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RESEARCH ARTICLE

Coaggregation of *Candida albicans*, *Actinomyces naeslundii* and *Streptococcus mutans* is *Candida albicans* strain dependent

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One sentence summary: Coaggregation between *Candida albicans, Actinomyces naeslundii* and *Streptococcus mutans.*
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ABSTRACT

Microbial interactions are necessarily associated with the development of polymicrobial oral biofilms. The objective of this study was to determine the coaggregation of eight strains of *Candida albicans* with *Actinomyces naeslundii* and *Streptococcus mutans*. In autoaggregation assays, *C. albicans* strains were grown in RPMI-1640 and artificial saliva medium (ASM) whereas bacteria were grown in heart infusion broth. *C. albicans*, *A. naeslundii* and *S. mutans* were suspended to give 10^6, 10^7 and 10^8 cells mL^{-1} respectively, in coaggregation buffer followed by a 1 h incubation. The absorbance difference at 620 nm (ΔAbs) between 0 h and 1 h was recorded. To study coaggregation, the same protocol was used, except combinations of microorganisms were incubated together. The mean ΔAbs% of autoaggregation of the majority of RPMI-1640-grown *C. albicans* was higher than in ASM grown. Coaggregation of *C. albicans* with *A. naeslundii* and/or *S. mutans* was variable among *C. albicans* strains. Scanning electron microscopy images showed that *A. naeslundii* and *S. mutans* coaggregated with *C. albicans* in dual- and triculture. In conclusion, the coaggregation of *C. albicans*, *A. naeslundii* and *S. mutans* is *C. albicans* strain dependent.

Keywords: aggregation; yeast form; hyphal form

INTRODUCTION

Autoaggregation is defined as the adherence ability of microorganisms belonging to the same species (Boris, Suarez and Barbes 1997), while coaggregation is the ability of genetically distinct microorganisms to adhere to each other (Ledder *et al.* 2008). Both autoaggregation and coaggregation have been classified as important mechanisms in the development of oral biofilms and postulated to provide protective mechanisms to the microbial inhabitants against shear forces that occur within the oral cavity. Aggregation contributes to the integration of new microbial species into biofilms, facilitating the exchange of genes and metabolic products that in turn supports survival of microorganisms against variable environmental conditions (Gibbon and Nygaard 1970; Bos, Van-der-Mei and Busscher 1996; Kolenbrander 2000; Kolenbrander *et al.* 2002; Rickard *et al.* 2003; Al-Ahmad *et al.* 2007; Ledder *et al.* 2008).

Furthermore, coaggregation has been shown to improve the colonization of oral epithelial cells by *C. albicans*, as preincubation of buccal epithelial cells with fimbriated strains of

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Escherichia coli or *Klebsiella pneumoniae* increases the adherence and subsequent attachment of *C. albicans* (Bagg and Silverwood 1986). Preadherence of *Streptococcus sanguinis* and *S. gordonii* to the hard surfaces of the oral cavity provides adhesion sites for *C. albicans*, which supports the importance of interkingdom interactions in the oral cavity (Jenkinson, Lala, Shepherd 1990; Bamford *et al.* 2009; Shirtliff, Peters and Jabra-Rizk 2009).

The oral microbiota comprises a wide variety of microorganisms such as yeasts (*C. albicans*) and bacteria (*Actinomyces* spp. and streptococci). *Candida* spp. that belong to kingdom fungi, especially *C. albicans,* have been found to colonize approximately 40–50% of healthy oral cavities (Manfredi *et al.* 2013). The number increases in immunocompromised patients with diseases such as AIDS and diabetes (Grimaudo, Nesbitt and Clark 1996; Thein *et al.* 2009). The human oral microbiome is also comprised of over 600 prevalent taxa at species level although only half of these have been cultured in the laboratory (Dewhirst *et al.* 2010). Among the important oral bacteria, *A. naeslundii* is an early oral colonizer that can constitute up to 27% of supragingival dental plaque (Nyvad and Kilian 1987; Li *et al.* 2004). The ability of this species to coaggregate with other oral microorganisms has been well recognized (Grimaudo, Nesbitt and Clark 1996; Li *et al.* 2001). *Streptococcus mutans*, an acidogenic and aciduric gram-positive oral bacterium, is widely regarded as a causative agent of dental caries (Peters *et al.* 2012).

The majority of *in vitro* studies of oral microbial coaggregation have assessed dual-species oral bacteria interactions (Cisar, Kolenbrander and McIntire 1979; Handley *et al.* 1985; Eke, Rotimi and Laughon 1989; Umemoto *et al.* 1999; Foster and Kolenbrander 2004; Shen, Samaranayake and Yip 2005; Rosen and Sela 2006; Ledder *et al.* 2008), and information of interkingdom interactions is limited. Further, as yet, no study utilizing artificial saliva medium (ASM) for the growth of *C. albicans* has been undertaken to assess interkingdom coaggregation. This is clinically relevant as *C. albicans* grows as yeast in ASM and as hyphae in RPMI-1640, and this dimorphism has a role in the virulence of the species (Arzmi *et al.* 2012, 2014). The yeast form of *C. albicans* can adhere to the host cell surfaces by the expression of adhesins, which trigger yeast-to-hyphae transition, followed by the expression of invasins by the hyphal form that mediate the uptake of the fungus by the host cell through endocytosis (Molero *et al.* 1998; Gow *et al.* 2011; Sudbery 2011; Mayer, Wilson and Hube 2013). In addition, research has also found that *S. salivarius* strain K12 preferred to coaggregate to the hyphal region of *C. albicans* than the yeast after 3 h incubation in RPMI-1640 at planktonic phase (Ishijima *et al.* 2012). A similar interaction was also observed between *S. gordonii* and *C. albicans* in which more bacteria coaggregated at the hyphal region of the yeast (Bamford *et al.* 2009).

The aim of the present study was to determine the coaggregation of *C. albicans*, *A. naeslundii* and *S. mutans* with the hyphotheses that autoaggregation and coaggregation are *C. albicans* strain dependent.

MATERIALS AND METHODS

Growth of microorganisms

C. albicans American Type Cell Culture (ATCC) 32354 (ALT1), ATCC MYA-2876 (ALT2), ATCC 90234 (ALT3), ATCC 18804 (ALT4), genotype A isolated from AIDS patient (ALC1), genotype B isolated from AIDS patient (ALC2), oral cancer isolate 1 (ALC3) and oral cancer isolate 2 (ALC4) were used in this study. *C. albicans* strains were subcultured on Sabauraud's dextrose agar (Difco, USA) and incubated at 37°C aerobically for 24 h.

To grow bacteria, stock cultures of *A. naeslundii* (NCTC 10301) and *S. mutans* (Ingbritt), provided by Oral Health Cooperative Research Centre, Melbourne Dental School, The University of Melbourne, were revived by subculturing onto blood agar (Difco, USA) and Todd-Hewitt yeast extract agar (Difco, USA), respectively. The agar plates were incubated at 37°C for 48 h.

Aggregation assay

A semiquantitative spectrophotometric assay based on that outlined by Ledder *et al.* (2008) and Nagaoka *et al.* (2008) was used to analyse the aggregation of the microorganisms. Initially, 24-h cultures of *C. albicans* grown aerobically in RPMI-1640 or 25% ASM (0.625 g L^{-1} type II porcine gastric mucin, 0.5 g L^{-1} bacteriological peptone, 0.5 g L^{-1} tryptone, 0.25 g L^{-1} yeast extract, 0.088 g L^{-1} NaCl, 0.05 g L^{-1} KCl, 0.05 g L^{-1} $CaCl_2$ and 0.25 mg mL^{-1} haemin, pH 7.0 supplemented with 2.5 mM DTT and 0.5 g L^{-1} sucrose) to stationary phase were harvested by centrifuging at 12 000 g for 5 min and washed twice using coaggregation buffer (0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 150 mM NaCl, 3.1 mM NaN_3 dissolved in 1 mM Tris buffer and adjusted to pH 7.0). The supernatant was discarded and the pellet resuspended in coaggregation buffer. A similar protocol was repeated for *S. mutans* and *A. naeslundii* except these microorganisms were grown in heart infusion broth (HIB) to stationary phase.

To determine autoaggregation, *C. albicans, A. naeslundii* and *S. mutans* were standardized in coaggregation buffer to give a final cell density of 10^6, 10^7 and 10^8 cells mL^{-1}, respectively in separate sterile 2 mL Eppendorf tubes that were equivalent to an optical density of 0.5 at 620 nm wavelength (OD_{620nm}). Each suspension was mixed thoroughly using a vortex mixer for 30 s and the OD_{620nm} at time (t) = 0 h was measured. The inoculum was incubated at room temperature for 1 h to allow aggregation and the OD_{620nm} was recorded. Sterile coaggregation buffer was used as the blank. Percentage aggregation was calculated using the following equation:

$$\begin{aligned}\%\ \text{Auto-aggregation} = ([&\mathrm{OD}_{620\mathrm{nm}}(t = 0\,\mathrm{h})\\ &- \mathrm{OD}_{620\mathrm{nm}}(t = 1\,\mathrm{h})]/\mathrm{OD}_{620\mathrm{nm}}(t = 0\,\mathrm{h})) \times 100\end{aligned}$$

Percentage autoaggregation was calculated for classification of autoaggregation; (1) high (more than 40%), (2) intermediate (30–40%) and (3) low autoaggregation (less than 30%).

A similar protocol was repeated for the study of coaggregation by inoculating *C. albicans*, *A. naeslundii* or/and *S. mutans* (interkingdom), and *A. naeslundii* and *S. mutans* (intrakingdom) into a sterile 2 mL Eppendorf tube with the same cell density as in the autoaggregation. The suspension was mixed thoroughly using a vortex mixer and the OD_{620nm} at $t = 0$ h was recorded. The suspension was incubated at room temperature for 1 h followed by the measurement of optical density at $OD_{620\ nm}$. The OD_{620nm} at time (t) = 0 h of dual culture and triculture were 1.0 and 1.5, respectively.

Coaggregation was assessed by measuring percentage coaggregation using the following equation:

$$\begin{aligned}\%\ \text{Co-aggregation} = ([&\mathrm{OD}_{620\mathrm{nm}}(t = 0\,\mathrm{h})\\ &- \mathrm{OD}_{620\mathrm{nm}}(t = 1\,\mathrm{h})]\ /\mathrm{OD}_{620\mathrm{nm}}(t = 0\,\mathrm{h})) \times 100\end{aligned}$$

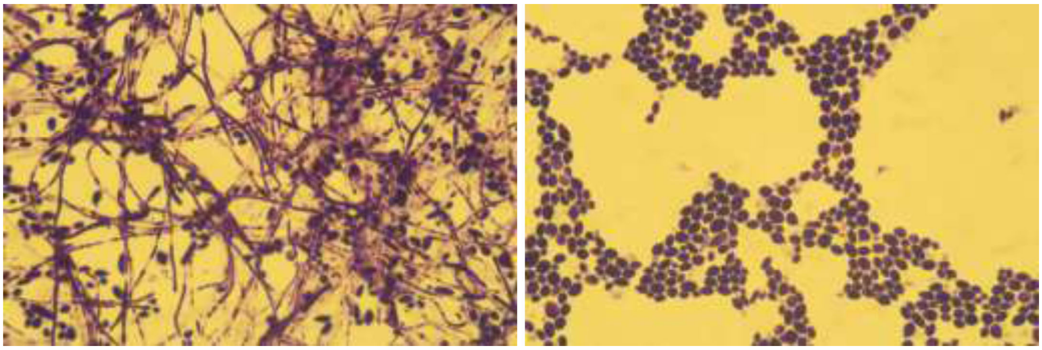


Figure 1. Gram-stained of *C. albicans* cultures observed under light microscopy at 1000× magnification. Left: *C. albicans* (ALT4) grown in RPMI-1640 after 24 h incubation at 37°C; >75% of *C. albicans* cells were present in hyphal form in this medium. Right: *C. albicans* (ALT4) grown in ASM after 24 h incubation at 37°C; 100% of *C. albicans* displaying yeast morphology in this medium.

Scanning electron microscopy (SEM) imaging

The 0 h and 1 h suspensions (100 μL sample) of a selected representative *C. albicans* strain, ALT4, *A. naeslundii* (NCTC 10301) and *S. mutans* (Ingbritt), prepared as above, were transferred onto cover slips and fixed with 1% osmium tetra-oxide (OsO_4) vapour. The specimens were dehydrated thoroughly in a freeze-drying system, sputter coated with palladium gold to a thickness of approximately 20 nm and observed using a scanning electron microscope (XL 30 Series, Philips, Japan).

Statistical analysis

All data were statistically analysed using SPSS software version 22.0 using independent *t*-test and considered statistically significant when $P < 0.05$.

RESULTS

Morphology of *C. albicans* in RPMI-1640 and ASM

C. albicans was shown to be predominantly in the hyphal form when grown in RPMI-1640 medium after 24 h incubation whereas the yeast form was the most observed in ASM after the same period of incubation (Fig. 1).

Autoaggregation

Variation in autoaggregation of RPMI-1640 grown *C. albicans* strains (hyphal growth) was observed with a group of four strains (ALT3, ALT4, ALC1 and ALC3) exhibiting high autoaggregation (over 40%), two strains (ALT1 and ALC4) exhibiting intermediate autoaggregation (30–40%) and two strains (ALT2 and ALC2) exhibiting low autoaggregation (Table 1; Fig. 2A). The autoaggregation values of *A. naeslundii* and *S. mutans* were also classified as low with 11.4 and 7.4%, respectively (Table 1).

Four strains of ASM-grown *C. albicans* (ALT2, ALT3, ALC1 and ALC4) (yeast growth) exhibiting intermediate autoaggregation while the remainder strains (ALT1, ALT4, ALC2 and ALC3) were classified as exhibiting low autoaggregation (Table 1; Fig. 2B).

There were four strains of *C. albicans* that exhibited significantly more autoaggregation when grown in RPMI-1640 (hyphal growth) (ALT1, ALT4, ALC1 and ALC3) compared to ASM (yeast growth) ($P<0.05$). Two strains (ALT2 and ALC2) showed significantly more autoaggregation when grown in ASM than RPMI-1640 ($P<0.05$) and two strains (ALT3 and ALC4) exhibited no difference in autoaggregation regardless of the media type (Fig. 2).

Interkingdom coaggregation

All strains of RPMI-grown *C. albicans* (hyphal growth) were found to coaggregate with *A. naeslundii* ranging from 9.9 ± 0.5% (ALT3) to 26.2 ± 0.4% (ALC3). Coaggregation of RPMI-grown *C. albicans* with *A. naeslundii* and *S. mutans* was also observed for all strains of the yeast ranging from 2.2 ± 0.3% (ALT3) to 17.0 ± 0.6% (ALC1). Our study showed that ASM-grown *C. albicans* strains (yeast form) coaggregated with *A. naeslundii* ranging from 9.6 ± 0.7% (ALT2) to 23.0 ± 0.1% (ALC3). ASM-grown *C. albicans* strains were observed to coaggregate *S. mutans* ranging from 9.9 ± 0.2% (ALT3) to 28.1 ± 0.1% (ALT4) (Table 1). Coaggregation of ASM-grown *C. albicans* with *A. naeslundii* and *S. mutans* were observed in all strains of the yeast ranging from 12.9 ± 0.4% (ALT2) to 25.8 ± 0.5% (ALT1) (Table 1).

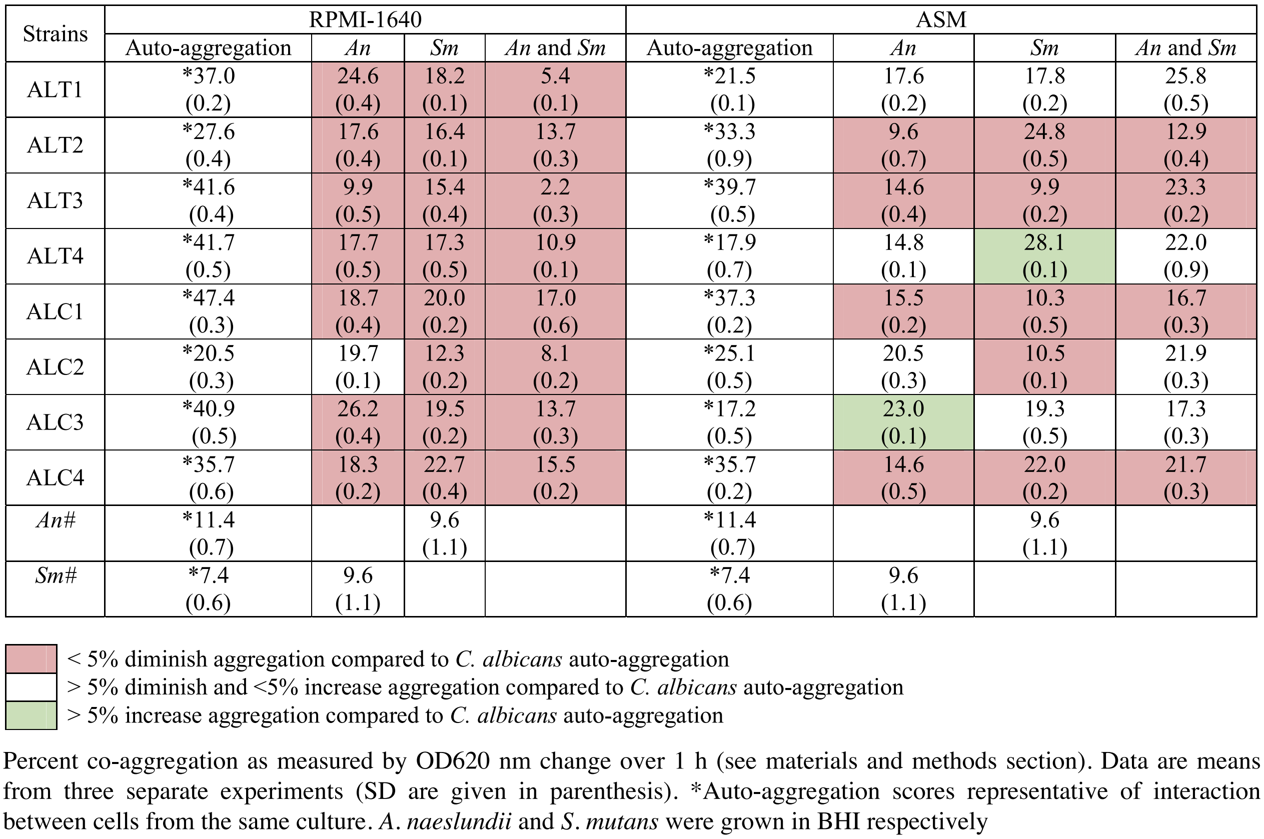
SEM analyses

SEM analysis of RPMI-grown *C. albicans* ALT4 strain exhibited autoaggregation in coaggregation buffer after 1 h incubation (Fig. 3A). Coaggregation was observed between *C. albicans* and *A. naeslundii* (Fig. 3B). In addition, an SEM image also revealed that *S. mutans* coaggregated with *C. albicans* mostly at the hyphal region of the yeast (Fig. 3C). The coaggregation of RPMI-grown ALT4 *C. albicans* with *A. naeslundii* and *S. mutans* showed that *A. naeslundii* and *S. mutans* were partially aggregating with *C. albicans* at the hyphal region. *A. naeslundii* was also observed to coaggregate with *S. mutans* (Fig. 3D).

SEM analysis showed that ASM-grown *C. albicans* ALT4 strain (yeast growth) had autoaggregation (Fig. 3E) and *A. naeslundii* was found to coaggregate on the yeast surface after 1 h incubation (Fig. 3F). Coincubation of ALT4 *C. albicans* with *S. mutans* revealed that there was interkingdom coaggregation between the two microorganisms with clumps of bacteria attached to the yeast surface of ALT4 *C. albicans* (Fig. 3G). In addition, an SEM image of the interaction between ASM-grown ALT4 *C. albicans* with both bacterial species showed that *A. naeslundii* and *S. mutans* coaggregated on the surface of the yeast. Finally, the image also revealed that *S. mutans* cells were coaggregating with *A. naeslundii* after 1 h incubation (Fig. 3H).

Taken together, the data demonstrate that the autoaggregation and interkingdom coaggregation of *C. albicans*, *A. naeslundii* and *S. mutans* are *C. albicans* strain dependent.

Table 1. Mono- and dual-culture aggregation scores of pairs of eight strains of RPMI-grown *C. albicans* (hyphal form), *A. naeslundii* and *S. mutans*.



Strains	RPMI-1640				ASM			
	Auto-aggregation	*An*	*Sm*	*An* and *Sm*	Auto-aggregation	*An*	*Sm*	*An* and *Sm*
ALT1	*37.0 (0.2)	24.6 (0.4)	18.2 (0.1)	5.4 (0.1)	*21.5 (0.1)	17.6 (0.2)	17.8 (0.2)	25.8 (0.5)
ALT2	*27.6 (0.4)	17.6 (0.4)	16.4 (0.1)	13.7 (0.3)	*33.3 (0.9)	9.6 (0.7)	24.8 (0.5)	12.9 (0.4)
ALT3	*41.6 (0.4)	9.9 (0.5)	15.4 (0.4)	2.2 (0.3)	*39.7 (0.5)	14.6 (0.4)	9.9 (0.2)	23.3 (0.2)
ALT4	*41.7 (0.5)	17.7 (0.5)	17.3 (0.5)	10.9 (0.1)	*17.9 (0.7)	14.8 (0.1)	28.1 (0.1)	22.0 (0.9)
ALC1	*47.4 (0.3)	18.7 (0.4)	20.0 (0.2)	17.0 (0.6)	*37.3 (0.2)	15.5 (0.2)	10.3 (0.5)	16.7 (0.3)
ALC2	*20.5 (0.3)	19.7 (0.1)	12.3 (0.2)	8.1 (0.2)	*25.1 (0.5)	20.5 (0.3)	10.5 (0.1)	21.9 (0.3)
ALC3	*40.9 (0.5)	26.2 (0.4)	19.5 (0.2)	13.7 (0.3)	*17.2 (0.5)	23.0 (0.1)	19.3 (0.5)	17.3 (0.3)
ALC4	*35.7 (0.6)	18.3 (0.2)	22.7 (0.4)	15.5 (0.2)	*35.7 (0.2)	14.6 (0.5)	22.0 (0.2)	21.7 (0.3)
An#	*11.4 (0.7)		9.6 (1.1)		*11.4 (0.7)		9.6 (1.1)	
Sm#	*7.4 (0.6)	9.6 (1.1)			*7.4 (0.6)	9.6 (1.1)		

🟥 < 5% diminish aggregation compared to *C. albicans* auto-aggregation
⬜ > 5% diminish and <5% increase aggregation compared to *C. albicans* auto-aggregation
🟩 > 5% increase aggregation compared to *C. albicans* auto-aggregation

Percent co-aggregation as measured by OD620 nm change over 1 h (see materials and methods section). Data are means from three separate experiments (SD are given in parenthesis). *Auto-aggregation scores representative of interaction between cells from the same culture. *A. naeslundii* and *S. mutans* were grown in BHI respectively

DISCUSSION

Coaggregation is a mechanism that induces the development of a complex architecture of oral biofilms, which assists the attachment of secondary colonizers such as *S. mutans* (Kolenbrander 2000; Min and Rickard 2009).

We have shown that interkingdom coaggregation was strain dependent. The coaggregation of the majority of RPMI-grown (hyphal growth) *C. albicans* strains, when grown with *S. mutans* and *A. naeslundii* either alone or in combination, resulted in variable coaggregation. The observed variability of coaggregation in *C. albicans* may be attributable to the different abundances of specific molecules that are important in adhesion and quorum sensing (e.g. farnesol) from different strains, which have been suggested to have a role in interkingdom interactions of *C. albicans* and bacteria (Morales and Hogan 2010). Furthermore, the variability of coaggregation observed in ASM-grown *C. albicans* (yeast growth) supports our hypothesis that the coaggregation of *C. albicans* to *A. naeslundii* and *S. mutans* is highly dependent on the individual yeast strain.

We have observed variability of coaggregation when ASM-grown *C. albicans* strains were coincubated with *S. mutans* and *A. naeslundii*. This variability suggests that *S. mutans* might have induced the formation of binding sites on the yeast surface that allow the coaggregation of *A. naeslundii* to ASM-grown *C. albicans* when cocultured. These results support our hypothesis that coaggregation is highly dependent on the *C. albicans* strain. It cannot be related to the production of glucan by *S. mutans* glucosyltransferases as no sucrose was present; however, it may be that specific proteins are induced on the surface of *C. albicans* due to the interaction with *S. mutans* that promotes further interaction with *A. naeslundii* (Holmes, Gopal and Jenkinson 1995; Koo *et al.* 2010; Falsetta *et al.* 2014). Further research is necessary to assess this hypothetical possibility.

It can be postulated that the observed variability in coaggregation may be related to that specific strain's ability to produce both non-specific (adhesins) and specific (lectin-saccharide) cell surface receptors (Kolenbrander and Williams 1981; Mcintire, Crosby and Vatter 1982; Rickard *et al.* 2003; Rosen and Sela 2006; Ledder *et al.* 2008). Previous studies have shown that the specific coaggregation between *C. albicans* and *A. naeslundii* is due to the presence of mannose-containing adhesin protein on the yeast cell surface (Grimaudo, Nesbitt and Clark 1996). This same study also showed variation in the coaggregation of *A. naeslundii* with four different yeast strains which supports the present study. Furthermore, other research has shown significant strain variation of the cell wall biogenesis in *C. albicans*, that may have a role in the observed variation in aggregation ability (Ragni *et al.* 2011). Further analysis of the cell wall structure of a range of *C. albicans* strains is necessary to fully elucidate the mechanism of this observed variability.

It has previously been suggested that, due to the limitation of nutrients present in RPMI-1640, growth in this media induces yeast–hyphae transition leading to predominant hyphal growth (Urban *et al.* 2006). Our light microscope images confirmed this with greater than 75% of *C. albicans* cells growing in hyphal form in RPMI-1640. No previous study has assessed the form of growth at SEM level when *C. albicans* is grown in ASM. The present study

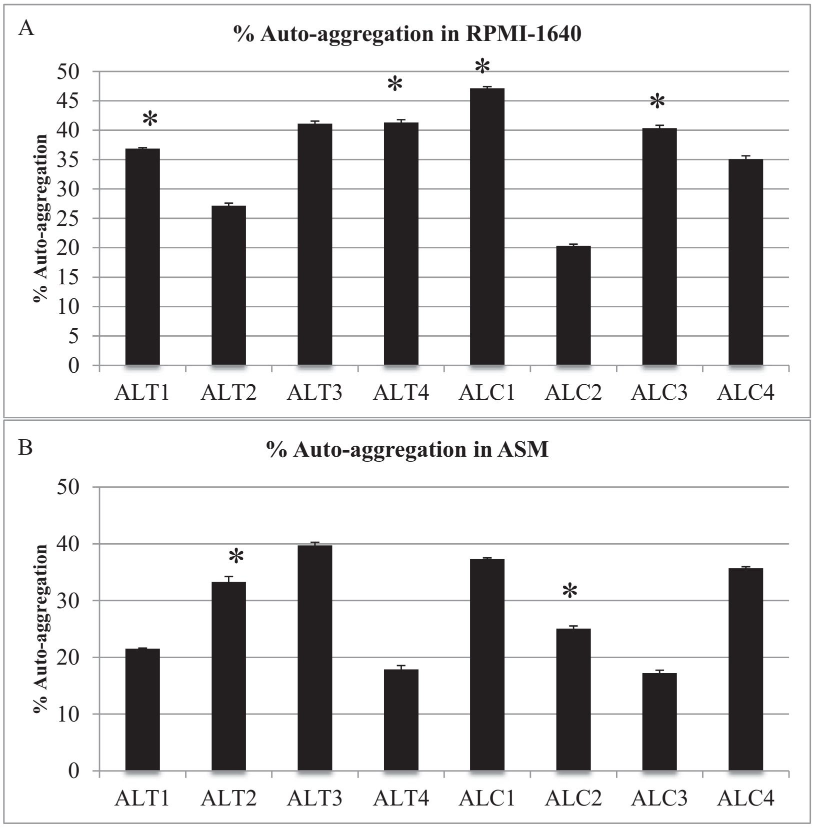


Figure 2. Percentage autoaggregation in RPMI-1640 (**A**) and ASM (**B**) grown *C. albicans* after 1 h incubation in coaggregation buffer. Data were analysed using independent *t*-test and considered as significantly different when $P < 0.05$. Asterisk indicates significantly more autoaggregation between the two growth media.

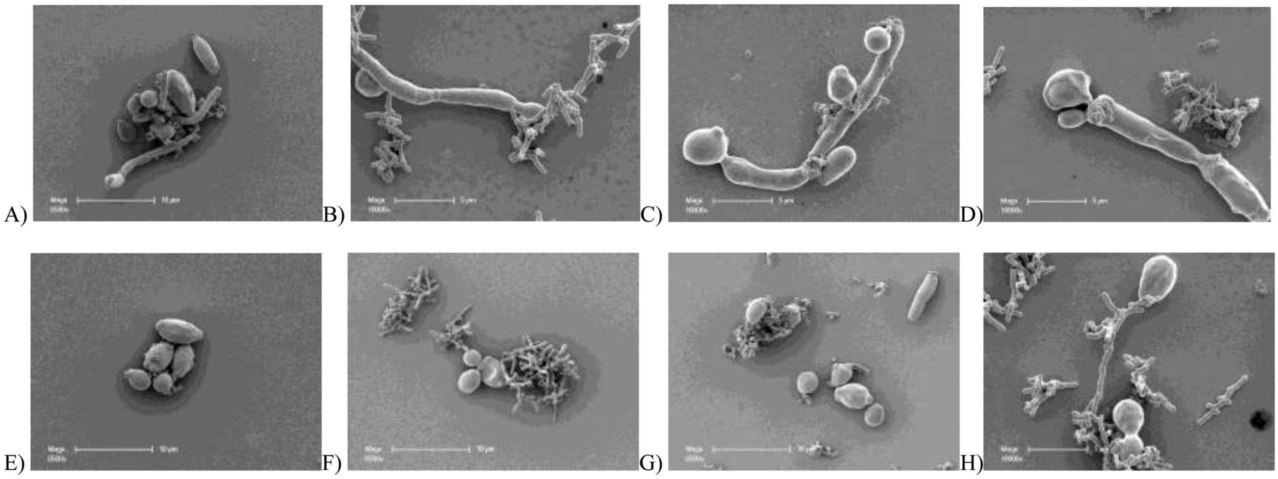


Figure 3. SEM of *C. albicans* autoaggregation (**A** and **E**), interkingdom interaction with *A. naeslundii* (**B** and **F**), *S. mutans* (**C** and **G**) and both bacteria (**D** and **H**). *C. albicans* was grown in RPMI-1640 (A–D) and ASM (E–H). Magnification is as shown on each image (6500× and 10 000×).

is the first to observe *C. albicans* cellular morphology in ASM using SEM imaging and we have shown that, similar to the light microscope observations, in this media *C. albicans* does not grow in hyphal form.

Future assessment of coaggregation of *C. albicans*, *A. naeslundii* and *S. mutans* requires animal studies to assess oral biological factors, such as salivary flow and immunological components that exist in the oral cavity, which may influence aggregation. These *in vivo* studies of coaggregation are likely to enhance our understanding of the mutual interaction of microorganisms in the oral cavity, a process likely to be critical in chronic infection and potentially oral carcinogenesis.

CONCLUSION

In conclusion, autoaggregation and interkingdom coaggregation of *C. albicans* have been shown to be strain dependent and this is likely to be important in polymicrobial oral biofilm formation.

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Conflict of interest. None declared.

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