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Author(s)	Bandara, HMHN; Yau, JYY; Watt, RM; Jin, LJ; Samaranayake, LP
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Escherichia coli and its lipopolysaccharide modulate *in vitro* *Candida* biofilm formation

H. M. H. N. Bandara, J. Y. Y. Yau, R. M. Watt, L. J. Jin
and L. P. Samaranayake

Correspondence

L. P. Samaranayake
lakshman@hkucc.hku.hk

Faculty of Dentistry, University of Hong Kong, Oral Biosciences, 5/F Prince Phillip Dental Hospital, 34 Hospital Road, Sai Ying Pun, Hong Kong SAR

Demystification of microbial behaviour in mixed biofilms could have a major impact on our understanding of infectious diseases. The objectives of this study were to evaluate *in vitro* the interactions of six different *Candida* species and a Gram-negative coliform, *Escherichia coli*, in dual-species biofilms, and to assess the effect of *E. coli* LPS on *Candida* biofilm formation. A single isolate of *E. coli* ATCC 25922 and six different species of *Candida*, *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Candida tropicalis* ATCC 13803, *Candida parapsilosis* ATCC 22019 and *Candida dubliniensis* MYA-646, were studied using a standard biofilm assay. Each *Candida* species was co-cultured with *E. coli* on a polystyrene surface and biofilm formation was quantified by a c.f.u. assay. The biofilm was then analysed by Live/Dead staining and fluorescence microscopy (confocal laser-scanning microscopy, CLSM), whilst scanning electron microscopy (SEM) was employed to visualize the biofilm architecture. The effect of *E. coli* LPS on *Candida* biofilm cell activity at defined time intervals was assessed with an XTT reduction assay. A significant quantitative reduction in c.f.u. counts of *C. tropicalis* (after 90 min), *C. parapsilosis* (after 90 min and 24 h), *C. krusei* (after 24 h) and *C. dubliniensis* (after 24 and 48 h) was noted on incubation with *E. coli* in comparison with their monospecies biofilm counterparts ($P < 0.05$). On the other hand, a simultaneous and significant reduction in *E. coli* cell numbers occurred on co-culture with *C. albicans* (after 90 min), and an elevation of *E. coli* cell numbers followed co-culture with *C. tropicalis* (after 24 h) and *C. dubliniensis* (after 24 h and 48 h) ($P < 0.05$). All quantitative findings were confirmed by SEM and CLSM analyses. By SEM observation, dual-species biofilms demonstrated scanty architecture with reduced visible cell counts at all stages of biofilm development, despite profuse growth and dense colonization in their single-species counterparts. Significantly elevated metabolic activity, as assessed by XTT readings, was observed in *E. coli* LPS-treated *C. tropicalis* and *C. parapsilosis* biofilms (after 48 h), whilst this had the opposite effect for *C. dubliniensis* (after 24 h) ($P < 0.05$). These data indicate that *E. coli* and *Candida* species in a mixed-species environment mutually modulate biofilm development, both quantitatively and qualitatively, and that *E. coli* LPS appears to be a key component in mediating these outcomes.

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INTRODUCTION

In nature, most micro-organisms do not exist as pure species and prefer a lifestyle of community growth with one or more other species in a complex community organization termed a biofilm. By definition, a biofilm is a complex functional community of one or more species of microbes encased in an extracellular polymeric network and attached to one another or to a solid surface (Samaranayake, 2006).

Abbreviations: CLSM, confocal laser-scanning microscopy; SEM, scanning electron microscopy.

A table of c.f.u. counts of *Candida* species and *E. coli* from biofilms at different time intervals is available as supplementary data with the online version of this paper.

According to the US National Health Institute and the Centers for Disease Control and Prevention, it has been estimated that more than 60% of all microbial infections are associated with biofilms (Lewis, 2001; Potera, 1999). Some examples of biofilm-related infections include cystic fibrosis-related infections, catheter-related infections, periodontal diseases and middle ear infections (Douglas, 2003).

Candida, a common commensal fungus of humans, may convert to an opportunistic pathogen causing superficial or deep infections in susceptible hosts including neonates, patients undergoing immunosuppressive or broad-spectrum antibiotic therapy, or those with indwelling catheters, human immunodeficiency virus infection and diabetes (Odds, 1987).

Indeed, *Candida* is considered the fourth most commonly isolated pathogen from bloodstream infections in US institutions (Banerjee *et al.*, 1991; Jones *et al.*, 1997; Pfaller *et al.*, 1998) and these infections appear to have, overall, the highest crude mortality (Crump & Collignon, 2000).

Constitutionally, a biofilm comprises either a single species (monospecies) or, more usually, multiple species of either bacteria alone or bacteria together with fungi such as *Candida* (multispecies) (Nobile & Mitchell, 2007). The architecture and functionality of such multispecies biofilms are complex and the biofilms exhibit interspecies relationships that have yet to be unravelled, as the organisms co-exist within a community with multifarious demands (Carlsson, 1997). Most of the work in the literature investigating such multispecies biofilms is based on multi-bacterial biofilms and there are few data on the interactions within fungal–bacterial biofilms. In one of the earliest studies, Nair & Samaranyake (1996a) demonstrated that adhesion of *Candida albicans* to denture acrylic surfaces was promoted by *Escherichia coli*, a Gram-negative bacterium. In subsequent studies, they reported that this coliform significantly suppressed the adhesion of *C. albicans* and *Candida krusei* to human buccal epithelial cells (Nair & Samaranyake, 1996b). More recently, the same group (Thein *et al.*, 2006) confirmed that coliforms such as *E. coli* and *Pseudomonas aeruginosa* can significantly compromise *C. albicans* biofilm formation in a dose-dependent manner.

The endotoxin LPS, a cell-wall constituent of all Gram-negative bacteria including *E. coli*, possesses immunomodulatory and pro-inflammatory properties (Morrison & Ryan, 1987; Rietschel *et al.*, 1982). The effect of LPS on *Candida* was examined by Akagawa *et al.* (1995) and Henry-Stanley *et al.* (2003), who noted that *Candida*-associated mortality of experimental animals was augmented by *E. coli* and its LPS. It has been stated that LPS itself has no direct effect on candidal growth (Palma *et al.*, 1992), but the immunomodulatory effects may be due to a secondary effect on polymorphonuclear leukocytes and monocytes (Cohn, 1978; Dahinden *et al.*, 1983; Henricks *et al.*, 1983). To date, the role of *E. coli* LPS on *Candida*–*E. coli* mixed biofilms has not been evaluated. Furthermore, most of the previous studies on interactions between *Candida* and bacteria in mixed biofilms focused only on *C. albicans*, and there are no studies to our knowledge relating to non-*albicans* *Candida* species biofilms in a mixed-species environment.

Hence, the aims of this study were to evaluate the interactions of *E. coli* and six different *Candida* species, *C. albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *Candida dubliniensis* and *C. krusei*, in dual-species biofilms and to assess the effect of *E. coli* LPS on the biofilm development of these *Candida* species.

METHODS

Experimental design. The study comprised two components. First, a series of experiments was conducted to evaluate the combined effect

of each of six different species of *Candida* and one isolate of *E. coli* on biofilm formation at three different time intervals. Secondly, a series of experiments was conducted to assess the effect of *E. coli* LPS on the biofilm development of the selected *Candida* species.

Micro-organisms. The following reference laboratory strains of *Candida* and *E. coli* were used: *C. albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. tropicalis* ATCC 13803, *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, *C. dubliniensis* MYA-646 and *E. coli* ATCC 25922. The identity of each organism was confirmed with the commercially available API 32C (for *Candida* strains) and API 20E (for *E. coli*) identification systems (bioMérieux). All isolates were stored in multiple aliquots at -20°C , after confirming their purity.

LPS. LPS purified from *E. coli* O111 : B4 (Ready Made solution, 1 mg ml^{-1} , $0.2\ \mu\text{m}$ filtered) was purchased from Sigma Aldrich and stored at $2-8^{\circ}\text{C}$ until use.

Growth media. Sabouraud dextrose agar (SDA) and yeast nitrogen base (YNB) solution supplemented with $100\ \text{mM}$ glucose were used for culturing *Candida* species, and blood agar, MacConkey agar and tryptic soy broth (TSB) were utilized for *E. coli* culture.

Microbial inocula. Prior to each experiment, *Candida* species and *E. coli* were subcultured on SDA and blood agar, respectively, for 18 h at 37°C . A loopful of this overnight growth was inoculated into YNB (*Candida*) or TSB (*E. coli*) medium and incubated for 18 h in an orbital shaker (75 r.p.m.) at 37°C . The resultant growth was harvested, washed twice in PBS and resuspended in PBS. PBS without calcium and magnesium ions was used to wash and rinse the suspended cells, as these ions facilitate cell binding and clumping, which would mask the mutual effects of *Candida* and *E. coli* during biofilm development. Concentrations of *Candida* species and *E. coli* were adjusted to 1×10^7 cells ml^{-1} by spectrophotometry and were confirmed by haemocytometric counting.

Biofilm formation. *Candida* biofilms were developed as described by Jin *et al.* (2004) with some modifications. Commercially available pre-sterilized, polystyrene, flat-bottomed, 96-well microtitre plates (Iwaki) were used. A standard cell suspension ($100\ \mu\text{l}$) of *Candida* species and *E. coli* (10^7 cells ml^{-1} , 1:1 ratio) was prepared and transferred into selected wells of a microtitre plate. The plate was incubated for 1.5 h at 37°C in an orbital shaker at 75 r.p.m. to promote microbial adherence to the surface of the wells. A volume of $100\ \mu\text{l}$ of monospecies controls of *Candida* species and *E. coli* was inoculated in an identical fashion. After the initial adhesion phase, the cell suspensions were aspirated and each well was washed twice with PBS to remove loosely adherent cells. A volume of $200\ \mu\text{l}$ TSB was added to each well and the plate was reincubated for 24 or 48 h. The wells were then washed two or three times at respective time intervals with PBS to eliminate traces of TSB. The bacterial–fungal interactions were studied at 90 min (adhesion phase), 24 h (colonization phase) and 48 h (maturation phase) time intervals.

Quantitative analyses

Spiral plating and c.f.u. assay. At the end of the adhesion (90 min), colonization (24 h) and maturation (48 h) phases, after washing as described above, $100\ \mu\text{l}$ PBS was added to each well and the biofilm mass was meticulously scraped off the well wall using a sterile scalpel (Jin *et al.*, 2004). The resultant suspension containing the detached biofilm cells was gently vortexed for 1 min to disrupt the aggregates, serially diluted and inoculated using a spiral plater onto SDA for *Candida* species and MacConkey agar for *E. coli*. The resultant c.f.u. ml^{-1} of yeast and bacteria were quantified after 48 h incubation at 37°C . Each assay was carried out in triplicate on three different occasions.

Evaluation of the effect of *E. coli* LPS using an XTT reduction assay. In the second part of the study, the effect of *E. coli* LPS on *Candida* species was evaluated. Freshly prepared LPS (1 mg ml⁻¹, Ready Made solution; Sigma Aldrich) and YNB supplemented with 100 mM glucose were mixed to yield a final concentration of 100 µg LPS ml⁻¹ in the medium of the test sample. LPS was replaced with sterile PBS in the control. *Candida* biofilms were formed in three different 96-well plates to evaluate the effect of *E. coli* LPS at 90 min, 24 h and 48 h as described below.

First, *Candida* suspension medium (100 µl each) was decanted after centrifugation, resuspended in test and control medium (see above), dispersed into selected wells of a 96-well plate at time 0 and incubated in an orbital shaker (75 r.p.m.) at 37 °C for 90 min for the adhesion assay. In the second assay, 200 µl test and control medium was added at 90 min to a different 96-well plate with pre-adherent *Candida* and incubated for 24 h for the initial colonization study. Finally, in the third assay, the latter procedure was repeated at 24 h for initially colonized *Candida* in a different 96-well plate and incubated for a further 24 h for the maturation phase study. At the end of the incubation period, the supernatant was removed and the wells were washed twice with sterile PBS to remove loosely adherent cells. A standard XTT reduction assay was performed, as described by Jin *et al.* (2004), to measure the metabolic activity of biofilms. In brief, commercially available XTT powder (Sigma) was dissolved in PBS to a final concentration of 1 mg ml⁻¹. The solution was filter-sterilized (0.22 µm pore-size filter) and stored at -70 °C. Freshly prepared 0.4 mM menadione solution was used for the XTT reduction assay. XTT solution was thawed and mixed with menadione solution at a ratio of 20:1 (v/v) immediately before the assay. Thereafter, 158 µl PBS, 40 µl XTT and 2 µl menadione solution were added to each well containing pre-washed biofilms and incubated in the dark for 3 h at 37 °C. Colour changes were measured with a microtitre plate reader (SpectraMax 340 tunable microplate reader; Molecular Devices) at 492 nm. All assays were carried out in triplicate on three different occasions.

Qualitative analyses Confocal laser-scanning microscopy (CLSM) (Jin *et al.*, 2005) and scanning electron microscopy (SEM) were used to observe the ultrastructure of the *Candida* and *E. coli* biofilms.

CLSM. Commercially available pre-sterilized flat-bottomed six-well plates (Iwaki) and pre-sterilized plastic coverslips (Thermanox; Nulge Nunc International) (Ramage *et al.*, 2001) were used to prepare biofilms as described above. Pre-sterilized coverslips were placed in the wells of a six-well plate, and suspensions of the monospecies or dual species were added. The plate was incubated for 90 min (adhesion phase) in an orbital shaker (75 r.p.m.) at 37 °C. Thereafter, the supernatant was removed, the wells were washed twice with PBS, fresh TSB was added and the plate was incubated for 24 h (initial colonization) or 48 h (maturation phase) under the same environmental conditions. At the end of each time interval, the pre-washed coverslips were stained with Live/Dead stain (Live/Dead BacLight Bacterial Viability kit; Invitrogen) (Jin *et al.*, 2005). The biofilm was then analysed by fluorescence microscopy (by CLSM).

SEM. For SEM, we grew single-species biofilms (*Candida* or *E. coli* alone), as well as *Candida* and *E. coli* mixed biofilms, on custom-made, tissue culture-treated, polystyrene coverslips as described above. At 90 min, 24 h and 48 h, selected coverslips were removed from the wells, washed twice with PBS and placed in 1% osmium tetroxide for 1 h. Samples were subsequently washed in distilled water, dehydrated in a series of ethanol washes (70% for 10 min, 95% for 10 min and 100% for 20 min) and air dried in a desiccator prior to sputter coating with gold. Specimens were mounted on aluminium stubs with copper tape and coated with gold under low pressure with an ion sputter coater (JFC1 100; JEOL). The surface topographies of the biofilm were visualized by SEM (Philips XL30CP) in high-vacuum mode at 10 kV and the images were processed.

Statistical analysis Statistical analysis was performed using SPSS software (version 16.0). A Mann-Whitney *U*-test was performed to compare the significant differences between the control and each test sample of the bacterial-candidal biofilm. Data from all *Candida* species and *E. coli* analyses at different time points were pooled and evaluated using a Wilcoxon matched-pairs test. The percentage modulation of candidal biofilm growth in the presence of *E. coli* and *E. coli* LPS in pooled data was compared using a χ^2 test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Candida and *E. coli* dual-species biofilm growth (supplementary Table S1 in JMM Online)

After 90 min of biofilm development with *E. coli*, a significant reduction in *Candida* cell counts was noted with *C. tropicalis* (18%, $P=0.010$) and *C. parapsilosis* (74%, $P=0.030$) compared with the controls, but this effect was not seen for *C. albicans*, *C. glabrata*, *C. dubliniensis* or *C. krusei* (Fig. 1a). Conversely, after 90 min, a significant reduction in the number of *E. coli* c.f.u. (44%, $P=0.047$) was observed in the presence of *C. albicans* but not with the other five *Candida* species (Fig. 1d).

After prolonged incubation for 24 h, a significant reduction in the counts of *C. krusei* (76%, $P=0.050$), *C. parapsilosis* (78%, $P=0.011$) and *C. dubliniensis* (69%, $P < 0.001$) was noted in dual-species biofilms with *E. coli* (Fig. 1b). However, *C. albicans*, *C. tropicalis* and *C. glabrata* counts were unaffected. On the other hand, the mean number of *E. coli* c.f.u. increased significantly in the presence of *C. tropicalis* (110%, $P=0.019$) and *C. dubliniensis* (11%, $P=0.017$) after 24 h, whilst the other four *Candida* species had no significant effect on *E. coli* numbers at this time point (Fig. 1e).

On further incubation for 48 h, *C. dubliniensis* growth was highly suppressed in dual-species biofilms with *E. coli* (85%, $P < 0.001$), whilst the remaining *Candida* species were unaffected (Fig. 1c). Simultaneously, the mean c.f.u. of *E. coli* increased dramatically in co-cultures with *C. tropicalis* (170%, $P=0.003$) and moderately so with *C. glabrata* (50%, $P=0.042$) (Fig. 1f). This effect on *E. coli* was not seen at this time point with the four remaining *Candida* species.

Despite these variable results at different time intervals, when data from all *Candida* species were pooled and analysed, a highly significant inhibition of *Candida* biofilm formation by *E. coli* was noted ($P < 0.005$). Furthermore, there appeared to be a simultaneous significant enhancement of *E. coli* biofilm development at 24 and 48 h time intervals triggered as a result of cohabitation with *Candida* ($P < 0.005$), particularly for *C. tropicalis*, *C. dubliniensis* and *C. glabrata*.

Effect of *E. coli* LPS on *Candida* biofilms (XTT reduction assay)

In the early biofilm development phase, after 90 min of incubation, none of the LPS-treated *Candida* species

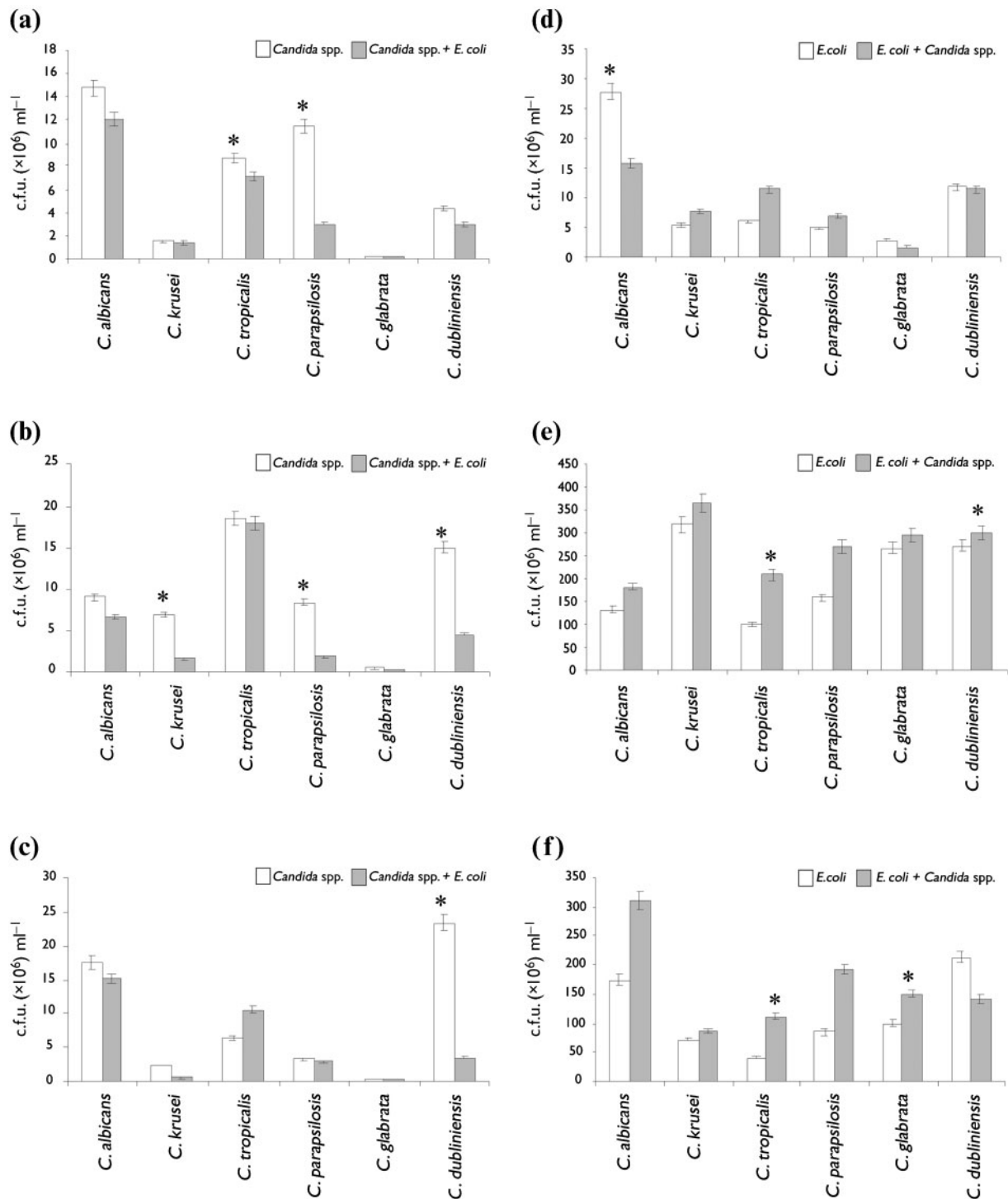


Fig. 1. Mean ($\pm 5\%$) c.f.u. counts of monospecies (*Candida* species or *E. coli*) and dual species (*Candida* species and *E. coli*) at different stages of biofilm formation. (a–c) Effect of *E. coli* on the biofilm formation of each *Candida* species at 90 min, 24 h and 48 h, respectively. Asterisks indicate a significant ($P < 0.05$) reduction in mean c.f.u. counts of *C. tropicalis* ($P = 0.010$) and *C. parapsilosis* ($P = 0.030$) at 90 min (a), *C. krusei* ($P = 0.050$), *C. parapsilosis* ($P = 0.011$) and *C. dubliniensis* ($P < 0.001$) at 24 h (b) and *C. dubliniensis* ($P < 0.001$) at 48 h (c) in the presence of *E. coli*. (d–f) Effect of each *Candida* species on the biofilm formation of *E. coli* at 90 min, 24 h and 48 h, respectively. A significant reduction ($P < 0.05$) in mean c.f.u. counts of *E. coli* was noted in the presence of *C. albicans* ($P = 0.047$) at 90 min (d) and a significant enhancement ($P < 0.05$) of mean c.f.u. counts of *E. coli* with *C. tropicalis* ($P = 0.019$) and *C. dubliniensis* ($P = 0.017$) at 24 h (e) and *C. tropicalis* ($P = 0.003$) and *C. glabrata* ($P = 0.042$) at 48 h (f). *, Significant difference between monospecies and dual-species biofilms at a given time point ($P < 0.05$).

demonstrated significant changes in metabolic activity compared with the controls, indicating that LPS has minimal or no effect on the biofilm metabolism at this stage. However, after 24 h, the metabolic activity of LPS-treated *C. tropicalis* biofilms increased significantly (19 %, $P=0.008$) in contrast to the biofilm activity of *C. dubliniensis*, which was significantly suppressed (4 %, $P=0.004$) (Table 1). LPS appeared to have no significant effect on the other four *Candida* species at this stage. On further incubation for 48 h, only LPS-treated *C. parapsilosis* biofilm demonstrated increased metabolic activity (24 %, $P=0.024$) compared with the controls (Table 1).

Despite the fact that direct comparisons of data from the c.f.u. assay and XTT reduction assay cannot easily be made, we compared the percentage modulation of candidal biofilm growth in the presence of *E. coli* and *E. coli* LPS in pooled data and found that there was no significant difference between the percentage modulation of *Candida* biofilms in the presence of *E. coli* or its LPS at any given time point.

CLSM

In 24 h dual-species biofilms, growth suppression of *Candida* by *E. coli* was clearly seen by CLSM with the Live/Dead stain, confirming the c.f.u. data. Thus, a few isolated *C. krusei* blastospores were seen among the cellular debris after 24 h (Fig. 2d) in contrast to its monospecies counterpart (Fig. 2c). Similarly, sparsely distributed *C. dubliniensis* blastospores were noted in dual-species biofilms (Fig. 2f) with *E. coli* after 48 h, confirming the above quantitative findings. Some dead cells and cellular

debris were also visible compared with the dense monospecies biofilm growth of the *C. dubliniensis* control (Fig. 2e).

With regard to *E. coli*, CLSM with Live/Dead stain indicated a reduction in *E. coli* cell numbers when incubated with *C. albicans* for 90 min, confirming the c.f.u. assay data. A considerable proportion of dead *E. coli* cells were visible in these dual-species biofilms (Fig. 2a, b).

When the relative proportions were quantified using image analysis software, all monospecies biofilms demonstrated a higher live:dead cell ratio compared with their dual-species counterparts (data not shown). This indicated quantitatively that there were fewer live cells and a higher number of dead cells in dual-species biofilms than in the monospecies biofilm.

SEM

In general, single-species biofilms of all *Candida* species grew well on the substrate on SEM observation, although species-specific growth variations were noted (Fig. 3). Thus, during the initial colonization phase of 24 h and the maturation phase of 48 h, single-species yeast biofilms characteristically exhibited profuse, dense growth, although extracellular matrix was scarcely visible (Fig. 3e). In contrast, at all stages of biofilm formation, dual-species biofilms demonstrated moderate growth with a reduction in the cellularity of the biofilm. Thus, in general, the dual-species biofilm had a reduced number of cell layers, a higher degree of cellular debris and had degrading yeast cells (Fig. 3b, d, f).

Table 1. Mean XTT values (\pm SD) of test (*E. coli* LPS-treated) and control (LPS-untreated) samples of different *Candida* species at 90 min, 24 h and 48 h

Significant differences ($P<0.05$) are shown in bold.

<i>Candida</i> species	Time interval	Test (+LPS)	Control (-LPS)	<i>P</i> value
<i>C. albicans</i>	90 min	0.579 \pm 0.091	0.569 \pm 0.094	0.795
	24 h	0.736 \pm 0.142	0.688 \pm 0.166	0.453
	48 h	1.251 \pm 0.041	1.205 \pm 0.099	0.312
<i>C. glabrata</i>	90 min	0.781 \pm 0.300	0.706 \pm 0.238	0.862
	24 h	0.920 \pm 0.316	0.961 \pm 0.303	0.326
	48 h	1.268 \pm 0.132	1.237 \pm 0.082	0.453
<i>C. tropicalis</i>	90 min	0.311 \pm 0.015	0.322 \pm 0.021	0.149
	24 h	0.362 \pm 0.055	0.303 \pm 0.037	0.008
	48 h	0.400 \pm 0.094	0.410 \pm 0.051	0.507
<i>C. parapsilosis</i>	90 min	0.232 \pm 0.046	0.254 \pm 0.066	0.248
	24 h	0.525 \pm 0.250	0.412 \pm 0.107	0.862
	48 h	1.053 \pm 0.220	0.851 \pm 0.268	0.024
<i>C. dubliniensis</i>	90 min	0.318 \pm 0.032	0.331 \pm 0.021	0.453
	24 h	0.566 \pm 0.008	0.588 \pm 0.023	0.004
	48 h	0.854 \pm 0.171	0.906 \pm 0.107	0.402
<i>C. krusei</i>	90 min	0.656 \pm 0.169	0.537 \pm 0.137	0.106
	24 h	1.075 \pm 0.353	1.024 \pm 0.313	0.862
	48 h	1.367 \pm 0.101	1.342 \pm 0.081	0.644

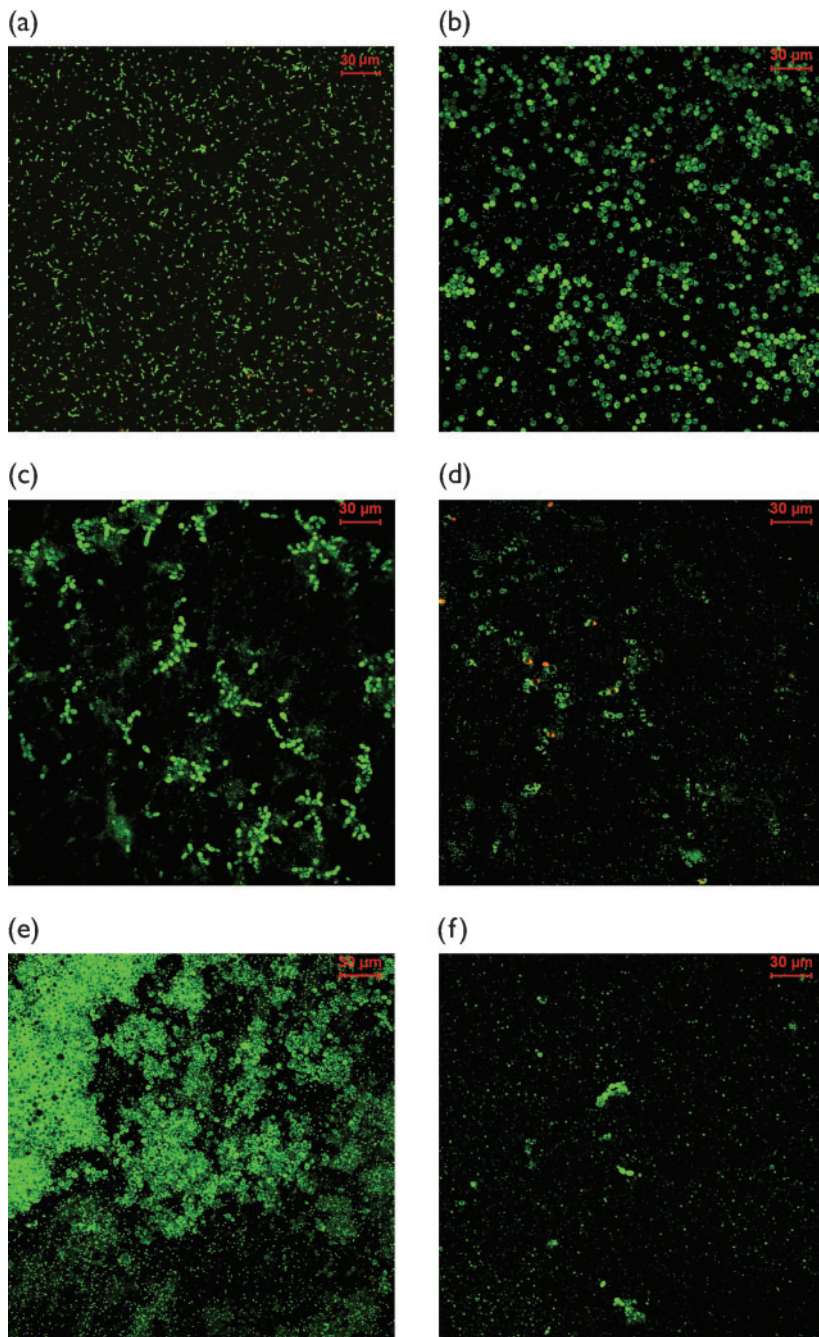


Fig. 2. CLSM images of monospecies (*Candida* species or *E. coli*) and dual species (*Candida* species and *E. coli*). Live cells are stained green and dead cells are red. (a) Adhesion of *E. coli* for 90 min. (b) Adhesion of *E. coli* in the presence of *C. albicans* for 90 min. Note the inhibition of *E. coli* adhesion by *C. albicans* in comparison with the monospecies counterpart. (c) Initial colonization of *C. krusei* for 24 h. (d) Initial colonization of *C. krusei* co-cultured with *E. coli* for 24 h. Note the inhibition of *C. krusei* colonization by *E. coli* compared with the *C. krusei* monospecies biofilm. (e) Maturation of *C. dubliniensis* for 48 h. (f) Maturation of *C. dubliniensis* when co-cultured with *E. coli* for 48 h. Note the suppression of *C. dubliniensis* maturation by *E. coli* in the dual-species biofilm.

With regard to *E. coli*, a few bacteria were seen and their density varied depending on the *Candida* species present and the different time intervals, and they had a less nondescript structure in comparison with the monospecies counterpart (Fig. 3d). Furthermore, the ability of *C. albicans* to suppress *E. coli* colonization in dual-species biofilms was clearly evident from SEM data (Fig. 3c, d).

DISCUSSION

Candida is a commensal yeast that colonizes oral, intestinal and vaginal mucosae of humans. The prevalence of

Candida species in oral cavities of healthy adults is approximately 50% (Samaranayake & MacFarlane, 1990) and *C. albicans* is the most common species followed by *C. tropicalis*. However, other *Candida* species such as *C. krusei*, *C. tropicalis*, *C. glabrata* and *C. parapsilosis* are also associated with oral infections (Samaranayake *et al.*, 1987; Webb *et al.*, 1998). *Candida* can colonize and develop into biofilms on animate as well as inanimate surfaces such as acrylic dentures, stents, shunts and prostheses (e.g. voice, heart valve, knee), implants (e.g. lens, breast, denture), endotracheal tubes, pacemakers and various types of catheter (Cardinal *et al.*, 1996; Gilbert *et al.*, 1996;

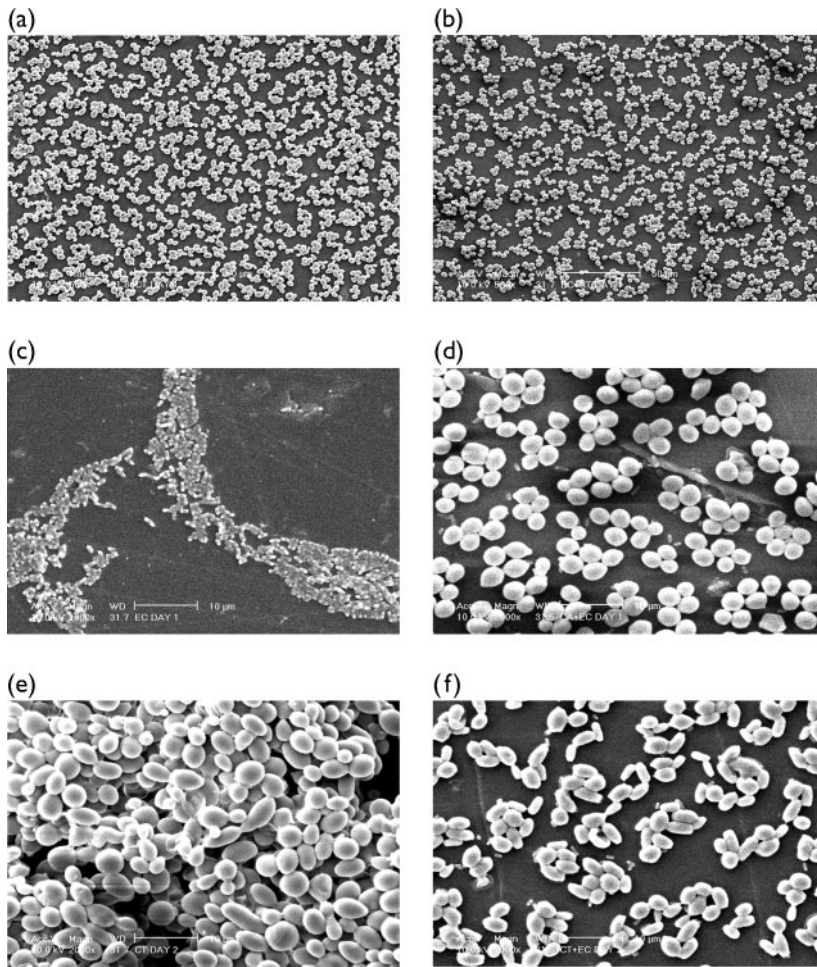


Fig. 3. SEM images of monospecies (*Candida* species or *E. coli*) and dual species (*Candida* species and *E. coli*). (a) Adhesion of *C. tropicalis* (90 min). (b) Adhesion of *C. tropicalis* co-cultured with *E. coli* (90 min). Note that *C. tropicalis* cells are smaller in size and less populous in comparison with the monospecies counterpart. (c) Adhesion of *E. coli* (90 min). (d) Adhesion of *E. coli* in the presence of *C. albicans* (90 min). Note that *E. coli* cell numbers are reduced and scantily distributed in comparison with the monospecies counterpart, and some cells are attached to *Candida* blastospores. (e) Initial colonization of *C. tropicalis* (24 h). (f) Initial colonization of *C. tropicalis* when co-cultured with *E. coli* (24 h). The *C. tropicalis* biofilm was less organized with a scanty distribution of cells in the dual-species biofilm, and some degrading yeast cells were also present. Magnification: $\times 500$ (a, b); $\times 2000$ (c–f).

Goldmann & Pier, 1993; Joly *et al.*, 1997; Leonhardt *et al.*, 1999; Penk & Pittrow, 1999; Reid *et al.*, 1992; Sen *et al.*, 1997).

Previous studies have shown that enterobacteria including *E. coli* are transient colonizers of the oral cavity and are more prevalent in Asian than in Western populations (10–15 %) (Samaranayake *et al.*, 1989; Sedgley & Samaranayake, 1994). *E. coli* is particularly prevalent in the mouth as a successional community during antibiotic therapy and also in immunocompromised patients and hospitalized patients. Despite the frequent association between *Candida* and *E. coli*, few data are available on the communal behaviour of these two organisms.

The principal aim of this study, therefore, was to evaluate the qualitative and quantitative effects of the transient oral colonizer *E. coli* on various stages of *in vitro* biofilm development by six different species of *Candida*. Our results indicated that both *Candida* and *E. coli* mutually modulate biofilm development to varying degrees at different stages of biofilm formation.

Using a c.f.u. assay, we report here, for the first time, the quantitative effect of *E. coli* on six different *Candida* species.

Our results indicated that *E. coli* had no significant effect on the adhesion of *C. albicans* to polystyrene surfaces, confirming the previous results of Nair & Samaranayake (1996a). They also demonstrated the enhanced adhesion of *Candida* to acrylic surfaces in the presence of *E. coli* ATCC 25922, irrespective of the bacterial load on the surface, and suppression of *C. krusei* adhesion in the presence of a high concentration of the coliform (10^5 and 10^6 cells ml^{-1}). Our results extend their work, as we noted that *E. coli* suppressed the adhesion of two other relatively common *Candida* species, *C. tropicalis* and *C. parapsilosis*. This implies that *Candida*–*E. coli* interactions related to adhesion and aggregation may be species-specific rather than strain-related. Further work with multiple strains of *Candida* from different species is required to confirm these findings.

Our results also implied that, on further prolonged incubation during the post-adhesion and biofilm development phase, *E. coli* suppresses the growth of *C. krusei*, *C. parapsilosis* (at 24 h) and *C. dubliniensis* (at both 24 and 48 h). For *C. albicans*, although Thein *et al.* (2006) noted a significant inhibition of this yeast at high concentrations of *E. coli* (2.5×10^6 , 5×10^6 and 1×10^7 c.f.u. ml^{-1}) at 24 h, we were unable to confirm this finding.

In the second part of this study, we evaluated the effect of *E. coli* LPS at different stages of biofilm formation on six different *Candida* species. Although the possible effect of LPS on the cellular layers of the biofilm is difficult to analyse with an XTT reduction assay, we tested the effect of LPS on three different growth phases of *Candida* biofilm development at 90 min, 24 h and 48 h, as these partially reflect the metabolic activity of the growth stages in the biofilm. Palma *et al.* (1992) were the first to study the anti-candidal effect of *E. coli* LPS *in vitro*, where graded concentrations of each LPS isolated from the cell walls of *E. coli*, *Serratia marcescens* and *Salmonella typhimurium* were added to neutrophils before addition of the target cells (*C. albicans*). They noted that LPS over a dose range of 1–1000 ng ml⁻¹ strongly enhanced the anti-candidal activity of human neutrophils, although LPS itself had no direct effect on candidal growth at any concentration used. In contrast, we have reported here that the LPS of *E. coli* had a direct modulatory effect on *in vitro* biofilm formation of specific *Candida* species, leading to growth stimulation of *C. tropicalis* and *C. parapsilosis* and inhibition of *C. dubliniensis*. This stimulatory/suppressive effect could be due to altered cell numbers or altered cellular activity in the formed biofilm, or both. However, such growth stimulatory/suppressive effects appeared to be related to the maturation period of the biofilm. More research is required to clarify this phenomenon.

Ultrastructural views of both monospecies and dual-species biofilms confirmed the results obtained from the c.f.u. assay. Generally, all monospecies biofilms of *Candida* were dense, confluent, relatively well defined and uniformly distributed, with minimal extracellular polymeric materials. In contrast, yeast growth in dual-species biofilms was less dense with cell clumps, dead cells and cellular debris indicating the effect of *E. coli*. In addition to the reduction in overall cell counts of *Candida* in dual-species biofilms with *E. coli*, all monospecies biofilms demonstrated a higher live:dead cell ratio compared with their dual-species counterparts on CLSM fluorescence image analysis. The dual-species biofilms were less viable than their monospecies variants, confirming the mutual inhibitory effect of *Candida* and *E. coli* during biofilm development.

A few workers have interpreted the clinical importance of such findings. Hummel *et al.* (1975), for instance, noted that many Gram-negative organisms including *Pseudomonas* and *E. coli* isolated from burns had a degree of inhibitory effect on *C. albicans*, although the effect of *E. coli* was the most consistent and reproducible. They also found that this activity was fungistatic and reversible.

In vitro studies have revealed that certain Gram-negative gut-associated organisms are frequently inhibitory towards *C. albicans* (Hummel *et al.*, 1975). Approximately 20% of *E. coli* strains were consistently inhibitory to the growth of *C. albicans*. These inhibitory *E. coli* were constantly more sensitive to a broad range of antibiotics than their non-inhibitory counterparts. Antibiotic therapy, therefore, may

encourage the overgrowth of fungi not only by reducing the number of Gram-negative organisms but also by selectively eliminating those that are most suppressive to fungal growth (Hummel *et al.*, 1975). Furthermore, viable *E. coli* and *E. coli* LPS may enhance the virulence of parenteral *C. albicans*, although there is a probable protective function of LPS in *Candida* infections initiated via the oral route (Henry-Stanley *et al.*, 2003).

In summary, our studies focused principally on the interactions of *Candida* and *E. coli* during different stages of biofilm development and indicate that *E. coli* has a significant growth inhibitory effect on specific *Candida* species at various stages of biofilm development. It is also clear that some *Candida* species are able to withstand this effect of *E. coli*. We also revealed the mutual modulatory effects between *Candida* and *E. coli* and its LPS. Further work is necessary to explain the molecular basis of these interactions and to understand the pathobiology of mixed bacterial–fungal interactions.

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