicans* strains used in this study (Table 1) were grown in Todd-Hewitt broth (THB) and yeast extract-peptone-dextrose plus uridine (YPDU) (2% dextrose, 2% bacto peptone, 1% yeast extract, and 80 mg/l uridine), respectively, in an atmosphere of 5% CO_2 at 37°C.

Construction and complementation of the *spaP* mutant

S. mutans' antigen I/II is encoded by *spaP* [14]. PCR ligation mutagenesis was used to construct the *spaP* mutant in *S. mutans* UA159 or UA159-GFP [15,16]. Primer pairs (Table 2) were used to amplify two flanking regions of *spaP*, and overlapping PCR was used to ligate an IFDC2 cassette into the flanking fragments[17]. The resulting PCR product was transformed into *S. mutans* UA159 or UA159-GFP. The *spaP* mutant transformants were selected on 10μg/ml erythromycin THB agar. To complement the *spaP* mutant, the full-length *spaP* gene was cloned into a shuttle vector pVPT-kan to produce *spaP*-pVPT-kan. A complemented strain was obtained by transforming this plasmid into the *spaP* mutant and selected on 1mg/mL kanamycin THB agar plates.

Biofilm formation assay

S. mutans and *C. albicans* single and two-species biofilms were grown in THB containing 1% sucrose. This condition was used to simulate ECC patients challenged with high dietary sugars. In brief, overnight cultures of *S. mutans* and *C. albicans* were sub-cultured into fresh THB and YPDU, grown to an OD_{470} of 0.6 and OD_{600} of 0.65, respectively, diluted 1:100, and aliquoted (200 μL) into a 96-well microtiter plate, and grown for 16 h at 5% CO_2 at 37 °C under static conditions [18]. Other growth conditions were also tested, whereby biofilms were grown in RPMI-1640 medium (Sigma) to promote biofilm formation of *C. albicans*. Biofilm biomass was measured at OD_{562} using crystal violet as described previously [19]. Each experiment was performed in duplicate and replicated three times.

Quantification of Colony Forming Units (CFU) of *S. mutans* and *C. albicans* in co-culture

Biofilms were gently washed with PBS to remove planktonic cells. *S. mutans* and *C. albicans* cells from mono-species or two-species biofilms were scraped from the bottom of each well and vortexed with 200 uL of PBS. Samples were sonicated in a water bath for 15 seconds and 30 seconds, alternately, 5 times. This treatment ensured the disruption of bacterial aggregates. The biofilm suspensions were serially diluted with PBS, plated on THB agar (total bacteria), in addition to selective media for *S. mutans* (Gold's media) and *C. albicans* (CHROMagar™), and incubated at 37°C with 5% CO_2. CFUs were counted after 24 h.

Fluorescence microscopy and confocal laser scanning microscopy (CLSM) analysis

S. mutans-GFP and *C. albicans* single and two-species biofilms were grown in an 8 well microscope slide (ibidi) under 5% CO_2 at 37°C for 16 h [15,20]. The biofilms were gently washed with PBS three times to remove any unattached cells and stained with calcofluor white to label *C. albicans* (Sigma-Aldrich). Biofilms were grown with either 1μM dextran-conjugated pHRodo red or dextran-conjugated Cascade Blue (Molecular Probes, Invitrogen) to monitor pH changes or glucan production, respectively[21]. The stained biofilms were examined using fluorescence microscopy and CLSM as reported [22]. Three independent experiments were performed, and the images displayed are representative of all studies. Bio-volume of biofilms were quantified by the program COMSTAT[23,24]. ImageJ was used to quantify fluorescence of acid (pHRodo red) and glucan (cascade blue) production.

Colonization of *Drosophila melanogaster*

Infection of *Drosophila* was performed as previously described[18, 22, 25]. This model was used to simulate sucrose-dependent bacterial colonization[18, 22, 25]. Briefly, mid-log phase wild-type *S. mutans*, *spaP* mutant, and *C. albicans* cells were harvested and re-suspended in 5% sucrose and adjusted to an OD_{600} of 2.5. The re-suspended cells (100 μL) were used to infect flies as previously described[18,22,25]. Flies were ground in an Eppendorf tube with 100 uL of PBS 2 days after infection with pipette tips and serial dilutions of the homogenates were plated onto THB agar for CFUs.

Statistical analysis

All data are expressed as mean±SD. Data were analyzed using the *t*-test. Differences were considered significant if $P < 0.05$.

RESULTS

Establishment of the *in vitro S. mutans* and *C. albicans* two species biofilm model

ECC is characterized as a carbohydrate-induced infectious disease, and high consumption of sucrose has been associated with an increased prevalence of ECC [26]. Accordingly, a sucrose-dependent biofilm model was utilized to mimic the cariogenic conditions associated with the oral cavity of ECC children. First, we established the biofilm time course in THB media with 1% sucrose using time points of 6, 12, 16, and 24 hours. In addition, wild-type *S. mutans* and the *spaP* mutant were compared to examine the role of antigen I/II of *S. mutans* in the two-species biofilms with *C. albicans*. Single-species biofilms of *S. mutans* and *C. albicans* were used as controls. There was no significant difference in the single-species biofilms between the wild-type, *spaP* mutant, or *spaP* complemented *S. mutans* (Fig. 1A). Total biofilm biomass significantly increased in the two-species biofilm consisting of *S. mutans* UA159 and *C. albicans* SC5314; however, this increase was abolished by the loss of *spaP* in *S. mutans,* and partially restored in the *spaP* complemented strain (Fig 1A), suggesting antigen I/II encoded by *spaP* is required for promotion of the two species biofilm. Biofilms grown at 6, 12, 16, and 24 hours showed similar trends in biofilm biomass for each strain in either single or dual species biofilms (Fig 1A). However, 24 hour-biofilms exhibited extremely dense biofilms due to the rapid development and maturation of biofilms under the rich medium, which made it difficult to analyze. As a result of these preliminary studies, we selected 16 hour biofilms for further *in vitro* studies. Consistent with data from previous studies using similar growth conditions, *C. albicans* did not form single species biofilms (Fig 1A) [27]. However, in RPMI-1640 medium, which supports the development of *C. albicans* biofilms, *C. albicans* formed robust single-species biofilms, but *S. mutans* biofilm formation was poor (Fig S1). This result does not represent the physiological conditions of ECC, since *S. mutans* is the primary colonizing microbe in cariogenic biofilms of ECC[26]. Clinical studies show a higher ratio of *S. mutans* than *C. albicans* in the dental plaque and saliva of children with severe early childhood caries (S-ECC)[28]. Therefore, we opted to use THB in order to form better *S. mutans* biofilms, which more closely represents the conditions of ECC.

S. mutans antigen I/II facilitates the incorporation of *C. albicans* in the two-species biofilm with *S. mutans*

To further characterize the dual species biofilm, and evaluate the contribution of *S. mutans* and *C. albicans* in the dual biofilm, we quantified each organism by colony-forming-units (CFUs). Neither wild-type *S. mutans*, the *spaP* mutant, nor the complemented strain displayed increased CFUs with the addition of *C. albicans* (Fig 1B). In contrast, significant numbers of *C. albicans* (CFUs) were detected in wild-type *S. mutans* biofilms, but drastically decreased in the *spaP* mutant biofilms (Fig 1C). As shown by the CFU data, the number of *S. mutans* is 100 times more than *C. albicans* in the co-species biofilm, which is similar to the ratio of the two species detected in S-ECC children[28]. We furthered examined single and two-species biofilms using CLSM. Structural analysis revealed that *S. mutans* forms larger aggregates when co-cultured with *C. albicans* compared to the single *S. mutans* biofilm (Fig 2A). In addition, *C. albicans* cells were predominantly clustered together and interspersed throughout the two-species biofilm, but did not directly co-localize with *S. mutans*. Similar to wild-type *S. mutans*, the *spaP* mutant also formed larger aggregates in the two-species biofilm with *C. albicans* compared to a single species biofilm (Fig 2A). However, loss of *spaP* resulted in fewer *C. albicans* present throughout the biofilm (Fig 2C). These data suggest that *C. albicans* can alter the structure of the two-species biofilm and *S. mutans* antigen I/II supports the incorporation of *C. albicans* into the biofilm with *S. mutans*.

Loss of *C. albicans* Als1 and Als3 does not affect dual-species biofilms with *S. mutans*

Als1 and Als3 proteins in *C. albicans* directly interact with antigen I/II-like protein, SspB, in *S. gordonii*, therefore, we tested whether single or double mutations in *als1* and *als3* would impact the two-species biofilm formation with *S. mutans*. As shown in Fig 3, loss of *als1*, *als3*, or both *als1* and *als3* did not affect biofilm formation with *S. mutans,* suggesting that Als1 and Als3 proteins are not required for *C. albicans* to interact with *S. mutans* in the presence of sucrose.

Acid production is promoted in the two-species biofilm

Acid production is critical for caries pathogenesis [29]. To determine if the presence of antigen I/II influences the biofilm pH, we used a dextran-conjugated pH probe (red) to monitor pH changes in single and two-species biofilms of wild-type and *spaP* mutant *S. mutans* in the presence of *C. albicans*. Fluorescence microscopy showed an increase in fluorescent intensity in the *S. mutans* and *C. albicans* two-species biofilm, indicative of a more acidic condition (Fig 4A and 4B). However, the increase in acidity was reduced in the *spaP* mutant two-species biofilm. Notably, there was no significant difference in fluorescent intensity between the wild-type and *spaP* mutant single species biofilms, suggesting antigen I/II has no effect on acid production in *S. mutans* (Fig 4A and 4B). Unexpectedly, in the two-species biofilm, the fluorescence of *S. mutans* was weaker than observed in the single *S. mutans* biofilm despite the comparable numbers of bacteria detected by CFUs (Fig 1C and 1D). This phenomenon is likely due to the fact that *S. mutans* cells were clustered and partially masked by the dense *C. albicans* population. Glucan is another key factor that helps *S. mutans* form biofilms and promotes the development of caries[30]. Therefore, a specific dextran-conjugated probe for *S. mutans* glucan was used to monitor changes in glucan production in the single

and two-species biofilms. There was no difference in the *S. mutans* glucan (blue) between single and two-species biofilms (Fig 4C and 4D). These results suggest that factors other than the presence of *S. mutans* glucans impact acid production within the two-species biofilms.

Antigen I/II is critical for colonization of *C. albicans* during co-infection with *S. mutans* in *Drosophila melanogaster*

To determine if antigen I/II influences the colonization of *S. mutans* and *C. albicans in vivo*, we employed a widely used *Drosophila* sucrose-dependent colonization model. Flies were infected with mid-log phase cells of wild-type *S. mutans*, *spaP* mutant, *C. albicans*, and *C. albicans* with either wild type *S. mutans* or the s*paP* mutant, respectively. There was no difference in colonization between wild-type *S. mutans* and the *spaP* mutant in single species infection. The two-species infection did not enhance colonization of wild-type *S. mutans* compared to the single *S. mutans* infection. However, colonization by the *spaP* mutant in the co-infection was significantly inhibited compared to the single *spaP* infected group (Fig 5A), suggesting a potential synergistic effect between *C. albicans* and antigen I/II *in vivo*. *C. albicans* colonized *Drosophila* at a low level in the single species *C. albicans* infection and during co-infection with the *spaP* mutant. Importantly, colonization of *C. albicans* significantly increased approximately fifteen-fold during co-infection with *S. mutans* (Fig 5B). These data demonstrate that antigen I/II supports the colonization of both *S. mutans* and *C. albicans* during co-infection in *Drosophila in vivo*.

DISCUSSION

C. albicans and *S. mutans* often coexist in pathogenic oral biofilms in ECC children; however, the underlying mechanism regarding their interaction is poorly understood. In our study, *C. albicans* and *S. mutans* formed enhanced two-species biofilms compared to a single *S. mutans* species biofilm *in vitro* and promoted the colonization of *C. albicans in vivo*. Interestingly, *S. mutans* cell number did not change between single and two-species biofilms *in vitro*, and only *C. albicans* numbers were increased in the two-species biofilms. However, the presence of *C. albicans* altered the *S. mutans* biofilm structure, which forms bigger aggregates, and may promote cariogenic potential. Prior studies have reported that *C. albicans* promotes the growth of *S. mutans* in the two-species biofilm, thus enhancing the biofilm formation[8]. In contrast, other studies reported that *C. albicans* and *S. mutans* display no synergistic effect, and some reports even document that *S. mutans* inhibits *C. albicans* growth and hyphae formation[31–34]. Different results may be due to the use of different biofilm conditions. In our model, we observed that many *C. albicans* cells were in yeast or pseudohyphae form (Fig 2), which is consistent with previous studies that demonstrate in low pH environments, *C. albicans* is typically in yeast or pseudohyphae form compared to hyphae form[35]. From a physiological standpoint, the yeast form of *C. albicans* may be more representative of *C. albicans* morphology under acidic, cariogenic conditions in the oral cavity. *S. mutans* dominating in co-species biofilms with fewer *C. albicans* is also consistent with clinical findings from ECC children[28]. Therefore, the model in our present study better mimics the conditions of cariogenic biofilms in ECC children. Overall, our studies demonstrate the dynamic interactions that exist between *C. albicans* and *S. mutans*. Our

findings represent a previously unknown cooperative interaction between *C. albicans* and *S. mutans*.

Sucrose is an important factor in two-species biofilms of *S. mutans* and *C. albicans*[29]. In the presence of sucrose, Gtfs, particularly GtfB, bind to the surface of *C. albicans* to produce large amounts of glucan, which provide strong binding sites for *S. mutans* [36]. Moreover, mannan of *C. albicans* was also involved in the binding to *S. mutans* GtfB[27]. However, additional factors important for the two-species interactions are unclear. In this study, we demonstrate that antigen I/II mediates the interaction between *S. mutans* and *C. albicans*. Loss of *spaP* coding for antigen I/II resulted in a significant reduction of *C. albicans* in the two-species biofilms both *in vitro* and *in vivo*. Interestingly, there was no change in the number of the *spaP* mutant cells recovered from the two-species biofilm *in vitro*, while the number of the *spaP* mutant cells were significantly decreased when co-infected with *C. albicans in vivo*. The reason for this inconsistency between our *in vitro* and *in vivo* data is unknown. It is plausible that the *Drosophila* immune response that facilitates clearance or reduced colonization of *C. albicans* in the absence of *spaP*, also simultaneously clears the *spaP* mutant during co-infection. Such clearance is protected by the two-species biofilm and the activity of antigen I/II. In fact, antigen I/II has been shown to be a potent immunomodulatory factor [12].

Antigen I/II family proteins are widely distributed on the cell wall of many streptococci and contain various adhesion epitopes associated with the interaction with other species [12]. The antigen I/II-like protein of *S. gordonii*, SspB, mediates adhesion with *C. albicans* via binding to *C. albicans* Als3 proteins[13,37]. However, *C. albicans* Als1 and Als3 proteins were not required for two-species biofilm formation between *C. albicans* and *S. mutans* in our current study. Als3 is a hyphae-specific protein, and therefore, is expressed on *C. albicans* hyphae and pseudohyphae, but not on yeast form[38,39]. Most of Als1 is localized on the tube and hyphae of *C. albicans*[40]. In the co-species biofilm, *C. albicans* were generally yeast and pseudohyphae, which explains why Als1 and Als3 are not involved in the interaction between *S. mutans* and *C. albicans* in our biofilm model. Although Als1 and Als3 have been reported to be important for the interaction with *S. gordonii*, *C. albicans* harbors six additional Als proteins (Als2, Als4, Als5, Als6, Als7, and Als9). Therefore, we do not rule out the possibility that other Als proteins may play a role in the interaction with *S. mutans*[41].

S. mutans glucan has been reported as a key factor for the interaction between *S. mutans* and *C. albicans* [27]. Interestingly, we did not observe any changes in *S. mutans* glucan production, suggesting there is an unknown factor involved in our model system. It was noteworthy that the two-species biofilms with *S. mutans* and *C. albicans* exhibited a lower pH compared to single *S. mutans* biofilms. Acidogenicity is critical for the pathogenesis of *S. mutans* in ECC children [8]. Increased production of acid in the *S. mutans* and *C. albicans* two-species biofilm would create a more cariogenic biofilm and promote the development of caries in ECC children. Both acid and glucan production are products of carbohydrate metabolism[42]. Glucan is the primary extracellular polysaccharide (EPS) of *S. mutans* and helps form the complex 3D architecture that allows the accumulation of acid[30]. However, in the present study, glucan production by *S. mutans* was not affected by *C. albicans*, suggesting that the change between wild-type *S. mutans* and the *spaP* mutant co-species biofilm is not

dependent on the function of glucosyltransferases that synthesize glucan. Alternatively, *C. albicans* may contribute to acid production. It has been reported that *C. albicans* can produce and tolerate acid, which can support the development of caries *in vivo* [43–44]. Of course, the hypothesis that *C. albicans* produces acid in a dual-species biofilm still needs further investigation.

In summary, our studies reveal a previously unknown dynamic interaction between *S. mutans* and *C. albicans*, and this activity is dependent on a well-known adhesin, antigen I/II. Our data support the notion that co-infection with *S. mutans* and *C. albicans* within the oral biofilm community may heighten pathogenicity by enhancing biofilm formation and acid production. Molecular mechanisms uncovered for the synergy exhibited between *S. mutans* and *C. albicans* would be beneficial for the design and development of targeted therapeutics that interrupt the pathogenesis of ECC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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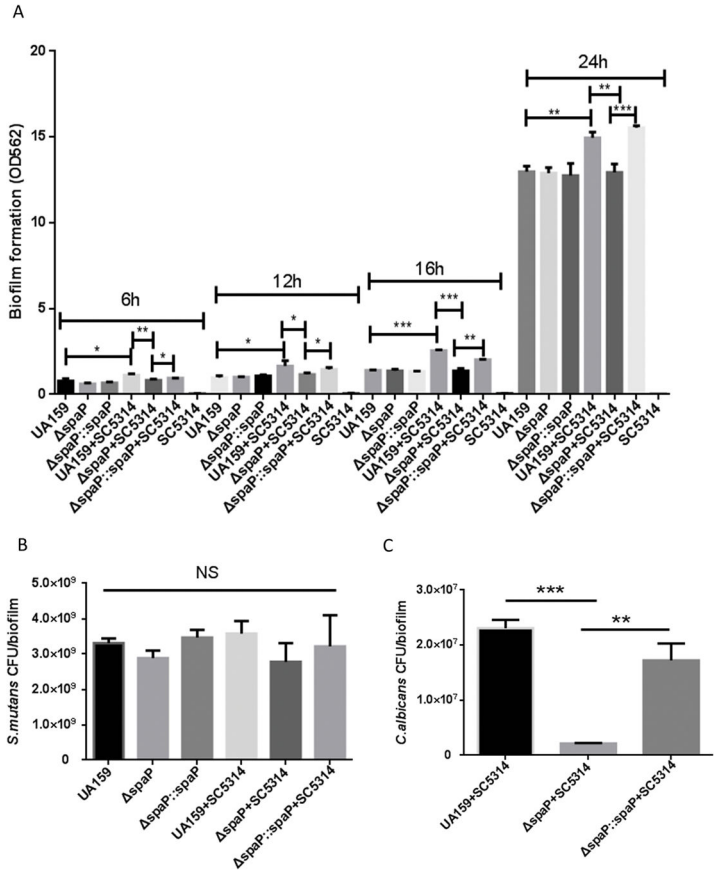

Figure 1. Antigen I/II promotes the adherence of *C. albicans* in mixed species biofilms with *S. mutans*

A. Crystal violet quantification of single and two-species biofilm biomass of wild-type *S. mutans*, *spaP* mutant, and *C. albicans* at 6, 12, 16, and 24 hours in THB and 1% sucrose B. CFU quantification of *S. mutans*, *spaP* mutant in single and two-species biofilms. C. CFU quantification of *C. albicans.* *(P<0.05), ** (P<0.005), *** (P<0.001),

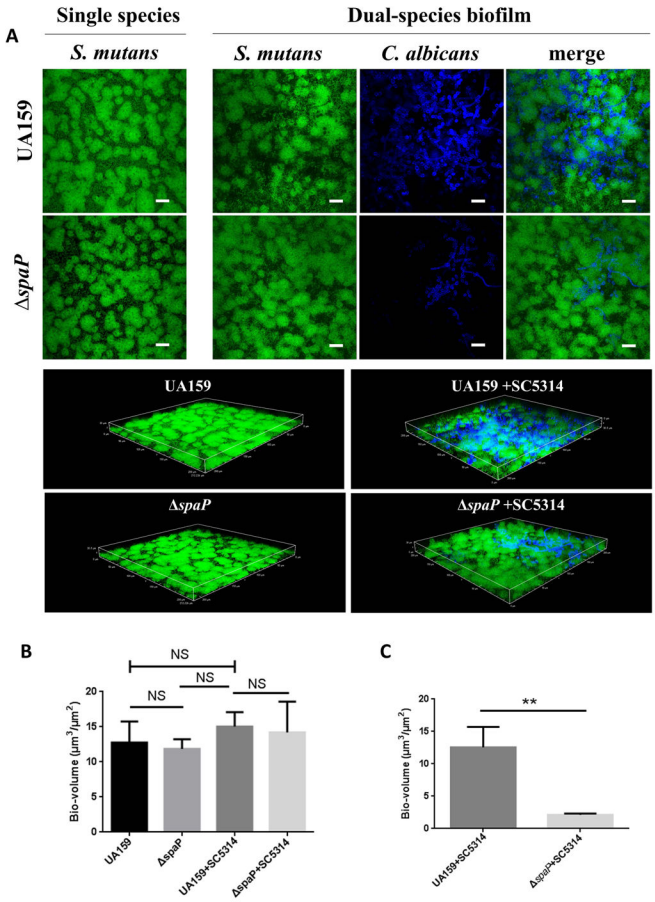

Figure 2. Confocal laser scanning microscopy studies of *S. mutans* and *C. albicans* single and co-species biofilms

A. Confocal scanning laser microscopy images of wild-type *S. mutans*, *spaP*, and *C. albicans* single and two-species biofilms at 60X magnification. *S. mutans* was labeled with green fluorescent protein (GFP) and *C. albicans* was stained with calcofluor white. Scale bar: 20 μM. All biofilms were grown for 16 hours in THB and 1% sucrose. B. COMSTAT2 analysis of biovolume in confocal images of *S. mutans*. C. COMSTAT2 analysis of biovolume in confocal images of *C. albicans*. ** (P<0.005), NS, not significant.

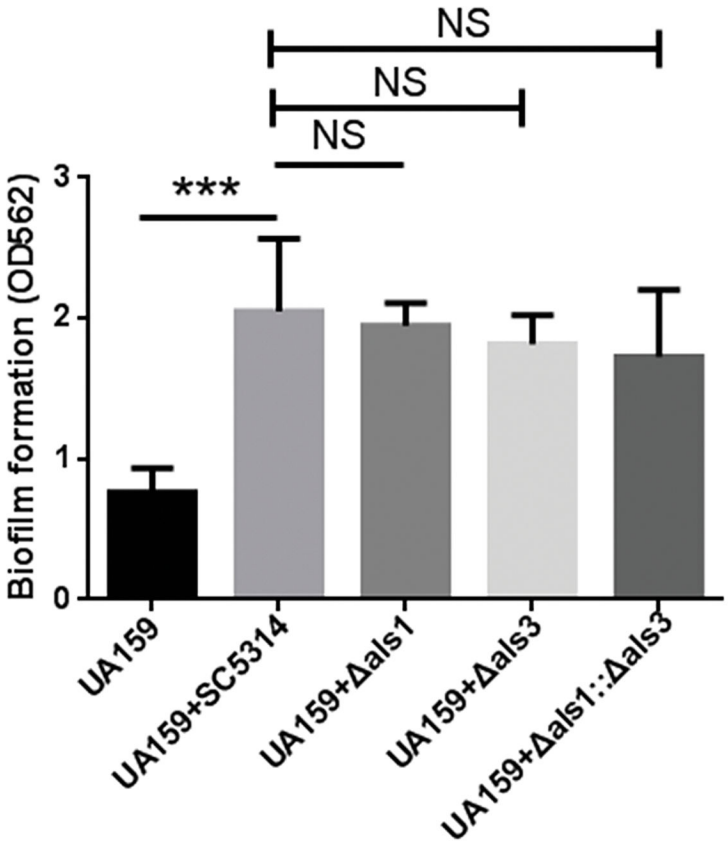

Figure 3. Als1 and Als3 do not play a role in the interaction between *S. mutans* and *C. albicans*

Crystal violet quantification of single and two-species biofilms of wild-type *S. mutans*, wild-type *C. albicans, als1* mutant, *als3* mutant and double mutant. NS(P>0.05).

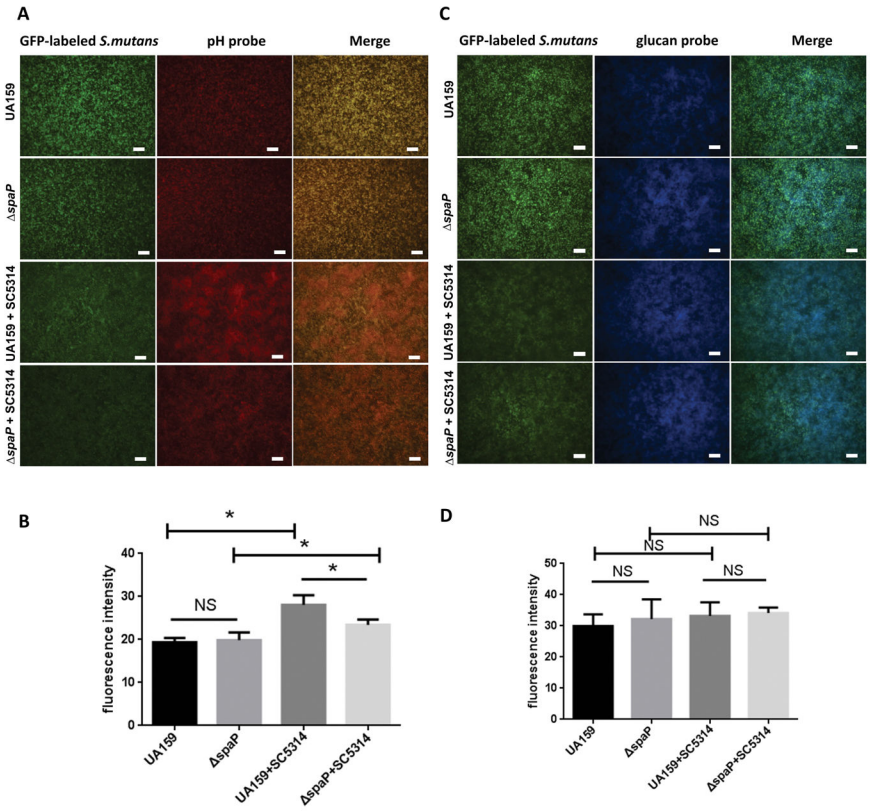

Figure 4. Acid production is promoted in the two-species biofilm of *S. mutans* and *C. albicans*.

A. Fluorescence microscopy images of wild-type *S. mutans*, *spaP*, and *C. albicans* single and mixed species biofilms at 10X magnification. *S. mutans* was labeled with green fluorescent protein (GFP) and acid production was monitored using a dextran-conjugated pHrhodo red probe. Scale bar: 200 μM. B. ImageJ analysis of fluorescence of pHrhodo red probe in 'A'. C. *S. mutans* glucan was monitored with cascade blue. Scale bar: 200 μM. D. ImageJ analysis of fluorescence of cascade blue in 'C'. *(P<0.05), NS, not significant.

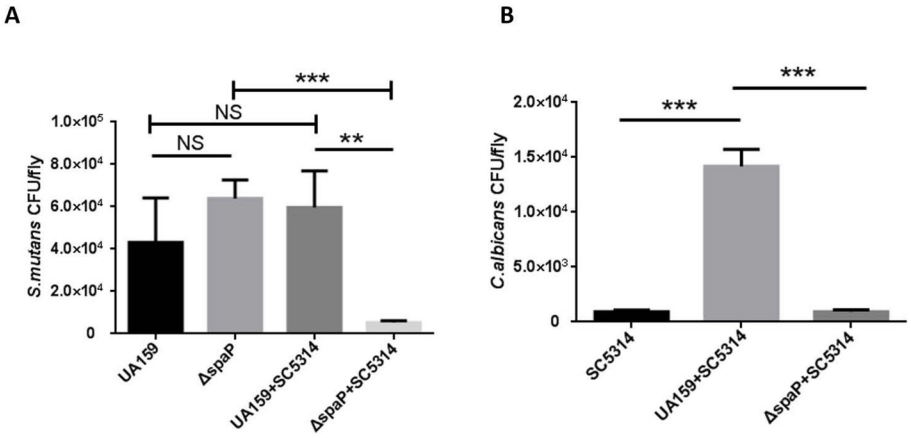

Figure 5. Antigen I/II is required for the colonization of both *S. mutans* and *C. albicans* during co-infection of *Drosophila melanogaster*

Single or co-infection of *Drosophila* with *S. mutans, spaP mutant, and C. albicans* for 48 hours. A. CFU quantification of wild-type *S. mutans*, the *spaP* mutant in single and two-species infection of *Drosophila*. B. CFU quantification of *C. albicans* in single and two-species infection of *Drosophila*. Data are representative of three experiments performed in triplicate. ** (P<0.005), *** (P<0.001), NS, not significant.

Table 1

Strains and plasmids used in this study

Strain	Description	Source
UA159	Wild-type *Streptococcus mutans*	[15]
Δ*spaP*	*S. mutans* antigen I/II mutant, Erm^R	This study
Δ*spaP*::Δ*spaP*	Antigen I/II complemented, Kan^R, Erm^R	This study
S. mutans-Gfp	Gfp-labeled *S. mutans*, Kan^R	[15,45]
Δ*spaP*-Gfp	Gfp-labeled UA159 antigen I/II mutant	This study
SC5314	Wild-type *Candida albicans*	[46]
Δ*als1*	*C. albicans als1* mutant	[47]
Δ*als3*	*C. albicans als3* mutant	[47]
Δ*als1*:Δ*als3*	*C. albicans als1*and *als3* double mutant	[47]
Plasmids		
pVPT	*E.coli-Streptococci shuttle* vetor, Kan^R	[48]

Table 2

Primers used in this study

spaP Wf-up	ACGGACAGGACGATATTATTTGGTA
spaP Wr-up	GAGTGTTATTGTTGCTCGGCATAAATCCTCCAAATCTGAATAAATC
spaP Wf-Down	GGTATACTACTGACAGCTTCTGACAGCATAGATATTACATTAGAATT
spaP Wr-Down	AACCACCATAAACCGAACGAACT
Complement-spaP -F-Sal	CATCAGTCGACGGCCAGAACATCTAGTCCAACT
Complement-*spaP*-R-Kpn	CATCAGGTACCTCAATCTTTCTTAGCCTTTAAGCCAAGC