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# Systemic *Staphylococcus aureus* infection mediated by *Candida albicans* hyphal invasion of mucosal tissue

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*Candida albicans* and *Staphylococcus aureus* are often co-isolated in cases of biofilm-associated infections. *C. albicans* can cause systemic disease through morphological switch from the rounded yeast to the invasive hyphal form. Alternatively, systemic *S. aureus* infections arise from seeding through breaks in host epithelial layers although many patients have no documented portal of entry. We describe a novel strategy by which *S. aureus* is able to invade host tissue and disseminate via adherence to the invasive hyphal elements of *Candida albicans*. *In vitro* and *ex vivo* findings demonstrate a specific binding of the staphylococci to the candida hyphal elements. The *C. albicans* cell wall adhesin Als3p binds to multiple staphylococcal adhesins. Furthermore, Als3p is required for *C. albicans* to transport *S. aureus* into the tissue and cause a disseminated infection in an oral co-colonization model. These findings suggest that *C. albicans* can facilitate the invasion of *S. aureus* across mucosal barriers, leading to systemic infection in co-colonized patients.

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## INTRODUCTION

Polymicrobial infections tend to be complex and result in aggressive forms of diseases that often exhibit increased

resistance to antimicrobials and impact therapeutic measures (Brogden, 2003; Harriott & Noverr, 2009; Jenkinson & Douglas, 2002; Klotz *et al.*, 2007; Lynch & Robertson, 2008). Yet, despite the gravity of such infections, studies of mechanisms underlying polymicrobial infections are in their infancy.

Among the vast number of human pathogens, the bacterial species *Staphylococcus aureus* and the fungal species *Candida albicans* are currently the second and third most commonly isolated bloodstream pathogens (Goetghebeur

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**Abbreviations:** d.p.i., days post-inoculation; PNA-FISH, peptide-nucleic acid fluorescent *in situ* hybridization; TSB, trypticase soy broth.

One supplementary video is available with the online Supplementary Material.

*et al.*, 2007; Klevens *et al.*, 2007; Perthroth *et al.*, 2007). In particular, *S. aureus* has gained considerable attention from the medical community due to its involvement in the increasing number of nosocomial and community acquired infections resulting in nearly half a million hospitalizations and 50 000 deaths each year in the USA alone (Goetghebeur *et al.*, 2007; Gordon & Lowy, 2008). This bacterial species is armed with an array of virulence factors including toxins and immunoavoidance strategies for invading and destroying host tissue during infection (Bien *et al.*, 2011; Ferry *et al.*, 2005). Despite its pathogenic potential, *S. aureus* is typically a non-invasive commensal and has been historically identified as a common nasopharyngeal resident, but is also found localized associated with moist skin areas of the axillae and groin. However, this microbial species has more recently been found to commonly exist in the oral cavity. *S. aureus* typically requires a breach in mucosal barriers to gain entry into the epithelium (Acton *et al.*, 2009; Ohara-Nemoto *et al.*, 2008; Smith *et al.*, 2003; Zimmerli *et al.*, 2009). Yet, it has been reported that a significant number of patients with staphylococcal bloodstream infections have no documented portal of entry (del Rio *et al.*, 2009).

Similarly, *C. albicans* is the most frequently encountered pathogenic human fungal species and commonly colonizes host mucosal and moist skin surfaces (Calderone & Clancy, 2012; Cannon & Chaffin, 2001). However, under conditions of immune dysfunction, this opportunistic microbe can rapidly transition from commensal to pathogen, causing an array of infections ranging from localized mucosal to severe systemic infections with high morbidity and mortality rates (Calderone & Clancy, 2012; de Repentigny *et al.*, 2004; Perthroth *et al.*, 2007). Oral candidiasis or thrush is the most common opportunistic infection in HIV-infected population with 80–90% of these individuals developing oropharyngeal candidiasis during the course of their illness (de Repentigny *et al.*, 2004; Fidel, 2006). In addition, recent longitudinal studies have shown that in the ageing population, *C. albicans* is even more frequently encountered in the oral cavity, especially in edentulous elderly populations (Budtz-Jørgensen *et al.*, 1996; Kulak-Ozkan *et al.*, 2002; Zaremba *et al.*, 2006). The success of this species as an opportunistic pathogen is the result of its repertoire of virulence factors, including the ability to switch between a yeast and hyphal morphology, a property crucial to its pathogenicity (Calderone & Clancy, 2012).

Several studies have reported the co-isolation of *S. aureus* and *C. albicans* from numerous biofilm-associated diseases such as periodontitis, denture stomatitis, cystic fibrosis, keratitis, ventilator-associated pneumonia and urinary tract and burn wound infections (Adam *et al.*, 2002; Baena-Monroy *et al.*, 2005; Costerton *et al.*, 1985; Cuesta *et al.*, 2010; Gupta *et al.*, 2005; Pate *et al.*, 2006; Tawara *et al.*, 1996; Timsit *et al.*, 2001; Valenza *et al.*, 2008). Although these studies only reported associations and did not prove causation, the frequency with which *S. aureus* and *C. albicans* are co-isolated merits further study. More

directly relevant to bacteraemia, a study by Klotz *et al.* (2007) investigating the incidence of candidal bloodstream infections in hospitalized patients reported that *C. albicans* was co-isolated with *S. aureus* in 20% of the cases. Further, animal studies by Carlson *et al.* (1983, 1985) demonstrated a significant increase in mortality in mice co-infected intraperitoneally with sublethal levels of *C. albicans* and *S. aureus*. This lethal synergism was also found in a more recent study by Peters & Noverr (2013) where co-infection led to a 40% mortality rate and increased microbial burden in the spleen and kidney. The interaction between *S. aureus* and *C. albicans* does not appear to be strain specific, and it is significantly higher than the interaction between *C. albicans* and other microbial species (*Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Bacillus subtilis*) (Peters *et al.*, 2010, 2012).

Collectively, these observations seem to indicate a potential synergy in virulence when these species co-exist in a host. These speculations were recently validated by our *in vitro* studies, where microscopic images revealed a complex physical interaction with *S. aureus* demonstrating high affinity to the hyphal elements of *C. albicans* (Peters *et al.*, 2010, 2012). Further analysis revealed that *S. aureus* bound specifically to the hyphae form of *C. albicans* and not to the yeast form (Peters *et al.*, 2010). The main *C. albicans* target for *S. aureus* binding was shown to be the agglutinin-like sequence 3 adhesin (Als3p). Purified recombinant Als3p has been shown to bind *S. aureus* in *in vitro* assays, and binding of *S. aureus* to the *C. albicans* hyphae is significantly reduced in a mutant of Als3p compared to WT or other adhesin-deficient mutant strains (Peters *et al.*, 2012). However, previous studies did not confirm this specific interaction by restoration of the WT binding phenotype through complementation of the *C. albicans* Als3p gene.

Given the propensity of *C. albicans* to adhere to and penetrate tissue via its invasive hyphae, combined with the high *in vitro* affinity of *S. aureus* to the hyphae, it was feasible to speculate that co-colonization with *C. albicans* may provide *S. aureus* with the means to gain entry into the vascular system (Cannon & Chaffin, 2001; Sudbery *et al.*, 2004). Since *S. aureus* has more recently been identified in the oral cavity along with *C. albicans*, we sought to test the effects of their co-colonization in the oral cavity using a standard murine model of candidiasis. Tongue histopathological analysis was performed and subepithelial co-penetration of the tissue by invasive *C. albicans* hyphae with adherent *S. aureus* was shown. This interaction was absent in mutants of the *C. albicans* Als3p hyphal protein but was restored when Als3p was restored through complementation. We developed a novel murine model of oral co-colonization to monitor the development and potential progression of mucosal co-colonization to systemic disease in an immunocompromised host. In this model, mice suffered disseminated staphylococcal disease with high morbidity and mortality only when co-colonized by *S. aureus* and *C. albicans*. While this ‘microbial

hitchhiking' phenomenon has been identified in other *in vitro* systems (Edwards *et al.*, 2006; Saito *et al.*, 2012), this is the first report, to our knowledge, of systemic disease associated with multi-Kingdom interactions. The clinical implications are far-reaching, especially if this microbial infectious synergy occurs in other areas where co-colonization can commonly occur such as the intestinal tract, genital tract, infected and devitalized skin or axillary or groin areas of the skin. This study also provides crucial insight into one of the complex mechanisms behind polymicrobial interactions.

## METHODS

**Strains.** The genotypes of the strains used can be found in the corresponding references: *C. albicans* strains SC5314 WT (SC5314) (Gillum *et al.*, 1984), *ALS3* mutant in SC5314 (*als3Δ/Δ*) (Phan *et al.*, 2007), *ALS3* mutant in SC5314 complemented (*als3Δ/Δ ALS3*) (Phan *et al.*, 2007), the methicillin-resistant *S. aureus* WT strains M2 (Brady *et al.*, 2006) and USA300 JE2 (Kennedy *et al.*, 2010). In order to determine potential adhesins on *S. aureus* to which *C. albicans* was binding, 25 different *S. aureus* mutant strains were selected based on a review of microbial surface components recognizing adhesive matrix molecules in *S. aureus* (Clarke & Foster, 2006). The parent strain USA300 JE2 and the 25 *S. aureus* mutant strains that were used in the experiment were obtained from The Nebraska Transposon Mutant Library from Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) and were constructed by the Center for Staphylococcal Research (CSR) at the University of Nebraska Medical Center (<http://app1.unmc.edu/fgx/>). A description of all strains used can be found in Table 1.

**Hyphal–bacterial attachment assay.** For all attachment assays carried out in order to identify the *S. aureus* adhesin responsible for *C. albicans* hyphal binding, an aliquot of a glycerol stock of *C. albicans* strain was grown and maintained on Sabouraud dextrose agar (BBL). Cultures were grown overnight in yeast–peptone–glucose (BBL) in an orbital shaker (120 r.p.m.) at 30 °C under aerobic conditions. Yeast cells were harvested and washed twice in sterile PBS. Hyphae formation was induced by first growing *C. albicans* as noted above on plastic Permaxox slides (Electron Microscopy Sciences) in polystyrene six well plates (Corning) in 3 ml RPMI 1640 for 3 h. Nonadherent hyphae were removed by gently washing the slides in PBS, followed by the addition of 3 ml of fresh RPMI 1640.

Starter cultures of the WT *S. aureus* strain JE2 and 25 different *S. aureus* mutants strains were grown in trypticase soy broth (TSB; Remel) and incubated overnight at 37 °C. Erythromycin (10 µg ml<sup>-1</sup>) was added into the TSB media for the *S. aureus* mutant strains. Fresh exponential-phase *S. aureus* starter cultures were grown by diluting the overnight culture 1:100 in fresh TSB for 3 h. Exponential-phase *S. aureus* cell suspensions were washed in PBS, diluted to an OD<sub>600</sub> of 0.1, and added to SC5314 *C. albicans* biofilms (WT and *als3Δ/Δ*). Dual-species biofilms were grown in RPMI 1640 buffered with HEPES and supplemented with L-glutamine (Invitrogen). Plates were placed on a rotary shaker to distribute the bacteria evenly and incubated for 1 h at 37 °C. Following incubation, non-adherent cells were removed by gently washing the slides in PBS and then examined using phase-contrast microscopy under a 100× oil-immersion objective. The total number of bacterial cells per field and attached bacteria per hyphae were counted. Per cent attachment was calculated by dividing the number of attached bacteria by the total number of bacteria. A total of ten random fields per coverslip were analysed. Each assay was done in triplicate.

**Real-time adhesion analysis.** Real-time adhesion was visualized using the Bioflux 200 system (Fluxion) combined with an EVOS *fl*

digital fluorescence microscope (AMG). To analyse adhesion of *S. aureus* to hyphae of SC5314 *C. albicans* in real-time, a GFP labelled *S. aureus* (*S. aureus*<sup>GFP</sup>) strain was used (Li *et al.*, 2011). *C. albicans* suspended at 10<sup>7</sup> cells ml<sup>-1</sup> in PBS was allowed to adhere to glass under static conditions at 37 °C. Following 2 h incubation, non-adherent cells were removed by flow with 0.2 Pa and yeast nitrogen base pH 7 containing 0.5% D-glucose (YNB) was flowed through the channel. The flow was stopped and the channel was incubated at 37 °C for 3 h to allow hyphae to form (Jarosz *et al.*, 2009), then YNB was removed by flow (0.2 Pa). Pilot experiments using different shear rates showed that hyphae remained attached to the glass surface up to a maximum of 2 Pa (not shown). Overnight cultures of *S. aureus*<sup>GFP</sup>, grown at 37 °C while shaking at 150 r.p.m., were harvested and resuspended in PBS. This suspension was flowed over the adherent hyphae of *C. albicans* at 0.2 Pa and images were obtained using an EVOS *fl* digital fluorescence microscope using a 10× objective and the appropriate filter set. Images were taken every 10 s for a total of 5 min and a movie was created at 2 frames s<sup>-1</sup>.

**Microscopic analysis.** Peptide-nucleic acid fluorescent *in situ* hybridization (PNA-FISH) staining and confocal scanning laser microscopy was utilized to visualize the architecture of mixed biofilms *in vitro* as described previously (Peters *et al.*, 2010). PNA-FISH employs fluorescent-labelled PNA probes to target the species-specific rRNA sequences in a specific FISH assay that enables whole cell visualization. Hybridization was performed following the manufacturer's protocol (Advandx) using a cocktail of Cy2-labelled *S. aureus* and FITC-labelled *C. albicans* PNA probe mixtures. Briefly, dual-species biofilms were grown on glass coverslips in polystyrene six well plates (Corning). *S. aureus* and *C. albicans* were diluted in PBS to an OD<sub>600</sub> of 0.1 and 1.0, respectively. This represented approximately 1 × 10<sup>8</sup> c.f.u. ml<sup>-1</sup> for *S. aureus* and 2 × 10<sup>7</sup> c.f.u. ml<sup>-1</sup> for *C. albicans*. Fifty microlitres of each cell suspension (representing a total of 5 × 10<sup>6</sup> c.f.u. of *S. aureus* and 1 × 10<sup>6</sup> c.f.u. of *C. albicans*) was added to wells containing 5 ml of RPMI 1640 buffered with HEPES and supplemented with L-glutamine (Invitrogen) and 5% heat-inactivated FBS (Hyclone) and plates were incubated for 24 h at 37 °C. Following incubation, coverslips were gently rinsed with PBS to remove non-adherent cells. Imaging was performed by confocal scanning laser microscopy with a Zeiss LSM 510 confocal microscope (Carl Zeiss) using FITC filter sets.

In addition, microscopy was also performed on excised mouse tongues using a previously described *ex vivo* model (Kamai *et al.*, 2002; Peters *et al.*, 2012). Briefly, tongues were excised from euthanized CD1 mice and placed into polystyrene well plates containing 5 ml of RPMI 1640 per well. *S. aureus* and *C. albicans* were added in an identical manner as in the *in vitro* attachment assay: 50 µl of cell suspensions of each microbe for a total of 5 × 10<sup>6</sup> c.f.u. of *S. aureus* and 1 × 10<sup>6</sup> c.f.u. of *C. albicans*. Tongues were then washed several times in sterile PBS, transferred to fresh RPMI 1640 and incubated overnight at 37 °C with 5% CO<sub>2</sub>. Tongues were then fixed, embedded in paraffin and tissue sections stained with PNA-FISH probes and examined by confocal microscopy. Fluorescent microscopy could not be performed on tissue recovered from animals infected *in vivo* due to interference in the hybridization process by host erythrocytes and inflammatory cells.

**Murine *in vivo* model of co-infection.** Strains were grown on appropriate growth media and mid exponential-phase cultures were used in experiments. Animal studies were approved by the University of Maryland Animal Care and Use Committee. The animal model for oral co-infection was designed based on an established protocol for oral candidiasis (Dwivedi *et al.*, 2011). Briefly, oral bacterial flora was suppressed by the addition of 300 µg ml<sup>-1</sup> ampicillin into the drinking water and mice (C57BL/6J) were immunosuppressed by three subcutaneous injections of 225 mg kg<sup>-1</sup> cortisone acetate, one

**Table 1.** Strains used in this study

Strain	Designation	Genotype	Reference
<i>S. aureus</i> M2	WT (SA)	ST30, <i>spa</i> type T019 and <i>agrIII</i>	Ali <i>et al.</i> (1997); Brady <i>et al.</i> (2006); Brady <i>et al.</i> (2007); Brady <i>et al.</i> (2011); Jabra-Rizk <i>et al.</i> (2006); Leid <i>et al.</i> (2002); Peters <i>et al.</i> (2010); Peters <i>et al.</i> (2012); Prabhakara <i>et al.</i> (2011a, b); Shirliff <i>et al.</i> (2002)
<i>S. aureus</i> JE2	JE2	USA300 JE2 – a plasmid cured strain from WT USA300-LAC	Kennedy <i>et al.</i> (2008); Kennedy <i>et al.</i> (2010); <a href="http://appl.unmc.edu/fgx/">http://appl.unmc.edu/fgx/</a>
	NE1	ΔSAUSA300_1327 – Cell Wall Protein (protein G-related albumin-binding (GA) module related to streptococcal <i>emb</i> adhesin)	
	NE26	ΔSAUSA300_0224 – <i>coa</i> (staphylocoagulase precursor)	
	NE33	ΔSAUSA300_2589 – <i>sasA</i> ( <i>Staphylococcus aureus</i> surface protein A and LPXTG-motif cell wall surface anchor family protein)	
	NE98	ΔSAUSA300_0548 – <i>sdrE</i> (SdrE protein binds host Factor H)	
	NE186	ΔSAUSA300_2441 – <i>fmbA</i> (fibronectin binding protein A)	
	NE286	ΔSAUSA300_0113 – <i>spa</i> (immunoglobulin G binding protein A)	
	NE295	ΔSAUSA300_0025 – hypothetical 5-nucleotidase family protein	
	NE332	ΔSAUSA300_1029 – <i>sasE</i> (iron transport associated domain protein)	
	NE391	ΔSAUSA300_2565 – <i>clfB</i> (clumping factor B)	
	NE432	ΔSAUSA300_0546 – <i>sdrC</i> (SdrC protein)	
	NE460	ΔSAUSA300_0955 – <i>atl</i> (autolysin)	
	NE472	ΔSAUSA300_2109 – <i>fmbB</i> (truncated FmtB protein)	
	NE510	ΔSAUSA300_2110 – <i>fmbB</i> (truncated FmtB protein)	
	NE543	ΔSAUSA300_0772 – <i>clfA</i> (clumping factor A)	
	NE557	ΔSAUSA300_1030 – <i>sirD</i> (iron transport associated domain protein)	
	NE728	ΔSAUSA300_2440 – <i>fmbB</i> (fibronectin binding protein B)	
	NE800	ΔSAUSA300_2581 – <i>sasF</i> (putative surface anchored protein)	
	NE825	ΔSAUSA300_2436 – hypothetical putative cell wall surface anchor family protein	
	NE1032	ΔSAUSA300_0136 – <i>sasD</i> (cell wall surface anchor family protein)	
	NE1075	ΔSAUSA300_1677 – <i>sasI</i> (cell wall surface anchor family protein)	
	NE1102	ΔSAUSA300_1028 – <i>sirH</i> (iron transport associated domain-containing protein)	
	NE1190	ΔSAUSA300_2256 – <i>Atl</i> (Bifunctional autolysin Atl [ <i>N</i> -acetylmuramoyl- $\gamma$ -alanine amidase/Endo-beta- <i>N</i> -acetylglucosaminidase)	
	NE1289	ΔSAUSA300_0547 – <i>sdrD</i> (SdrD protein)	
	NE1558	ΔSAUSA300_0774 – <i>empbp</i> (secretory extracellular matrix and plasma binding protein)	
	NE1561	ΔSAUSA300_1370 – <i>ebpS</i> (cell surface elastin binding protein)	
<i>C. albicans</i> SC5314	SC5314	<i>URA3 ALS3 ARG4 HIS1 URA3 ALS3 ARG4 HIS1</i>	Gillum <i>et al.</i> (1984)
<i>C. albicans</i> CAYF178U	<i>als3A/A</i>	<i>ura3Δ::λimm434::URA3-IRO1 als3::ARG4 arg4::hisG his1::hisG ura3Δ::λimm434 als3::HIS1 arg4::hisG his1::hisG</i>	Phan <i>et al.</i> (2007)
<i>C. albicans</i> CAQTP178U	<i>als3A/A ALS3</i>	<i>ura3Δ::λimm434::URA3-IRO1 als3::ARG4::ALS3 arg4::hisG his1::hisG ura3Δ::λimm434 als3::HIS1 arg4::hisG his1::hisG</i>	Phan <i>et al.</i> (2007)

given prior to inoculation and the other two given following inoculation 2 days apart. Anaesthetized animals were orally administered with standardized cell suspensions of *C. albicans* (100  $\mu$ l of a  $6 \times 10^8$  c.f.u.  $\text{ml}^{-1}$  suspension administered on a small cotton pad), *S. aureus* (50  $\mu$ l of a  $1 \times 10^8$  c.f.u.  $\text{ml}^{-1}$  suspension), or in combination (four mice per group in triplicate) on days 1 and 3, and were

continually exposed to *C. albicans* ( $6 \times 10^6$  c.f.u.  $\text{ml}^{-1}$ ) through drinking water. During the course of the experiments, animals were weighed every 3 days, and on day 6 post-infection, were euthanized and tongues and kidneys were harvested. Tissue homogenates were assessed for microbial burden (cells  $\text{g}^{-1}$  tissue) by culturing on selective microbial media. Mice exhibiting greater than 20% weight



loss were euthanized and considered deceased, as established as a humane end point by the University of Maryland Institutional Animal Care and Use Committee.

**Statistical analysis.** Experiments were performed in triplicate. Data analysis was performed using the two-tailed Student's *t*-test, the Log Rank Test, or the Mann–Whitney U Test (two tailed), where appropriate. Values of  $P < 0.05$  were considered statistically significant.

**Ethics statement.** The experiments in this study were approved under Protocol 1111010 of the Dental School IACUC committee and adhered to Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals (Animal Welfare Assurance A3200-01) and United States Department of Agriculture Animal Welfare Act & Animal Welfare Regulations.

## RESULTS

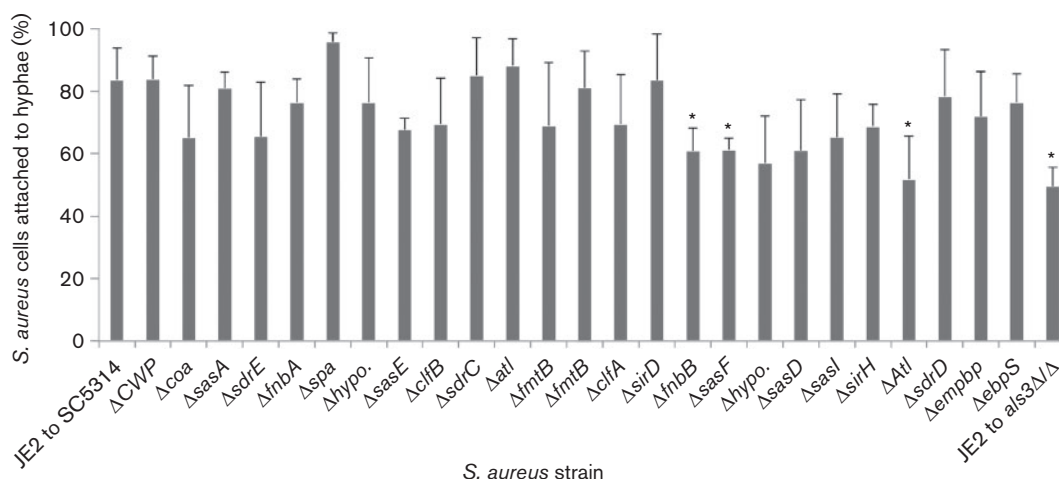
### Hyphal–bacterial attachment assay: *in vitro* analysis of polymicrobial biofilm development using confocal and time lapse microscopy

As colonization and biofilm formation is a prerequisite for the development of the pathogenic process, *in vitro* studies were performed in order to visualize the interaction between *S. aureus* and *C. albicans* strains as they co-existed in a polymicrobial biofilm. Mixed-species biofilms of *S. aureus* and *C. albicans* mutant and complemented strains were grown *in vitro* and visualized by PNA-FISH staining. Microscopic images of the mixed biofilms paralleled those from our previous studies (Peters *et al.*, 2010, 2012) where

a significant decrease in *S. aureus* adherence to the hyphae of the *C. albicans* strain lacking the Als3p (*als3Δ/Δ*) was observed compared to the strains expressing the receptor. Additionally, there was no appreciable difference between the WT *C. albicans* or the *als3Δ/Δ* *ALS3* complemented strain (data not shown).

Importantly, the initial adherence of *S. aureus* to the hyphae leading to the development of a mature biofilm was monitored and captured in real-time by fluorescent microscopy in a biofilm flow system. A movie was created of compiled images taken every 10 s over a 5 min time frame (Video S1, available in the online Supplementary Material). As can be seen, immediately upon entry into the flow cell, *S. aureus*<sup>GFP</sup> rapidly and specifically adheres to the hyphae attached to the surface of the flow cell. When a higher cell density of *S. aureus* is flowed through the system, a more rapid and dramatic adhesion is seen resulting in development of a mature mixed-species biofilm within 5 min of initiation of the adherence process.

In order to determine the specific staphylococcal factor responsible for the hyphal binding, WT *S. aureus* strain JE2 and 25 different *S. aureus* strains mutated in previously described adhesins were individually added to SC5314 *C. albicans* biofilms (see Fig. 1, Table 1 for strain designations). The percentage of cells attached to the hyphae of SC5314 was determined for each mutant and compared to the hyphal attachment percentage of WT *S. aureus* co-cultured with either SC5314 or the SC5314 *als3Δ/Δ*. It was found that the percentage of WT JE2 cells binding to hyphae was that same as that seen for the staphylococcal strain M2 (84 % vs 82 %



**Fig. 1.** Hyphal binding assay for determining the *S. aureus* adhesin responsible for *C. albicans* hyphae binding. The WT *S. aureus* strain JE2 and 25 different *S. aureus* mutants strains in previously described adhesins, were grown in TSB, added to *C. albicans* hyphae, and incubated for 3 h. Non-adherent hyphae were removed by gently washing the slides in PBS, and then examined using phase-contrast microscopy under a 100× oil-immersion objective. The total number of bacterial cells per field and attached bacteria per hyphae were counted. Attachment (%) was calculated by dividing the number of attached bacteria by the total number of bacteria. A total of 10 random fields per coverslip were analysed in triplicate ( $n=3$ ). Error bars indicate SD. \* denotes statistically significant ( $P < 0.05$ ) reductions in binding ability compared to the WT *S. aureus* and *C. albicans* attachment (JE2 to SC5314) as evaluated by a two-tailed Student's *t*-test.

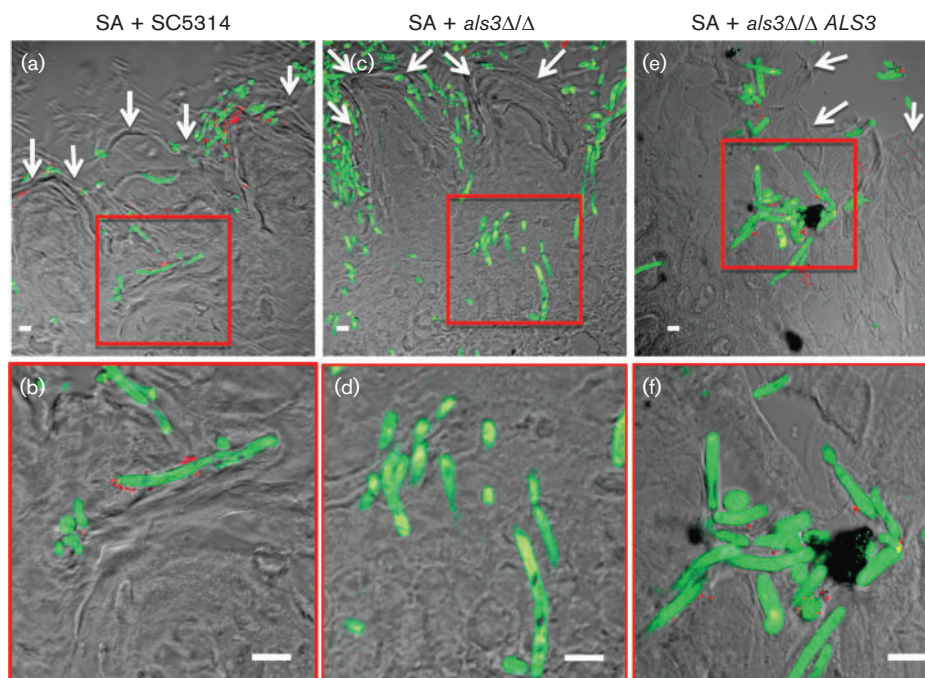
respectively) (Peters *et al.*, 2012). The use of JE2, a *S. aureus* strain that is phylogenetically divergent from the M2 strain (Diep *et al.*, 2006; Harro *et al.*, 2013), provides broader support for the idea that this *S. aureus*–*C. albicans* interaction is species-specific and not just strain specific.

Several of the staphylococcal mutants tested had a statistically significant reduction in their ability to bind *C. albicans*, including NE728 ( $\Delta$  in fibronectin binding protein B) with 62 % attachment, NE800 ( $\Delta$  in *sasF*, a putative surface anchored protein) with 61 % attachment and NE1190 ( $\Delta$  in *Atl*, a putative *N*-acetylmuramoyl-L-alanine amidase) with 52 % attachment. In addition, five other adhesin mutants showed a similarly reduced ability (52–65 % attachment) to bind *C. albicans*, although these differences were not statistically significant due to larger SD. The creation of single, double, triple, or octuple mutants in these adhesins has been shown to eliminate the ability of *S. aureus* to both form biofilms and survive *in vivo* (Bose *et al.*, 2012; Chen *et al.*, 2013; Kenny *et al.*, 2009; Mazmanian *et al.*, 2000; Palmqvist *et al.*, 2005). Therefore, these *S. aureus* mutants are no longer able to systemically spread in cases of co-infection with *C. albicans* regardless of the mechanism of deep tissue delivery. Since it cannot be concluded whether a lack of systemic disease by *S. aureus* is a product of reduced *C. albicans* hyphal binding or an inability to survive within

the host, subsequent studies to determine the combination of staphylococcal adhesins required for hyphal interactions *in vivo* were not possible.

### Microscopic analysis

To investigate the role of Als3p in the invasive mechanism of co-colonization, experiments were performed where both micro-organisms were allowed to interact on excised tongues infected *ex vivo*. Similarly to the images of abiotic surfaces, *S. aureus* was seen strongly adhering to the *C. albicans* hyphae with the Als3p receptor (WT SC5314 and *als3Δ/Δ ALS3*; Fig. 2b, f) but only minimally to the hyphae lacking the receptor *als3Δ/Δ* (Fig. 2d). However, in all three *C. albicans* strains, *S. aureus* and *C. albicans* were seen forming a polymicrobial biofilm on the surface of the tongue (indicated by the white arrows in Fig. 2a, c, e). Importantly, in addition to mere co-colonization, this model demonstrated tissue co-penetration of *S. aureus* along with the invasive hyphae of *C. albicans* into the subepithelium (Fig. 2a, b, e, f), a process dependent on Als3p as *S. aureus* did not co-penetrate with *als3Δ/Δ* (Fig. 2c, d). We did notice that the staphylococcal hyphal binding was markedly reduced in these studies compared to the *in vitro* analysis of polymicrobial biofilm development using confocal



**Fig. 2.** Microscopic images (low and high magnification) of sections of tongues infected and stained with probes specific to *S. aureus* (red) and *C. albicans* (green) demonstrating hyphal invasion of tissue and bacterial presence. Hyphae of Als3p-expressing *C. albicans* strains (WT SC5314 and complemented strain *als3Δ/Δ ALS3*) are seen penetrating the epithelium with *S. aureus* (SA) adhering to and thus co-invading the tissue with the hyphae (a, b, e, f). In contrast, although the hyphae of the *C. albicans* Als3p mutant (*als3Δ/Δ*) penetrated the tissue and SA adhered to the border of the tongues, no bacteria were seen adhering to the hyphae or detected in the subepithelium (c, d). White arrows denote tongue outer surface and scale bars represent 10  $\mu$ m.

microscopy. These differences may have been due to salivary components and structural complexities of the tongue environment, as well as the tongue's ability to produce host defence factors, such as  $\beta$ -defensins (Weinberg *et al.*, 1998).

### Murine *in vivo* model of oral co-colonization

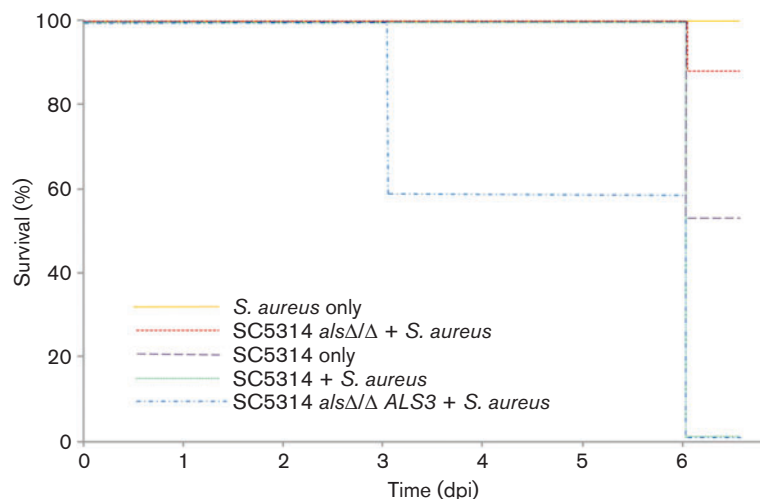
An animal model of microbial co-colonization was developed to investigate the pathogenic implications of microbial interactions on mucosal tissue in immunocompromised hosts. Mouse oral cavities were inoculated with both *S. aureus* and *C. albicans* alone or in combination, and tongues and kidneys were harvested from euthanized animals. In addition, the *C. albicans als3Δ/Δ* mutant and *als3Δ/Δ ALS3* complemented strain were inoculated as well, either alone or in combination with *S. aureus*. Clinical presentation of the harvested tongues was consistent with advanced candidiasis and diagnosis was confirmed by histopathology.

None of the animals colonized with *S. aureus* alone exhibited significant symptoms of disease or weight loss, and all survived until the conclusion of the experiment (Fig. 3). Mice infected with *C. albicans* alone exhibited symptoms of oral candidiasis and half of the mice required early euthanization due to significant weight loss (Fig. 3). However, none of these mice exhibited symptoms of systemic infection, suggesting the weight loss was solely due to the oral candidiasis, and not

because of an invasive infection. There was no significant difference in the survival among all three of the *C. albicans* strains administered alone (data not shown).

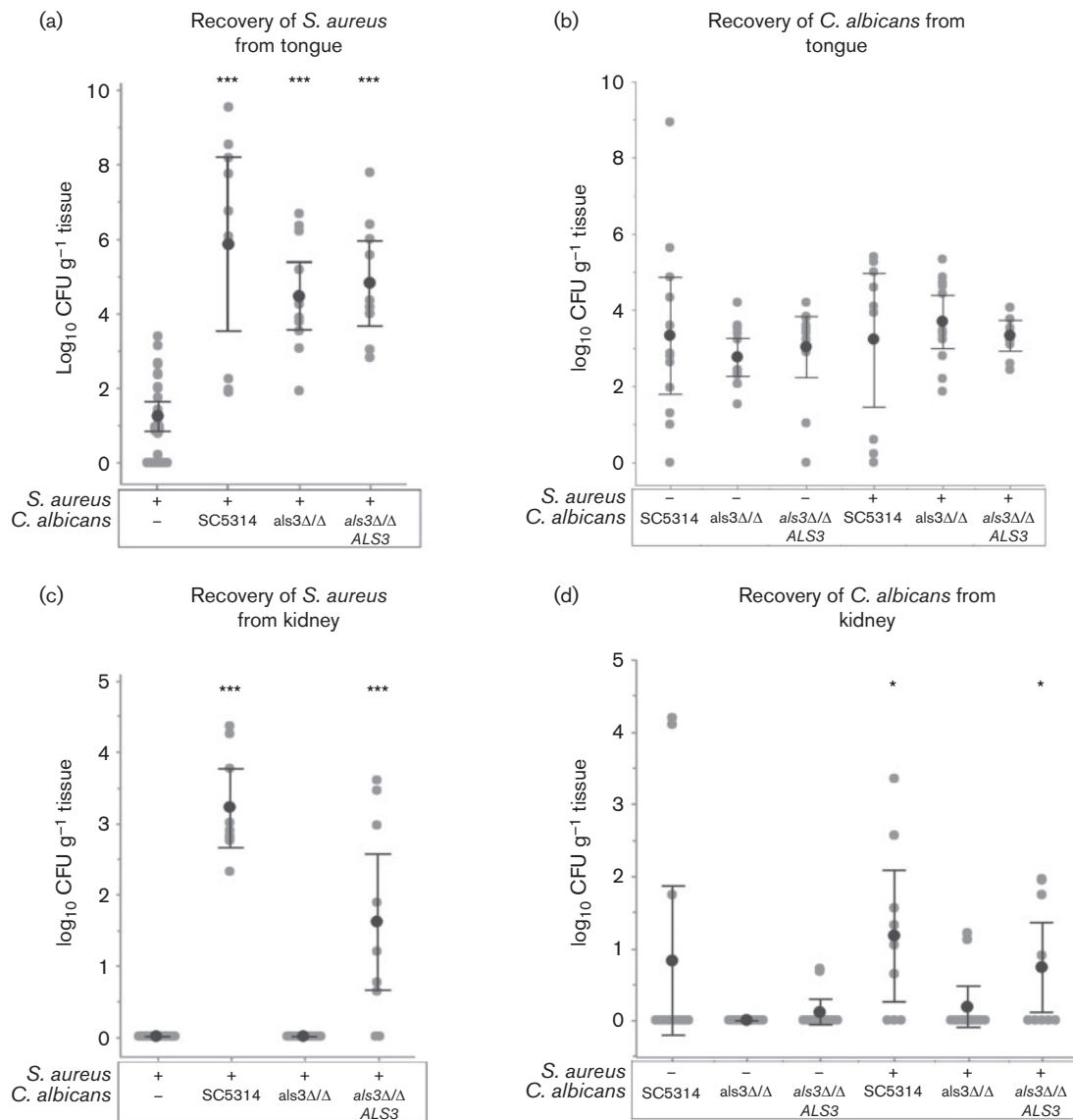
As opposed to the animals orally inoculated with either single species, those simultaneously inoculated with *S. aureus* and *C. albicans* containing an intact Als3p (WT SC5314 or SC5314 *als3Δ/Δ ALS3* complemented) exhibited signs of systemic infection, including dehydration and lethargy. Additionally, the mice in this group showed significant loss of body weight, requiring early euthanization and halting of experiments at 6 days post-inoculation (dpi), as per the University of Maryland Institutional Animal Care and Use Committee humane end point guidelines. Significantly, all of these animals succumbed to their infection prior to the end of the experiment as determined by the humane end points (Fig. 3). In contrast, none of the animals in the group co-colonized with *S. aureus* and *C. albicans* lacking Als3p exhibited evidence of systemic infection and the majority (83%) of these mice survived until the conclusion of the experiment (Fig. 3).

In order to diagnose the cause of morbidity in the sick animals, the tongues and kidneys were harvested and cultured to identify microbial presence. There was no significant difference in c.f.u. of *C. albicans* recovered from the tongues, regardless of which *C. albicans* strain was used and whether it was inoculated alone or co-inoculated with *S. aureus*, suggesting that *C. albicans* does not exhibit any deficiency



**Fig. 3.** Mortality in mice orally colonized with either *S. aureus* or *C. albicans* individually or in combination. Animals were weighed 1, 3 and 6 days post-inoculation (dpi). Those animals exhibiting >20% weight loss (defined by the University of Maryland Institutional Animal Care and Use Committee as a humane end point in rodents), were considered deceased and were euthanized. Mice co-colonized with *S. aureus* and *C. albicans* (SC5314 + *S. aureus*) showed a significant decrease in survival vs *S. aureus* alone or *C. albicans* alone as determined by the Log Rank Test where  $P < 0.05$  is considered significant. Additionally, there was no significant difference between survival in mice colonized with *S. aureus* alone and mice co-colonized with the Als3p mutant (Log Rank Test  $P = 0.197$ ). The dramatic weight loss and associated mortality seen in co-colonized mice was restored once Als3 expression was complemented in the *als3Δ/Δ ALS3* strain. All experiments were performed with at least four mice per group. Survival in SC5314 *als3Δ/Δ* alone and SC5314 *als3Δ/Δ ALS3* alone were not significantly different than survival with SC5314 alone (data not shown).





**Fig. 4.** Microbial burden in the tongue and kidneys in mice orally colonized with either *S. aureus* or *C. albicans* WT (SC5314), Als3p mutant (*als3* $\Delta/\Delta$ ), and Als3p complemented (*als3* $\Delta/\Delta$  *ALS3*) individually, or in combination. Three days post-infection, tissue was extracted, homogenized and cultured for assessment of microbial presence based on number of organisms recovered per gram of tissue. Number of microbes recovered is reported as  $\log_{10}$  of c.f.u. per gram of respective tissue. (a, c) *S. aureus* recovered from the tongues (a) and kidneys (c) of infected mice. (b, d) *C. albicans* recovered from the tongues (b) and kidneys (d) of infected mice. Both microbes were recovered from tongues of mice under all conditions tested. However, *S. aureus* was only recovered from kidneys of mice co-colonized with either WT *C. albicans* or mutant *C. albicans* that had been complemented for Als3p expression. All experiments were performed in triplicate in groups of four mice per group. In addition to all data points, the mean of the log of c.f.u./gram is shown. Error bars indicate 95% confidence intervals. Significance was determined using the Mann–Whitney U Test to compare each polymicrobial condition to the monomicrobial control infection. Asterisks denotes statistical significance versus the mono-microbial control infection according to the Mann–Whitney U Test. \* indicates  $P < 0.05$  and \*\*\* indicates  $P < 0.001$ .

in colonizing the tongue in the absence of Als3p (Fig. 4b). *S. aureus*, on the other hand, exhibited enhanced colonization of the tongue when co-inoculated with any of the *C. albicans* strains as compared to *S. aureus* given alone (Fig. 4a).

However, the clinical symptoms indicating systemic disease were corroborated by the recovery of *S. aureus* and *C. albicans* from the kidneys in strains with a functional Als3p protein compared to the *als3* $\Delta/\Delta$  (Fig. 4c, d). High numbers

of *S. aureus* were recovered from mice co-infected with *C. albicans* expressing intact Als3p (WTSC5314 or SC5314 *als3Δ/Δ ALS3* complemented) and no *S. aureus* was found in the kidneys of mice inoculated without *C. albicans* or with *C. albicans* lacking Als3p (Fig. 4c). Additionally, there were significant c.f.u. of *C. albicans* recovered from the kidneys of mice co-inoculated with *S. aureus* and the WT *C. albicans*. This is in contrast with mice inoculated with *C. albicans* alone or co-inoculated with *S. aureus* and *C. albicans* lacking Als3p, where most mice had no *C. albicans* present in their kidneys (Fig. 4d).

The data presented in Fig. 4 suggests that, as seen in Fig. 2, *S. aureus* and *C. albicans* are able to form a dense polymicrobial biofilm on the epithelial surface of the tongue with or without the expression of Als3p. However, *S. aureus* is unable to enter the bloodstream and disseminate in the absence of Als3p, ostensibly due to the lack of binding to penetrating hyphae as shown in Fig. 2. Collectively, these findings demonstrate that co-colonization of the oral mucosa by *C. albicans* and *S. aureus* in immunocompromised animals may lead to systemic *S. aureus* infections with high morbidity and mortality.

## DISCUSSION

Polymicrobial diseases represent the clinical manifestations induced by the presence of multiple microbial species that may act synergistically to cause complex infectious processes (Brogden & Gerberding, 2002; Pittet *et al.*, 1993; Tuft, 2006). Yet, despite the gravity of such infections, areas of study in polymicrobial diseases and particularly those involving vastly diverse pathogens such as fungi and bacteria are in their infancy (Peleg *et al.*, 2010; Shirliff *et al.*, 2009). *C. albicans* and *S. aureus* are frequent colonizers of human mucosal surfaces and exhibit a strong ability to adhere to host tissue and develop drug tolerance. However, studies investigating the implications of their interaction as they co-colonize host mucosal tissue have not been previously performed.

*S. aureus* avidly and specifically adheres to hyphae of *C. albicans* *in vitro*, as illustrated using static and time lapse microscopy (supplementary movie V1). Adhesion is followed by tissue penetration of *S. aureus* along with the invasive hyphae (Fig. 2). Although no single adhesin of *S. aureus* was found to be solely responsible for hyphal binding, three staphylococcal adhesin mutants (*AfnpB*, *ΔsasF*, and *ΔAtl*) were found to have significantly reduced hyphal binding compared to the WT strain (Fig. 1). FnpB (fibronectin binding protein B) is one of two important fibronectin binding proteins in *S. aureus*. FnpB is known to be upregulated in host tissues, and helps facilitate binding to eukaryotic cells (Menziez, 2003). Interestingly, the other structurally related *S. aureus* fibronectin binding protein, FnpA, did not appear to be involved in hyphal binding (Fig. 2), suggesting that the minor structural differences between FnpB and FnpA may influence the ability to bind *C. albicans* hyphae. SasF (staphylococcus aureus surface

protein F) is a highly conserved cell surface-associated adhesin (McCarthy & Lindsay, 2010). SasF has been shown to be upregulated in response to linoleic acid (Kenny *et al.*, 2009), which is one of the major endogenous fatty acids in *C. albicans* (Nigam *et al.*, 2011). The third mutant with significantly reduced binding was a mutation of Atl, an uncharacterized protein that is annotated as a putative *N*-acetylmuramoyl-L-alanine amidase, and likely functions as an *S. aureus* autolysin (Diep *et al.*, 2006).

In addition, five other staphylococcal mutants had similar reductions in binding (although these were not statistically significantly different from the WT strain). This was not surprising considering the redundant nature of adhesin binding partners in *S. aureus* (Clarke & Foster, 2006). Interestingly, neither the JE2 WT strain binding to the Als3p deficient mutant nor any of the *S. aureus* mutants binding to the WT *C. albicans* showed a reduction in binding to less than 50%. This may be due to the expression of other proteins on *C. albicans* hyphae that can have minor roles in *S. aureus* adherence. One such protein is Als1, which was shown in an earlier study to have a role, albeit a much smaller role compared to Als3p, in binding *S. aureus* *in vitro* (Peters *et al.*, 2012). However, the clear difference between the *C. albicans als3Δ/Δ* mutant and the WT strain, both in terms of hyphal binding as shown in Fig. 2 and dissemination as shown in Fig. 4, suggest that differential *in vivo* regulation of these other *C. albicans* proteins may reduce their contributions to *S. aureus* binding during an actual infection.

This interaction with staphylococcal adhesins and Als3p may not be surprising since a number of *S. aureus* adhesins are predicted to have significant 3D homology to Als3p and other Als proteins (Nobile *et al.*, 2008; Salgado *et al.*, 2011). This homology is also demonstrated in cross protection studies in which immunization with *C. albicans* Als3p has been shown to provide protection from *S. aureus* challenge in animal models of infection (Ibrahim *et al.*, 2013; Lin *et al.*, 2009). Since the self-self binding and clumping due to Als proteins have been well documented, the binding of staphylococcal adhesins to Als3p may mimic this self-agglutination (Lipke *et al.*, 2012; Ramsook *et al.*, 2010).

In order to determine the *in vivo* implications of this Als3p-dependent interaction, we developed a novel murine model to demonstrate the potential progression of mucosal co-colonization into disseminated systemic disease. In our animal model, mice co-colonized with *C. albicans* and *S. aureus* succumbed to systemic bacterial infection, *S. aureus* was recovered from the kidneys, and Als3p was crucial for this systemic dissemination (Fig. 3 and 4). As expected, colonization with *S. aureus* individually did not result in death or any signs of significant morbidity, as *S. aureus* typically requires a breach in host surface barriers to invade (Acton *et al.*, 2009; Smith *et al.*, 2003; Veeh *et al.*, 2003).

While the enhanced colonization of the tongue by *S. aureus* in the presence of all three *C. albicans* strains (Fig. 4a) may not correlate to the pattern of colonization seen in the

kidney (Fig. 4c), this is likely to reflect the fact that the *C. albicans* factors responsible for co-colonization are different from the one required for hyphal binding. As shown in an earlier study, the co-culture of *C. albicans* and *S. aureus* together causes reciprocal changes in protein expression (Peters *et al.*, 2010). Additionally, although *S. aureus* has reduced binding to *C. albicans* lacking Als3p, it does not show a reduction in polymicrobial biofilm formation (Peters *et al.*, 2012). Instead, what is likely to be happening *in vivo* is that *S. aureus* and *C. albicans* are still able to form a dense biofilm on the epithelial surface of the tongue, but are unable to attach to penetrating hyphae. This is what is observed in Fig. 2.

It is important to note that the mutant strain lacking Als3p did not exhibit a defect in hyphal formation (Fig. 2). This is significant, since we propose that Als3p does not affect *C. albicans* pathogenesis when present as a mono-species infection, but instead is important for synergy with *S. aureus*. Also, while the mutant lacking Als3p had reduced colonization of the kidneys in co-colonization experiments, Als3p has previously been shown to be dispensable for *C. albicans* pathogenesis in an intravenous challenge model (Cleary *et al.*, 2011). This suggests that the increased presence of the WT *C. albicans* in the kidneys in comparison to the mutant is also mediated by this synergy with *S. aureus*. As mentioned previously, *S. aureus* can produce an array of virulence factors (Bien *et al.*, 2011; Ferry *et al.*, 2005) that are likely to be helping to further mediate tissue lysis and facilitate dissemination of both *S. aureus* and *C. albicans* into the bloodstream. The fact that deletion of Als3p does not alter the formation of hyphae (a necessary prerequisite for *S. aureus* attachment; Peters *et al.*, 2010), nor affects the virulence of *C. albicans* in the bloodstream, suggests that its role in our model is solely due to *S. aureus* attachment. Therefore, the inability of the mutant to mediate a bacterial or fungal systemic disease further confirms the specificity of Als3p in the invasion process.

As our animal model involved the establishment of a polymicrobial infection in mice receiving immunosuppression, it is most directly relevant to cases of co-colonization in immunocompromised individuals. Immunocompromised hosts represent a significant and appropriate patient population to model, as these patients frequently suffer from infections and diseases of the oral mucosa and bloodstream that involve *C. albicans* and *S. aureus* (Bassetti *et al.*, 2012; Delorenze *et al.*, 2013; Dongari-Bagtzoglou *et al.*, 2009; Marukutira *et al.*, 2014; Olczak-Kowalczyk *et al.*, 2012; Stammler Jaliff *et al.*, 2014; Yehia *et al.*, 2011). Additionally, a recent pair of studies found that among HIV positive individuals, *S. aureus* could be isolated from the oral cavity of 92% of individuals while *C. albicans* was isolated from 73% of the study subjects (Back-Brito *et al.*, 2009, 2011), suggesting our model is highly relevant clinically. Furthermore, due to the continued rise in the prevalence of HIV (Hall *et al.*, 2008) and the continued increase in patients living with transplants and receiving immunosuppression (Hall *et al.*, 2008; Port *et al.*, 2007), the

population of immunocompromised patients at risk for co-colonization with *S. aureus* and *C. albicans* continues to expand.

While this ‘hitchhiking’ phenomenon of systemic infection stemming from staphylococcal–candidal interaction has yet to be established in a human host, we propose that this may be due to a failure to look for it. It is feasible to speculate that in a significant number of the 40–50% of cases of staphylococcal bloodstream infections with no clearly established portal of entry (del Rio *et al.*, 2009), oral candidiasis may have been the underlying cause. Should this phenomenon occur in a vulnerable human host harbouring both species, as often occurs on mucosal surfaces, the medical consequences are likely to be significant, as reflected by the high rate of morbidity and mortality observed in our co-colonized animals (Fig. 3).

Although this study focused on *C. albicans* and *S. aureus*, it is important to note that in the oral cavity, *C. albicans* co-exists with the myriad of bacterial species commonly residing the various niches of this complex environment (Bagg & Silverwood, 1986; Brehm-Stecher & Johnson, 2003; Holmes *et al.*, 1995, 1996; Jenkinson *et al.*, 1990; Klotz *et al.*, 2007; Silverman *et al.*, 2010). The oral cavity is colonized by an ever-changing population (Lazarevic *et al.*, 2010) of over 900 bacterial species and therefore, it may also be conceivable that similar phenomena with clinical relevance may also occur between *C. albicans* and oral bacteria (Zaura *et al.*, 2009). This has been seen in a recent study in which *Streptococcus oralis* colonization of the oral and gastrointestinal tract was augmented in the presence of *C. albicans* and resulted in increased size and frequency of thrush lesions in a murine model of dual infection (Xu *et al.*, 2014). As our knowledge of interspecies interactions on niches expands, the role of *C. albicans* in pathogenesis might prove to be more intricate than currently recognized.

A potential limitation of the study is that this interaction was only evaluated in oral cavity instead of in other body locales where these microbes, particularly *S. aureus*, have been isolated, including the nasopharynx, moist skin areas and the genital tract. While the model used in this study has relevance, since both of these microbes have been co-isolated in the oral cavity, we look forward to future studies where we can evaluate this interaction in the mouse nasopharynx and skin models. Also worth noting is the possibility of gastrointestinal (GI) colonization to contribute to this ‘hitchhiking’ phenomenon. Although our study focused on the oral mucosa, based on the oral administration of the microbes there was potential for the co-colonization of the GI tract to occur as well. *C. albicans* and *S. aureus* have both been shown to be able to colonize the GI tract (Acton *et al.*, 2009; Prieto *et al.*, 2014), and GI candidiasis can occur in immunocompromised individuals, although it is much less common than oral candidiasis (Vazquez & Sobel, 2002). Although our current study failed to examine this potential route of invasion, we feel that based on the requirement that Als3p be present in order to

produce systemic *S. aureus* bacteraemia, any dissemination occurring through the gut mucosa is likely to be occurring through the same proposed model of Als3p binding and hitchhiking. We hope to clarify the potential role this important mucosal site may play in this phenomenon in future studies.

In summary, this study elucidates a synergistic interaction between the bacterial pathogen *S. aureus* and the opportunistic fungal pathogen *C. albicans* during polymicrobial biofilm growth in the oral cavity, for which we propose the term 'microbial hitchhiking'. The interactions between these diverse and important commensals and opportunistic pathogens hold significant clinical implications, and therefore characterizing their complex interactions is a major step in understanding the nature of their co-existence in the host. Although retrospective and prospective clinical studies are warranted, our findings advocate screening of critically ill patients for oral candidal colonization as a measure for the prevention of systemic bacterial infections.

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